Paracrine Regulation of Human Prostate Cancer Cell Growth and Function.

A thesis submitted for the degree of Doctor of Philosophy
in
the Faculty of Medicine.

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This Thesis is Dedicated to
Antonella & Luca

my life would had been
cold without them

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I never thought my work as that of an individual. I rather believe in being part of a work team, where different skills and aptitudes concur to get better results. This is the main reason why these acknowledgements would appear rather long.

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ABBREVIATIONS USED.

$2OHE_1$	2-hydroxyoestrone
$2OHE_2$	2-hydroxyoestradiol
$2MeOE_1$	2-methoxyoestrone
3α/3β-diols	3α/3β-androstanediols
3α/3β-HSD	3α/3β-hydroxysteroid dehydrogenase
4OH-A	4-hydroxyandrostenedione
5α-Adione	androstanedione
$16\alpha OHE_1$	16α-hydroxyoestrone
17βHSD	17β-hydroxysteroid dehydrogenase
A	androsterone
Å	angström unit
ACTH	adrenocorticotropic hormone
ADP	adenosine diphosphate
AR	androgen receptors
Arg	arginine
Asn	asparagine
Asp	aspartic acid
ATP	adenosine triphosphate
α2M	α_2-macroglobulin
bp	base pairs
BPH	benign prostatic hyperplasia
BSA	bovine serum albumin
^{14}C	carbon label
℃	degrees centigrade
Ca^{2+}	calcium ion
cAMP	cyclic adenosine monophosphate
CAS	cell analysis system
CCE	catecholoestrogens
cDNA	complementary deoxyribonucleic acid
cGMP	cyclic guanosine monophosphate
Ci	curie
cm	centimetre

CO_2	carbon dioxide
COMT	catechol-O-methyltransferase
cpm	counts per minute
CR	conversion rate
CT-FCS	charcoal treated-FCS
Cys	cysteine
DAG	diacyl glicerol
DAB	diaminebenzidine
DCC	dextran coated charcoal
DEPC	diethylpyrocarbonate
DES	diethylstilbestrol
DHT	dihydrotestosterone
Δ^4Ad	androstenedione
Δ^5Adiol	androstenediol
DHA	dehydroepiandrosterone
DHAS	dehydroepiandrosterone sulphate
DHICT-FCS	dialysed heat-inactivated charcoal treated FCS
DHT	dihydrotestosterone
DMBA	7,12-dimethylbenz(a)anthracene
DMSO	dimethylsulphoxide
DNA	deoxyribonucleic acid
DNase	deoxyribonuclease
dNTP	deoxynucleotides triphosphates
DTT	dithiothreitol
E_1	oestrone
E_1S	oestrone sulphate
E_2	oestradiol
E_3	oestriol
EDTA	ethylenediamine tetra-acetic acid
EGF	epidermal growth factor
EGFR	epidermal growth factor receptor
EpiA	epiandrosterone
ER	oestrogen receptor
ERE	oestrogen response elements
FCS	foetal calf serum
FGF	fibroblast growth factor

FITC	fluorescein isothiocyanate
fmol	femtomoles
g	gravitational force
GAG	glycosaminoglycan
GAP	GTPase activating protein
GBq	giga bequerel
GDP	guanosine diphosphate
Glu	glutamic acid
Gly	glycine
GR	glucocorticoid receptor
GTP	guanosine triphosphate
^3H	tritium label
HBD	hormone-binding domain
HEPES	hydroxyethyl-piperazine ethanesulphonic acid
HPLC	high performance liquid chromatography
HRE	hormone response elements
HRT	hormone replacement therapy
hsp	heat shock protein
ICA	immunocytochemical assay
IGF-I & II	insulin-like growth factor I & II
IGFBPs	insulin-like growth factor binding proteins
IGFR	insulin-like growth factor receptor
IgG	immunoglobulin G
IP_3	inositol triphosphate
kb	kilobase
K_d	dissociation constant
K_m	Michaelis constant
kDa	kiloDaltons
LAP	latency-associated peptide
LBA	ligand binding assay
LE_2	lipoidal oestradiol
LHRH	luteinizing hormone releasing hormone
L-TGFβ_1	latent-transforming growth factor β_1
M	molar
M-6P	mannose-6-phosphate
ma	milliamperes

mCi	milliCurie
mg	milligram
Mg^{2+}	magnesium ion
µg	microgram
min	minute(s)
ml	millilitre
µl	microlitre
mm	millimetre
mM	millimolar
µM	micromolar
µm	micrometre
mmol	millimoles
MMTV	mouse mammary tumour virus
MOPS	3-(N-morpholino)propanesulphonic acid
M_r	relative molecular mass
MR	mineralcorticoid receptor
mRNA	messenger ribonucleic acid
MVGF	myxoma virus growth factor
M.W.	molecular weight
µm	micrometre
µM	micromolar
NAD	nicotinamide adenine dinucleotide
NADPH	nicotinamide adenine dinucleotide phosphate
ND_{50}	neutralising dose 50
nm	nanometre
nM	nanomolar
NMU	N-nitrosomethylurea
NRK	normal rat kidney
OC	oral contraceptives
OHPs	organic hydroperoxide
PAGE	polyacrilamide gel electrophoresis
PAP	peroxidase-anti peroxidase
PBP	prostate binding protein
PBS	phosphate buffered saline
PBS-A	Dulbecco's phosphate buffered saline
PCR	polymerase chain reaction

PDGF	platelet-derived growth factor
PDGFR	platelet-derived growth factor receptor
pg	picogram
Pg	progesterone
PgR	progesterone receptor
PI-3K	phosphatidyl insositol 3-kinase
PIP_2	phosphatidyl inositol 4,5-diphosphate
PKC	protein kinase C
PLC	phospholipase C
pM	picomolar
$pp60^{c\text{-}src}$	Rous sarcoma virus transforming gene product
pRb	retinoblastoma gene protein product
PRFs	peptide regulatory factors
R1881	methyltrienolone
RBA	relative binding affinity
RNA	ribonucleic acid
RNase	ribonuclease
RP-HPLC	reverse phase-HPLC
RRT	relative retention times
RSV	Rous sarcoma virus
RT-PCR	reverse transcriptase-PCR
S	svedberg unit
SD	standard deviation
SDS	sodium dodecyl sulphate
SE	standard error
Ser	serine
SFGF	Shope fibroma virus growth factor
SH2	src homology-2
SHBG	sex hormone binding globulin
SRE	steroid response elements
SSC	standard saline citrate
SSV	simian sarcoma virus
T	testosterone
TAM	tamoxifen
TCA	trichloroacetic acid
Tfm	testicular feminization

TGFα	transforming growth factor α
TGFβ	transforming growth factor β
$TGF\beta_1$-BP	transforming growth factor β_1-binding protein
Thr	threonine
Tris	tris(hydroxymethyl)amino methane
TRPM-2	testosterone-repressed prostate message-2
TUR	transurethral resection
UGE	urogenital epithelium
UGM	urogenital mesenchyme
UGS	urogenital sinus
UV	ultraviolet (light)
V	volt
VGF	vaccinia virus growth factor
v / v	volume for volume
w / v	weight for volume

FIGURES & TABLES.

SUPPLIERS.

Reagent	Source
Acquamount	BDH
Amphotericin B	Gibco BRL Ltd.
[^{14}C]Androstenedione	Du Pont de Nemours
AR gene	HUMARA
Bradford reagent	BioRad Ltd.
Calf Thymus DNA	Sigma
DEPC-treated water	Gibco BRL Ltd.
Diethylstilbestrol	Sigma
Dihydrotestosterone	Sigma
Dithiothreitol	Sigma
DNase I	Gibco BRL Ltd.
EGF	Sigma
[^{125}I]-EGF	Du Pont de Nemours
ERICA kit	Abbott
Eukitt® mounting medium	Bio-Optica
Foetal calf serum	Gibco BRL Ltd.
Gelatin	Sigma
Hoechst 33258	Sigma
Methyltrienolone (R1881)	Du Pont de Nemours
[^{3}H]Mibolerone	Amersham
Mibolerone	Amersham
Nonidet P-40	BDH Ltd.
17β-Oestradiol	Sigma
[^{3}H]17β-Oestradiol	Du Pont de Nemours
Penicillin/Streptomycin	Gibco BRL Ltd.
PgRICA kit	Abbott
Ready-Gel	Beckman
Ready-Flow III	Beckman
Reverse transcriptase	Boehringer
Ribonuclease A	Sigma
RNase H	Boehringer

RNazol™ B	Biotecx Lab.
RPMI-1640 medium	Gibco BRL Ltd.
Nonidet P-40	BDH Ltd.
Sheep serum	SAPU
Superscript II RT	Gibco BRL Ltd.
Taq-DNA-polymerase	Promega
[^{3}H]Testosterone	Du Pont de Nemours
Testosterone	Sigma
TGFα	Sigma
TGFß$_1$	Sigma
[^{125}I]-TGFß$_1$	Du Pont de Nemours
[^{3}H]Thymidine	Du Pont de Nemours
Transferrin	Sigma
Triamcinolone acetonide	Amersham
Trypsin:EDTA	Gibco BRL Ltd.
Tween 20	Sigma

Suppliers for any other specific material or apparatus are indicated throughout the text. All other basic chemicals were purchased from Sigma and BDH. All plasticware used in cell culture was obtained from Costar Italia, Nunc or Bibby Ltd.

Summary

This thesis mainly deals with steroid-growth factor interaction in the regulation of growth and steroid metabolism of LNCaP, DU145 and PC3 human prostate cancer cell lines. Growth response to both androgens and oestrogens and to TGFα and $TGF\beta_1$ were investigated; in addition, content and status of steroid (AR and ER) and growth factor (EGFR and TGFβR) receptors were assessed using multiple approaches, including ligand binding assay, immunocytochemistry and reverse transcriptase-PCR (RT-PCR). Furthermore, immunofluorescent staining was used to evaluate expression of EGF, TGFα and $TGF\beta_1$ and of the 27 kDa heat shock protein (hsp27) as a marker of oestrogen sensitivity. On the other hand, patterns of both testosterone (T) and oestradiol (E_2) metabolism were studied using incubation of cultured cells with labelled steroid precursor and reverse phase-HPLC (RP-HPLC) analysis of precursor degradation and formation of metabolic products. Possible influence of both TGFα and $TGF\beta_1$ on rates and direction of metabolism of both steroids was also determined.

Growth of LNCaP cells was significantly stimulated by physiological concentrations of the two major androgens (T and dihydrotestosterone: 30.2% and 33.8% respectively, $P<0.03$) and of E_2 (65.8%, $P=0.009$), using stringent culture conditions. Interestingly, increasing concentrations of E_2 (0.01-100 nM) induced a significant inhibition of the proliferative activity of PC3 cells (55.2% at 100 nM E_2, $P<10^{-6}$), while neither androgen

significantly affected growth of this cell line. In contrast, DU145 cells proved insensitive to the addition of either androgens or oestrogens.

Presence of androgen binding sites in prostate tumour cell lines was ascertained using radioligand binding assay and RT-PCR approaches. High affinity AR were detected in both soluble and pellet fractions of LNCaP and DU145 cells, whilst PC3 cells showed only nuclear AR. These results were also supported by PCR system, where ampifiable AR mRNA was found in all three cell lines.

Multiple evidence for high affinity sites of oestrogen binding in LNCaP cells was obtained: i) biochemical assay allowed the detection of high affinity, low capacity binding sites in both soluble and nuclear cell fractions; ii) immunocytochemical and immunofluorescent assays showed a consistently intensive staining for both ER and PgR, as well as hsp27; iii) the RT-PCR system documented the presence of normal or a variant ER mRNA in PC3 cells; the latter, which lacks the entire exon 4, has been recently characterised in our laboratories in human mammary carcinoma cells. Presence of ER in PC3 cells was also documented by biochemical and cytochemical assays as well as, indirectly, by hsp27 staining. However, the relative estimate of ER expression displayed levels significantly and consistently lower than those found in LNCaP cells. This finding was confirmed using the RT-PCR approach, where transcript levels for both normal and variant ER mRNA were proportionally lower than in LNCaP cells. Conversely, DU145 cells were found to be

consistently ER-negative using biochemical and cytochemical assays, hsp27 staining and RT-PCR system.

The evidence that the E_2-induced growth was completely reversed in LNCaP cells by the addition of the pure antioestrogen ICI-182,780, clearly suggests that E_2 acts via its own receptor. The possibility that the inhibitory effect exerted by E_2 on growth of PC3 cells could be mediated via an increase of TGFß production was also supported by the fact that use of a neutralising antibody raised against $TGF\beta_1$ produced a three-fold increase of cell growth; this effect was almost completely abolished after addition of 100 nM E_2. However, Northern blot analysis did not reveal any increase of $TGF\beta_1$ mRNA following E_2 administration in this cell line.

Growth of PC3 cells was significantly stimulated by TGFα (36% at 50 ng/ml, $P<0.003$) and inhibited by $TGF\beta_1$ (55% at 5 ng/ml, $P<10^{-6}$) after 48 hours exposure in routine medium. Proliferative activity of DU145 cells was minimally affected by TGFα, but significantly inhibited by $TGF\beta_1$ (28% at 5 ng/ml, $P<0.009$). In contrast, LNCaP cells proved to be poorly sensitive, at least in the short-term, to either growth factor.

Radioreceptor assay showed presence of high affinity binding sites for EGF in all three cell lines and for TGFβ in DU145 and PC3 cells, whilst no detectable site of TGFβ binding was found in LNCaP cells. DU145 cells displayed the highest EGFR content, LNCaP the lowest; TGFβR were expressed in greater amounts in PC3 than in DU145 cells. EGFR binding data were also confirmed using Western blot analysis, the DU145 cells having EGFR

expression levels higher than PC3 and LNCaP cells. In addition, immunofluorescent staining revealed high amounts of both EGFR and TGFα, and fairly high EGF in DU145 and, to a lesser extent, in LNCaP cells; by contrast, PC3 cells exhibited low levels of both receptor (EGFR) and ligands (EGF, TGFα), but appreciable levels of endogenous $TGF\beta_1$. Overall, these results suggest a differential sensitivity to TGFα and $TGF\beta_1$ by prostate cancer cells; TGFα response seems to be not proportional to the EGF-R content of individual cell lines, while $TGF\beta_1$ response appears to be inversely related to the androgen-sensitivity of cells.

The ability of human prostate cancer cells to metabolise steroids was assessed using an approach which allows the evaluation of conversion metabolic rates after administration of labelled steroid precursor (T or E_2) at physiological concentration (0.5-10 nM) for various exposure times. Analysis of both precursor degradation and formation of metabolic products was carried out using reverse phase-HPLC (RP-HPLC) and "on line" radioactive detection after either 24 or 72 h incubation. Overall, different prostate cancer cells degraded T and E_2 quite differently, alternatively favouring reductive or oxidative metabolic pathways. In particular, both LNCaP and DU145 cells retained high levels of unconverted T, with a limited production of Δ^4-Ad and its 17-keto derivatives (if any) and relatively high amounts of DHT and 3α-diol; similarly, these cell lines maintained the most part of E_2 undegraded, with a limited E_1 formation. In contrast, PC3 cells quickly degraded T and exhibited high formation rates of Δ^4Ad and 17keto metabolites, whilst neither

DHT nor 3α-diol were detected after either short or longer incubation times; equally, E_2 was rapidly oxidised to E_1, with an appreciable production of $16\alpha OHE_1$. No detectable formation of steroid conjugates was noted in either PC3 or DU145 cells, whilst a remarkable production of T conjugates (43 to 57%) was observed in LNCaP cells at 24 h and, to a far lesser extent (<10%), at 72 h.

The effects of both TGFα and $TGF\beta_1$ on rates and direction of T and E_2 metabolism were explored. They varied markedly depending on the cell line studied. Either growth factor enhanced reductive pathways of both T and E_2 metabolism in LNCaP cells, while oxidative pathways were respectively increased and reduced by TGFα and only moderately affected by $TGF\beta_1$. In DU145 cells, TGFα caused a rise in the extent of both T and E_2 oxidative reactions, with a corresponding decrease of the reductive pathway; $TGF\beta_1$ paralleled the effects of TGFα on T metabolic pathways, but had an opposite, minor influence on E_2 metabolism in this cell line. Finally, T metabolic pathways were minimally affected by either growth factor in PC3 cells. Both TGFα and $TGF\beta_1$, reduced the conversion of E_2 to E_1 in PC3 cells and decreased $16\alpha OHE_1$ formation, with an additional increase of the oxidative way. The influence exerted by TGFs on the overall direction of steroid metabolism in prostate tumour cells, although apparently moderate, could be important in determining the actual concentration of biologically active metabolites of steroids and, therefore, may be relevant also to their growth regulation.

These results are used as the basis for a model of prostate cancer progression, whether hormone-sensitive or insensitive.

INTRODUCTION.

1. Steroids and Cancer

1. Steroids and Cancer

1.1. Historical Overview

In the last 50 years the relationship between hormones and cancer has been extensively investigated, but only poorly elucidated. In particular, the potential involvement of steroids in the phenomena of tumour initiation, promotion and progression remains today a dilemma. Notwithstanding, the bond existing between steroid hormones and some human neoplasias was already grasped since the eighteenth century, long time before the discovery of specific receptor proteins as essential intermediaries of steroid action at target tissue level.

After the early observations by Hunter [Hunter, 1786] on the dependence of prostatic function and development on gonadal androgens, much work accumulated on the use of hormonal manipulation as a tentative intervention therapy in patients having prostate cancer [White, 1895; Cabot, 1896]. Subsequent pioneering studies (reviewed by [Sharifi & Kiefer, 1987]) eventually led Charles Huggins and colleagues [Huggins & Hodges, 1941; Huggins *et al.*, 1941] to conclude that androgen withdrawal is an effective approach in the management of prostate cancer. On the other hand, in a well-known work, Beatson [Beatson, 1896] reported that some premenopausal patients with advanced breast cancer experience both subjective and objective responses following the ablation of ovaries. A link,

although indirect, between gonadal function and tumour growth was therefore established.

Since then, an enormous amount of experimental work has been accruing, but so far clinicians are still at a loss in the management of most human endocrine-related cancers. The reason for this failure is not unique and mainly lies in the restricted knowledge of several key phenomena, including malignant transformation, control of tumour cell growth, mechanisms of neoplastic progression.

Concerning steroids, the situation is confused by the complexity inherent in the nature of these hormones. Firstly, although steroids are normally categorised in several classes, their activity is rather expressed by a continuous spectrum of biological effects, a significant extent of overlapping being generally encountered. In the second place, different steroid classes may give rise to either synergistic or antagonistic actions in target tissues; examples in point are given by the well-known antagonism between oestrogens and androgens in both human breast and prostate [Huggins & Clark, 1940], and between oestradiol and progesterone in uterine tissues [Courrier, 1950]. Finally, steroid action appears to be multifaceted by the presence of numerous biologically active metabolites, particularly oestrogens, having a wide range of specific, even opposite, effects [Dao, 1979]. Formation of these metabolic products may easily occur in target tissues, wherein the expression of several steroid enzyme activities has been recently documented; it is enough to consider the relevance currently stressed on

catecholoestrogens (CCE) in human breast tissues or the paramount importance of dihydrotestosterone (DHT) in prostate development and function, in humans.

Additionally, it is now well established that tissue response to steroids varies considerably among tissues, in both qualitative and quantitative terms. At the beginning of fifties, Dorfman [Dorfman, 1948] indicated that different breeds of chicks exhibit a substantially divergent comb-growth response to testosterone. Later, Bardin and colleagues [Bardin *et al.*, 1970] revealed that the lack of androgen response in normally androgen-sensitive tissues of mice or rats having the testicular feminization syndrome is due to the absence of specific binding sites required to elicit androgen action. This stimulating area has been pursued extensively, yielding a large body of evidence relevant to the understanding of the molecular biology of steroid action in regulation of both normal and malignant cell growth.

1.2. Oestrogens and Breast Cancer

For more than half a century, oestrogens have been implicated as causative agents in tumour development. This assumption has been corroborated by the evidence that chronic exposure to sex steroids may produce either benign or malignant growth at different organ sites in mammals, including primates and man [Li & Nandi, 1990]. However, data suggesting that physiological levels of steroids are carcinogenic in humans are

considerably sparse. This is not surprising in view of the "multihit" nature of breast cancer development: several years before clinical evidence, breast tumours originate from an initial transformation induced by carcinogen(s) and a subsequent lag period, of extremely variable length, where different promoters, either systemic or local, may act in concerted pathways whose net effect finally leads the committed cells to grow.

1.2.1. Oestrogens and carcinogenesis in animal models

Data concerning oestrogen-induced tumours in experimental animals appear of relevance, not only in theoretical but also in practical terms.

A wide range of neoplastic forms has been produced in different animal species (including mouse, hamster, rat, rabbit and dog) following oestrogen administration; however, the pharmacological doses used, as well as the duration of exposure, often extended to a significant portion of the life span of the species involved, would suggest that these data be taken with some caution.

Probably, the oestrogen-induced tumours in hamster kidney and liver represent the experimental models most extensively investigated in hormonal carcinogenesis [Li & Li, 1984]. In particular, the Syrian hamster kidney seems to be an ideal system, since it behaves as an oestrogen-responsive tissue [Li & Li, 1978; Li & Li, 1981] and since spontaneous renal tumours are virtually non-existent in hamster [Pour *et al.*, 1979]. In this model, oestrogens induce multiple bilateral renal tumours

in either intact or castrated male Syrian hamsters, at an incidence that approximates 100% [Li & Li, 1987]; on the other hand, renal tumorigenesis can be completely prevented through the concomitant administration of antioestrogens, progesterone or androgens [Antonio *et al.*, 1974]. It has been suggested that oestrogens can affect neoplastic transformation through both hormonal and metabolic activity, the former being defined as receptor-mediated effects on growth or synthesis of induced proteins, the latter as the generation of biologically active metabolites and their subsequent interaction with cellular macromolecules [Li & Li, 1990].

Studies on breast cancer oncogenesis in rodents have been based on mammary carcinoma experimentally induced by chemicals and viruses [Moore *et al.*, 1979; Nicholson *et al.*, 1988]. In particular, 7,12-dimethylbenz(a)anthracene (DMBA) and mouse mammary tumour virus (MMTV) model systems have been used. In either model, rates of tumour development and growth appear to be under hormonal control. Studies on the DMBA-induced tumours, pioneered by Charles Huggins [Huggins *et al.*, 1961], have shown that the highest proportion of developing tumours occurs when the carcinogen is administered to virgin rats soon after the beginning of oestrus, namely between 40 and 46 days of age; malignant transformation appears to affect mainly the terminal end buds (TEB) of the glandular tree, whilst the more differentiated alveolar buds (AB) are seemingly insensitive to DMBA [Russo & Russo, 1987]. On the other hand, there is evidence that formation of DMBA-induced

mammary cancer in rats is inhibited by castration of animals prior to DMBA administration; in addition, most tumours (>85%) produced in intact animals are hormone-dependent and regress after oophorectomy [Nicholson *et al.*, 1988]. The process of MMTV induction of breast tumours in rodents has been extensively investigated. The retrovirus is transmitted to neonatal mice through breast milk, commonly leading to development of mammary cancer when animals are 6 to 9 months old. It is of interest to note that reverse transcribed DNA of MMTV contains a peculiar regulatory sequence known as long terminal repeat (LTR) which, when integrated, may interact with adjacent genes by subverting their normal regulatory processes [Dickson *et al.*, 1984; van de Vijver & Nusse, 1991]; these genes includes sequences, known as hormone-responsive elements (HREs), which determine the cell responsiveness to hormones and are specifically recognised by hormone receptors [Ponta *et al.*, 1985]. Thus, insertion of MMTV at different integration sites may well alter the function of those genes which are believed to primarily mediate hormone action.

Mammary tumours can also be produced in rats exposed to N-nitrosomethylurea (NMU) [Gullino *et al.*, 1975]. NMU-induced tumours have been reported to be hormone-dependent and endowed with receptors specific to oestrogens, progesterone, androgens and prolactin [Arafah *et al.*, 1980]. In this system, tumour regression can be observed after ovariectomy or following treatment with either antioestrogens or luteinizing hormone-releasing hormone analogues [Rose *et al.*, 1980].

Interestingly, tumour growth can be reactivated with oestradiol administration [Pruitt *et al.*, 1979], indicating that this tumour model is oestradiol-dependent. In addition, the finding that both oestrogen and progesterone receptors are significantly reduced after ovariectomy, whilst prolactin receptor levels are almost unaffected [Arafah *et al.*, 1980] reinforces the oestrogen-dependence of NMU-induced mammary carcinoma while it partly abridges the potential role of prolactin, at least in this experimental model.

1.2.2. Risk factors for breast cancer

A constellation of factors influences the risk for breast cancer [Bland, 1987]. They include reproductive experience, ovarian activity, dietary habit and specific endocrine components.

Variables related to reproductive life have been consistently associated with risk for female breast cancer. Most of them involve an either absolute or relative hyperoestrogenism as the critical element (for a review see [Zumoff *et al.*, 1975]). Over the last thirty years, breast cancer risk has been in turn ascribed to an excess of total oestrogens, to an excess of oestrogens not counteracted by progesterone or to an unbalance among several oestrogen fractions. All these hypotheses originated from both epidemiological and clinical observations on the relationship between reproductive factors and the incidence of breast cancer.

Overall, epidemiological data suggest that the younger the age at first birth, the greater the protection from breast cancer acquired. It has been previously reported that nulliparity only slightly raises the risk of developing breast cancer [MacMahon *et al.*, 1970], whilst the birth of the first child after the age of 30 produces a relative risk of 2.2 to 2.7 [Brinton *et al.*, 1983]. Apparently, the age of 35 may represent a crucial point; in fact, any full-term pregnancy before this age confers some degree of protection [Trichopoulos *et al.*, 1983]. Women over the age of 30 at the time of their first birth have a risk greater than women who never become pregnant. However, increasing parity seems to have a significant protective effect, which is maintained even when adjusted for age at first birth and other risk factors [Pathak *et al.*, 1986]. The evidence that early pregnancy is associated with a reduced breast cancer could imply that it either modifies some etiological factors responsible for breast cancer or changes breast tissue itself by making it less susceptible to malignant transformation. It has been documented that a first pregnancy has a lasting impact on the hormonal milieu of individual female; for instance, a rise of circulating oestriol and decreases in dehydroepiandrosterone (DHA), its sulphate (DHAS) and prolactin levels can all persist many years after delivery [Musey *et al.*, 1987]. Firstly, Huggins and Jensen [Huggins & Jensen, 1955] and later Lemon [Lemon *et al.*, 1966] designed the hypothesis of the "impeded oestrogens" to explain the protective role exerted by pregnancy on breast cancer development. Since oestriol (E_3) was thought to oppose the action of oestradiol (E_2) and oestrone (E_1),

the authors postulated that the higher E_3 excretion levels found in pregnant women may counterbalance the carcinogenic potential of both E_2 and E_1. Lemon therefore introduced the "E_3 quotient", namely the ratio of urinary E_2 plus E_1 to E_3, to discriminate high and low risk subjects in Asian and North American women having a substantially different incidence of breast cancer. However, this hypothesis has been subsequently contradicted by a number of experimental data. In first place, Clark and associates [Clark *et al.*, 1977] indicated that E_3 exhibits the same oestrogenic potency as E_2 and E_1 when used for longer exposure intervals. Secondly, the higher E_3 quotient found in women, like Japanese, at low risk of breast cancer should be regarded as a consequence of reduced levels of E_2 and E_1 rather than the effect of an E_3 excess [Zumoff, 1981]. Nevertheless, Lemon's observations are praiseworthy since they have drawn our attention to steroid excretion patterns as a possible reflection of the endocrine status of both normal and diseased breast tissues. Data coming from our laboratory have, for instance, revealed a bimodal expression of steroid excretion profiles in breast cancer patients, which is significantly related to both steroid receptor content of primary tumours and overall prognosis of patients [Castagnetta *et al.*, 1981; Castagnetta *et al.*, 1985; Castagnetta *et al.*, 1986a].

The possibility that an imbalance between oestrogens and progesterone is in some way related to breast cancer development has been previously introduced by Sommers [Sommers, 1955]. The author observed a noticeable incidence of

hyperplastic lesions in endometria obtained at autopsy from women who died of breast cancer, suggesting that this could be due to the action of unopposed oestrogens. This evidence was further refined by Grattarola [Grattarola, 1964] who documented that breast cancer patients denote endometrial patterns significantly different from those found in healthy controls. In particular, almost 80% of breast cancer patients exhibited some endometrial abnormalities (proliferative endometrium, adenomatous or atypical hyperplasia) which are suggestive of anovulatory cycles; by contrast, more than 70% of normal controls displayed a progestative endometrium. This evidence was initially attributed to an oestrogen excess not compensated by progesterone, which would give rise to anovulatory cycles. On this basis, subsequent work by Sherman and Korenmann [Sherman & Korenman, 1974] connected several risk factors for breast cancer (nulliparity, obesity, early menarche and late menopause) to the presence a luteal insufficiency, i.e. lack of a full progesterone phase or an oestrogen "window" (see below). However, data coming from clinical research have not always confirmed this conjecture. On one hand, studies on either infertile women with a history of progesterone deficiency or anovulatory women indicated respectively a 5.4 and a 3-4 fold increase of breast cancer risk [Cowan *et al.*, 1981; Coulam & Annegars, 1983]. On the other hand, normal luteal phases were found in both women at high genetic risk of breast cancer and a group of premenopausal breast cancer patients [McFayden *et al.*, 1976].

Many epidemiological investigations have indicated that bilateral ovariectomy before the age of 40 significantly reduces the risk of developing breast cancer [Trichopoulos *et al.*, 1972; Helmrich *et al.*, 1983; Irwin *et al.*, 1988]. On the other hand, early menarche and late menopause produce a small increase (from 1.4 to 3.3 times) of risk [Roush *et al.*, 1987]. This evidence indicates that ovarian function plays a continuing role throughout a woman's reproductive life; in particular, number of physiological ovarian cycles before first pregnancy may provide a clue to unravel the intimate relationship existing between age at menarche, age at first pregnancy and breast cancer. Korenmann's "oestrogen window" hypothesis is mainly based on the view that unopposed oestrogen stimulus is the most favourable state for breast tumour induction (the "open" window) and that the extent of risk depends upon the length of oestrogen exposure (how long the window is "open") [Korenman, 1980]. Following this hypothesis, breast susceptibility to carcinogen action declines as soon as a normal luteal secretion of progesterone occurs, becoming minimal during pregnancy. The pubertal years prior to the establishment of regular ovulatory menstrual cycles and the perimenopausal interval of waning follicle maturation and ovulation represent the two main "open window" periods. Several factors, like obesity, late pregnancy, earlier menarche and delayed menopause may prolong these periods and therefore be associated with an increased susceptibility of breast tissues to environmental carcinogens. To corroborate this hypothesis, Korenmann considered evidence

coming from separate studies on atomic bomb survivors of Hiroshima and Nagasaki, and on tubercolotic women receiving repeated chest fluoroscopies. In the A-bomb survivors, no increase of breast cancer incidence was seen in children who were exposed to irradiation before the age of 10, whilst a conspicuous risk exposure was observed between 10 and 14 years, namely just before menarche, suggesting that cells are being transformed at a time of non-opposed oestrogen stimulus. Over that age, irradiation-induced risk decreased rapidly, but it seemed to reappear after the age 50, i.e. around menopause [Tokunaga *et al.*, 1979]. In the repeatedly fluoroscoped women having tubercolosis, the highest risk increment over un-x-rayed controls was found in the 15-20 years age group, while no increase was observed after the age of 30 [Hrubec *et al.*, 1989]. However, other reports evidently conflict this theory. In particular, Anderson *et al.* [Anderson *et al.*, 1982] observed that the peak of proliferative activity of breast cells occurs in the luteal phase of the menstrual cycle, under the influence of both oestrogen and progesterone. Although the oestrogen window hypothesis has been partly contradicted by other clinical and epidemiological evidence, it appears meaningful mainly because has stressed the key role of endocrine environment in breast cancer development.

Despite the ever-growing literature on the relationship between diet and cancer, data are often contradictory and difficult to decipher. Different case-control studies have reported a significant association between obesity and increased breast

cancer risk in post-menopausal years [Byers & Graham, 1984]. Initially, Siiteri and colleagues [Siiteri *et al.*, 1974] suggested that this increase could be ascribed to E_1. This assumption was mainly based on the evidence that E_1 is the main circulating oestrogen in post-menopausal women and that the aromatisation of androstenedione to E_1 preferentially takes place in the adipose tissue which could therefore represent a significant source of oestrogens in the obese post-menopausal female. It is unclear whether or not obesity and fat intake are independently associated with breast cancer risk, but it seems likely that an excess of caloric intake may be important in post-menopausal breast cancer because it affects body fat levels [Byers & Graham, 1984]. However, the potential role of E_1 in human breast cancer development remains to be established.

Several epidemiological studies have suggested that a diet rich in fiber and complex carbohydrates is associated with a decreased risk of breast cancer [Kolonel *et al.*, 1981; Brisson *et al.*, 1989; van't Veer *et al.*, 1990]; in particular, women consuming grain fiber during their teens have a reduced risk in both young and old age [Pryor *et al.*, 1989]. It is well recognised that either incidence or mortality rates of hormone-related cancers, including breast, are considerably higher in the western world compared to Asian and some east European countries, where vegetarian or semivegetarian diet is prevailing [Rose *et al.*, 1986]. Although it has been earlier ascertained that dietary fiber reduces the enterohepatic circulation of oestrogens [Adlercreutz *et al.*, 1986], the overall mechanism by which diet may affect

breast cancer development remains largely unknown. Recently, the discovery of phytoestrogens and other oestrogenically active compounds in human urine [Adlercreutz *et al.*, 1987] has disclosed a new scenery for investigating this potentially important issue. Phytoestrogens, such as lignans and isoflavones, are diphenolic plant constituents which are excreted in large amounts by subjects consuming vegetables, soybeans and whole-grain products. The finding that urinary levels of these compounds are significantly lower in breast cancer patients with respect to omnivorous and vegetarian controls [Adlercreutz *et al.*, 1982] has encouraged researchers to explore the likelihood that dietary weak oestrogens exert a protective role for breast cancer. For instance, it has been recently suggested [Adlercreutz, 1990] that both lignans and isoflavones stimulate synthesis of sex hormone binding globulin (SHBG) in human liver; the SHBG increase would in turn produce a reduction of either the albumin-bound or the free fraction of sex hormones (oestradiol, testosterone), rescuing breast tissues from an exceeding stimulus of biologically available steroids. Many questions about the relationship between dietary oestrogens, their biological effects and sex steroid metabolism and function in man remain open; however, data accumulated thus far seem to reinforce the epidemiological view that western diet is one of the main factors responsible for the higher incidence of breast and other hormone-related cancers in western countries.

1.2.3. Plasma and tissue oestrogens

Bioavailable oestrogens in blood

Since methods for measuring blood oestrogens became available, much attention has been devoted to investigate their plasma levels in relation to breast cancer. In man, the most part (between 40 and 50%) of circulating E_2 is bound to SHBG with high affinity; the majority of the remainder is loosely and non-specifically bound to serum albumin, leaving a very small fraction (less than 2%) totally free of protein binding. From early studies, evaluating plasma levels of total oestrogens in breast cancer patients and healthy controls in both pre- and postmenopausal women, no clear picture emerged. In a comprehensive study, England and colleagues [England *et al.*, 1974], taking into account the ovulatory status of subjects, found only a marginally significant increase of blood E_2 in premenopausal breast cancer patients with respect to both normal women and women with benign breast disease; a large scattering of values was also encountered in cancer cases. On the other hand, Sherman *et al.* [Sherman *et al.*, 1979] failed to show any significant difference in the mean E_2 levels between controls and disease-free breast cancer patients in premenopause. Even more conflicting results have been obtained in postmenopausal women concerning blood concentrations of E_2, E_1 and E_1 sulphate (E_1S).

The development of suitable methods to isolate and estimate the different fractions of circulating oestrogens has

enabled researchers to attempt to assess the E_2 fraction which can cross plasma membranes and be available to a tissue, i.e. the "biologically active" fraction. It has been generally assumed that not only free E_2, but also some protein-bound E_2 may be accessible for tissue uptake, provided that dissociation from binding protein occurs rapidly (within seconds) in relation to the transit time of a capillary bed. However, Pardridge and colleagues [Pardridge & Landow, 1984] pointed out that dissociation constant of steroid binding to albumin measured *in vitro* is much lower than that calculated *in vivo*, i.e. steroids seem to be more loosely bound than expected from *in vitro* studies; the higher dissociation rate found *in vivo* could imply a conformational change of binding protein which facilitates the release of the ligand. Following data obtained on rat liver and brain (reviewed by [Pardridge, 1981]), it has been suggested that, as far as glandular breast tissue is concerned, also the albumin-bound E_2 fraction may well be available [Siiteri *et al.*, 1982].

Vihko and Apter [Vihko & Apter, 1986] reported that girls having early menarche show a greater increase of serum E_2 and significantly lower concentrations of SHBG with respect to other girls; this was apparent since the age of 10 and persisted up to five years after menarche. It may be that the resulting higher E_2 to SHBG ratio may account for the early appearance of menarche. However, it may also be relevant to an increased breast cancer risk in these girls.

Siiteri and associates [Siiteri *et al.*, 1981] showed that breast cancer patients have an overall increase of bioavailable E_2 in their blood, suggesting that this condition may favour breast cancer genesis. This evidence was subsequently confirmed by several case-control studies [Moore *et al.*, 1982; Reed *et al.*, 1983; Langley *et al.*, 1985; Siiteri *et al.*, 1986; Takatani *et al.*, 1987] which documented higher concentration of non-protein-bound E_2 in breast cancer with respect to normal women; however, the differences between the two groups were always very small, the case to control ratio of free E_2 ranging from 1.11 to 1.41. A comparison of British women (representing a high-risk population) with Japanese women (representing a low-risk population) revealed that the latter had higher levels of SHBG-bound E_2 (supposed to be not biologically available) than did the British counterparts [Moore *et al.*, 1983]. However, the same authors were unable to confirm this trend in five different racial groups living in Hawaii, in spite of a five-fold range of incidence rates of breast cancer [Goodman *et al.*, 1988].

In a prospective study on the Guernsey Island population, Bulbrook *et al.* collected blood samples from 5000 ostensibly healthy women over the age of 35; volunteers were therefore screened for breast cancer by palpation and mammography. Preliminary results indicated that plasma levels of both free and albumin-bound E_2 were significantly higher in subjects who subsequently developed breast cancer (named precancer cases) with respect to age-matched controls [Moore *et al.*, 1986]; in addition, SHBG amounts in precancer cases were at the lower

ends of control ranges. Unfortunately, as the study progressed, the differences between cases and controls became statistically not significant, the SHBG levels being found within the normal range. Bulbrook *et al.* [Bulbrook *et al.*, 1988] proposed that this could be a reflection of tumour growth rates, in that rapidly growing tumours, which would originate in women having lower SHBG and higher free E_2 levels, would have an earlier clinical appearance than tumours growing more slowly, which conversely associate with SHBG levels at the upper end of the normal range. Thus, the overall incidence of breast cancer is not reflected by different plasma SHBG levels, simply the age of clinical detection being younger in patients who have lower SHBG plasma levels.

Although methods to estimate SHBG are uncomplicated and reliable, many case-control studies comparing breast cancer patients and matched controls have yielded results largely at variance [Meyer *et al.*, 1986; Ota *et al.*, 1986; Takatani *et al.*, 1987]. This has been ascribed to the fact that these studies disregard tumour growth rates [Bulbrook *et al.*, 1989]. This rather simplistic interpretation neglects other potentially effective elements. In first place, it has been shown that SHBG is relatively unstable in frozen plasma samples in long-term storage conditions; this may produce a significant increment of the free E_2 fraction [Siiteri *et al.*, 1986] and may account for the extremely conflicting reports in the existing literature. Secondly, there is increasing evidence that SHBG-bound E_2 can also enter cells. Immunocytochemical studies have revealed presence of SHBG within various steroid target tissues, including prostate

[Bordin & Petra, 1980] and human breast cancer [Tardivel-Lacombe *et al.*, 1984]. Experimental observations on human prostate and decidual endometrium, using ^{125}Iodine-labelled SHBG, are suggestive for SHBG binding sites on the membrane of oestrogen target cells [Strel'chyonok *et al.*, 1984; Hryb *et al.*, 1985]. In particular, SHBG seems to bind decidua membranes only when complexed to oestradiol, but not when it is devoid of steroid or carrying testosterone [Hryb *et al.*, 1990]; this evidence has been recently confirmed in premenopausal human endometrium, where SBHG affinity for its receptor increases in presence of oestradiol and is reduced by androgens [Fortunati *et al.*, 1991]. These findings would imply that, if also SHGB-bound E_2 may be accessible to a tissue, then a decrease of SHBG levels in cancer patients will not necessarily turn in a greater biovailable E_2 fraction.

Bulbrook *et al.* [Bulbrook *et al.*, 1988], showed that levels of circulating androgens appear to be correlated to the age of breast cancer appearance. The authors postulated that androgens act as growth inhibitors; since the normal range for plasma steroids is five-fold, balance between growth inhibiting androgens and growth promoting oestrogens may comprise a ten-fold range, wherein physiological levels of each steroid may yet be important to clinical manifestations of breast cancer. However, simple measurement of plasma concentrations of steroids may be in any event misleading unless we precisely define levels of biologically available hormone; mechanisms controlling the release of both albumin- and SHBG-bound E_2 to plasma

membranes are only poorly understood and there is obvious hazard in extrapolating data coming from animal model systems to human breast. Anyway, a central question which remains to be established is to what extent hormone plasma levels reflect concentration and endocrine environment of tissues. As discussed in the successive sections, either normal or cancer target tissues appear endowed with key enzyme activities of steroid metabolism and exhibit levels of endogenous steroids which are about ten up to one hundred (or more) times higher than the respective plasma values. Therefore, increases of 0.25 to 0.65% in plasma free E_2 are highly unlikely to represent a critical factor in determining the level of bioavailable oestrogen to the tumour cells.

Oestrogens in breast tissues

A variety of steroids and their derivatives have been analytically identified and measured in both normal and cancerous human breast [Bonney *et al.*, 1983a; Vermeulen *et al.*, 1986]; they include E_2, E_1, E_1S, testosterone (T), DHA and DHAS, androstenedione (Δ^4Ad), androstenediol (Δ^5Adiol) and progesterone (Pg). Numerous investigations have shown that either concentration or profile of oestrogens in blood and breast tissues are substantially divergent in humans [van Landeghem *et al.*, 1985a; Vermeulen *et al.*, 1986]. In particular, similar concentrations of breast tissue E_2 are present in pre- and postmenopausal women, in spite of the conspicuous fall of the respective plasma levels after menopause [Thijssen *et al.*, 1986];

in addition, either pre- or postmenopausal breast tissues display E_2 concentration significantly higher than that found in plasma [van Landeghem *et al.*, 1985a]. Therefore, the lack of correlation between E_2 levels in tissue and circulation suggests that local factors are principally responsible for the oestrogen content and pattern within the breast. Selective, against-gradient uptake from blood, local biosynthesis and metabolism, or binding to low affinity-high capacity binding sites have been in turn implied.

There is evidence that a positive arteriovenous gradient across mammary cancer tissues exists for Δ^4Ad, DHA, T and Pg, whilst it is not significant for both E_2 and E_1 [Vermeulen & Deslypere, 1989]. Previous studies, however, indicated that breast tumours exert an active uptake of oestrogens from the bloodstream [Deshpande *et al.*, 1976]. James and colleagues [James *et al.*, 1987], using an elegant experimental model, demonstrated that E_1 accumulates in breast cancer tissues following simultaneous infusion of ^{14}C-E_1 and ^{3}H-Δ^4Ad immediately prior to surgery. Nevertheless, the proportion of labelled E_1 in cancer tissue which derived from Δ^4Ad (i.e. produced by the aromatase enzyme system) was extremely variable, ranging from 0% up to 89%; this would suggest that local factors may differently regulate endogenous production of oestrogens in individual tumours. Recently, Blankenstein *et al.* [Blankenstein *et al.*, 1992] have shown that the maintenance of intratumoural E_2 levels cannot be accounted for solely by a tumour-directed gradient of aromatase substrates and their precursors in fatty tissue surrounding tumour mass; the authors

concluded that mechanisms which sustain E_2 concentration in breast cancer tissues are not substrate-driven, the Δ^4A d concentration being found much lower than the reported K_m for the respective aromatase activity ($\Delta^4Ad--->E_1$). As far as intratissue E_1 levels are concerned, it has been suggested that circulating E_1S may provide a major source for E_1 accumulation in human breast. Infusion studies, using either ^3H-E_1S or E_1-^{35}S, have demonstrated that E_1S, as such, does not contribute to the E_1 content of breast tumours in postmenopausal women, unless it is earlier hydrolysed to E_1 by sulphatase enzyme [Purhoit *et al.*, 1992]. This supports the view that breast sulphatase levels may be important in determining the actual E_1 intratumour concentration.

Previous reports on populations at high incidence of breast cancer have indicated that the E_2 content of tumour tissues is higher than that found in normal breast, while this difference is less pronounced concerning E_1 [van Landeghem *et al.*, 1985a; Thijssen *et al.*, 1987]. Comparison of E_2 and E_1 concentrations in normal and malignant human breast tissues from either pre- or postmenopausal women has revealed that a significantly greater amount of E_2 is present in cancerous specimens with respect to non-malignant samples, irrespective of age and plasma levels [van Landeghem *et al.*, 1985a; Thijssen *et al.*, 1986]. In addition, a statistically significant accumulation of E_2 in oestrogen receptor (ER) positive tumours with respect to ER negative ones was observed; however, no significant correlation was found between E_2 concentration and ER levels. Therefore, it seems highly

unlikely that elevated tumour E_2 may be dependent on selective uptake from blood or binding to specific receptor sites.

More recently, Thijssen and colleagues [Thijssen *et al.*, 1990] compared E_2 and E_1 concentrations in normal and malignant breast tissues obtained from Dutch and Polish women, the latter representing a population at a relatively low incidence of breast cancer; interestingly, while E_2 levels displayed the same pattern in the two groups (significantly higher amount in tumour tissues from either pre- or postmenopausal patients than in controls), the E_1 concentration was approximately 50% lower in malignant breast tissues from Polish women with respect to Dutch women of the corresponding menopausal status. This suggests that endogenous E_1 levels may be related to breast cancer incidence, but further investigations are needed to confirm whether or not E_1 is involved in determining the different incidence of breast cancer seen in distinct geographical areas.

Overall, the experimental and clinical evidence discussed so far speaks for the apparent autonomy of human breast cancer tissues in maintaining elevated endogenous E_2 irrespective of available oestrogen in blood. On a biochemical point of view, balance between biosynthetic and catabolic processes could eventually regulate the intratumoural E_2 levels. However, this is not the whole story. Recently, Adams *et al.* [Adams *et al.*, 1988a] suggested that human breast stromal fibroblasts secrete a polypeptide which increases the conversion of E_1 into E_2 (reductive pathway) in breast epithelial cells; in addition, insulin-

like growth factors I & II (IGF-I & II) have been shown to enhance this pathway, while they do not affect the opposite direction (E_2--->E_1, oxidative pathway) in MCF7 human breast cancer cells [Singh & Reed, 1991]. These observations emphasise that local factors may modulate, in a paracrine fashion, key enzymes of steroid metabolism and therefore determine the actual amount of E_2 to which breast epithelial cells are exposed.

1.2.4. Hydroxylated oestrogens

In humans, the metabolism of E_2 is mostly oxidative, consisting of an initial conversion to E_1 through the generation of a keto function on the 17β-hydroxy group. The subsequent metabolic fate of E_2 is essentially determined by the activity of two cytochrome P450-dependent enzymes, which hydroxylate the parent oestrogen at either the C-16α or the C-2 position. Hydroxylation at the C-2 or the C-4 position leads to production of the so-called catecholoestrogens (CCE, namely 2- and 4-hydroxy-E_1 and E_2), while the C-16α reaction yields in sequence 16α-hydroxy-E_1 (16αOHE_1) and E_3. The C-2 and C-16α hydroxylations represent mutually exclusive pathways of E_2 metabolism, since it is highly unlikely that both reactions occur on the same molecule. These cytochrome P450 enzyme activities are generally unsaturated, thus any increase in one pathway will actually withdraw substrate from the other, resulting in a reduced product formation by the competed pathway.

Although CCE formation is commonly believed to be a terminal metabolic step, yielding biologically inactive

metabolites, several pieces of evidence suggests that this is not so. In first place, early studies suggested that 2-hydroxylated oestrogens may be potentially important for catecholaminergic function in the brain [Fishman & Norton, 1975]; secondly, there is convincing evidence that 4-hydroxylated metabolites may behave as long-acting oestrogens [Weisz, 1991]. The assumption that 2-hydroxylation produces peripherally inactive, or mildly anti-oestrogenic derivatives, is based mainly on studies where cultured cells are exposed to high concentrations of CCE under conditions which favour their oxidative degradation and induce cell damage [Schneider *et al.*, 1984]. However, recent in vivo studies have supported the anti-oestrogenic effect of $2OHE_2$ [Michnovicz *et al.*, 1986; Galbraith & Michnovicz, 1989]. On the other hand, $16\alpha OHE_1$ has been shown to be an exceptionally potent uterotrophic agent with respect to the same E_2 [Martucci & Fishman, 1979]. This ketol has an extremely low affinity for SHBG; thus, although circulating levels of $16\alpha OHE_1$ in man are low, its biological impact may be exaggerated by a larger availability to target tissues. A unique property of $16\alpha OHE_1$ is the ability of covalently binding to primary aminogroups of proteins [Yu & Fishman, 1985]; unlike all 17-keto oestrogens, which form only transient Schiff bases with aminogroups of various proteins, $16\alpha OHE_1$ spontaneously undergoes a subsequent non-enzymatic Heyns rearrangement, yielding a 16-keto-17-amino derivative which covalently binds proteins. The driving force of this rearrangement lies in the generation of a 16-keto function instead of the one 17-keto, indicating that $16\alpha OHE_1$ and its more

labile epimer 16βOHE$_1$ are the sole natural oestrogens with the potential of forming covalent bonds; 16βOHE$_1$, however, is rapidly transformed in 17-ketoE$_2$ which is unlikely to survive intact the Schiff base formation, thus leaving 16αOHE$_1$ as the only oestrogen metabolite capable of covalently binding proteins by this route. Ultimately, since 2- and 16α-hydroxylation represent two opposing, mutually exclusive pathways, a relative change in one with respect to the other would shift the oestrogen balance within a given target tissue toward either a proestrogenic or an antioestrogenic state.

Several investigations have indicated that 2-hydroxylase activity can easily be modified by a variety of stimuli, in humans; by contrast, 16α-hydroxylation appears to be refractory to most influencing factors in many experimental conditions. In an elegant model, using E$_2$ specifically labelled at the C-2, C-16α, or C-17α position, Schneider *et al.* [Schneider *et al.*, 1982] assessed the extent of both reactions in breast cancer patients and matched controls by measuring the release of ^{3}H into the body water pool. Interestingly, 16α-hydroxylation exhibited significantly higher (50%) values in patients with respect to controls; this evidence could not be biased by menopausal status of subjects since the overall metabolic pattern was previously found to be time invariant [Fishman *et al.*, 1980]. However, in order to exclude the possibility that this difference could be a consequence of tumour formation, the authors extended these studies to women at high familial risk of breast cancer. Women at high breast cancer risk displayed a significant increase in the

extent of 16α-hydroxylation with respect to matched subject at normal risk [Osborne *et al.*, 1988]. In support to this observation, studies using mice of different strains, all aged 6-10 weeks (long before the spontaneous appearance of tumours), showed that the percent of 16α-hydroxylation was closely related to the incidence of subsequent mammary tumours. In particular, strains such as the C3H/OuJ and the RIII, having a cancer incidence which approximates 100%, displayed 20% extent of 16α-hydroxylation, while in strains like the C57Br/Cd7, at zero incidence of mammary tumours, the percent of the reaction was much lower (below 4%). In this respect, suitable cross-nurturing studies have explored the relationship existing between the MMTV and 16α-hydroxylation in the aforementioned mice strains. These studies showed that the extent of the reaction dropped by half in strains whose MMTV-positive status was reversed to negative by appropriate foster nurturing from birth; on the contrary, normally MMTV-negative C57Br/Cd7 strain, made MMTV-positive by nurturing on C3H/OuJ mothers, exhibited a significantly increased 16α-hydroxylation [Bradlow *et al.*, 1985].

Complements to this literature come from tissue culture studies on the terminal duct lobular units (TDLU) [Telang *et al.*, 1989]; the latter have been reported to be the morpho-functional site where mammary tumours originate. In these studies TDLU were isolated from areas adjacent to breast tumours (TDLU-H) and from tissues of reductive mammoplasty (TDLU-L); in addition, organ cultures of terminal end buds (TEB) from mice strains at different incidence of breast cancer were also

investigated. Measurement of either 2- and 16α-hydroxylation or ras oncogene p21 expression revealed higher levels of both 16α-hydroxylation and ras oncogene in TDLU-H and in TEB from mice strains at high breast cancer risk with respect to TDLU-L and TEB from strains at low incidence of mammary tumours.

All this body of evidence provided a stimulating background for development of preventive strategies based on dietary or pharmacological reduction of breast cancer risk. Although 16α-hydroxylation behaves in a constitutive fashion, being commonly refractory to alteration, it seems reasonable that a significant enhancement of 2-hydroxylation, shunting the shared substrate (E_1) away from the production of both 16αOHE_1 and E_3, would be biologically equivalent to a decrease of 16α-hydroxylation. Numerous factors have been shown to markedly increase 2-hydroxylation in man; they include cigarette smoking, exercise, drugs like cimetidine, different dietary habits (high protein and low fat diets, fish oil diet). Unfortunately, none of them suits their clinical use as preventive agents against breast cancer. Recently, however, Bradlow *et al.* [Bradlow *et al.*, 1991] have suggested that the indole-3-carbinol (I3C), which is present in cabbages and in other vegetables of the cruciferous family, could represent an appropriate candidate for this kind of intervention manoeuvre. Preliminary results obtained either in vitro, on MCF7 human breast cancer cells, or in vivo, on both mice and human volunteers, indicated that I3C induces a two to four-fold increase of 2-hydroxylation which is

sufficient to decrease formation of 16α-hydroxylated metabolites.

Overall, this CCE hypothesis has not yet encountered general consensus, nor has it been fully defined. However, it draws attention to the importance of tissue specific processing of oestrogens for local potentiation and diversification of their function and yields a possible new approach to prevention of breast cancer.

1.2.5. Exogenous oestrogens

Oral contraception

Combined estro-progestogen oral contraceptives (OC) were first marketed in Britain in 1960, consisting of early preparations at relatively high hormone dosage; over the subsequent decades, noticeable changes in the OC formulation have occurred, reducing the steroid dose to as little as 20 μg oestrogen or as little as 50 μg of a new generation of progestagens having higher bio-potency and reduced affinity for androgen receptors (AR). The increasingly large number of women assuming OC steroids, combined with the conviction that steroids provoke or promote abnormal breast growth, has provided for years a source of major concern to such a point that this has become one of the most investigated areas of epidemiology. Since the early eighties, results of several case-control and cohort studies have been reported (for a review see [Vessey, 1988]). Firstly, the Royal College of General Practitioners (RCGP) and the Oxford Family Planning Association (OFPA) cohort studies, respectively

involving the follow-up of 23,000 and 9,653 women using OC, have indicated no significant differences in breast cancer incidence between users and non-users [Royal College of General Practitioners, 1974; Vessey *et al.*, 1977]; the same evidence came from the Walnut Creek and the US Nurses Health studies [Ramcharan *et al.*, 1980]. However, these early investigations primarily included married women who used OC mainly to space out their children; this could overlook any excess risk of breast cancer linked to prolonged use of OC in younger women delaying their first pregnancy. Since 1979, when this possibility was initially suggested, 12 case-control studies on the use of OC in young women have been published; most of them concentrated on OC use before the first term birth (BFTB) or before age 25 years. Some studies [Pike *et al.*, 1983; Meirik *et al.*, 1986; McPherson *et al.*, 1987] reported an increased risk of breast cancer in early OC users, while other studies [Stadel *et al.*, 1985; Paul *et al.*, 1986] did not. Reasons for these conflicting findings may be many-sided. In first place, two major sources of bias (reviewed by [Skegg, 1988]) should be mentioned: 1) women having breast cancer may recollect their OC use better than controls; 2) women taking the pill would experience a more careful breast surveillance than non-users, leading to an earlier tumour detection or even to discovery of cancers that would otherwise have remained hidden. Additionally, the wide range of pilss taken, in terms of both ratio and overall dose of oestrogens and progestins, provide confounding elements. Subsequent large studies, however, have all contradicted that OC may increase

breast cancer risk, even in long-term users. In particular, the Cancer and Steroid Hormone study , from the Centers for Disease Control (CDC) in Atlanta, has emphasised that there was no increased risk in women before the age of 20 or 25; this was true even in those using OC for more than 15 years, irrespective of the hormone dose or type of preparation [The Cancer and Steroid Hormone Study, 1986]. Further, more recent results from the WHO [WHO Collaborative Study of Neoplasia and Steroid Contraceptives, 1990] and the UK national case-control [UK National Case-Control Study Group, 1989] studies, and the review and meta-analysis of the existing data [Romieu *et al.*, 1990], continue to be inconsistent. Although this evidence is very reassuring, there still is the possibility that a potential risk of breast cancer in young OC users may persist into older age, when the disease becomes more prevalent; more, a long, as yet unobserved, latent effect may well be involved, bearing in mind the long dormant phase of human breast cancer. Therefore, data in the next two decades are awaited with interest to ascertain whether or not OC use may have a lasting adverse influence on breast cancer risk, especially in women aged over 45 years.

Hormone replacement therapy

Hormone replacement therapy (HRT) is commonly used to relieve the symptoms of the menopause and, given for much longer periods, to offer a clinically useful degree of cardiovascular protection and to prevent or limit bone loss. In the United States, HRT has become very popular since 1960s; suffice it to say that

in 1974 the widely used Premarin®, a conjugated equine oestrogen preparation, was the fourth most frequently prescribed drug in the country [Kennedy *et al.*, 1985]. Considering that the number of postmenopausal women in our society is vast and that a considerable proportion of these women will seek the benefits of the oestrogen replacement therapy, it is an obvious, basic concern to determine the potential risk of breast cancer in these subjects; however, due to the magnitude of the problem, even a small increase in risk will actually produce several thousands of women dying of breast cancer. Therefore, it seems essential to provide a highly accurate measure of breast cancer risk in women receiving HRT. Unfortunately, this can only assessed through observational studies, but the fact that the risk extent (if any) is highly likely to be small makes this task very difficult to achieve.

Over the past fifteen years, 21 case-control studies and 10 cohort studies have tried to weigh breast cancer risk in relation to HRT (for a review see [Mann, 1992]). Overall, no sign of a dramatic increase of risk attributable to HRT use has been reported; none the less, variation in the relative risks observed is markedly large, much greater than the expected divergence among different studies. This could be the outcome of several bias factors, including preceding differences in breast cancer risk or diverse therapeutic regimens used in these studies. It is worth mentioning that only a small proportion of women were followed for more than 10 years and that differences in duration of therapy, dosage and type of preparation used may account at

least for some of the conflicting results obtained thus far. Some reports have shown a consistent and significant trend of parallel increase in relative breast cancer risk (up to 2.32) and duration of treatment (up to 20 years or more) [Brinton *et al.*, 1986; Ewertz, 1988; Bergkvist *et al.*, 1989a]; other case-control studies, however, have failed to find such an association [La Vecchia *et al.*, 1986; Nomura *et al.*, 1986; Colditz *et al.*, 1990]. The reason for this distinction is mostly unknown, but a likely explanation resides in the criteria used for selecting appropriate control groups; the latter, in fact, essentially fall in two general categories: hospitalised patients and healthy population controls. The finding that hospital-based studies could not find any risk increase, while the studies using healthy controls did find, would suggest that using hospitalised subjects, which are more likely to be prescribed elective drugs for lengthy periods, may cause an underestimate of the true relative risk. Concerning dose of HRT regimens, women receiving less than 0.625 mg/day of conjugated oestrogens did not show any appreciable increase of breast cancer risk; use of higher doses ($\geq$1.25 mg/day), however, was found to associate to a rise, although small ($\leq$2.0), of the relative risk [Dupont & Page, 1991a]. Interestingly, two out of the three studies which found a significantly increased risk in long-term HRT users were European; this appears of importance in view of the fact that oestradiol compounds are far more commonly used in Europe than in North America, where conjugated oestrogens are widespread.

In the attempt to better explore the likelihood that HRT use adversely affects breast cancer risk, a limited number of meta-analyses have also been reported. This approach, which combines risk estimates from different studies into a single one, has however the inconvenience of being unable to deem properly the methodological strengths and shortcomings of individual studies. The meta-analysis carried out by the CDC in Atlanta concluded that, across all studies involved, there is evidence of a duration-related increase of breast cancer risk in women who used HRT, but that this increase is limited (10% for each 5 years treatment) [Steinberg *et al.*, 1991]; Dupont and Page [Dupont & Page, 1991b], in another meta-analysis, found an even smaller risk increase.

Recently, attention has been drawn also on the effects of HRT on breast cancer mortality, rather than on incidence. Based on the Swedish cohort study of Bergkvist and coworkers [Bergkvist *et al.*, 1989a; Bergkvist *et al.*, 1989b], Ross and Bernstein [Ross & Bernstein, 1991] have estimated that, up to 6 years of treatment, mortality rates of breast cancer in HRT users and non-users are comparable, but thereafter (8-10 years use) the mortality gradually increases in users, attaining an overall excess of 40%. This evidence may seriously alarm women against HRT use, but it should be kept in a more appropriate context. Ross and associates [Ross *et al.*, 1989], using a simple risk-benefit mortality model for HRT, indicated that over 100,000 women using conjugated equine oestrogens for more than 10 years, the net expected effect is to have 300 fewer deaths; this represents an approximately 17% reduction in the annual mortality from all

causes. The authors concluded that, in terms of mortality rates, the overall risk-benefit equation balances strongly in favour of benefit.

Although the balance of the evidence, coming from the plethora of reports, is rather reassuring, there is a need for definitive studies to precisely measure the risks of contemporary HRT preparations, instead of chaotic data collection which merely serve to leave us puzzled!

1.3. Androgens and Prostate Cancer

Over the last 50 years the endocrinology of the human prostate has been dominated by the belief that development, growth and maintenance of morphology and functional activity of the normal adult gland are largely dependent upon testicular secretion of testosterone, the main circulating androgen in man. This concept essentially matured from the original observation on androgen-dependence of human prostate cancer, that is to say that, as often happens in science, knowledge of physiological processes may well be refined by learning more on pathological condition.

Although the use of orchidectomy as palliative measure against severe cases of enlarged prostates was first suggested in the last decade of the nineteenth century [White, 1895; Cabot, 1896], the cornerstone of our understanding of prostatic cancer is represented by the work of Dr. Charles Huggins and his colleagues [Huggins & Hodges, 1941; Huggins *et al.*, 1941]; these

surgeons initially established the benefits of androgen withdrawal in patients having advanced prostatic carcinoma. Since then, several other endocrine manoeuvres, including treatment with oestrogens, progestins, antiandrogens or luteinizing hormone releasing hormone (LHRH) superagonists, have been in turn proposed to achieve a decline of androgenic stimuli. Notwithstanding, after an initial objective response rate of 80-90%, patients experiencing these hormonal manipulations fail to respond any further and relapse within one or two year, with a median survival of six months after first recurrence [Whitmore, 1973; Lepor *et al.*, 1982]. It is therefore frustrating to notice that prognosis for prostate cancer patients is not fundamentally changed from when Maximilian Bruch commented, one and half a century ago, that the disease was "serious, of bad prognosis, commonly lethal and rarely cured, even with excision" and also observed that "the cure employed is palliative" [Manci & Gardner, 1986]. It is now clear that factors other than androgens are likely to be involved in the regulation of abnormal prostatic growth and that the "micromilieu" which hosts both epithelial and stromal prostate cells could represent a crucial site where steroid hormones and peptide growth factors may directly and reciprocally influence tumour development and growth.

1.3.1. Carcinogenic role of androgens

The potential role of androgens in carcinogenesis of the human prostate has been extensively investigated. Although

development of prostatic cancer has been classically viewed as a multi-step process, involving progression from low histologic grade, small, latent carcinoma towards large, higher grade, metastasising carcinoma, recent experimental evidence suggests that a variety of pathogenetic pathways may exist [Bosland, 1992].

Different rat strains have been used as fruitful model systems to explore the possible contribution of steroid hormones (especially androgens and oestrogens) to development of prostate tumours. A significant association between androgen administration and tumour formation has been revealed in Noble (NBL) rats [Noble, 1977], in AXC [Shain *et al.*, 1975], Wistar (W) [Bosland *et al.*, 1983] and Lobund-Wistar (L-W) [Pollard *et al.*, 1982] rats. In the latter, for instance, incidence of spontaneous prostate cancer has been reported to be partly enhanced by testosterone (T), but highly significantly increased when rats were treated with T following a single-dose injection of N-nitrosomethylurea (NMNU) [Pollard & Luckert, 1986]. This finding has been confirmed in W rats, where a single injection of a prostatic carcinogen, such as 3,2'-dimethyl-4-aminobiphenyl (DMAB), DMBA or NMNU, given after sequential treatment with cyproterone acetate and testosterone propionate, produced adenocarcinomas of the dorsolateral region (DLP) of the prostate gland [Bosland *et al.*, 1990]. Previous reports [Pour & Stepan, 1987] indicated that continuous administration of testosterone to W-derived MRC rats, following treatment with the carcinogen N-nitrosobis(2-oxopropyl)amine (BOP), induced a high proportion

of prostatic carcinoma (over 60%) in the DLP, regardless of whether the rats were orchiectomized or not; by contrast, using short-term administration of testosterone, a significantly lower incidence of tumours was observed. On this basis, the authors concluded that testosterone may play a role in the initiation of prostatic carcinogenesis, whereas the promotional phase is likely to be governed by interaction of androgen with other factors; this conjecture, however, appears to be rather speculative in the view that many variables (strain of rats, length and type of exposure to both carcinogen(s) and hormone, dosage and route of administration, etc) may critically affect the end result in these experimental models. In this respect, trenbolone (a synthetic androgen) and testosterone have been found to have only a weak promoting potential on Syrian hamster embryo cells or even an anti-transforming effect when associated with DMBA [Lasne *et al.*, 1990]. Furthermore, other studies have failed to see any prostatic carcinoma in castrated F344 rats after administration of DMAB during peak cell proliferation induced by dietary methyltestosterone [Shirai *et al.*, 1987]. The same authors observed that when intact F344 rats were treated with DMAB and ethinyl oestradiol instead of methyltestosterone, a large proportion (85.7%) of animals displayed prostatic tumours, while this occurred in only 5.2% of rats receiving DMAB alone [Shirai *et al.*, 1988]. This evidence is also supported by the observation of el Abed and colleagues [el Abed *et al.*, 1987] on Sprague-Dawley rat strain sensitive to DMBA treatment; the authors, measuring serum levels of testosterone, androstenedione, progesterone and

its 17-hydroxy derivative, E_2 and E_1 after DMBA treatment, found that the preovulatory surge of both oestrogens on proestrus days was significantly increased, while no such stimulation was seen for any other steroid at any time of the oestrus cycle. Another confirmation comes from Bosland [Bosland, 1992] who found that chronic administration of E_2 to rats in combination with low-dose testosterone results in development of low-grade prostate carcinomas which exclusively originate from the dorsolateral and the anterior prostate. This evidence has also been strengthened by the previous finding that high incidence rates of prostate adenocarcinoma can be obtained in NBL rats treated for 18 months or longer with silastic implants of testosterone and oestrogens [Drago, 1984]. Most interestingly, Pollard and colleagues [Pollard *et al.*, 1987] investigated the individual role of testosterone and DHT as tumour promoting agents in prostate cancer-susceptible L-W rats. The authors found that 3 month old rats, treated for 14 months with subcutaneous depots of testosterone or DHT, only testosterone administration produced a 24% incidence of cancer, while no tumour formation was observed in DHT-treated rats; in the latter the significant reduction in weight of testes, the dramatic fall of plasmatic testosterone and the absence of spermatogenesis, all suggested an antiandrogenic effect of DHT. More recently, Leav *et al.* [Leav *et al.*, 1989] compared cell proliferation and histology of both ventral (VP) and DLP prostate lobes in intact NBL rats after testosterone or DHT were administered separately or in combination with various oestrogens for 16 weeks. Using a

stathmokinetic in vivo metaphase-arrest technique, the authors showed that combined treatment of rats with testosterone and E_2 produced florid dysplasia and markedly increased the mitotic index of the DLP region; by contrast, either joint administration of DHT and oestrogens or treatment with T or DHT alone respectively provoked only mild proliferative lesions in DLP or simply maintained the morphological integrity of both VP and DLP regions. It was concluded that protracted androgen-supported oestrogen stimulation of DLP appears to be essential to achieve an important increment of the overall proliferative activity in this prostate area.

Although the prostate gland is commonly considered as the prototypic androgen-dependent tissue, there is convincing evidence that oestrogens *per se* may induce division and differentiation of prostatic epithelial cells in many species [Leav *et al.*, 1978; Schulze & Barrack, 1987]. In addition, presence of oestradiol binding sites has been detected in both rat [Swaneck *et al.*, 1982; Mobbs & Johnson, 1986] and human [Donnelly *et al.*, 1983; Ekman *et al.*, 1983] prostate. Keeping with the above indications, it would seem reasonable, as the present thesis will also suggest, to reconsider a major role for oestrogens in both normal and malignant prostate growth.

1.3.2. Analytical epidemiology

While several risk factors for breast cancer have been clearly identified and, at least partly, weighed, the same does not apply to cancer of the human prostate; results from either case-

control or cohort studies are often confusing and contradictory. Some factors, such as urbanisation, family history and marital status show only a weak relationship with risk of prostate cancer, while several others, including socioeconomic status, sexual and social habits, religion, are inconsistent in this respect (for a review see [Boyle & Zaridze, 1993]). However, some epidemiological information appears to be well established.

In the first place, prostate cancer is a disease of the older age; its incidence significantly increases with age in a virtually logarithmic fashion. This is consistently true, regardless of race or geographical area. The finding that both prostatic carcinoma and benign prostatic hyperplasia (BPH) are conditions rarely present in men under the age of 50, has stimulated researchers to investigate the possible etiological relationship between the two diseases. In particular, the fact that nodular hyperplasia was frequently associated with foci of prostate cancer in autoptic material, eventually led Armenian and his colleagues [Armenian *et al.*, 1974] to consider the likelihood that BPH may be a risk factor for the subsequent development of malignancy. However, there is an overall consensus that BPH does not represent a premalignant condition and that prostatic cancer and BPH do not have a common aetiology [Greenwald *et al.*, 1974], being derived from separate regions of the prostate gland which are thought to be governed by a totally different hormonal milieu. BPH in fact develops from the periurethral glands pertaining to the upper segment of the urethra above the verumontanum [McNeal, 1972;

Blacklock & Bouskill, 1977], whereas prostatic carcinoma arises in the peripheral zone of the "true" prostate [McNeal, 1975].

Secondly, there is a one hundred-fold variation in incidence of prostatic cancer throughout the world, the highest incidence occurring in Scandinavia and North America, the lowest in Asian countries; the age-adjusted rate in various parts of the United States is 10 to 30 times higher than in Japan and up to 125 times that in China [Skeet, 1976; Miller, 1988]. Additionally, a striking difference in both incidence and mortality rates of prostate cancer exists between the black and the white populations of the United States [Muir *et al.*, 1988]. The reasons for these racial and geographical differences remain today largely unknown, but it is clear that environmental or dietary factors may be aetiologically important. Migration studies have indicated that mortality rates for prostate cancer in Japanese who become domiciled in United States increases to half that of the indigenous American people; the age standardised mortality ratio of white Americans to Japanese, which normally is 10:1, gradually decreases to 5:1 in the first generation and to 1.4:1 in the second [Haenzel & Kurihara, 1968]. This evidence emphasises the relevance of environmental as opposed to racial factors in the aetiology of human prostatic tumours.

Studies on the relationship between diet and risk for prostate cancer appear of special interest, since nutrition may well influence the hormone balance, especially of steroids. The finding that prostate cancer occurs at higher frequency in Western countries, where there is the greatest *per capita* food

consumption, suggests an association between calorie intake and cancer development [Howell, 1974]. In particular, populations at high risk of prostate cancer show a higher proportion of animal fat intake with respect to those at low risk [Armstrong & Doll, 1975]; furthermore, a positive association between relative risk and fat consumption has been observed in different ethnic groups of Hawaii, having different incidence of prostate cancer [Heshmat *et al.*, 1985]. In a prospective study on Japanese men, a significant increase of age standardised death rate of prostatic cancer (from 4.9 up to 11.8) was found in subjects at diet rich in milk, meat and eggs, as opposed to a low intake of green and yellow vegetables [Hirayama, 1979]; the author suggested that vitamin A, present in the vegetables, may confer some degree of protection from development of prostate cancer. It is worth mentioning here that incidence of prostate cancer in Japan is today markedly increasing, approaching that of United States and Western Europe; this has been ascribed to the Westernization of Japanese dietary patterns. In a recent case controlled study on one hundred Japanese men with prostate cancer, Oishi *et al.* [Oishi *et al.*, 1988] have revealed that low daily intake of betacarotene and vitamin A is associated with an increased risk (respectively, 2.13 and 1.94); the same is true for frequent intake of bread (2.4 relative risk), which is considered typical of Westernized diets.

Although it has been reported that diets relatively high in meat and fat intake produce an increase of plasma androgens, it is still unclear whether or not different dietary habits may

directly affect the hormonal micromilieu of steroid target tissues, such as prostate.

1.3.3. Circulating androgens

If the potential role of plasma oestrogens in breast cancer development is still unclear, an even greater uncertainty applies to circulating androgens and carcinoma of the prostate.

As for oestrogens, the free fraction of plasmatic androgens represents only a minor proportion (less than 3%) of the total, while a 30 to 40% is bound to albumin and the remaining to SHBG [Vermeulen *et al.*, 1971]; the latter has a much higher affinity for testosterone than for oestradiol, but only a limited capacity of steroid binding [Vermeulen & Verdonck, 1968].

Measurements of serum concentration of testosterone have failed to demonstrate convincing abnormalities in prostate cancer patients [British Prostate Study Group, 1979; Ghanadian, 1982]. This riddle is yet complicated by the fact that plasmatic testosterone is mostly protein-bound [Anderson, 1974] and that its concentration is age-dependent [Vermeulen *et al.*, 1972]; therefore, studies comparing prostate cancer patients and healthy subjects should combine measurement of the free fraction of serum testosterone and careful age matching. However, there is suggestion that age-related variations in both free and protein-bound plasma testosterone may well be variable in different groups of subjects, in such a way that parallel studies, even after proper age matching, could lead to largely conflicting data [Levell *et al.*, 1985].

Various studies have been focusing on plasma concentrations of sex steroids, especially testosterone, comparing healthy subjects with either BPH or prostate cancer patients. Rannikko and Adlercreutz [Rannikko & Adlercreutz, 1983] found that serum levels of free E_2 are lower in men having prostatic carcinoma and in young controls with respect to BPH patients, while the mean free T was significantly higher in controls than in the other two groups. The authors suggested that the observed age-dependent increase of plasma E_2 and the resulting higher E_2 to free testosterone ratio in men with BPH may play a protective role against prostate cancer. On the other hand, Hulka *et al.* [Hulka *et al.*, 1987] interpreted the modest depression of serum testosterone and E_2 found in prostate cancer patients as a result of the malignant process rather than a favouring condition. The apparent paradox of men with prostatic carcinoma having relatively low rather than high plasma testosterone concentration has been also supported using isotope dilution techniques [Meikle *et al.*, 1987]; measurements of plasma androgens, oestrogens, SHBG, follicle-stimulating hormone (FSH) and luteinising hormone (LH) showed no significant differences between prostate cancer patients, their brothers and unrelated controls.

As mentioned above, it is known that black Americans have the highest rate of prostate cancer throughout the world [Muir *et al.*, 1988]. The finding that the 2:1 black-to-white ratio in prostate cancer incidence becomes evident around the age 45, namely at the earliest appearance of prostatic carcinoma, apparently implies that factors responsible for this difference

may act early in life. To test this presumption, Ross and colleagues [Ross *et al.*, 1986] embarked on a study of white and black college students in Los Angeles, looking at both total and free serum testosterone in matched subjects. The authors found that adjusted levels of the total and the free fraction of plasma testosterone were higher (15% and 13%, respectively) in blacks than in whites. However, the percent of free testosterone increased only by 0.08% (from 2.50% to 2.58%) in the black population; in addition, when age was included in the multivariate analysis, the mean serum testosterone was virtually the same in the two groups. It was concluded that the 15% increase of circulating testosterone found in the black population could account for the 2:1 ratio in prostate cancer incidence between blacks and whites, provided that this increase persists with ageing. However, several arguments speak against this implication. In the first place, there is evidence that the age-dependent rise of serum testosterone in prostate cancer patients may be due to an increase of the protein-bound component [Zumoff *et al.*, 1982]. Secondly, more recent studies have revealed no significant difference in serum testosterone concentrations between young adult Japanese (who represent a low-risk population) and young adult American whites and blacks [Ross *et al.*, 1992]. Similarly, a population-based nested case-control study on prediagnostic serum levels of both androgens and oestrogens, showed no statistically significant difference in hormone concentration of subjects who subsequently developed

prostate cancer with respect to healthy controls, matched for age and race [Hsing & Comstock, 1993].

In conclusion, no definitive evidence for a principal role of circulating androgens in prostate cancer has been so far provided. In this context, it should be pointed out that plasmatic levels of androgens may not reflect those of biologically active androgen metabolites within prostatic tissues; more, the contribution of androgens to oestrogen action or their role as oestrogen precursors in both normal or diseased human prostate gland should also be carefully considered.

1.3.4. Prostatic androgens

It is generally agreed that androgenic stimulation of prostate target cells is essentially exerted by DHT; the latter originates from the conversion of testosterone through a 5α-reductase enzyme activity. Although one could expect that the intracellular concentration of DHT may primarily depend on blood supply of circulating testosterone, there is a persuasive argument that plasma androgens do not substantially determine the intraprostatic steroid patterns; in fact, no correlation has been found between plasma and tissue levels of any particular steroid, neither in malignant nor in benign human prostate [Klein *et al.*, 1991]. This evidence emphasises the pivotal role of local factors in regulation of intratissue levels of androgens, particularly of DHT; they include activity of key enzyme systems and content of specific, high-affinity sites of androgen binding.

Formation, accumulation and deactivation of intraprostatic DHT are mainly governed by the balance between the 5α-reductase enzyme, which presides over the conversion of testosterone to DHT, and the 3α/3β-hydroxysteroid dehydrogenase (3α/3β-HSD), which directs the generation of 3α/3β-androstanediols (3α/3β-diols). In the regular prostate the mean levels of testosterone, DHT and 3α-diol are reported to be 0.2, 1.6 and 1.7 ng/gr of tissue, respectively.

Early studies, aiming to ascertain differences of intraprostatic androgens between normal and hyperplastic prostate tissues, suggested that androgen metabolic profiles are not dissimilar in the two groups [Morfin *et al.*, 1978]. However, significantly increased levels of DHT in benign with respect to normal specimens were initially reported [Siiteri & Wilson, 1970]; this finding was subsequently confirmed by others [Geller *et al.*, 1976; Hammond, 1978]. In addition, formation of 3α/3β-diols appeared to be remarkably reduced in prostatic hyperplasia, the ratio of DHT to 3α/3β-diols being significantly increased compared to normal tissues [Bruchovsky & Lieskovsky, 1979; Krieg *et al.*, 1979]; this evidence substantiated the hypothesis that the higher amounts of DHT found in BPH may be a consequence not only of an altered 5α-reductase activity, but also of an increase in DHT reconversion from 3α/3β-diols. This, however, has not been confirmed by others, who found that the concentration of 3α-diol is not reduced in BPH tissues [Bartsch *et al.*, 1990]. A major concern which has often hampered BPH studies is the difficulty to obtain normal prostatic samples for

comparison with hyperplastic tissues. Walsh and colleagues [Walsh *et al.*, 1983] indicated that DHT levels in human BPH are not supranormal provided that specimens are obtained by surgery; the authors observed that DHT concentration in normal prostatic tissue removed at autopsy, as done in the previous studies, is five times less than that present in normal prostates obtained from patients who underwent surgery for bladder cancer. Therefore, it was concluded that use of autoptic material as a source of regular prostate specimens could seriously abridge the accuracy of androgen estimate and therefore entangle the value of this observation. This, however, does not entirely contradict the "DHT" theory or the more recent "oestrogen-androgen imbalance" theory, which still maintain convincing grounds to be further explored.

Concerning prostatic carcinoma, comparisons of steroid concentrations in noncancerous prostates did not show any systematic cancer-associated changes [Klein *et al.*, 1991]. Although a significant decrease of 5α-reductase activity has been previously reported, leading, as expected, to significantly increased tissue levels of testosterone, DHT levels appeared to be not reduced in the malignant prostate [Bartsch *et al.*, 1990; Klein *et al.*, 1991]. This evidence induced Geller and coworkers [Geller *et al.*, 1984] to postulate that, in spite of a lower 5α-reductase, DHT accumulation may still occur in prostate cancer tissues mainly because of nuclear AR binding; the authors found that DHT tissue content is related to both degree of differentiation of the tumour tissue and duration of patients' response to endocrine

therapy, thus representing an useful pointer of the androgen-responsiveness of a given prostatic carcinoma. It would imply that quantitatively unchanged and functionally intact AR are present in prostate cancer tissues, but this has been not unequivocally demonstrated. Some studies have failed to find such an association between histology and AR content of prostate cancer [Trachtenberg *et al.*, 1982; van Aubel *et al.*, 1985], even though nuclear AR have been proposed as a better prognostic indicator [Ghanadian *et al.*, 1981]. Several methodological and biological elements may critically influence the results of AR assay in prostatic tissues and therefore jeopardise any clear interpretation of the existing data. However, since intraprostatic DHT concentration is far exceeding (10 to 20 times) that of AR, it seems unlikely that nuclear binding alone may account for DHT accumulation in prostate tumours. In this respect, other local non-steroidal components, including peptide growth factors and stromal contribution, could act in combination with enzymatic activities and protein binding to generate the actual DHT amount within individual prostates.

2. Steroid Function in Target Tissues and Cells

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2.1. The Steroid Receptor Superfamily: Structure and Function

Our knowledge on molecular mechanisms of steroid hormone action has been enormously expanding in the last 20 years, especially since molecular biology techniques became available. This led to the description of a wide family of receptors, called the steroid receptor superfamily, where steroid receptor proteins represent only a moiety of a much larger group of distinct molecules. Therefore, it would seem to be more appropriate to term them *nuclear receptor family*, to indicate that they primarily act via nuclear events. This family in fact includes also the receptors for thyroid hormone T_3, vitamin D_3, retinoic acid and oncogenes, such as *v-erb* A. The main implications concerning structure and function of this class of regulatory proteins have been recently surveyed by two exemplary reviews [O'Malley, 1990; King, 1992].

2.1.1. The domain structure of receptors

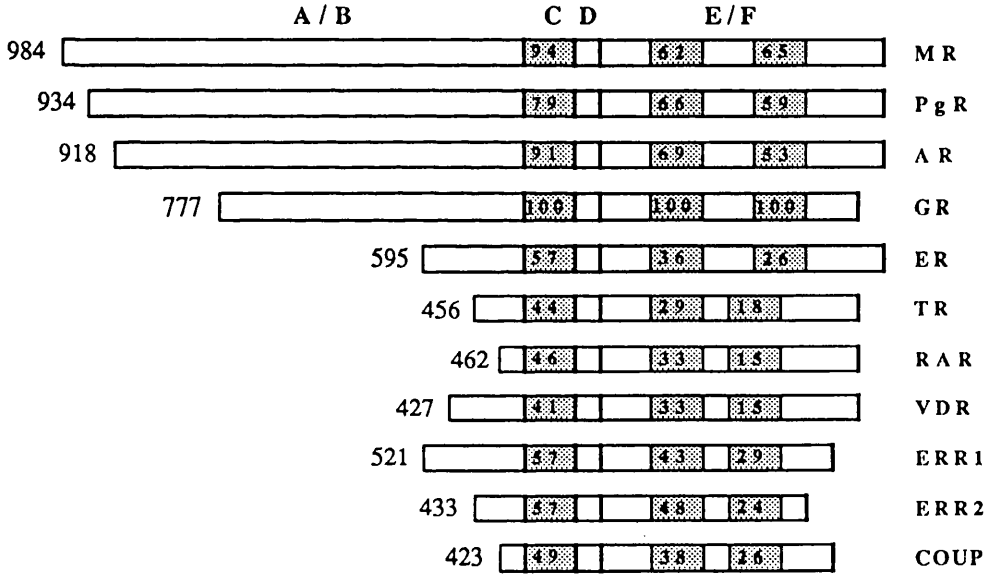
As full cDNAs for all major steroid receptors have been cloned and sequenced, it has been possible to precisely define their structure. Based on the cDNA data, computer comparisons of amino acid sequences showed that several receptor proteins share regions of close homology, while other regions reveal

striking differences. On the other hand, mutational analysis, which tests function of genetically altered receptor proteins, allowed separate functions to be assigned to specific regions (domains) of individual molecules. To date, four major domains (steroid binding, DNA binding, nuclear localisation and transactivation domains) are commonly identified, having some degree of functional overlap; a schematic presentation of the receptor domain structure is reported in Figure 1.

Steroid binding domain

This fairly large domain is located at the C terminus of the receptor molecule. Although some single amino acid changes may produce a variable loss of binding [Carson-Jurica *et al.*, 1990; Danielson, 1991], the highly conserved structure of this region suggests that it must remain intact for high affinity ligand binding. It is known that androgen, progestin, glucocorticoid and mineralcorticoid receptors have a closer ligand specificity than the oestrogen receptors (ER); this is reflected in the higher degree of homology present within the former group as opposed to that existing between the whole group and the ER. It is singular that so far the precise role of ligand in the overall steroid receptor pathway is still unclear. Earlier studies have indicated specific structural-based properties of ligand which seem to be required for optimal binding and biological activity [King & Mainwaring, 1974]. However, there is evidence to support the concept that the receptor protein contains a pocket which is suited to accommodate the ligand; it is thought that steroid

Figure 1. The nuclear receptor superfamily. Schematic representation of the domain structure (A/B to E/F) of steroid receptors and other related proteins. The boxed numbers indicate the percent of homology in both the DNA-binding (C) and the ligand-binding (E/F) domains using the human glucocorticoid receptor as reference point. The numbers to the left of each bar represent the number of amino acid residues in that receptor. Linear representation (top to bottom) of human receptors for mineralcorticoid (MR), progesterone (PgR), androgen (AR), glucocorticoid (GR), oestrogen (ER), thyroid hormone (TR), retinoic acid (RAR), vitamin D (VDR), oestrogen-related receptors 1 and 2 (ERR1 and ERR2) and the orphan receptor chicken ovalbumin upstream promoter (COUP).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33
15
VDR
521
57
43
29
ERR1
433
57
48
24
ERR2
423
49
38
26
COUP

induces conformational changes in the receptor which lead to its dimerization, the latter process being presumed to be essential for subsequent nuclear processing of steroid-receptor complex. Other mechanisms however may also contribute to steroid-receptor interaction dynamics, including dissociation of heat shock proteins [Catelli *et al.*, 1990] and protein phosphorylation [Carson-Jurica *et al.*, 1990].

DNA binding domain

This important region contains particular amino acid sequences which are primarily responsible for specific recognition of the cognate steroid response elements (SRE) in the genomic DNA adjacent to target genes. Although the structure of this domain manifests close homologies, being highly conserved (40 to 90%) among different receptor classes, subtle differences in amino acid sequences determine the gene specificity of a given receptor protein. Of special interest are the so called "zinc fingers", which consist of stretches of about 25 amino acids each containing four cysteine residues disposed in such a way that they form a loop or finger to hold one molecule of zinc [Evans, 1988; Beato, 1989]; steroid receptors have two such zinc fingers in the DNA binding domain. The first (N-terminal) finger is thought to contain primary information for specific nucleotide binding in the major groove of the DNA helix; the second may stabilise the receptor-DNA complex by ionic interactions with phosphate groups in the DNA backbone [O'Malley, 1990; Parker & Bakker, 1991]. The relevance of this region to the specificity of DNA binding has

been dramatically demonstrated by domain-swapping experiments, where the interchange of DNA binding domains of the glucocorticoid receptors (GR) and ER produced stimulation of glucocorticoid sensitive genes by oestradiol and viceversa [Green & Chambon, 1991].

Nuclear localisation domains

Two additional sets of amino acids C-terminal to the DNA binding domain have been indicated as site of nuclear localisation signals, since mutations of these sequences may vary the subcellular localization of the receptor [Guichon *et al.*, 1989]. Two such domains have been identified in the GR, while the ER contains only one. This region may be relevant to the intracellular distribution of the unliganded receptor. In fact, it has been suggested that presence of cytoplasmic GR may be due to the second localization domain, while other steroid receptors, having one domain only, are mostly nuclear [Picard *et al.*, 1990].

Transactivation domains

Molecular data support the existence of at least two different regions, located at both sides of the DNA binding domain, which may markedly influence the overall competence of the receptor mechanism [Fawell *et al.*, 1990; Tasset *et al.*, 1990]. These regions have been defined as transactivation domains, implying that a target gene is regulated by a receptor protein which represents a product of a different gene. Although transactivation domain(s)

appear necessary for transcriptional activation, the latter process seems to require the combination of all the receptor compartments (DNA binding, ligand binding, dimerization, etc.).

2.1.2. The steroid response elements (SREs)

Structure and function of target genes for steroid hormones have been investigated as appropriate methodologies became available. These genes contain short (about 15 base pairs) nucleotide sequences, generally located within their 5' flanking region upstream to the start site of transcription, which are called steroid (SRE) or hormone (HRE) response elements. The SRE consists of two half-sites of about 6 nucleotides having a dyad axis of symmetry (inverted repeats or palindromic sequences) around a few central base pairs of random composition [O'Malley & Tsai, 1992]. Androgen, progestin, glucocorticoid and mineralcorticoid receptors share similar SREs, while the oestrogen response element (ERE) differs from the former by a two-base-pair change in the middle of each half-site. Steroid receptors bind to the respective SRE as dimers, with an affinity (in the nanomolar order) adequate to influence transcription [Tsai *et al.*, 1988]. Once bound, the receptor dimer may associate with another dimer (or transcription factor) at an adjacent SRE site to establish a more stable complex, having a much higher binding affinity (dissociation constant (K_d) approximately 10^{-11}M) [Tsai *et al.*, 1989].

The most distinctive point of SRE is that they commonly belong to a category of genetic *cis* elements called enhancers or,

in only limited cases, silencers, depending on whether they stimulate or inhibit transcription. The SREs are separated from the transcription start site by a DNA segment which may be rather long; it is thought that this DNA portion can form loops so that SRE and RNA initiation sites become juxtaposed.

The finding that receptor proteins can bind to any DNA led to postulate a "search and find" model, where the receptor, thanks to weak associations with non-specific sequences, glides along the DNA strand until it arrives at the specific SRE site for high affinity binding [Yamamoto, 1985]. Alternatively, unliganded receptors may be at or near the specific DNA site at all times, ready to bind the ligand and form an active complex, as it happens with the T3 receptor [Damm *et al.*, 1989].

Recently, much attention has also been drawn on the interaction of receptor with other nuclear proteins. It has been observed that the difficulty encountered in the past to produce specific transcriptional activation by addition of ligand to a cell-free system was mainly due to the lack of additional protein factors, distinct from receptor and RNA polymerase [Kalff *et al.*, 1990]. An example is given by the regulation of the collagenase gene by glucocorticoids. Binding of a protein complex, named AP1, to an enhancer sequence similar to, but distinct from, HRE appears to be the principal mechanism of activating collagenase gene trasncription. The AP1 binds to DNA as a dimer, consisting of either two oncogenes, *jun* and *fos*, or two subunits of *jun* [Gutman & Wasylyk, 1991]. It is possible that monomers of both GR and *jun* associate, giving rise to inactive heterodimers.

Therefore, cross-talk between AP1 and nuclear receptor dynamics by heterodimer formation may depict unsuspected ways by which different signalling pathways can influence each other.

2.1.3. The orphan receptors

One of the most intriguing observation on the steroid receptor superfamily of related genes is represented by its unexpected large size and by the disparate nature of its members. After cDNAs for the major steroid receptors have been cloned and sequenced, screening of DNA libraries by cross-hybridisation using cDNA probes revealed a large number of receptoroids, not corresponding to any known receptor molecule. These proteins have been termed orphan receptors as they still wait for both ligand and function. The existence of two such molecules was firstly reported by Evans and associates [Giguere *et al.*, 1988] who termed them ERR (oestrogen-receptor related) I and II. Since then, a number of orphan receptor sequences have been published, none of them having a recognised function. It is not compulsory that all these putative receptors have a ligand and exert a precise functional role, but this is likely for at least some of them since they are expressed in cells as full-processed cytoplasmic messenger RNAs [O'Malley, 1990]. An interesting member of this singular family has been recently discovered by Wang *et al.* [Wang *et al.*, 1987]. This factor promotes initiation of transcription of the chicken ovalbumin gene by RNA polymerase and has therefore been called COUP-TF (chicken ovalbumin

upstream promoter-transcription factor). It contains surprising homologies with other members of the nuclear receptor superfamily in both DNA and ligand binding domains. Probably, the most relevant feature of COUP-TF is that for the first time a promoter regulatory protein is recognised as a legitimate component of the receptor superfamily, where only enhancer regulatory factors have been thus far included.

It is exciting to speculate, as insinuated by Bert O'Malley, that orphan receptors may have endogenous ligands and that these ligands may act as indigenous hormones to the specific target cells. If this is true, a new scenario will open up, where presence of intracrine regulatory systems may have a crucial role in the functional control of the cell.

2.2. Sites of Steroid Binding

In the early sixties, the synthesis of radiolabeled oestrogens allowed to better investigate the mechanism of hormone action at target tissue level. In particular, Glascock and Hoekstra [Glascock & Hoekstra, 1959] documented a selective accumulation of tritiated hexoestrol in reproductive organs of immature goats and sheep. Further, Folca *et al.* [Folca *et al.*, 1961], after administration of labeled hexoestrol to women having advanced breast cancer, found that patients whose tumours showed a remarkable uptake of radioactive oestrogen had a favourable response to subsequent adrenalectomy. The

availability of labelled oestradiol having high specific activity enabled Jensen and Jacobson to demonstrate that radioactivity concentrated almost exclusively in tissues which were commonly thought to be targets for oestrogen action [Jensen & Jacobson, 1962]. Subsequently, Mobbs [Mobbs, 1966] showed that the uptake of tritiated oestradiol by DMBA-induced rat mammary cancer tissues is much higher in oestrogen-dependent tumours than in those having an independent growth. Similarly, *in vitro* binding of labeled oestradiol was observed only in breast tumour specimens from either rats [Terenius, 1968] or patients [Jensen *et al.*, 1967] who responded to endocrine treatment. All these findings, albeit indirect, indicated that target tissues would contain specific components which selectively bind oestrogens.

The first direct evidence for ER in target tissues has been provided by Toft and Gorski [Toft & Gorski, 1966] who isolated and characterised ER proteins from the rat uterus. The vast majority of earlier reports on ER were based on the sucrose density gradients studies. This procedure permits separation of different receptor forms by centrifugation through concentration gradients of a sucrose solution, the sedimentation constant (i.e. the rate of sedimentation per unit gravitational field) of a receptor protein being expressed in Svedberg (S) units. Using this approach, two major ER forms have been identified, having sedimentation constants of about 4S and 8S, depending on the ionic strength of extraction and fractionation buffer. The 8S form was usually isolated at low ionic strength, while the 4S form may dissociate from the 8S form using ionic strength of buffer

approaching 0.4M KCl [Wittliff *et al.*, 1972]. Current understanding is that the 4S "salt-extracted" receptor represents activated, DNA-bound receptor and that the 8S form coincides in the inactive receptor-heat shock protein complex (see later).

The first demonstration of high affinity binding proteins for androgens dates back to 1969, when Fang *et al.* [Fang *et al.*, 1969] revealed the presence of a cytoplasmic component (named α-protein) of the rat ventral prostate, having a sedimentation coefficient of 3.5S, which was able to bind DHT following administration of labeled testosterone to animals. Concurrently, Mainwaring [Mainwaring, 1969] and Unhjem *et al.* [Unhjem *et al.*, 1969] documented the presence of another cytoplasmic protein, binding specifically DHT, with a 8S coefficient. Both proteins have been thought to represent different forms of the same receptor molecule, the 8S component being presumably the result of aggregation of smaller subunits [King & Mainwaring, 1974].

2.2.1. Heterogeneity of steroid binding sites

It is well recognised that steroid hormones bind to sites in the target cells other than the specific receptor. This binding has been generally ascribed to contamination by serum albumin or α-fetoprotein [Michel *et al.*, 1974]. However, presence of cytoplasmic low-affinity binding sites for oestrogens, which cannot be accounted for contamination, was originally reported by Clark and associates in the rat uterus [Eriksson *et al.*, 1978; Markaverich & Clark, 1979]. The authors in fact identify type I and II sites of oestradiol binding, the former being characterised

by high affinity (K_d < 0.5-1.0 nM) and low capacity (1 pmol/uterus) of hormone binding, the latter by greater (four fold) concentration values and by an affinity for oestradiol which is about 40 times lower (K_d > 20 nM) than that of type I sites. It has been observed that type II sites are responsible for up to 50% of the oestrogen bound in the rat uterus [Peck *et al.*, 1973]. The precise role for these secondary sites is still obscure. They may serve as storage proteins to accumulate biologically available steroid in target cells. A second class of low-affinity sites of oestrogen binding has also been described in the nuclei of uterine cells. These nuclear type II sites have a much lower affinity (K_d form 10-30 nM) and a greater capacity of oestradiol binding. Markaverich and Clark [Markaverich & Clark, 1979] observe that high levels of type II sites are maintained by a single injection of oestradiol for as long as 72 h, while type I sites rapidly decline to basal levels by 24 h. The absence of nuclear type II sites from organs such as pituitary and hypothalamus, which do not grow in response to oestrogens, suggests that these sites may be involved in growth stimulation of target tissues by oestrogens [Kelner & Peck, 1981].

Multiple binding sites for other steroid hormones have also been reported, suggesting that they represent a general feature of steroid receptor proteins. Our laboratories have reported the presence of both type I and II androgen binding sites in both benign hyperplasia and cancer of the human prostate [Castagnetta *et al.*, 1992a]. This evidence confirms previous data

obtained either *in vivo* [Pertschuk *et al.*, 1985] or *in vitro* [Turcotte *et al.*, 1988].

There is sufficient consensus that type I sites are commonly identified at K_d values in the range of 10^{-11} to 10^{-10}M [Hawkins *et al.*, 1980; Wittliff, 1984], while type II receptors are generally confined to K_d in the nanomolar order. However, several receptor studies, based on radioligand binding assay, use higher ligand concentration ranges and reveal K_d values which may be reasonably interpreted as defining type II sites only. In this respect, the value of type I and II sites of oestrogen binding is not merely conceptual, but it holds a functional significance. In fact, numerous studies have indicated that only type I ER correlate to both prognosis and response to endocrine therapy of human breast and endometrial cancer patients [Barnes *et al.*, 1979; Leake *et al.*, 1981; Wittliff, 1984; Castagnetta *et al.*, 1987a; Castagnetta *et al.*, 1989b]. This is especially true when the assessment of a functional receptor status is addressed by measuring ER and progesterone receptors (PgR) in both cytosols and nuclei [Thorsen, 1979; Castagnetta *et al.*, 1983; Leake & Habib, 1987; Castagnetta *et al.*, 1987b].

2.3. Models for Steroid Action

Historically, most of the current knowledge on steroid hormone action derived from studies carried out in the late 1950s at the laboratories of Gerald Mueller and Elwood Jensen [Mueller *et al.*, 1958], suggesting that oestrogens may complex with an intracellular protein and therefore play a role in regulating nuclear function of a target cell by interacting directly with its genetic apparatus. In the subsequent years much effort has been made to elucidate the mechanism of action of steroids.

2.3.1. The old model

The classic "two-step" model originated from the early work of Gorski [Gorski *et al.*, 1968] and Jensen [Jensen *et al.*, 1968]. It states that the native unliganded receptor is located in the cytosol and that, after binding to steroid, the hormone-receptor complex is translocated to the nucleus to bring about a nuclear response. This model was first developed for oestrogens, but therefore extended to other steroid hormones [Liao & Fang, 1969; O'Malley *et al.*, 1970; Rousseau *et al.*, 1973]. It was mainly based on the observation that homogenates of oestrogen target tissues (such as uterus, pituitary, oviduct, etc.) from immature or ovariectomised animals contained unoccupied ER mostly in the cytosolic fraction, while very little unliganded receptor was found in the nucleus [Shyamala & Gorski, 1969]. In addition, after administration of labelled oestrogen, the majority of radioactivity was found in the nucleus as hormone-receptor

complex, with a corresponding loss of cytoplasmic receptor [Gorski *et al.*, 1968; Jensen *et al.*, 1968].

The phenomenon by which the empty receptor acquires a high affinity for nuclear component as a consequence of steroid binding has been named *activation*. In the case of ER, this process has been shown to be accompanied, in the rat uterus, by an increase in the sedimentation constant from 4S to 5S [Notides & Nielsen, 1974]. The activation of the unoccupied receptor is thought to represent a temperature-dependent phenomenon, being obtained in vitro by incubation of target cell cytosols with hormones at 20-37 °C; use of low temperatures (0-4 °C) gives rise to non-activated steroid-receptor complexes, which do not bind nuclear elements. The temperature-dependent step has been also referred to as *transformation*, indicating that this process converts the receptor into a transformed form having higher affinity for polyanions like DNA, RNA and proteins [Yamamoto & Alberts, 1972]. Gschwendt and Kittstein [Gschwendt & Kittstein, 1980] proposed to restrict the term *activation* to the rise in nuclear affinity of receptor and to use the term *transformation* to illustrate the change in the sedimentation coefficient. Contrary to oestrogens, activation of other steroid hormone-receptor complexes does not involve an increase of the sedimentation constant. In the case of androgens, activation of DHT-receptor complex is followed by a decrease of the sedimentation rate from 3.8S to 3.0S [Liao, 1975]. The same is true for PgR complex of both hamster [Chen & Leavitt, 1979] and rabbit [Saffran *et al.*, 1976].

2.3.2. The equilibrium model

The terms cytosol and cytoplasm have been very often mistakenly confused. The cytosol (or soluble) fraction commonly derives from a rough cellular disruption (like homogenisation) and consists of all soluble components of sheared cells, including some nucleoplasmic proteins and various components loosely attached to insoluble structures. Sheridan and colleagues [Sheridan *et al.*, 1979], in a very elegant experiment series, suggested that, on the basis of a comparison between autoradiographic and biochemical dilution studies, the unoccupied steroid receptors are distributed in equilibrium between nuclear and cytoplasmic compartments. The authors showed that use of aqueous or non-aqueous methods to measure both soluble and nuclear receptor dramatically changed the results of assay; in fact, the non-aqueous procedure mainly preserved the receptor in the nuclear pellet, while using the aqueous approach receptor was recovered mostly in the soluble fraction [Sheridan *et al.*, 1981]. This was true either for ER or PgR. On this basis, Martin and Sheridan [Martin & Sheridan, 1982] proposed that, in intact target cells, the unoccupied steroid receptors are partitioned in equilibrium between nucleus and cytoplasm according to the free water content of each cell compartment. Further to this model, several concurrent reports contributed to severely challenge the old translocation model. Siiteri *et al.* [Siiteri *et al.*, 1973] found that, in the immature rat uterus, both the 4S and 5S ER forms could be detected at all

times in the nucleus after two hours exposure to oestrogen; the authors suggested that the activation process takes place in the nuclear compartment and that it may well require the presence of DNA to occur. Zava and McGuire [Zava & McGuire, 1977] proposed that, in MCF7 human mammary cancer cells, the unoccupied ER is located in the nucleus; similarly, McCormack and Glasser [McCormack & Glasser, 1980] observed that in dispersed uterine cells in culture the amount of empty receptor recovered from the nuclear fraction was much higher than expected. Finally, thyroxin and 1,25-dihydroxy vitamin D receptors were found to be nuclear proteins, irrespective of the presence of ligand [Oppenheimer *et al.*, 1976; Walters *et al.*, 1980].

2.3.3. The plasma membrane model

Originally, Pietras and Szego [Pietras & Szego, 1979] showed that homogenisation of uterine cells in hypotonic buffers produced evidence of cytosol ER, while use of buffers containing 0.25M sucrose led to the recovery of unoccupied receptors mostly from the particulate fraction. On this basis, the authors postulated that oestrogen is captured by plasma membrane-associated receptors and that the oestrogen-receptor complex is therefore internalised by pinocytosis and transferred to the nucleus via lysosomes. However, some experimental work has indicated that, in a physiological state, steroid uptake by target cells is a nonsaturable process, not limiting to the specific receptor binding [Müller & Wotiz, 1979]. This would imply that

the cellular transport of steroid is not a primary factor, regardless of the subcellular localization of receptor. In this respect, the affinity of steroid for the lipidic component of plasma-membrane may be relevant only to the intracellular diffusion and transit of hormone.

2.3.4. The new model

Despite the above disparate observations, the concept of the old model was in force until mid 1980s, when two separate lines of investigation provided convincing evidence that steroid receptor is a nuclear protein at all time whether or not the ligand is bound.

Welshon and associates [Welshons *et al.*, 1984] applied a cell enucleation technique to examine the intracellular localization of ER. Using centrifugation-cytochalasin B-induced enucleation the authors separated cytoplasm from the nucleus of the GH_3 cells, a clonal cell line originated from a rat pituitary tumour [Tashjian *et al.*, 1970]. The latter, following homogenisation procedures, has been found to contain cytosolic ER, which incur nuclear translocation after exposure to oestrogen [Haug *et al.*, 1978]. Enucleation technique gives rise to cytoplasm-derived element, called *cytoplast*, and to the remainder of the cell, named *nucleoplast*, which contains the nucleus and a rim of cytoplasm. Both structures are surrounded by a plasma membrane and can be reunited to yield a viable cell [Veomett *et al.*, 1974]. The cytoplast fraction of the enucleated GH_3 cells consistently exhibited low levels of unoccupied ER, whilst the

majority of receptor was recovered from the nucleoplast fraction, namely from a combination of cells lacking variable amount of cytoplasm and a few whole cells. In particular, the receptor content of cytoplasts represented approximately 10% of that found in the whole cells plus nucleoplast fraction.

The subcellular localization of steroid receptors has also been investigated using immunocytochemistry. Early studies documented the presence of unoccupied ER in the cytosol fraction of human breast tumour tissues [Greene & Jensen, 1982; Raam *et al.*, 1982]. Originally, Greene and colleagues reported on a new set of monoclonal antibodies raised against the ER purified from the MCF7 human mammary cancer cell line [Greene, 1984]. The antibody specificity ranged from recognition of the human ER only, to cross-reactivity with a variety of both mammalian and avian oestrogen receptors. Using these antibodies, a positive staining was found exclusively in nuclei of hormone-responsive tissues, including human breast tumours and uterus, and rabbit uterus [King & Greene, 1984]. The apparent divergence between this evidence and the aforementioned immunocytochemical studies has been ascribed to the use of polyclonal antibodies, or to unsuitable fixation and sectioning techniques in previous reports. Other studies have demonstrated the presence of unoccupied PgR in the nucleus of both mammalian tissues [Perrot-Applanat *et al.*, 1985] and in the chick oviduct [Gasc *et al.*, 1984]. However, worth mentioning, Gasc and colleagues observed that while two polyclonal antibodies (IgG-G3 and IgG-RB) showed nuclear localization of PgR, a monoclonal antibody (BF4) reacted

with a component of the 8S cytosolic receptor moiety, which seemed to be either cytoplasmic or both cytoplasmic and nuclear [Gasc *et al.*, 1984]. Immunocytochemical studies on GR have revealed either cytoplasmic or cytoplasmic plus nuclear immunoreactivity in target cells [Govindan, 1980; Papamichail *et al.*, 1981; Antakly & Eisen, 1984]. Androgen receptors (AR) have been also localised in nuclei of both rat and human prostate using monoclonal antibodies [Chang *et al.*, 1989]; however, Demura and associates [Demura *et al.*, 1988] detected immunocytochemically AR in both cytoplasm and nucleus of benign and malignant human prostate, although the receptor specificity of the monoclonal antibody used was not reported.

Contrary to the plasma-membrane model, neither enucleation nor immunocytochemical studies have confirmed presence of oestrogen binding on the surface of target cells.

On the basis of the bulk of data coming from both enucleation of intact cells and immunocytochemistry of ER, a new model for steroid hormone action was designed [Gorski *et al.*, 1984; Gorski & Hansen, 1987]. In this model, oestrogen diffuses passively into the target cell, crosses the cytoplasm and reaches the nucleus, where the unoccupied ER is thought to reside immobilised through association to structural nuclear element(s). After high affinity binding of oestrogen to a specific site of the receptor, the resulting complex incurs a dramatic conformational change which is believed to be the "one and only" essential step of the primary cellular response to oestrogens [Gorski & Hansen, 1987]. This rearrangement induces a variation in the affinity of

receptor for nuclear components, allowing increase and/or decrease in the expression of an assortment of tissue-specific genes, which eventually leads to modification of the cell physiology [Yamamoto, 1985].

The site of steroid receptor synthesis is mostly the cytoplasm; since this seems to be a rather continuous process, which can be modulated by a variety of factors, it is reasonable to expect that some receptors are normally found in the soluble fraction of target cells.

An interesting contribution to this subject comes from studies on the dynamics of AR in prostate cells. Hiipakka and Liao [Hiipakka & Liao, 1984], based on isotope-chasing experiments, suggested that the AR recycling is a slow process which occurs between chromatin-bound and cytosolic forms. The authors observed that incubation of minced ventral prostate from castrate rats with actinomycin D and 3'-deoxyadenosine (3'-dA), which selectively inhibit transcription and processing of newly synthesised RNA, produces opposite results. In fact, in the presence of actinomycin D, the AR complex appeared to be trapped as a chromatin-bound form, while, using 3'-dA, the level of cytosolic AR complex increased with a concomitant decrease of the chromatin-bound form to a minimum [Hiipakka & Liao, 1988a]. Armed with this evidence, Liao and associates have proposed a model by which steroid-receptor complexes and ribonucleoproteins may interact to mutually facilitate their release from the nucleus; continued association of receptors to newly transcribed RNA could also assist the utilisation of RNA

into the cytoplasm [Hiipakka & Liao, 1988b]. This would imply that some the cytoplasmic receptor may also derive from recycling of the steroid-receptor complexes which have moved to cytoplasm from nucleus in conjunction with RNA as part of a processing mechanism.

In conclusion, unless unequivocal evidence of subcellular localization of steroid receptors becomes available, it appears hazardous to neglect any indication coming from the steroid action models proposed thus far. Rather, it seems appropriate to point out with Jack Gorski that "we are still unable to present a cogent and detailed model of steroid hormone action at molecular level".

2.4. Heat Shock Proteins

The heat-shock phenomenon was originally discovered in the fruit fly *Drosophila* and described as the massive synthesis of a small number of proteins (heat shock proteins, hsps) in response to a few degrees rise above the normal growth temperature (for a review see [Lindquist & Craig, 1988]). This phenomenon is today known to be common to most organisms, both prokaryotic and eukaryotic. The pattern of heat-induced proteins varies greatly, but three major species can be identified on the basis on their molecular mass: the 80-90, the 60-75 and the 15-30 kDa proteins. Although initially identified on the basis of their specific induction during the cellular response to heat

shock, hsps are also induced under a variety of other stress conditions and, more, most of them are normally present in substantial amount in unstressed cells. Therefore, the terms "heat shock proteins" and "stress proteins", which have been in turn proposed to describe this relatively large class of molecules, both appear inappropriate and "hsps" will be used simply to describe the family.

The relevance of hsps to the cell physiology is still debated, but it is widely accepted that they all share protein folding and chaperone functions with a larger family of proteins that have been highly conserved during evolution (reviewed by [Gething & Sambrook, 1992]).

The interest for steroid receptors-hsps interactions originated from the observation of Toft and Gorski [Toft & Gorski, 1966] that cytosolic ER of the rat uterus sedimented at 9S in sucrose gradient centrifugation analysis. Subsequently, all steroid receptors were found to sediment as 8-10S (untransformed) complexes in steroid-free cells, while they were recovered mainly as 4S (transformed) forms in the presence of the specific hormone [Sherman, 1984]. In the late 1970s the composition of the 9S complexes was carefully investigated, leading to the evidence that both progesterone and glucocorticoid untransformed receptor complexes contain a major protein component of approximately 90 kDa [Puri *et al.*, 1982; Housley & Pratt, 1983]. The latter was hence identified as the 90-kDa heat shock protein, hsp90 [Sanchez *et al.*, 1985; Schuh *et al.*, 1985]. In the last decade, the appreciation that hsp90 is not the only

receptor-associated protein has clearly emerged. Rather, data coming from several studies suggest that other hsps complex to unliganded steroid receptors and that the 9S form is a core unit of a larger structure [Pratt *et al.*, 1992].

2.4.1. Hsp association to steroid receptors

At least three different hsps (hsp90, hsp70, hsp56) have been found to associate with steroid receptors in large heterocomplexes. These complexes, however, are commonly present in cell cytosol regardless of the presence of receptors [Perdew & Whitelaw, 1991].

Hsp90

This is an ubiquitous, conserved protein which is present in relatively high amount in eukaryotic unstressed cells [Schlesinger, 1990]. It complexes to several steroid receptors, including ER, PgR and GR [Pratt, 1987]. There is convincing evidence that hsp90 interacts with the hormone-binding domain (HBD) of the GR, although it is still unclear whether HBD includes a minimal hsp90 binding site or a diffuse region of the domain is involved [Pratt *et al.*, 1988]. In addition, the ER HBD has been found to be necessary but not sufficient for hsp90 binding, indicating that other contact sites out of the HBD (probably including the DNA-binding domain) are required for association.

It has been proposed to subdivide the steroid receptor superfamily into three classes according to different properties of hsp90 binding. Class I includes the receptors for thyroid

hormone, retinoic acid and vitamin D. The unliganded receptors in this class are not recovered from the cell in association to hsp90, but bind tightly to nuclear structures requiring high salt conditions to be extracted [Dalman *et al.*, 1990; Dalman *et al.*, 1991]. By contrast, receptors in classes II (GR and mineralcorticoid receptors, MR) and III (ER, PgR, AR) give rise to stable associations with hsp90. However, while class II receptors require hsp90 for high-affinity steroid binding, class III receptors are retained in nuclear "docking" complexes where they incur specific conformational changes for hormone binding independent of hsp90 [Pratt, 1993].

Several studies suggest that hsp90 behaves as a dimer, with a stoichiometry of two molecules to each molecule of receptor [Minami *et al.*, 1991]. Murine hsp90 is composed of two distinct isoforms which represent products of two different genes [Mendel & Orti, 1988]. It has been postulated that GR interact more directly with one of the two hsp90 molecules, giving rise to an asymmetrical GR-hsp90 complex [Lefebvre *et al.*, 1989]. Interestingly, the only study available on the stoichiometry of ER-hsp90 association indicates that, contrary to both GR and PgR heterocomplexes, the 9S ER heterocomplex consists of a dimer of the hormone-binding protein coupled to a dimer of hsp90 [Redeuilh *et al.*, 1987]. This could be a reflection of the presence of a strong dimerization site in the HBD of ER, which is thought to be required for DNA binding.

Mendel *et al.* [Mendel *et al.*, 1986] revealed that exposure of intact mouse thymoma cells to glucocorticoid causes the

dissociation of the GR from the 90-kDa protein. This evidence was confirmed later by Denis and associates [Denis *et al.*, 1988] who demonstrated that, during this temperature-dependent process, GR proceeds from a non-DNA-binding state towards a DNA-binding form. Although this phenomenon has been examined in detail only for GR, it seems likely the both adrenocorticoid sex steroid receptors undergo comparable changes following dissociation from hsp90.

Hsp70

This hsp family consist of either constitutive or inducible proteins which share common properties, such as ATP-binding. They have been in turn implied as catalysts of protein assembly and shown to possess protein unfoldase activity, which is relevant to protein transport across plasma membranes. The first report of an association between hsp70 and steroid receptors dates back to 1984, when Wrange and coworkers [Wrange *et al.*, 1984] isolated a 72-kDa protein linked to GR in the rat liver. Recently, hsp70 has been shown to represent an important component of steroid receptor heterocomplexes reconstituted in rabbit reticulocyte lysate system (see below) and its unfoldase activity is reputed to be an essential requirement for hsp-receptor complex assembly [Hutchison *et al.*, 1992]. Additionally, Sanchez *et al.* [Sanchez *et al.*, 1990a] suggested that hsp70 may also play a role in the transit of steroid receptors across nuclear membrane.

Hsp56

Since its first recognition as a 59-kDa component of rabbit PgR, ER, AR and GR heterocomplexes [Tai *et al.*, 1986], hsp56 has met an increasing interest for several reasons. In first place, it was shown to be immuno-adsorbed jointly with hsp90 and hsp70 in the rat uterus cytosol. It was therefore proposed that these three proteins are associated with each other to form a cytosolic complex independent of the presence of receptor [Sanchez *et al.*, 1990b]. Secondly, hsp56 has been recognised as a member of the immunophilin proteins, which bind immunosuppressive agents, such as cyclosporin A and FK506 [Tai *et al.*, 1992]. Although nomenclature of this protein has generated some confusion because of the diverse molecular weights reported in different species, it is now clear that the receptor-associated 54-59 kDa proteins coincide with hsp56 and that the latter is a novel, moderately abundant heat-shock protein [Sanchez, 1990].

Hsp 27

As for hsp56, a group of small proteins, in the range of 24-30 kDa, have been reported to be involved in cellular thermotolerance and response to a miscellany of both growth and differentiation factors. In particular, recent studies have revealed that the 28-kDa protein found in MCF7 human mammary cancer cells is the same as the oestrogen-regulated 24-kDa protein and the mammalian hsp27 [Faucher *et al.*, 1993]. Hsp27 has been shown to be phosphorylated in response to various stress conditions, including heat [Zhou *et al.*, 1993]. However, there is

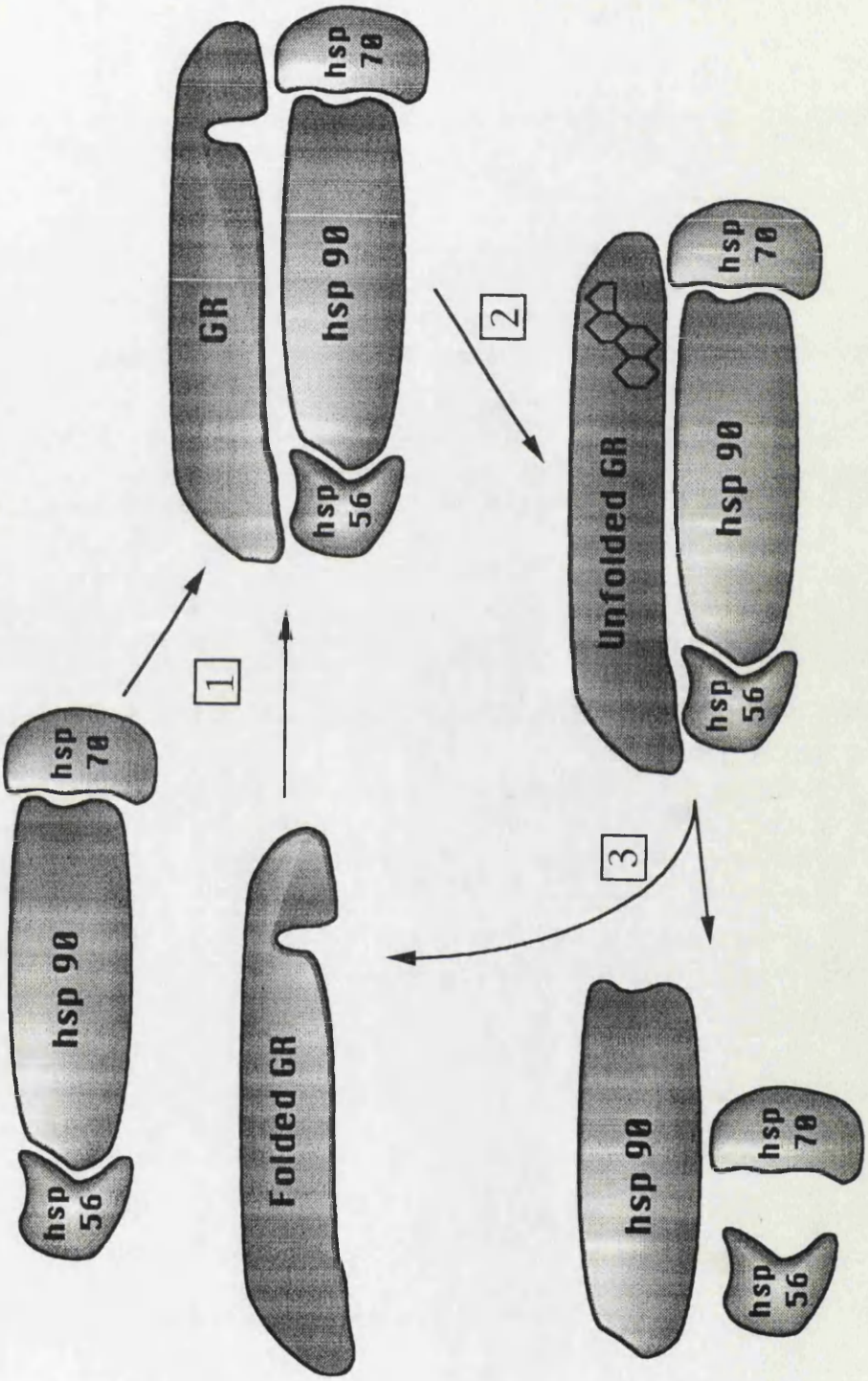
some suggestion that phosphorylation at serine residues 82 and 78, which respectively represent a major and a minor phosphorylation site of the molecule, may activate hsp27 functions linked to growth signalling pathways in unstressed cells [Landry *et al.*, 1992]. In addition, hsp27 has been shown to behave itself as a serine kinase phosphoprotein, which is likely to be involved in a major signal transduction cascade [Zhou *et al.*, 1993]. Interestingly, immunological evidence has indicated that oestrogen-regulated hsp27 and the ER-associated 29-kDa protein (p29) are the same molecule [Ciocca & Luque, 1991]. The p29 phosphoprotein was previously characterised as a cytosolic component of both breast and endometrial cancer tissues which is quantitatively and qualitatively related to ER but not to other steroid receptors or other binding proteins [King *et al.*, 1987]. It was proposed that p29 represents a non-hormone-binding component of the receptor mechanism which complexes to ER under certain conditions, such as treatment with ammonium sulphate [Cano *et al.*, 1986]. This would suggest that hsp27 is another component of larger cytosolic complexes of hsps. However, this comparison appears to be unrewarded since p29/hsp27 has been found almost exclusively in ER positive tissues, whilst larger hsps (hsp90, hsp70) are encountered also in hormone-insensitive cells, regardless of the presence of steroid receptors. Although several points favour the idea that hsp27 is implicated in the oestrogen action, it is still doubtful whether this protein participates in the processes of association and

dissociation of ER heterocomplexes, or it is rather related to the receptor activation (dimerization?) and DNA binding phenomena.

2.4.2. Models for receptor heterocomplex assembly

The evidence that several hsps exist in large complexes, irrespective of the presence of both steroid receptors and ligands, suggests that these proteins may interact in a spatially organised and temporally co-ordinated manner [Pratt, 1993]. Recently, it has became possible to reconstitute purified receptors into heterocomplexes by means of an enzymatic system in rabbit reticulocyte lysate [Denis & Gustafsson, 1989]. Using this system, the GR heterocomplex formed appeared to be functionally identical to the native non-DNA-binding 9S complex. This heterocomplex was also found to consist of other proteins in addition to both hsp90 and hsp70. This was true for both PgR and GR, the former including p23 [Smith *et al.*, 1992], the latter containing hsp56 [Hutchison *et al.*, 1993]. It has been suggested that steroid receptors might associate with a preformed complex in the lysate, which includes several hsps. In this respect there is evidence for an essential role of hsp70 in the receptor association with hsp90. In fact, Smith *et al.* [Smith *et al.*, 1992] reported that pretreatment of the lysate with a monoclonal antibody raised against hsp70 inhibited hsp90 binding to PgR. An elemental multistep model has been recently proposed for GR

Figure 2. Model for heterocomplex assembly. A hypothetical multistep model for glucocorticoid receptor (GR)/heat shock proteins (hsps) heterocomplex formation is illustrated. Initially (step 1), the folded GR binds to a preformed hsp complex consisting of two molecules of hsp90 and one molecule each of hsp56 and hsp70. The latter subsequently catalyses the unfolding of the GR hormone binding domain (HBD) through its intrinsic unfoldase activity, allowing the GR to acquire a high affinity steroid-binding conformation which is further stabilised via hsp90 (step 2). Dissociation of the steroid-free GR from hsp complex is accompanied by refolding of the HBD and possible recycling of the GR into another heterocomplex assembly process (step 3).
(Adapted from [Pratt, 1993 #296]).


hsp 70
hsp 90
hsp 56
GR
hsp 70
hsp 90
hsp 56
1
2
Unfolded GR
hsp 70
hsp 90
hsp 56
3
Folded GR
hsp 90
hsp 70
hsp 56

heterocomplex assembly (see Figure 2). In this model hsp90 and hsp70 behave as a tandem unit, where hsp70 recognises the folded state of receptor and favours an ATP-dependent process which allows the receptor to associate with the complex (step 1). Then, hsp70 causes the unfolding of the receptor HBD (step 2), with a subsequent hsp90-mediated stabilisation of the unfolded receptor in a high-affinity steroid-binding state (step3). Finally, high salt dissociation of the hormone-free heterocomplex is paralleled by refolding of the receptor HBD and loss of the steroid-binding conformation. Hsp90 role seems therefore devoted to maintain the receptor HBD in a metastable, partially unfolded state. In this respect, the steroid receptor heterocomplexes may retain receptors during transit to and within the nucleus. These complexes may therefore act as "transportosomes", wherein receptors remain "docked" until specific hormone binding triggers their dissociation [Pratt, 1993].

3. Steroid Formation and Metabolism

3. Steroid Formation and Metabolism

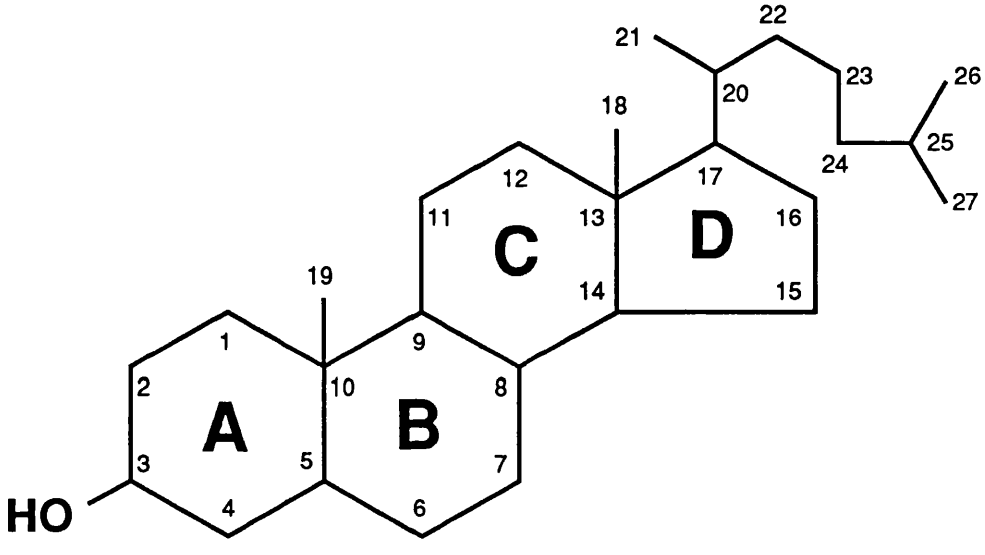
3.1. Steroidogenetic Pathways

All steroid hormones are of basically similar structure and share common biosynthetic pathways, although relatively minor chemical changes lead to striking diversities in their biochemical activity. In this respect, steroid-producing organs - specifically the adrenal gland, ovary, testis and placenta - which are endowed with key enzyme activities of steroidogenesis, are characterised by more or less complete blocks at certain reaction sites so that the compound preceding the block represents the major secretory product of a single tissue.

As illustrated in Figure 3, the basic structure of steroids is represented by a perhydrocyclopentanephenanthrene nucleus, which is composed of three 6-carbon rings (A, B and C) and one 5-carbon ring (D). This molecule contains 6 centres of asymmetry and may give rise to 64 possible isomers, some of which are inactive, through changes in the position of only one substituent. The A, B and C rings are not planes, but they commonly assume a "chair" conformation; aromatisation of the ring A to form a ring of the benzene type changes its conformation to a flat structure.

The basic building block in steroidogenesis is represented by cholesterol, a 27-carbon atoms molecule (see Figure 3), whose basic structure is called cholestane. Apart from the placenta, all steroidogenic tissues can actively synthesise cholesterol from the

Figure 3. Basic structure of steroid hormones. All steroids derive from the 27 carbon molecule of cholesterol (here illustrated) through a series of enzymatic reactions which eventually lead to formation of corticoids, and the three sex steroid classes (progestins, androgens, oestrogens). These share the perhydrocyclopentanephenantrene nucleus, consisting of three 6-carbon rings (A, B and C) and one 5-carbon ring (D).


HO
A
B
C
D
1
2
3
4
5
6
7
8
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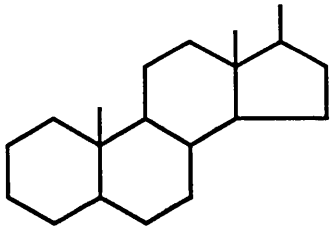
two-carbon molecule of acetate, even if the major resource remains the blood supply. Sex steroids are subdivided into three main classes, according to the number of carbon atoms they possess (see Figure 4). The 21-carbon series includes corticoids and progestins, and the basic structure is the pregnane nucleus. The 19-carbon series corresponds to all androgens and is based on the androstane nucleus, whereas the oestrogens are 18-carbon steroids, based on the oestrane nucleus.

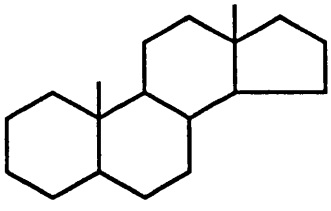
During steroidogenesis, the number of carbon atoms progressively reduces from cholesterol to the various steroid hormones which are in turn synthesised. This process involves several enzymatic reactions which produce cleavage of a side chain (desmolase reaction), conversion of hydroxyl groups into ketones or viceversa (dehydrogenase reaction), addition of OH groups (hydroxylation), formation or reduction of double bonds respectively by removal or addition of hydrogens.

The first metabolic step, which occurs in the mitochondria and requires NADPH and molecular oxygen, is represented by the conversion of cholesterol into pregnenolone (3β-hydroxy-pregn-5-en-20-one). This step involves hydroxylation at both the C20 and C22 positions (20- and 22-hydroxylase enzymes), with subsequent cleavage of the side chain (20,22-desmolase). Once pregnenolone is formed, further steroid synthesis may follow two alternative pathways, namely either the Δ^5-3β-hydroxysteroids or the Δ^4-3-ketone pathway. The former (the Δ^5 pathway) proceeds through the 17-hydroxypregnenolone and the dehydroepiandrosterone (DHA), the latter (the Δ^4 pathway) by

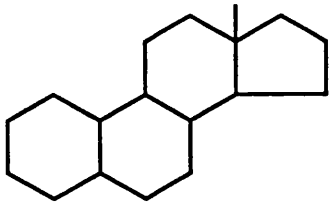
Figure 4. The three classes of steroid hormones. Steroids are divided into 3 main groups depending on the number of carbon atoms they possess: the 21-carbon series, including corticoids and progestins, the 19-carbons series, including all androgens and the 18-carbon series, represented by oestrogens.



Progestins
Corticoids
(21 carbons)



Androgens
(19 carbons)



Oestrogens
(18 carbons)

way of progesterone and 17α-hydroxyprogesterone. In the Δ^4 pathway, two enzyme activities govern the conversion of pregnenolone to progesterone: the 3β-hydroxysteroid dehydrogenase and the Δ^{4-5} isomerase; they transform the 3-hydroxyl to a ketone group and transfer the double bond from the 5-6 to the 4-5 position. Once the Δ^{4-5} ketone is formed, progesterone is hydroxylated at the 17 position to yield 17α-hydroxyprogesterone, which represents the immediate precursor of the C19 series of androgens. Following peroxide formation at C20 and epoxidation of both the C19 and C20 carbons, the side chain is split off to produce androstenedione. Intervention of the 17β-hydroxysteroid dehydrogenase (17βHSD) determines the reduction of the 17-ketone to the 17β-hydroxyl androgen testosterone. Either androgen (androstenedione and testosterone) can be rapidly converted by microsomal enzymes to the corresponding C18 phenolic steroid oestrogens (oestrone and oestradiol) in a process referred to as aromatisation. This process includes hydroxylation of the angular 19-methyl group, followed by loss of the 19-carbon as formaldehyde and aromatisation of the A ring (dehydrogenation). Alternatively, in the Δ^5 pathway, pregnenolone can be directly converted to the Δ^5-3β-hydroxy C19 steroid DHA through 17α-hydroxylation and subsequent desmolase cleavage of the side chain. However, irrespective of the precursor source, C19 Δ^4-3-ketone substrates may proceed to oestrogen formation, as indicated above. The latter aromatisation step mainly takes place in the ovary, but

also, though to a lesser extent, in both testis and the adrenal gland.

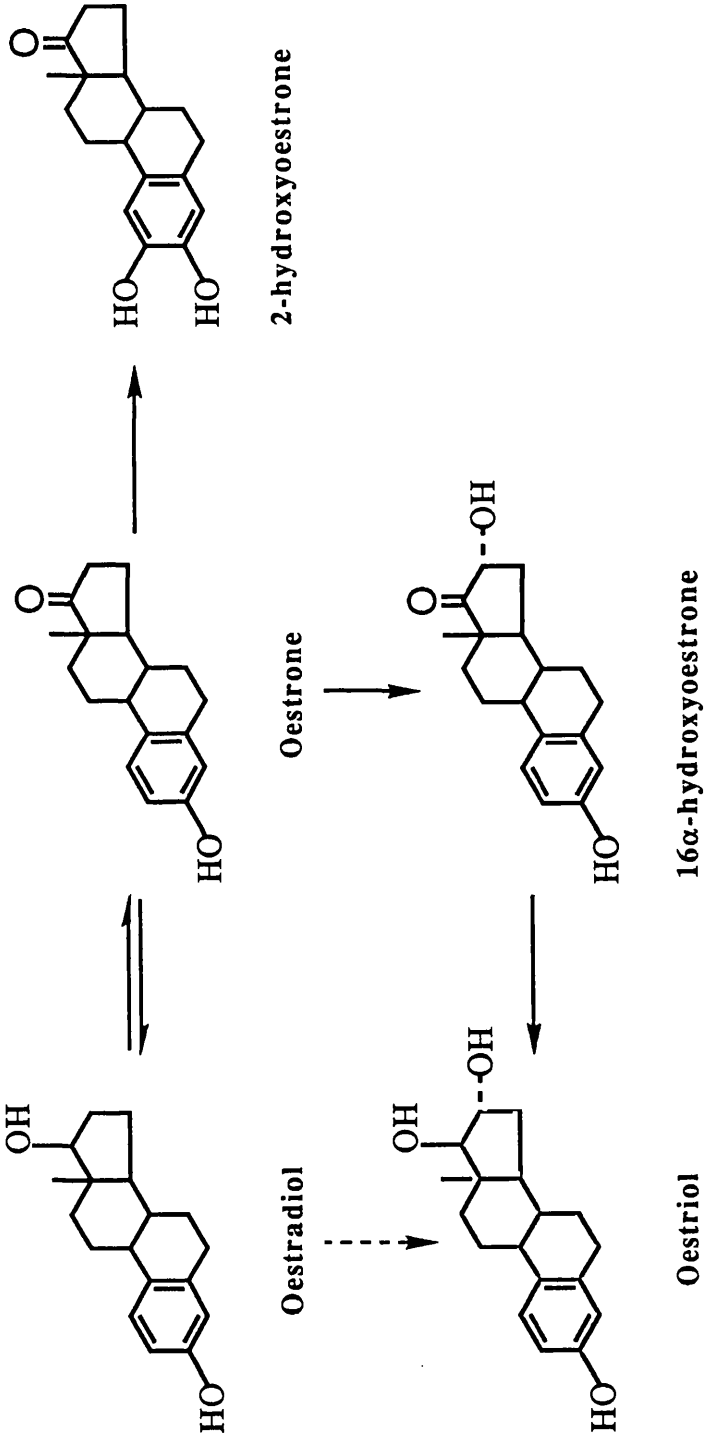
3.2. Local Metabolism

Although the main circulating sex steroids, namely testosterone in man and oestradiol or oestrone respectively in pre- or post-menopausal woman, may act directly on target tissues, peripheral metabolism frequently gives rise to biologically active metabolites which may assume an important role in regulation of both normal and malignant cell growth. Much attention has been drawn in recent years to pathways of steroid formation and degradation, including conjugate steroids, in peripheral target tissues, especially human breast and prostate. In particular, key enzyme activities have been isolated and purified, their genes have been sequenced and possible regulatory mechanisms have also been explored. This has allowed a better understanding of basic rules which determine the actual levels of active hormones in target cells in different conditions, including cancer.

3.2.1. Oestrogens

Unlike the C19 and C21 steroids, the metabolism of oestradiol is primarily oxidative, the main pathway being the oxidation at C17β position to yield oestrone (E_1) (see Figure 5). This conversion, like that of testosterone to androstenedione, is

Figure 5. Pathways of oestradiol metabolism. The metabolism of oestradiol in target tissues primarily includes the reversible conversion to oestrone via the 17β-hydroxysteroid dehydrogenase (17βHSD) enzyme. This is commonly followed by two mutually exclusive reactions, which consist of hydroxylation at the C16α or at C2 positions, to yield respectively 16α-hydroxyoestrone or 2-hydroxyoestrone. The former may be further converted by 17βHSD to oestriol, the latter can also be metabolised by catechol-O-methyltransferase to its methoxy derivative 2-methoxyoestrone. Possible direct formation of oestriol from oestradiol is also considered (hatched line).


2-hydroxyoestrone
Oestrone
16α-hydroxyoestrone
Oestradiol
Oestriol

the only freely reversible reaction (E_2 originates from E_1 in the reductive pathway), and is governed by the 17βHSD enzyme system. It is commonly followed by two mutually exclusive pathways which consist of hydroxylation at the C16α or at C2 positions, to give respectively 16α-hydroxyoestrone ($16\alpha OHE_1$) or 2-hydroxyoestrone ($2OHE_1$). The former may be further converted by 17βHSD to oestriol (E_3), the latter can also be metabolised by catechol-O-methyltransferase (COMT) to its methoxy derivative 2-methoxyoestrone ($2MeOE_1$) .

The concept that oestrogens, like testosterone, may need to be converted to biologically active derivatives for full expression of hormone action was first introduced by Fishman and Norton [Fishman & Norton, 1975]. The authors suggested that 2-hydroxylated oestrogens (catecholoestrogens, CCE) may well mediate the parent hormone's action, having properties distinct but relevant to the physiology or pathophysiology of a target organ. Today, it is recognised that CCE can be generated by at least three distinct microsomal, P450-mediated enzymatic functions, namely 2-, 4- and 2/4-hydroxylases, which produce both 2- and 4-hydroxy derivatives of either E_2 or E_1. These hydroxylase activities appear to be independently expressed and differentially regulated in several target tissues [Weisz, 1991].

As already discussed (see 1.2.4.), while the oestrogenic potency appears to be reduced by 2-hydroxylation, it may be preserved or even enhanced by hydroxylation at the C4 position. This could be also ascribed to the slower rate of dissociation of $4OHE_2$ from the oestrogen receptor (ER), although the biological

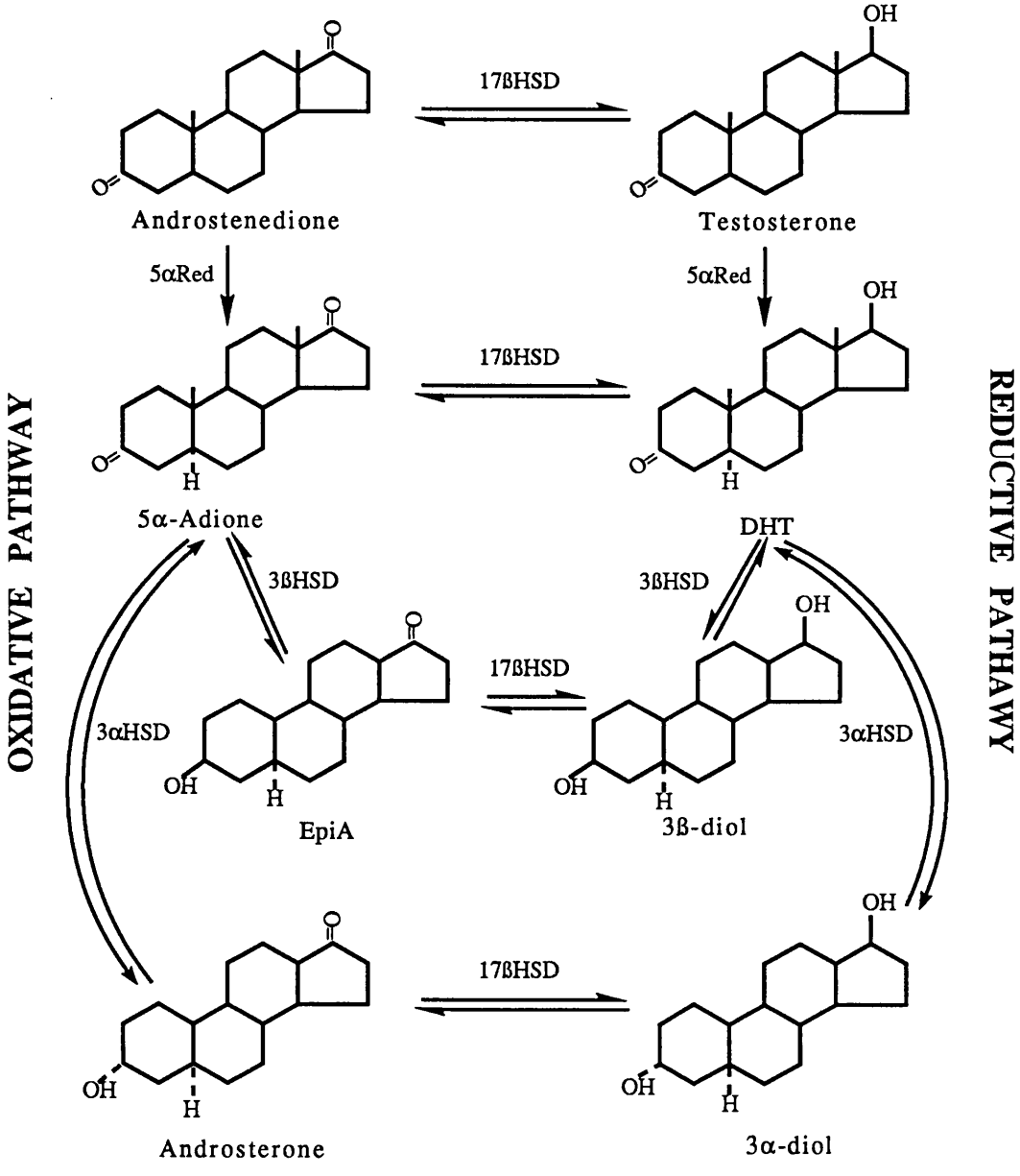
significance of such a prolonged association remains to be clarified. Interestingly, the 2/4-hydroxylase enzyme, which appears to be the only hydroxylase activity expressed in human breast organoids, has been found to be dependent on the availability of organic hydroperoxide (OHPs) [Bui & Weisz, 1988]. CCE, like other hydroquinones, represent suitable substrates for redox cycling, by which potentially mutagenic free radicals are generated [Liehr *et al.*, 1986]. Such a process, while using OHPs, also generates them through the lipid peroxidation caused by free radicals. It has been proposed that, in the presence of both oestrogens and appropriate P450, the availability of OHPs may initiate a viscious cycle where peroxide-dependent CCE formation may provide further fuel for redox cycling by generation of free radicals and the resulting maintenance of OHPs supply [Weisz, 1991]. This cascade, however, would occur only in the case that cellular mechanisms protecting against oxidative damages have been overwhelmed. Such a system could support the potential role of oestrogens as either initiators or promoters of cancer.

3.2.2. Androgens

In some androgen target tissues, such as prostate, seminiferous tubules, epididymis and some cutaneous regions, circulating testosterone is commonly converted into its biologically active derivative 5α-dihydrotestosterone (DHT) through a 5α-reductase enzyme system. This reductive pathway is an essential step as androgen action on development and growth of these tissues can be elicited. DHT has long been

recognised as the principal intranuclear androgen in human prostate and skin [Anderson & Liao, 1968; Gomez & Hsia, 1968]. DHT may be in turn converted by means of the 3α/3β-hydroxysteroid dehydrogenase (3α/3βHSD) to 3α- or 3β-androstanediols, which exhibit an androgen potency even greater than that of testosterone in specific target tissues [Wilson & Walker, 1969; Ito & Horton, 1971]. On the other hand, testosterone can be converted to Δ^4-androstenedione (oxidative pathway) through the 17βHSD enzyme system. The latter, as far as androgen metabolism is concerned, presides over the crosslink between the oxidative and the reductive pathways (see Figure 6). Androstenedione is generally considered to behave as a weak androgen, mainly because it lacks the hydroxyl group at the 17 position. It is commonly metabolised to its 5α-reduced derivative androstanedione (5α-androstan-3,17-dione) by means of the 5α-reductase enzyme activity and further deactivated to either androsterone or its epimer epiandrosterone respectively by the action of 3α or 3βHSD. Alternatively, androstenedione can be reduced to the 5β-androstanedione and subsequently degraded to etiocolanolone. The latter, together with androsterone and epiandrosterone, are thought to represent catabolic products (17-ketosteroids) having very little (if any) androgen potency. They can be readily eliminated, mostly with urine but also with bile, following conjugation with glucuronic or sulphuric acids in liver and, to a lesser extent, in kidney. These metabolites may be also

Figure 6. Pathways of androgen metabolism. Both reductive and oxidative pathways are illustrated. In the former, testosterone is converted to dihydrotestosterone (DHT) via the 5α-reductase (5α-Red) enzyme; DHT can be in turn metabolised to 3α/3β-androstanediols (3α/3β-diols) through the 3α/3β-hydroxysteroid dehydrogenase enzyme (3α/3βHSD). In the latter, testosterone is oxidised to androstenedione via the 17β-hydroxysteroid dehydrogenase (17βHSD); androstenedione can be further converted to 5α-reduced metabolites of the 17keto series, including 5α-androstanedione (5α-Adione), androsterone and its epimer epiandrosterone (EpiA), through 5αRed and 3α/3βHSD enzymes. As can be seen, the 17βHSD activity presides over the cross-link between reductive and oxidative pathways of androgen metabolism.


OXIDATIVE PATHWAY
REDUCTIVE PATHAWY
17ßHSD
Androstenedione
Testosterone
5αRed
5αRed
17ßHSD
5α-Adione
DHT
3ßHSD
3ßHSD
17ßHSD
3αHSD
3αHSD
EpiA
3ß-diol
17ßHSD
Androsterone
3α-diol

converted to their 17β-hydroxy derivatives, to give rise to either 3α or 3β androstanediols.

3.2.3. Steroid conjugates

Metabolism of blood-borne steroid hormones also includes their conjugation to form extremely polar water-soluble derivatives, such as sulphates and glucuronides. This metabolism produces steroid conjugates which can be freely eliminated through urine and/or bile. There is still some question as to whether these polar metabolites serve other biological functions. In the past, the enzymic reactions which control formation of steroid conjugates were assumed to be the sole prerogative of intrasplancnic organs, such as liver and kidney. However, it has been observed that these reactions may well occur also in peripheral target tissues and cells [Pack & Brooks, 1970; Buirchell & Hähnel, 1975].

Sparse experimental evidence indicated that other forms of steroid conjugates, which are even less polar than the free parent hormones, exist. In particular, formation of acetate and fatty acid esters of testosterone was documented in rat mammary and brain homogenates [King *et al.*, 1964; Kishimoto, 1973]. Although this was first ascribed as an artifact of *in vitro* incubation, more recent reports have consistently revealed the presence of endogenous hydrophobic steroid derivatives, especially oestrogens, termed lipoidal steroids.

Oestrogens

There is convincing evidence that ovaries may secrete both oestradiol and oestrone either as free steroids or as sulphate esters [Giorgi, 1967]. Oestrogen sulphates represent the major form of circulating oestrogens during the menstrual cycle [Nunez *et al.*, 1977], in postmenopausal women [Roberts *et al.*, 1980; Wyllie *et al.*, 1984; Kyprianou & Isaacs, 1989a] and in the human foetal life [Pasqualini & Kincl, 1986]. Firstly Robbins and Lipman [Robbins & Lipman, 1957] discovered that specific enzyme activities (sulphotransferases or sulphokinases) may catalyse the transfer of a sulphate group to a variety of substrates, including hormones, from the so-called "active sulphate". This compound, commonly termed PAPS, was shown to consist of a high energy phosphosulphate group esterified (through the phosphate) at the 5'-position of 3'-phosphoadenosine. The steroid sulphotransferases represent a class of enzymes which includes both 3β-hydroxysteroid [Adams & Edwards, 1968] and oestrogen [Adams, 1967] sulphotrasnferases. Presence of oestrogen sulphurylation activity was initially reported in both bovine adrenal gland [Adams & Poulos, 1967] and porcine uterus [Pack & Brooks, 1974]. Concomitant experimental evidence indicated that human mammary carcinoma tissues contain steroid sulphokinase enzyme activities [Adams, 1964]. In particular, Dao and Libby [Dao & Libby, 1971] demonstrated that steroid sulphurylation is significantly correlated with both prognosis and response to hormone-ablative manoeuvres, such as adrenalectomy, in late-stage breast cancer patients. The authors,

comparing dehydroepiandrosterone (DHEA) and 17β-oestradiol (E_2) as substrates for tumour sulphotransferases, found that a lower ratio of DHEA sulphate (DHEA-S) to E_2 sulphate is associated with a poor prognosis and response to adrenalectomy. In addition, Leung and colleagues [Leung *et al.*, 1973] showed a close correlation between levels of oestrogen sulphurylation and content of cytosolic ER in human primary breast tumour tissues. This original observation was confirmed by ensuing studies where high sulphotransferase activity was found almost exclusively in ER positive/progesterone receptor (PgR) positive human breast cancer tissues [Adams *et al.*, 1979; Pewnim *et al.*, 1980; Wilking *et al.*, 1980; Tseng *et al.*, 1983] and cells [Adams *et al.*, 1990]. Similar results have been obtained in human endometrium, where both sulphotransferase and 17βHSD enzyme activities were found to be under progesterone control [Tseng & Liu, 1981; Brooks *et al.*, 1983].

The finding that circulating oestrone sulphate (E_1S) may be readily transformed into free oestradiol in both mammary [Wilking *et al.*, 1980] and uterine [Pack & Brooks, 1970] tissues indicates that E_1S may act as oestrogen precursor in target cells containing the enzyme activity necessary to hydrolyse it (sulphatase). The same applies also to plasma DHEA-S [Prost *et al.*, 1984]. Sulphatases are membrane-bound enzymes which, contrary to what observed with sulphokinases, do not appear to be significantly correlated with either ER or PgR status of human breast tumours [Prost *et al.*, 1984]. However, studies on cultured mammary cancer cells have shown that labelled E_1S is largely

hydrolysed in hormone-dependent cell lines (MCF7, T47D) to produce free oestradiol, whilst this conversion is significantly lower in hormone-independent (MDA-MB231, MDA-MB436) cells [Pasqualini *et al.*, 1990].

Overall, the above studies support the concept that peripheral target tissues, such as breast and endometrium, may actively hydrolyse circulating steroid sulphates to yield biologically active oestrogens. The latter can be in turn sulphurylated to provide a storage pool which may contribute to maintain elevated intracellular oestrogen in hormone-dependent tissues, even in absence of an adequate blood supply of steroids.

In 1977 Hochberg and colleagues reported on the isolation of a new class of steroid conjugates, called lipoidal steroids [Hochberg *et al.*, 1977]. These nonpolar derivatives were first identified in the bovine adrenal cortex as naturally occurring metabolites of the Δ^5-3β-hydroxysteroids, pregnenolone, dehydroisoandrosterone and 17α-hydroxypregnenolone (reviewed by [Hochberg & Lieberman, 1980]). More recently, hydrophobic derivatives of oestradiol have been found to be produced by human breast tumour tissues [Schatz & Hochberg, 1981] and human mammary cancer cells in culture [Adams *et al.*, 1986]. They mainly consist of long-chain fatty acids esterified to oestradiol at the 17β position [Abul-Hajj, 1982]. Fatty acid esters of the adrenal-derived oestrogen 5-androstene-3β,17β-diol (Δ^5Adiol) have been also identified in human breast cancer cells [Adams *et al.*, 1990]. Perhaps, the most important feature of these alkyl esters of steroids resides in their superior biological

potency, as opposed to the polar steroid conjugates which have very weak or null hormonal activity. In this respect, lipoidal oestrogens represent the endogenous analogs of synthetic esters of oestrogens which have been used for decades as long-acting hormonal drugs. Lipoidal oestrogens are in fact resistant to metabolism and capable of producing a prolonged stimulation of sensitive cells.

Among all the oestrogen conjugates, lipoidal oestradiol (LE_2) is the only to maintain a full oestrogenic potency. Several experimental observations have strengthened the assumption that LE_2 may be involved in the oestrogen-primed function at target tissue level. In this respect, is there possible that this nonpolar steroid is itself oestrogenic or that it plays a role in the oestrogen action by affecting the intracellular equilibrium between oestradiol and oestrone.

Androgens

The significance of androgen conjugates was initially recognised in the early 1930s, when acid hydrolysis of human urine was found to release compounds which stimulate capon comb growth [Adler, 1934]. Subsequent studies demonstrated that adrenal DHEA-S is an important precursor of the 5α-reduced androgen conjugates in urine [van de Wiele *et al.*, 1963]. In addition, after intraveous administration of labelled testosterone to experimental animals, both androstanediols and androsterone were detected in the urine mostly as glucuronides and sulphates [Mauvais-Jarvis *et al.*, 1968].

Conjugated androgens commonly include glucuronide and sulphate conjugates of DHT, androsterone and androstanediol. Although both 3α and 3β forms of androstanediol exist, little or no 3β-reduced androgens are being found in serum or urine after administration of labelled testosterone or DHT [Mahoudeau *et al.*, 1971]. Formation of glucuronide and sulphate androgens can take place at the 17-carbon position of DHT, the 3-carbon position of androsterone and at either the 3- or the 17-carbon position of androstanediol. It must be also pointed out that the parent unconjugated steroids are readily interconverted and that this process has been shown to occur not only in the liver but also in peripheral target tissues, such as skin and prostate [Gomez & Hsia, 1968]. Chung and Coffee [Chung & Coffey, 1978] investigated formation of androgen glucuronides in minces of both normal and hyperplastic human prostate after incubation with testosterone. The authors found that testosterone glucuronide predominates in normal tissues, while DHT glucuronide prevails in hyperplastic tissues.

Although the more obvious concept is that androgen conjugation represents merely a process for hormone inactivation and excretion, the likelihood that androgen conjugates could serve as reservoir for back conversion to biologically active metabolites is still claimed. The latter possibility has recently received some support from the evidence that human genital skin contains sulphatase activity [Kaufman *et al.*, 1990]. Huot and Shain [Huot & Shain, 1988] indicated that both normal AXC/SSh rat ventral prostate and clonally derived AXC/SSh rat

prostate cancer cells actively metabolise either oestrone sulphate or oestrone glucuronide to oestrone, whilst neither normal nor neoplastic prostate cells have the ability to convert DHEA-S to DHEA. However, both benign and malignant human prostate tissues have been shown to possess steroid sulphatase enzymes, including those which preside over the production of free DHEA by cleavage of DHEA-S [Klein *et al.*, 1988]. The same authors observed that cancerous prostatic tissues retain substantial 3α/3βHSD activity, which yields androstenedione from DHEA, concluding that human malignant prostate may be able to use at least androstenedione as substrate for direct production of biologically active androgens. Interestingly, aromatase activity also has been found in human prostate [Stone *et al.*, 1986]. In this context, the finding that tamoxifen counteracts the stimulatory effect of DHEA on growth of MCF7 human breast cancer cells [Di Monaco *et al.*, 1991] appears to be worth mentioning. This evidence suggests that DHEA effects may be also mediated via aromatisation of this androgen to oestrone or oestradiol. The likelihood that prostate tumour cells could use circulating androgens (specifically DHEA or its sulphate) to produce intraprostatic oestrogens through the aromatase pathway deserves attention, especially as oestrogens may have a role in both benign and aberrant growth of the human prostate.

3.3. Peripheral Uptake and Metabolism of Steroids

Although the blood supply is assumed to be the major source of tissue steroids, the ability of peripheral target tissues, such as breast and prostate, to withdraw circulating steroids, to accumulate them against gradient and to give rise to biologically active metabolites, once again emphasises the cardinal importance of local microenvironment in driving the overall metabolic function of individual tissues. Additionally, mechanisms which are involved in the regulation of such a complex apparatus of enzymatic activities are likely to be dependent on local factors; the latter may direct the metabolic fate of steroids based on the actual cell requirement rather than on the potentially available steroids from circulation. This has recently led Labrie [Labrie, 1991] to introduce the new concept of "intracrinology", signifying the intracellular processes relevant to the hormonal control of a target tissue, as opposed to endocrinology as the central homeostasis of the whole endocrine system. This scenario is even more intimate to the neoplastic condition, where diffusible trophic factors are primarily responsible for growth control of target cancer cells, especially in the hormone-refractory disease.

3.3.1. Oestrogen uptake and metabolism in human breast

Biosynthesis of active oestrogens by breast tissues commonly includes transformation of plasma-derived steroid precursor, such as androgen and oestrone sulphate, into

oestradiol. The mechanisms involved in the local production of oestrogens in breast tumour tissues have been investigated using tissue homogenates, tissue cultures and animal models. Three main enzyme systems have been identified: the aromatase, the sulphatase/sulphokinase and the 17βHSD.

Aromatase

The process by which an androgen precursor is converted to oestrogens is called aromatisation. Concerning human breast tissues, this process is mostly represented by transformation of circulating androstenedione to E_1 through the aromatase. Most of our knowledge on the enzymology of this transformation comes from studies on the human plancental aromatase. This enzyme complex consists of two protein components, a flavoprotein NAPH-cytochrome P-450 reductase and a member of the cytochrome P-450 gene superfamily known as aromatase P-450 [Thompson & Siiteri, 1974]. The aromatisation of the steroid aliphatic A ring into an aromatic ring is unique in biology and is exemplified only in the oestrogen biosynthesis [Fishman, 1988]. This reaction is completely stereospecific and involves the loss of both the C19 methyl group and the 1β and 2β hydrogens, while those at 1α and 2α positions are retained. It is conceivable that this precise sequence cannot be accomplished in one single step, but is rather achieved by three separate hydroxylation processes. However, it is still uncertain whether three different enzymes or one enzyme having three distinct catalytic sites are involved.

Aromatisation of androgens to yield oestrogens occurs in several sites in the human, mainly the ovaries in woman and the testes in man; quite importantly, however, this reaction may also take place in other tissues, such as prostate and fat. Presence of aromatase activity has been repeatedly documented in both normal and tumour breast tissues [Miller *et al.*, 1982; Santen, 1986; Vermeulen *et al.*, 1986; James *et al.*, 1987]. The proportion of aromatase-containing tumours is ranging between 60 and 85% [Miller, 1986; Lipton *et al.*, 1987], the measured activity being comparable or higher than that found in other peripheral tissues [Abul-Hajj *et al.*, 1979]. Aromatase enzyme has been also detected in breast fat [Perel & Killinger, 1979], as well as the adipose tissue from other body sites [Grodin *et al.*, 1973].

While in premenopausal women the major source of oestrogen is represented by the ovary, in postmenopause conversion of circulating androstenedione, mostly of adrenal origin, to E_1 could substantially contribute to the oestrogen content of tumour breast tissues. This assumption has been questioned by several investigators who claimed that kinetic characteristics of the aromatase enzyme make this route a relatively unimportant contributor to intratumour oestrogen [Bradlow, 1982; Tilson-Mallet *et al.*, 1983]. As already discussed, however, results from *in vitro* assay of enzyme activities should be taken with caution since they may not precisely predict *in vivo* events. Using precise isotopic kinetic techniques *in vivo*, Santen and coworkers calculated that the rate of aromatisation of androstenedione to E_1 ranged from 0.5 up to 10% in

postmenopausal women [Santen, 1988]. In an elegant experiment series, James and colleagues [James *et al.*, 1990] observed that, following simultaneous infusion of ^{3}H-androstenedione and ^{14}C-oestrone to postmenopausal breast cancer patients, the aromatisation route appeared to be the major biosynthetic pathway of oestrogens in some but not all cases. On the other hand, the observation that plasma levels of E_2 in patients with advanced breast cancer are substantially reduced by administration of 4-hydroxy-androstenedione, a potent "suicide" inhibitor of aromatase, clearly speaks in favour of the view that aromatase is an important source of endogenous oestrogen [Coombes *et al.*, 1984].

Conflicting reports exist on the relationship between the extent of the aromatase activity and the ER status of breast tumours. Most studies failed to find a significant association [Li & Adams, 1981; Tilson-Mallet *et al.*, 1983], whereas others detected even higher aromatase activity in the receptor negative tissues. However, Miller and O'Neill [Miller & O'Neill, 1987], using the technique of product isolation, showed a statistically significant tendency of aromatase to associate with the presence of oestrogen receptors. Similarly, Vermeulen and colleagues [Vermeulen *et al.*, 1986] reported a positive correlation of aromatase activity and the receptor content of breast tumours. In addition, patients whose tumours contain detectable levels of aromatase are more likely to respond to anti-aromatase treatment, particularly aminoglutethimide [Miller & O'Neill, 1989].

An exciting piece of evidence comes from studies of O'Neill and Miller [O'Neill & Miller, 1987]. They found that adipose tissue derived from breast tumours exhibits significantly higher aromatase activity with respect to breast fat taken from women having benign breast disease. Furthermore, the authors compared aromatase levels in adipose tissue from the periphery of each breast quadrant of twelve consecutive mastectomy samples from patients having breast cancer. Interestingly, the highest level of aromatase activity was constantly found in the quadrant which contained clinically manifest tumour, whilst the quadrant with the lowest activity never showed macroscopic tumour. This evidence lends itself to some conjecture. Although fat from breast quadrants bearing tumour might have the highest aromatase activity because of microscopic tumour involvement, no neoplastic foci could be demonstrated in the fat adjacent to that taken for aromatase assay. Rather, it has been suggested that breast tumours may secrete polypeptide factors which can in turn induce or stimulate the aromatase activity in the surrounding adipose tissue [Simpson & Mendelson, 1989]. Alternatively, a regional increase of aromatase activity could represent a pre-cancer condition, whereby locally increased E_2 concentration may lead to malignant growth at that site. The authors concluded that, irrespective of any possible explanation, this evidence suggests that breast tumour development is associated with significant changes of steroid metabolism in the local environment.

Sulphatase/Sulphokinase

Another important source of oestrogen for peripheral target tissues is provided by circulating E_1S. During the human menstrual cycle, plasma levels of E_1S are 5 to 10 times those of unconjugated oestrogens (E_1 and E_2), while E_1S represents the major circulating oestrogen in the postmenopausal age. Remarkable amounts of E_1S are being found in mammary cancer tissues [Hawkins *et al.*, 1985]; correspondingly, several reports have indicated that both sulphatase and sulphokinase enzymes are present in human breast tumours [Prost *et al.*, 1984; Santner *et al.*, 1984]. Therefore, there is appropriate background to assume that sulphate and unconjugated oestrogens are in a continuous equilibrium in breast cancer cells.

Sulphatase enzyme appears to be rather ubiquitous, being found in adipose, benign and cancerous human breast tissues [Hawkins *et al.*, 1985]. Similarly, sulphokinase activity can be detected in several types of breast samples, although different forms of the enzyme appear to be present [Adams *et al.*, 1979].

Hydrolysis of E_1S to E_1 through breast sulphatase may represent a major route by which intracellular E_1, once produced, is further converted to E_2 by the 17βHSD activity. There is evidence that sulphatase levels in human breast tumour tissues are much higher than those of aromatase [Santner *et al.*, 1984; Santner *et al.*, 1986]. Although the substrate affinity of sulphatase is lower than that of aromatase, the amount of oestrogen formed via the sulphatase pathway is about ten times greater than that produced through aromatase [Santner *et al.*,

1984]. In order to ascertain whether or not the sulphatase pathway holds a biological significance, Santner and colleagues [Santner *et al.*, 1986] measured the effects of both E_1S and E_2 on colony formation of NMU rat mammary tumour cells (which are both oestrogen- and prolactin-dependent). At 10^{-8}M, E_1S induced an increase of number of colonies which was comparable to that obtained after exposure to 10^{-10}M E_2; similarly, 10^{-6}M E_1S produced about the same effect as 10^{-8}M E_2. In addition, Santen [Santen, 1986] observed that rats bearing NMU mammary tumours experience a complete tumour regression after castration, while intact animals double tumour size over a period of 14 days; using an Alzet mini-pump as delivery system for steroid hormones, the author found that castrated rats exhibited a reappearance of tumour following infusion with either 100 nmol/hour E_2 or 1000 nmol/hour E_1S. The latter was shown to be converted to E_2 in this system. This evidence clearly supports the view that circulating E_1S may behave as a biologically active oestrogen, provided that the target cell retains the enzyme apparatus which is required to yield free E_1 and hence to convert it to E_2.

Further complements to this issue come from *in vitro* studies. Initially, Vignon and associates [Vignon *et al.*, 1980] demonstrated that MCF7 human breast cancer cells convert E_1S to E_1 and that this pathway is responsible for the oestrogenic effect of E_1S on protein synthesis. More recently, Pasqualini *et al.* [Pasqualini *et al.*, 1990] indicated that different oestrogen-3-sulphates (E_1-3S, E_2-3S and E_3-3S) may exert important biological

effects on several mammary cancer cells in culture, while oestrogen-17-sulphates appear to be inactive. Presumably, cells may actively hydrolyse sulphates at the C3 but not the C17 position. In addition, after 24 hours incubation with ^{3}H-E_1S, most of precursor is converted to unconjugated E_2 in hormone-dependent cell lines (MCF7, T47D), whilst very little conversion (if any) occurs in hormone-independent mammary cancer cells (MDA-MB231 and MDA-MB436). It was concluded that sulphatase activity seems to be strictly related to the hormonal status of cells. Previous *in vivo* studies, however, did not show any significant association of sulphatase activity to the oestrogen or PgR status of the tumour or to the hormonal status of breast cancer patients [Prost *et al.*, 1984]. Additionally, reasonable uncertainty remains on whether hydrolysis of E_1S and DHEA-S is governed by one single enzyme or two separate sulphatase activities are involved. Studies on MCF7 human breast cancer cells documented that these reactions are mediated by different enzymes, having distinct kinetics [MacIndoe *et al.*, 1988]. Parenthetically, the same authors found that DHEA-S significantly inhibits, in a non-competitive fashion, the hydrolysis of physiological E_1S in intact MCF7 cells.

As reported above (see 3.2.3. Steroid conjugates: *Oestrogens*), breast tumour tissues and cells contain sulphokinase activity, though at levels by one order of magnitude lower than sulphatase. High sulphating activity has been found to be invariably associated with oestrogen receptor positive tumours, whereas receptor negative ones commonly have much lower

activity [Pewnim *et al.*, 1980]. This observation has been confirmed by studies on mammary cancer cell lines [Adams *et al.*, 1988b], where 3-sulphates of both E_2 and E_1 represented the major products of E_2 metabolism in oestrogen receptor positive MCF7 and ZR75-1 cells, while production of oestrogen sulphates in receptor negative cells MDA-MB231 and MDA-MB330 was either significantly lower or not detectable. This evidence, combined to the finding that oestrogen sulphates do not bind to oestrogen receptors, insinuates that the sulphokinase enzyme and the receptor may be functionally related.

17β-hydroxysteroid-dehydrogenase (17βHSD)

The 17βHSD enzyme system is responsible for the interconversion of E_2 and E_1, and for that of testosterone and androstenedione and DHEA and Δ^5androstenediol. However, data gained with the human placental 17βHSD have indicated that the affinity for E_2 is much higher than that for testosterone, the K_m values being 10 μM and 250 μM, respectively [Luu-The *et al.*, 1990a]. This enzyme activity is also normally present in several other tissues, including testis, kidney, liver, skin and ovary. Its inherent complexity is also mirrored in the presence of multiple enzyme forms, having distinct co-factor requirements, kinetics of reaction and subcellular distribution [Pollow *et al.*, 1977]. In addition, the equilibrium between E_2 and E_1, that is to say between the reductive way (E_1--->E_2) and the oxidative way (E_2-->E_1), has been shown to vary between *in vitro* [Bonney *et al.*, 1983a] and *in vivo* [McNeill *et al.*, 1986a] conditions.

In postmenopausal women, breast tumour tissues show an invariably higher (up to 20 times) E_2 concentration with respect to E_1 [James *et al.*, 1986]; conversely, E_1 is the predominant circulating steroid at that age. This evidence strongly suggests that some mechanisms would either permit E_2 accumulation in breast tumours or increase the intratumour conversion of E_1 to E_2. James and associates [James *et al.*, 1990], using infusion of radiolabelled oestrogens in postmenopausal breast cancer patients before surgery, observed that administration of either ^{3}H-E_2 or ^{3}H-E_1 produced intratumour levels of E_2 which always exceeded those of E_1. This indicates that the preferred metabolic route of radioactive oestrogens was towards E_2. Furthermore, the authors showed that the proportion of intratumour E_2 was significantly related to the plasma levels of DHEA-S, suggesting that the latter steroid could in some way modulate the direction of E_2 metabolism in breast tissues. This assumption is corroborated by the evidence that some adrenal androgens, including Δ^5-androstenediol, DHEA and its sulphate, may inhibit in a noncompetitive manner the 17βHSD activity of both human breast and endometrium [Bonney *et al.*, 1983b]. On the other hand, induction of 17βHSD activity (oxidative way) by progestins has been documented in human endometrium and in normal breast cells in culture [Tseng & Gurpide, 1975; Prudhomme *et al.*, 1984]. In the latter, however, the progestin-induced stimulation of the E_1 formation seems to occur exclusively in the presence of the same E_2, suggesting that an oestrogen-priming effect is

prerequisite for progestin action on 17βHSD activity in normal human breast [Mauvais-Jarvis *et al.*, 1990].

Circumstantial data from the literature indicate that 17βHSD levels in breast tumour tissues are related to the stage of disease. Beranek *et al.* [Beranek *et al.*, 1985] showed that the extent of 17βHSD activity in breast fat of women having breast cancer is positively and significantly associated to the size of the tumour. Furthermore, Miller and O'Neill [Miller & O'Neill, 1989] reported a significantly higher 17βHSD activity (oxidative way) in breast fat from patients whose tumours have colonised axillary lymph nodes, as compared to node-negative patients. Therefore, it would seem that a more advanced stage of disease (in terms of both tumour size and nodal involvement) and a poorer prognostic expectation are associated with a raise of 17βHSD activity in breast fat.

Additional interest comes from studies of Vermeulen and Deslypere [Vermeulen & Deslypere, 1989]. The authors found that 17βHSD activity (both directions) is remarkably higher in ER positive than in ER negative human mammary cancer; however, the oxidative pathway (E_2--->E_1) was always prevailing with respect to the reductive pathway (E_1--->E_2) in both ER positive and negative tumours. This would lead to E_1 accumulation in tumour tissues, irrespective of the receptor status. Thus, we are facing a seeming paradox in that the significantly and consistently higher E_2 concentration found in ER positive breast tumour tissues [van Landeghem *et al.*, 1985a; Thijssen *et al.*, 1986], as well as the greater E_1 content observed in ER negative

ones [Abul-Hajj, 1979] do not match the supposed prevalence of the oxidative way of 17βHSD (favouring E_1 accumulation) in these tissues. Once again, the lack of correlation between levels of endogenous oestrogens and both extent and direction of key enzyme activities of oestrogen metabolism is rather disappointing. However, it ought to be emphasised that, from a methodological standpoint, measurement of such a complex and dynamic structure as an enzyme system using crude extract and classical enzymology approach is doubtless far more artificial and puzzling than the quantitation of individual steroids in tumour tissue homogenates. Relevant points arise from studies on E_2 metabolism *in vitro* in human oestrogen-responsive and unresponsive mammary and endometrial cancer cells. Several groups [Castagnetta *et al.*, 1986b; Adams *et al.*, 1988b; Pasqualini *et al.*, 1990; Carruba *et al.*, 1994] have in fact found a significant association between the ER status of cells and their metabolic behaviour, in that ER-positive hormone responsive cells favour the reductive pathway of 17βHSD leading to E_2 accumulation, while ER-negative non-responsive cells favour E_2 oxidation to E_1. This pattern has been consistently shown in our laboratories using variable incubation times with physiological concentrations of labelled E_2 or E_1 used as precursors and subsequent HPLC analysis to separate and identify radioactive steroid metabolites [Castagnetta *et al.*, 1991a]. This procedure, which is highly sensitive and reproducible, allows us to simultaneously measure multiple enzyme activities of both oestrogen and androgen metabolism in intact cultured cells; it also allows us to evaluate a

wide spectrum of steroid metabolites as either conjugated (sulphates and glucuronides) or unconjugated compounds. Kinetic parameters relevant to the main enzyme activities of steroid metabolism have proved to be far from those assessed using classical enzymology, suggesting that the latter approach is unlikely to predict the factual enzyme function *in vivo*.

Preliminary observation has also suggested that 17βHSD activity may substantially change in different types of breast tissues [Santner *et al.*, 1986]. Both membrane-bound and soluble activities have been shown to occur in breast fat and glandular tissue [Tait *et al.*, 1989]. In particular, different soluble forms, three in the adipose tissue and two in the glandular tissue of human breast, have been encountered. James and colleagues [James *et al.*, 1990] embarked on a study of both soluble and membrane 17βHSD forms in normal and tumour breast tissues and cells. Both adipose and glandular breast specimens revealed at least two different soluble activities, having molecular weights of 30 and 70 kDa and markedly distinct substrate and/or co-factor requirements. Using Sephadex G-200 chromatography, in the presence of E_2 as substrate and NAD or NADPH as co-factor, the authors found that the 30-kDa form is predominant in both breast adipose tissue and isolated stromal cells, whereas in MCF7 and T47D human epithelial mammary cancer cells the 70-kDa form prevails. Both C19 and C21 steroids, including DHEA-S and progesterone, significantly inhibited the 30-kDa enzyme activity, whilst they did not affect the higher molecular weight form. On the other hand, kinetic analysis of the membrane-bound 17βHSD

activity, revealed that in normal and cancerous breast tissues the K_m for E_2 ranged from 1 to 5 μM, while a lower affinity (K_m of 25-40 μM) enzyme was present in the membrane fraction of both fibroblasts and epithelial MCF7 or T47D cancer cells. The addition of conditioned medium from cultures of breast fibroblasts to MCF7 cells induced a significant increase in the conversion of E_1 to E_2 (reductive way), with a corresponding rise in the activity of the soluble 30-kDa form of the 17βHSD, while very little effect on the oxidative direction (E_2--->E_1) was seen. In summary, the above evidence indicates that at least two major forms of both soluble and membrane-bound 17βHSD exist and that these two forms can be distinguished on the basis of molecular weights, substrate affinity and specificity, co-factor requirements. In addition, the 30-kDa form seems to be susceptible to a variety of stimuli, while the 70-kDa form appears to be refractory to these factors. Interestingly, the 30-kDa form is likely to correspond to the 17βHSD reductive activity, while the precise functional role of the 70-kDa form is more obscure. It might be that the latter form governs the oxidative 17βHSD pathway or that it presides over C19 steroid oxo-reductive pathways. A molecular biology approach has recently allowed isolation of two distinct 17βHSD mRNAs [Poutanen *et al.*, 1992] and two "in tandem" human 17βHSD genes, located on chromosome 17, have been sequenced [Luu-The *et al.*, 1990b]. All this body of evidence supports the view that 17βHSD is a complex of multiple enzyme moieties having different kinetics, cellular localisation and regulatory function of E_2 metabolism.

3.3.2. Enzymes of androgen metabolism in human prostate

It has long been recognised that DHT accumulates in nuclei of prostate target cells and that androgenic regulation of prostatic cell function is principally mediated through DHT binding to specific receptor sites [Anderson & Liao, 1968; Bruchovsky & Wilson, 1969]. Since DHT is not a significant product of either gonadal or adrenal secretion, it is clear that local metabolism is primarily involved in the androgen action. Therefore, studies of the enzyme functions which influence intracellular DHT levels may hold a striking interest, especially in the view that both benign hyperplasia and cancer of the human prostate remain, at least in part, under endocrine control (for a review see [Griffiths *et al.*, 1991]).

Unfortunately, several aspects limit the biological interpretation of the *in vitro* enzyme analysis. First of all, there is evidence that distinct enzyme forms, having different properties and subcellular localisation, may exist in either normal or pathological human prostate [Rennie *et al.*, 1983; Hudson, 1984]. In second place, the quantitative estimates of the enzyme kinetic data (K_m and V_{max}) are currently carried out under conditions that are optimised to yield maximum conversion rates. This condition largely differs from physiological environment of pH, temperature, co-factor, substrate concentration, and enzyme inhibitors, *in vivo*. For prostate studies, it has been proposed that, given the generally low substrate concentration compared to the observed K_m, the velocity of the reaction is directly proportional

to and therefore better expressed by the ratio V_{max}/K_m [Krieg *et al.*, 1983].

Sulphatase

As already discussed for oestrogen metabolism in human breast tumour tissues (see 3.3.1.: *Sulphatase/Sulphokinase*), this enzyme complex catalyses the cleavage of steroid sulphates, such as DHEA-S and E_1S. It is normally present in various human peripheral tissues, including liver, lung, brain, endometrium and placenta. Early studies have documented that human prostate may readily hydrolyse both DHEA-S and E_1S [Farnsworth, 1973; Carlström *et al.*, 1980] and therefore use these steroid precursors for further conversion to biologically active metabolites of both androgens and oestrogens. However, some question remains on whether cleavage of DHEA-S or E_1S is governed by one or two different enzyme activities; some authors concluded that one only enzyme is involved [Klein *et al.*, 1989], but the diverse K_m (μM) and V_{max} (nmol/h/mg DNA) values therein reported (respectively, E_1S: 8.7 and 47.4; DHEA-S: 4.3 and 8.4) suggest otherwise. Since plasma levels of both DHEA-S and E_1S are elevated and the ratio V_{max}/K_m for this enzyme activity is comparatively high in prostatic tissues, even low uptake by prostatic cells could substantially contribute to the intratissue pool of unconjugated steroids. Indeed, higher tissue content of both DHEA and E_1 with respect to plasma levels has been found in human prostate [Bartsch *et al.*, 1986]. It is still unclear,

however, whether these steroids act as precursors or may exert a direct effect on prostate cell function.

3β-hydroxysteroid-dehydrogenase/$\Delta^{4,5}$-isomerase

This isomerase enzyme complex is commonly present at high activity in steroidogenic organs. It presides over conversion of both DHEA to androstenedione and Δ^5Adiol to testosterone. Firstly, Harper and colleagues [Harper *et al.*, 1974] detected isomerase activity in human prostate, although the extent of reaction was very low. More recent studies [Bartsch *et al.*, 1990], using HPLC separation of steroid metabolites, showed that human BPH tissues convert DHEA to androstenedione in the presence of NAD as co-factor. However, the low isomerase activity consistently found in both normal and pathological human prostate suggests that this reaction is rate-limiting.

5α-reductase

With no doubt this enzyme system holds a central role in the androgenic regulation of both regular and diseased prostate. It was first described by Farnsworth and Brown [Farnsworth & Brown, 1963] and subsequently found mainly in the nuclear membrane fraction of prostatic tissues [Houston *et al.*, 1985; Bruchovsky *et al.*, 1988]. It converts Δ^4-steroids, including testosterone and androstenedione, to the respective 5α-reduced derivatives, i.e. DHT and 5α-androstanedione (5α-Adione).

The intracellular DHT concentration is primarily regulated by the 5α-reductase enzyme in conjunction with the 3α/3βHSD,

which catalyses the reduction of DHT to 3α and 3β-diols. These two enzyme systems have been shown to possess significantly higher V_{max}/K_m ratio values than all other enzymes involved in prostatic androgen metabolism [Bartsch *et al.*, 1990].

Previous studies on castrated rats treated for two weeks with silastic implants releasing testosterone, androstenedione or DHT, revealed that, regardless of the androgen infused, prostate tissues always contained high levels of DHT [Bartsch *et al.*, 1983]. This suggests that androgen metabolism in rat prostate is almost exclusively directed towards DHT and that other enzyme activities (e.g. 17βHSD) concur in this process. Both epithelial and stromal components of the prostate may significantly contribute to DHT formation, the 5α-reductase activity being found to be evenly distributed between the two compartments [Sirett *et al.*, 1980; Bolton *et al.*, 1981].

The question whether or not alterations in DHT formation and degradation are involved in development of both BPH and prostatic carcinoma may be properly addressed by studies comparing androgen metabolism and content in normal, hyperplastic and cancerous prostatic tissues. In the past, these studies have been crucially hampered by the complication of obtaining suitable specimens of normal prostate. Further, the evergrowing practice to use transurethral resectomy, instead of open surgery, as palliative, disobstructing manoeuvre in prostate cancer patients has made it hard to get undamaged cancer samples of appropriate size. Nonetheless, a number of studies have investigated either DHT content or 5α-reductase activity in

BPH and prostate cancer tissues as opposed to regular prostate. Unfortunately, results from these studies are largely at variance.

DHT levels were earlier reported to be significantly (3 to 4 fold) higher in BPH than in normal human prostate [Geller *et al.*, 1976; Hammond, 1978; Morfin *et al.*, 1978; Isaacs *et al.*, 1983]. This evidence, however, was subsequently challenged by Walsh and colleagues [Walsh *et al.*, 1983], who did not find a supranormal tissue content of this androgen in hyperplastic prostate. If correct, this finding would suggest that, in spite of the reduction of circulating testosterone with increasing age, DHT levels in BPH remain unaltered through a compensatory increase of the 5α-reductase activity. The latter possibility, however, is still debated. By contrast, more recent reports [Bruchovsky *et al.*, 1988; Bartsch *et al.*, 1990] documented that normal and hyperplastic prostate exhibit comparable levels of 5α-reductase activity. It should be pointed out that this apparent discrepancy may well derive from methodological pitfalls. In fact, while some earlier studies used conditions which approximate the *in vivo* state (fresh prostate tissue slices unsupplemented with pyridine-nucleotide co-factors), others measured enzyme activities in more artificial environments (tissue homogenates supplemented with NADPH as co-factor), which do not allow function of the regulatory mechanisms existing in intact cells and the utilisation of endogenous co-factors. Bartsch and colleagues [Bartsch *et al.*, 1987] claimed that, since 5α-reductase activity found in BPH tissues was similar to that observed in normal prostate, differences in DHT concentration between the two groups should

be better ascribed to other age-related events. In particular, it has been suggested that androgen receptors could be responsible for increased nuclear "trapping" of DHT in BPH, postulating that at that site DHT is protected from further metabolism since the 3α/3βHSD is mainly located outside the nucleus [Bartsch *et al.*, 1990]. This assumption, however, is rather unlikely since either DHT concentration (in the order of pmol/mg DNA) or extent of 5α-reductase activity (in the order of nmol/h/mg DNA) are far exceeding the androgen receptor content (in the order of fmol/mg DNA) of BPH tissues.

There is overall consensus that 5α-reductase activity is distinctly decreased in human prostatic carcinoma with respect to both normal and hyperplastic tissues [Bartsch *et al.*, 1990; Klein *et al.*, 1991]. This phenomenon may be also a reflection of either the preponderance of epithelial cancer cells having comparatively low enzyme levels or the process of de-differentiation leading to loss of 5α-reductase activity. More importantly, however, overall decrease of 5α-reductase in prostate cancer tissues may be the outcome of reduced enzyme activity in intracarcinomatous stromal cells, wherein substantial amounts of 5α-reductase are located. This issue has been recently approached by Klein *et al.* [Klein *et al.*, 1991], who measured hydroxyproline concentration to approximately quantify the stromal contribution to the overall tumour volume. Although a significantly decreased hydroxyproline content was found in prostate cancer tissues with respect to surrounding benign areas, this reduction was too small to account for the

remarkable differences observed between 5α-reductase activity of benign and malignant samples. More, epithelial and stromal compartment revealed parallel contribution to the overall losses of this enzyme activity in prostate tumours. Surprisingly, in spite of the decrease of 5α-reductase, unchanged or even higher DHT concentrations have been found in malignant with respect to benign prostate tissues [Bartsch *et al.*, 1990; Klein *et al.*, 1991]. On the other hand, intratumour testosterone levels were significantly higher than in nonmalignant prostate. This finding has been interpreted once more as a consequence of DHT accumulation in nuclei of prostate cancer cells through binding to intact androgen receptors. However, the same uncertainty manifested above for BPH also applies here.

This conflicting body of evidence is further complicated by the fact that human prostate might contain several types of 5α-reductase isoenzymes [Rennie *et al.*, 1983]. Martini and associates [Martini *et al.*, 1990] demonstrated that ageing decreases conversion of testosterone to DHT in the rat prostate, without altering the production of 5α-reduced derivatives of the 17-keto series, namely 5α-Adione and androsterone (A), from Δ^4-androstenedione (Δ^4Ad). In addition, the authors found that administration to rats of 4-hydroxy-4-androstene-3,17-dione (4OH-A), an inhibitor of both 5α-reductase and aromatase prostatic enzymes, significantly reduces formation of the 17-OH series metabolites (DHT and 3α/3β-diols), while the 5α-reduction yielding the derivatives of the 17-keto series is even increased. The latter observation was further corroborated by the finding

that, using Δ^4Ad as labelled precursor in either presence or absence of 4OH-A, the conversion to 5α-reduced metabolites of 17-keto series was not significantly inhibited. Therefore, it was concluded that rat prostate may contain two distinct 5α-reductase isoenzymes, having differential sensitivity to both ageing and 4OH-A.

Presence of two separate 5α-reductases in nuclei of prostatic stromal and epithelial cells has been recently reported [Hudson, 1987]. The apparent K_m values for stromal enzyme were found to be 10-fold higher than those of the epithelial form, indicating that the latter has greater substrate affinity; on the contrary, stromal activity exhibited V_{max} values up to 5 times those observed for the epithelial enzyme, suggesting that either stromal 5α-reductase activity has higher velocity of substrate-enzyme complex degradation or its tissue concentration is greater. Support to the possibility that different forms of 5α-reductase activity coexist in the epithelial compartment itself comes from recent *in vitro* studies carried out in our laboratories on human prostate cancer cell lines. Incubation of hormone-responsive LNCaP cells with tritiated testosterone used as precursor yielded comparatively high DHT formation (reductive pathway), also accompanied by production of Δ^4Ad and 5α-reduced compounds of the 17-keto series (5α-Adione and A). By contrast, androgen nonresponsive PC3 cells, under exactly the same experimental conditions, metabolised a high proportion of testosterone mainly to Δ^4Ad, with a consequent conversion to both 5α-Adione and A (oxidative pathway), while no detectable

DHT formation was observed [Castagnetta *et al.*, 1994]. This pattern was consistently obtained between 24 and 96 hours incubation. This evidence indicates that LNCaP and PC3 cells, although both possess 5α-reductase activity, preferentially turn testosterone metabolism to the reductive or the oxidative pathway, namely towards DHT or Δ^4Ad formation, respectively. This suggests that at least two distinct 5α-reductase isoforms exist and that they may be differently expressed or regulated in these cell lines.

Aromatase

Although it has been suggested that oestrogens may participate in the pathogenesis of prostate diseases, including BPH and cancer, patterns of oestrogen formation and metabolism in the human prostate are still uncertain. Since levels of circulating oestrogens in the older male are about one hundred times lower than those of testosterone, it seems more likely that prostatic tissues use androgen blood supply for local oestrogen production. Therefore, attention has been drawn to the presence of aromatase enzyme in the prostate. Thus far, however, results from several studies are highly controversial in this respect [Smith *et al.*, 1982; Stone *et al.*, 1986]. Nonetheless, more recent reports have readdressed this issue using new, accurate methodologies. Fishman [Fishman, 1988] observed that normal human prostate actively aromatises Δ^4Ad to yield both E_1 and E_2 and that this activity is substantially increased in BPH tissues. Correspondingly, Martini and colleagues [Martini *et al.*, 1990]

evaluated aromatase activity in pools of human BPH tissue homogenates using the amount of tritium released in the aqueous phase as a measure of the conversion of 1β-^{3}H-androstenedione to E_1. The authors found that this enzyme activity is present at a significant rate in BPH tissues, with an affinity which is lower than that reported for other tissues (rat brain, human placenta). Moreover, BPH aromatase proved to be highly sensitive to 4OH-A, which is known to inhibit the aromatisation process in placental microsomes [Brodie *et al.*, 1987].

These data reinforce the view that androgen-derived oestrogens may basically contribute to the hormonal microenvironment of prostatic tissues and therefore be potentially important in development of pathological entities.

17β-hydroxysteroid dehydrogenase

Presence of 17βHSD activity has been earlier reported in both normal [Acevedo & Goldzieher, 1965a] and diseased [Acevedo & Goldzieher, 1965b] human prostate gland. It is mainly located in the microsomial fraction of prostate epithelial cells, although is also present in the stromal compartment. This enzyme complex, other than governing E_2 and E_1 interconversion, directs the crosslink between oxidative and reductive pathways of androgen metabolism. In the reductive side, it converts some 17-ketosteroids, including DHEA, androstenedione and E_1, to the respective 17β-hydroxy derivatives, namely Δ^5Adiol, testosterone and E_2. It is worth noting that cleavage of both DHEA-S and E_1S, followed by 17β-reduction to yield respectively

Δ^5Adiol and E_2, eventually lead to formation of oestrogenic steroids, suggesting that this pathway is mainly involved in regulation of oestrogen milieu in the prostate. In a recent study, Tunn and colleagues [Tunn *et al.*, 1993] determined the kinetic parameters of the 17βHSD enzyme complex in testosterone and Δ^4Ad interconversion in epithelial and stromal compartments of normal and hyperplastic human prostate. Both K_m and V_{max} values obtained are in favour of the presence of a heterogeneous enzyme system, with a relative prevalence of the reductive side (Δ^4Ad--->testosterone) of the reaction, particularly in BPH stroma. At this site the balance of this reversible transformation is thus potentially shifted towards testosterone. However, since the capacity of the conversion is much lower than that of other prostatic enzymes, such as 5α-reductase, it remains unclear whether testosterone formation from Δ^4Ad significantly contributes to the intraprostatic levels of this androgen, *in vivo*.

4. Peptide Growth Factors

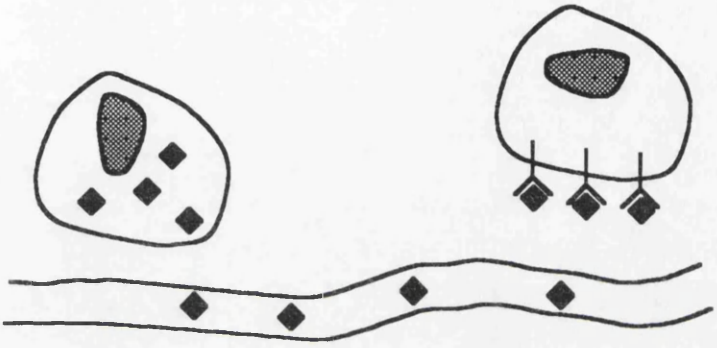
4. Peptide Growth Factors

4.1. Peptide Regulatory Factors (PRFs)

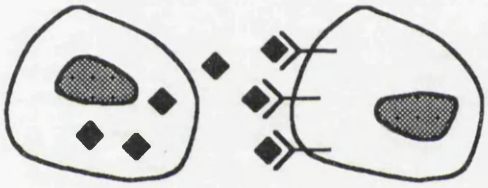
The term PRFs describes a class of peptide signalling factors which share certain basic properties: (a) low molecular weight (usually below 80 kDa); (b) specific high-affinity cell surface receptors; (c) short or intermediate range of action at very low concentration (10^{-9} to 10^{-11}M) (d) involvement in the control of cell differentiation and/or proliferation. For clarity, these "growth factors" would be kept apart from cytokines, which are commonly defined as a subset of peptides having immunoregulatory effects. However, this distinction is becoming blurred since many cytokines have been shown to affect also growth of non-immune cells.

PRFs are commonly thought to play a central role in the so-called *paracrine* and *autocrine* regulation [Sporn & Todaro, 1980], as opposed to the endocrine control of cell growth and differentiation exerted by circulating hormones (see Figure 7). In the *paracrine* and *autocrine* loops, PRFs act respectively on the immediate vicinity cells or upon the producing cells themselves. The *autocrine* model was initially proposed as a mechanism by which transformed cells may escape the growth dependence on exogenous supply of both hormones and growth factors. However, it is now clear that many normal cell types produce PRFs that can actively regulate their own growth and

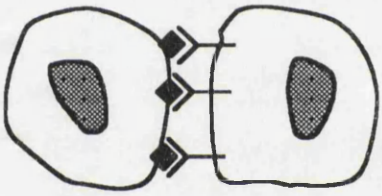
Figure 7. Models for cell growth control. Different mechanisms by which hormones or polypeptide growth factors regulate growth of target cells are illustrated. In the *endocrine* mode of action, hormones are produced by cells in distant organs and act upon target cells following delivery into the bloodstream. In the *paracrine* and *autocrine* loops, peptide growth factors act respectively on the immediate vicinity cells or upon the producing cells themselves. Two additional models are here depicted. The *juxtacrine* model involves cell-cell contacts whereby growth-regulating signals are primed through binding of membrane growth factors to specific cell surface receptors on adjacent cells. The *intracrine* model describes the intracellular mode of action of indigenous regulatory molecules which are produced and act inside the cell.



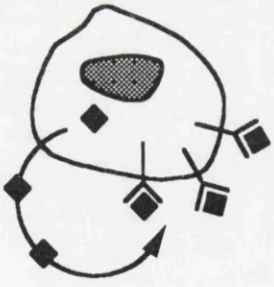
ENDOCRINE



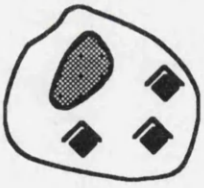
PARACRINE



JUXTACRINE



AUTOCRINE



INTRACRINE

differentiation. Additionally, there is evidence that autostimulatory signals may act within the cellular compartments where protein synthesis occur. This has led to introduce the term "*intracrine*" to describe the intracellular mode of action of regulatory molecules produced inside the cell. An example comes from the "orphan receptors" (see 2.1.3.), namely from intracellular binding proteins that may interact with endogenous, as yet unknown, ligands as potential intermediaries in the functional control of the cell. Furthermore, cell-cell contacts, such as attachment to the extracellular matrix, may imply growth-regulating signals which are mediated through specialised cell adhesion molecules and their specific cell surface ligands [Liotta, 1986]. Overall, this bidirectional regulation can be described as "juxtacrine", indicating that intercellular connections may prime growth regulatory mechanisms through the filamentous system of the cytoskeleton.

In many instances, PRFs represent multifunctional molecules, having a range of biological effects [Sporn & Roberts, 1988]. Two main events generally occur. Firstly, post-receptor signalling pathways (see 4.3.) produce a variation of gene expression; in the second place, ligand-receptor complexes are internalised and degraded, leading to down-regulation of the receptor function.

This dogma, however, has been recently challenged by the suggestion that some PRFs may also act directly at specific nuclear sites [Burwen & Jones, 1987]. Another important concern is that the effects of individual PRFs are crucially dependent on

the background set by other signalling molecules; this implies that a target cell would integrate the assortment of signals received to yield a response coherent with its context. In addition, different PRFs may have divergent or additional effect on the same signalling pathway. The matter is further complicated by the indication that some PRFs can down-regulate other unrelated receptors (a process termed transmodulation) [Zachary & Rozengurt, 1985] or induce the production of other PRFs [Balkwill, 1989]. Overall, this makes much more difficult to predict confidently the net effect of specific PRFs.

In terms of cell biology, the cancer phenomenon can be described as a disorder of the cell social behaviour in multicellular organisms. This could reflect errors in external regulatory signals or a subversion of intracellular control mechanisms at genetic level. These two apparently opposed views coincide in that any alteration in the cellular genome organisation causes a distortion of the recognition of and response to external signals regulating cell growth and differentiation. In this framework, PRFs studies might contribute to the understanding of processes responsible for the appearance of a transformed phenotype.

Abnormalities of both PRFs production and membrane receptors may well lead to disorders of cell growth. Various experimental systems have documented that immortalised cell lines secrete substantial amounts of PRFs in their culture medium and that this activity may enhance cell proliferation via an *autocrine* loop [Sporn & Roberts, 1985]. The "conditioned"

medium obtained from these cultures has also been shown to promote growth of cells of totally unrelated lineages or even induce, at least transiently, a transformed phenotype in some nonmalignant cells in culture [Derynck, 1988]. On the other hand, any increase of number and/or affinity of specific cell surface receptors may result in an enhanced response to locally available PRFs. In addition, structural alterations may give rise to a constitutively active form of the receptor (e.g. truncated receptor) in the absence of ligand [Weiner *et al.*, 1989]. It is also conceivable that the intracellular signalling cascade may be altered in such a way that cellular response to normal concentrations of PRFs is exaggerated or that cell is locked in a continuous proliferative state in absence of external signals [Robertson, 1988]. This would effect the ability of cells to grow in vitro on minimal requirements of both medium an serum. Finally, cells may escape a negative growth control normally exerted by some PRFs through loss of specific membrane receptors or block of the inhibitory signal at some later point [Roberts *et al.*, 1988].

4.2. The Epidermal Growth Factor (EGF) Family

Although the first description of EGF activity dates back over 30 years, the subsequent attempts to ascertain its precise biological role and mechanism of action have remained mostly fruitless. In the early 1960s, when Rita Levi-Montalcini and

Stanley Cohen were investigating the control of nerve cell development and growth [Levi-Montalcini & Cohen, 1960], they found that snake venom containing phosphodiesterases, added to tumour cell extracts to exclude involvement of nucleic acids, exhibited itself a nerve growth factor-like activity. This evidence eventually led Cohen [Cohen, 1962] to describe this activity in mouse submaxillary gland extracts as responsible for premature eyelid opening and incisor eruption ("tooth-lid factor") when injected into neonatal mice. The present name EGF first appeared few years later, when Cohen [Cohen, 1965] documented that this molecule also promotes epidermal proliferation and keratinisation in organ cultures of embryonic skin. Following optimisation of both isolation and purification procedures, sufficient amount of mouse EGF (mEGF) was then available to determine its primary sequence [Savage *et al.*, 1972]. It was found to consist of 53 amino acids, with an approximate molecular weight of 6054, and to contain six cysteine residues. The latter represent the sites of three disulphide bonds, specifically located between Cys_6-Cys_{20}, Cys_{14}-Cys_{31} and Cys_{34}-Cys_{42}, which create three main peptide loops [Savage *et al.*, 1973]. All these three bonds seemed to be required for full biological potency of EGF [Taylor *et al.*, 1972].

Concomitantly, independent studies led to the purification of urogastrone, a substance which proved to be effective in treatment of experimentally induced ulcers in dogs, from urine of pregnant women (at low frequency of ulcers) [Gregory, 1975]. Subsequent comparative analysis of amino acid compositions

persuaded Gregory to conclude that urogastrone was the human homologue (hEGF) of mEGF.

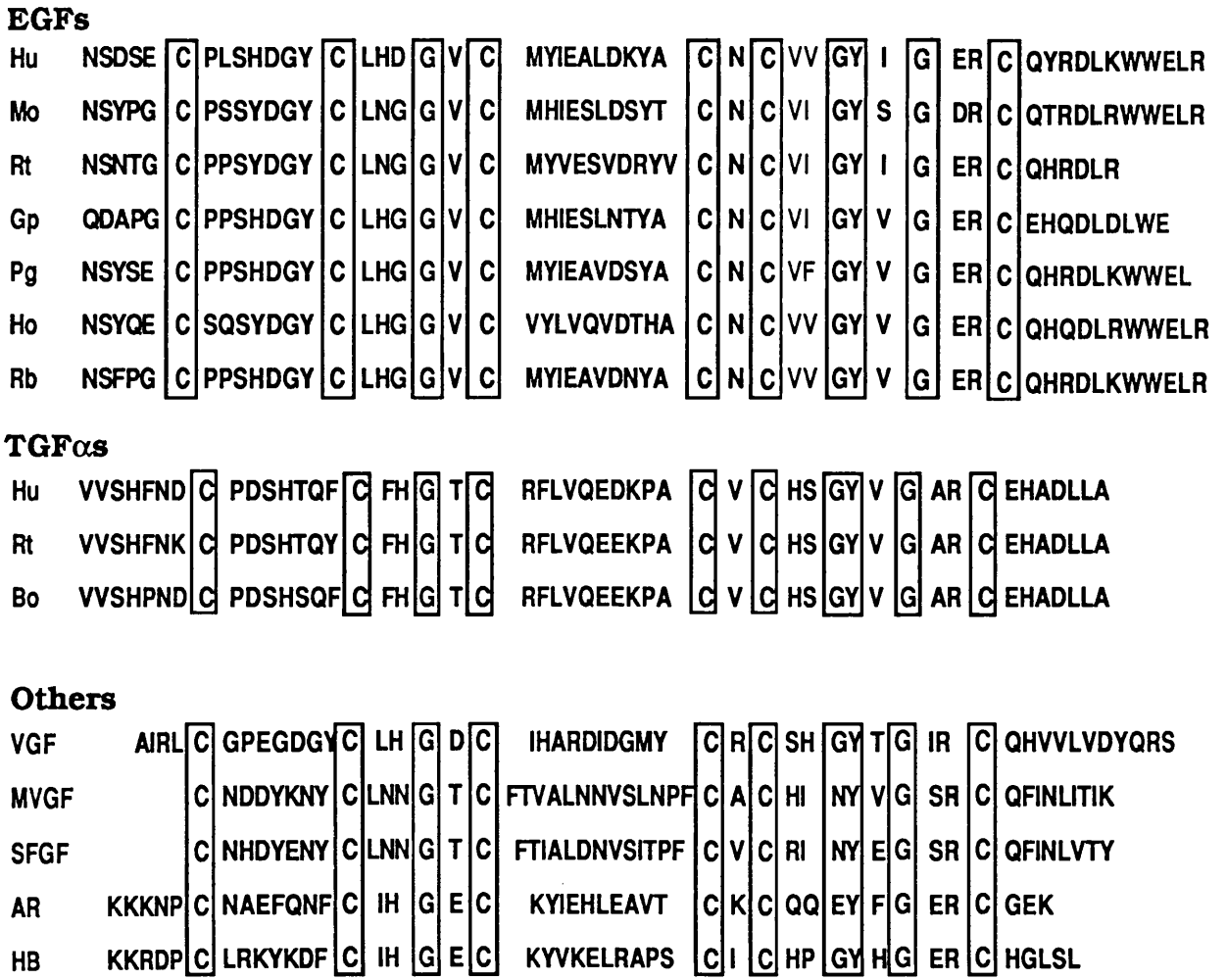
Initially, De Larco and Todaro [De Larco & Todaro, 1978] reported that retroviral transformation of 3T3 fibroblasts with the Moloney sarcoma virus was associated with the release into the culture medium of a mitogenic substance which was referred to as "sarcoma growth factor" (SGF). Further studies revealed that SGF consisted of two distinct components, termed transforming growth factor α (TGFα) and β (TGFβ). The former was found to be structurally homologous to EGF and characterised by binding to EGF receptor, the latter potentiated the clonal effects of TGFα on growth of normal rat kidney (NRK) cells, in culture. Subsequently, rat TGFα was purified from conditioned media of feline sarcoma virus (FeSV)-transformed cells and its amino acid sequence determined [Marquardt *et al.*, 1984]. TGFα is a 6-kDa polypeptide whose 50 amino acid primary structure displays homology to EGF; this includes preservation of the six cysteine residues and the resulting three disulphide bonds, with some additional conservation most notably in the third loop.

More recent reports have deduced the primary sequence of EGF from other species, including rat, guinea-pig and pig [Simpson *et al.*, 1985; Pascall *et al.*, 1991]. Comparison of the known EGF sequences points to some interesting clues (see Figure 8). Firstly, the six cysteine and other amino acid (G_{18}, G_{36}, Y_{37}, G_{39}, R_{41} and L_{47}) residues are invariant. Overall, there is 50% homology among EGF sequences, the most highly conserved region being extended from Ser_9 throughout Glu_{24}. In addition,

both N and C termini appear to be unimportant for biological activity, while a core of (4-48)EGF in all species would be sufficient in this respect. Similarly, TGFα sequence is very highly conserved in different species (human, rat, bovine) and shows 33-44% homology with EGF (see Figure 8).

Apart from the different species-specific subtypes, it is now clear that both EGF and TGFα belong to an extended family of related proteins which share the invariant spacing of six cysteine residues and the ability to bind and activate the EGF receptor. For instance, the newly discovered amphiregulin, isolated from conditioned medium of MCF7 cells treated with phorbol ester, was found to be structurally and functionally related to both EGF and TGFα [Shoyab *et al.*, 1989]. The same was true for other two proteins, the Schwannoma-derived growth factor [Kimura *et al.*, 1990] and the heparin-binding EGF-like growth factor [Higashiyama *et al.*, 1991]. Furthermore, a series of poxvirus growth factors show considerable (25-30%) homology to EGF. They include vaccinia virus growth factor (VGF) [Reisner, 1985], myxoma virus growth factor (MVGF) [Upton *et al.*, 1987], molluscum contagiosum [Porter & Archard, 1987] and Shope fibroma virus growth factor (SFGF) [Chang *et al.*, 1987]. Additionally, the EGF structural motif is also encountered in a variety of proteins not formally classified as PRFs, as well as in an assortment of distant species. This amazing array of naturally occurring EGF-like domains makes it difficult to imagine a satisfactory interpretation of their functional role. They could represent sequence motifs used to generate a number of similar

Figure 8. Amino acid sequence alignment of epidermal growth factor receptor (EGFR) ligands. Several members of the EGF family or related factors which are known to activate EGFR are included from both human and other species. Conserved residues (notably cysteine residues) are boxed. Hu, human; Mo, mouse; Rt, rat; Gp, guinea pig; Pg, pig; Ho, horse; Rb, rabbit; Bo, bovine; VGF, vaccinia virus growth factor; MVGF, myxoma virus growth factor; SFGF, Shope fibroma virus growth factor; AR, amphiregulin; HB, heparin-binding EGF-like growth factor.


EGFs
Hu NSDSE C PLSHDGY C LHD G V C MYIEALDKYA C N C VV GY I G ER C QYRDLKWWELR
Mo NSYPG C PSSYDGY C LNG G V C MHIESLDSYT C N C VI GY S G DR C QTRDLRWWELR
Rt NSNTG C PPSYDGY C LNG G V C MYVESVDRYV C N C VI GY I G ER C QHRDLR
Gp QDAPG C PPSHDGY C LHG G V C MHIESLNTYA C N C VI GY V G ER C EHQDLDLWE
Pg NSYSE C PPSHDGY C LHG G V C MYIEAVDSYA C N C VF GY V G ER C QHRDLKWWEL
Ho NSYQE C SQSYDGY C LHG G V C VYLVQVDTHA C N C VV GY V G ER C QHQDLRWWELR
Rb NSFPG C PPSHDGY C LHG G V C MYIEAVDNYA C N C VV GY V G ER C QHRDLKWWELR
TGFαs
Hu VVSHFND C PDSHTQF C FH G T C RFLVQEDKPA C V C HS GY V G AR C EHADLLA
Rt VVSHFNK C PDSHTQY C FH G T C RFLVQEEKPA C V C HS GY V G AR C EHADLLA
Bo VVSHPND C PDSHSQF C FH G T C RFLVQEEKPA C V C HS GY V G AR C EHADLLA
Others
VGF AIRL C GPEGDGY C LH G D C IHARDIDGMY C R C SH GY T G IR C QHVVLVDYQRS
MVGF C NDDYKNY C LNN G T C FTVALNNVSLNPF C A C HI NY V G SR C QFINLITIK
SFGF C NHDYENY C LNN G T C FTIALDNVSITPF C V C RI NY E G SR C QFINLVTY
AR KKKNP C NAEFQNF C IH G E C KYIEHLEAVT C K C QQ EY F G ER C GEK
HB KKRDP C LRKYKDF C IH G E C KYVKELRAPS C I C HP GY H G ER C HGLSL

protein structures, vestigial copies of an ancestral gene product devoid of any actual function or common precursors for as yet unknown growth factors.

4.2.1. Biosynthetic routes

Early reports indicated that several high molecular weight (9, 28, 30 and 74-kDa) forms of EGF exist [Taylor *et al.*, 1974; Frey *et al.*, 1979]. More recently, with the advent of recombinant DNA technology, it has been revealed that EGF mRNA is approximately 4750 base pairs and that it encodes a large precursor (called preproEGF) of 1217 amino acids (128-kDa) [Gray *et al.*, 1983; Scott *et al.*, 1983]. Correspondingly, cloning of rat cDNAs showed that TGFα is encoded by a 4.8-kilobase mRNA that includes a short 5' untranslated region of 150 nucleotides, a long 3' untranslated region of approximately 3900 nucleotides and an intermediate coding region in between [Lee *et al.*, 1985a; Blasband *et al.*, 1990]. The latter sequence encodes a precursor (termed proTGFα) 159 or 160 amino acids long.

The first 25 amino acids from the N terminus of the preproEGF are thought to behave as a "pre-signal" sequence. The mature (1-53)EGF is represented by residues 976-1029 of the precursor, although the latter remarkably includes seven more repeating EGF-like domains with some degree (20-40%) homology to EGF [Scott *et al.*, 1983]; interestingly, the most conserved region in these EGF-like segments corresponds to the 29-37 span in the "authentic" (1-53)EGF. The extreme C terminus of preproEGF shows a 20 amino acid hydrophobic region

(residues 1039-1058), down-sequence from the "authentic" EGF by just ten residues. It has been suggested that this motif acts as a transmembrane domain, in a alpha helical conformation, whose function is to arrange the bulk of the precursor as a membrane-bound glycoprotein having both carbohydrate and EGF-like domains on the external surface of the cell membrane [Scott *et al.*, 1985]. Although the most obvious concept is that EGF, under this form, is available to extracellular protease activities for local cleavage of the mature EGF, it is intriguing to speculate that preproEGF may even directly participate in cell-to-cell or cell-to-extracellular matrix interactions while it is held within the cell membrane. Using an EGF mini-gene expression system, it has been shown that the membrane-anchored form of EGF is capable of stimulating EGF receptors on adjacent cells [Dobashi & Stern, 1991].

By analogy, proTGFα has been shown to contain both a signal peptide and a second extremely hydrophobic region having the characteristics of a transmembrane domain [Derynck *et al.*, 1984]. It has been therefore suggested that proTGFα is an integral membrane glycoprotein from which the mature growth factor is cleaved through the action of an elastase-like enzyme on alanine-valine bonds at both termini. As postulated for preproEGF, these membrane-spanning precursor forms may provide a mechanism whereby growth factor action can be limited to adjacent cells or, alternatively, behave as cell recognition or adhesion molecules. There is recent convincing evidence that proTGFα binds to and activates EGF receptor on

adjacent cell surfaces, leading to immediate downstream signal transduction [Brachmann *et al.*, 1989; Wong *et al.*, 1989]. In addition, this intracellular signalling cascade has been found to result in the stimulation of DNA synthesis and transformation of NRK cells [Blasband *et al.*, 1990].

4.2.2. Tissue distribution and functional implications

The enormous literature concerning structural and functional features of both EGF and TGFα has been recently surveyed by two excellent reviews [Fisher & Lakshmanan, 1990; Prigent & Lemoine, 1992]. Although very often studies on intratissue EGF have been constrained by the low levels of either immunoreactive EGF or EGF mRNA, several reports have immunohistochemically or biochemically detected significant amounts of EGF in a range of human tissues [Kasselberg *et al.*, 1985; Kajikawa *et al.*, 1991]. Apart from submandibular salivary gland, these include different sites of the gastrointestinal tract, anterior pituitary, bone marrow, mammary gland, uterus, ovary and placenta.

The possibility that TGFα could be detected also in normal tissues was realised soon after its discovery [Roberts *et al.*, 1981]. Significant levels of either TGFα or its transcript have been found mainly in normal self-renewing epithelial tissues (e.g. skin and gastrointestinal tract) [Derynck *et al.*, 1984]. In addition, TGFα mRNA has been identified in epithelial cells from various body sites, including anterior pituitary [Samsoondar *et al.*, 1986], skin keratinocytes [Coffey *et al.*, 1987], female genital tract [Kommoss

et al., 1990], brain [Wilcox & Derynck, 1988] and others. There is today a great deal of evidence suggesting that TGFα is implicated in the process of normal development. Earlier reports showed that relatively high levels of TGFα mRNA are present in crudely dissected rat foetuses at days 8 and 9 of gestation [Lee *et al.*, 1985b]. Subsequent *in situ* hybridisation, however, revealed that this evidence was mainly a result of contaminating maternal decidua, rather than a contribution of the embryo itself [Han *et al.*, 1987]. Interestingly, TGFα expression in decidual tissues displayed an apparent gradient, the highest levels being found in the immediate proximity to the embryo. Since both decidua and embryo tissues contain EGF receptor [Chegini & Rao, 1985], it is conceivable that maternal TGFα may stimulate decidual growth and embryonic development respectively via autocrine and/or paracrine mechanisms.

4.2.3. Biological effects and cancer growth

Both *in vivo* and *in vitro* studies have demonstrated that, apart from growth regulation, EGF may have profound effects on mechanisms involved in differentiation, motility and function of target cells. For instance, implants of EGF pellets induced formation of ductal end buds in mouse mammary gland and superseded oestrogen and progesterone requirement for normal development of mammary stem cell layer [Coleman *et al.*, 1988]. Several reports also indicate that EGF may have a role in the central nervous system [Fallon *et al.*, 1984]. In particular, EGF has

been shown to support growth of cultured telencephalic neurones from the rat brain [Morrison *et al.*, 1987].

TGFα has been shown to stimulate cell migration [Barrandon & Green, 1987], angiogenesis [Schreiber *et al.*, 1986] and bone resorption [Stern *et al.*, 1985]. Additionally, TGFα stimulates both growth and migration of keratinocytes in wound healing [Schultz *et al.*, 1987]

Most of our present knowledge of the effects of both EGF and TGFα is based on *in vitro* studies. The range of either epithelial or mesenchymal cultured cells which are influenced by EGF and TGFα is astonishing. Although obvious care should be placed in extrapolating the role of these growth factors in normal and malignant cell growth *in vivo*, the pleiotrophic action of EGF and TGFα *in vitro* suggest a myriad of functions of these molecules *in vivo*. However, it should be emphasised that net effects may be both dependent on the availability of adequate EGF receptor and related to interaction with both hormones and other growth factors. In particular, it is known that bombesin, PDGF and TGFβ act in synergy with either EGF or TGFα [Rozengurt, 1986].

Insofar as cancer cell growth is concerned, there is evidence that TGFα is involved in malignant transformation, while this association is less clear for EGF.

Overproduction of both TGFα and its mRNA has been observed in transformed cultured cells [Derynck *et al.*, 1987] and in several human solid tumours, including breast [Barrett-Lee *et al.*, 1990] and prostate [LLoyd *et al.*, 1992]. Transfection of NRK

or mouse mammary epithelial cells with TGFα plasmids may produce full transformation, as assessed by anchorage-independent growth and tumourigenicity in nude mice [Watanabe *et al.*, 1987; Shankar *et al.*, 1989]. However, concurrent overexpression of EGF receptors may be required for TGFα-induced transformation of 3T3 fibroblasts [Di Marco *et al.*, 1989].

Although the potential role of EGF in neoplastic transformation is still obscure, there is a large body of literature supporting a tumour promotional activity for EGF (reviewed by [Stoscheck & king, 1986]). Elevated expression of EGF has been found in a large proportion (68%) of prostatic tumours [Fowler *et al.*, 1988], while high levels of EGF-like activity have been described in breast, endometrial and ovarian carcinomas [Bauknecht *et al.*, 1989]. Some earlier work [Kurachi *et al.*, 1985] suggested that EGF has a likely role in the onset and subsequent growth support of mouse mammary tumour.

Most importantly, however, the overall involvement of EGF and TGFα in the induction, promotion and progression of malignant cells from endocrine target tissues (such as breast and prostate) should be considered in a much wider context where the combined action of these and other peptide growth factors with steroid hormones has to be carefully taken into account. There is good evidence that production of both EGF and TGFα are under control of oestrogens and androgens respectively in human breast and prostate tumour tissues and cells (for pertinent reviews see [Lippman & Dickson, 1989; Griffiths *et al.*, 1991]).

4.3. The Transforming Growth Factor β (TGFβ) Superfamily

The main reason why research on TGFβ has attracted so fervid scientific excitement in the last two decades mainly lies in some characteristics unique to this peptide family. Firstly, unlike many other growth factors, TGFβs are largely expressed by a variety of both normal and transformed cells. Secondly, there is an exceptionally high conservation of TGFβs structure in several species. Finally, TGFβs have been implicated in a range of biological activities, including embryonic development, tissue remodelling, inflammation and wound repair, angiogenesis, immune response, apart from control of cell proliferation (reviewed by [Roberts & Sporn, 1990]). In this respect, TGFβs can be considered the prototype of multifunctional signalling molecules.

TGFβ was first described as a factor capable of inducing colony formation of NRK fibroblasts in soft agar in the presence of EGF and originally referred to as sarcoma growth factor, SGF [De Larco & Todaro, 1978]. Since similar activities were found in acid-ethanol extracts of several tumour cells, the name was changed to "transforming growth factor" [Roberts *et al.*, 1980]. It was subsequently revealed that the original SGF activity was composed by two entirely different peptides, which were hence termed TGFα and TGFβ [Anzano *et al.*, 1983].

Today, it is known that TGFβs belong to a multi-gene family of related peptides which share 30 to 40% amino acid homologies

and conservation of 7 cysteine residues. These peptides include inhibins and activins [Ying, 1988], Müllerian inhibitory substance [Josso & Picard, 1986], bone morphogenetic proteins [Wozney *et al.*, 1988], *Drosophila* decapentaplegic protein [Panganiban *et al.*, 1990], the mouse Vgr-1 gene product [Lyons *et al.*, 1989a] and the GDF-1 factor [Lee, 1990]. It has been suggested that many of these peptides act in orchestrated patterns during the embryonic development [Lyons *et al.*, 1989b], but the broad range of the biological activities exerted suggest that they are important components of several basic cellular processes.

The cellular and molecular biology of TGFβ has been extensively reviewed by recent symposia, to which the reader may rely on for reference*.

4.3.1. Biosynthesis and structure

Initially, TGFβ ($TGF\beta_1$) was purified to homogeneity from human platelets and placenta, partly sequenced and finally cloned from a human placental cDNA library [Derynck *et al.*, 1985]. These studies revealed that TGFβ was a disulphide-linked homodimer of 25 kDa, composed of two monomers of 112 amino acids each (12.5 kDa). Further studies, however, showed that monkey kidney BSC-1 cells released in their culture medium a

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* Transforming Growth Factor-ßs. Chemistry, Biology and Therapeutics. K.A. Piez & M.B. Sporn (eds). Annals of the New York Academy of Science. volume 593, 1990 (379pp).
Clinical Applications of TGF-ß. G.R. Bock & J. Marsh (eds). Ciba Foundation Symposium 157, Wiley , Chichester, 1991 (254pp).

TGFβ-related molecule, having negative autocrine growth effects [Tucker *et al.*, 1984]; this factor was later isolated and found to be identical to a second form of TGFβ, called $TGF\beta_2$, which had been previously isolated from bovine bone [Seyedin *et al.*, 1987] and human glioblastoma cells [Wrann *et al.*, 1987]. It was therefore clear that at least two different TGFβ forms (the original $TGF\beta_1$ and $TGF\beta_2$) exist. In the past few years, three more TGFβ species have been cloned by low stringency hybridisation from mammalian ($TGF\beta_3$) [Derynck *et al.*, 1988], chicken ($TGF\beta_4$) [Jakowlew *et al.*, 1988] and frog ($TGF\beta_5$) [Kondaiah *et al.*, 1990] cDNA libraries. Overall, there is 70-80% homology in the different TGFβ monomers, with a complete preservation of the 9 cysteine residues throughout, while the sequence of each mature TGFβ type is very highly conserved (98-100%) between species. $TGF\beta_1$, 2 and 3 have been found to share most of their activities, while the biological significance of the other two forms remains to be established. All these three human isoforms are thought to exist prevalently as homodimers; however, biologically active heterodimers, consisting of one chain each of $TGF\beta_1$ and $TGF\beta_2$, have been found in porcine platelets [Cheifetz *et al.*, 1987] and it would be not surprising if this were also true for human TGFβs.

$TGF\beta_1$ is synthesised as a larger 391 amino acid precursor (preproTGFβ1) of which the sequence of the mature $TGF\beta_1$ monomer comprises the C-terminal end region. Similarly, $TGF\beta_2$ and $TGF\beta_3$ derive from precursor forms respectively of 414 and 412 amino acids, the mature forms being represented by

disulphide-bond homodimers of 24 kDa [ten Dijke *et al.*, 1990]. However, only 30% homology occurs in the proregion of these different precursors. Analysis of Chinese hamster ovary (CHO) cells transfected with $TGF\beta_1$ precursor cDNA revealed that both mature and precursor forms of $TGF\beta_1$ are held in a protein complex (90-110 kDa), which consists of $preproTGF\beta_1$, the pro-region of $TGF\beta_1$ precursor and the mature $TGF\beta_1$ [Gentry *et al.*, 1987]. The proregion of the precursor form includes three main potential sites of glycosylation (Asn 82, Asn 136, Asn 176) which are thought to be essential for $TGF\beta_1$ processing and secretion [Miyazono & Heldin, 1989; Sha *et al.*, 1989]. In particular, the first two glycosylation sites contain mannose-6-phosphate (M-6P) residues which may capacitate the precursor to bind to M-6P/insulin-like growth factor II cell surface receptors [Purchio *et al.*, 1988]. This suggests that $preproTGF\beta_1$ may function differently from the mature peptide and that it may be delivered to lysosomes where its proteolytic cleavage occurs. Glycosylation sites have been found also in other TGFβ precursor types, specifically three in $TGF\beta_2$, four in $TGF\beta_3$ and one in $TGF\beta_4$. Interestingly, all TGFβ precursor forms, apart from $TGF\beta_2$, show an Arg-Gly-Asp (RGD) sequence proximal to the cleavage site which is thought to represent a recognition motif for extracellular matrix receptors of the integrin family [Ruoslahti & Pierschbacher, 1987].

An important feature of several TGFβs is that they exist in many tissues as high molecular weight latent complexes, which can be activated by transient acidification or proteolytic

treatment [Lyons *et al.*, 1988; Pircher *et al.*, 1988]. Latent TGFβ$_1$ (L-TGFβ$_1$) complex has been purified from human platelets and structurally characterised [Miyazono *et al.*, 1988]. Under non-reducing conditions it displays a molecular weight of 210 kDa and is found to consist of at least three distinct components (13, 40 and 125-160 kDa, respectively) using reducing conditions. The 13-kDa component represents the TGFβ$_1$ monomer, while the 40-kDa component corresponds to the N-terminal remnant of the precursor, lacking the hydrophobic signal sequence (residues 1-29). The third 125-160-kDa component is a novel subunit, distinct from previously described proteins, which has been termed TGFβ$_1$-binding protein (TGFβ$_1$-BP). The assembly of these components to yield the 210-kDa L-TGFβ$_1$ complex includes a series of disulphide bonds which bridge them each other; a tentative model is illustrated in Figure 9. As can be seen, the complex consists of a mature TGFβ$_1$ homodimer noncovalently associated to a dimeric form of the 40-kDa N-terminal remnant of the TGFβ$_1$ precursor (latency-associated peptide, LAP). The latter is disulphide-linked to the TGFβ$_1$-BP which is most likely monomeric. Recombinant TGFβ$_1$, 2 and 3 have been shown to occur as smaller latent complexes (approximately 105 kDa) which lack TGFβ$_1$-BP; this indicates that LAP is sufficient for latency [Brown *et al.*, 1990].

There is a subtle perplexity on the relationship existing between the TGFβ precursor forms, on one hand, and the latent TGFβ complex on the other. To my best knowledge, there is no

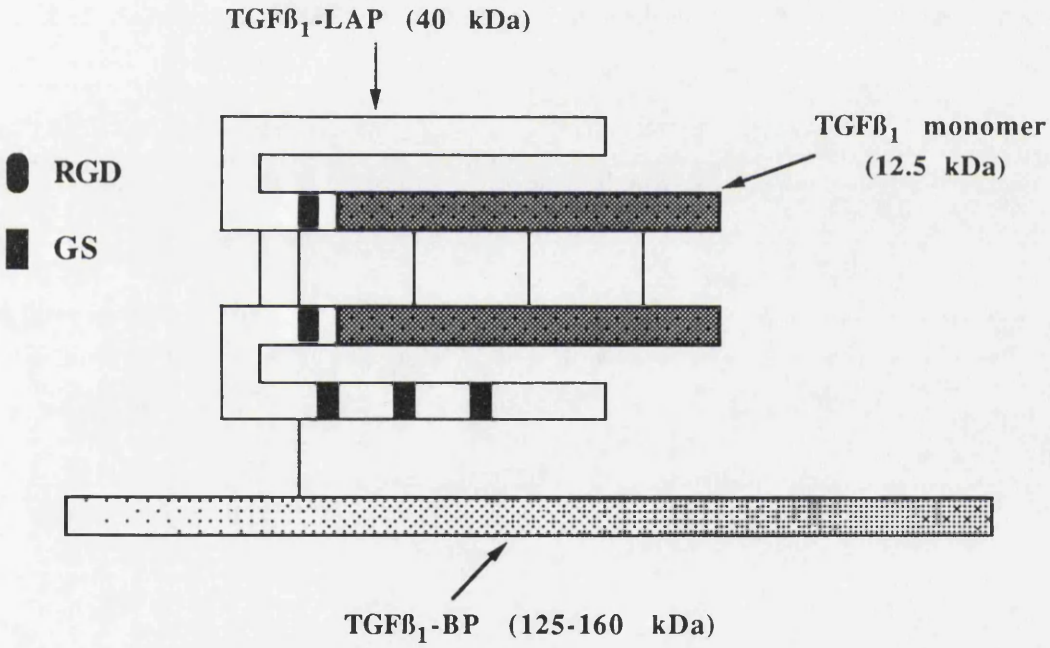
evidence in the literature on how these two entities relate each other, curiously authors tend rather to keep them separated.

TGFβ-LAPs are peptides consisting of approximately 300 amino acids that, likewise their parent precursors, contain at least three potential N-glycosylation sites and some RGD sequences (see Figure 9). The presence of these peculiar domains suggests that LAPs, alone or in complexes, may interact with specific cellular binding proteins different from the TGFβ receptors.

An interesting complement to this issue comes from the analysis of $TGF\beta_1$-BP. The latter is a 125-160 glycoprotein which has been found to contain multiple EGF-like repeats [Miyazono *et al.*, 1988]. Similarly, $TGF\beta_1$-BP purified from human foreskin fibroblasts showed a 64% of its sequence to be occupied by 16 EGF-like fragments and by three copies of a distantly related eight-cysteine motif [Kanzaki *et al.*, 1990]. The functional role of $TGF\beta_1$-BP remains to be clarified. Addition of $TGF\beta_1$-BP does not inhibit $TGF\beta_1$ binding to its own receptor, suggesting that it is not required for latency. However, $TGF\beta_1$-BP may stabilise the latent complex or take part in the biosynthesis and processing of the mature $TGF\beta_1$. Additionally, free $TGF\beta_1$-BP forms have been shown to occur in some cell types, including human platelets. This evidence, combined to the peculiar structure of this large protein (including multiple EGF-like motifs), suggests that it may also participate *in vivo* to processes unrelated to the TGFβ action.

Finally, the active forms of TGFβ can also be bound and inactivated by α_2-macroglobulin (α2M), a serum protease

Figure 9. Latent transforming growth factor-β_1 (TGFβ_1) complex. In this schematic representation a mature TGFβ_1 homodimer is disulphide-bonded to a dimeric form of the 40-kDa N-terminal remnant of the TGFβ_1 precursor (the so called latency-associated peptide, LAP). The latter is also linked by disulphide bonds to a relatively large protein, the TGFβ_1-binding protein (TGFβ_1-BP) which is monomeric. Presence of three potential N-glycosylation sites (GS) and some Arg-Gly-Asp (RGD) sequences in the LAP molecule is also indicated (for details see text). (Modified from [Miyazono, 1991 #297])


TGFß1-LAP (40 kDa)
TGFß1 monomer
(12.5 kDa)
RGD
GS
TGFß1-BP (125-160 kDa)

inhibitor [Huang *et al.*, 1988]. TGFβ-α2M complexes have been found in serum [O'Connor-McCourt & Wakefield, 1987], but is still unclear whether these complexes function like carriers to release bioactive TGFβ at relevant target sites or α2M acts locally as a scavenger for undesirable TGFβ activity, *in vivo*.

4.3.2. Biological activity

In an imaginative description, Sporn and Roberts [Sporn & Roberts, 1988] have represented the biological activity of TGFβ and other growth factors, like symbols or letters in an alphabet of a biological regulatory language, is always contextual. Indeed, TGFβ is a pointed example of multifunctional signalling peptide working in the intricate network of communication between and within cells.

It must be pointed out, however, that the multiplicity of TGFβ actions is also based on the balance of the different TGFβ isoforms in individual tissues. Although most *in vitro* assays showed nearly equivalent activity of the three human TGFβ isoforms, the evidence that in certain systems one isoform exhibits effects several times greater than the others indicates that isoform switching may have significant consequences at specific target sites. In addition, TGFβs themselves have been shown to participate in the differential regulation of TGFβ isoform expression. For instance, Bascom and colleagues [Bascom *et al.*, 1989a] have documented that in murine AKR-2B cells TGF-β_1 down-regulates expression of the mRNA for both TGFβ_2 and TGFβ_3, while TGFβ_2 up-regulates expression of all TGFβ mRNAs.

This differential regulation finds its molecular basis in the different location of the TGFβ genes (chromosomes 19, 1 and 14 respectively for $TGF\beta_1$, 2 and 3) and, especially, in the very different character of their 5' flanking regions [Roberts *et al.*, 1991].

Perhaps, the most striking effects of TGFβs are those on cell growth and differentiation and on extracellular matrix formation. Both of them are relevant to many other TGFβ actions, including embryogenesis, angiogenesis, inflammation and tissue repair, immune response, bone remodelling and cartilage formation.

Epithelial differentiation and growth inhibition

Inhibition of epithelial cells is often paralleled by their terminal differentiation. While $TGF\beta_1$ potently inhibits growth of rat intestinal, mink lung and human bronchial epithelial cells in the presence or absence of mitogens, it concomitantly induces their differentiation [Massui *et al.*, 1986; Kurokawa *et al.*, 1987].

Both $TGF\beta_1$ and $TGF\beta_2$ are the most potent growth inhibitors known for a wide range of cultured cells, including epithelial, endothelial and many myeloid cells [Bascom *et al.*, 1989b]. All the three human TGFβ species intensely inhibited growth of keratinocytes by inducing a reversible growth arrest in the G_1 phase of the cell cycle [Shipley *et al.*, 1986; Graycar *et al.*, 1989]. Since keratinocytes have specific cell surface receptors for TGFβs, release remarkable amounts of TGFβ into the culture medium and respond to addition of TGFβ, it has been proposed that $TGF\beta_1$ acts in this context as a negative autocrine growth

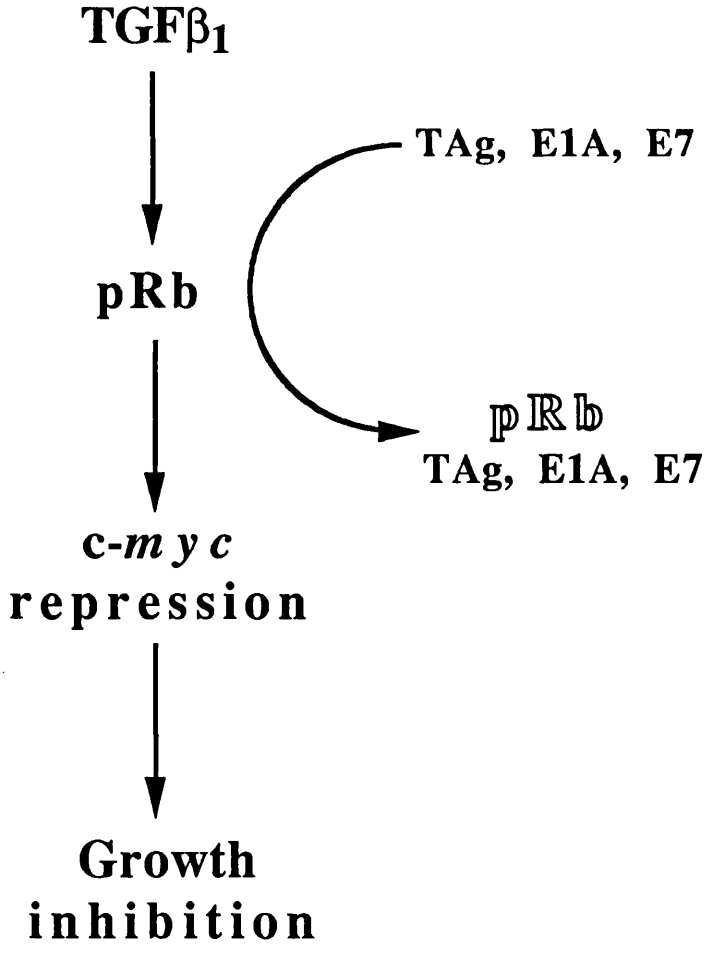
regulator. Although TGFβ_1 does not seem to interfere with early events produced by other peptide growth factors, such as the EGF induction of the *c-fos* proto-oncogene, EGF stimulation of DNA synthesis is prevented by addition of TGFβ_1 in the late G_1, namely long after early EGF-induced events have occurred [Like & Massagué, 1986]. Furthermore, TGFβ_1 inhibits entry of the cell into the S phase when added at any time up to the G_1/S boundary [Pietenpol *et al.*, 1990a]. Subsequent studies have indicated that TGFβ_1 participates in a growth inhibitory pathway which involves the proto-oncogene *c-myc* and the protein product (pRb) of the retinoblastoma tumour suppressor gene. It is known that early *c-myc* induction is necessary for the recruitment of quiescent cells into the G_1 phase [Bascom *et al.*, 1989b]. Knock-out of the *c-myc* gene using antisense oligonucleotides intensely inhibits proliferation of mouse keratinocytes [Pietenpol *et al.*, 1990b]. However, unlike other immediate early genes, elevated *c-myc* expression persists over the G_1 and during the S phases of the cell cycle [Coffey *et al.*, 1988]. Interestingly, addition of TGFβ_1 to EGF-stimulated mouse keratinocytes at any point of the G_1 phase or during the early S phase rapidly reduced expression of both *c-myc* transcript and protein [Moses *et al.*, 1991]. It was proposed that TGFβ_1 action is mediated via a protein that interacts with a specific *cis* element of the 5' regulatory region of the *c-myc* gene, leading to inhibition of the transcriptional initiation of this gene [Pietenpol *et al.*, 1990b]. Further studies have indicated that this protein component is likely to correspond to pRb. The latter is 105-kDa

cell cycle regulatory factor, having presumptive growth suppressor activity, which is differentially expressed in multiple phosphorylated forms throughout the different cell cycle phases [De Caprio *et al.*, 1989]. The underphosphorylated forms prevail during G_1 and in the growth-arrested state, while hyperphosphorylated forms are encountered in the S and G_2/M phases. On this basis it has been suggested that underphosphorylated pRb is responsible for growth suppression and that TGFβ_1 may prevent further scheduled pRb phosphorylation, leading to cell arrest in the late G_1 [Laiho *et al.*, 1990a]. In addition, it has been demonstrated that underphosphorylated pRb associates with transforming proteins of several DNA tumour viruses, such as large T antigen (TAg) of the SV40 [De Caprio *et al.*, 1988], E1A of adenovirus [Whyte *et al.*, 1988] and E7 of human papilloma virus -16 [Dyson *et al.*, 1989]. This association has been shown to result in a block of the growth suppressive activity of pRb [De Caprio *et al.*, 1988] and in the prevention of the inhibitory effect of TGFβ_1 on both cell proliferation and *c-myc* expression [Pietenpol *et al.*, 1990a]. A model has been therefore proposed whereby TGFβ_1-induced growth suppression is mediated by the repression of the *c-myc* gene via pRb (see Figure 10).

Extracellular matrix formation

The action of TGFβ on many cell types, both normal and neoplastic, commonly implies the up-regulation of several cell adhesion molecules [Massagué, 1990]. In particular, TGFβ

Figure 10. Model for transforming growth factor β_1-induced growth inhibition. TGFβ_1 is thought to activate the protein product (pRb) of the retinoblastoma tumour suppressor gene by preventing its hyperphosphorylation during the cell cycle. Underphosphorylated pRb interacts with a specific *cis* element of the 5' regulatory region of the *c-myc* gene, leading to inhibition of the transcriptional initiation of this cellular oncogene [Pietenpol, 1990b #298]. The latter has been shown to be necessary for the recruitment of quiescent cells into the G_1 phase [Bascom, 1989 #299]. Therefore, TGFβ_1 could induce a repression of the *c-myc* gene through activation of pRb, leading to the observed growth arrest of cells in the late G_1 phase of the cell cycle. This assumption is also supported by the evidence that association of underphosphorylated pRb with transforming proteins of several DNA tumour viruses, such as large T antigen (TAg) of the SV40, E1A of adenovirus and E7 of human papilloma virus-16, results in a block of the growth suppressive activity of pRb and in the prevention of the inhibitory effect of TGFβ_1 on both cell proliferation and *c-myc* expression [Pietenpol, 1990a #300].
(Modified from [Moses, 1991 #301]).


TGFβ1
TAg, E1A, E7
pRb
pRb
TAg, E1A, E7
c-myc
repression
Growth
inhibition

increases the expression of genes encoding for both fibronectin and different types of collagen [Ignotz & Massagué, 1986], as well as production of other extracellular matrix components, including tenascin, osteonectin, thrompospondin, etc. [Massagué, 1990]. In addition, TGFβ down-regulates expression of various collagenase-like enzymes [Kerr *et al.*, 1988], but increases the synthesis of proteolytic inhibitors, such as plasminogen activator inhibitor-1 (PAI-1) [Laiho *et al.*, 1986]. The likely net effect is represented by an accumulation of extracellular matrix, as observed following intradermal administration of TGFβ [Roberts *et al.*, 1986]. TGFβ has also been shown to control the expression of the repertoire of the integrin family of cell-extracellular matrix and cell-cell adhesion receptors [Heino *et al.*, 1989]. Thus, TGFβ may dramatically affect the adhesive behaviour of the cell and therefore be primarily involved in those processes wherein cell migration, homing and settlement are essential.

Other activities

The developmental role of TGFβs is supported by its spatial and temporal expression in the developing embryo. For instance, in the mouse developing palate, $TGF\beta_3$ mRNA is expressed very early in the medial edge epithelial cells, while $TGF\beta_1$ mRNA expression emerges in the same cells at a slightly later stage; in contrast, $TGF\beta_2$ mRNA is confined to mesenchymal cells [Fitzpatrick *et al.*, 1990].

In the bone remodelling process TGFβ potently stimulates chemotaxis, proliferation and terminal differentiation of

committed osteoblasts [Noda & Camilliere, 1989], while it inhibits osteoclast formation and activity [Chenu *et al.*, 1988]. In this context, however TGFβ synergises with other locally produced factors; these include the osteoinductive factor (OIF) and a family of related proteins called bone morphogenesis proteins (BMP) [Wozney *et al.*, 1988]. Recently, it has been documented that oestrogens stimulate TGFβ production in bone cells [Komm *et al.*, 1988]. The value of this evidence is twofold. Firstly, it may explain why menopausal oestrogen withdrawal also results in osteoclastic bone resorption and reduced bone formation. In second place, the finding that $TGF\beta_1$ is an oestrogen-regulated growth factor in human mammary cancer cells [Knabbe *et al.*, 1987] and that it produces an increased cell adhesion via up-regulation of extracellular matrix, may provide a background for the well known propensity of breast tumours cells to metastasise to bones. Similarly, production of $TGF\beta_2$ by human prostatic adenocarcinoma cells [Danielpour *et al.*, 1989] may be implicated in complex mechanisms whereby osteoblastic metastases are preferentially formed during prostate tumour progression.

TGFβ has also marked negative effects on the immune system, in that it inhibits thymocyte proliferation and opposes the action of some immunoregulatory agents, such as interleukins [Kehrl *et al.*, 1986]. By contrast, TGFβ is chemotactic to macrophages, stimulates their function [Wahl *et al.*, 1987] and has been shown to promote angiogenesis [Roberts & Sporn, 1990].

The latter activities seem to be essential for TGFβ action in tissue repair. It is worth noting here that wound healing and

cancer share common molecular and cellular mechanisms, as in: "a wound is a tumour that heals itself" [Haddow, 1972] and "tumours are wounds that do not heal" [Dvorak, 1986]. Wound healing is a complex but orderly process of repair which involves several processes, e.g. inflammation, cell migration and proliferation, synthesis of extracellular matrix, angiogenesis and tissue remodelling [Peacock, 1984]. Intuitively, TGFβ is a likely candidate to actively participate in wound repair since it regulates many of these processes. In animal models, TGFβ increases the rate of healing and the breaking strength of the repaired tissue and enhances angiogenesis and consequent blood flow to dermal wounds directly or indirectly through local release of other growth factors (reviewed by [Amento & Beck, 1991]).

4.4. The Platelet-Derived Growth Factor (PDGF)

The PDGF is an ubiquitous peptide growth factor having potent mitogenic activity on a number of both normal and transformed cells. Its discovery is directly linked to studies on serum as a necessary growth requirement for cultured cells. Originally, it was noted that several cell types, notably 3T3 fibroblasts and smooth muscle cells, grew in culture medium supplemented with serum (i.e. the soluble fraction of clotted blood), but their proliferation rate was markedly reduced in presence of plasma (namely the non-cellular fraction of the

whole blood). The evidence that normal growth could be restored by supplementing plasma with an extract of platelets (which break down during blood clotting) clearly indicated that these cells contained a major growth factor for 3T3 and smooth muscle cells and eventually lead Ross and colleagues [Ross *et al.*, 1974] to baptise it as platelet-derived growth factor.

4.4.1. Structure, isoforms and coding genes

After valorous attempts (because of the very low concentrations found in the starting material), human PDFG (hPDGF) was finally purified to homogeneity from platelets [Heldin *et al.*, 1979]. It was found to be a homodimer of approximately 30 kDa, consisting of two different peptide chains, denoted A and B, respectively of 16,000 and 14,000 molecular weight. The dimer is highly disulphide-bonded, both within and between each chain, by a total of 16 cysteine residues, with a 60% homology between the A and B chains. The latter are encoded by two different genes, localised on different chromosomes. The A-chain gene contains at least 7 exons spanning 22-24 kilobases of genomic DNA and is located on the proximal long arm of chromosome 7 [Betscholtz *et al.*, 1986]. It generates three different mRNAs (1.9, 2.3 and 2.8 kb) which are thought to give rise to various forms of the A-chain. The gene for B-chain corresponds to the c-*sis* proto-oncogene, the cellular homologous of the viral oncogene encoded by simian sarcoma virus (SSV) [Doolittle *et al.*, 1983]. The different chromosomal localisation of the two genes justified the evidence that PDGF was

secreted by cell types other than platelets as biologically active homodimers of the individual chain. These PDGF isoforms (PDGF-AA and PDGF-BB) were respectively identified in SSV-transformed cells [Owen *et al.*, 1984] and in a human osteosarcoma cell line [Heldin *et al.*, 1986].

4.4.2. Mitogenic action, transformation and function *in vivo*

Early studies showed that PDGF alone was unable to properly stimulate DNA synthesis by cultured 3T3 fibroblasts, unless platelet-deprived plasma was supplemented [Pledger *et al.*, 1978]. This clearly indicated that PDGF synergises with other plasma-borne factors, some of which were shown to have insulin-like activity (somatomedins) [Stiles *et al.*, 1979]. It is now recognised that PDGF acts, jointly with other growth factors like EGF and fibroblast growth factor (FGF), as a "competence" factor in the first part of the G_1 cell cycle phase; other factors (particularly the somatomedin insulin-like growth factor I, IGF-I) act later in the G_1 phase as "progression" factors. Both "competency" and "progression" steps are required to allow the quiescent (G_0) cells to enter the G_1 phase and to proceed along until the restriction point of the late G_1 is reached [Pledger *et al.*, 1978]. It has been shown that PDGF produces an early induction of cellular proto-oncogenes, such as c-*fos* and c-*myc*, suggesting that this effect is necessary for progression of cells through the cell cycle (reviewed in [Stiles, 1983]).

Many diploid cells have been found to produce both PDGF chains. On this basis, it has been postulated that PDGF may play

a role in the pathophysiology of a number of tissues. Smooth muscle cells and placental cytotrophoblasts actively synthesise PDGF and contain specific cell surface receptors; this suggests that PDGF may function as an autocrine regulator for both vessel wall and placental growth [Seifert *et al.*, 1984; Nilsson *et al.*, 1985]. By contrast, endothelial cells and activated macrophages produce PDGF but fail to respond to it since they lack PDGF receptors [Di Corleto & Bowen-Pope, 1983; Martinet *et al.*, 1986]; this would imply that PDGF act on the neighbouring cells in a paracrine fashion.

Equally, expression of both A and B-chain mRNAs or production of PDGF-like activity have all been observed in a variety of human tumour cell lines (reviewed by [Ross *et al.*, 1986]). While lineages derived from mesenchymal neoplasias and gliomas produce and respond to PDGF, carcinoma and leukaemia cells do not express PDGF receptors but secrete PDGF, suggesting that a paracrine regulation of the surrounding stromal cells could be in force.

The functions of PDGF *in vivo* extend over a wide range of biological effects (for a review see [Antoniades, 1991]). Either the hPDGF heterodimer or the two PDGF homodimers are potent mitogens and chemoattractants for a number of diploid cells, including fibroblasts, arterial smooth muscle cells and osteoblasts. In addition, PDGF enters important aspect of cellular metabolism, such as protein, lipid and prostaglandin synthesis. Finally, it appears to be involved in the pathophysiology of some

important processes, like mammalian embryogenesis, wound healing and osteogenesis, fibrosis and atherosclerosis.

4.5. The Insulin-Like Growth Factors (IGF)

IGFs are a structurally related family of circulating peptides having profound effects on both growth and differentiation of many cell types. The term IGFs was originally introduced to denote their 50% homology to the proinsulin. They were initially discovered as a growth hormone (GH)-dependent serum factor which stimulates *in vitro* sulphation of cartilage proteoglycans [Salmon & Daughaday, 1957]. Subsequently, it was found that they may act in endocrine, paracrine and/or autocrine fashion and that, unlike most peptide growth factors, circulate in blood bound to various specific binding proteins (reviewed by [Froesch *et al.*, 1985; Baxter, 1986]).

4.5.1. Structure and molecular biology

Two major IGFs, termed IGF-I and IGF-II, have been thus far identified and characterised [Rinderknecht & Humbel, 1978a; Rinderknecht & Humbel, 1978b]. IGF-I and II are single chain polypepdtides respectively of 70 (M_r 7649) and 67 (M_r 7471) amino acids which share 60-70% homology each other. In comparison to proinsulin, the sequence homology is confined to both A and B domains, while the connecting C peptides are different, being shorter in IGFs (8-12 amino acids with respect to

35 in the proinsulin); furthermore, both IGF-I and II have an additional C-terminal D domain of 6-8 amino acids. This is, however, an overly simplistic picture. Several larger and smaller variants of the parent IGF-I and II have been in fact isolated from disparate sources, including human tissue extracts and body fluids, or predicted on the basis of cloning studies [Herington, 1991]. The physiological significance of these relatively low abudant forms remains to be established

In the last five years, molecular biology techniques have allowed to determine cDNA sequences, mRNA transcripts, genomic structures and chromosomal localisation of the genes encoding for both IGF-I and II (reviewed by [Daughaday & Rotwein, 1989]). The IGF-I gene has been recently sequenced and subsequently denominated IGF-IA [Rotwein *et al.*, 1986a] It was found to encode the complete amino acid sequence (B, C, A and D domains) plus a N-terminal 5' signal sequence and a 3' sequence of 105 nucleotides encoding a C-terminal extension (the E peptide) of 35 amino acids. Later, Rotwein [Rotwein, 1986b] identified a second human IGF-I cDNA (termed IGF-IB) having a longer (77 amino acids) E peptide chain. This double expression is probably a product of an alternative splicing of the primary mRNA transcribed from a single IGF-I gene. The latter is located on the long arm of the chromosome 12 and consists of five exons, the IGF-IA being derived from exons 1, 2 3, and 5, the IGF-IB from exons 1,2, 3 and 4. Four major mRNAs have been identified in both rat and human tissues. Their size ranges from 0.8 up to 7.5 kb, mostly depending on the length of the 3' untranslated

region [Murphy *et al.*, 1987]. Expression of IGF-I mRNAs occurs in both adult and foetal tissues, where it appears to be under control of GH and other hormonal factors; e.g. oestrogens have been shown to markedly increase the IGF-I mRNA levels in the rat uterus [Murphy & Friesen, 1988]. Characterisation of both human and rat IGF-II genes revealed that they are located on the short arm of chromosome 11, adjacent to the related insulin gene [Bell *et al.*, 1985]. Structural analysis indicated that, differently from the IGF-I gene, the human IGF-II gene is composed of eight exons, of which the first five are non-coding sequences 5' to the signal peptide, while exons 6, 7 and 8 encode for IGF-II. As with IGF-I, multiple forms of IGF-II mRNA have been identified, ranging from 2 to 6 kb in size, in both foetal and non-hepatic adult tissues [de Pagter-Holthuizen *et al.*, 1988]. Contrary to IGF-I, GH is only a minor regulator of IGF-II expression, while developmental status seems to be of utmost importance.

4.5.2. IGF binding proteins

A distinctive feature of IGFs is that these polypeptide growth factors circulate bound to specific, high-affinity binding proteins (IGFBPs). This evidence, however, has been recently found to be common to other growth factors, e.g. PDGF [Raines *et al.*, 1984]. The IGFBPs compose a family of related polypeptides which have been recently cloned and characterised. At least five different IGFBPs have been so far identified [Baxter & Martin, 1989a]. IGFBP-1 has been cloned as a 25-28 kDa protein having equivalent binding affinities for IGF-I and II [Brewer *et al.*,

1988], while IGFBP-2 was originally isolated from BRL-3A foetal rat liver cell line and subsequently found to be a non-glycosylated protein of 35 kDa which binds IGF-II with higher affinity than IGF-I [Binoux *et al.*, 1982]. The IGFBP-3 has been shown to participate in the major circulating IGFBP complex, the 150 kDa acid-labile, GH-dependent binding protein [Baxter & Martin, 1989b]. This complex consists of the IGFBP-3 glycosylated protein, detected as a characteristic doublet of 40 and 43 kDa on SDS-PAGE, an acid-labile component (ALS) of 84 kDa and IGF-I or II. It seems that ALS combines to a pre-formed IGFBP-3/IGF complex and that IGFBP-3 cannot bind to ALS in the absence of IGF. More recently, two additional IGFBPs (4 and 5) have been isolated and cloned, but their biological signicance and mechanisms of regulation are still unknown.

Comparison of the individual amino acid sequences indicates that all known IGFBPs share marked conservation throughout different species (particularly rat and human) and striking homology (over 50%) one to another. Conserved residues are generally clustered in the N and C termini, all the 18 cysteines being preserved at both sites. IGFBP-1 and 2 show a peculiar RGD (Arg-Gly-Asp) sequence in the C-terminal domain and IGFBP-3 and 4 also contain electrostatically comparable sequences. This feature, which is common to other growth factor precursors and associated proteins (see also 4.3.1.), is of special interest since it seems to represent a recognition motif for extracellular matrix receptors of the integrin family [Ruoslahti & Pierschbacher, 1987]. It is still unclear whether IGFBPs act

indirectly by influencing IGF action or they have a functional role as such. In the first instance IFGBPs, which bind both IGF-I and II in a stoichiometric manner, may increase the half-life and reduce the clearance of IGFs from circulation [Cohen & Nissley, 1976]. However, whether IGFBPs limit the IGF access to extravascular space or they rather enhance IGF delivery and availability to target tissues (? via RGD) remains to be clarified. Alternatively, IGFPBs might be involved in paracrine/autocrine functions, also independent from the IGF action.

4.5.3. Biological activity

The IGFs feature a wide spectrum of biological effects on many tissues and cells, *in vitro* (reviewed by [Froesch *et al.*, 1985; Baxter, 1986]). These effects are only assumed to take place *in vivo*, because relevant studies have been crucially hampered in the past by the difficulty in obtaining adequate supply of IGFs. The effects of IGFs, especially IGF-I, on cell proliferation appear to be cell cycle-dependent. In fact, they require prior exposure to other "competence" factors, such as PDGF or FGF, to allow the cell to cross the G_0/G_1 boundary (see also 4.4.2.). In this respect, IGF-I has been defined a "progression" factor, whose potent mitogenic effects eventually lead to a rapid increase in DNA synthesis [Stiles *et al.*, 1979]. If cell proliferation is at steady state or is unrealisable, IGF-I may act as a powerful stimulator of cellular differentiation, as it occurs in the ovarian granulosa cells [Hutchinson *et al.*, 1988]. IGF-I has been shown to stabilise specific mRNAs in Balb/C 3T3

cells, suggesting that it may increase the abundance of labile mRNAs and proteins by preventing their degradation [Zumstein & Stiles, 1987].

4.6. Growth Factor Receptors

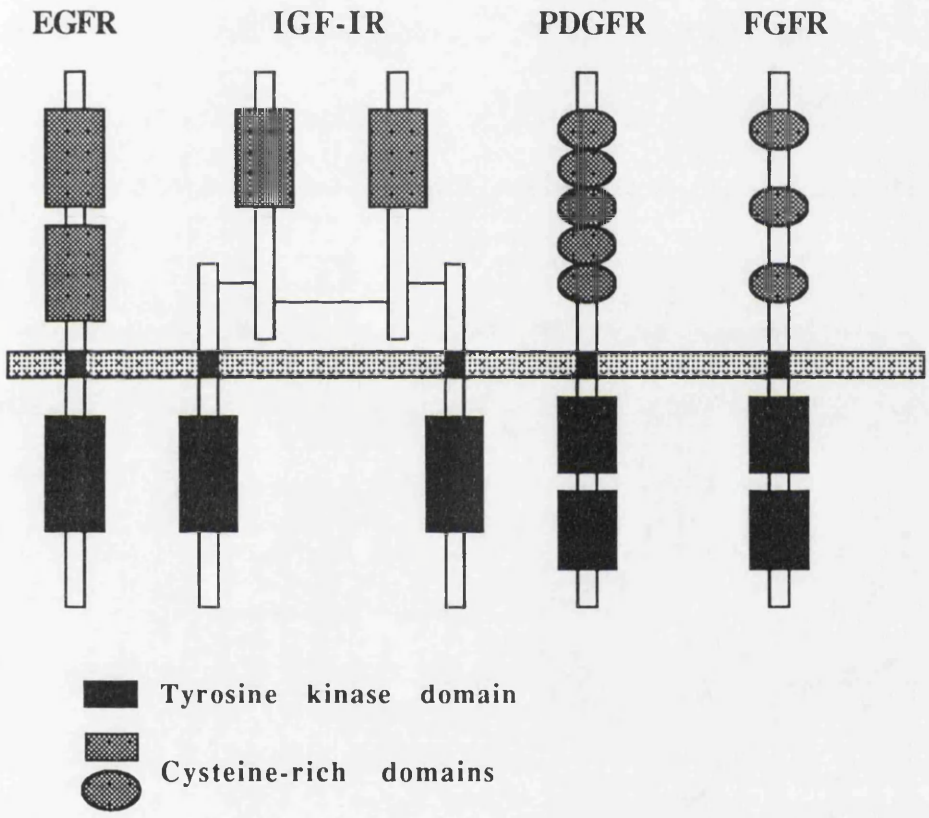
The myriad of biological effects induced by peptide growth factors mostly depends upon their interaction with specific, high-affinity cell membrane receptors. Binding of a growth factor to its cognate receptor on the external surface of the cell triggers a cascade of intracellular events which eventually leads to stimulation of DNA synthesis.

The molecular analysis of known growth factor receptors reveals that they fall into defined categories having distinct structural and functional peculiarities. They include simple transmembrane receptors, as in the tyrosine kinase, the serine/threonine kinase, the tyrosine phosphatase and the multichain "cytokine" families, or the unrelated G-protein associated seven-helical domain class of receptors, called serpentine receptors.

4.6.1. The tyrosine kinase family

Although addition or removal of phosphate groups normally occur on either threonine or serine residues, binding of specific growth factors, such as EGF, PDGF or IGF-I and II, to their cell surface receptors leads to rapid phosphorylation of the

Figure 11. The tyrosine kinase family of growth factor receptors (for details see text).


EGFR
IGF-IR
PDGFR
FGFR
Tyrosine kinase domain
Cysteine-rich domains

tyrosine residues of a particular set of cellular proteins. Structural analysis has revealed that the tyrosine kinase activity is located within the intracellular domain of the receptor (see Figure 11) and that ligand binding is required for phosphorylation of specific substrates, including the receptors themselves and a number of key intracellular regulatory enzymes.

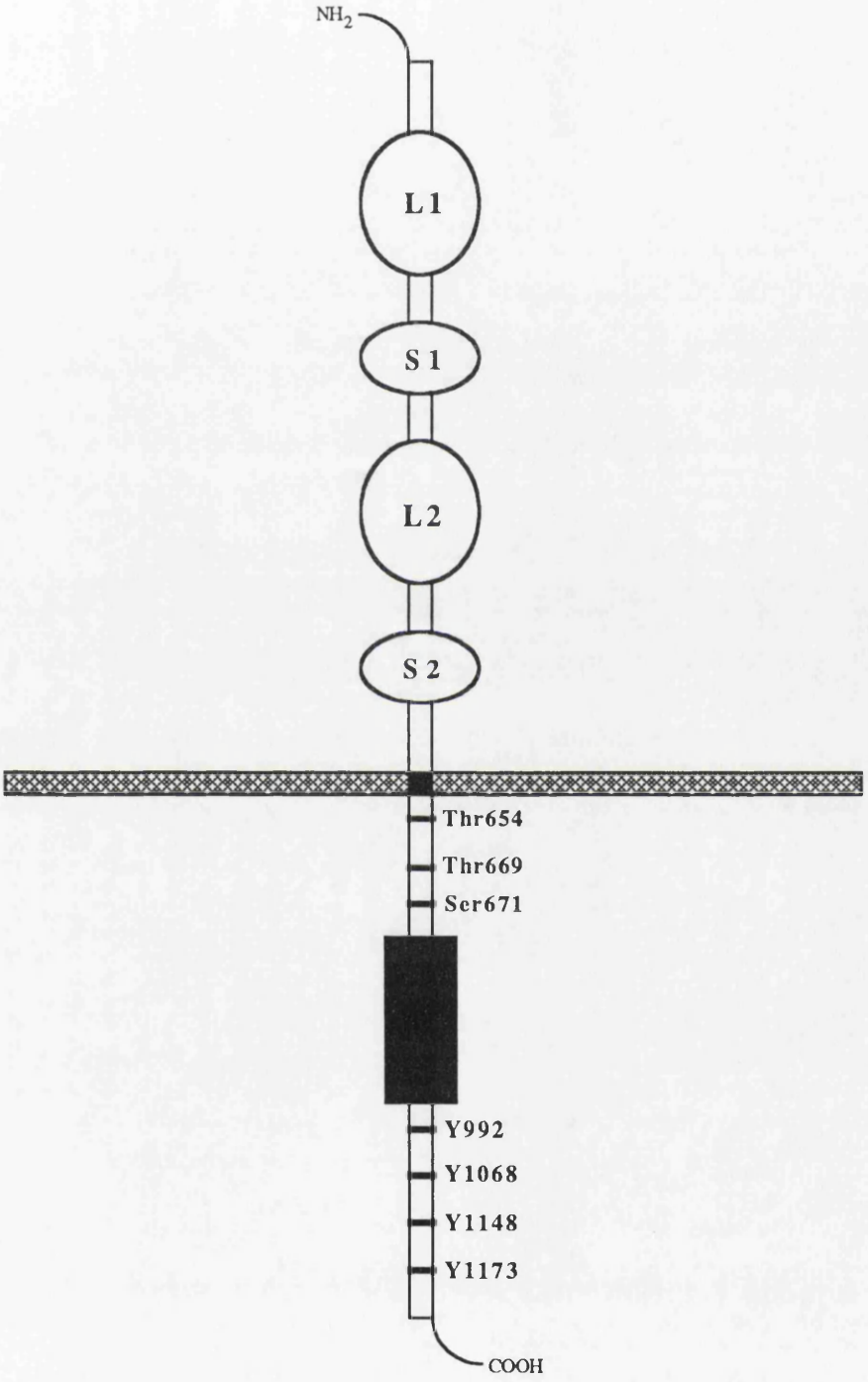
The EGF receptor (EGFR)

The availability of large amounts of pure EGF and the use of the squamous carcinoma cell line A431, which overexpresses EGFR, has made possible the purification of this receptor [Wrann & Fox, 1979]. Both cDNA sequence and genomic structure of EGFR have been determined [Ullrich *et al.*, 1984; Haley *et al.*, 1987].

The EGFR is a 170 kDa (1186 amino acids) single chain membrane glycoprotein consisting of three separate domains: a 621 amino acid extracellular domain which is responsible for EGF binding and contains a high proportion of cysteine residues clustered in two distinct regions; a hydrophobic 23 amino acid transmembrane region; and a 542 amino acid intracellular region containing a highly conserved tyrosine kinase domain.

The extracellular domain comprises about one half of the entire EGFR sequence and is thought to interact with EGF on a stoichiometric basis. Bajaj and colleagues [Bajaj *et al.*, 1987] have proposed a model for tertiary structure of this domain (see Figure 12). It includes two large variable regions (L1 and L2), each L subdomain being followed by one small cysteine-rich

Figure 12. The epidermal growth factor receptor (EGFR). This schematic representation exemplifies the EGFR domain composition. According to a recent model for the tertiary structure of EGFR, the external domain (621 amino acids) includes two large variable regions (L1 and L2), each followed by one small cysteine-rich region (S1 and S2). The short hydrophobic transmembrane domain (23 amino acids) continues in a composite intracellular domain (542 amino acids). This consists of a tyrosine kinase region (reported in black) and a further 250 amino acid tail at the C terminus containing specific tyrosine residues (Y992, Y1068, Y1148, Y1173) which serve as receptor autophosphorylation sites. The two threonine residues (Thr654 and Thr669) and the serine residue (Ser671) in between the transmembrane and the kinase domains represent phosphorylation sites for other growth factor-stimulated kinases, leading to a negative regulation EGFR activity.
(Adapted from [McDonald, 1989 #302]).


NH2
L1
S1
L2
S2
Thr654
Thr669
Ser671
Y992
Y1068
Y1148
Y1173
COOH

region (S1 and S2). Compact folding of both L and S subdomains, also involving formation of disulphide bonds, confers stability to the structure and might account for the unusual resistance of this receptor domain to proteases.

The transmembrane domain of EGFR is a highly hydrophobic region containing a stretch of basic residues at the cytoplasmic end which may serve as a "stop" sequence to anchor the polypeptide chain to the plasma membrane. Since 20 amino acids are enough to cross the 30 Å cell membrane, it is thought that this 24 amino acid domain forms an α-helix.

The cytoplasmic domain of the EGFR can be roughly subdivided in a 50 amino acids intracellular space, a 250 amino acid tyrosine kinase domain and a further 250 amino acid tail at the C terminus. The latter region contains three major tyrosine residues (Y1068, Y1148, Y1173) which serve as receptor autophosphorylation sites [Downward *et al.*, 1985], although additional site(s) (Y992) have been recently reported [Walton *et al.*, 1990]. The autophosphorylation process appears to be relevant to procure maximum biological activity of the receptor [Helin *et al.*, 1991]. The tyrosine kinase region is featured by the presence of an ATP binding site and a catalytic site that controls phosphoryl transfer to tyrosine residues of specific intracellular proteins (see below). The smaller bridging region in between the transmembrane and the kinase domains is also functionally important since it includes at least two threonine residues (Thr654 and Thr669) that can be phosphorylated by other growth factor-stimulated kinases, leading to a negative

regulation of ligand-induced tyrosine kinase activity and receptor internalisation [Lin *et al.*, 1986; Northwood *et al.*, 1991]. These phosphorylation sites, jointly with an additional serine residue (Ser671) in the same region, may provide a mechanism whereby the same EGF or other growth factors (notably PDGF) may down-modulate the EGFR activity [Heisermann *et al.*, 1990].

The EGFR is naturally present at around 10^4-10^5 sites/cell on the membrane of normal cells, while some transformed cells overexpress EGFR (10^6-10^7 sites/cell). It is widely accepted that EGF binding induces receptor aggregation in clusters on the cell surface. The most likely model foresees that ligand-receptor interaction occurs mainly at the two large subdomains (L1 and L2) of the extracellular region of the receptor through hydrophobic contacts. This may cause dramatic conformational changes in both the ligand and the receptor and probably produce intermolecular allosteric effects which eventually lead to receptor dimerization [Schlessinger, 1988]. This would bring the tyrosine kinase domains of two receptor monomers into close proximity allowing reciprocal phosphorylation and activation. Further to ligand binding, the EGF-receptor complex is captured by clathrin-coated pits and internalised within 30 minutes. This leads to lysosomal receptor degradation, with negligible receptor recycling. Since full signal transduction takes much longer (8 hours), the cell must therefore initiate an active synthesis of EGFR to avoid receptor depletion at its surface. Although endocytosis and degradation of the receptor are ligand-induced processes, they do not seem to affect the biological activity of

EGFR; in fact, substances that inhibit either receptor degradation or formation of the coated pits respectively leave the EGF-induced mitogenic response unchanged [Savion *et al.*, 1980] or even increase it [Maxfield *et al.*, 1979].

The PDGF receptors (PDGFR)

PDGF receptors have been found on the membrane of most mesenchymal connective-tissue-forming cells, whilst they have not been observed on epithelial or arterial endothelial cells. Initial work postulated the presence of a single class PDGF receptor which was first described as a 180 kDa transmembrane glycoprotein with an external PDGF-binding domain and an intracellular region containing a split tyrosine kinase domain [Huang *et al.*, 1982]. Subsequent studies, using pure recombinant PDGF isoforms, revealed that this receptor, called PDGFR-β, binds to PDGF-BB and hPDGF heterodimer respectively with high and lower affinity, while it does not recognise the PDGF-AA homodimer [Hart *et al.*, 1988]. The latter binds instead to a second receptor form, termed PDGFR-α, which is also able of binding the two other PDGF isoforms [Heldin *et al.*, 1988]. Cloning studies revealed that PDGFR-β is synthesised as a 160 kDa precursor that mature to the 180 kDa glycoprotein [Keating & Williams, 1987], while the PDGFR-α derives from a 140 kDa precursor which is converted to a 170 kDa mature form [Claesson-Welsh *et al.*, 1989]. The α and β receptor forms share about 40% homology each other and have been found to be products of two separate genes. It has become increasingly

evident that PFGFR-α and β represent distinct receptor subunits that are brought together by one of the three PDGF isoforms [Seifert *et al.*, 1988]. The receptor subunits are thought to be "floating" within the cell plasma membrane and to acquire a stable dimeric conformation following binding to a PDGF isoform. The α-subunit can bind any PDGF chain (A or B), while the β-subunit can bind only the B chain; as a result, PDGF-BB will bind any combination of the two receptor subunits (αα, αβ, ββ), PDGF-AB will bind to either αα or αβ receptors and PDGF-AA can bind solely to the αα form [Seifert *et al.*, 1988]. Since several cell types differentially express α and β subunits, the mitogenic effects of PDGF will depend upon the presence of adequate amount of individual PDGF isoforms and, especially, on the relative number of the different receptor subunits on the cell surface.

The extracellular region of both PDGF receptors contains five immunoglobulin-like domains, suggesting that they are related to the immunoglobulin superfamily. At the intracellular end, PDGF receptors show a tyrosine kinase domain split into two parts by a variable-length insertion sequence with little or no homology to other kinases. Interestingly, deletion of this insert sequence produces a mutated form of receptor which retains the kinase activity, but is unable to transduce the mitogenic signal [Escobedo & Williams, 1988]. Phosphorylation of several tyrosine residues of the intracellular domain of the receptors occurs as a result of PDGF binding and is thought to be important for signal transduction [Ek *et al.*, 1982]. Cell surface PDGF receptors have a

half-life of approximately 3-4 hours which decreases to 30 minutes following PDGF binding and receptor aggregation. Once formed, PDGF-receptor complex is internalised within 1-2 minutes in endosomes and subsequently degraded into lysosomes, the breakdown products of PDGF being found in the extracellular space after 15-30 minutes.

The IGF receptors (IGFR)

Response of target cells to IGF-I and II is mediated by three distinct receptor types, precisely the type I and II IGFR and the insulin receptor. High homology exists between the type I and insulin receptors, while the type II receptor is structurally and functionally unrelated [Rechler & Nissley, 1986]. The type I IGFR is a 300 kDa complex which results from a heterotetrameric arrangement of two dimers each consisting of disulphide-bonded $\alpha\beta$ subunits (see Figure 11). The two α subunits are responsible for IGF binding and are extracellular, being linked by disulphide bonds to the β subunits. The latter cross the cell membrane with a shorter N terminal tail in the extracellular space and a cytoplasmic region which contains the tyrosine kinase domain [Nissley & Rechler, 1984]. Autophosphorylation of the type I IGFR itself has been shown to occur at three main phosphorylation sites (Y1146, Y1150 and Y1151) and is reputed to be involved in the signal transduction process [Czech, 1989]. There is a high degree of functional specificity for the type I receptor binding, the relative affinities being IGF-I>IGF-II»insulin, whilst the

insulin receptor binds insulin>IGF-I>IGF-II [Nissley & Rechler, 1984].

In contrast, the type II IGFR is quite different from both type I and insulin receptors in that it consists of a monomeric glycosylated polypeptide of 260 kDa which lacks either tyrosine kinase activity or phosphorylation sites. It binds IGF-II with greater affinity than IGF-I, but does not recognise insulin at all. Recently, cloning of type II receptor has revealed that it is identical to the cation independent mannose 6-phosphate (M-6P) receptor [Tong *et al.*, 1987]. The receptor is mostly extracellular, with a very short cytoplasmic tail, and is composed of 15 repeats of the M-6P binding subunit. The type II IGF receptor seems to behave as a multifunctional protein, capable of binding not only IGF-II and M-6P, but also other peptides such as the latent $TGF\beta_1$ [Purchio *et al.*, 1988] (see also 4.3.1.). The role of type II receptors in mediating the IGF-induced mitogenic action is unclear. Although most studies have indicated that this effect is mediated solely by type I receptors, there is increasing evidence that the type II/M-6P receptors may be implicated in specific pathways of signal transduction, like via the GTP-dependent coupling proteins [Nishimoto *et al.*, 1989].

Other receptors

Two member of the human proto-oncogene *erb*B family are worthy of mention here. Both c-*erb*B-2 and c-*erb*B-3 proto-oncogenes have been identified probing human genomic libraries with the v-*erb*B oncogene from the avian erythroblastosis

retrovirus [Yamamoto *et al.*, 1986; Kraus *et al.*, 1989]. The latter encodes for a truncated version of EGFR (the GP74$^{v\text{-}erbB}$ protein) having a short N-terminal tail of 61 amino acids and lacking also the 32 amino acid residues at the C-terminal end of the receptor, which contains the major autophosphorylation sites of EGFR [Beug *et al.*, 1988]. However, it has been shown that the v-*erb*B protein retains intrinsic tyrosine kinase activity and that it incurs autophosphorylation at as yet unknown sites [Kris *et al.*, 1985]. In contrast, the predicted structure of both human c-*erb*B-2 and c-*erb*B-3 protein products revealed colinearity with most of the EGFR sequence, with an extracellular region arranged as a pocket to accomodate a putative polypeptide ligand [Coussens *et al.*, 1985]. A remarkable degree of homology is present between the two proto-oncogene products and with EGFR, mostly within the tyrosine kinase domain (respectively 82 and 60% with respect to EGFR). The c-*erb*B-2 protein has been also referred to as HER-2 based on its similarity with the human EGF receptor; it represents the human counterpart of the rat *neu* protein, the product of an oncogene associated with neuroblastomas [Schechter *et al.*, 1984].

The potential role of both c-*erb*B-2 and c-*erb*B-3 in the transmission of a mitogenic signal is obscure. There is however evidence that activation of the EGFR may produce EGFR/c-*erb*B-2 heterodimers and result in cross-phosphorylation of the c-*erb*B-2 protein in cells that express both receptors [King *et al.*, 1988]. In this respect, the EGFR and c-*erb*B-2 may act in a synergistic fashion to induce transformation of rodent cells, although it

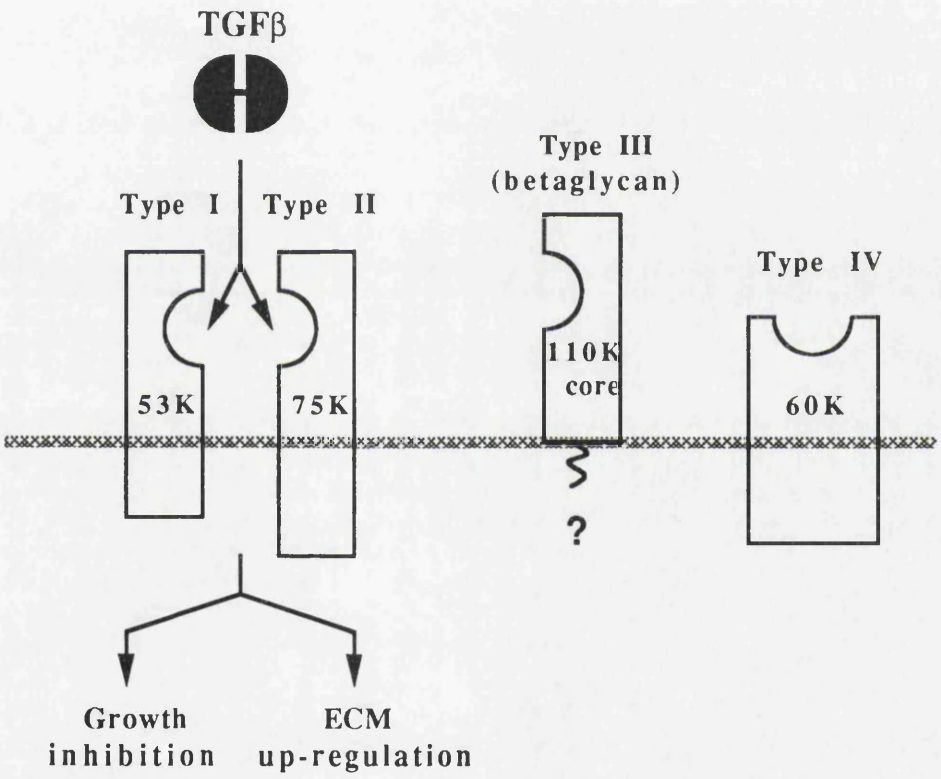
seems likely that tyrosine kinases of the two receptors may recognise either common or separate, specific substrates. On the other hand, the potential role of c-*erb*B-3 in transduction of a mitogenic signal is presently unknown; however, expression of c-*erb*B-3 transcripts has been detected in both human mammary MDA-MB361 and squamous A431 carcinoma cell lines, being seemingly restricted to cells of epithelial or neuroectodermal origin [Plowman *et al.*, 1990].

4.6.2. The TGFβ receptors

Many cell types express on their membranes multiple binding sites for TGFβ, having distinct affinity for the different TGFβ isoforms (reviewed by [Massagué *et al.*, 1990]). Four different cell surface binding proteins have been thus far identified, having the high affinity and specificity characteristics pertinent to physiologically significant receptors [Segarini, 1991]. They have been termed TGFβ receptor type I, II, III (or betaglycans) and IV (see Figure 13).

The type I and II receptors share common characteristics. Both are glycoproteins of approximately 53 and 70-85 kDa, respectively, and contain complex N-linked carbohydrate. They exhibit a 10 to 20-fold decreasing affinity of $TGF\beta_1 > TGF\beta_2$ binding and are ubiquitously distributed in an assortment of both normal and transformed cells from different species, including human. Nevertheless, peptide mapping studies have documented striking diversities in the ligand-binding domain of these two receptor forms [Cheifetz *et al.*, 1986]. Interestingly,

Figure 13. Transforming growth factor-β (TGFβ) binding proteins. This diagram illustrates the four TGFβ receptor forms so far identified. Both type I (53 kDa) and II (75 kDa) receptors are thought to mediate the TGFβ-induced growth inhibition and up-regulation of the extracellular matrix components. The betaglycan (type III) receptor (280 to 330 kDa) is an integral membrane proteoglycan containing a core unit of approximately 110-120 kDa which binds both $TGF\beta_1$ and $TGF\beta_2$ with equal affinity; it may have a role in presentation of TGFβ to functionally active receptors or in its delivery to the extracellular space. The type IV receptor has been recently isolated from GH_3 rat pituitary tumours cells; its functional role is presently unknown.
(Modified from [Massagué, 1990 #64]).


TGFβ
Type I
Type II
Type III
(betaglycan)
Type IV
53K
75K
110K
core
60K
?
Growth
inhibition
ECM
up-regulation

studies on chemically induced TGFβ-resistant mutants derived from mink lung epithelial cells (Mv1Lu) have revealed that class R mutants, which have selectively lost expression of functional type I receptors, become totally insensitive to all known TGFβ effects, including growth suppression and extracellular matrix formation [Boyd & Massagué, 1989]. While this suggested that type I receptors are primarily involved in the TGFβ-specific transmembrane signalling, a cognate role for the type II receptor was initially reputed unlikely. However, studies on recently established cell clones where type I and II receptors are simultaneously altered, indicated that either receptor is involved in signal transduction, possibly as component of a single receptor complex [Laiho *et al.*, 1990b].

The type III receptor is a high molecular weight complex of 280 to 330 kDa on SDS-PAGE which has been termed betaglycan [Andres *et al.*, 1989]. It is present on the cell surface of a variety of cell types, although its distribution is not as broad as that of type I and II receptors. The most distinctive feature of betaglycan resides in its heterogeneous composition: it is an integral membrane proteoglycan consisting of a 200 kDa glycosaminoglycan (GAG) chain component and a 10 kDa N-linked glycans bound to a core unit of approximately 110-120 kDa [Cheifetz *et al.*, 1988a]. The 200 kDa component is a hybrid of two GAGs, heparan sulphate and chondroitin sulphate [Segarini & Seyedin, 1988], which do not bind TGFβ and are not required for functional expression of the receptor on the cell surface [Cheifetz & Massagué, 1989]. The core unit is responsible for TGFβ binding

that, contrary to type I and II receptors, occurs at equal affinity for both $TGF\beta_1$ and $TGF\beta_2$. A soluble form of betaglycan which lacks membrane anchor has been found in serum, extracellular matrices and in the culture medium of several cell types [Andres *et al.*, 1989]. Although most TGFβ effects are commonly thought to be mediated solely by type I and II receptors, whether and how betaglycan is implicated in TGFβ action remains to be established.

The type IV receptor has been recently isolated from GH_3 rat pituitary tumours cell as a binding protein that associates with other members of the TGFβ superfamily, such as inhibin and activin [Cheifetz *et al.*, 1988b]. Its functional role is presently unknown, but it seems conceivable that it mediates $TGF\beta_1$ and inhibin/activin regulation of FSH release by the pituitary.

Unfortunately, the signalling cascade downstream of TGFβ interaction with cell surface receptors is ill-defined. After receptor binding, TGFβ is rapidly internalised and degraded by lysosomal enzymes [Massagué & Kelly, 1986], but it is unclear whether this represents an essential step for TGFβ-induced changes. Some experimental evidence suggested that TGFβ may activate several intracellular signalling pathways, including protein kinase C (PKC), phosphatidylinositol and GTP-dependent binding proteins (see below), but there is no indication that this activation is directly dependent on TGFβ binding to its receptors.

4.7. Post-Receptor Signalling Pathways

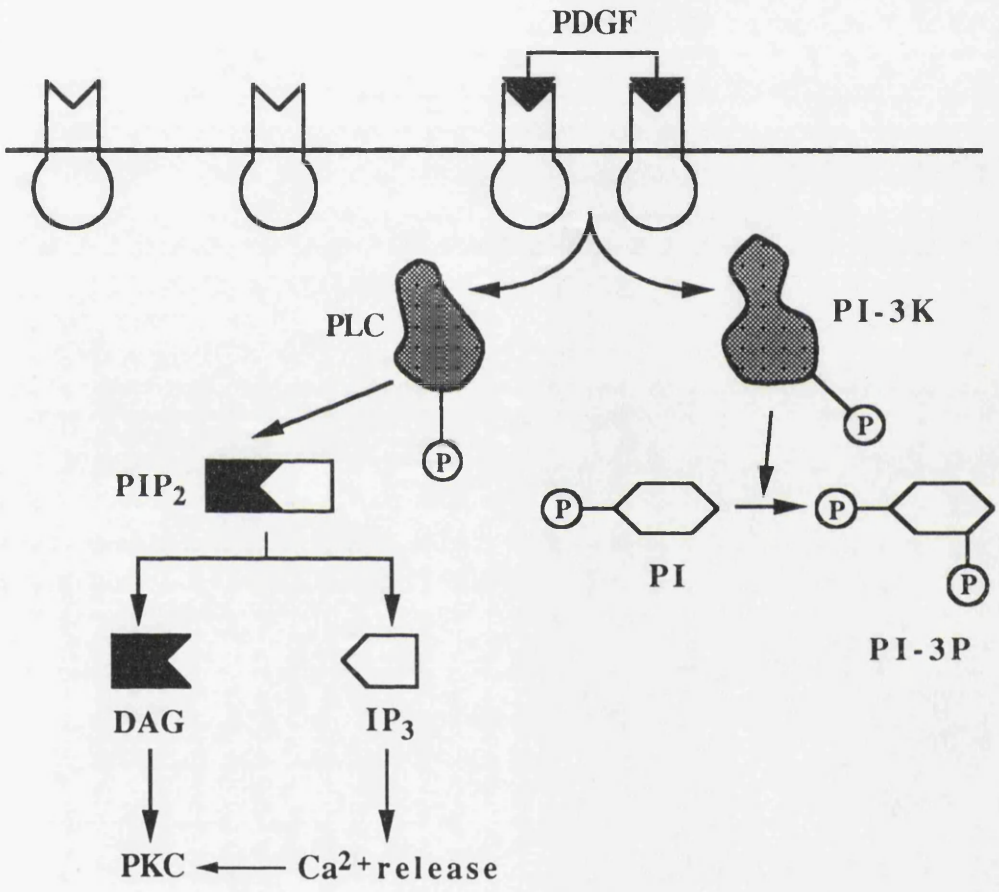
4.7.1. Tyrosine kinase-associated pathways

As previously described (see 4.6.1.), several growth factor receptors (including EGFR, PDGFR and IGFR) contain a cytoplasmic domain having intrinsic tyrosine kinase activity.

A search for selected proteins which are thought to be likely candidates for physiological tyrosine kinase substrates revealed that, following binding of PDGF to cell surface receptors, a number of cellular proteins are phosphorylated on tyrosine within seconds. Most of these proteins may be phosphorylated directly by the PDGFR tyrosine kinase, becoming physically attached to the receptor as a consequence of its dimerisation and autophosphorylation (see Figure 14). Notably, four distinct proteins have been found to associate with the tyrosine kinase domain of the PDGFR: the phospholipase C (PLC), the phosphatidyl insositol 3-kinase (PI-3K), the $pp60^{src}$ encoded by the *src* proto-oncogene and the ras GTPase activating protein (ras-GAP). Activation of this protein set results in a generation of small molecules, called second messengers, which are potent mediators of signals dispatched to distant sites in the cell. They include the quintessential second messenger cyclic AMP (cAMP), cyclic GMP (cGMP), inositol triphosphate (IP_3), diacyl glicerol (DAG) and calcium ions (Ca^{2+}).

As illustrated in Figure 14, after the cytoplasmic domain of PDGFR undergoes autophosphorylation, the PLC is activated and produces cleavage of the phosphatidyl inositol 4,5-diphosphate

Figure 14. Tyrosine kinase receptor signalling pathways. PDGF-induced post-receptor pathways are here exemplified. After ligand-induced PDGFR dimerization and autophosphorylation, subsequent activation of phospholipase C (PLC) produces cleavage of the phosphatidyl inositol 4,5-diphosphate (PIP_2) into diacyl glycerol (DAG) and inositol triphosphate (IP_3). The latter causes a rise of intracellular calcium (Ca^{2+}) levels which in turn induces the stimulation of various cellular enzymes and concurs with DAG in the activation of the protein kinase C (PKC). On the other hand, liganded PDGFR stimulates the phosphatidyl inositol 3-kinase (PI-3K) activity, that adds phosphates to the ring of phosphoinositol at position 3, yielding an active product (PI-3P) which is thought to play an important role in the cell growth cycle.


PDGF
PLC
PI-3K
P
P
P
P
P
PIP2
PI
PI-3P
DAG
IP3
PKC
Ca2+release

(PIP_2) into IP_3 and DAG [Wahl *et al.*, 1989]. The IP_3 diffuses through the cytoplasm and causes release of Ca^{2+} from membranous storage compartments where it is usually sequestered by binding to a specific receptor located on the endoplasmic reticulum; the resulting rise of the intracellular calcium in turn induces the activation of various cellular enzymes. In addition, IP_3 may enter a number of metabolic pathways, including further phosphorylation to yield IP_4, IP_5 and IP_6 or dephosphorylation to inositol from which phosphatidyl insositol can be resynthesised. On the other side, DAG combines with Ca^{2+} in the activation of another key growth-regulating enzyme, the protein kinase C (PKC, see Figure 14), which phosphorylates both serine and threonine residues on dozens of target proteins. Another consequence of PDGFR autophosphorylation is the stimulation of the IP-3K activity (see Figure 14); the latter adds phosphates to the ring of phosphoinositol at position 3, yielding an active product which is thought to play a crucial role in the cell growth cycle [Coughlin *et al.*, 1989].

The $pp60^{v\text{-}src}$ is the aberrant protein product of the avian Rous sarcoma virus (RSV), the very first oncogene product identified [Collett *et al.*, 1978]. It is a membrane protein kinase which phosphorylates selected tyrosine residues on both its own molecule and a panel of unrelated proteins. Its normal cousin, the $pp60^{c\text{-}src}$, also has a kinase activity which conversely is latent until activated by various external stimuli, including PDGF binding.

The ras-GAP is a multifunctional protein which represents a site of crosstalk between the PDGFR and the ras signalling pathway, one of the most important growth regulatory circuitries in the cell. This protein is required for the activation of GTPase belonging to the ras family of membrane-associated G-proteins [Ellis *et al.*, 1990]. The ras protein, termed $p21^{ras}$, exists in two states, active and inactive, which shift one to another respectively through alternative binding of GTP and GDP nucleotides. The inactive, GDP-bound $p21^{ras}$ is proded to release the captive GDP molecule by ligand-activated growth factor receptors through as yet unknown mechanisms. Once released, GDP is immediately substituted by a GTP molecule via a GDP/GTP exchange factor, causing the switch of $p21^{ras}$ to an active state. The latter, however, persists a few seconds since GTP is rapidly transformed into GDP by loss of a phosphate and therefore $p21^{ras}$ again acquires an inactive mode. In contrast, the mutant oncogenic $p21^{ras}$ has lost the ability to convert GTP into GDP, hence maintaining a prolonged excited state and behaving as a lasting source of growth-stimulatory signals.

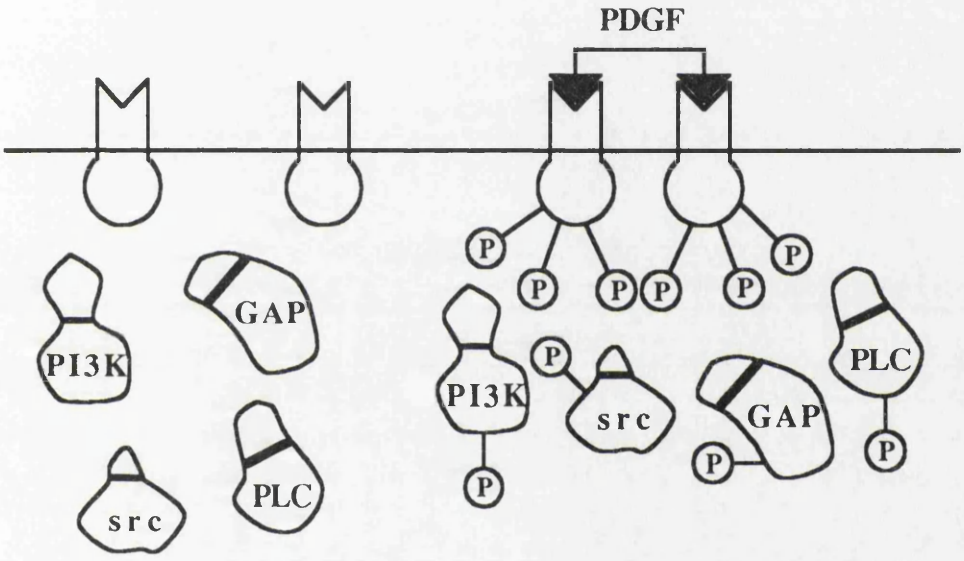
Association of all these four putative protagonists of post-receptor signalling pathways with the cytoplasmic domain of tyrosine kinase receptors appears to involve a short sequence of around 100 amino acids (termed SH2: src homology-2) which is capable of recognising phosphorylated tyrosines and the surrounding residues on the insert region of the split kinase domain of PDGFR or more distal autophosphorylated sites in other growth factor receptors [Koch *et al.*, 1991]. Once created,

receptor phosphotyrosines attract and bind these proteins through the SH2 domain, producing their activation via either phosphorylation or subtle changes in protein folding (see Figure 15).

4.7.2. The protein kinase C (PKC)

The PKC represents a multigene family of closely related membrane serine/threonine protein kinases [Nishizuka, 1984]. Structural analysis has revealed that different PKCs share a number of conserved domains [Nishizuka, 1988]. In particular, the N terminus of the molecule contains a C1 region, which is thought to be responsible for enzyme inhibition, and a C2 region, which confers calcium-dependence to PKC. It is of interest to note that in the presence of DAG the calcium levels required for PKC activation drop to those normally found within the cell, with a consequent induction of PKC activity and phosphorylation of a set of cellular proteins. The involvement of PKC in the transduction of mitogenic signals is outlined by the evidence that the phorbol esters, potent mitogens for quiescent cells, act as non-metabolisable analogues of DAG, causing an unrelenting stimulation of PKC activity and a likely priming of mechanisms controlling the entry of the cell from G1 into the S cell cycle phase. However, identification of potential substrates for PKC activity has been difficult. One interesting clue is that PKC phosphorylates membrane receptors, such as the EGFR, inducing a loss of binding affinity and a consequent inhibition of the EGFR-linked tyrosine kinase activity [Lin *et al.*, 1986].

Figure 15. The SH_2 proteins. The physical association of phosphatidyl insositol 3-kinase (PI-3K), phospholipase C (PLC), $pp60^{v\text{-}sr}$ (src) and ras-GAP (GAP) proteins with the cytoplasmic domain of tyrosine kinase receptors is shown. These four putative components of post-receptor signalling pathways contain a short sequence of around 100 amino acids (termed SH2: src homology-2; marked in face-bold) which is capable of recognising phosphorylated tyrosines and the surrounding residues on the insert region of the split kinase domain of PDGFR or other growth factor receptors, but that ignores the partner receptor if the critical tyrosine residues are not phosphorylated. Once phosphotyrosines are created, the receptor will attract and bind these proteins, producing their activation via either phosphorylation or subtle changes in their protein folding.



SH_2 proteins

Overall, it appears that phopshorylation of specific amino acid residues on selected proteins is a cardinal process in growth signalling pathways wherein both tyrosine and serine/threonine kinases concur to activate multiple cellular substrates. Using this simple mechanism, an extraordinary array of intracellular "molecular switches" can be turned "on" and "off" in seconds, allowing the cell to maintain continuously a social behaviour.

5. Cancer of the Human Prostate

5. Cancer of the Human Prostate

5.1. Prostatic Carcinoma: A Difficult Case

Unlike both oestrogen and progesterone receptors in breast and endometrial cancer, the use of androgen receptors both as prognostic indicators and predictors of response of prostate cancer patients to endocrine treatment has been crucially hampered by either methodological pitfalls in the assay or the heterogeneous composition of most tumour tissues; the situation is further complicated by developmental and functional features inherent in the human prostate gland that make it highly heterogeneous in nature. Secondly, little is known on the mechanisms whereby such tumour types invariably tend to progress from an endocrine towards a hormone-refractory status. Continued growth of prostate cancer is achieved through intricate pathways where steroid hormones and other polypeptide growth factors act directly or in a paracrine/autocrine fashion in the growth control of cancer cells, but also serve as mediators of cellular interactions between stromal and epithelial compartments as like as with the extracellular matrix. Finally, insensitivity of androgen-independent tumour growth to endocrine manoeuvres is also reflected in the failure of androgen ablation to induce programmed cell death, as it is seen in hormone-dependent prostatic epithelial cells. A more complete knowledge of all the above processes may well help to design

new effective treatments which would encompass the diverse susceptibility to conventional therapies developed by prostate cancer cells during tumour progression.

5.1.1. Epidemiological background

Incidence of prostate cancer varies greatly throughout the world, being the highest in the black population of the United States and the lowest in the Asian populations of China and India [Zaridze *et al.*, 1984]. The Western European countries range between the upper and the mean position, with Norway, Sweden and Switzerland having the highest rates. Epidemiological data (reviewed by [Griffiths *et al.*, 1987]) give an alarming idea on the dimensions of the problem: in the United States more than 90,000 new cases of prostatic cancer present annually, resulting in approximately 26,000 deaths each year [Sondik, 1988]. Overall, statistical data also indicate that during the last decade incidence rates have been dramatically rising worldwide, to such a point that prostate cancer has become the most prevalent cancer and the second principal cause of cancer death in man [Carter & Coffey, 1990]. However, mortality rates for prostate cancer are remarkably lower than the incidence rates and the increasing prevalence of this tumour type is accompanied by a smaller increase in deaths [Zaridze *et al.*, 1984].

It is recognised that the human prostate has a uniquely high occurrence of latent microcarcinomas. This is of interest, since autopsy studies have revealed that prevalence of latent carcinomas appears to be associated with the mortality rates of

clinical prostate carcinoma [Breslow *et al.*, 1977]. It has been postulated that tumour initiation does not differ in populations having high or low mortality rates, but that geographical differences in both prevalence of latent prostatic cancer and mortality rates could be ascribed to a diverse weight of tumour promoting factors.

5.1.2. Morphofunctional implications

The heterogeneous composition of both normal and diseased human prostate is well established. Evidence coming from anatomy or embryological studies suggests that the prostate gland is composed of distinct regions having different functional activity and sensitivity to both androgens and oestrogens. Initially, McNeal proposed that the prostate gland contains three major glandular regions, the central zone (CZ), the transition zone (TZ) and the peripheral zone (PZ), and some important nonglandular areas concentrated in the anteromedial portion of the gland [McNeal, 1972]. These different regions can also be separated topographically. In fact, the TZ together with periurethral glands surround the urethra proximal to the veromontanum and represent the "inner" prostate, while the CZ and PZ comprise the remainder of the gland, the "outer" prostate. Comparative anatomy studies on primates also support this view, in that the cranial and caudal prostatic lobes of the rhesus monkey appear to be homologous to the human CZ and PZ, respectively [Blacklock & Bouskill, 1977]. It is noteworthy that human BPH mostly derives from the TZ, whilst prostate cancer

typically originates in the PZ and may subsequently spread to both the CZ and the periurethral region. Additionally, foci of intraductal dysplasia, a prostatic lesion that is thought to be premalignant [McNeal, 1986], have been found to associate with PZ microcarcinomas [McNeal, 1993]. Although prostate tumours may occasionally originate from TZ, either histological or clinical features of these tumours point to the possibility that their biological potential is different from the PZ tumours [Blacklock, 1982; McNeal, 1992].

This histological distinction is not merely instructive, but holds a precise functional significance. For instance, significantly higher tissue concentrations of DHT have been documented in the periurethral than in the outer prostate [Siiteri & Wilson, 1970]. Furthermore, presence of cytosol androgen receptors (AR) has been more frequently revealed in the PZ region (71%) with respect to periurethral tissue (39%) from both benign and malignant human prostate [Bowman *et al.*, 1986].

Another major concern pertains to the procedures routinely used to obtain prostate cancer specimens suitable for AR assays. Since transurethral resection (TUR) is currently performed with increasing frequency in place of retropubic prostatectomy, it has been argued that the heat generated by the cutting loop of resectoscopes may seriously deteriorate the AR content of the resected chippings [Kyprianou *et al.*, 1986]; however, this problem can readily be encompassed by selecting large uncharred specimens [Bowman *et al.*, 1985]. In addition, studies recently carried out in our laboratories have suggested that TUR

specimens represent suitable material for receptor studies, provided that only cutting current is employed, while the coagulating current is confined to the final part of the resection [Castagnetta *et al.*, 1991b]. It should be emphasised that, due to the uneven distribution of AR throughout normal and diseased human prostate, sampling condition during TUR or other bioptic manoeuvres may critically influence the outcome of biochemical and biological assays. Based on normal anatomy, specimens taken from the beginning of TUR would correspond to the periurethral region, while the most peripheral part of the resection is most likely to be PZ tissue; further, perineal or transrectal biopsy will initially encounter the PZ and subsequently the CZ region. This, together with the undoubted regional and histological variation in both steroid receptor content and hormone sensitivity of prostatic tissues, implies that results from morphological and biochemical studies should be also interpreted by the light of the different areas sampled.

5.1.3. Molecular biology of androgen action

It is universally recognised that conversion of circulating testosterone into its biologically active derivative DHT represent an essential step for androgen action in prostate tissues. High affinity binding of DHT to intracellular AR primes a sequence of events that eventually lead to changes in the expression of a specific set of androgen-responsive genes.

The human AR belongs to the steroid receptor superfamily (see 2.1.), a section of a larger multigene family of ligand-

inducible trans-acting regulatory factors [Parker, 1986]. It is a 110 kDa protein encoded by a single gene that spans over 90 kilobases and is located on the chromosome X [Lubahn *et al.*, 1988]. The human AR gene consists of 8 exons, of which one large exon gives rise to the N-terminal domain of the receptor and 5 exons divide out the sequence coding for the hormone-binding domain [Brinkmann *et al.*, 1989].

Studies on the main secretory protein of the rat ventral prostate, the prostate binding protein (PBP), have provided a significant insight into the androgenic interaction at genetic level. The PBP (also referred to as prostatein) is a tissue-specific glycoprotein consisting of three polypeptides (C1, C2 and C3) encoded by three highly homologous genes [Parker *et al.*, 1982]. Its synthesis is profoundly depressed by castration and can be restored after androgen administration [Heyns *et al.*, 1977]. The homology existing between the DNA-binding domain of AR and that of other steroid receptor classes accounts for the fact that AR recognise hormone response elements (HRE, see 2.1.) specific to glucocorticoids or progestins in the tyrosine aminotransferase (TAT) gene [Denison *et al.*, 1989] and the MMTV long terminal repeat region [Ham *et al.*, 1988]. Similarly, the PBP genes contain putative androgen response elements which are responsible for androgenic modulation of their expression [Rushmere *et al.*, 1987]. Androgen-responsive genes also include those encoding for secretory proteins that are being used to monitor prostatic disease, specifically the acid phosphatase [Yeh *et al.*, 1987] and the kallikrein-like prostate specific antigen [Digby *et al.*, 1989].

A deeper knowledge of mechanisms whereby androgens regulate expression of responsive genes directly involved in the growth control of prostatic cells appears essential for evolution of both basic and clinical research relevant to the malignant human prostate.

5.1.4. Multifarious growth regulation

Androgens are thought to be necessary for prostate development and maintenance of both cell number and functional activity in the adult gland. However, a direct role for androgens in the control of growth of prostatic cells is still questionable. Androgen-dependence of both pre- and post-natal development of the prostate is illustrated by the evidence that administration of testosterone to immature male rats produces an accelerated prostatic development [Berry & Isaacs, 1984], whilst deficiency of either AR or the 5α-reductase enzyme results in a rudimental prostate or in its complete absence [Imperato-McGinley, 1984]. In addition, the presence of androgenic steroids preserves the normal size of the adult gland through a balance between cell renewal and cell death, although exogenous androgens do not induce its enlargement. However, it has been suggested that other controlling factors are required for accomplishment of androgen-promoted proliferation of prostatic cells [Bruchovsky *et al.*, 1975].

There is substantial evidence that steroid hormones other than androgens may be significantly involved in the growth control of normal or pathological human prostate. In the first

place, oestrogens have been implicated in the development of BPH, while their role in human prostate cancer is still debated (see [Griffiths *et al.*, 1989]). Previous work indicated that oestrogens may exert a direct effect on the proliferative activity of prostate epithelial cells, an action which has its unique morphological counterpart in the so-called "squamous metaplasia" [Bern, 1963]. Further studies have shown that oestradiol, alone or synergizing with androgens, may be relevant to the genesis of both benign prostatic hyperplasia and prostatic carcinoma [Wilson, 1980; Wilding, 1992]. This has led to the hypothesis of androgen-supported oestrogen-enhanced stimulation of prostate epithelium as an essential requirement to overcome factors that normally limit cell proliferation [Leav *et al.*, 1989]. Additionally, high affinity oestrogen binding sites have been identified in normal, benign and cancerous human prostatic tissues [Murphy *et al.*, 1980; Ekman *et al.*, 1983]. In particular, immunocytochemical studies have revealed that oestrogen receptors are mostly located in the stromal compartment of prostatic tissues, arguing that stromal cells may represent the primary site of oestrogen action in the prostate [Chaisiri & Pierrepoint, 1980]. This view has been further supported by the finding that oestrogens apparently lack mitogenic activity on cultured prostatic epithelium [Eaton & Pierrepoint, 1982]. However, several *in vitro* studies have shown that oestradiol stimulates growth of androgen-dependent LNCaP human prostate epithelial cancer cells at levels comparable or

even greater than those observed after androgen administration [Schuurmans *et al.*, 1988a; Sonnenschein *et al.*, 1989].

Although a role for glucocorticoids in the pathogenesis and progression of human prostate cancer has not yet been defined, their implication in prostate growth and function is suggested [Griffiths *et al.*, 1979]. Glucocorticoid receptors (GR) have been found in both rat and human prostatic tissues; their presence may account for the glucocorticoid stimulation of prostatic epithelial cells observed *in vitro* [McKeehan *et al.*, 1984]. Glucocorticoids seem to counteract some prostate processes induced by castration, such as weight loss and decrease of PBP secretion. This suggests that glucocorticoids may assume an important role when androgen regulation of prostatic tissues is in some way impaired.

The conviction that androgens could not be solely responsible for prostatic cell proliferation has stimulated research on the possible involvement of diffusible local factors in both normal and aberrant prostate growth. A number of prostate-derived growth factors have been identified in human BPH and prostate tumour tissues and isolated from prostatic secretions [Parrish *et al.*, 1984; Matuo *et al.*, 1987]. In particular, substantial amounts of EGF-like activity have been detected in human prostatic tissues and fluids [Elson *et al.*, 1984; LLoyd *et al.*, 1992]. Additionally, presence of EGFR has been documented in both human and rat ventral prostate. In the latter, androgens down-regulate expression of EGFR, suggesting that castration may result in EGFR induction and consequently prevent prostatic

regression [Traish & Wotiz, 1987]. In contrast, synthesis of EGFR is stimulated by androgens in LNCaP human prostate epithelial cancer cells [Schuurmans *et al.*, 1988a]; this evidence directly hints at the likely implication of prostate stromal compartment in the epithelial EGFR regulation by androgens. Studies on human prostate cancer have revealed an inverse relationship between EGFR and AR, with a developing prevalence of high versus low affinity sites of EGF binding in poorly differentiated tumours [Davies & Eaton, 1989].

Several human prostate cancer cells in culture synthesise and release into the culture medium polypeptide growth factors. In particular, DU145 and PC3 cells produce PDGF-AA and PDGF-BB, in spite of the absence of specific cell surface receptors [Sitaras *et al.*, 1988]. Furthermore, the effects of peptide growth factors on proliferation of prostate tumour cell lines have been investigated. Notably, either growth stimulation by EGF and TGFα [Eaton *et al.*, 1988] or inhibition by TGFβ [Wilding *et al.*, 1989a] have been observed.

Unfortunately, very little information exists on the interaction between steroid hormones and peptide growth factors in the regulation of prostatic cancer cell proliferation and function. A clearer understanding of these processes, in either androgen-responsive or androgen-independent states, appears fundamental to appropriately address expanding areas of basic research and for development of new growth factor-based therapies for human prostate carcinoma.

5.1.5. Mesenchymal-epithelial interactions

The potential role of mesenchymal-epithelial interaction in the development of both BPH and cancer of the human prostate was initially realised at histomorphological level by the pioneering studies of Franks [Franks, 1956]. Later on, using an *in vitro* model, Franks and colleagues observed that DNA synthesis in prostatic epithelial cells depends on the presence of adjacent stromal and myoepithelial cells [Franks *et al.*, 1970].

In the more recent years, this area of investigations has been dominated by the impressive work of both Gerald Cunha's and Leland Chung's groups (for relevant reviews see [Cunha *et al.*, 1987] and [Chung *et al.*, 1991]).

In the first place, Cunha and colleagues indicated that the balance between stromal and epithelial compartments is essential for direction of prostate development and growth. Using elegant procedures, the authors separated the endodermal urogenital sinus (UGS), from which prostate epithelial outgrowths (prostatic buds) originally emerge, into mesenchyme (UGM) and epithelium (UGE) and found that the amount of UGM present is primarily responsible for the final size of the adult gland [Chung & Cunha, 1983]. In addition, increase of prostatic growth *in situ* was observed when UGM or intact UGS were grafted directly into the prostate of intact adult male rats [Chung *et al.*, 1984].

It has been proposed that a tight interaction exists between neuroendocrine networks residing primarily in the mesenchyme and key enzymes of androgen metabolism basically located in the epithelium of the human prostate [Chang & Chung, 1989; Higgins

& Gosling, 1989]. The role of mesenchyme as a mediator of androgen action upon prostatic epithelium has been established in mesenchyme and epithelium tissue recombination experiments using mice affected by testicular feminization (Tfm) syndrome. In the latter condition, the external genitalia are feminized and the prostate completely fails to develop as a consequence of defective or absent androgen receptors [Ohno, 1977]. Four possible combinations of Tfm or wild-type UGM and UGE were grafted into intact male hosts to achieve a physiological androgenic milieu. Prostatic morphogenesis occurred only in the presence of the wild-type UGM, whilst no prostatic differentiation was seen using Tfm UGM, irrespective of the epithelium type (Tfm or wild-type UGE) used [Cunha *et al.*, 1980]. The androgen-dependent formation of prostate observed using the wild-type UGM and Tfm UGE combination indicates that androgen action would be mediated by receptor-positive stromal cells, since Tfm epithelium remains AR-negative throughout prostate induction [Shannon & Cunha, 1984]. Additionally, using this combination, androgen-induced DNA synthesis was found to be equivalent to that obtained in completely wild-type prostates, supporting the view that androgens regulate a variety of epithelial features via androgen-induced mediators of stromal origin [Cunha & Chung, 1981]. This assumption is also supported by the fact that androgens stimulate DNA synthesis in epithelial prostatic tissue in organ culture only in the presence of stroma, whereas androgen-induced growth of cultured isolated epithelial cells is not yet established [Sandberg & Kadohama, 1980].

In a recent model system, morphologically and biochemically well characterised prostatic fibroblast and epithelial cell lines derived from both embryonic and adult prostate glands have been inoculated together *in vivo* or co-cultured *in vitro* [Camps *et al.*, 1990]. Results obtained so far appear noteworthy. Firstly, coinoculation of athymic mice with a rat foetal UGM cell line (rUGM) and androgen-independent PC3 human prostate epithelial cancer cells resulted in rUGM stimulation of PC3 tumour growth in the male mice; this suggests that androgen-sensitive growth can be induced in androgen-unresponsive epithelial cells by interaction with relevant mesenchymal cells [Chung *et al.*, 1991]. In the second place, the marginally tumourigenic LNCaP human prostate cancer cell line can be stimulated to form carcinomas in the male athymic mice by the concomitant inoculation of non-tumourigenic prostate or bone fibroblasts; in contrast, LNCaP cells did not form tumours in either castrated male or female hosts [Gleave *et al.*, 1991]. Additionally, conditioned media from cultured bone and prostate fibroblasts remarkably enhanced growth of LNCaP *in vitro*. This evidence indicates that normal fibroblasts may accelerate prostate epithelial tumour formation in the presence of an androgenic microenvironment and that this action is likely to be mediated by diffusible, paracrine growth factors.

5.1.6. Cell apoptosis: a self-program to die

In the past the concept of cell death was mistakenly identified as a pathological process, termed necrosis, whereby the

cell dies because of physical or chemical external injuries. This "accidental" cell death typically tends to assume specific morphological and biochemical characteristics, also depending on the causative agent. However, further investigations made clear that a morphologically distinct, spontaneous form of cell death occurs naturally in several tissues under various conditions. In the early 1970s, Kerr and coworkers [Kerr *et al.*, 1972] christened this physiological process of cell death as apoptosis, from the ancient Greek word describing the falling off of leaves from trees or petals from flowers. The fact that cell death is spontaneously occurring generated the idea that it is self-programmed and led to introduce the term "programmed cell death" as an alternative expression to name this process (reviewed by [Schwartzman & Cidlowski, 1993]).

In the last two decades an increasing number of reports have indicated that programmed cell death occurs in phylogenetically distant organisms during embryonic and foetal development, tissue differentiation and growth. Furthermore, addition or removal of appropriate regulatory factors induces apoptosis in many hormone- and growth factor-responsive tissues, including prostate, breast, uterus, thymus, adrenal cortex. It is also known that apoptosis occurs in several human tumours spontaneously or in consequence of treatment; examples include breast cancer, prostate cancer, leukaemias, pancreatic cancer and many others.

In the programmed cell death a certain number of specific signals activate the cell to undergo an energy-dependent

mechanism of death or, by paraphrasing, the cell commits suicide without any apparent reason in an otherwise friendly microenvironment, as opposed to the cell which is murdered by its hostile milieu in the case of necrosis. Activation of the programmed cell death may depend upon either the presence of a specific inducer (e.g. glucocorticoids in the thymocyte death [Wyllie, 1980]) or the loss of a normally active repressor (as when reduction of serum ACTH leads to cell death in the zona reticularis of the adrenal gland [Wyllie *et al.*, 1973]). Once activated, programmed cell death proceeds along an orderly sequence of morphological and biochemical events which ultimately lead to an irreversible degradation of genomic DNA and cell fragmentation. The structural changes that eventually lead to cell death are rather stereotypic and initially consist of loss of cell junctions, condensation of nucleus and cytoplasm, and chromatin coalescence into one or several large masses; this is followed by nuclear breaking and cellular rupture into numerous membrane-bound smooth-surfaced fragments, called apoptotic bodies, which contain a variety of morphologically intact cytoplasmic organelles and some nuclear fragments. The apoptotic bodies are subsequently phagocytosed and rapidly digested by the nearby macrophages or epithelial cells.

Androgen withdrawal induces specific biochemical and molecular changes in the normal prostate which finally lead to cell death. After castration, the levels of serum testosterone dramatically fall below 2% of normal controls; as a consequence, prostatic DHT also drops to less than 5% of intact controls, leading

to both variation in the nuclear AR function and a novel expression of genes encoding for a specific set of proteins. These genes include the testosterone-repressed prostate message-2 (TRPM-2) gene [Montpetit *et al.*, 1986], the Ca^{2+} responsive *c-fos* [Buttyan *et al.*, 1988] and the *c-myc* [Quarmby *et al.*, 1987] protooncogenes, the hsp70 gene [Buttyan *et al.*, 1988] and the TGFβ gene [Kyprianou & Isaacs, 1989a]. Initially, castration-induced apoptosis of prostatic cells involves enzymatic degradation of genomic DNA into fragments consisting of nucleosomal oligomers lacking intranucleosomal breaks. This DNA fragmentation is due to the activation of a Ca^{2+}/Mg^{2+}-dependent endonuclease, induced by the increase of intracellular calcium levels further to androgen ablation [Wyllie *et al.*, 1984]. The process of DNA degradation is complete one day only after castration and it appears to be an irreversible commitment step for viable cells to die [Kyprianou *et al.*, 1991]. The importance of perturbation of cellular calcium homeostasis in the prostatic cell death is first of all suggested by the evidence that a rapid, sustained increase of intracellular calcium is the earliest detectable event in cells undergoing apoptosis. More importantly, the artificial rise of intracellular calcium concentration by means of calcium ionophores (such as A23187) or ionomycin induces prostatic cell death [Martikainen & Isaacs, 1990]. Conversely, comparison of castrated rats receiving prostate implants of either placebo or nifedipine, a calcium channel blocker, showed that castration-induced prostatic involution is significantly slower in the nifedipine-treated rats than in the placebo-treated group

[Martikainen & Isaacs, 1990]. During days 2-7 after castration, an increasing expression of specific genes is seen in the involuting rat ventral prostate. In particular, the TRMP-2 mRNA appears 2 days after castration and peaks at a 400-fold level at day 4 [Montpetit *et al.*, 1986], while TGFβ mRNA increases by approximately 10-fold since the first day and is maximally expressed (about 40-fold) by 4 days postcastration [Martikainen *et al.*, 1990]. Similarly, mRNA for the c-*myc* protooncogene raises up to 6-fold in the regressing prostate, apparently as a component of a cascade induction of c-*fos*, c-*myc* and hsp70 transcripts [Buttyan *et al.*, 1988].

Basically, tumour growth depends upon the balance of cell division and cell death, a greater cell proliferation being required for continued neoplastic growth. Therefore, effective treatments could be designed aiming to either reduce the rate of cancer cell proliferation or augment the rate of cell death, eventually leading to a negative balance in favour of cell death and to the involution of the tumour. Since most of the presently available chemotherapeutic agents act on proliferating cancer cells, the effectiveness of cancer therapy will be greater in tumours having high proliferation rates. However, in the presence of a low rate of cell death, it is highly unlikely that chemotherapy could lessen the proportion of dividing cells below that of dying cells, also because the maximum tolerated therapeutic intensity is generally not high enough to achieve a significant reduction of cell proliferation [Schackney *et al.*, 1978]. Therefore, in order for chemotherapy to be effective the rates of both cell proliferation

and death in a given tumour must be high [Tubiana & Malaise, 1975]. An additional obstacle comes from the fact that currently used cytotoxic drugs affect only proliferating (cycling) cells, while cells in a quiescent state (G_0) or cells dividing late after exposure to the chemotherapeutic agents may repair the damage undergone and survive the treatment. Thus, a major hope is to develop new pharmacological therapies which can kill cancer cells regardless of their cell cycle state. This is exactly what occurs in the normal rat prostate following androgen ablation. In the intact adult male the supply of circulating androgens is enough to retain rates of cell proliferation and death in equilibrium, avoiding either overgrowth or involution of the gland. Since the proportion of dividing cells in the normal adult prostate of intact male rats is approximately only 2% per day, a 20% increase of the daily rate of cell death caused by androgen ablation, coupled to the decrease of cell proliferation, will result in the fact that after 7 days castration over 70% of the rat prostatic cells have died [Isaacs, 1984]. This clearly indicates that the cell death induced by androgen ablation mostly involves non-dividing, G_0 cells.

Coming to human prostate cancer, the vast majority of patients experience an often dramatic, beneficial response to androgen ablation therapy; unfortunately, most of them subsequently develop a hormone-refractory disease where any second-line antiandrogen treatment is ineffective, no matter how aggressively given [Eaton & Griffiths, 1990]. There is convincing evidence that this failure is a reflection of the heterogeneous

composition of primary prostate tumours, wherein androgen-dependent and -independent cancer cells are likely to coexist well before any endocrine treatment is initiated [Isaacs & Coffey, 1981].

Several experimental data confirm the assumption that programmed cell death is activated also in androgen-dependent prostate tumour cells following androgen ablation. In this respect, the case of transplantable PC82 human prostatic cancer cell line is enlightening [Kyprianou *et al.*, 1990]. When PC82 cells are grafted into intact male nude mice they give rise to actively growing tumours. If the host mice undergo castration, the proportion of dividing cells in the PC82 population decreases about 7-fold, while the rate of cell death increases up to 11-fold, so that the tumour rapidly regresses. At the molecular level, these changes are paralleled by DNA fragmentation and by the increasing expression of both TRPM-2 and TGFβ genes. Conversely, if exogenous androgens are administered to the castrated mice, DNA degradation ceases, TRPM-2 and TGFβ transcripts decline to pre-castration levels and tumour growth resumes.

Unfortunately, androgen-independent prostatic cancer cells have lost the program of cell death in response to androgen ablation. However, using a panel of cell lines derived from the Dunning R3327 androgen-independent prostate tumours, it has been shown that these cells retain the major part of the pathways leading to programmed cell death. For instance, treatment *in vitro* of the Dunning AT-3 cell line with non-

androgenic ablative agents inducing a "thymine-less death" causes the induction of the TRMP-2 gene and the activation of the Ca^{2+}/Mg^{2+}-dependent endonuclease with the resulting fragmentation of genomic DNA [Kyprianou & Isaacs, 1989b]. Once again, a crucial shortcoming of this type of anticancer drug is that they require cell proliferation to be effective. Thus, although apoptosis can readily be induced in androgen-dependent prostate cancer cells by androgen ablation and proliferating androgen-independent cells can be controlled using the presently available cytotoxic agents, the key aspect remains the management of quiescent androgen-independent cell clones. In this respect, development of non-androgenic (growth factor-based?) strategies able to activate the pathways of programmed death in nondividing androgen-independent prostate tumour cells is awaited.

6. Aims of Thesis

6. Aims of Thesis

The main goal of the present thesis was to explore the activity of both sex steroid hormones and transforming growth factors α and β in the regulation of growth and metabolism of prostate cancer cells, *in vitro*.

To this end, three human prostate tumour cell lines, LNCaP, DU145 and PC3, each having distinct status of steroid sensitivity, have been used as model systems.

The effects of both androgens (T and DHT) and oestradiol on cell growth rates have been weighed, also in relation to mechanisms responsible for accomplishment of hormone action. In addition, status and content of steroid receptors (AR and ER) has been determined using multiple approaches, including ligand binding assay, immunocytochemical assay, reverse transcriptase-PCR of mRNAs, and through immunofluorescent staining of the hsp27, which appears to be quantitatively and qualitatively related to a functional ER machinery.

Growth response of prostate cancer cells to either TGFα or $TGF\beta_1$ has been assessed in different experimental conditions. Furthermore, presence of membrane receptor sites for both EGF and TGFβ has been evaluated using radioreceptor assay and, for EGFR, Western blot analysis. Expression of endogenous EGF, TGFα and $TGF\beta_1$ has also been estimated by immunofluorescent staining.

Analysis of rates and direction of steroid metabolism has been carried out through incubation of cultured cells with labelled steroid precursor (T or E_2) and reverse phase-HPLC estimation of both precursor degradation and product's formation. Possible paracrine influence of either TGFα or TGFβ_1 on the extent of both individual reactions and overall reductive and oxidative pathways of either metabolism have also been inspected.

The results have been used as the basis for models of hormone-sensitive and insensitive prostate cancer, allowing suggestions of how new therapies might be devised.

MATERIALS AND METHODS.

1. Materials

1. Materials

1.1. In Vitro Systems

1.1.1. Cell lines

LNCaP.FGC (passage 19), DU145 (passage 59) and PC3 (passage 16) human prostate cancer cell lines all were purchased from the American Type Culture Collection (Rockville, MD).

The LNCaP cell line (Figure 16A) was originally established from a lymph node metastasis of a human prostatic adenocarcinoma [Horoszewicz *et al.*, 1980]. Cultured cells grew in monolayers loosely attached to the surface of plastic culture dishes, with a mean population doubling time of 60 h. Early studies showed that LNCaP cells contained specific androgen- and oestrogen-binding proteins in the cytosol; nuclear androgen receptors were also detected [Horoszewicz *et al.*, 1983]. In addition, cells responded in a dose-dependent manner to DHT, with a parallel rise in the production of the prostatic acid phosphatase. Karyology studies revealed that this cell line is aneuploid, having a wide range (33 to 91) of chromosome number, at least 7 different marker chromosomes and a modal number of 76 chromosomes after 32 months growth *in vitro*;

The DU145 cell line (Figure 16B) was originated from a brain lesion of a human metastatic prostatic carcinoma [Stone *et al.*, 1978]; the brain metastasis was described as a

A

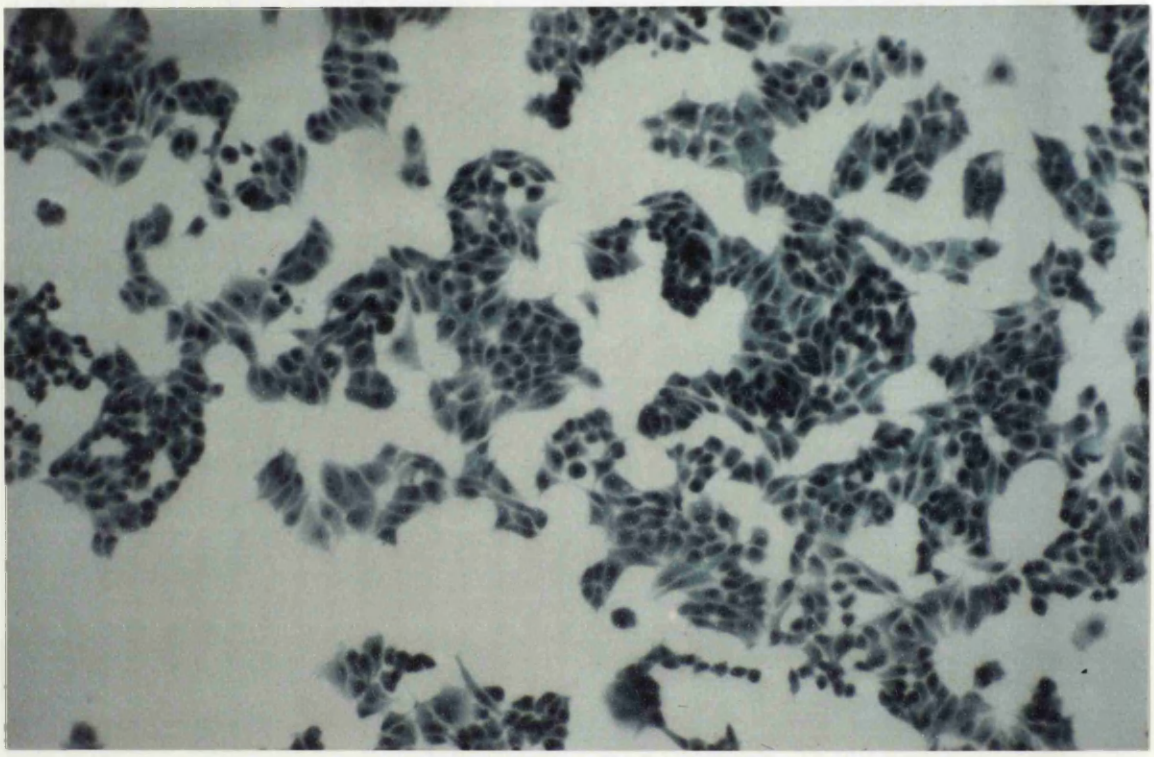
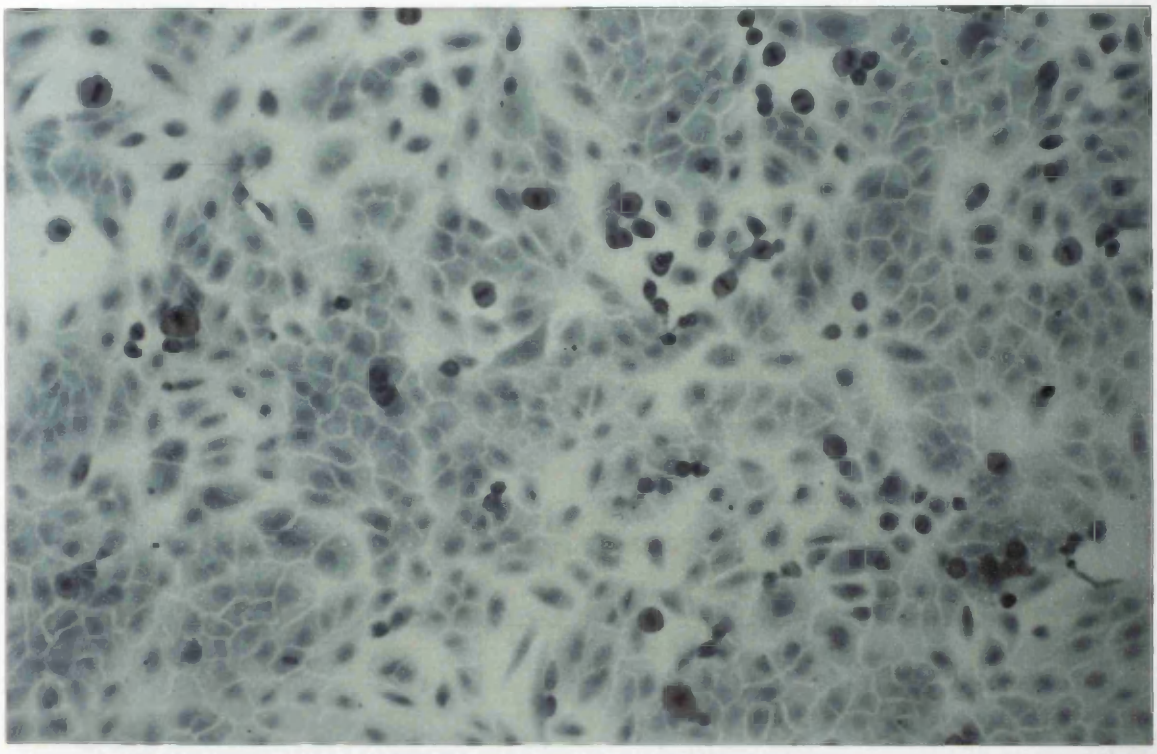
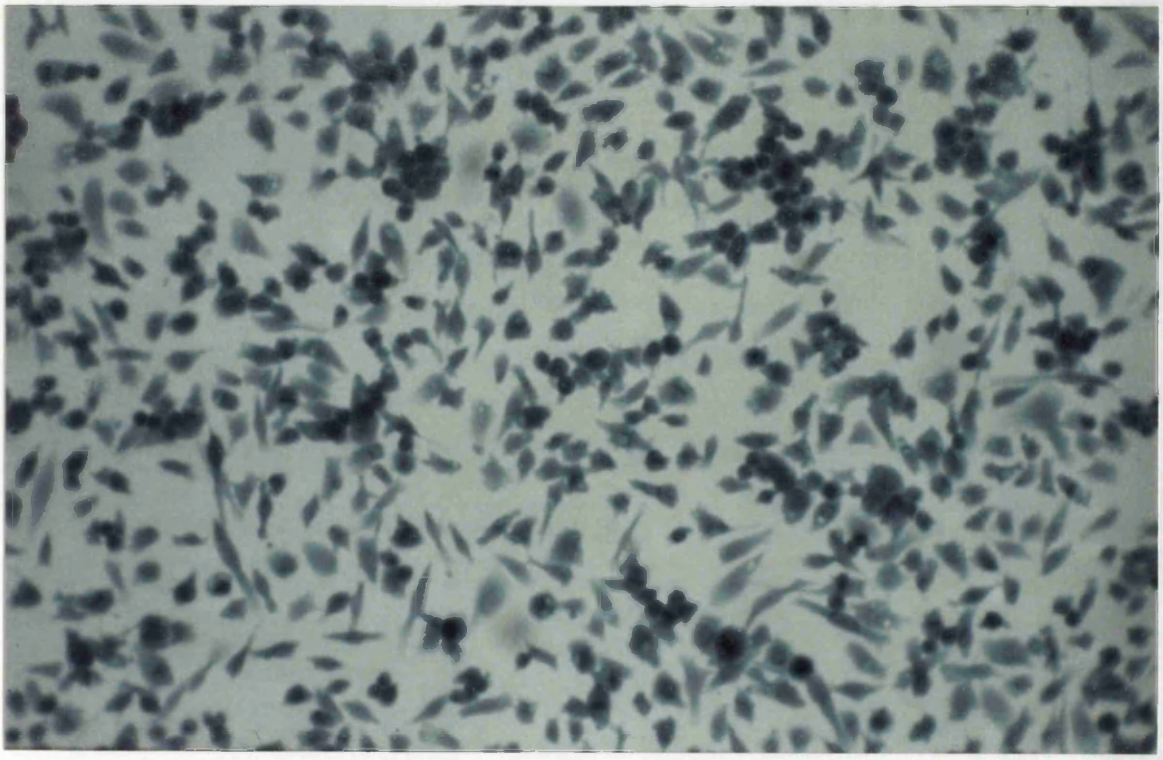


Figure 16. Microphotographs of LNCaP (A), DU145 (B) and PC3 (C) cells in culture (x40 magnification, Papanicolau staining).

B



C



moderately differentiated adenocarcinoma tissue, with foci of poorly differentiated cells. The DU145 cells were reported to be an apparently non hormone-sensitive cell line, growing as isolated islands in culture with an average doubling time of 34 h. Karyotype analysis at transfer 57 revealed that cells were aneuploid, having a hypotriploid modal chromosome number (64), several minute chromosomes and 3 marker chromosomes.

The PC3 cell line (Figure 16C) were derived from a vertebral metastasis of a poorly differentiated human prostatic carcinoma [Kaighn *et al.*, 1979]. Cultured PC3 cells were described as having epithelial-like morphology, with numerous grape-like aggregates of loosely attached viable cells. Growth of cells was insensitive to a variety of factors, including DHT, EGF, FGF and insulin, with a population doubling time of approximately 33 h. Chromosome studies at passage 17 showed a bimodal frequency distribution of chromosome number in the subtriploid range, with two major peaks at 60 and 69 chromosomes. In addition, subsequent karyotypic analysis demonstrated that chromosomes 2, 3, 5, 15 and Y were consistently missing, while at least 11 different marker chromosomes were observed [Ohnuki *et al.*, 1980].

1.1.2. Cell culture

For routine maintenance, cells were grown in RPMI-1640 medium, containing 5 µg/ml phenol red and supplemented with 10% foetal calf serum (FCS), 2 mM L-glutamine and antibiotics (100 UI/ml Penicillin, 100 µg/ml Streptomycin, 0.25 µg/ml

Amphotericin B) - all from GIBCO BRL (Uxbridge, Middlesex, UK). Cells were harvested using trypsin:EDTA (1:5 v/v, GIBCO BRL) and subcultured at 1:4 to 1:5 ratio in 100 mm plastic dishes (Costar Italia srl, Milan) at 37°C in a humidified atmosphere of 5 % CO_2 in air. Both culture medium and cells were routinely tested for mycoplasma contamination using either infusion in mycoplasma broth and enriched nutrient agar or Hoechst 33258 fluorescent staining method. For all experiments cells having a narrow range of passage number were used (LNCaP = 21-24, DU145 = 61-63, PC3 = 18-20).

1.2. Antibodies and Antisera

The D5 antibody and the human ER cDNA were kindly provided by R.J.B. King (Guildford University, UK) and by P. Chambon (Strasbourg, F), respectively.

Neutralising antibody against TGFß$_1$ (rabbit, polyclonal) was purchased from British Biotechnology Ltd. (Abingdon, UK).

Polyclonal (rabbit, BG48) antibody against EGF receptor was generously supplied by W. Gullick (ICRF, Oncology Group, London).

Monoclonal antibodies against EGF, TGFα and TGFβ$_1$ were purchase from Oncogene Science Ltd (Manhasset, NY).

Fluorescent (FITC conjugate) anti-mouse IgG (sheep) and anti-rabbit IgG (donkey) antisera were kindly supplied by SAPU (Carluke, Scotland).

ADDENDUM

The identity of LNCaP cells was also assessed by immunocytochemical assay of PSA, in both standard conditions and following PSA stimulation by 1,25$(OH)_2D_3$ (vitamin D_3). (Skowronski et al., Endocrinology 1993, 132: 1952-1960.)

2. Experimental Procedures

2. Experimental Procedures

2.1. Growth Studies

Growth rates of LNCaP, DU145 and PC3 human prostate tumour cell lines were measured in routine RPMI medium supplemented with 10% FCS. Cells ($1\text{-}5x10^4$) were seeded onto 6-well tissue culture plates (9.5 cm^2, Costar) and left undisturbed for 24 h. Thereafter, cells were grown up to 12 days with a medium change every 3 days. DNA estimation was performed as described later in this section (see 2.10.) at day 2, 4, 6, 8, 10 and 12 on triplicate wells for each observation time. The average population doubling time was calculated by dividing the $\log_{10}$ of the average DNA content per well by the $\log_{10}2$.

2.2. Radioligand Binding Assay of AR and ER

Steroid receptor (AR and ER) content and status of human prostate cancer cells was determined by means of radioligand binding assay, as extensively described elsewhere [Castagnetta *et al.*, 1987a; Castagnetta *et al.*, 1992a]. Cells (70-80% confluent), growing in 162 cm^2 plastic flasks (Costar) in routine medium, were harvested and pelleted by centrifugation at 120xg for 5 minutes at 4°C. The cell pellet was then washed twice in ice-cold PBS-A (170 mM NaCl, 3.4 mM KCl, 2 mM Na_2PO_4, pH 7.4), resuspended in 4 ml of HED buffer (10 mM Hepes, 1.5 mM EDTA, 0.5 mM Dithiothreitol, pH 7.4) and gently teflon-glass homogenised for 2x10 sec bursts at a setting of 600-800 rpm on

a Potter S cooling system homogeniser (B.Braun Biotech GmbH, Germany). As routinely carried out in our laboratories for both ER and AR assay in either breast or prostate tissues [Castagnetta *et al.*, 1987a; Castagnetta *et al.*, 1992a], cell homogenates were spun at 800xg for 5 min at 4°C to separate the soluble (supernatant) from the nuclear (pellet) cell fraction. The supernatant was further centrifuged at 2000xg for 5 min at 4°C to remove cell debris; the nuclear pellet was subsequently washed three times in ice-cold buffered saline (10 mM Hepes, 150 mM NaCl, pH 7.4) and resuspended to the original volume in the same saline. Aliquots (150 µl) of each cell fraction were therefore incubated overnight at 4°C against increasing concentrations (from 0.1 up to 5 nM) of radioligand, namely [17α-methyl-^{3}H]mibolerone for AR and [2,4,6,7-^{3}H]17β-oestradiol for ER. In the case of AR, a 100 fold excess of unlabeled mibolerone was used to measure displacement of androgen binding; a constant concentration (10^{-7}M) of cold triamcinolone acetonide was also used to hamper mibolerone binding to glucocorticoid receptors. In the case of ER, a 100 fold excess of unlabeled diethylstilbestrol (DES) was used for competition studies; in addition, a 100 fold excess of unlabeled synthetic androgen methyltrienolone (R1881) was used to measure possible displacement of oestradiol binding from AR. After incubation, DCC absorption and filtration methods were used to separate bound from unbound ligand in soluble and nuclear fraction, respectively. For the soluble fraction, each tube was mixed for 15 min with 0.5 ml of DCC (0.15% w/v Charcoal,

0.0015% w/v Dextran) suspension in HES buffer (10 mM Hepes, 1.5 mM EDTA, 250 mM Sucrose, pH 7.4) to strip the unbound radioligand. Samples were centrifuged at 3000xg for 5 min at 4°C. One ml aliquots of the resulting supernatant were counted in a LS1801 β-counter (Beckman Inc., Irvine, CA, USA) using Ready Gel™ (Beckman) as scintillation cocktail. For the nuclear fraction, 100 µl aliquots of the nuclear suspension were filtered through Whatman GF/C glass fiber filters (Whatman Ltd., Maidstone, UK) using a Millipore apparatus (Millipore SpA, Milan, Italy), the unbound ligand being washed out using saline. Filters were then removed, dried overnight at room temperature and counted for radioactivity. All the experiments to measure AR and ER cell contents were done in triplicate. Binding data were finally processed as described below.

2.3. Analysis and Expression of Receptor Data

Receptor data from both saturation and competition studies were analysed and processed using Scatchard analysis and a modification (©OncoLog 2.2) of a least-square fit routine [Leake *et al.*, 1987], run on an IBM-PC, yielding both dissociation constant (K_d) and concentration values (fmol/ml homogenate); the latter were expressed either as fmol/mg protein or DNA or as number of sites per cell, for any cell compartment. Data were also analysed using a model for one or two binding sites, depending on the best fit achieved. Protein and DNA cell contents were determined using the Bradford [Bradford, 1976] and the modified Burton methods [Katzenellenbogen & Leake, 1974], respectively.

2.4. Reverse Transcriptase-PCR of AR

Expression of AR mRNA in LNCaP, DU145 and PC3 cells was examined using reverse transcription of cellular RNA and PCR amplification of the reaction products. Cells grown in 100 mm cell culture dishes (56 cm^2, Costar) until 80-90% confluent were washed twice with PBS-A and lysed directly on dishes using 3 ml of RNazol™ B (Biotecx Lab., Houston, TX). Cell lysates were therefore harvested and total RNA extracted according to the recommendations of the supplier.

The precipitated RNA was dissolved using 100 µl of diethylpyrocarbonate (DEPC)-treated water and heated to 60 °C for 15 minutes to ensure complete solubility. In order to avoid contamination of genomic DNA, RNA preparations were diluted to a final concentration of 1 µg/ml and aliquots (1 µl) incubated with 10U DNase I (GIBCO BRL) for 15 minutes at room temperature. Digestion was terminated by the addition of 1 µl of 20 mM EDTA and DNase I heat inactivated by incubation at 65 °C for 15 minutes. Reverse transcription was performed using a modification of the Superscript preamplification system (GIBCO BRL) methodology. Briefly, 1 µl of random hexamers (50 ng/µl) was added to each digest; samples were then mixed gently and heated to 70 °C for 10 minutes. After cooling on ice for 5 minutes, one set of RNA duplicates received 8 µl of the reaction cocktail, consisting of 3 µl DEPC-treated water, 1 µl of 10x reaction buffer (200 mM Tris-HCl - pH 7.4, 500 mM KCl, 25 mM MgCl, 1 mg/ml bovine serum albumin, BSA), 1 µl of 10 mM deoxynucleotides

triphosphates (dNTP) mix, 2 µl of 0.1M dithiothreitol (DTT), 1 µl of Superscript II RT (200 U/µl). The second set of duplicates received the same reaction cocktail, except one µl more DEPC-treated water instead of Superscript II RT. After gentle mixing, all samples were incubated at 45 °C for 50 minutes and the reaction terminated by heating to 70 °C for 15 minutes. Excess RNA was eliminated through incubation for 20 minutes at 37 °C with 1 µl RNase H (2U/µl, Boehringer, Mannheim, Germany). PCR conditions used were a modification of those previously reported by others [Allen *et al.*, 1992]. Shortly, the amplification reaction was carried out in a total volume of 100 µl containing the reverse transcribed cDNA, 10 pmol (1 µM) each of the 24-mer 5'-GCTGTGAAGGTTGCTGTTCCTCAT-3' as sense primer and the 24-mer 5'-TCCAGAATCTGTTCCAGAGCGTGC-3' as antisense primer, 10x PCR buffer (100 mM Tris-HCl - pH 9.0, 500 mM KCl, 1.0% (w/v) Triton X-100), 10% (v/v) dimethylsulphoxide (DMSO), 5U Taq-DNA-polymerase (Promega, Madison, WI). PCR was carried out using a Hybaid Omnigene thermocycler in 40 cycles, each comprising 45 seconds at 93 °C, 1 minute at 60 °C and 1 minute at 72 °C. The primer set used identifies a 280 bp target locus from nucleotides 229 to 508 of exon 1 of the human AR gene (HUMARA, GenBank; accession number M21748), which includes a unique, highly polymorphic CAG trimeric repeat (short tandem repeat, STR) encoding 11-31 glutamine residues.

Aliquots (16 µl) of PCR products were electrophoresed for 2 h at 50 W through a 2% agarose gel containing 1x TBE buffer (89 mM Tris-borate, 89 mM boric acid, 2 mM EDTA, pH 8.0). The gel

was then stained with 1 μg/ml ethidium bromide for 20 minutes at room temperature, destained in water by 2x15 minutes washes and finally photographed using a Polaroid land camera on a long wave UV light box.

2.5. Immunocytochemistry of ER and PgR

Presence of both ER and PgR was investigated in LNCaP, DU145 and PC3 cells by a modification respectively of ER-ICA and PgR-ICA methods, using the commercially available Abbott (Divisione Diagnostici, Rome, Italy) kits. Cells were grown in routine medium directly onto 2-well Lab-Tek® Tissue Culture Chamber Slides™ (Nunc, Naperville, IL) until 60-80% confluent. Medium was aspirated off, cells washed twice in ice-cold PBS-A for 5 minutes and fixed for 10 minutes at 4°C using 3.7% (v/v) formaldehyde solution in PBS (150 mM NaCl, 10 mM Na_2HPO_4, 2.0 mM KH_2PO_4, pH 7.4). After 5 minutes rinse in PBS at 4°C, wells were exposed for 4 minutes to methanol and for 3 minutes to acetone at -20°C, with two subsequent rinses in PBS for 5 minutes. Non-specific binding sites were blocked through incubation of slides with goat serum for 20 minutes in a humidified chamber at room temperature. The excess of blocking serum was then removed and slides incubated for 24 h at 4°C with the primary rat monoclonal anti-ER or anti-PgR antibodies (0.1 μg/ml). Parallel control wells received normal rat IgG under identical conditions. After 2 washes of 5 minutes in ice-cold PBS, slides were incubated in a humidified chamber at room temperature with the bridging antibody (goat anti-rat) and then

exposed to the rat PAP-complex, each step being for 30 minutes. Slides were washed twice as before and the solution of the chromogen substrate (diaminebenzidine, DAB) added for 15 minutes under the above conditions. After 5 minutes washing under gentle running of distilled water, all slides were counterstained for 10 minutes with 0.2% (w/v) ethyl green in O.1M sodium acetate buffer (pH 4.0). Finally, slides were rinsed in distilled H_2O, air dried and, after 2x1.5 minutes dips in butanol and 3X2 minutes dips in xylene, mounted using the Eukitt® mounting medium (Bio-Optica, Milan, Italy). Abbott ER-ICA and PgR-ICA control slides were simultaneously processed and used as positive controls. For each condition, 100 randomly selected fields from at least 3 different slides were analysed. Analysis of the receptor staining was performed using the quantitative oestrogen/progesterone analysis application (release 2.0) for CAS™200 Image Analyser (Becton-Dickinson Italia SpA, Milan), which automatically yields percent of positively stained nuclei and measures the intensity of staining (positive stain); the latter was defined as the summed optical density for the positive receptor nuclear area over the summed total optical density of all the nuclei expressed as a percentage. Percentage values of <30%, from 30 to 60% and >60% respectively identified weak, moderate and strong stain intensities.

2.6. Reverse Transcriptase-PCR of ER

Presence of ER mRNAs in prostate cancer cell lines was investigated using reverse transcriptase-PCR, as recently

established in our laboratories [Pfeffer *et al.*, 1993]. Cells grown to near confluence in 100 mm cell culture dishes (56 cm^2, Costar) were washed twice with PBS-A, lysed directly on dishes in 3 ml of RNazol™ B (Biotecx Lab., Houston, TX) and harvested using a rubber policeman. Total RNA was extracted from ZR75-1, MCF7 and MDA-MB231 mammary carcinoma cell lines and from prostate cancer cells according to the manufacturer's protocol (see Section 2.4.). One µg RNA of each cell line was reverse transcribed at 42°C for 45 minutes using 25 pmol (2.5 µM) of a 15-mer antisense primer downstream of the region to be amplified and avian myeloblastosis virus reverse transcriptase (Boehringer, Mannheim, Germany) in PCR-buffer (10 mM Tris-HCl pH 8.3, 1.5 mM $MgCl_2$, 50 mM KCl, 0.005% Tween-20, 0.005% Nonidet P-40, 10 mg/ml gelatin) containing the dNTP at 10 mM each. cDNA was used for amplification without further purification. PCR was carried out with Taq-DNA-polymerase (Promega, Madison, WI) using 25 pmol (2.5 µM) each of the 24-mer 5'-CATAACGACTATATGTGTCCAGCC-3' (nucleotides 937-962, exon 3) oestrogen receptor cDNA sequence [Greene *et al.*, 1986] as sense and the 24-mer 5'-AACCGAGATGATGTAGCCAGCAGC-3' (nucleotides 1596-1572, exon 6) as antisense primers in 30 cycles at 65°C, 72°C and 94°C of 30 sec each. The amplification products were then electrophoresed on a 1.4% agarose gel, photographed under UV-illumination and blotted onto nylon membranes by alkaline transfer [Ausubel *et al.*, 1994]. For Southern hybridisation experiments, a human oestrogen receptor cDNA, generously gifted by Pierre Chambon (Strasbourg, France),

was cut from pSG5 plasmid, radiolabelled with ^{32}P by random-primed labelling (Boehringer) and used as a probe following standard procedures [Ausubel *et al.*, 1994].

2.7. Hsp27 Immunostaining

Immunofluorescence of the 27 kDa HSP27, which is reported to be qualitatively and quantitatively related to ER in several human tissues [King *et al.*, 1987], was also used as a possible index of functional ER. To this end cells grown directly onto 8-well Lab-Tek® Tissue Culture Chamber Slides™ (Nunc, Naperville, IL) were fixed at -20 °C for 15 min in acetone:methanol (1:1, v/v). Cells were then incubated for 30 min at room temperature with 10% (v/v) sheep serum (SAPU, Carluke, Scotland) in PBS to suppress non specific binding. After 5 washes in BSA/PBS (0.1%, w/v), cells were incubated for 2 h at room temperature in presence of a monoclonal (mouse) D5 antibody (1:100) raised against a ER component purified from human myometrium [Coffer *et al.*, 1985]; duplicates wells received PBS/BSA only. After incubation, cells were washed 5 times as before and specific binding of D5 antibody visualised through a second step FITC-conjugated anti-mouse antiserum (1:40). Following 2 h incubation at room temperature in the dark, cells were washed, the plastic upper chambers removed and slides coverslip-sealed using Acquamount (BDH, Dorset, UK) mounting medium. Immunofluorescence was visualised within 24 h using a Leitz Orthoplan fluorescent microscope.

2.8. Preparation of Dialysed, Heat-Inactivated, Dextran Coated Charcoal Treated-FCS (DHICT-FCS)

One hundred ml aliquots of FCS were dialysed (12 kDa molecular weight cut-off) for 48 hours at 4°C against 4x1 litre changes of HBSS Hank's modified buffer (1.3 mM $CaCl_2$, 5.4 mM KCl, 0.5 mM $MgCl_2$, 0.5 mM $MgSO_4$, 137 mM NaCl, 4 mM $NaHCO_3$, 0.4 mM NaH_2PO_4, pH 7.2). The dialysed serum was transferred to a glass container and heat inactivated at 56 °C for one hour in a water bath. The serum was then cooled to 4°C and added to a pellet of dextran coated charcoal (DCC: 0.25% (w/v) Charcoal, 0.0025% (w/v) Dextran) previously suspended in PBS. The solution was stirred at 4°C for 45 minutes and then centrifuged at 10,000xg for further 45 minutes at 4°C. The supernatant was finally filtered through 0.22 µm filters (Millipore SpA, Milan, Italy) to ensure sterility.

2.9. Steroid Responsiveness

To examine growth response of human prostate tumour cells to various concentrations of either androgens (DHT and T) or oestrogen (E_2), cells were cultured for a week in RPMI medium supplemented with 10% DHICT-FCS, with medium changes every 72 h. Subconfluent cell monolayers were rinsed twice in PBS-A, harvested by trypsinisation and seeded onto 6-well tissue culture plates (9.5 cm^2, Costar) at a density of 5-10 x 10^4 cells/well in routine medium. After 24 h (day 0), cells were washed twice with PBS-A and medium replaced with phenol red-free RPMI medium, supplemented with 10% DHICT-FCS, containing increasing concentrations (from 10^{-11} up to 10^{-7}M) of

T, DHT or E_2 in ethanol (0.1% final concentration); control wells received vehicle (ethanol, 0.1%). At day 3, medium was removed and replaced with fresh medium as before. After 6 days, medium was discarded and DNA estimation performed as described below.

In separate experiments, PC3 cells were exposed to E_2 ($1x10^{-11}$ up to $1x10^{-7}$M) in presence or absence of a anti-TGFß$_1$ neutralising antibody (chicken, polyclonal; ND_{50} = 2-3 µg/ml; British Biotechnology, Oxon, UK) at a final concentration of 9.2 µg/ml; parallel wells were incubated with the anti-TGFß$_1$ antibody alone, control wells received vehicle (ethanol, 0.1%) only. After different incubation times (24, 72, 144 h) medium was discarded and DNA content measured as hereafter described.

2.10. DNA growth assay

Estimation of the DNA cell content was performed using a Hoechst 33258 microassay, as extensively described elsewhere [Leake & Habib, 1987]. Briefly, cells from growth studies or hormone responsiveness or TGFα/TGFß$_1$ (see below, 2.16.) experiments were washed twice with PBS-A. Cells were then solubilised for 30 minutes at 37 °C in 3 ml of pre-warmed 0.2% (w/v) sodium dodecyl sulphate (SDS) in ETN buffer (10 mM EDTA, 10 mM Tris+HCl, pH 7.0). Aliquots (100 µl) of each sample were then transferred to RT-30 test tubes to which 3 ml of ETN buffer containing Hoechst 33258 (100 ng/ml) and RNase A (5 ng/ml), were added. Tubes were thoroughly vortexed and incubated for 30 minutes at room temperature in the dark.

Fluorescent enhancement at 450 nm was finally measured through a Hitachi Perkin-Elmer MPF-24 fluorescent spectrophotometer (Perkin-Elmer, Norwalk, CT), using an excitation wavelength of 360 nm and both slit widths at 5 nm. Cell DNA content was finally calculated on a calibration curve constructed using stock DNA solution. Using this method DNA content and cell number have been shown to be linearly correlated [Leake & Habib, 1987].

2.11. Tritiated Thymidine Uptake

Separate experiments were carried out in order to assess whether or not growth stimulation of LNCaP cells by oestradiol is mediated through ER. Cells were grown for a week in RPMI medium supplemented 10% charcoal treated-FCS (CT-FCS) and then seeded onto 6-well tissue culture plates (9.5 cm^2, Costar) at a density of $2x10^5$ cells/well in phenol red-free RPMI medium plus 5% CT-FCS. Cells were left undisturbed for 48 h and then additioned with 0.01, 1 or 100 nM oestradiol with or without 100 nM ICI-182,780, a pure synthetic antioestrogen [Wakeling *et al.*, 1991]. Parallel wells were exposed to ICI-182,780 alone; control wells received vehicle (ethanol 0.1%) only. Cells were then incubated for 6 days, with a medium change at day 3. After the incubation period, [methyl-^{3}H]Thymidine (specific activity 248 GBq/mmol, Du Pont de Nemours Italiana SpA, Milan) was added (74 KBq/well) in RPMI for 6 h. After the pulse, medium was discarded and cells were fixed in methanol for 10 minutes at -20°C to minimise cell losses. Cells were then rinsed twice with

PBS-A and washed three times using 2 ml of ice-cold 10% trichloroacetic acid (TCA) for 10 minutes. Cell monolayers were subsequently solubilised using 1 ml of 0.1% SDS in 0.3N NaOH and duplicate aliquots (400 µl) of acid-precipitable material from SDS solution were counted for incorporated radioactivity in a ß-counter after acidification using 10% (v/v) acetic acid. All experiments were performed in triplicate.

2.12. Northern Blot Analysis of TGFβ$_1$ mRNA

Specific experiments were carried out to investigate TGFß$_1$ mRNA expression and possible regulation by E_2 in PC3 cells. Cells were grown to near confluence in 10-cm plastic dishes in routine medium. After two washes with PBS-A, cells were trypsinised and plated at appropriate densities in 75 cm^2 tissue culture flasks. After 24 h, medium was replaced with serum-free, phenol red-free RPMI medium, supplemented with 20 µg/ml transferrin (Sigma-Aldrich, Milan, Italy) and 400 µg/ml BSA fraction V (Sigma-Aldrich), containing 10^{-11} to 10^{-7}M E_2 in ethanol (0.1%); duplicate flasks received ethanol only. After varying incubation times (6, 24 and 72 h), cell monolayers were washed three times with ice-cold PBS and lysed directly into the culture flasks using 3 ml/dish of RNAzol™ B (Biotecx Laboratories, Houston, TX); RNA was therefore extracted from cells following the recommendations of the supplier (see Section 2.4.). Ten µg of total RNA were electrophoresed in 3-(N-morpholino) propanesulphonic acid (MOPS) buffer at 30 volts for 16 h in a cold room through a 1.2% agarose gel containing 6.6%

formaldehyde. The filters were hybridised using DNA probes to human TGFß$_1$ (British Biotechnology) consisting of chemically synthesised single stranded oligonucleotides. The latter were a cocktail of antisense sequences complementary to exons 6, 7A and 7B. Probes were end-labelled by T4-Polynucleotide Kinase using $\gamma[^{32}P]$-dATP. Hybridisation was carried out at 60 °C for 16 h in 1M NaCl, 50 mM Tris-HCl (pH 7.5), 10% Dextran Sulphate, 1% SDS. Filters were subsequently washed for 5 min at room temperature in 2x standard saline citrate (SSC) and for 15 min at 60 °C in 2x SSC/0.1% SDS. Filters were finally autoradiographed for 20 h at -70 °C using Kodak X-OMAT AR film.

2.13. Growth Factor Binding Studies

Presence of high affinity binding sites for both EGF and TGFβ$_1$ in prostate cancer cells was assessed by means of radioreceptor assay. Cells were seeded onto 24-well plates at a density of $5x10^4$ cells/well in routine medium. After overnight incubation in a 5% CO_2 atmosphere at 37 °C, cells were washed three times with PBS and grown to near confluence in RPMI medium with 1% FCS. Cells were then washed three times using PBS/BSA (0.1% w/v) and incubated for 2 hours at 25 °C in 200 µl binding buffer (RPMI without phenol red and FCS, with 0.1% BSA) containing 300 pM ^{125}I-EGF or 100 pM ^{125}I-TGFß$_1$ (Du Pont de Nemours Italiana SpA, Milan) plus various concentrations of unlabeled growth factor (EGF = 0.15 to 40 nM; TGFß$_1$ = 2 pM to 20 nM); ranges of cold EGF or TGFß$_1$ were selected according to previous binding studies on prostate tumour tissues and cells

[Traish & Wotiz, 1987; Schuurmans *et al.*, 1988a; Wilding *et al.*, 1989a]. Separate wells were used for DNA and protein estimation. After four washes with ice-cold PBS/BSA, cells were solubilised for 30 minutes at 37 °C using 500 µl of SDS (0.1% w/v). Duplicate aliquots of the cell lysates were finally counted in a gamma-counter (LKB, Surrey, UK). Results of quadruplicate experiments were processed using Scatchard analysis and a least square fit routine, as described above (see Section 2.3.).

2.14. Immunofluorescence of EGFR, EGF, TGFα and TGFβ$_1$

Expression of both EGFR and endogenous EGF, TGFα and TGFβ$_1$ in LNCaP, DU145 and PC3 prostate cancer cell lines was studied using fluorescent immunostaining. Cells were seeded onto 8-well Lab-Tek® Chamber Slides™ (Nunc Inc., Naperville, IL) at a density of $2x10^4$ cells per well and left undisturbed at 37 °C in a humidified CO_2 incubator for 24-48 hours. Excess culture medium was then aspirated and cells washed three times with PBS-A. Cells were fixed at -20 °C for 10 minutes using acetone:methanol (1:1, v/v) and air dried. To suppress non specific binding, cells were incubated for 30 minutes at room temperature with 10% (v/v) sheep serum (SAPU, Carluke, Scotland) in PBS. Following incubation, cells were washed five times using 0.1% (w/v) BSA in PBS. The latter was removed and aliquots (200 µl/well) of primary antibodies appropriately diluted (EGF-R 1:200; EGF and TGFα 1:7; TGFß$_1$ 1:200) using PBS/BSA, were added. To assess non specific fluorescence, duplicate wells received PBS/BSA only. The slides were incubated for 2 hours at room temperature in a

humidified chamber. Following incubation, the primary antiserum was aspirated and cells washed five times as before. Aliquots (200 µl) of FITC fluorescent secondary antibodies (SAPU), diluted 1:100 using PBS/BSA, were then added to each well and incubated for 2 hours in a humidified chamber in the dark. After incubation, cells were washed again five times, the plastic upper chambers were removed and coverslips sealed on slides using Acquamount (BDH, Dorset, UK) mounting medium. Finally, immunofluorescence was visualised within 24 hours using a Leitz Orthoplan fluorescent microscope.

2.15. Western Blot Analysis of EGFR

The expression of EGFR in prostate cancer cell lines was also investigated using western blot analysis. Cells grown in 162 cm^2 plastic flasks (Costar) were washed twice with ice-cold PBS, harvested in 10 ml PBS using a rubber policeman and transferred to 12 ml centrifuge tubes. Cells were then pelleted by centrifugation at 1000xg for 10 minutes at 4 °C. The cell pellet was washed twice with 1 ml PBS and lysed using 1 ml lysis buffer (50 mM Tris-HCl - pH 6.8, 150 mM NaCl, 5 mM EDTA, Nonidet P-40 0.5% v/v, 1 mM phenylmethylsulphonylfluoride, PMSF). After gentle resuspension, cell lysates were transferred to eppendorf polytubes, left on ice for 1 h and centrifuged at 1000xg for 20 minutes at 4 °C. To the resulting supernatant was added 200 µl sample buffer (62.5 mM Tris-HCl - pH 6.8, 5% w/v glycerol, 2% w/v SDS, 0.001% bromophenol blue), and the mix was boiled for 3-5 minutes and briefly cleared by centrifugation.

Aliquots (50 µl) of the supernatant were used for protein estimation using the Lowry method [Lowry *et al.*, 1951]. Samples were loaded at a concentration of 10 to 200 µg protein.

Polypeptides were resolved by a 12% SDS/polyacrylamide gel electrophoresis (SDS/PAGE) in Tris/Glycine buffer (25 mM Tris, 192 mM Glycine, 0.1% w/v SDS) using a MiniSub-Cell™ apparatus (Bio-Rad Italiana, Milan) set at a constant current of 40-60 milliamperes (ma) until the bromophenol blue tracker dye was 0.5 cm from the bottom of the gel. Where appropriate, gels were stained for protein in 0.1% (v/v) coomassie brilliant blue R250, 50% (v/v) methanol and 10% (v/v) acetic acid for 1 h at room temperature; the molecular weight (MW) of the resolved polypeptides was determined by calibration with a set of protein markers (myosin, MW 200,000; α2-macroglobulin, MW 170,000; phosphorylase B, MW 97,400; glutamate dehydrogenase, MW 55,400; lactate dehydrogenase, MW 36,500) run on parallel lanes of the same gel. Destaining was accomplished by repeatedly washing the gels with 50% methanol (v/v) and 10% acetic acid (v/v). Proteins resolved by SDS/PAGE were transferred electrophoretically onto nitrocellulose membrane (Hybond N) in transfer buffer (25 mM Tris, 192 mM Glycine, 0.01% w/v SDS, 20% v/v methanol, pH 8.3) using a Bio-Rad Trans-Blot™ cell at a constant current of 400 ma for 3 h or 40 ma for 16-20 h. In order to verify the quality of transfer and to mark the positions of molecular weight standards, blots were stained for 5 minutes with 0.1% (w/v) Amido Black dissolved in 45% (v/v) methanol plus 10% (v/v) acetic acid and destained using 90% (v/v)

methanol/2% (v/v) acetic acid. Following transfer, blots were blocked through incubation with 10 ml TBST buffer (10 mM Tris-HCl - pH 8.2, 150 mM NaCl, 0.05% v/v Tween 20) containing 2% (w/v) non-fat dry milk for 1 h at room temperature. The blots were then washed five times using TBST for a total of 1 h and incubated with the BG48 anti-EGFR primary polyclonal antibody (1:200 dilution in TBST) for 16 h at 4 °C. After five washes as before, specific binding was revealed through addition of 0.1 μCi/ml [^{125}I]-Protein A in TBST and incubation for 1-2 h at room temperature. After incubation, removal of the unbound radiolabel was achieved by a further five washes in TBST. Finally, blots were air dried and exposed for one week to Kodak X-omat S film with intensifying screens at -70 °C.

2.16. TGFα and TGFß Dose-Response Experiments

The effects of either TGFα or TGFβ_1 on growth of LNCaP, DU145 and PC3 cells have been compared. Cells growing in log phase were seeded onto 6-well tissue culture plates (Nunc) at a density of $2x10^5$ cells per well in routine culture medium to parallel experimental conditions used for the EGFR binding assay. After 24 hours, increasing concentrations of either TGFα (from 0.5 to 50 ng/ml) or TGFß$_1$ (from 0.05 to 5 ng/ml) were added. After a further 24 or 48 hours incubation, medium was discarded and DNA estimation carried out as described before (see Section 2.9.).

Additional experiments were designed to inspect response of cells to either growth factor in the presence of steroid-

deprived, dialysed FCS, namely under experimental conditions identical to those used for hormone responsiveness studies. Cells in the exponential growth phase were harvested and plated on 6-well tissue culture plates (Costar) at a density of $1\text{-}2x10^5$ cells/well in routine culture medium. After 24-48 h, medium was removed and substituted with phenol red-free RPMI medium supplemented with 10% DHICT-FCS, containing various concentrations of TGFα (0.5-50 ng/ml) and TGFß$_1$ (0.05-5 ng/ml) (day 0). Cells were then incubated for 48 h or, after a medium change at day 3, for 6 days. Following incubation, medium was discarded and DNA cell content measured as described in Section 2.9.

2.17. Steroid Metabolism

Methodological approach and procedures used to measure metabolic pathways of steroids in *in vitro* systems have been previously established and optimised [D'Agostino *et al.*, 1984; D'Agostino *et al.*, 1985; Castagnetta *et al.*, 1986c]. Cells growing in log phase were harvested by trypsinisation, counted in a hemocytometer and plated onto 60 mm cell culture dishes at a density of $0.5\text{-}2x10^6$ cells/dish. After 24-48 h, cells were washed twice with PBS-A and the medium substituted with FCS-free, phenol red-free RPMI medium, in order to avoid any interfering factor that might modify the metabolic ability of cells. After a further 24 h, medium was replaced with the same experimental medium containing $1\text{-}10x10^9$M radioactive testosterone ([1,2,6,7-^{3}H(N)]testosterone; specific activity: 92.1 Ci/mmol; DuPont de

Nemours Italiana SpA, Milan) or oestradiol ([6,7-^{3}H]oestradiol; specific activity: 48 Ci/mmol; DuPont de Nemours) as precursors. The latter were both periodically checked and purified using high-performance liquid chromatography (HPLC) prior to experimental use. Following either 24 or 72 h incubation, medium was transferred to sterile plastic tubes (Costar®, Cambridge, MA) and stored at -80°C until analysis; to prevent oxidation of hydroxylated oestrogen metabolites, 1 mM ascorbic acid was added to the medium when appropriate. Cells were washed three times using PBS-A and solubilised in 3 ml of 0.1% SDS at 37°C for 15-30 minutes. Aliquots (100 µl) of the cell lysates were therefore used to estimate DNA content, as previously described (see 2.9.).

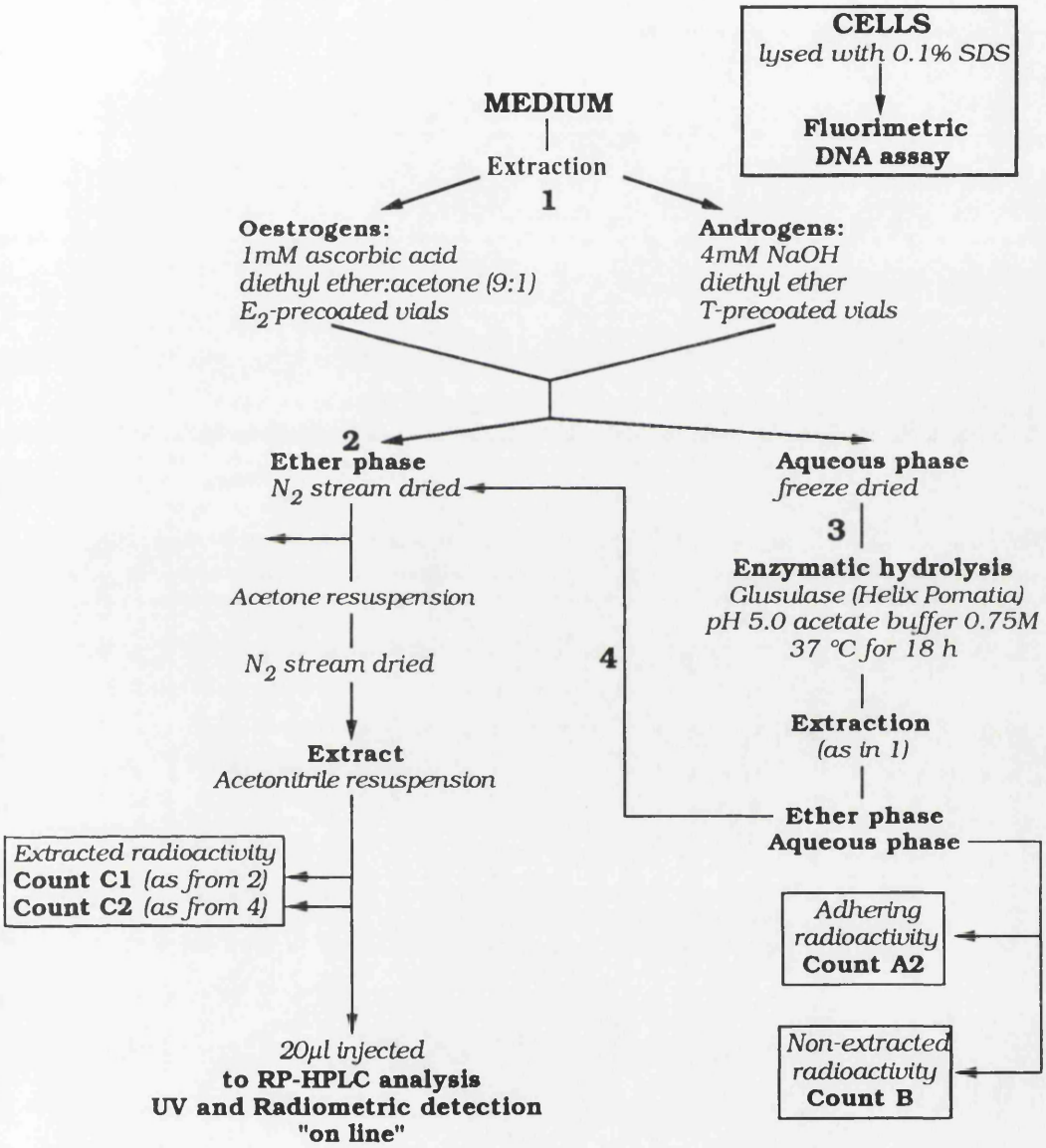
For time-course experiments, triplicate dishes of PC3 cells ($2x10^{6}$ cells/dish) were incubated in the presence of 50 nM tritiated T for 30 min, 2, 8 and 24 h, under exactly the same experimental conditions. Medium and cells were then processed as described above. In addition, separate experiments were carried out to measure conversion rates of labelled androstenedione ($6x10^{-6}$M, [4-^{14}C]androst-4-ene-3,17-dione; specific activity: 55 mCi/mmol; DuPont de Nemours) in PC3 cells after 24 h incubation; both experimental conditions and analytical procedures used were identical to those herein described for both testosterone and oestradiol metabolism.

All the experiments were done in triplicate.

2.17.1. Extraction procedures

The procedures used for extraction and subsequent HPLC analysis of both androgens and oestrogens are summarised in Figure 17. Steroid extraction was carried out on the incubation medium, since it has been shown that the steroid profile of the surrounding medium accurately reflects any steroid metabolism by the intact cells [Castagnetta *et al.*, 1986b]. To prevent loss of radioactivity, all the glassware was precoated prior to sample manipulation with 4 µg of the same radioinert steroid. One ml aliquot of medium was transferred to a scintillation vial to assess the total radioactivity (TR) processed per ml. Steroid extraction was performed in glass vials on 1 ml aliquots of the incubation medium using 10 ml of diethyl ether for androgens or 10 ml of diethyl ether:acetone (9:1, v/v) for oestrogens. To facilitate androgen extraction, the pH was raised to approximately 10.0 by addition of 20 µl of 2M sodium hydroxide, since this increases the preferential partitioning of free androgen metabolites in the ether phase. Samples were thoroughly mixed by gentle shaking in a water-bath at 4°C for 30 minutes and then left standing for 5-10 minutes. The aqueous phase was transferred to separate glass tubes and freeze dried in a SVC100H Speed Vac® evaporator-concentrator (Savant Instruments Inc., Farmingdale, NY) for about 2 h. The dried extracts were resuspended using 970 µl of acetate buffer (0.75 M, pH 5.0), containing 30 µl of Glusulase enzyme mixture (DuPont Co., Wilmington, DE) and incubated at 37°C for 18 h to hydrolyse steroid conjugates (sulphates and glucuronides). Following incubation, samples were

Figure 17. Flow diagram of procedures used for steroid extraction and chromatographic separation. The chart summarises the methodologies used for extraction of either free or conjugate steroids (androgens and oestrogens) from the incubation medium of prostate cancer cells in steroid metabolism experiments (for full details of methodologies see Materials and Methods: 2.17.).


CELLS
lysed with 0.1% SDS
Fluorimetric
DNA assay
MEDIUM
Extraction
1
Oestrogens:
1mM ascorbic acid
diethyl ether:acetone (9:1)
E2-precoated vials
Androgens:
4mM NaOH
diethyl ether
T-precoated vials
2
Ether phase
N2 stream dried
Aqueous phase
freeze dried
3
Enzymatic hydrolysis
Glusulase (Helix Pomatia)
pH 5.0 acetate buffer 0.75M
37 °C for 18 h
Extraction
(as in 1)
Ether phase
Aqueous phase
4
Acetone resuspension
N2 stream dried
Extract
Acetonitrile resuspension
Extracted radioactivity
Count C1 (as from 2)
Count C2 (as from 4)
Adhering
radioactivity
Count A2
Non-extracted
radioactivity
Count B
20µl injected
to RP-HPLC analysis
UV and Radiometric detection
"on line"

transferred to glass vials and extracted again as before: the resulting aqueous phase was read for non-extracted radioactivity (B), while the ether phase was processed as hereafter described to extract hydrolysed steroids. The two ether phases (free and hydrolysed steroids) were evaporated to dryness under gentle nitrogen stream at 42°C in a water-bath, resuspended three times with 2 ml acetone and transferred to separate tubes to be desiccated again as before; the empty vials from both ether phases received 10 ml of scintillation cocktail (Ready Gel™, Beckman) which was then counted for radioactivity adhered to walls (A1 and A2) in a β-counter (Beckman Instruments Italia, Milan). The dried extracts were finally stored at -20°C until chromatographic analysis. Extracts of either free or hydrolysed steroids were resuspended in 30 µl of a mixture consisting of 20 µl acetonitrile and 10 µl acetic acid 0.2M in the case of androgens, or 10 µl acetonitrile, 10 µl acetic acid and 10 µl equilin (500 µg/ml) used as internal standard, in the case of oestrogens. Twenty µl of the resulting solution were used for HPLC analysis (see below), while 5 µl were read in a β-counter to measure the radioactivity respectively extracted for each steroid fraction (C1 and C2). The extraction efficiency was finally calculated as follows:

$$\textbf{Extraction Efficiency} = \frac{(C1+C2)\times 6}{(C1+C2)\times 6+(A1+A2+B)}$$

2.17.2. Chromatographic Analysis

Extracted steroids were chromatographically analysed in reverse phase (RP)-HPLC, using a Beckman model 324 HPLC system equipped with an UV detector (model 160), set at 280 nm or 214 nm respectively for oestrogens and androgens, and with an "on-line" Flo-One/beta (model IC) three-channel radioactive detector (Radiomatic Instruments, High Wycombe, UK). The latter allows to detect simultaneously signals coming from two separate radioactive counting channels (i.e. for ^{3}H and ^{14}C radioisotopes, at efficiencies of 42% and 81%, respectively, under stopped-flow conditions) and from the UV detector. Steroids were separated under isocratic condition using an Ultrasphere ODS (Beckman) column (250x4.6mm I.D., 5µm particle size), thermostated at 20°C. An optimised mobile phase, consisting of acetonitrile:tetrahydrofuran:0.05M citric acid (31:9:60, v/v) or of acetonitrile:0.05M citric acid (45:55, v/v), at a flow rate of 1 ml/minute, was used to separate respectively androgen and oestrogen metabolites in a total analysis time of approximately 30 minutes. Identification of individual metabolites was achieved by simultaneous UV evaluation of cold steroid standards; relative retention times (RRT) for authentic androgens and oestrogens are reported in Table 1. Radiometric detection was performed using a 1 ml flow cell and Ready-Flow III (Beckman) as scintillation mixture at a flow rate of 3 ml/min. Routine data integration was achieved by a Flo-One/beta F1B IC program (Radiomatic, Tampa, FL) and computed in net cpm, after correction for both sample residence time and background subtraction (40 and 70 cpm

Table 1. Trivial names, abbreviations and relative retention times (RRT) of authentic steroid standards in reverse phase-high performance liquid chromatography (RP-HPLC)

Trivial Name	Abbreviation	RRT	Id. No.
Androgens (A)			
16αhydroxy-testosterone	16αOH-T	0.35	
19hydroxy-androstenedione	19OHA	0.44	
Androstenediol	Δ^5Adiol	0.96	
Testosterone	**T** (i.s.)	**1.00**	(1)
Androstenedione	Δ^4Ad	1.18	(2)
3β-androstanediol	3β-diol	1.28	
Dehydroepiandrosterone	DHEA	1.36	
Epiandrosterone	EpiA	1.58	(3)
Dihydrotestosterone	DHT	1.66	(4)
3α-androstanediol	3α-diol	1.75	(5)
5α-androstanedione	5α-Adione	2.09	(6)
Androsterone	A	2.41	(7)
Oestrogens (B)			
Oestriol	E_3	0.22	
16α–Hydroxyoestrone	16α0HE_1	0.35	(1)
2-Hydroxyoestradiol	2OHE_2	0.43	
4-Hydroxyoestradiol	4OHE_2	0.47	
2-Hydroxyoestrone	2OHE_1	0.55	
4-Hydroxyoestrone	4OHE_1	0.65	
Oestradiol	E_2	0.70	(2)
4-Methoxyoestradiol	4MeOE_2	0.76	
2-Methoxyoestradiol	2MeOE_2	0.86	(3)
Equilin	**Eq** (i.s.)	**1.00**	
Oestrone	E_1	1.09	(4)
4-Methoxyoestrone	4MeOE_1	1.21	
2-Methoxyoestrone	2MeOE_1	1.33	

RP-HPLC analysis: Ultrasphere-ODS column; mobile phases used: A=Acetonitrile:Tetrahydrofurane:0.05M citric acid (31:9:60, v/v); B=Acetonitrile:0.05M citric acid (45:55, v/v). Flow rate: 1 ml/min. i.s.: internal standard; Id. No.: peak identification number.

All experiments, unless otherwise indicated, were performed three times, each time in triplicate (N=9), using 6-well tissue culture plates. Statistical analysis was carried out through the InStat™ GraphPad Software (version 2.01) run on a Macintosh LCii computer. This statistical package automatically tests the assumption of equal variances within and between data groups through an F-test, allowing appropriate use of parametric or non-parametric tests to compare population means or medians.

respectively for ^{3}H and ^{14}C). Both precursor degradation and formation of metabolic products were finally expressed either as percentages of conversion rate (%CR), i.e. as the percent of total radioactivity recovered, or as pmol/ml for any incubation time.

2.18. Studies on Modulation of Steroid metabolism by TGFα and TGFβ$_1$

These experiments were specifically designed to explore possible modulation of rates and direction of steroid metabolism by both TGFα and TGFβ in human prostate cancer cells, maintaining exactly the same experimental conditions used in studies on steroid metabolism. To this end, cells ($0.5\text{-}1x10^{6}$) were incubated for 72 h in phenol red-free, FCS-free RPMI medium containing the radioactive steroid ($1\text{-}5x10^{-9}$M tritiated testosterone or oestradiol) in presence or absence of either TGFα or TGFβ$_1$, at the dose of 50 and 5 ng/ml, respectively. These dose levels were selected on the basis of the maximum effect observed on cell proliferation yields. After incubation, cells and medium were processed and analysed as described in the 2.15. chapter.

2.19. Statistics

The two-tailed Student's *t* test, the F test and the Spearman correlation test (all 95% confidence limits) were used for statistical comparisons.

EXPERIMENTAL.

1. GROWTH CHARACTERISTICS

Growth curves of prostate tumour cell lines are shown in Figure 18. Overall, population doubling times, as calculated during the log phase growth (i.e. days 4 to 6 over a 12 days observation interval), was approximately 36 h for LNCaP (passage 23), 23 h for DU145 (passage 62) and 32 h for PC3 (passage 19) cells.

The DNA content of the three cell lines ranged approximately from 30 to 60 pg DNA/cell.

2. STEROID RECEPTORS

2.1. Androgen Receptors (AR)

2.1.1. Ligand binding assay

The presence of both soluble and nuclear sites of androgen binding in human prostate cancer cell lines has been assessed by means of radioligand binding assay. Dissociation constant (K_d) and concentration values of AR in either cell fraction are shown in Table 2. Overall, K_d values fell well below the cut-off value of 1 nM (ranging from 0.10 and 0.37 nM), ensuring that purely type I (high affinity, limited capacity) androgen binding sites were identified [Castagnetta *et al.*, 1992a]. In addition, presence of both soluble and nuclear type II (lower affinity, greater capacity) AR was also revealed. Typical Scatchard plots of type I and II

Figure 18. Growth curves of human prostate cancer cells. Mean population doubling times were 36 h for LNCaP, 23 h for DU145 and 32 h for PC3 cells, as calculated in the exponential growth phase (days 4 to 6). Values represent mean ±SD (bars) of triplicate wells. For methodological details see text (Material and Methods: 2.1.).

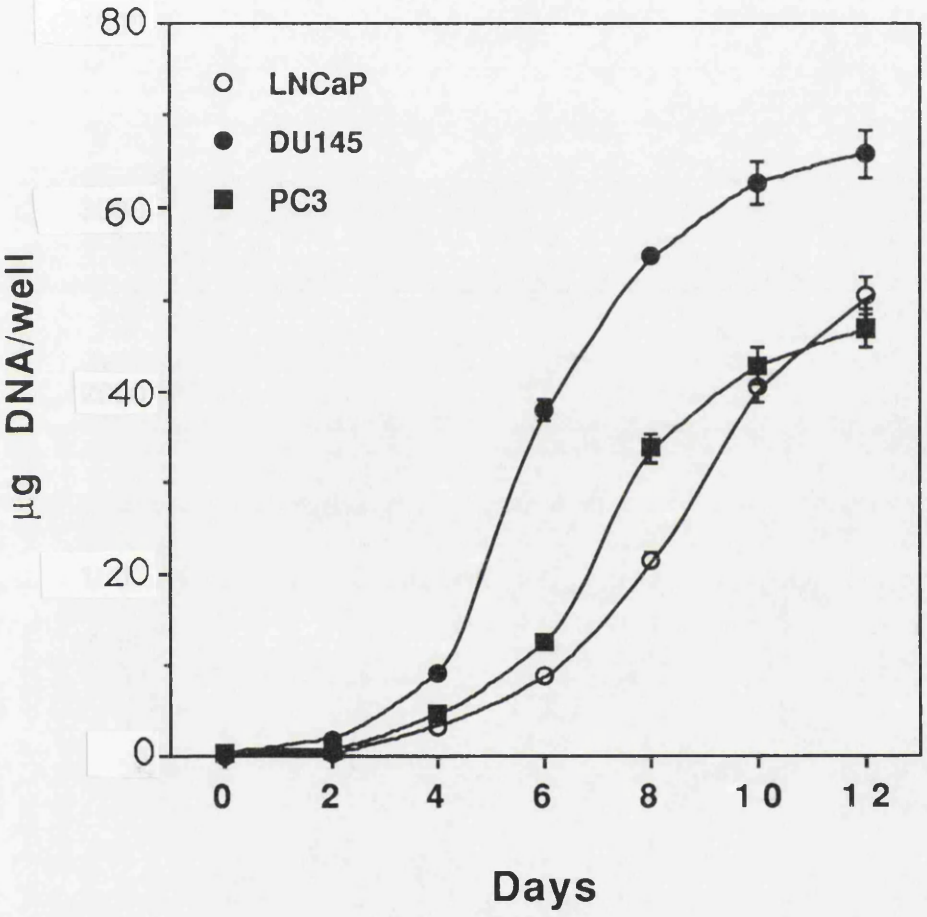

80
60
40
20
0
µg DNA/well
LNCaP
DU145
PC3
0
2
4
6
8
10
12
Days

Table 2. Type I androgen receptor content of LNCaP, DU145 and PC3 human prostate cancer cell lines.

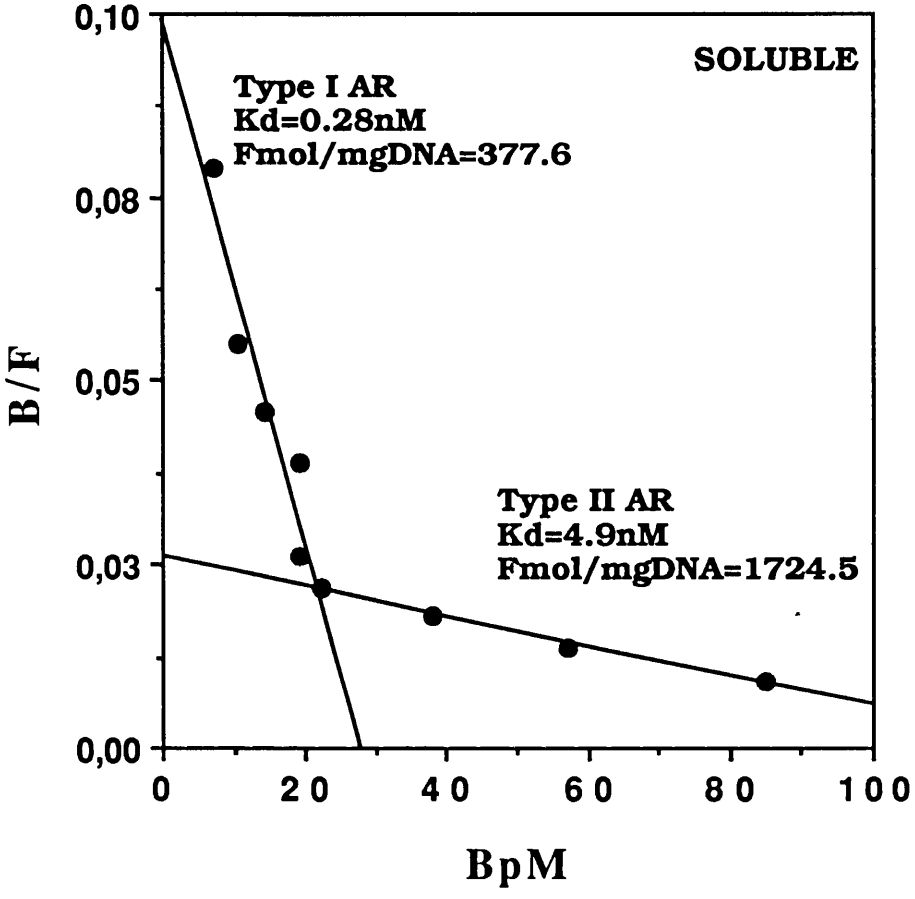
	Soluble Fraction		Nuclear Fraction	
	K_d (nM)	Fmol/mg DNA	K_d (nM)	Fmol/mg DNA
LNCaP	0.37 ±0.11	372.5 ±183.5	0.18 ±0.05	69.1 ±13.0
DU145	0.24 ±0.09	334.3 ±194.2	0.22 ±0.09	84.0 ±17.9
PC3	ND	ND	0.10 ±0.02	44.1 ± 5.5

Values represent mean ±SD of triplicate assays. K_d = dissociation constant; ND = not detectable.

AR in LNCaP and DU145 cells (soluble and nuclear) and in PC3 cells (nuclear only) are shown in Figures 19 to 21.

In the soluble fraction, LNCaP and DU145 cells exhibited similar mean levels of type I AR (see Table 2); this was also true when receptor concentrations were expressed as site number per cell (13,922 ±5,142 vs 13,294 ±5,794) or as fmol/mg cytosol protein (14.8 ±7.3 vs 14.3 ±8.3). Type II AR were detected in both LNCaP and DU145 cells, with mean K_d and concentration values respectively of 5.6 nM and 108.6 fmol/mg protein in LNCaP and of 7.3 nM, and 124.2 fmol/mg protein in DU145 cells. Neither type I nor type II AR were detectable in the soluble fraction of PC3 cells.

Figure 19. Ligand binding assay of androgen receptor (AR) in LNCaP cells. Typical Scatchard plots of both soluble (top) and nuclear (bottom) androgen binding are shown. Presence of two sites of androgen binding was determined according to the significantly better ($P<0.05$, F test) fit achieved for the two-sites model. Each data point was performed in triplicate. K_d = dissociation constant, BpM = bound picomolar, B/F bound over free. For methodological details see text (Materials and Methods: 2.2. and 2.3.).


SOLUBLE
Type I AR
Kd=0.28nM
Fmol/mgDNA=377.6
Type II AR
Kd=4.9nM
Fmol/mgDNA=1724.5
B/F
0,10
0,08
0,05
0,03
0,00
0
20
40
60
80
100
BpM

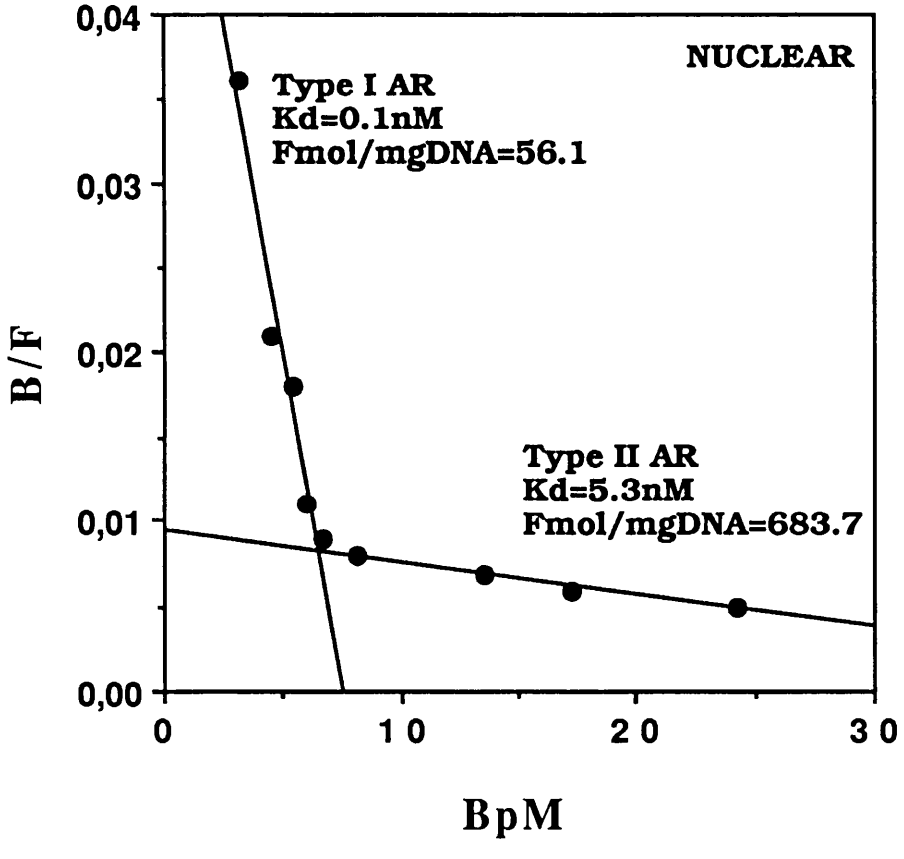
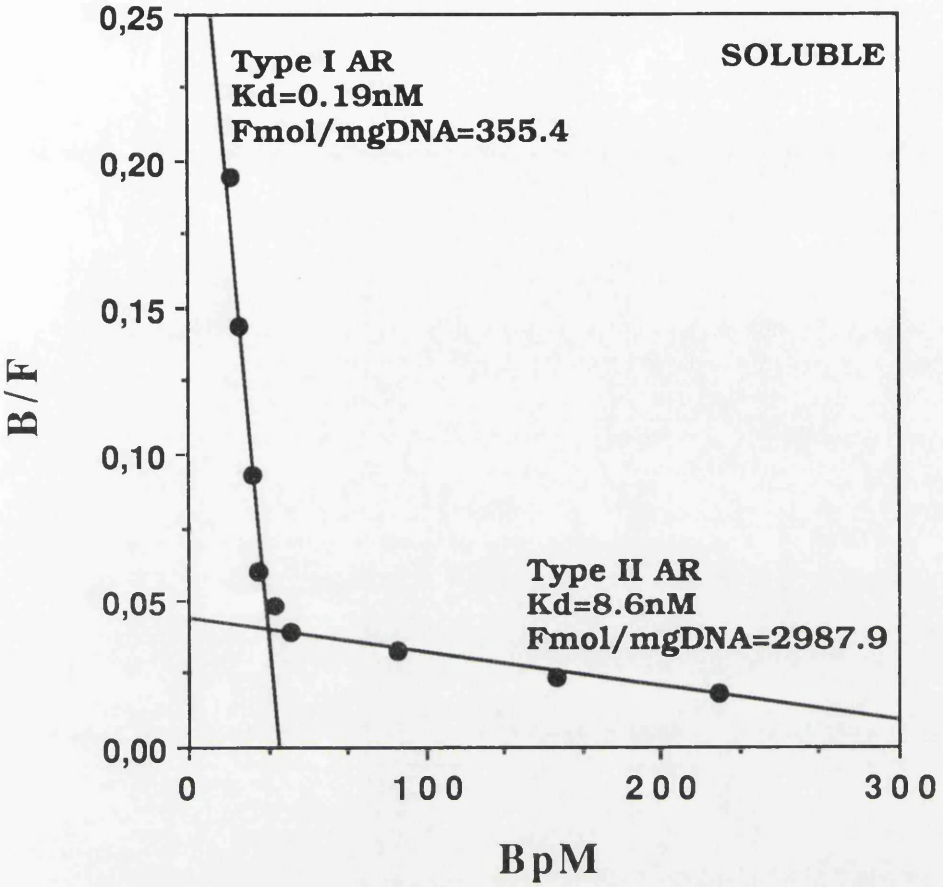

NUCLEAR
Type I AR
Kd=0.1nM
Fmol/mgDNA=56.1
Type II AR
Kd=5.3nM
Fmol/mgDNA=683.7
B/F
0,04
0,03
0,02
0,01
0,00
0
10
20
30
BpM

Figure 20. Ligand binding assay of androgen receptor (AR) in DU145 cells. Typical Scatchard plots of both soluble (top) and nuclear (bottom) androgen binding are shown. Presence of two sites of androgen binding was determined according to the significantly better ($P<0.05$, F test) fit achieved for the two-sites model. Each data point was performed in triplicate. K_d = dissociation constant, BpM = bound picomolar, B/F bound over free. For methodological details see text (Materials and Methods: 2.2. and 2.3.).


SOLUBLE
Type I AR
Kd=0.19nM
Fmol/mgDNA=355.4
Type II AR
Kd=8.6nM
Fmol/mgDNA=2987.9
B/F
0,25
0,20
0,15
0,10
0,05
0,00
0
100
200
300
BpM

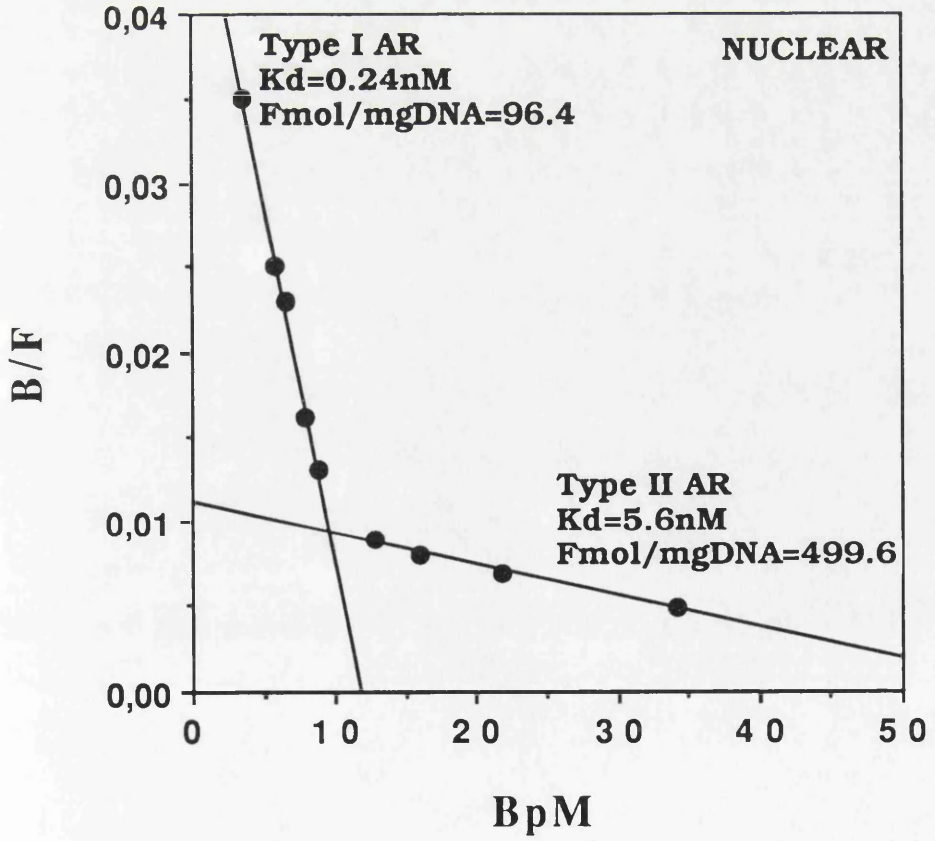
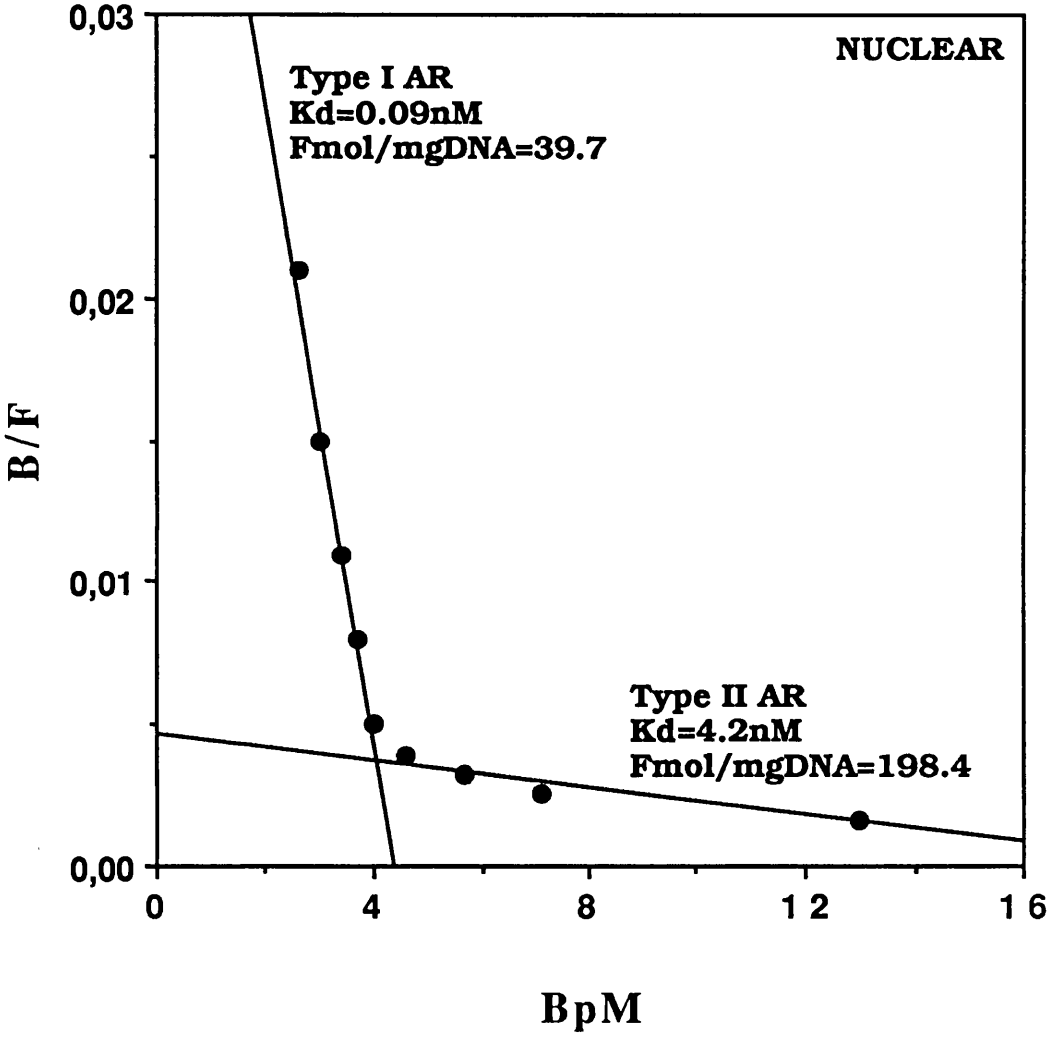

NUCLEAR
Type I AR
Kd=0.24nM
Fmol/mgDNA=96.4
Type II AR
Kd=5.6nM
Fmol/mgDNA=499.6
B/F
0,04
0,03
0,02
0,01
0,00
0
10
20
30
40
50
BpM

Figure 21. Ligand binding assay of androgen receptor (AR) in PC3 cells. A typical Scatchard plot of nuclear androgen binding is shown. No detectable AR were found in the soluble fraction. Presence of two sites of nuclear androgen binding was determined according to the significantly better ($P<0.05$, F test) fit achieved for the two-sites model. Each data point was performed in triplicate. K_d = dissociation constant, BpM = bound picomolar, B/F bound over free. For methodological details see text (Materials and Methods: 2.2. and 2.3.).


NUCLEAR
Type I AR
Kd=0.09nM
Fmol/mgDNA=39.7
Type II AR
Kd=4.2nM
Fmol/mgDNA=198.4
B/F
0,03
0,02
0,01
0,00
0
4
8
12
16
BpM

As regards the nuclear fraction, all three cell lines displayed the presence of type I receptors, the highest concentration being found in DU145 and LNCaP cells (3,419 ±728 and 2,666 ±502 sites/cell, respectively), the lowest in PC3 cells (1,572 ±196 sites/cell). Nuclear type II androgen binding sites were also found in all cell lines, with K_d values ranging from 3.3 up to 6.8 nM and binding site concentrations of 155.7. to 798.2 fmol/mg DNA.

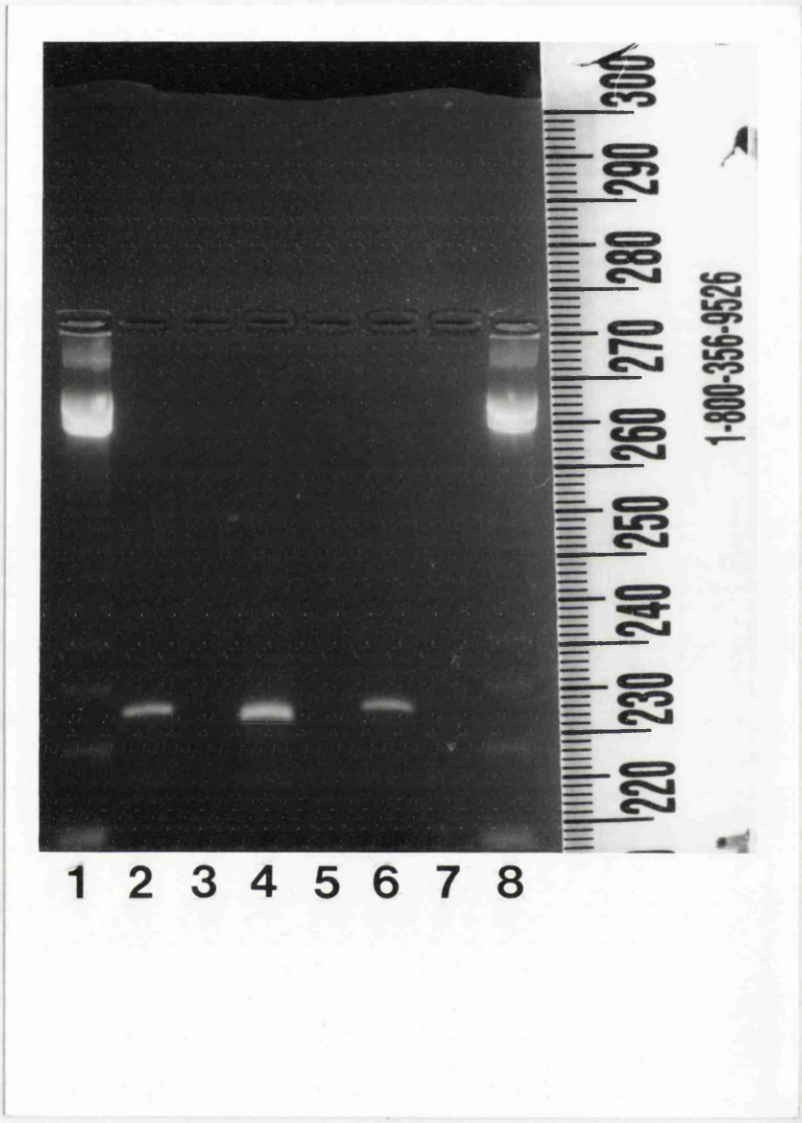
It is noteworthy that while LNCaP and DU145 cells show both soluble and nuclear type I AR, the absence of detectable AR from the soluble fraction of PC3 cells seems to indicate an impaired receptor machinery [Castagnetta *et al.*, 1990]. However, DU145 cells, which contain apparently functional AR, failed to grow in response to either T or DHT (see below: 3.1.).

2.1.2. Reverse transcriptase-PCR (RT-PCR)

Inspection of human AR mRNA expression in LNCaP, DU145 and PC3 prostate tumours cells was accomplished by means of reverse transcriptase-PCR system. This approach allows identification of very small amounts of specific messenger RNAs through reverse transcription and subsequent amplification of the reverse-transcribed DNA.

Using a primer set consisting of a sense and an antisense primer corresponding to a sequence in exon 1 of the human AR gene (HUMARA, nucleotides 229 to 508), DNA amplification gave rise to one single PCR product of the expected length (around 280bp) in all the three cell lines. As shown in Figure 22, agarose

Figure 22. Reverse transcriptase-polymerase chain reaction (RT-PCR) of androgen receptors in human prostate tumour cells. Ethidium bromide-stained 2% agarose gel of amplification products obtained from one μg of total RNA in presence or absence of reverse transcriptase (RT). All RNA samples were treated with DNase I prior to reverse transcription. The reference ladder is expressed in base pairs (bp). Lane 1: 123 bp ladder; lane 2: DU145 +RT; lane 3: DU145 -RT; lane 4: LNCaP +RT; lane 5: LNCaP -RT; lane 6: PC3 +RT; lane 7: PC3 -RT; lane 8: 123 bp ladder.


280bp-
1 2 3 4 5 6 7 8
300
290
280
270
260
250
240
230
220
1-800-356-9526

gel electrophoresis of the amplification products revealed the presence of a strong band in each cell line, with minor diversities in the relative mobility; the latter is likely to be a reflection of the highly polymorphic STRs present in exon 1. Although the semiquantitative nature of PCR does not permit any conclusive inference, scrutiny of the ethidium bromide-stained agarose gel indicates that, given the same amount of starting template (1 μg of total RNA for each cell line), LNCaP cells yielded the greatest amount of the amplification product, while DU145 and PC3 cells respectively showed intermediate and lower levels of expression. Additionally, possible contamination by genomic DNA was eliminated by treatment of the RNA preparations with DNase I and double-checked by omitting the addition of reverse transcriptase (Superscript II RT) in duplicate RNA preparations. The absence of any detectable amplification product from samples not receiving Superscript II RT (see Figure 22) clearly demonstrates that no genomic DNA is present and that, therefore, PCR products derive exclusively from the extracted RNA.

2.2. Oestrogen Receptors (ER)

2.2.1. Ligand binding assay

ER content and status of human prostate cancer cell lines were evaluated by means of the radioligand binding method. Presence of both type I and type II sites of oestrogen binding was documented in the soluble and nuclear fractions of LNCaP

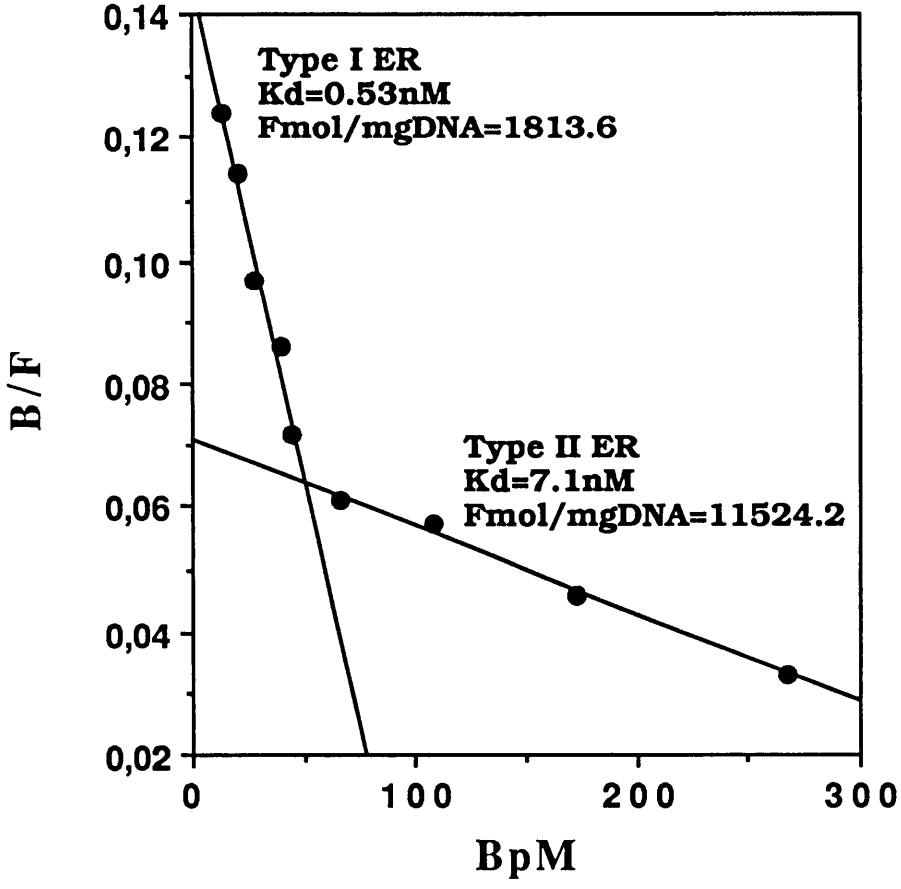
cells. In PC3 cells, the soluble fraction did not show any detectable oestrogen binding site, while presence of both type I and type II ER was revealed in the nuclear fraction. By contrast, neither soluble nor nuclear oestrogen binding was revealed in DU145 cells. K_d and concentration values for type I ER in LNCaP and PC3 cells are reported in Table 3. As can be seen, K_d values were below the cut-off of 0.55 nM (range of 0.16 to 0.49 nM), indicating that type I (high affinity, limited capacity) ER are detected in both soluble and nuclear compartments [Castagnetta *et al.*, 1987a; Castagnetta *et al.*, 1992b]. Figures 23 (A and B) and 24 respectively illustrate typical Scatchard plots and saturation curves for type I ER in LNCaP (soluble and nuclear) and PC3 (nuclear only) cells.

Table 3. Type I oestrogen receptor content of LNCaP and PC3 human prostate cancer cell lines.

	Soluble Fraction		Nuclear Fraction	
	K_d (nM)	Fmol/mg DNA	K_d (nM)	Fmol/mg DNA
LNCaP	0.49 ±0.05	2123 ±368.1	0.25 ±0.03	328.1 ±48.2
PC3	ND	ND	0.16 ±0.04	118.8 ± 22.3

Values represent mean ±SD of triplicate assays. K_d = dissociation constant; ND = not detectable.

Figure 23A. Ligand binding assay of oestrogen receptor in the soluble fraction of LNCaP cells. Typical Scatchard plot (top) and tracer ligand saturation curve (bottom) of oestrogen binding. BpM = bound picomolar; B/F = bound over free. ■ = total, ● = specific, ▲ = non-specific binding of tritiated oestradiol, as calculated from competition studies using a 100 fold excess of unlabelled diethylstilbestrol. Each data point was performed in triplicate.


Type I ER
Kd=0.53nM
Fmol/mgDNA=1813.6
Type II ER
Kd=7.1nM
Fmol/mgDNA=11524.2
B/F
BpM
0,14
0,12
0,10
0,08
0,06
0,04
0,02
0
100
200
300

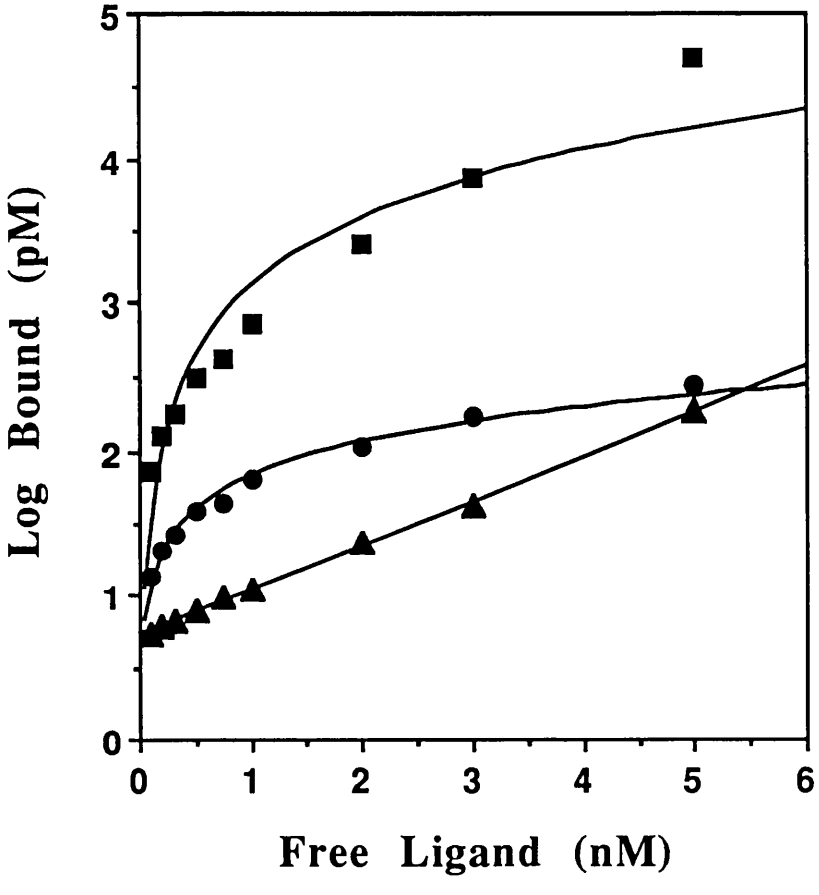
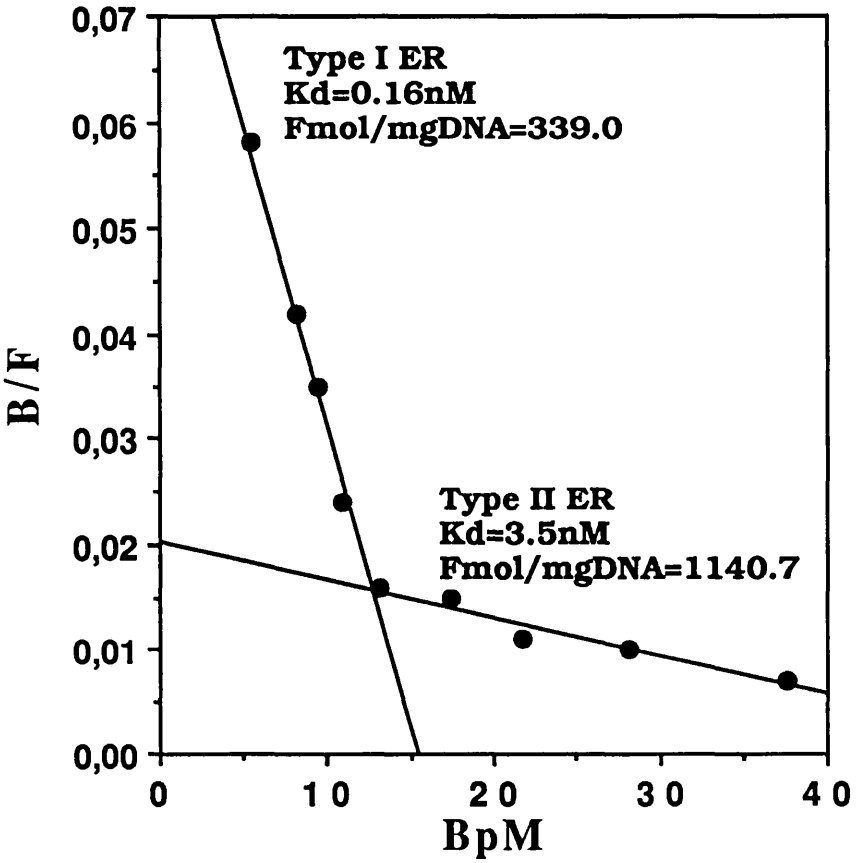

Log Bound (pM)
Free Ligand (nM)
0
1
2
3
4
5
6

Figure 23B. Ligand binding assay of oestrogen receptor in the nuclear fraction of LNCaP cells. Typical Scatchard plot (top) and tracer ligand saturation curve (bottom) of oestrogen binding. BpM = bound picomolar; B/F = bound over free. ■ = total, ● = specific, ▲ = non-specific binding of tritiated oestradiol, as calculated from competition studies using a 100 fold excess of unlabelled diethylstilbestrol. Each data point was performed in triplicate.


Type I ER
Kd=0.16nM
Fmol/mgDNA=339.0
Type II ER
Kd=3.5nM
Fmol/mgDNA=1140.7
B/F
BpM
0,07
0,06
0,05
0,04
0,03
0,02
0,01
0,00
0
10
20
30
40

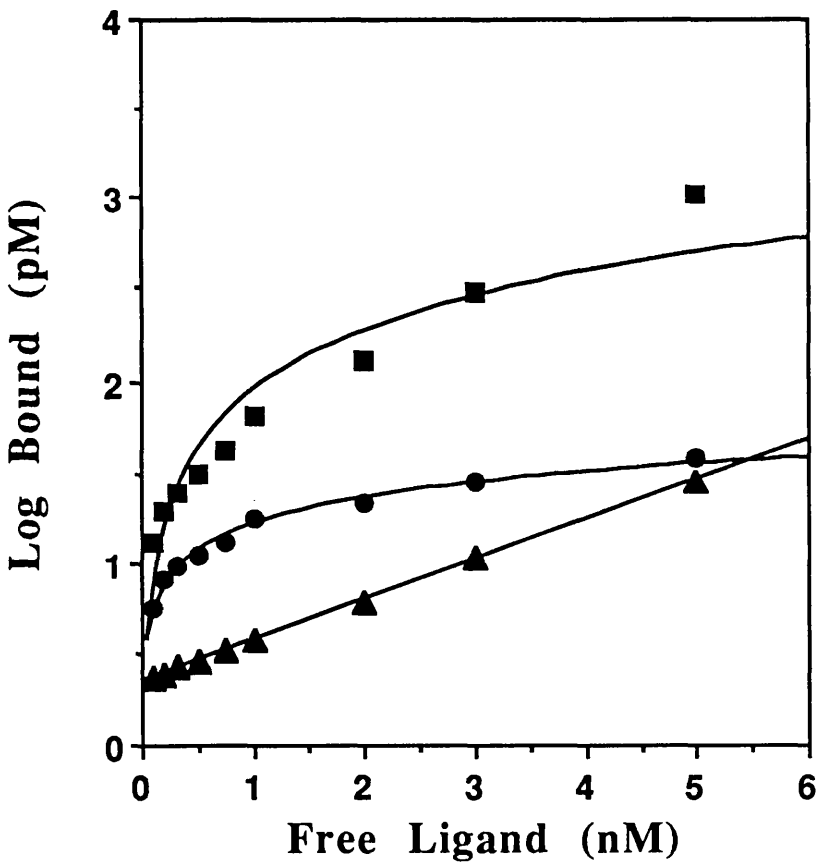
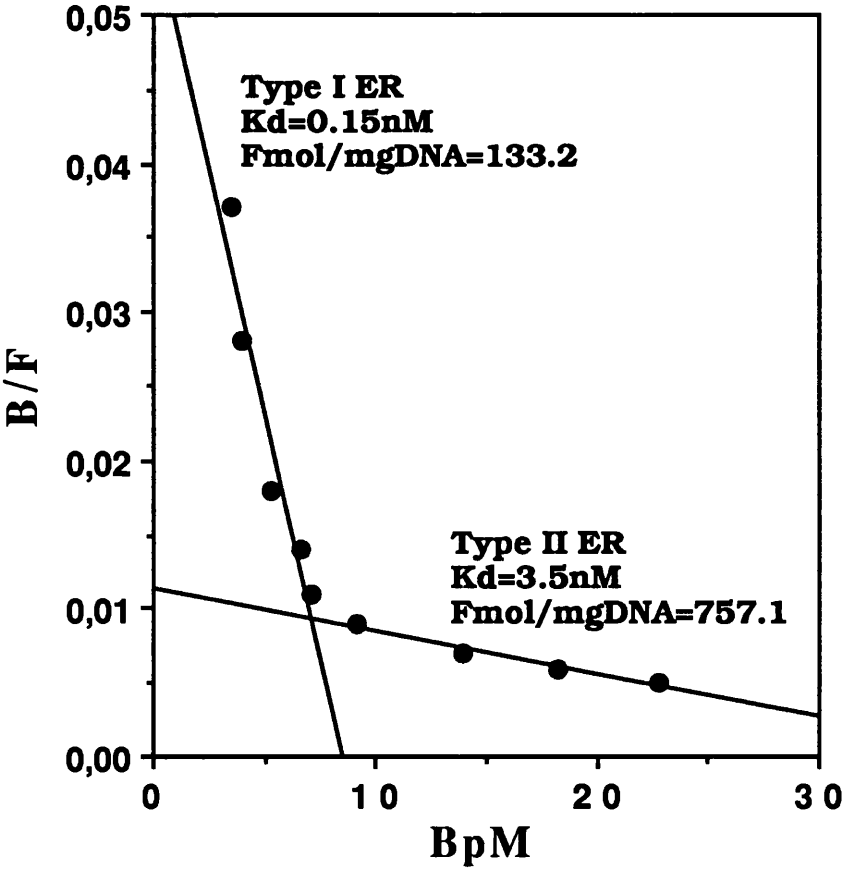
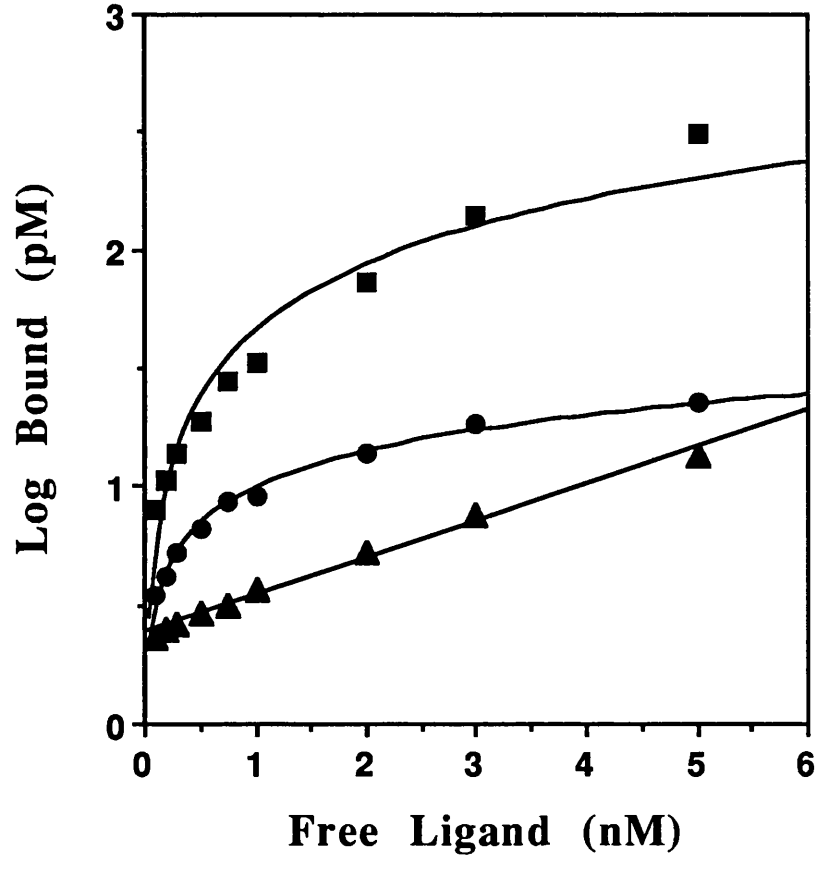

Log Bound (pM)
Free Ligand (nM)
0
1
2
3
4
5
6

Figure 24. Ligand binding assay of oestrogen receptor in the nuclear fraction of PC3 cells. Typical Scatchard plot (top) and tracer ligand saturation curve (bottom) of oestrogen binding. BpM = bound picomolar; B/F = bound over free. ■ = total, ● = specific, ▲ = non-specific binding of tritiated oestradiol, as calculated from competition studies using a 100 fold excess of unlabeled diethylstilbestrol. Each data point was performed in triplicate.


Type I ER
Kd=0.15nM
Fmol/mgDNA=133.2
Type II ER
Kd=3.5nM
Fmol/mgDNA=757.1
B/F
BpM
0,05
0,04
0,03
0,02
0,01
0,00
0
10
20
30


Log Bound (pM)
Free Ligand (nM)
3
2
1
0
0
1
2
3
4
5
6

In LNCaP cells, the soluble fraction displayed type I receptor concentrations ranging from 1,814 to 2,531 fmol/mg DNA, with a mean number of 79,755 ±2,657 binding sites/cell, corresponding to 51.3 ±3.9 fmol/mg cytosol protein. The nuclear fraction revealed a much lower type I ER content (ranging from 275.5 up to 370.0 fmol/mg DNA); this was also true when ER concentrations were expressed in terms of site number/cell (mean value of 13,737 ±1,158). In PC3 cells, no detectable type I ER were found in the soluble fraction. In the nuclear fraction, PC3 cells showed lower type I ER fmolar concentrations (in the range of 96.4-132.3 fmol/mg DNA) than LNCaP cells, with a mean number of 4,037 ±556.3 sites/cell.

Type II ER were also found in both cell fractions of LNCaP cells and in the nuclear compartment of PC3 cells (see Table 4).

Table 4. Type II oestrogen receptor content of LNCaP and PC3 human prostate cancer cell lines.

	Soluble Fraction		Nuclear Fraction	
	K_d (nM)	Fmol/mg DNA	K_d (nM)	Fmol/mg DNA
LNCaP	7.24 ±0.30	11784.8 ±3867.2	7.02 ±0.35	1942.2 ±513.0
PC3	ND	ND	4.42 ±0.87	848.9 ± 223.7

Values represent mean ±SD of triplicate assays. K_d = dissociation constant; ND = not detectable.

In LNCaP cells, soluble type II receptors exhibited mean K_d and concentration values respectively of 7.2 ±0.3 nM and 11,785 ±3,867 fmol/mg DNA (equivalent to 391.4 ±135.4 fmol/mg protein); the nuclear fraction showed similar K_d values (meanly 7.02 ±0.35 nM), but a much lower type II ER content, the mean concentrations being 1,942 ±513 fmol/mg DNA. The PC3 cells exhibited slightly lower type II nuclear ER content (ranging from 854.8 to 2,053.3 fmol/mg DNA) and K_d values (range of 3.5 to 4.6 nM), while no detectable type II binding was found in the soluble fraction.

2.2.2. Immunocytochemistry of ER and PgR

Immunocytochemical assay for both ER and PgR in human prostate cancer cells was carried out using the commercially available ER-ICA and PgR-ICA kits (Abbott) and subsequent quantitative image analysis through the CAS™200 Image Analyser (Becton-Dickinson Italia SpA, Milan). As illustrated in Figure 25, LNCaP cells stained positively for both ER and PgR, the percent of positive nuclei being 66% and 59%, respectively. The intensity of staining was consistently strong (positive stain over 80% for both ER and PgR), with a coefficient of variation lower than 15% for either receptor. On the other hand, PC3 cells displayed very low ER positivity (see Figure 26), with about 25% of positive nuclei having a weak to moderate degree of staining (48% positive stain), while all cells were PgR-negative. The DU145 cells proved negative for both ER and PgR.

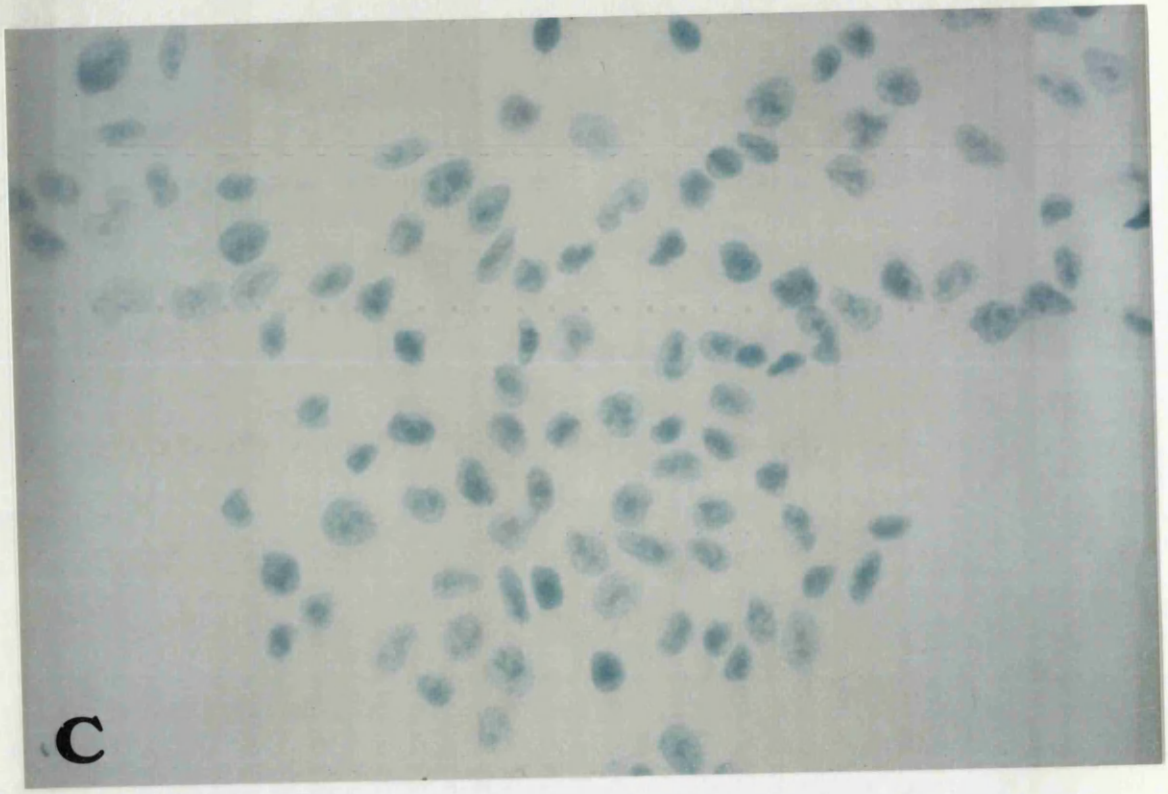
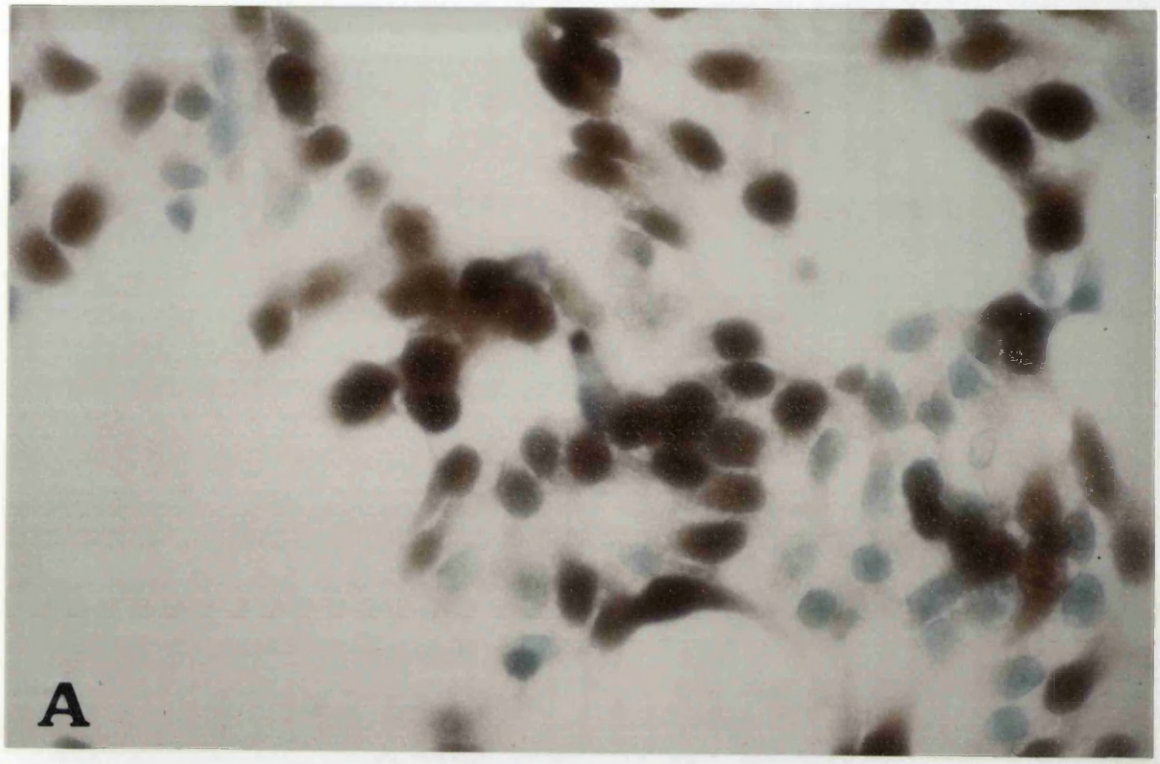


Figure 25. Immunocytochemistry of oestrogen (ER) and progesterone (PgR) receptors in LNCaP human prostate cancer cells. A : LNCaP/ER. B : LNCaP/PgR (x40 final magnifications). Immunocytochemical assay of both ER and PgR was carried out using a modification respectively of ER-ICA and PgR-ICA Abbott kits. Cells were incubated for 24 h at 4°C with primary monoclonal anti-ER or anti-PgR monoclonal antibodies and the reaction visualised through a peroxidase-anti peroxidase (PAP) system using the diaminobenzydine as chromogen substrate.

C Negative control, receiving 0.1 µg/ml normal rat IgG (x40).


A

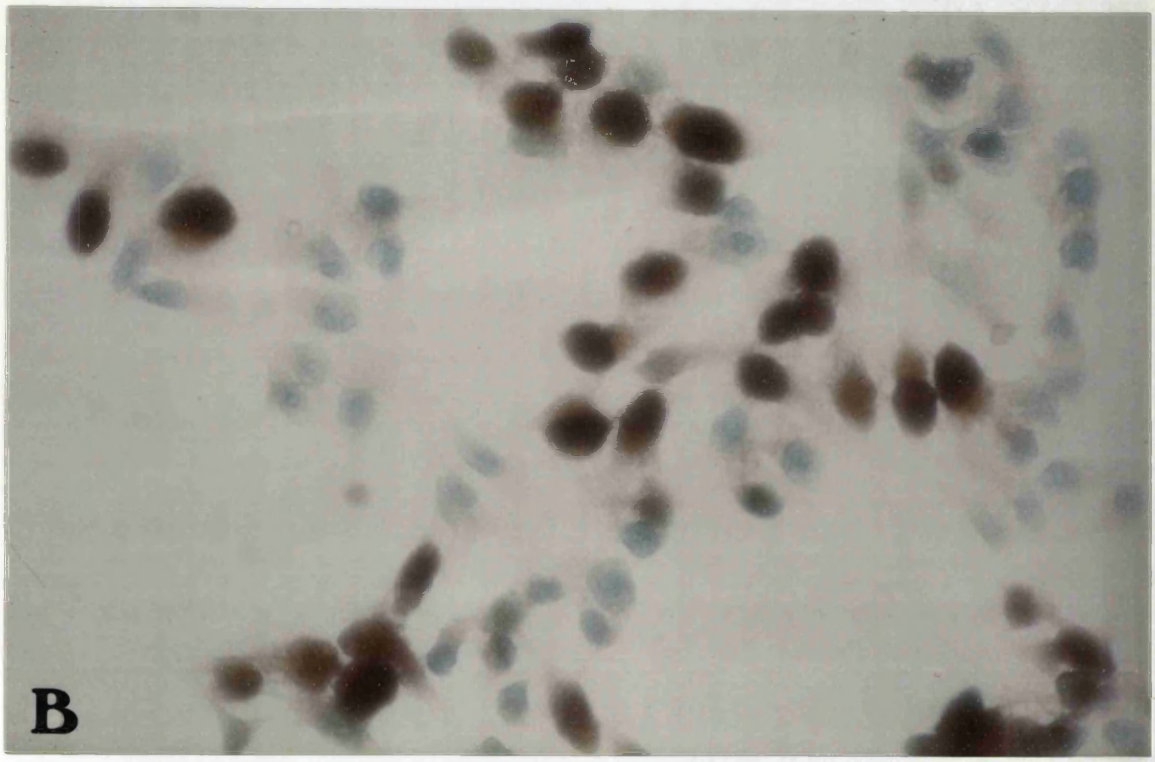
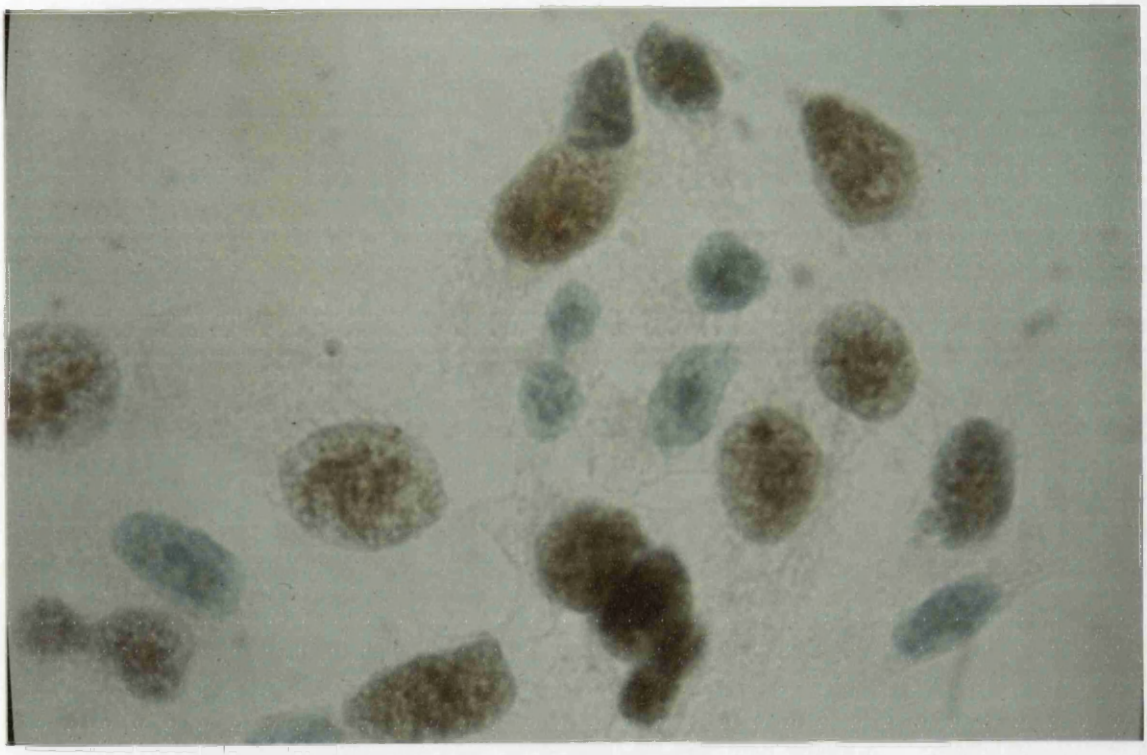

B

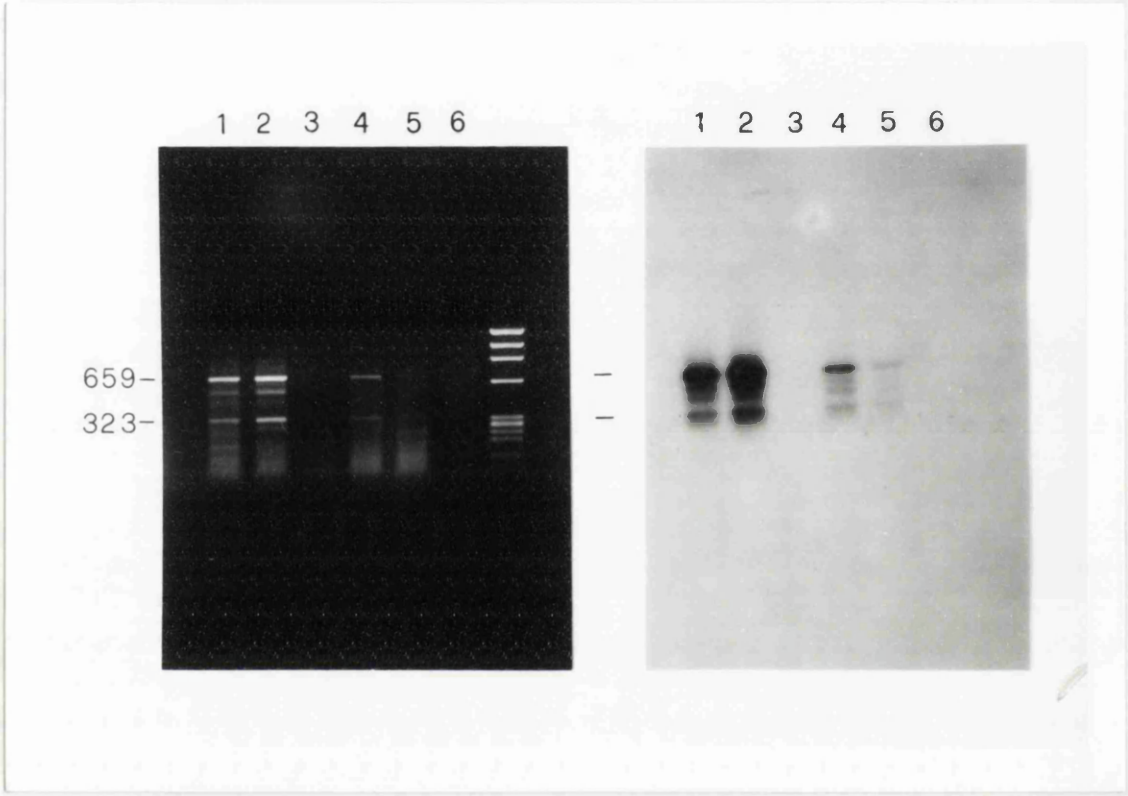
Figure 26. Immunocytochemistry of oestrogen receptors (ER) in PC3 prostate cancer cells (x40 final magnification). Immunocytochemical assay of ER was carried out using a modification of the ER-ICA Abbott method. Cells were incubated for 24 h at 4°C with primary monoclonal anti-ER monoclonal antibody and the reaction visualised through a peroxidase-anti peroxidase (PAP) system using the diaminobenzydine as chromogen substrate.



2.2.3. Reverse transcriptase-PCR (RT-PCR)

Presence of mRNAs for ER in prostate tumour cells was investigated through reverse transcription and PCR amplification of total RNA extracted from LNCaP, DU145 and PC3 cells, using the breast cancer cell lines ZR75-1 and MCF7 as positive and MDA-MB231 as negative controls. Southern hybridisation was also used to confirm identity of the amplification products. For PCR amplification, an ER cDNA was used as template, with a sense primer corresponding to a sequence in exon 3 and an antisense primer corresponding to a sequence in exon 6. PCR products obtained from either mammary or prostate cancer cell lines were separated on a 1.4% agarose gel, as shown in Figure 27 (left panel). As determined by comparison with the ϕx-HaeIII marker fragments, two major bands of 659 and 323bp were observed in ZR75-1, MCF7, LNCaP and PC3 cells, whereas no detectable amplification product was found in the oestrogen non responsive, ER-empty MDA-MB231 cell line nor in DU145 prostate cancer cells (not shown). Results from Southern hybridisation analysis, using the complete ER-cDNA as a probe, revealed hybridisation of the two major bands and some minor bands in between (Figure 27, right panel). The 659bp band corresponds well to the expected length (nucleotides 937 to 1596 of the ER sequence) of a normal ER mRNA. The 323bp band originates from a variant ER mRNA, lacking the entire exon 4; this variant, which is a likely product of alternative splicing, has recently been isolated and characterised from human breast cancer cell lines in our laboratories [Pfeffer *et al.*, 1993]. The bands of intermediate

Figure 27. Reverse transcriptase-polymerase chain reaction (RT-PCR) of oestrogen receptors in LNCaP and PC3 prostate cancer cells. *Left panel.* Ethidium bromide staining of agarose gel electrophoresis of PCR amplification products obtained from one μg total RNA: lane 1 = ZR75-1; lane 2 = MCF7; lane 3 = MDA-MB231; lane 4 = LNCaP; lane 5 = PC3 cells; lane 6 = reverse transcription-PCR of a RNA-free control sample. Marker lane = ϕx-174-HaeIII digest fragments (length given in base pairs). *Right panel.* Southern blot analysis of the PCR-amplified DNA from the left panel. Samples were hybridised using a ^{32}P-labelled human oestrogen receptor cDNA as a probe.


1 2 3 4 5 6
659–
323–
1 2 3 4 5 6

length are of unknown origin, although one amplification product shows a length compatible with a putative variant messenger lacking exon 5 which would also be amplified by the primer set used; using a nested primer set with an antisense primer in exon 4, a single band was observed in all cases (not shown). Although reverse transcriptase-PCR is by no means precisely quantitative, the relative amount of the ER messengers was reproducibly different in the cell lines studied. Equivalent expression of both ER mRNAs was observed in the two oestrogen responsive mammary cancer cell lines and, though at lower levels, in the androgen sensitive LNCaP cells. In contrast, both mRNAs appeared to be expressed in far lower but detectable amounts in androgen non-responsive PC3 cells. Under the same conditions, no reaction could be detected in the oestrogen non-responsive human breast cancer cell line MDA-MB231; therefore, expression of the ER mRNA in PC3 cells is clearly significant.. Moreover, digestion of the reaction products by the restriction enzyme Hind III, which cuts once in the amplified region (ER exon 4), yielded identical fragments of the expected size (not shown).

2.3. Immunostaining of the 27 kDa Heat Shock Protein (hsp27)

Immunofluorescence of the 27 kDa hsp27, which has been reported to be qualitatively and quantitatively correlated to ER in several human tissues [King *et al.*, 1987], was used as an indication of functional ER in human prostate cancer cells. Immunostaining was obtained using a D5 monoclonal antibody raised against hsp27 and visualised through a FITC-conjugate

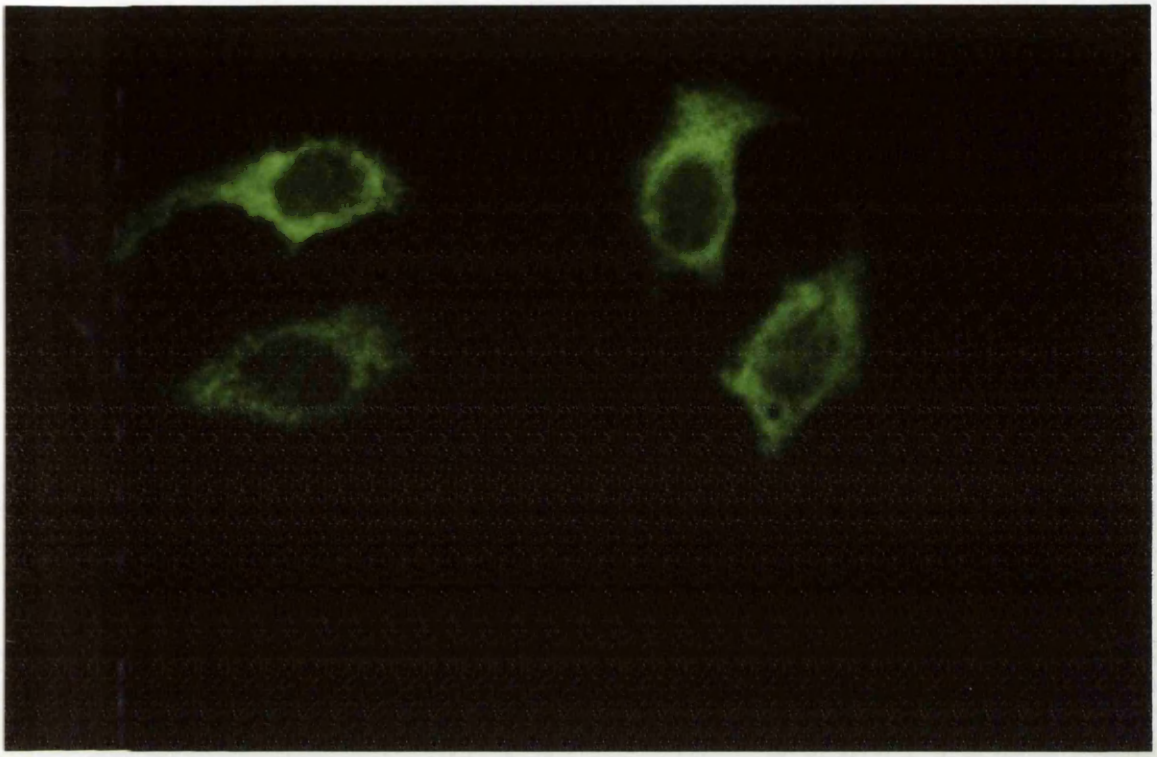
secondary antiserum. A positive staining was revealed in both LNCaP and PC3 cells, whilst DU145 were hsp27-negative. In LNCaP, the majority (over 85%) of cells showed an intensive stain, mostly located in the cytoplasm, which was consistently strong (see Figure 28, top). Conversely, PC3 cells displayed a positive staining mainly occurring in nuclei, although some cytoplasmic staining was also present (see Figure 28, bottom); percent of positive cells was lower than that seen in LNCaP cells (approximately 50%), with minor discrepancies in stain intensity. Control wells from either cell line not receiving primary D5 antibody stained very poorly, indicating that hsp27 staining was specific.

3. HORMONE RESPONSIVENESS

3.1. Response to Androgens

In order to evaluate the effects of either T or DHT on growth of human prostate cancer cell lines, cells were grown for 6 days in RPMI medium supplemented with 10% DHICT-FCS in the presence of increasing concentrations of androgens and the growth response measured as the DNA content of exposed cells with respect to controls. Using such stringent experimental conditions, growth of both DU145 and PC3 cells was minimally affected by any dose of T and DHT used (see Figure 29, top and centre respectively). The effects of T and DHT on growth of LNCaP cells are shown in Figure 29, bottom. As can be seen, both

Figure 28. Immunofluorescence of the 27 kDa heat shock protein (hsp27) in human prostate cancer cells. Top: LNCaP cells; Bottom: PC3 cells. Expression of hsp27 was visualised using a D5 primary monoclonal antibody and a secondary step FITC-conjugated antiserum (x50 final magnifications).



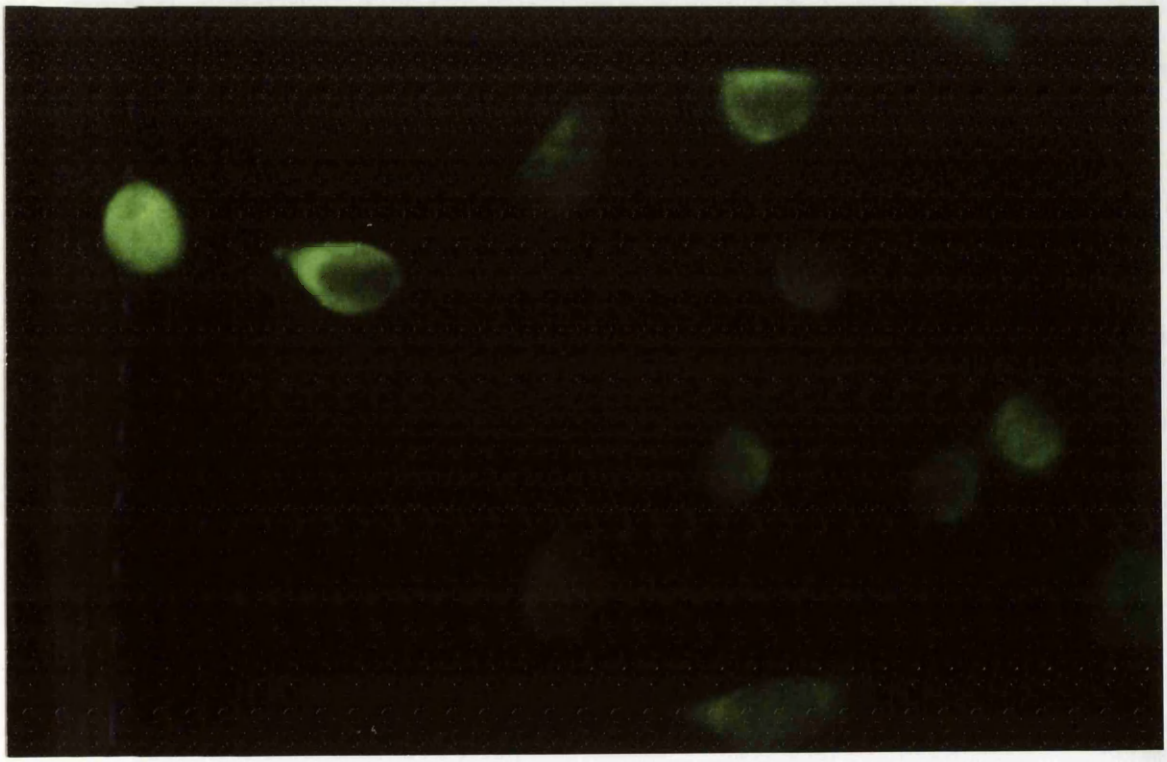
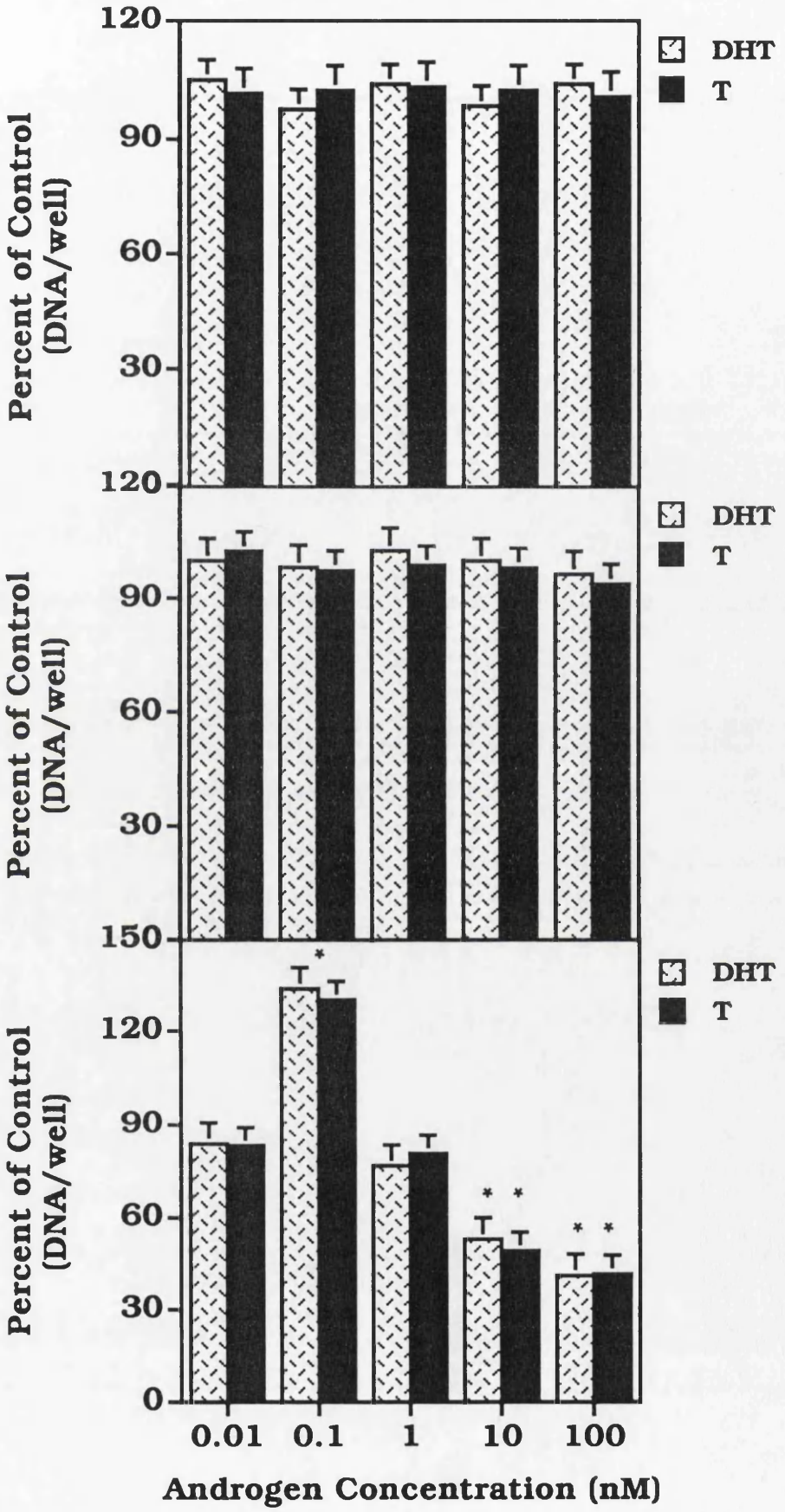


Figure 29. Androgen response of human prostate cancer cells. DU145 (top), PC3 (centre) and LNCaP (bottom) cells growing in RPMI medium supplemented with 10% DHICT-FCS were exposed for 6 days to increasing concentration (from 0.01 up to 100 nM) of dihydrotestosterone (DHT) and testosterone (T). Values represent mean ±SD (bars) of triplicate experiments. *P<0.03 and **P<0.004 with respect to control (Student's *t* test, 95% confidence limits).


Percent of Control (DNA/well)
120
90
60
30
120
DHT
T
Percent of Control (DNA/well)
90
60
30
150
DHT
T
Percent of Control (DNA/well)
120
90
60
30
0
DHT
T
0.01
0.1
1
10
100
Androgen Concentration (nM)

0.01 and 1 nM doses of either T or DHT produced a slight, not significant decrease of the cell growth. However, DNA content of LNCaP cells significantly increased following addition of $1x10^{-10}$M T or DHT (respectively 30.2% and 33.8% above control, $P<0.03$). Higher doses of either androgen produced a progressive reduction (from 19.4 up to 58.8%) in DNA content of this cell line, which was highly significant at both 10^{-8} and 10^{-7}M doses ($P<0.004$).

3.2. Response to Oestradiol

The effects of E_2 on growth of LNCaP, DU145 and PC3 cells were investigated under exactly the same experimental conditions used for androgens. As illustrated in Figure 30, growth of androgen responsive, AR-positive LNCaP cells was significantly stimulated by the addition of E_2 (peak of 65.8% at 1 nM; $P=0.009$). Conversely, a highly significant inhibition of cell proliferation was seen at 100 nM E_2 dose (40%, $P=0.006$).

In contrast, growth of DU145 cells was totally unaffected by any E_2 concentration used (see Figure 30).

Surprisingly, androgen unresponsive, AR-negative PC3 cells displayed a significant, dose-related inhibition of growth, with a maximal effect at 10^{-7}M E_2 (55.2% with respect to control, $P<10^{-6}$; see Figure 30 and, in more detail, Figure 31A); this effect was also significant at lower (even at 0.01 nM) E_2 doses and became evident after 72 h at concentrations higher than 1 nM E_2 (not shown). It is noteworthy that this negative growth regulation is cognate to that observed in PC3 cells after addition

Figure 30. Oestrogen response of human prostate cancer cells. LNCaP, DU145 and PC3 cells growing in RPMI medium supplemented with 10% DHICT-FCS were exposed for 6 days to increasing concentration (from 0.01 up to 100 nM) of 17β-oestradiol (E_2). Values represent mean ±SD (bars) of triplicate experiments. Degrees of significance (P) with respect to controls: LNCaP: 1 nM <0.01, 100 nM <0.006. PC3: 0.01 nM <0.0002, 0.1 and 1 nM <0.00002, 10 nM <0.0006, 100 nM $<10^{-6}$ (two-tailed Student's *t* test, 95% confidence limits). No significant differences were found for DU145 cells at any E_2 concentration used.

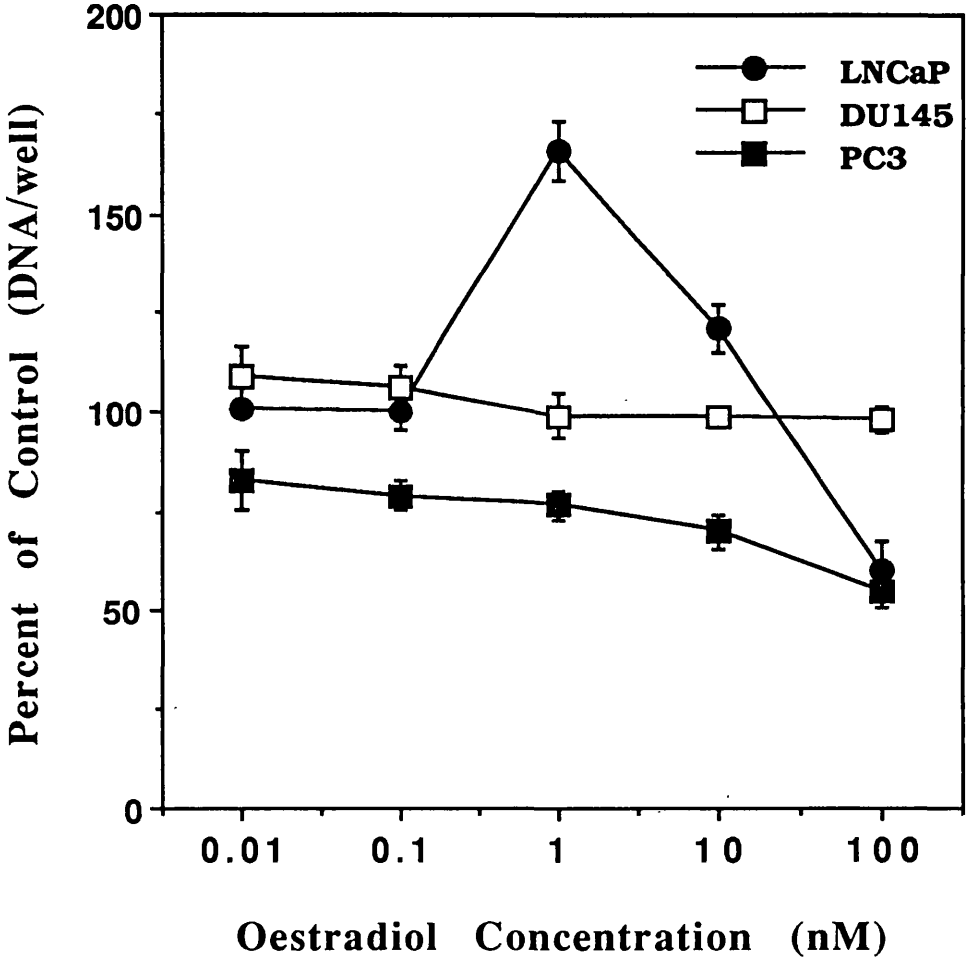
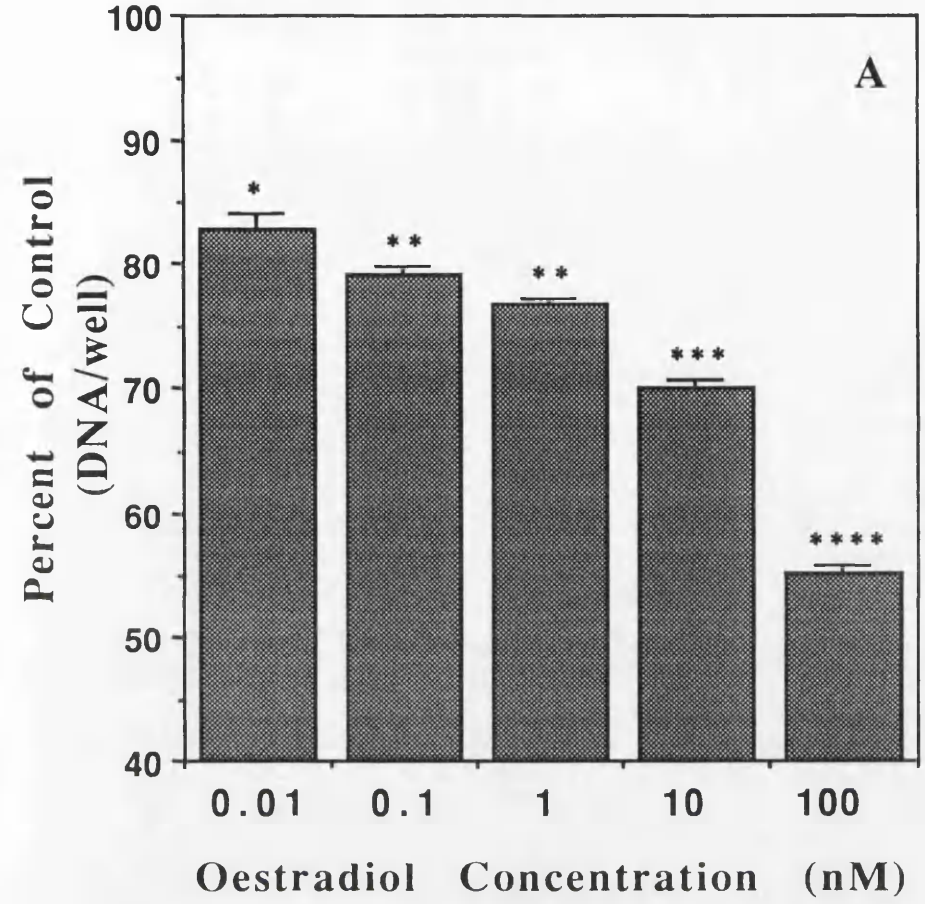
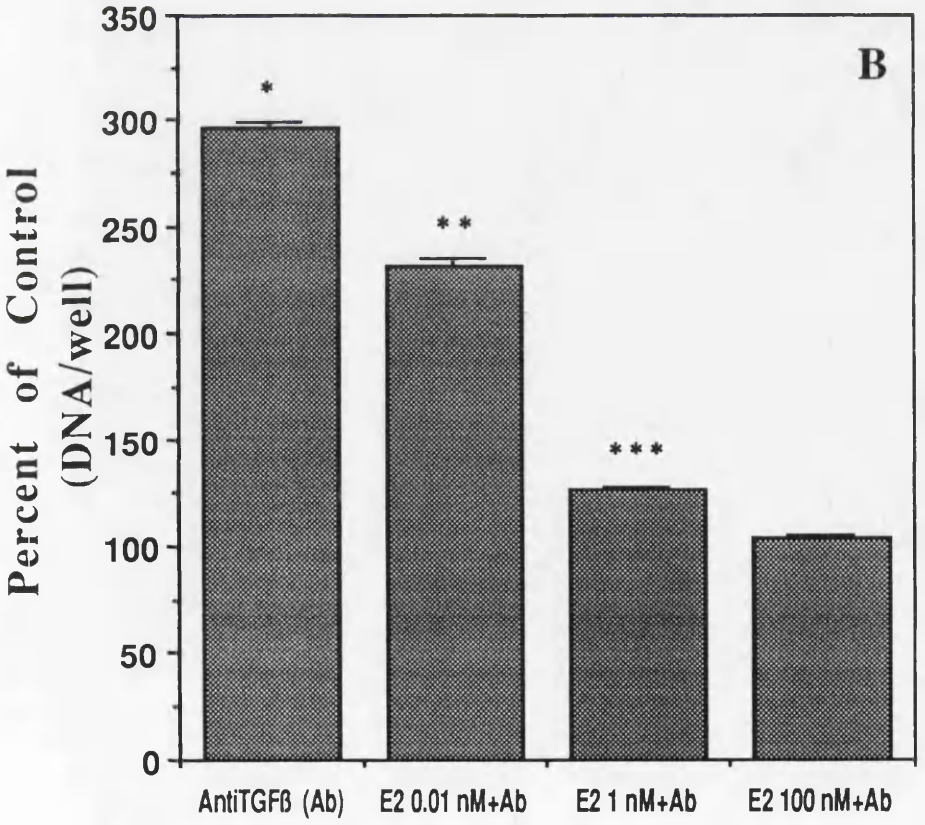

200
150
100
50
0
Percent of Control (DNA/well)
LNCaP
DU145
PC3
0.01
0.1
1
10
100
Oestradiol Concentration (nM)

Figure 31. Growth effects of oestradiol and anti-TGFβ_1 antibody in PC3 cells. Cells growing in RPMI medium supplemented with 10% DHICT-FCS were exposed for 6 days to (A) increasing concentration (from 0.01 up to 100 nM) of 17β-oestradiol (E_2) or (B) anti-TGFβ_1 (Ab) antibody (9.2 μg/ml) ±E_2 (0.01, 1 and 100 nM). Values represent mean ±SE (bars) of triplicate experiments.(A): *, P<0.0002; ***, P<0.0006**; P<0.00002; ****, P<0.00001. (B): *, P<0.00001; **, P<0.0001; ***, P<0.008 (two-tailed Student's *t* test, 95% confidence limits). No significant difference was found in comparison of Ab+100 nM E_2 with respect to control (P=0.435).


A
Percent of Control (DNA/well)
100
90
80
70
60
50
40
*
**
**
***
****
0.01
0.1
1
10
100
Oestradiol Concentration (nM)

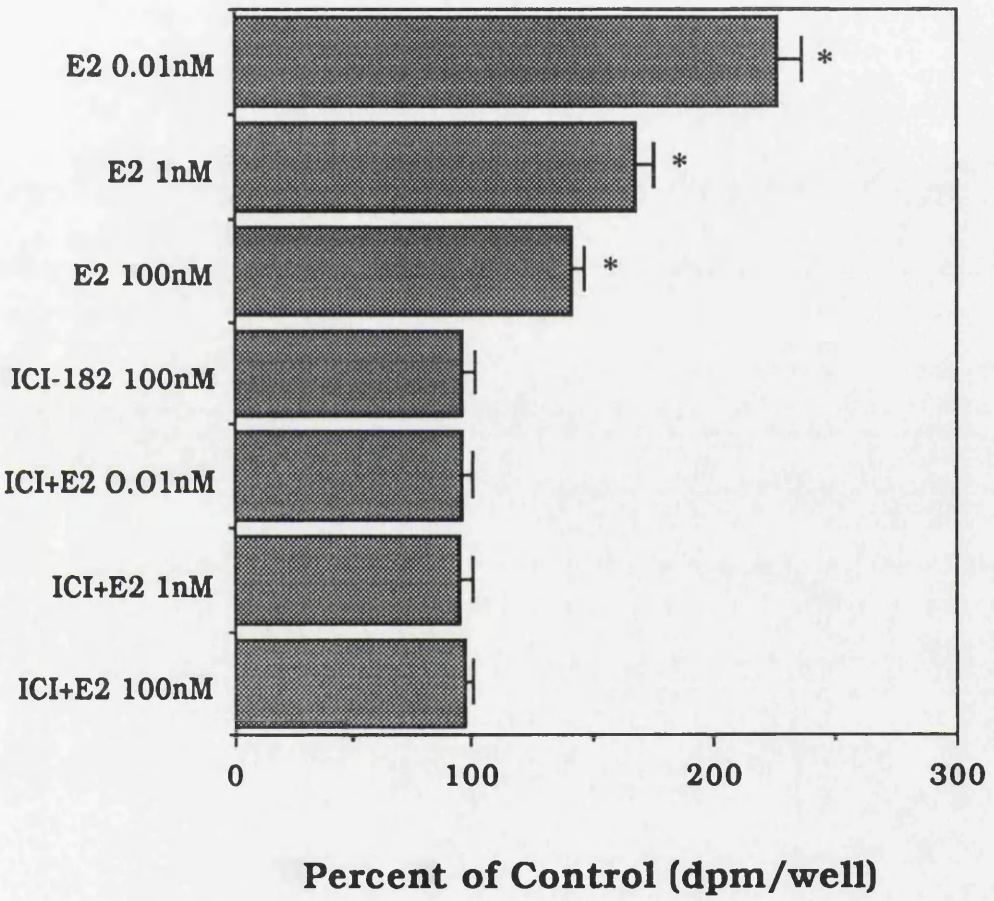

B
Percent of Control (DNA/well)
350
300
250
200
150
100
50
0
*
**
***
AntiTGFß (Ab)
E2 0.01 nM+Ab
E2 1 nM+Ab
E2 100 nM+Ab

of 1 ng/ml TGFß$_1$ (54.4% of control), under exactly the same experimental conditions (see below). In order to test whether the E_2-induced growth inhibition could be mediated via TGFβ, PC3 cells were incubated with a polyclonal antibody which neutralises the biological activity of TGFß$_1$. Exposure to the anti-TGFβ antibody produced a large increase of growth of PC3 cells, which was near 300% of control after 6 days; this effect was opposed by the addition of E_2 (10^{-11} to 10^{-7}M), being almost completely reversed at the dose of 100 nM (see Figure 31B).

3.3. Tritiated Thymidine Uptake

Separate experiments were carried out in order to ascertain whether or not E_2-induced effects on growth of LNCaP cells are mediated via its own receptor. ^{3}H-thymidine incorporation was measured in LNCaP cells pre-cultured for a week in steroid-deprived medium. As shown in Figure 32, after 6 days exposure to different E_2 concentrations (0.01, 1 and 100 nM), with or without addition of the pure antioestrogen ICI-182,780, growth of LNCaP cells was significantly increased in presence of all E_2 doses, with a peak stimulation of over 120% above control at the 0.01 nM dose ($P<0.00001$). Simultaneous addition of 100 nM ICI-182 completely abolished the E_2-induced growth stimulation at any oestradiol concentration, while ICI-182 itself did not affect cell proliferation yields. These results seemingly contradict those obtained measuring DNA content of LNCaP cells exposed for 6 days to E_2 in RPMI medium supplemented with 10% DHICT-FCS, whereby 1 nM E_2 only singificantly stimulated cell

Figure 32. Effects of oestrogen and anti-oestrogen ICI-182,780 on tritiated thymidine uptake by LNCaP cells. Cells were exposed for 6 days in steroid-deprived medium to increasing concentrations of E_2 ±ICI-182 or to ICI alone; cell proliferation was measured through a 6 h pulse of ^{3}H-thymidine as described in the Materials and Methods section (see 2.11.). Values represent mean dpm ±SD (bars) of triplicate experiments. *P<0.00001, two-tailed Student's *t* test (95% confidence limits).

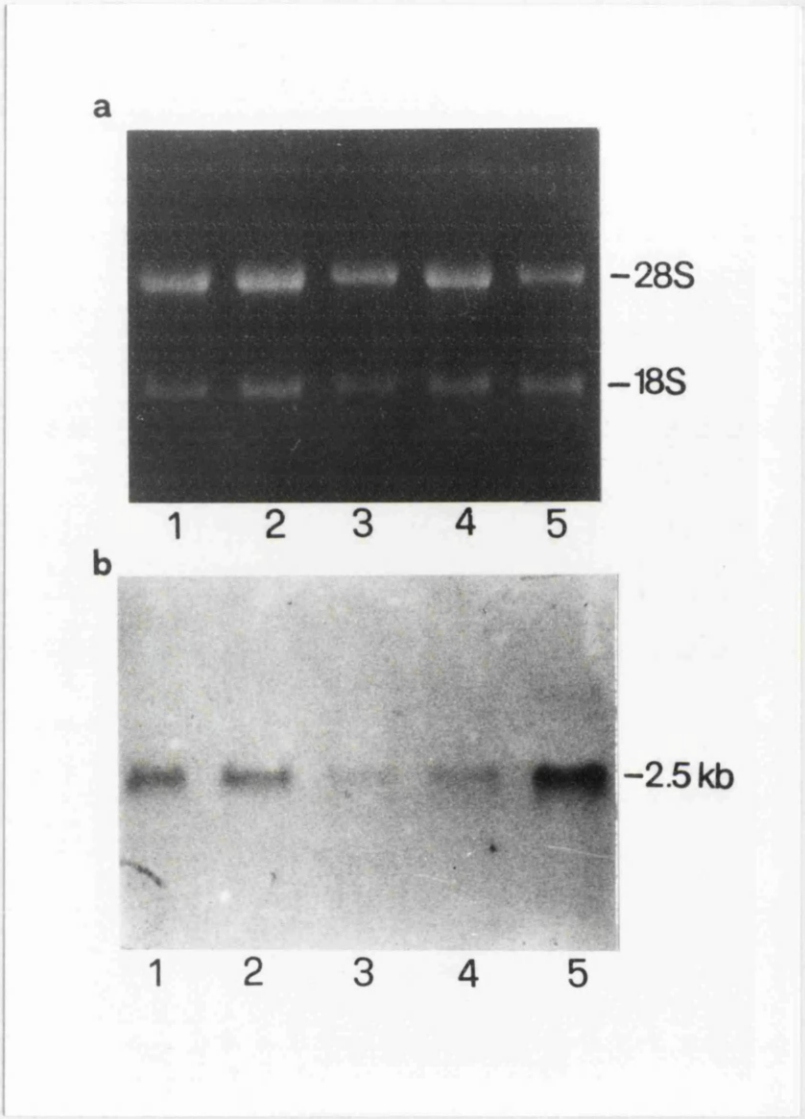

E2 0.01nM
E2 1nM
E2 100nM
ICI-182 100nM
ICI+E2 O.O1nM
ICI+E2 1nM
ICI+E2 100nM
*
*
*
0
100
200
300
Percent of Control (dpm/well)

proliferation. This apparent discrepancy, however, could be ascribed to the different experimental conditions used (10% DHICT-FCS vs 5% CT-FCS as RPMI supplements).

3.4. Northern Blot Analysis of TGFβ$_1$ mRNA

Aiming to verify the likelihood that E_2 inhibits growth of PC3 cells through induction of TGFß$_1$, PC3 cells were exposed to different E_2 concentrations for various intervals (from 6 up to 72 h) and possible effects on TGFß$_1$ mRNA investigated through Northern blot analysis. Results from 72 h experiments are shown in Figure 33a. As it can be seen, the ethidium bromide staining of ribosomal RNA revealed less amount of total RNA loaded on lanes 3 and 4 of gel (respectively, 1 and 100 nM E_2). The relative level of TGFß$_1$ mRNA is shown in Figure 33b. Although lanes 3 and 4 apparently contained less amounts of TGFß$_1$ mRNA, no significant difference was observed between controls (lane 1) and E_2 0.01, 1 and 100 nM (lanes 2, 3 and 4, respectively) when carefully compared to the ethidium bromide picture. This was also true at shorter incubation times (6 and 24 h, not shown). Human fibroblasts (lane 5) used for comparison showed higher level of TGFß$_1$ mRNA expression.

Figure 33. Northern blot analysis of transforming growth factor-β_1 (TGFβ_1) messenger RNA in PC3 cells: effects of oestrogen. Cells were incubated with increasing concentrations of oestradiol (from 10^{-11} up to 10^{-7}M) in serum-free medium for 72 h prior to RNA extraction. Ten μg of total RNA were loaded onto each lane and blots probed against a cocktail of specific TGFβ_1 oligonucleotides. (a) Staining with ethidium bromide of ribosomal RNA is shown to verify the amount of total RNA loaded on gel. (b) Autoradiograms of blots hybridised with TGFβ_1 probes. Lane 1 represents a control sample receiving vehicle only (ethanol 0.1%); the effects of 100 nM, 1 nM and 0.01 nM oestradiol are shown in lanes 2, 3 and 4, respectively. RNA from normal human fibroblasts is shown in lane 5.


a
28S
18S
1 2 3 4 5
b
2.5 kb
1 2 3 4 5

4. GROWTH FACTOR RECEPTORS AND CONTENT

4.1. Growth Factor Receptors

Radioreceptor assay of either EGF-R or TGFβ-R in prostate tumour cell lines was carried out through incubation with ^{125}I-EGF or ^{125}I-TGFß$_1$ in the presence of increasing concentrations of unlabeled growth factor.

Results of Scatchard analysis of EGF-R content of the three cell lines are reported in Table 5.

Table 5. Epidermal growth factor receptor levels in LNCaP, DU145 and PC3 human prostate cancer cell lines.

	K_d (nM)	Fmol/mg P	Sites/cell
LNCaP	0.16 ±0.06	107 ±11	43,046 ±3,444
DU145	0.32 ±0.09	541 ±67	187,654 ±6,011
PC3	0.17 ±0.03	392 ±36	99,200 ±4,415

Values represent mean ±SD of quadruplicate experiments.
K_d = dissociation constant; P = protein

All LNCaP, DU145 and PC3 cells exhibited high affinity binding sites for EGF, with K_d and receptor concentration values ranging

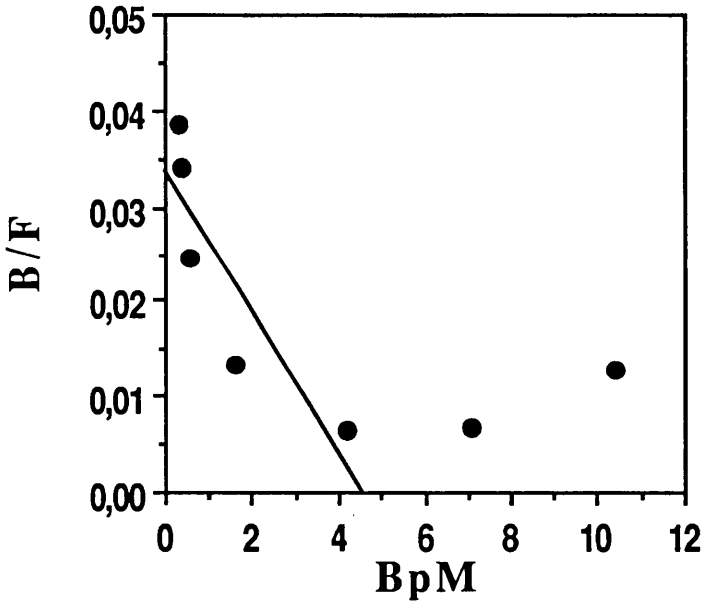
from 0.16 to 0.32 nM and from 43,046 to 187,654 sites/cell, respectively. Although presence of low affinity EGF binding sites in human tumour tissues and cells has been previously reported by others [REFS], no clear evidence for lower affinity sites of EGF binding in the prostate cancer cell lines studied was obtained. As can be seen in Table 5, EGF-R concentrations appeared, however, much higher in the DU145 cells, being respectively two and four times greater than in PC3 and LNCaP cells. Scatchard plots of EGFR in the three cell lines are illustrated in Figure 34.

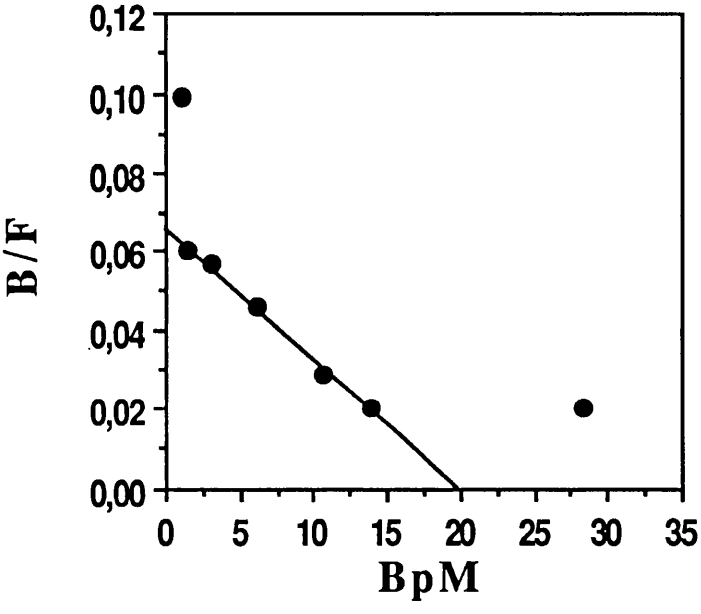
Presence of high-affinity TGFß$_1$ receptors was revealed in both DU145 and PC3 cells (for respective Scatchard plots see Figure 35), whilst no detectable high-affinity site of TGFß$_1$ binding was found in LNCaP cells. DU145 cells showed mean concentration values of 45.9 ±4.1 fmol/mg protein, corresponding to 3,023 ±376 binding sites/cell, with a K_d of 7.9 ±2.5 pM. PC3 cells displayed higher receptor content, with mean values of 95 ±8 fmol/mg protein (corresponding to 4,745 ±732 sites/cell) and a K_d in a lower picomolar range (4.5 ±0.6 pM). In addition, low affinity sites of TGFß$_1$ binding were found in DU145 cells (see Figure 35, top), with mean K_d and concentration values of 91.1 ±15.8 pM and 16,959 ±1,189 sites/cell, respectively.

4.2. Immunofluorescence for EGFR, EGF, TGFα and TGFβ$_1$

Presence and expression of EGF-R, EGF, TGFα and TGFß$_1$ in LNCaP, DU145 and PC3 cells was investigated through fluorescent immunocytochemical staining using specific antibodies, either monoclonal or affinity-purified polyclonal, raised against the

Figure 34. Radioreceptor assay of epidermal growth factor receptors (EGFR) in human prostate cancer cells. Scatchard plots of specific high affinity EGF binding in LNCaP (top), DU145 (centre) and PC3 (bottom) cells are shown. Each data point was performed in quadruplicate. Both dissociation constant (K_d) and concentration values are reported in Table 5. r: LNCaP=0.881, DU145=0.998, PC3=0.906. BpM = bound picomolar, B/F bound over free. For methodological details see text (Materials and Methods: 2.13.).


0,05
0,04
0,03
0,02
0,01
0,00
B/F
0
2
4
6
8
10
12
BpM


0,12
0,10
0,08
0,06
0,04
0,02
0,00
B/F
0
5
10
15
20
25
30
35
BpM

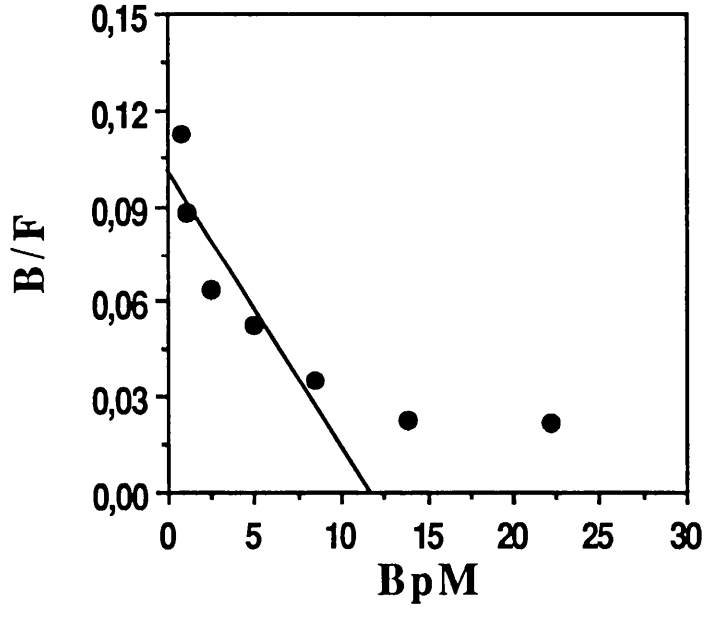
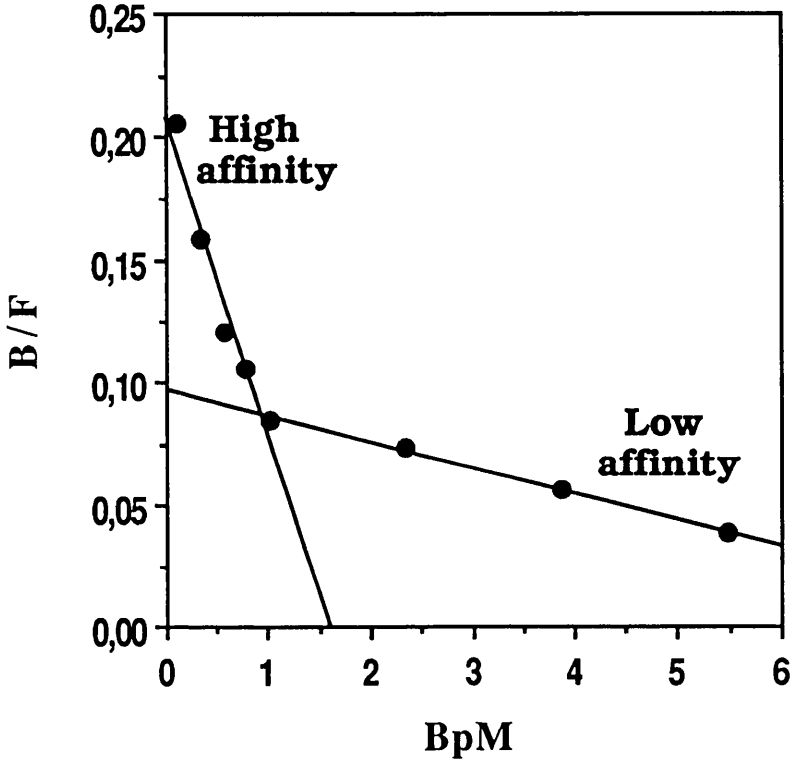
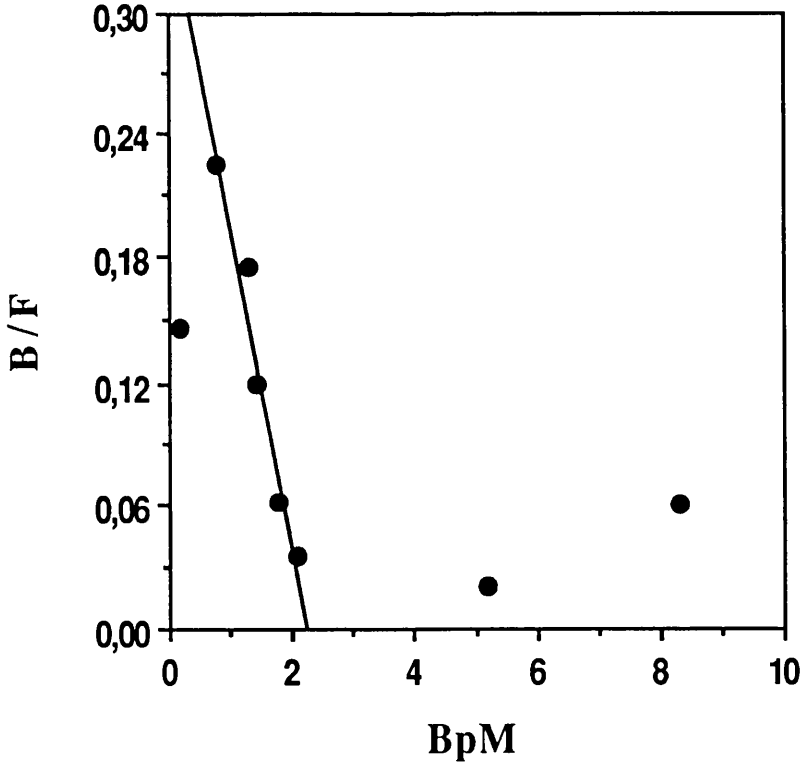

0,15
0,12
0,09
0,06
0,03
0,00
B/F
0
5
10
15
20
25
30
BpM

Figure 35. Radioreceptor assay of transforming growth factor-β_1 (TGFβ) receptors in human prostate cancer cells. Scatchard plots of high affinity TGFβ binding in DU145 (top) and PC3 (bottom) cells are shown. DU145: K_d=6.9 pM; 2,925 sites/cell; r=0.978. PC3: K_d=4.1 pM; 4,526 sites/cell; r=0.981. Presence of low affinity TGFβ sites in DU145 cells is also illustrated: K_d=84.9 pM; 15,863 sites/cell; r=0.936. Each data point was performed in quadruplicate. BpM=bound picomolar, B/F=bound over free.


0,25
0,20
0,15
0,10
0,05
0,00
B / F
High affinity
Low affinity
0
1
2
3
4
5
6
BpM


0,30
0,24
0,18
0,12
0,06
0,00
B / F
0
2
4
6
8
10
BpM

selected antigens. Specifically bound antibodies were visualised by addition of a second step FITC-conjugated antiserum against immunoglobulins of the relevant species (rabbit, mouse). Results are summarised in Table 6.

Table 6. Intensities of immunofluorescent stain for growth factor (GF) and growth factor receptor (GFR) content of human prostate cancer cells.

	GF/GFR			
	EGFR	EGF	TGFα	TGFβ$_1$
LNCaP	++	+	++	(+)
DU145	+++	++	+++	ND
PC3	(+)	ND	(+)	+

ND = not detectable. (+) = weakly positive.

Intensive stain for EGF-R (Figure 36A) and TGFα was seen in DU145. TGFα stained slightly less in LNCaP cells (Figure 36B); by contrast, PC3 cells displayed a weak staining for both TGFα and EGF-R. EGF was also detected in both DU145 and LNCaP cells, although at lower stain intensity, while PC3 cells were negative for EGF expression. TGFß$_1$ stained poorly in LNCaP cells and not at all in DU145 cells, but showed a positive stain, though not intense, in PC3 cells (Figure 36C). In all cases, the very low levels

A

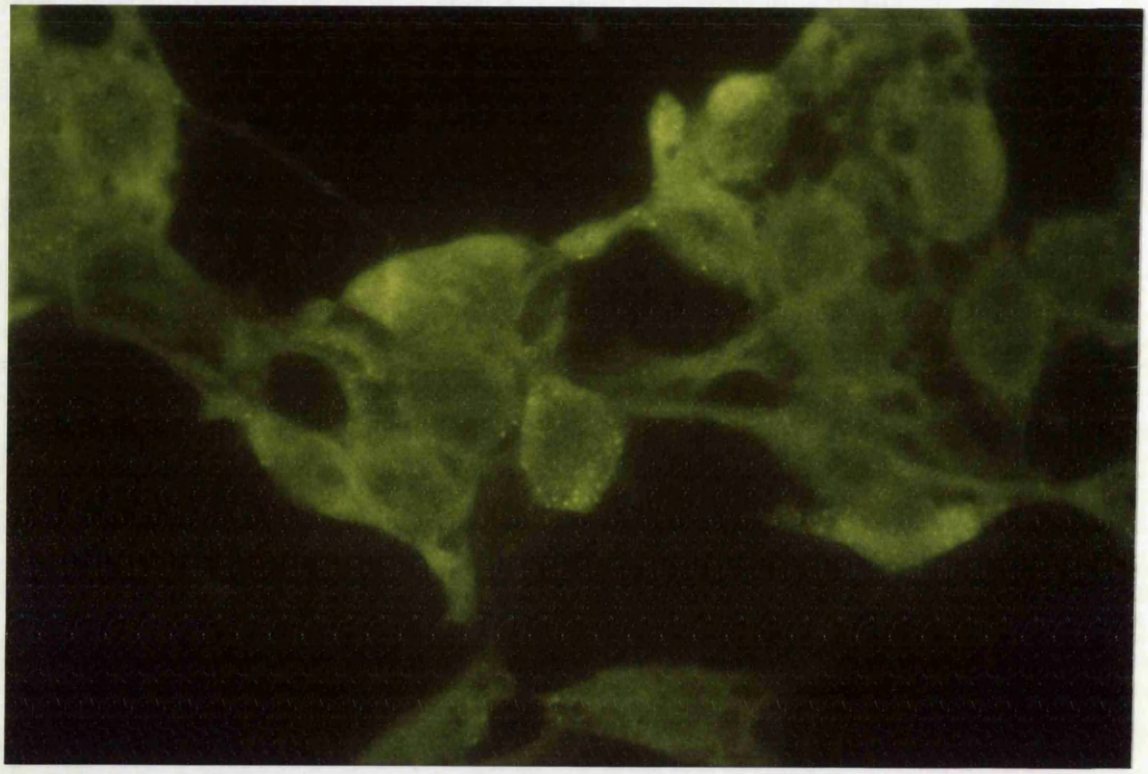
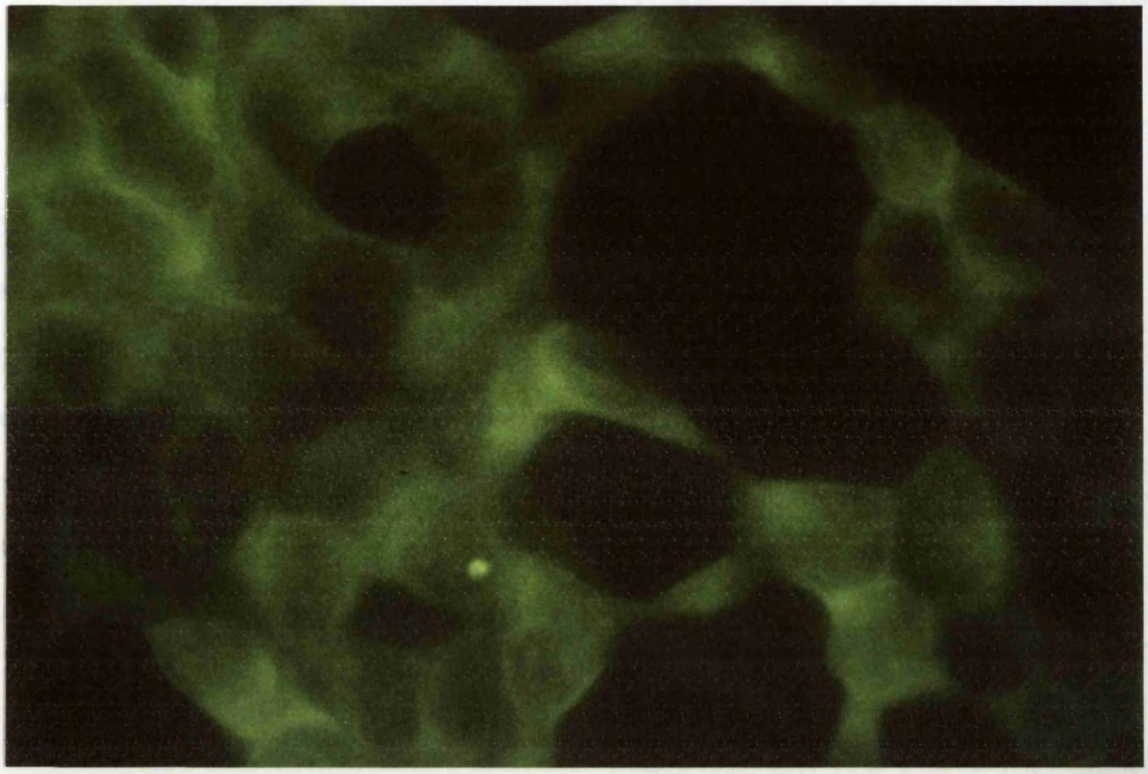
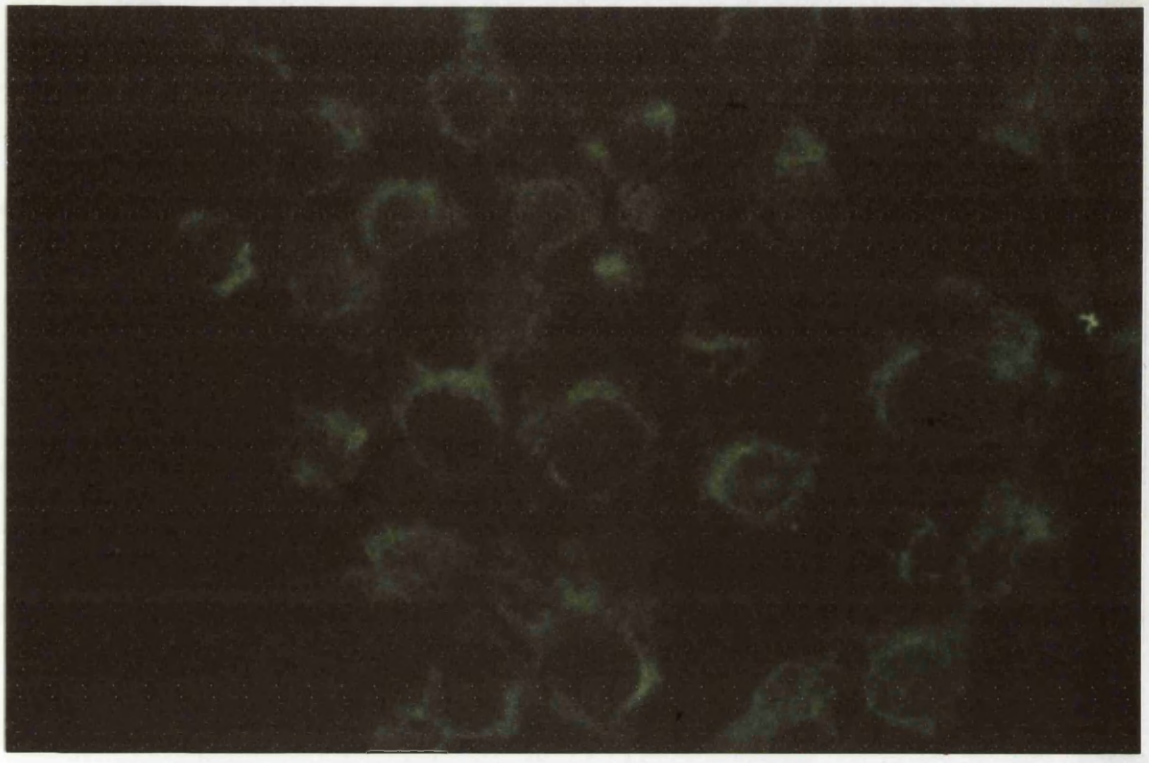


Figure 36. Immunofluorescence of growth factor and growth factor receptors in prostate cancer cells. The immunofluorescent staining of (A) EGF-R in DU145 cells, (B) TGFα in LNCaP cells and (C) $TGF\beta_1$ in PC3 cells is illustrated (x50 final magnification). Cell monolayers were exposed to primary polyclonal (EGF-R) or monoclonal (EGF and TGFα) antibodies and specific binding visualised through a second-step FITC-conjugate antiserum. Results are summarised in Table 5.

B



C



of non-specific binding observed substantiated the specific nature of staining.

4.3. Western Blot Analysis of EGFR

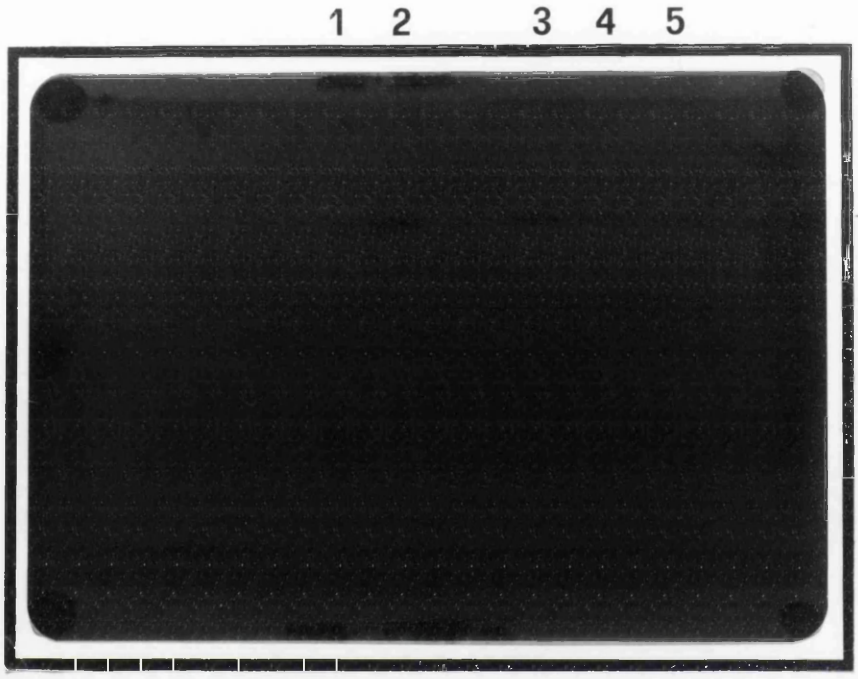
Expression of cell surface EGF-R in LNCaP, DU145 and PC3 cells was also evaluated through Western blotting analysis, using a specific polyclonal antibody BG48 raised against the synthetic peptide 2E (residues 985-996) and labelling of specific binding with [^{125}I]-Protein A. Amido Black staining of blots allowed also verification of the quality of transfer and comparison of molecular weights with selected standards proteins (not shown). As illustrated in Figure 37, autoradiograms showed higher levels of EGFR expression in DU145 cells, intermediate in PC3 and lower in LNCaP cells, overall in agreement with Scatchard plot data, but partly in contrast to the fluorescent staining. EGFR levels of prostate cancer cells were lower than those found in EGFR-rich A431 squamous carcinoma cells, but markedly higher than in MCF7 human mammary cancer cells.

5. RESPONSE TO GROWTH FACTORS

5.1. Growth response to TGFα and TGFß$_1$

The effects of various concentrations of both TGFα and TGFß$_1$ on growth of prostate cancer cells after either 24 or 48 h incubation were firstly assessed on cells grown in routine culture

Figure 37. Western blot analysis of epidermal growth factor receptor (EGFR) in prostate cancer cells. The autoradiogram shows specific EGFR binding using the anti-EGFR polyclonal antibody BG48 and [^{125}I]-protein A as secondary species labelling. Lane 1: MCF7 cells; lane 2: A431 cells; lane 3: DU145 cells; lane 4: LNCaP cells; lane 5: PC3 cells. Sample loading of 200 μg membrane protein for MCF7 cells, 10 μg for A431 cells and 100 μg each for DU145, LNCaP and PC3 cells.


1 2 3 4 5
170K

medium in parallel experimental conditions to those used for EGF-R and TGFβ-R binding studies.

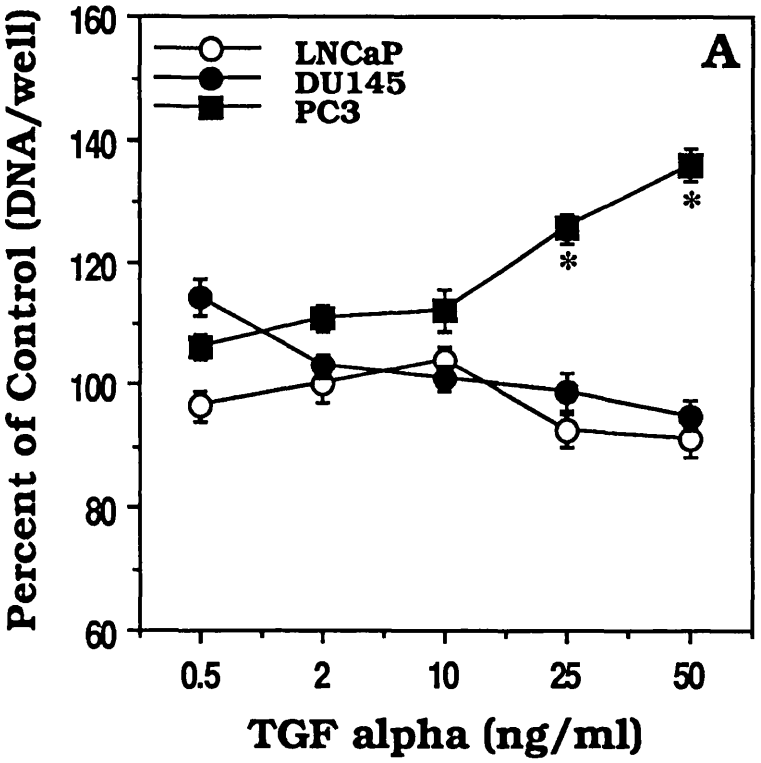
At 24 hours (data not shown), neither LNCaP nor DU145 cells exhibited growth stimulation in response to increasing concentrations (from 0.5 up to 50 ng/ml) of TGFα. In contrast, PC3 cells showed a dose-dependent increase (as measured by DNA content), reaching a maximum of about 18% above control at 50 ng/ml dose.

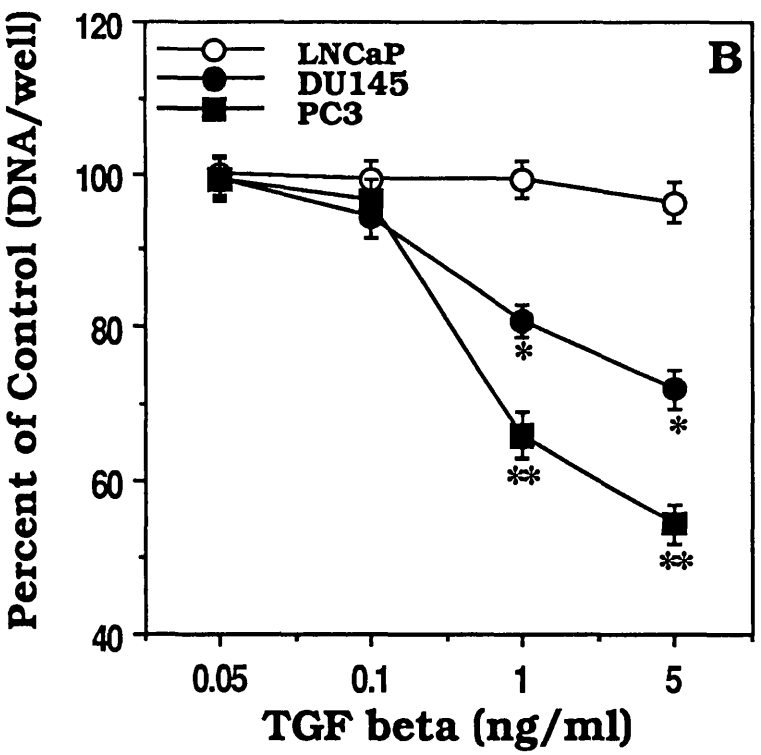
After 48 hours exposure (see Figure 38A), LNCaP cells remained insensitive to TGFα at all doses tested; conversely, PC3 cells showed a more pronounced dose-related increase of growth, which was about 36% at the 50 ng/ml dose ($P<0.003$). DU145 cells revealed only a slight rise of DNA content at the dose of 0.5 ng/ml, whilst the other doses did not affect growth of this cell line.

As regards TGFß$_1$, LNCaP cells seemed to be insensitive to increasing concentrations (from 0.05 to 5 ng/ml) of this growth factor over 24 hours exposure. PC3 cells, on the contrary, showed a remarkable decrease of DNA content (25.6% and 23.7% at 1 and 5 ng/ml, respectively). Similarly, DU145 cells exhibited reduction in growth which was very close to that found in PC3 cells, being respectively of 24.6% and 22.3% at 1 and 5 ng/ml doses.

After 48 hours exposure (see Figure 38B), the data appeared consistent with those obtained at 24 hours. LNCaP still exhibited no response to any TGFß$_1$ dose, while PC3 cells showed a significant decrease of growth with respect to control (maximal at the 5 ng/ml dose - 54.5% of control, $P<10^{-6}$), which was twice

Figure 38. Growth response of prostate cancer cells to transforming growth factor-α (TGFα) and -β_1 (TGFβ_1). Cells were grown in routine medium and exposed for 48 h to increasing concentrations of either (A) TGFα (0.5-50 ng/ml) or (B) TGFβ_1 (0.05-5 ng/ml). Values represent mean ±SD (bars) of triplicate experiments. (A): *$P<0.03$ and **$P<0.003$; (B): *$P<0.009$ and **$P<10^{-6}$, with respect to controls (two-tailed Student's *t* test, 95% confidence limits).


A
LNCaP
DU145
PC3
Percent of Control (DNA/well)
160
140
120
100
80
60
0.5
2
10
25
50
TGF alpha (ng/ml)
*
*
*


B
LNCaP
DU145
PC3
Percent of Control (DNA/well)
120
100
80
60
40
0.05
0.1
1
5
TGF beta (ng/ml)
*
*
**
**

the effect seen at 24 hours. On the other hand, DU145 cells displayed a much less pronounced reduction in DNA content between 24 and 48 hours, being only 28.2% at 48 hours after the same dose of 5 ng/ml ($P<0.009$).

Additional experiments were carried out to investigate the growth effects of both TGFα (10 and 50 ng/ml) and TGFß$_1$ (1 and 5 ng/ml) after 48 h and 6 days exposure using DHICT-FCS supplemented RPMI medium, therefore under experimental conditions identical to those used for evaluating response of cells to steroids (androgens and oestrogen) and closer to those used in studies on steroid metabolism (see below).

Concerning TGFα, neither 48 h nor 6 days exposures of LNCaP cells to either 10 or 50 ng/ml significantly affected cell proliferation (see Figure 39, top). Surprisingly, DU145 cells showed a decrease of growth that peaked at 50 ng/ml after 6 days (25.2% with respect to control; see Figure 39, centre). Similarly, PC3 cells lost the growth stimulation seen after 48 h in routine medium (35.9% at 50 ng/ml), showing a slight reduction (less than 15%) of their DNA content after 6 days exposure at the 50 ng/ml TGFα dose (see Figure 39, bottom).

Concerning TGFβ$_1$, results from both 48 h and 6 days experiments confirmed that LNCaP cells are insensitive to this growth factor, only a minor decrease of growth (around 15%) being found at 5 ng/ml after a 6 days interval (see Figure 40, top). At 48 h exposure, the DU145 cells displayed a decrease of growth following addition of 1 and 5 ng/ml TGFβ$_1$ which was equivalent to that seen at the same doses after 48 h in routine

Figure 39. Growth response of human prostate cancer cell lines to transforming growth factor-α (TGFα). LNCaP (top), DU145 (centre) and PC3 (bottom). Cells were grown in RPMI medium supplemented with 10% DHICT-FCS and exposed for 48 h or 6 days to selected concentrations of TGFα (10 and 50 ng/ml). Values represent mean ±SD (bars) of triplicate experiments.

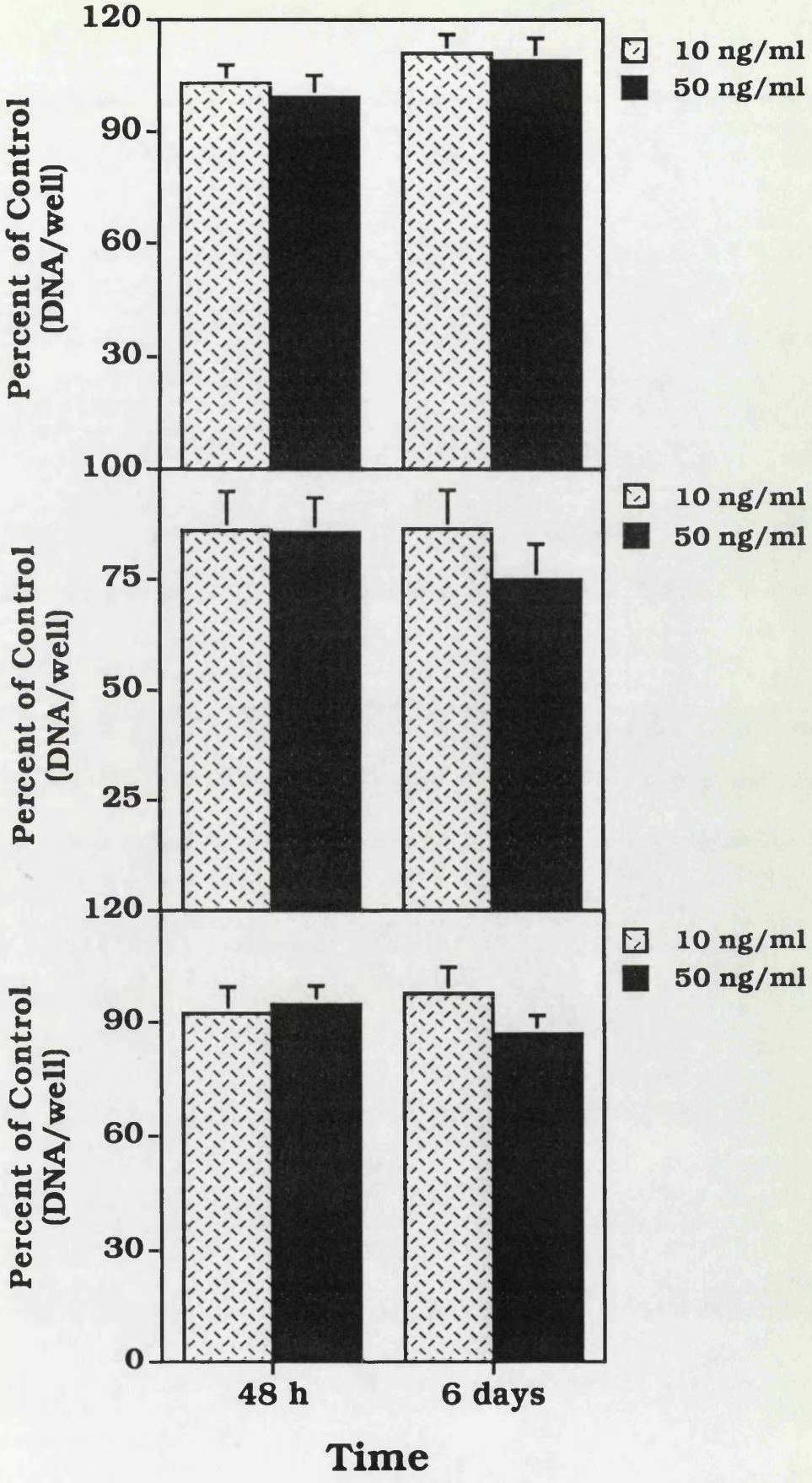
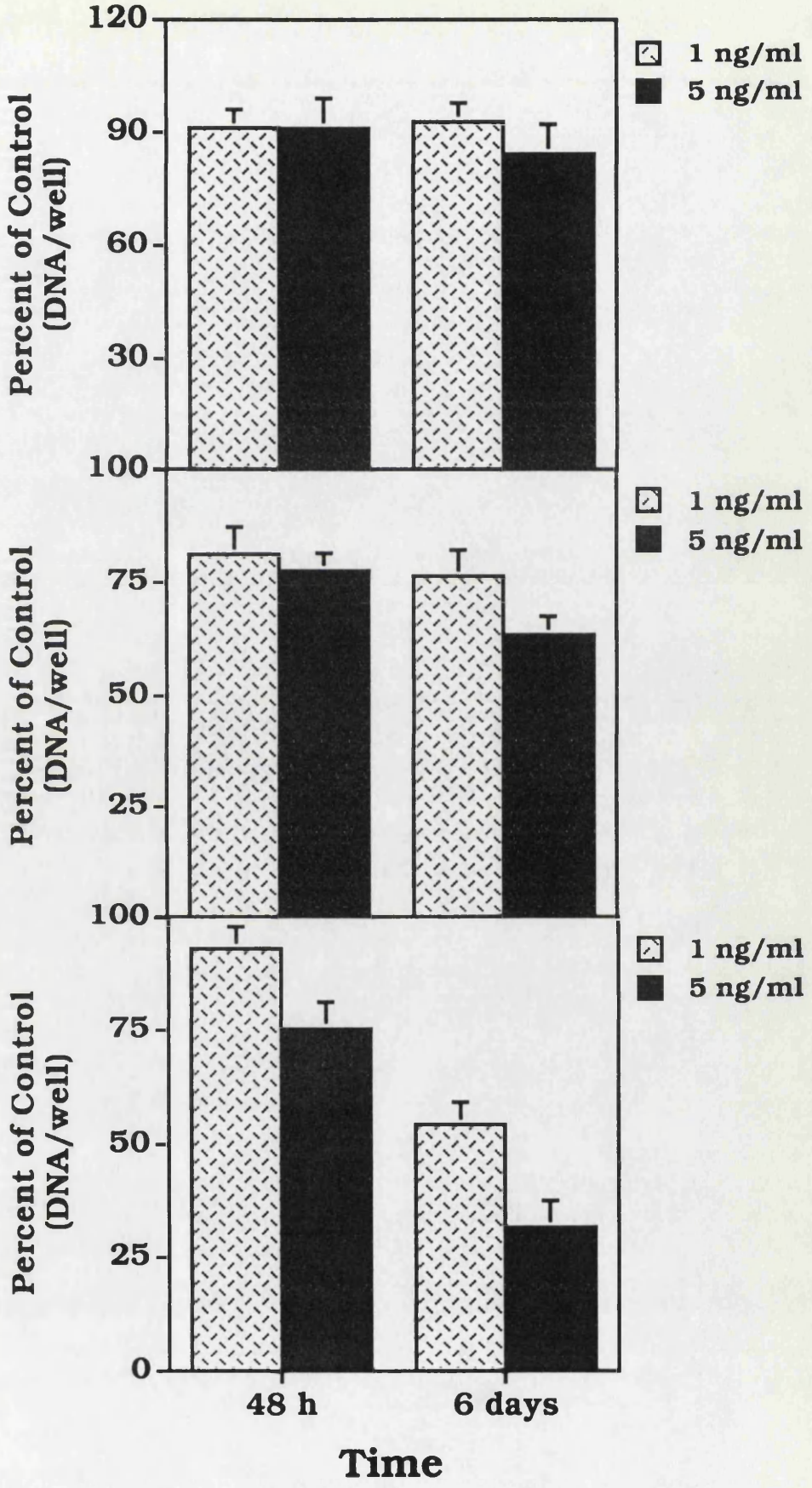

120
90
60
30
100
Percent of Control
(DNA/well)
10 ng/ml
50 ng/ml
75
50
25
120
Percent of Control
(DNA/well)
10 ng/ml
50 ng/ml
90
60
30
0
Percent of Control
(DNA/well)
10 ng/ml
50 ng/ml
48 h
6 days
Time

Figure 40. Growth response of human prostate cancer cell lines to transforming growth factor-β_1 (TGFβ_1). LNCaP (top), DU145 (centre) and PC3 (bottom). Cells were grown in RPMI medium supplemented with 10% DHICT-FCS and exposed for 48 h or 6 days to selected concentrations of TGFβ_1 (1 and 5 ng/ml). Values represent mean ±SD (bars) of triplicate experiments.


120
90
60
30
100
Percent of Control
(DNA/well)
1 ng/ml
5 ng/ml
100
75
50
25
Percent of Control
(DNA/well)
1 ng/ml
5 ng/ml
100
75
50
25
0
Percent of Control
(DNA/well)
1 ng/ml
5 ng/ml
48 h
6 days
Time

medium; this trend was further confirmed at 6 days incubation, a greater inhibition of growth (36.8% with respect to control) being observed at the 5 ng/ml dose (see Figure 40, centre). In contrast, PC3 cells exhibited a marked lessening (about one half) of the effects previously found at 48 h using routine medium at either $TGF\beta_1$ dose (1 ng/ml: 17.2% vs 34.1% and 5 ng/ml: 24.8% vs 45.5% with respect to routine medium conditions). After 6 days exposure, PC3 cells showed a further significant decrease of their DNA content, with a maximal effect seen at 5 ng/ml (over 68% below control, see Figure 40, bottom).

6. STEROID METABOLISM

6.1. Androgens

Measurement of multiple enzyme activities of testosterone metabolism in human prostate cancer cell lines was carried out using physiological concentration of tritium-labelled precursor(s) administered to living cultured cells for variable incubation times and RP-HPLC separation and identification of radioactive androgens.

After 24 h incubation, AR positive LNCaP and DU145 cells, on the one hand, and androgen unresponsive, AR-poor, PC3 cells, on the other, displayed divergent patterns of testosterone metabolism (see Table 7).

Table 7. Testosterone conversion rates in human prostate cancer cells: percent values of undegraded precursor (^{3}H-T) and products formed after 24 h incubation.

	^{3}H-T	DHT	3α-diol	Δ^4Ad	5α–Adione	A	EpiA
LNCaP	81.7 ±2.6	1.8 ±0.2	0.87 ±0.17	11.6 ±0.9	3.7 ±0.3	*	*
DU145	86.1 ±2.8	6.9 ±0.7	0.76 ±0.12	4.1 ±0.6	1.3 ±0.4	ND	ND
PC3	11.3 ±0.6	ND	ND	60.9 ±2.1	18.3 ±1.3	2.9 ±0.1	6.0 ±0.7

Values are expressed as percentages (mean ± SD) of total radioactivity detected in triplicate experiments. Cells (0.5-1x10^6) were incubated with 1-2x10^{-9}M labelled testosterone as precursor, in FCS free, phenol red free-RPMI medium for 24 h and the incubation medium processed as described in the Materials and Methods section (see 2.17.). (*) negligible amounts occasionally detected in the free fraction only (see Figure 41). ND = not detectable. For abbreviations see Table 1.

In fact, as also illustrated in Figures 41 and 42 respectively, T remained mostly unconverted in both LNCaP and DU145 cells, the percent of labelled T being 81.7 ±2.6 for LNCaP and 86.1 ±2.8 for DU145 cells. A relatively high DHT formation was revealed in both cell lines, with a higher extent of the reaction in DU145 (mean 6.9 ±0.7%) with respect to LNCaP cells (mean 1.8 ±0.2%); a small proportion of 3α-androstanediol (3α–diol) was also seen at a nearly equivalent extent (0.87 and 0.76%) in both cell lines.

Figure 41. Typical HPLC profile of androgen metabolism in LNCaP cells. Cells ($1x10^6$) were incubated with $1x10^{-8}$M tritiated testosterone for 24 h and both precursor degradation and product formation analysed through RP-HPLC and radioactive detection. Crude cpm values were: (1) T, 309,983; (2) Δ^4Ad, 48,608; (3) EpiA, 1,971; (4) DHT, 4,298; (5) 3α-diol, 2,592; (6) 5α-Adione, 13,686; (7) A, 2,006. For abbreviations, relative retention times and peak identification number see Table 1, Androgens.

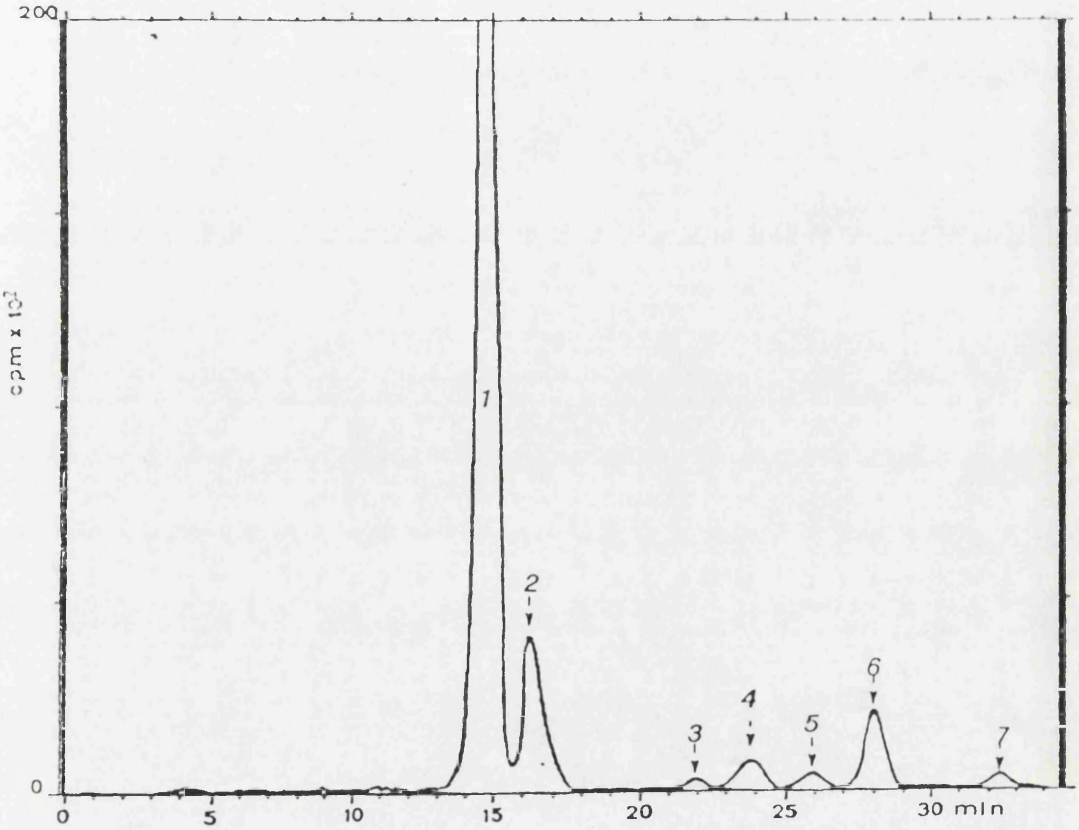
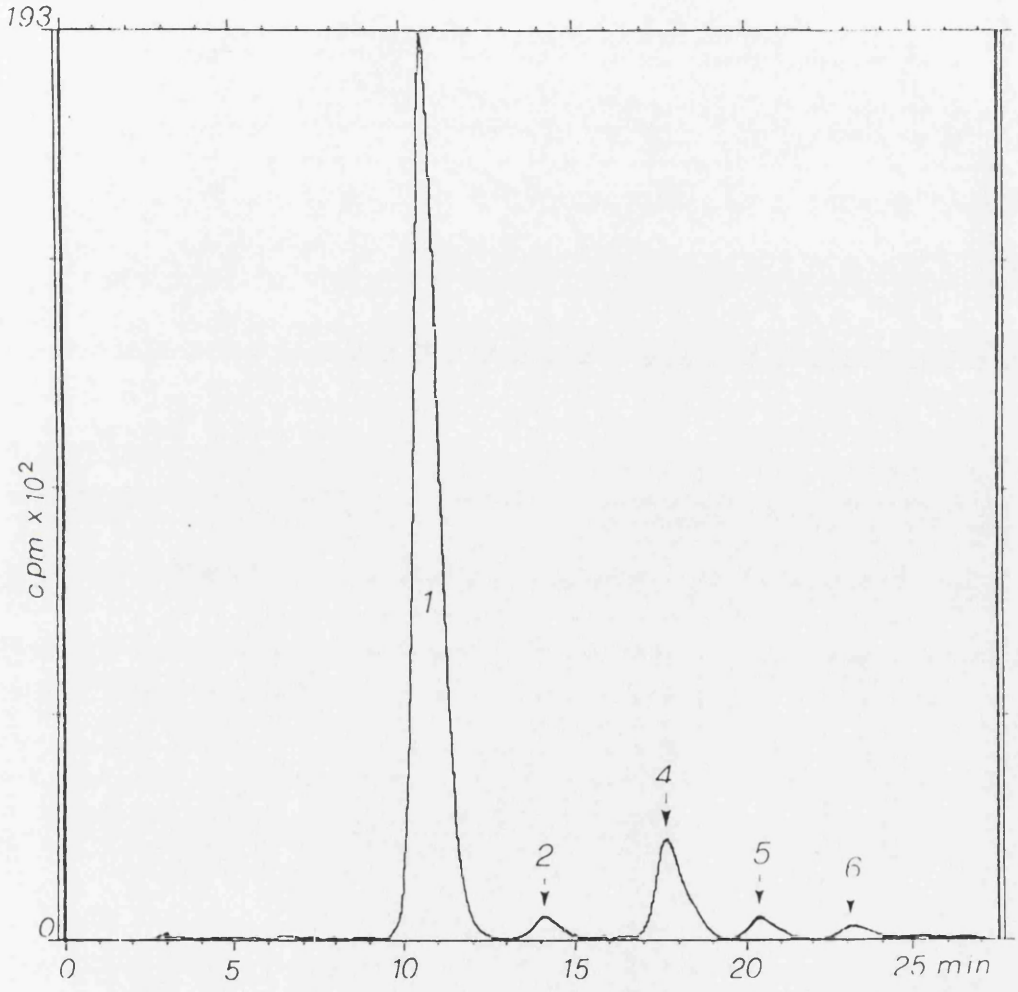

200
0
cpm x 10²
1
2
3
4
5
6
7
0
5
10
15
20
25
30 min

Figure 42. Typical HPLC profile of androgen metabolism in DU145 cells. Cells ($5x10^5$) were incubated with $5x10^{-9}$M tritiated testosterone for 24 h and both precursor degradation and product formation analysed through RP-HPLC and radioactive detection. Crude cpm values were: (1) T, 162,694; (2) Δ^4Ad, 5,167; (4) DHT, 21,895; (5) 3α-diol, 5,783; (6) 5α-Adione, 2,859. For abbreviations, relative retention times and peak identification number see Table 1, Androgens.


193
cpm x 10²
0
1
2
4
5
6
0
5
10
15
20
25 min

Conversely, the production of androstenedione (Δ^4Ad) and its 5α-reduced derivative 5α-androstanedione (5α-Adione) was greater in LNCaP (respectively, 11.6 and 3.7%) than in DU145 cells (respectively, 4.1 and 1.3%). Occasionally, negligible amounts of both androsterone (A) and its epimer epiandrosterone (EpiA) were found in LNCaP cells (see Figure 41).

In contrast, PC3 cells exhibited high conversion rates of T, the percent of unconverted precursor being only 11.3 ±0.6 after 24 h. Formation of metabolic products preferentially followed the oxidative way, with a remarkable conversion of T to Δ^4Ad (60.9 ±2.1%) and a consequent formation of the 5α-reduced derivatives of the 17-keto series, 5α-Adione, A and EpiA (Figure 43). Overall, these three androgen metabolites accounted for more than 27% of total radioactivity detected by 24 h.

The prevalence of the oxidative pathways of androgen metabolism in this cell line was further confirmed by experiments using ^{14}C-labelled Δ^4Ad as labelled precursor (see Figure 44). In fact, the most part (89.6%) of Δ^4Ad remained unconverted after 24 h incubation, with a negligible (less than 1%) formation of T and an appreciable production of the 17-keto derivatives 5α-Adione, A and EpiA (overall more than 9% as a sum). Although the proportion of 17-keto metabolites formed from Δ^4Ad is smaller than that seen after 24 h incubation with ^{3}H-T (over 27%), this evidence clearly indicates that the direction of androgen metabolism in PC3 cells is totally shifted in favour of the oxidative way.

Figure 43. Typical HPLC profile of androgen metabolism in PC3 cells. Cells ($1x10^6$) were incubated with $1x10^{-8}$M tritiated testosterone for 24 h and both precursor degradation and product formation analysed through RP-HPLC and radioactive detection. Crude cpm values were: (1) T, 28,687; (2) Δ^4Ad, 236,990; (3) EpiA, 29,565; (6) 5α-Adione, 81,262; (7) A, 10,895. For abbreviations, relative retention times and peak identification number see Table 1, Androgens.

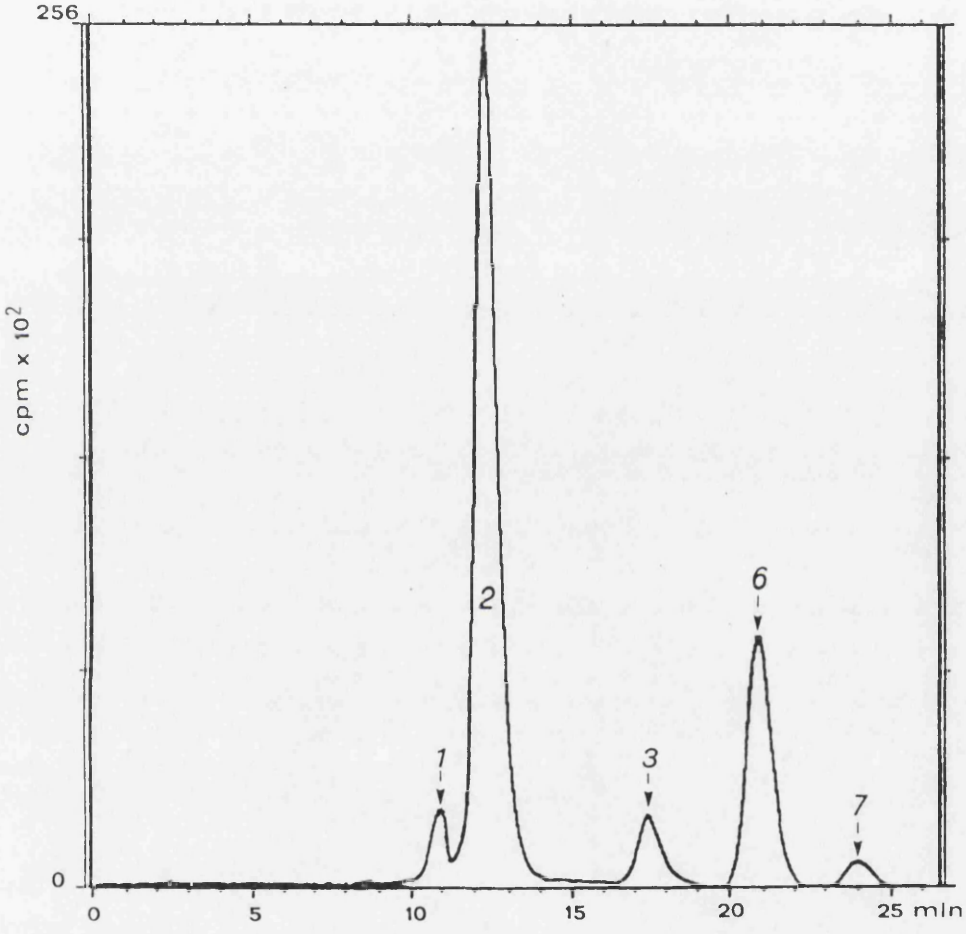
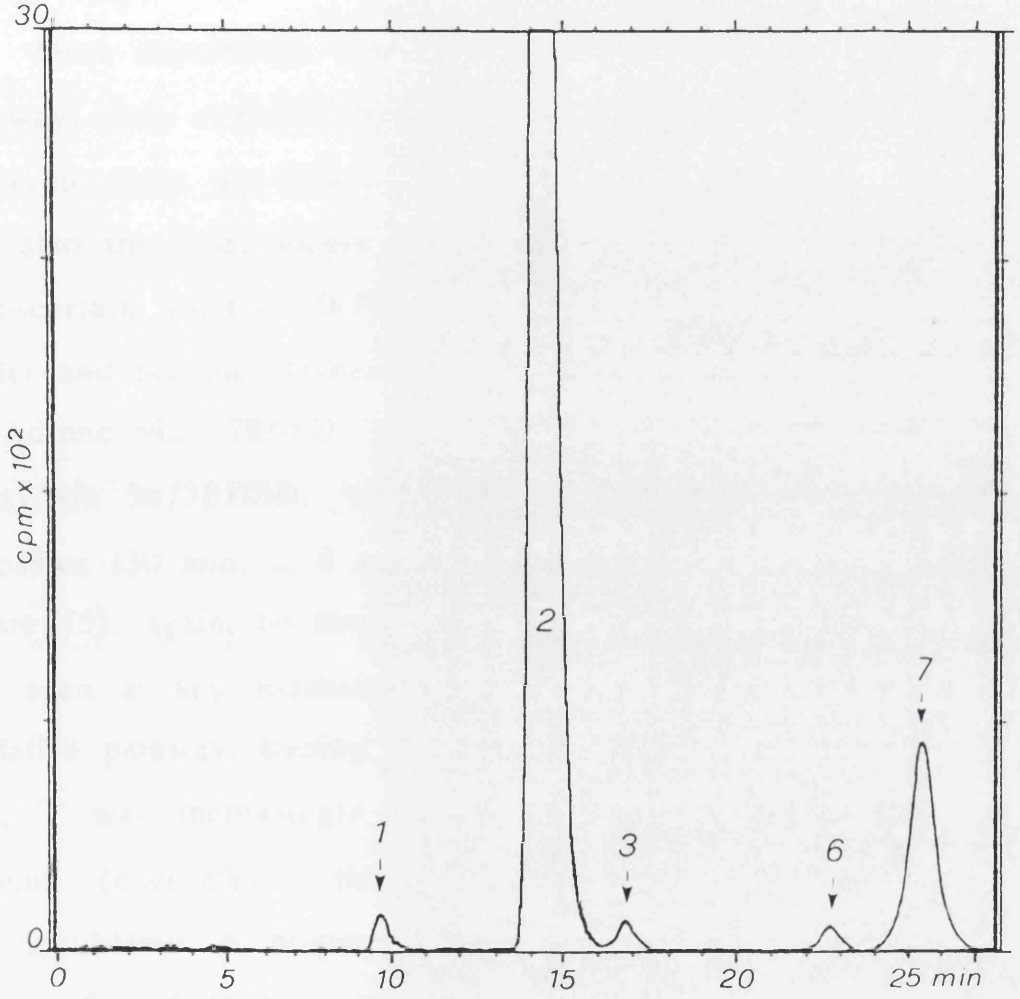

256
0
cpm x 10^2
1
2
3
6
7
0
5
10
15
20
25 min

Figure 44. HPLC profile of androstenedione metabolism in PC3 cells. Cells ($2x10^6$) were incubated for 24 h in the presence of carbonated androstenedione ($6x10^{-6}$M) and rates and direction of metabolism analysed by means of RP-HPLC and radioactive detection. Crude cpm values were: (1) T, 2,322; (2) Δ^4Ad, 216,868; (3) EpiA, 1,962; (6) 5α-Adione, 1,838; (7) A, 18,434. For abbreviations, relative retention times and peak identification number see Table 1, Androgens.


30
0
cpm x 10²
1
2
3
6
7
0
5
10
15
20
25 min

Notably, however, a good proportion (range: 43 to 57%) of radioactive androgens was present in conjugate form (either sulphate or glucuronide) in LNCaP cells, mostly (over 90%) as unconverted T. By contrast, no conjugate formation could be observed in either DU145 or PC3 cells, as also confirmed by the high percent values of extraction efficiency (87% and 90% respectively).

Most importantly, no detectable DHT formation (reductive pathway) was revealed by 24 h in PC3 cells; as previously observed from our laboratories [Castagnetta *et al.*, 1991c], this was also true after longer exposure intervals (72-96 h). In order to ascertain whether DHT formation in PC3 cells may occur earlier and become clandestine because of its rapid conversion to 5α-Adione via 17ßHSD, or to 3α/3β-androstanediols (3α/3ß-diols) via 3α/3βHSD, time-course experiments using shorter exposures (30 min, 2, 8 and 24 h) in sequence were set up (see Figure 45). Again, no measurable DHT or 3α/3ß-diols production was seen at any incubation time, while the prevalence of the oxidative pathway, leading to Δ^4Ad formation, was confirmed. In fact, T was increasingly converted to Δ^4Ad, an appreciable amount (over 8%) of this metabolite being found after only 30 min incubation. A proportional increase of 5α-Adione was also seen at 8 and 24 h. Formation of Δ^4Ad plus 5α-Adione was inversely and significantly related to the proportion of metabolised T ($r=-0.976$, $P<0.03$; Spearman correlation test).

Results from 72 h experiments were confirmatory of those obtained after 24 h incubation (see Table 8). In fact, both LNCaP

Figure 45. Time-course of testosterone metabolism in PC3 cells. Cells ($2x10^6$) were incubated with 50 nM tritiated testosterone as precursor for various intervals (from 30 minutes up to 24 hours) to inspect both testosterone degradation and formation of metabolic products. The histograms illustrate the proportion of undegraded testosterone (open bars) and that of androstenedione (hatched bars) plus 5α-androstanedione (black bars) formed against time. No DHT formation was observed at any incubation time. Values represent mean ±SD from triplicate experiments. For methodological details see text (Materials and Methods: 2.17.).

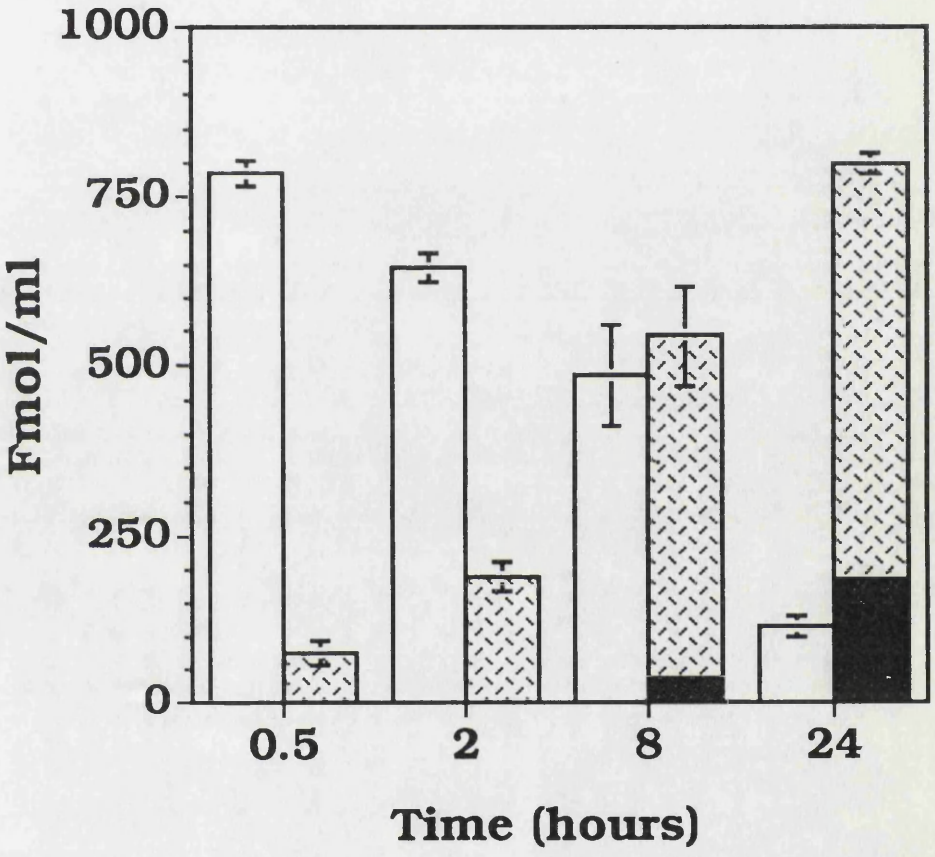

1000
750
500
250
0
Fmol/ml
0.5
2
8
24
Time (hours)

Table 8. Testosterone conversion rates in human prostate cancer cells: percent values of undegraded precursor (^{3}H-T) and products formed after 72 h incubation.

	^{3}H-T	DHT	3α-diol	Δ^4Ad	5α–Adione	A	EpiA
LNCaP	55.4 ±1.8	3.3 ±0.3	14.0 ±0.8	14.2 ±0.7	0.5 ±0.1	10.3 ±0.6	2.3 ±0.3
DU145	54.6 ±1.1	13.2 ±0.6	11.0 ±0.8	13.3 ±0.8	2.1 ±0.4	3.1 ±0.2	2.9 ±0.3
PC3	2.9 ±0.4	ND	ND	46.5 ±1.7	24.8 ±1.2	10.9 ±0.9	14.9 ±0.8

Values are expressed as percentages (mean ± SD) of total radioactivity detected in triplicate experiments. Cells (0.5-1x10^6) were incubated with 1-2x10^{-9}M labelled testosterone as precursor, in FCS free, phenol red free-RPMI medium for 72 h and the incubation medium processed as described in the Materials and Methods section (see 2.17.). ND = not detectable. For abbreviations see Table 1.

and DU145 cells maintained over 50% of labelled T unconverted, while a more pronounced formation of either DHT or 3α-diol was observed. These two 5α-reduced metabolites accounted for a considerable proportion (LNCaP, 17.3%; DU145 14.2%) of total radioactivity detected in both cell lines. On the other hand, formation of Δ^4Ad increased noticeably between 24 and 72 h in DU145 cells (from 4.1 to 13.3%) and, to a lesser extent, in LNCaP cells (from 11.6 to 14.2%), with a consequent rise of 5α-reduced

metabolites of the 17-keto series (A and EpiA). Despite this, production of androgen derivatives through the oxidative way (including Δ^4Ad, A and EpiA) never exceeded 30% of radioactive androgens in either LNCaP or DU145 cells.

In contrast, PC3 cells showed a further increase of T degradation, the proportion of unconverted precursor being only 2.9% of the total. Formation of Δ^4Ad was remarkable (46.5%), though decreased with respect to 24 h experiments (60.9%). This is not surprising in the view of the markedly enhanced conversion of Δ^4Ad to its derivatives 5α-Adione (24.8%), A (10.9%) and EpiA (14.9%). These metabolites, in fact, accounted for approximately 50% of total radioactivity detected; notably, oxidative metabolism produced over 95% of all radioactive androgens found in PC3 cells.

A negligible proportion (less than 10%) of conjugate (either sulphate or glucuronide) T was occasionally found in LNCaP cells after 72 h incubation. Conversely, as in 24 h experiments, no conjugate formation could be detected in either DU145 or PC3 cells.

6.2. Oestrogens

Enzyme activities of oestradiol metabolism in human prostate cancer cell lines were inspected through incubation of cultured cells with a physiological concentration of labelled precursor (tritiated E_2) and RP-HPLC analysis. Similarly to testosterone metabolism (see Section 6.1.), divergent patterns of

oestradiol metabolism were encountered in LNCaP, DU145 and PC3 cells.

Table 9. Oestradiol conversion rates in human prostate cancer cells: percent values of undegraded precursor (^3H-E_2) and products formed after 24 h incubation.

	^3H-E_2	E_1	16αOHE_1	2MeOE_2
LNCaP	88.5 ±2.6	10.3 ±0.8	ND	1.2 ±0.3
DU145	93.2 ±2.1	6.6 ±0.7	ND	ND
PC3	6.3 ±0.4	89.6 ±2.9	3.8 ±0.3	ND

Values are expressed as percentages (mean ± SD) of total radioactivity detected in triplicate experiments. Cells ($0.5\text{-}1x10^6$) were incubated with $1\text{-}2x10^{-9}$M labelled oestradiol as precursor, in FCS free, phenol red free-RPMI medium for 24 h and the incubation medium processed as described in the Materials and Methods section (see 2.17.). ND = not detectable. For abbreviations see Table 1.

As reported in Table 9 and respectively illustrated in Figures 46 and 47, after 24 h incubation both LNCaP and DU145 cells maintained the most part of ^3H-E_2 unconverted (respectively 88.5% and 93.2%), a relatively minor oxidation to E_1 being observed in both cell lines (10.3% in LNCaP and 6.6% in DU145 cells); additionally, LNCaP cells formed very little 2MeOE_2. In contrast, PC3 cells (see Figure 48) showed a large conversion of E_2 to E_1 (approximately 90%), with only a small part (less than

Figure 46. Typical HPLC profile of oestrogen metabolism in LNCaP cells. Cells ($1x10^6$) were incubated with $1x10^{-8}$M tritiated oestradiol for 24 h and both precursor degradation and product formation analysed through RP-HPLC and radioactive detection. Crude cpm values were: (2) E_2, 157,778; (3) $2MeOE_2$, 2,244; (4) E_1, 18,342. For abbreviations, relative retention times and peak identification number see Table 1, Oestrogens.

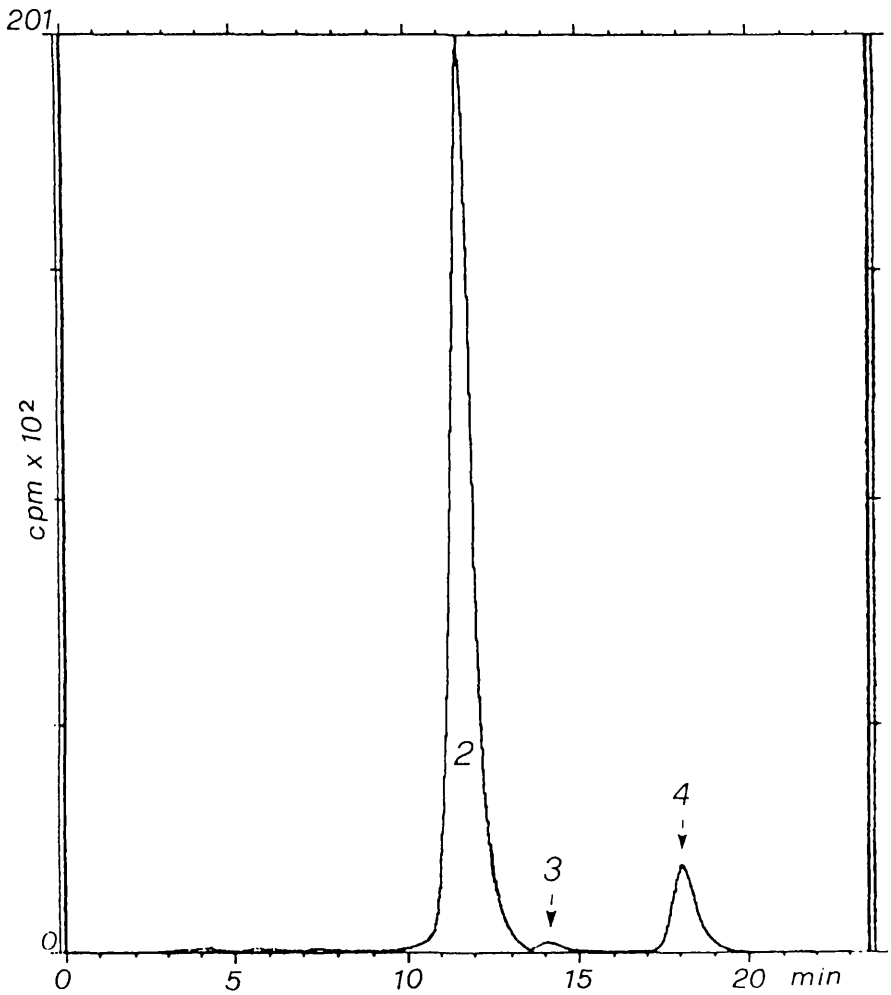

201
cpm x 10²
0
2
3
4
0
5
10
15
20
min

Figure 47. Typical HPLC profile of oestrogen metabolism in DU145 cells. Cells ($5x10^5$) were incubated with $1x10^{-9}$M tritiated oestradiol for 24 h and both precursor degradation and product formation analysed through RP-HPLC and radioactive detection. Crude cpm values were: (2) E_2, 19,387; (4) E_1, 1,275. For abbreviations, relative retention times and peak identification number see Table 1, Oestrogens.

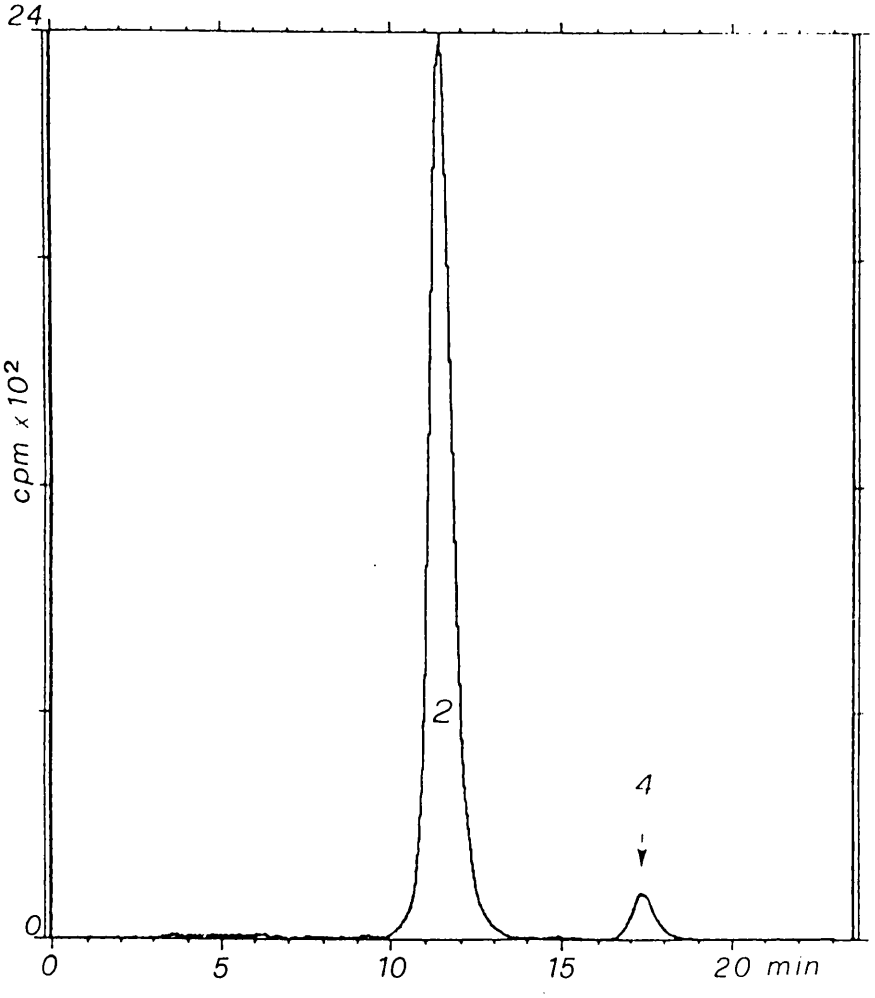
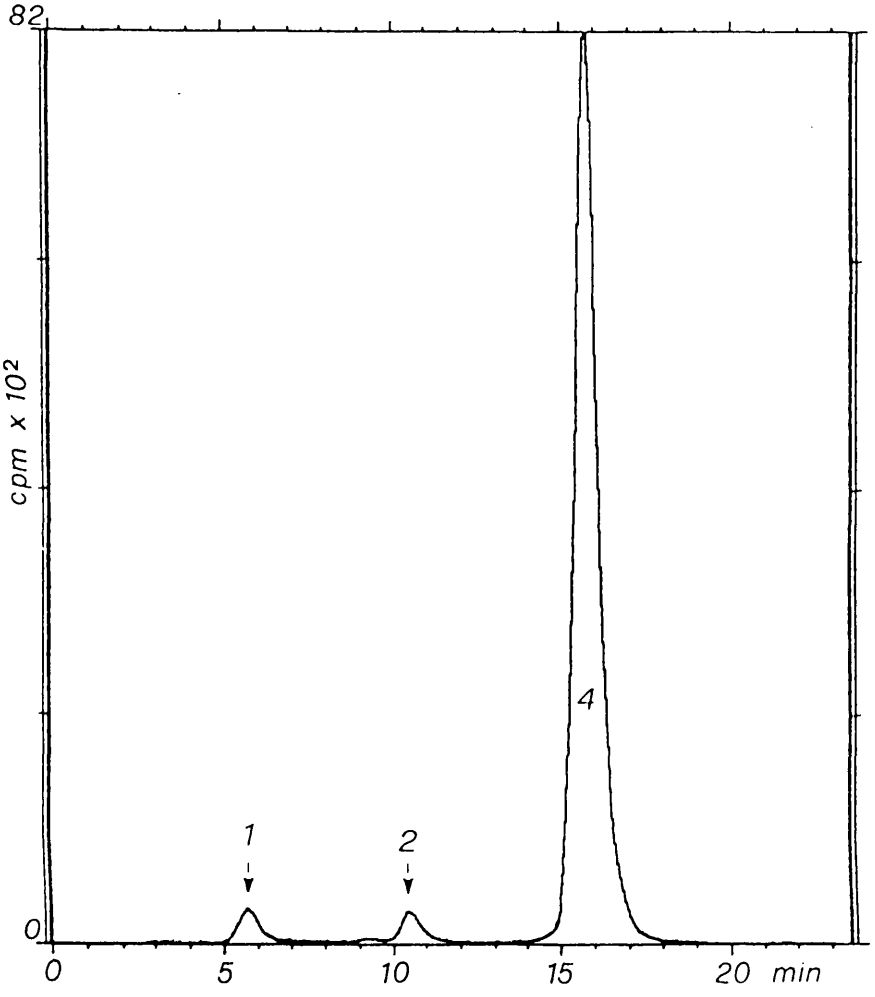

24
cpm x 10²
0
2
4
0
5
10
15
20 min

Figure 48. Typical HPLC profile of oestrogen metabolism in PC3 cells. Cells ($5x10^5$) were incubated with $5x10^{-9}$M tritiated oestradiol for 24 h and both precursor degradation and product formation analysed through RP-HPLC and radioactive detection. Crude cpm values were: (1) 16αOHE_1, 2,973; (2) E_2, 2,450; (4) E_1, 68,573. For abbreviations, relative retention times and peak identification number see Table 1, Oestrogens.


82
cpm x 10²
0
1
2
4
0
5
10
15
20 min

7%) of the precursor remaining undegraded; interestingly, an appreciable amount (3.8%) of 16αOHE$_1$ was also found in this cell line.

Results of 72 h experiments were largely in accordance to those obtained by 24 h (see Table 10).

Table 10. Oestradiol conversion rates in human prostate cancer cells: percent values of undegraded precursor (^{3}H-E$_2$) and products formed after 72 h incubation.

	^{3}H-E$_2$	E$_1$	16αOHE$_1$
LNCaP	70.6 ±1.8	28.9 ±1.2	ND
DU145	85.1 ±1.1	14.5 ±0.9	ND
PC3	3.6 ±0.7	85.6 ±1.6	10.5 ±0.8

Values are expressed as percentages (mean ± SD) of total radioactivity detected in triplicate experiments. Cells (0.5-1x10^6) were incubated with 1-2x10^{-9}M labelled oestradiol as precursor, in FCS free, phenol red free-RPMI medium for 72 h and the incubation medium processed as described in the Materials and Methods section (see 2.17.). ND = not detectable. For abbreviations see Table 1.

Although, as expected, the extent of E$_1$ formation increased in both LNCaP and DU145 cells (28.9% in the former and 14.5% in the latter), the most part of ^{3}H-E$_2$ remained unconverted (respectively 70.6% and 85.1%). Conversely, PC3 cells showed a further precursor degradation, the proportion of remaining ^{3}H-E$_2$

being only 3.6%; nonetheless, the proportion of the E_1 produced did not change significantly (85.6% vs 89.6% at 24 h), because of the marked increase in $16\alpha OHE_1$ formation (up to 10.5% of the total).

7. MODULATION OF STEROID METABOLISM BY TGFα AND TGFβ

7.1. Testosterone Metabolism

The influence of TGFα and $TGF\beta_1$ on testosterone metabolism in prostate cancer cells was initially investigated through simultaneous addition of labelled T and a selected concentration of the relevant growth factor and incubation for a 72 h interval.

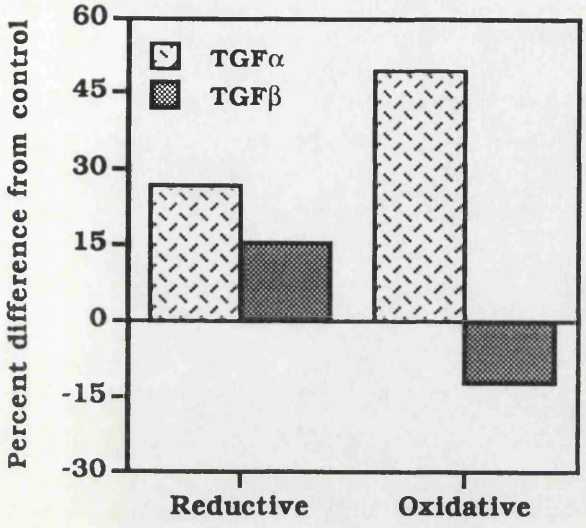
In LNCaP cells (see Figure 49, bottom), $TGF\beta_1$ did not significantly affect the proportion on unconverted T, whilst TGFα reduced it by more than 20%. Both growth factors produced an increase of both DHT and 3α-diol formation (reductive metabolism), which was approximately 3-fold greater for TGFα (35.1% for DHT and 12.5% for 3α-diol). On the other hand, a noticeable rise of both Δ^4Ad (21.4%) and its derivatives, including A and EpiA (over 80% as sum), was seen following TGFα administration; by contrast, $TGF\beta_1$ induced a decrease of these 17-keto metabolites that, however, never exceeded 15% from control. Overall, comparison of reductive (DHT plus 3α-diol) and oxidative (Δ^4Ad and A+EpiA) metabolism in LNCaP cells (see

Figure 49, top) indicated that both TGFα and TGFβ$_1$ caused an extension of the reductive pathways, which was greater for TGFα (26.7%) than for TGFβ$_1$ (15.4%); conversely, TGFα induced a remarkable increase (nearly 50%) of the oxidative metabolism, while TGFβ$_1$ slightly reduced formation of 17keto androgens (12.2% less than control).

The DU145 cells showed a different picture (see Figure 50, bottom). In fact, although neither growth factor substantially modified the extent of T degradation, both TGFα and TGFβ$_1$ produced a reduction, though not significant, of DHT formation (respectively 5.9% and 10.1%). Conversely, production of Δ^4A d was significantly stimulated (respectively 107.3% and 51.7%) by either factor, while a much less pronounced increase (15.7%) of A+EpiA was found with TGFβ$_1$ only. Overall, reductive metabolism was substantially decreased by TGFα (23.5% from control) and only slightly reduced by TGFβ$_1$ (6.2%), while both growth factors caused an equivalent increase (around 26%) of the oxidative metabolism (see Figure 50, top).

Testosterone metabolism was minimally affected by either TGFα or TGFβ$_1$ in PC3 cells (see Figure 51, bottom). The 21% decrease of unconverted T observed with TGFα is of minor importance since in this cell line the proportion of unmetabolised precursor after 72 h incubation is very small and, therefore, even little variations in T conversion rates may result in apparently significant differences of T degradation. Apart from a quite limited increase seen in Δ^4Ad formation with both TGFα and TGFβ$_1$, the oxidative metabolism in PC3 cells was unaffected by

Figure 49. Effects of TGFα and TGFβ$_1$ on androgen metabolism in LNCaP cells. Selected doses of TGFα (50 ng/ml) and TGFβ$_1$ (5 ng/ml) were added to experimental medium containing 1x10^{-8}M tritiated testosterone and cells (1x10^6) incubated for 72 h. Rates and direction of metabolism were subsequently assessed through RP-HPLC and radioactive detection. The diagram at bottom shows the percent of reaction for any single metabolite detected with respect to control (0). The chart at the top illustrates the overall influence of either growth factor on both reductive and oxidative pathways of androgen metabolism, respectively expressed as the sum of (DHT+3α-diol) and of (Δ^4Ad+EpiA+A).


60
45
30
15
0
-15
-30
Percent difference from control
TGFα
TGFβ
Reductive
Oxidative

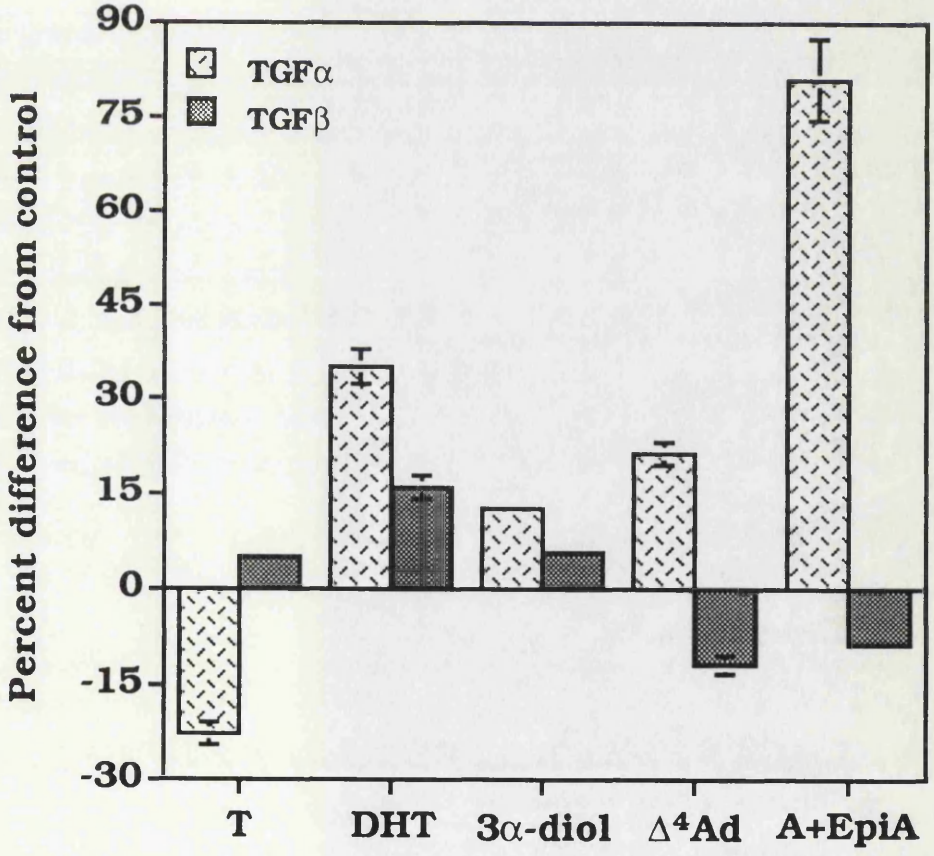
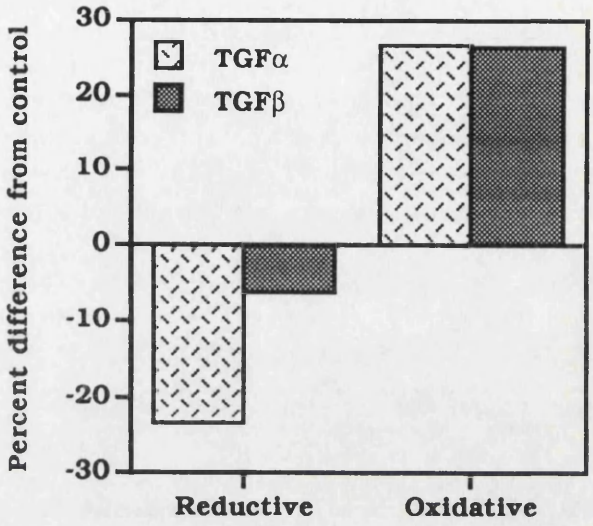

90
75
60
45
30
15
0
-15
-30
Percent difference from control
TGFα
TGFβ
T
DHT
3α-diol
Δ4Ad
A+EpiA

Figure 50. Effects of TGFα and TGFβ_1 on androgen metabolism in DU145 cells. Selected doses of TGFα (50 ng/ml) and TGFβ_1 (5 ng/ml) were added to experimental medium containing $1x10^{-8}$M tritiated testosterone and cells ($1x10^6$) incubated for 72 h. Rates and direction of metabolism were subsequently assessed through RP-HPLC and radioactive detection. The diagram at bottom shows the percent of reaction for any single metabolite detected with respect to control (0). The chart at the top illustrates the overall influence of either growth factor on both reductive and oxidative pathways of androgen metabolism, respectively expressed as the sum of (DHT+3α-diol) and of (Δ^4Ad+EpiA+A).


Percent difference from control
30
20
10
0
-10
-20
-30
TGFα
TGFβ
Reductive
Oxidative

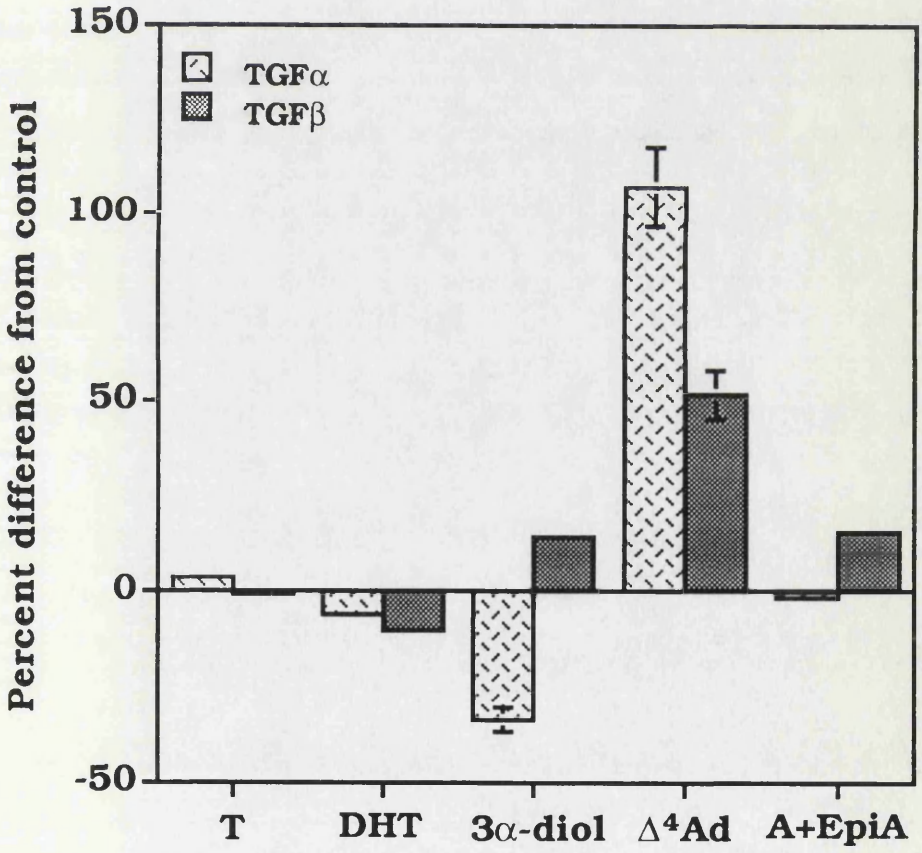
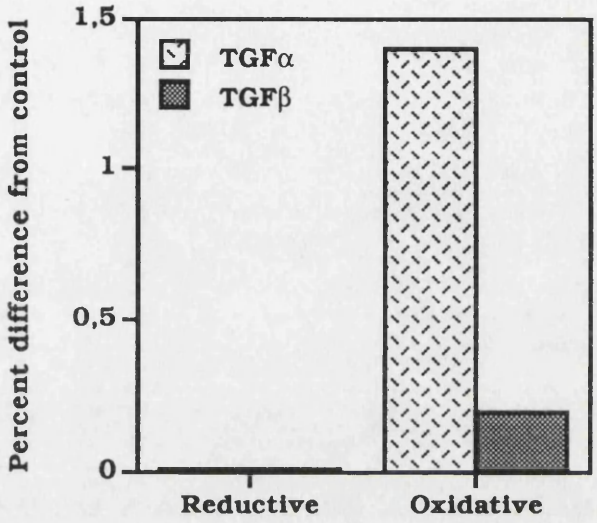
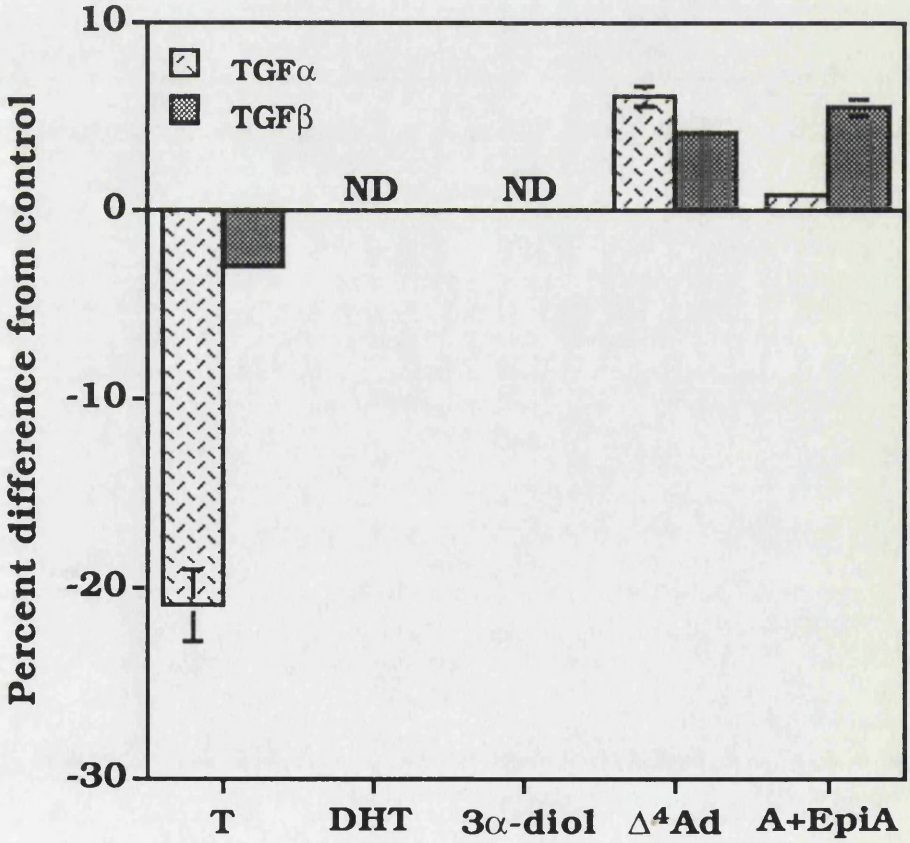

Percent difference from control
150
100
50
0
-50
TGFα
TGFβ
T
DHT
3α-diol
Δ4Ad
A+EpiA

Figure 51. Effects of TGFα and TGFβ$_1$ on androgen metabolism in PC3 cells. Selected doses of TGFα (50 ng/ml) and TGFβ$_1$ (5 ng/ml) were added to experimental medium containing 1x10^{-8}M tritiated testosterone and cells (1x10^6) incubated for 72 h. Rates and direction of metabolism were subsequently assessed through RP-HPLC and radioactive detection. The diagram at bottom shows the percent of reaction for any single metabolite detected with respect to control (0); ND = not detectable. The chart at the top illustrates the overall influence of either growth factor on both reductive and oxidative pathways of androgen metabolism, respectively expressed as the sum of (DHT+3α-diol, if any) and of (Δ^4Ad+EpiA+A).


Percent difference from control
1,5
1
0,5
0
TGFα
TGFβ
Reductive
Oxidative


Percent difference from control
10
0
-10
-20
-30
TGFα
TGFβ
ND
ND
T
DHT
3α-diol
Δ4Ad
A+EpiA

either growth factor, while the reductive metabolism remained virtually absent (see Figure 51, top).

7.2. Oestradiol Metabolism

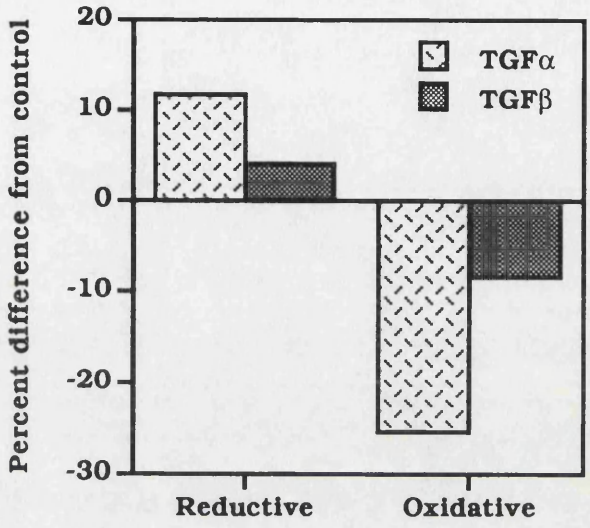
The effects of both TGFα and TGFβ$_1$ on oestradiol metabolism in prostate tumour cell lines were also compared.

In LNCaP cells, where only E_1 formation was seen after 72 h incubation, both TGFα and TGFβ$_1$ caused a slight increase of the proportion of unconverted E_2 (11.7% and 4.1%, respectively), with a proportional decrease of E_1 production (25.4% and 8.4% from control: see Figure 52).

A reverse picture was observed in DU145 cells (see Figure 53, bottom). In fact, either growth factor slightly reduced the proportion of undegraded E_2 (5.3% and 5.7% for TGFα and TGFβ$_1$, respectively). However, while TGFα induced a remarkable increase of E_1 formation (33.8%), TGFβ$_1$ caused its reduction (9.1%); interestingly, an appreciable amount of E_3 (6.2%) was found following TGFβ$_1$ administration. Consequently, the reductive metabolism (expressed as the sum of E_2 and E_3) was only somewhat altered by both TGFα and TGFβ$_1$, whilst the oxidative pathway (namely E_1 formation) was enhanced by TGFα and relatively reduced by TGFβ$_1$ (see Figure 53, top).

Although in PC3 cells, after 72 h incubation, the most part E_2 is converted to E_1, both TGFα and TGFβ$_1$ significantly augmented oestradiol oxidation in this cell line (see Figure 54, bottom). In fact, either growth factor induced a further degradation of E_2 (22.7% and 25.1% for TGFα and TGFβ$_1$,

Figure 52. Effects of TGFα and TGFβ_1 on oestrogen metabolism in LNCaP cells. Selected doses of TGFα (50 ng/ml) and TGFβ_1 (5 ng/ml) were added to experimental medium containing $1x10^{-8}$M tritiated testosterone and cells ($1x10^6$) incubated for 72 h. Rates and direction of metabolism were subsequently assessed through RP-HPLC and radioactive detection. The diagram at bottom shows the percent of reaction for any single metabolite detected with respect to control (0). The chart at the top illustrates the overall influence of either growth factor on both reductive and oxidative pathways of oestrogen metabolism, respectively expressed as the sum of unconverted E_2+2MeOE_2 and the extent of E_1 formation.


Percent difference from control
20
10
0
-10
-20
-30
TGFα
TGFβ
Reductive
Oxidative

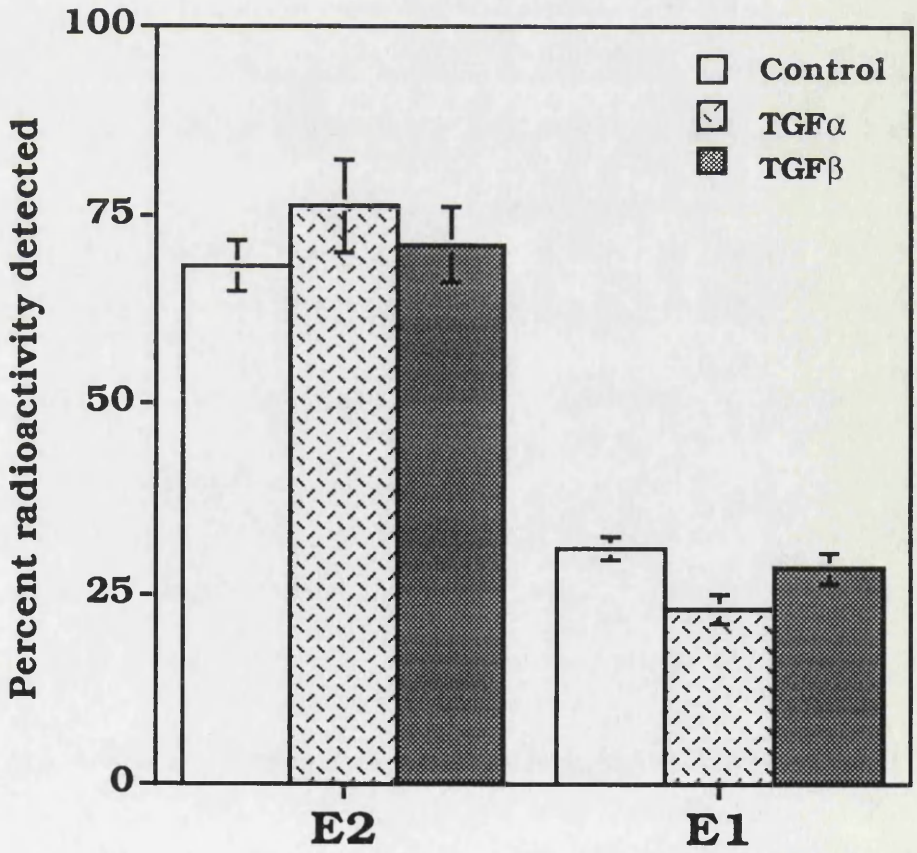
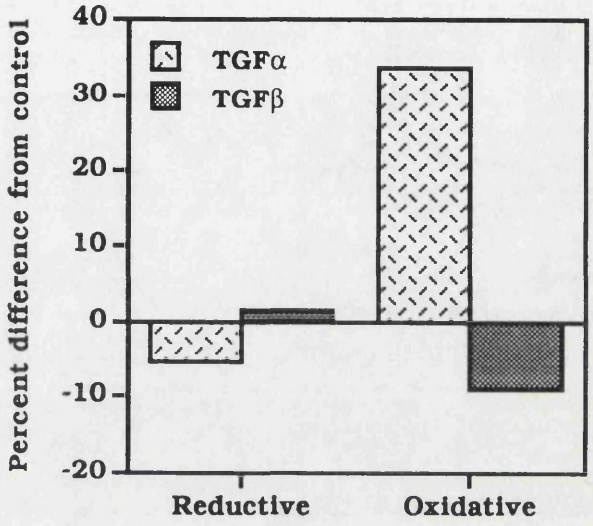

Percent radioactivity detected
100
75
50
25
0
Control
TGFα
TGFβ
E2
E1

Figure 53. Effects of TGFα and TGFβ$_1$ on oestrogen metabolism in DU145 cells. Selected doses of TGFα (50 ng/ml) and TGFβ$_1$ (5 ng/ml) were added to experimental medium containing 1x10^{-8}M tritiated testosterone and cells (1x10^6) incubated for 72 h. Rates and direction of metabolism were subsequently assessed through RP-HPLC and radioactive detection. The diagram at bottom shows the percent of reaction for any single metabolite detected with respect to control (0). The chart at the top illustrates the overall influence of either growth factor on both reductive and oxidative pathways of oestrogen metabolism, respectively expressed as the proportion of unconverted E_2 and the extent of E_1 formation.


Percent difference from control
40
30
20
10
0
-10
-20
TGFα
TGFβ
Reductive
Oxidative

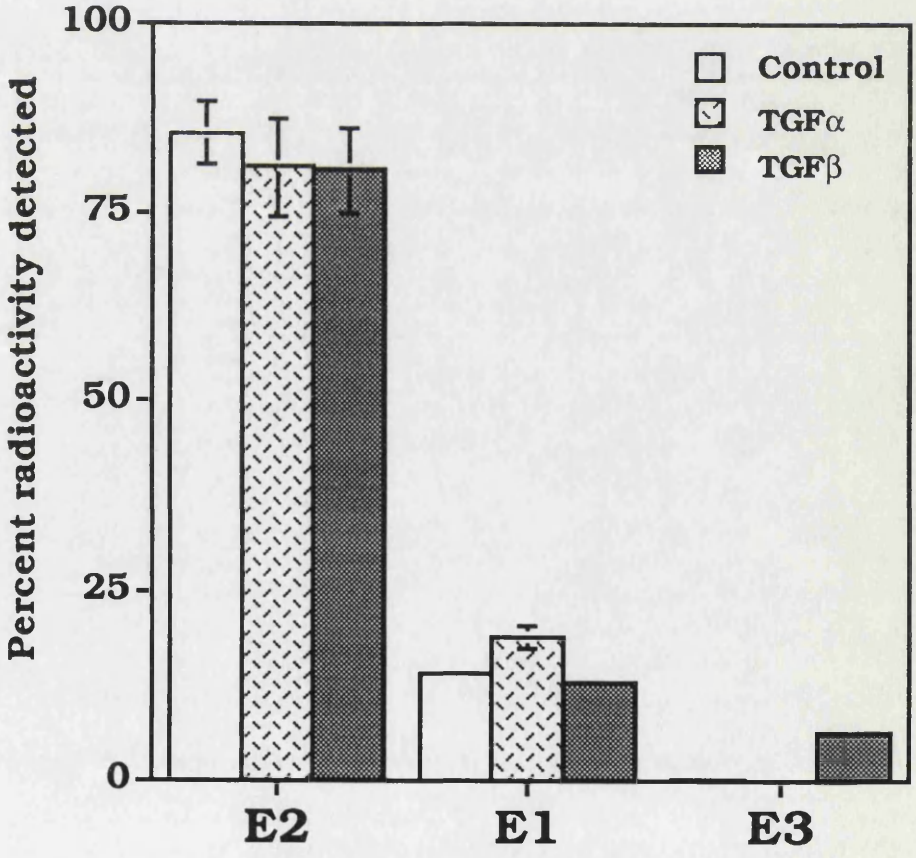
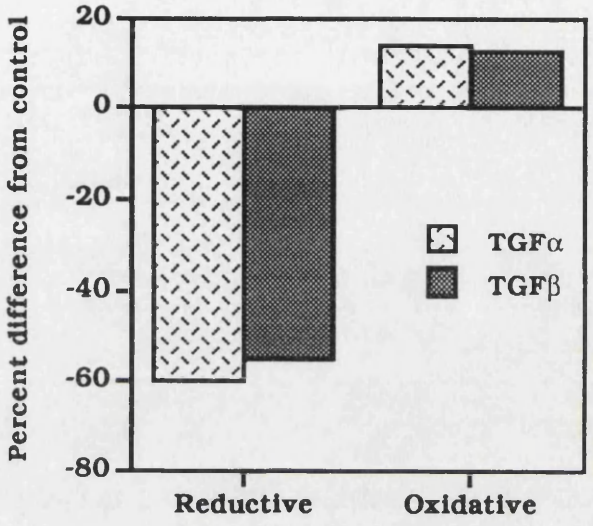
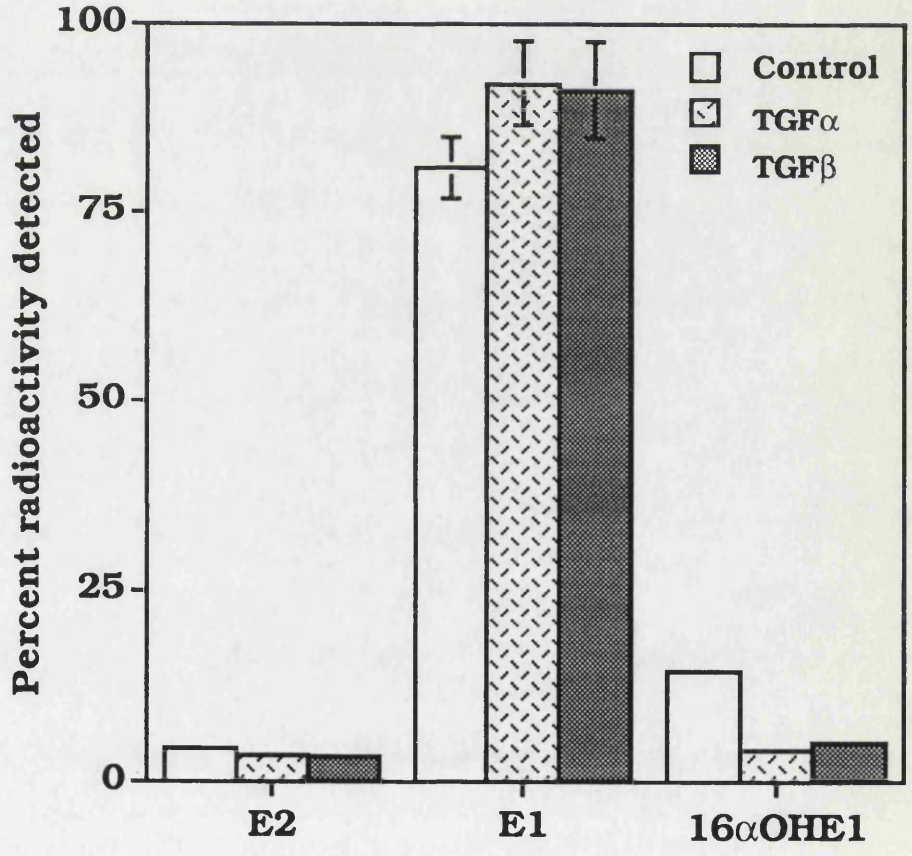

Percent radioactivity detected
100
75
50
25
0
Control
TGFα
TGFβ
E2
E1
E3

Figure 54. Effects of TGFα and TGFβ$_1$ on oestrogen metabolism in PC3 cells. Selected doses of TGFα (50 ng/ml) and TGFβ$_1$ (5 ng/ml) were added to experimental medium containing 1x10^{-8}M tritiated testosterone and cells (1x10^6) incubated for 72 h. Rates and direction of metabolism were subsequently assessed through RP-HPLC and radioactive detection. The diagram at bottom shows the percent of reaction for any single metabolite detected with respect to control (0). The chart at the top illustrates the overall influence of either growth factor on both reductive and oxidative pathways of androgen metabolism, respectively expressed as the sum of unconverted E_2+16αOHE_1 and the extent of E_1 formation.


Percent difference from control
20
0
-20
-40
-60
-80
TGFα
TGFβ
Reductive
Oxidative


Percent radioactivity detected
100
75
50
25
0
Control
TGFα
TGFβ
E2
E1
16αOHE1

respectively). This was accompanied by a parallel increase of E_1 formation (nearly 13% for both factors) and a significant decline of $16\alpha OHE_1$ production (71.3% for TGFα and 64.3% for $TGF\beta_1$). Therefore, reductive E_2 metabolism (expressed as the sum of E_2 and $16\alpha OHE_1$) was remarkably lessened by either TGFα (nearly 60%) or $TGF\beta_1$ (55.1%), while both factors yielded a correspondent increase of E_2 oxidation to E_1 (14.0% and 12.8% respectively for TGFα and $TGF\beta_1$) (see Figure 54, top).

DISCUSSION.

1. Models for Prostate Cancer Studies

As emphasised in the Introduction (see Section 5.1.1.), the incidence of human prostate cancer has been continuously increasing during the last decade, so that prostatic carcinoma has become the most prevalent neoplasm and the second principal cause of cancer death in man [Carter & Coffey, 1990]. The shortage of suitable tools for either screening or early diagnosis as well as the limited knowledge on both the aetiology and the regulation of development and growth of prostate tumour, burden this cancer with a unfavourable prognosis and leave clinicians at a loss in the management of patients.

In the past, prostate studies have been critically obstructed by two major problems: in the first place, the difficulty in obtaining suitable tissue specimens; secondly, the lack of appropriate *in vitro* models. Despite the numerous efforts made since the early 1970s, the establishment of prostate tumour cells in long-term tissue culture has been considerably rare. Initially, isolation of prostatic epithelial cell lines from primary prostate cancer was complicated by the heterogeneous composition of primary lesions and by the objective limitations in achieving physical separation and identification of the cell type(s) grown *in vitro*. This has led researchers to derive epithelial cells from metastatic tissue, where mostly tumour cells are likely to be present. Despite this, prostate cancer cell lines provided with a genuine pedigree have been rare; for instance, both the early MA160 [Fraley *et al.*, 1970] and the EB33 [Okada & Schröder,

1974] prostate tumour cells have been shown to be contaminated by the ubiquitous HeLa cervical carcinoma cells. More recently, however, the isolation of the DU145 [Stone *et al.*, 1978] and PC3 [Kaighn *et al.*, 1979] cell lines, followed by the LNCaP cell line [Horoszewicz *et al.*, 1980], has allowed investigators to use these cells as useful models respectively for hormone-independent and hormone-dependent human prostate cancer. Although data from cultured prostate epithelial cells should be considered with caution, especially because of the lack of a physiological stromal compartment, most of the present knowledge on the growth regulation of human prostate cancer has been gained with *in vitro* systems. The use of both conditioned media and transplantable prostatic tumour cell lines may partly override the intrinsic limits of these systems and provide important additional approaches to completion of the understanding of malignant prostate development and growth.

2. Steroid Function in Human Prostate Cancer Cells

2.1. Response to steroids

Several studies have explored the effects of steroid hormones on growth of cultured human epithelial prostate cells. In the present work, using stringent experimental conditions (RPMI medium supplemented with 10% DHICT-FCS), LNCaP cells show growth response to DHT (33.8% peak stimulation at 10^{-10}M, after 6 days exposure), while DU145 and PC3 prove to be

insensitive; this is in agreement to the existing literature [Stone *et al.*, 1978; Kaighn *et al.*, 1979; Horoszewicz *et al.*, 1983]. As also previously reported by others [Sonnenschein *et al.*, 1989], LNCaP cells exhibit a significant growth inhibition when exposed to higher (10^{-9} to 10^{-7}M) DHT concentrations, with a maximum decrease of over 58% at 10^{-7}M. This evidence apparently fits with the two-step mechanism designed by Sonnenschein group on the growth control exerted by androgens on LNCaP cells [Olea *et al.*, 1990]. In this model, low concentrations ($\leq 10^{-10}$M) of either androgens or other steroidal compounds (including oestrogens, progestins and antiandrogens) induce a growth stimulation of LNCaP cells through removal of a putative plasma-born inhibiting protein, called androcolyone I (step 1). At higher concentrations ($>10^{-9}$M), only androgens trigger a specific AR-mediated effect which results in a inhibition of growth of LNCaP cells (step 2). However, a recent report documented that growth of a LNCaP supersensitive subline (LNCaP-ss) in serum-free medium is significantly stimulated by both very low (10^{-16}-10^{-14}M) and higher (10^{-9}-10^{-7}M) DHT concentrations [Kirschenbaum *et al.*, 1993].

Under the same experimental conditions, growth of LNCaP human prostatic epithelial cancer cells is significantly stimulated by a physiological concentration (1 nM) of oestradiol, while it is significantly inhibited at a higher concentration (100 nM) (see Figure 30). Contrary to what observed with androgens, where this biphasic behaviour has been repeatedly reported by others [Berns *et al.*, 1986; Sonnenschein *et al.*, 1989], this inhibition is

here reported for the first time. These data confirm previous studies in that the oestradiol-induced increase of LNCaP proliferation is comparable or even greater than that produced by androgens [Schuurmans *et al.*, 1988a; Sonnenschein *et al.*, 1989; Iguchi *et al.*, 1990]. Conversely, growth of DU145 cells is minimally affected by the addition of any E_2 concentration.

Interestingly, E_2 shows a clear inhibitory effect on growth of human prostate cancer PC3 cells; this is true also at lower concentration, within physiological range (see Figure 30 and 31A). At higher doses (1 nM or more) this effect becomes evident before 6-days exposure. Again, the data in this thesis are the first to show that E_2 inhibits growth of epithelial prostate cancer cells is provided; this may represent an alternative explanation for the efficacy of the oestrogen therapy in advanced prostatic cancer, wherein androgen-independent tumour cells are likely to be largely prevalent. Since the early 1940s, the high response rates (70-80%) achieved in prostate cancer patients using a synthetic oestrogen, the orally active DES, have been ascribed to the fact that, after DES administration, circulating testosterone falls to the levels found in castrates [Turkes *et al.*, 1988]. Recent experimental data have, however, emphasised that intravenous stilbestrol diphosphate, and oestrogens in general, may exert a direct cytotoxic effect on prostate tumours which could explain the response to oestrogens seen in patients having metastatic and otherwise hormone-refractory disease [Ferro, 1991].

2.2. Steroid receptors

Presence of binding sites for both androgens and oestrogens in LNCaP, DU145 and PC3 cells has been investigated using multiple approaches.

In the first place, radioligand binding assay reveals that both type I (high affinity, limited capacity) and type II (lower affinity, greater capacity) sites of androgen binding are present in soluble and nuclear cell fractions of LNCaP and DU145 cells, while PC3 cells exhibit only nuclear type I AR (see Table 2). Based on previous experience by others [Thorsen, 1979; Leake & Habib, 1987] and our own group [Castagnetta *et al.*, 1983; Castagnetta *et al.*, 1987b; Castagnetta *et al.*, 1992a], the absence of type I receptors from one cell fraction is likely to denote an impaired mechanism of action of steroids. Recently, it has been observed that nuclear type I AR are widespread in human prostate cancer tissues and therefore their potential value as predictors of both prognosis and response to endocrine treatment of patients is questionable; the same applies to type II AR, that are almost ubiquitously distributed in either cell fraction [Castagnetta *et al.*, 1991b; Castagnetta *et al.*, 1992a].

Preliminary data obtained using the reverse transcriptase-PCR (RT-PCR) system support the results of biochemical assay of AR. In fact, using this approach, a portion of exon 1 of the human AR gene is amplifiable in LNCaP, DU145 and PC3 cells (See Figure 22); this evidence is strongly suggestive of the presence of AR mRNA in all three cell lines. Assuming that the same amount of template target within the total RNA extracted from cells gives

rise to equivalent amplification and synthesis of cDNA in the three cell lines, the LNCaP cells yield the greatest PCR product, with DU145 and PC3 cells respectively having intermediate and lower levels.

While androgen-responsive LNCaP cells are universally recognised as AR-endowed, though with a point-mutated form of AR, conflicting data in the literature exist on the presence of AR in androgen independent human prostate cancer cells. Although radioligand binding assay has been frequently used to measure AR content of cultured prostate tumour cells, data concerning DU145 and PC3 cells are surprisingly sparse. In this study, following the original observation of Clark and Peck for oestrogen receptors [Clark & Peck, 1979], use of ligand concentrations ranging from 0.1 up to 5 nM allows definition of an experimental window which covers both type I and at least part of type II receptors and therefore enables a proper study of the heterogeneity of steroid binding sites. This is of primary concern, since clinical data strongly support the view that type I receptors are essential intermediaries of steroid hormone action, while the potential role of type II binding sites remains unclear [Barnes *et al.*, 1979; Leake *et al.*, 1981; Castagnetta *et al.*, 1989b]. Overall, apart from one report [Veldscholte *et al.*, 1990a], most studies have used a ligand concentration range (from 1 up to 25 nM) such that mainly type II (low affinity, greater capacity) AR are highly likely to be identified [Horoszewicz *et al.*, 1983; Berns *et al.*, 1986; Sonnenschein *et al.*, 1989; Olea *et al.*, 1990]. This inconsistency is also reflected in the higher K_d and concentrations

values of cytosolic AR commonly obtained in LNCaP cells (ranges: K_d, 0.9-3.2 nM; concentration, 67-920 fmol/mg protein) as opposed to those herein reported for type I receptors (ranges: K_d, 0.23-0.41 nM; concentration 11.3-24.1 fmol/mg protein).

Previous work suggested that either DU145 or PC3 cells lack AR mRNA or protein, although no alteration in the structure of androgen receptor gene could be detected [Tilley *et al.*, 1990; Trapman *et al.*, 1990]. However, recent studies have documented that normal AR mRNA is present, though at low levels, in PC3 cells [Culig *et al.*, 1993]. In addition, Brolin and colleagues [Brolin *et al.*, 1992], comparing cytochemical and biochemical AR assays in prostate cancer cell lines, found that, using immunohistochemistry, the majority (75%) of DU145 cells was AR positive, although they failed to reveal detectable AR sites with biochemical techniques.

Soluble and nuclear ER content of prostate tumour cells has been investigated by means of radioligand binding assay. Both type I and II ER are present in both cell fractions of LNCaP cells; conversely, PC3 cells show presence of both receptor types in the nuclear fraction only, while the soluble compartment does not display any detectable site of oestrogen binding (see Tables 3 and 4). The DU145 cells apparently lack both cytosolic and nuclear ER.

Several studies have investigated the steroid receptor content of LNCaP cells using the radioligand binding assay method. Early work by Horoszewicz and colleagues [Horoszewicz *et al.*, 1983] revealed presence of cytosolic oestrogen receptors in

LNCaP cells. Some inconsistency, however, emerged from further studies which failed to detect ER from either nuclear extract or cytosol fraction of LNCaP cells [Berns *et al.*, 1986; Sonnenschein *et al.*, 1989]. For this reason, the oestradiol-induced increase of LNCaP proliferation has been for years ascribed to the presence of a point-mutated form of androgen receptor, having an increased affinity for progestagenic and oestrogenic steroids [Veldscholte *et al.*, 1990a]. In fact, the relative binding affinity (RBA) observed for oestradiol ranged from 0.9 up to 4.3% when compared to that of the synthetic androgen R1881 [Schuurmans *et al.*, 1988a; Sonnenschein *et al.*, 1989; Veldscholte *et al.*, 1990a; Veldscholte *et al.*, 1990b]. Thus, we are facing a seeming paradox: the very low RBA values reported for oestradiol could not, in fact, match its remarkable effect on growth of LNCaP cells. The present data clearly indicate that both type I (high affinity, limited capacity) and type II (lower affinity, greater capacity) oestrogen binding proteins are present in both cell fractions of LNCaP cells and that their levels are comparable to those found in other oestrogen-responsive cancer tissues [Castagnetta *et al.*, 1987a; Castagnetta *et al.*, 1987b] and cells [Lo Casto *et al.*, 1983; Castagnetta *et al.*, 1986b]. Besides, our results are consistent with the original definition of biochemical and functional features relevant to distinct sites of oestrogen binding [Clark & Peck, 1979] and, more, with the discriminant value of type I ER in both breast and endometrial cancer patients [Castagnetta *et al.*, 1987a; Castagnetta *et al.*, 1992b]. It must be pointed out that most of previous studies used higher ligand concentrations (from 2 up to

20 nM) [Sonnenschein *et al.*, 1989], even as single point assay [Berns *et al.*, 1986], so selecting an experimental window which does not allow quantitation of high affinity, low capacity binding sites.

The expression of ER at the mRNA level in prostate cancer cells has been determined using the highly sensitive RT-PCR; the combined Southern blot analysis enhances the sensitivity and confirms the identity of the amplification products. Using this approach, expression of a normal ER mRNA in LNCaP cells and, though in far lower amounts, in PC3 cells is illustrated here for the first time (see Experimental, Section 2.2.3.; see Figure 27); in contrast, no detectable amplification product(s) is seen in DU145 cells. Furthermore, a rather abundant expression of a variant ER mRNA lacking the entire exon 4 is also observed in LNCaP and at much lower extent in PC3 cells. This variant has been previously characterised in our laboratories from oestrogen responsive mammary carcinoma cell lines, suggesting that it is a likely product of alternative splicing [Pfeffer *et al.*, 1993]. It is noteworthy that this variant is jointly expressed with the normal messenger only in ER positive, oestrogen-responsive MCF7 and ZR75-1 mammary carcinoma cell lines, whilst both mRNAs are absent from ER negative, non responsive MDA-MB231 cells. Although RT-PCR does not allow an accurate estimation of the relative amount of the ER transcripts, it can be deduced that equivalent levels of expression of both normal and variant ER mRNA are present in the two oestrogen-responsive mammary carcinoma cell lines and in LNCaP cells, whilst PC3 cells show

much lower amounts of both the amplification products. This may account for the apparent lack of ER in the soluble fraction of the latter cell line using biochemical assay. However, the fact that no reaction product could be observed in the oestrogen non-responsive human breast cancer cell line MDA-MB231 under the same conditions, clearly indicates that the expression of the ER mRNAs in PC3 cells is significant. It is worth mentioning that the amplification primers (24-mers) used show 9 and 10 mismatches in the androgen receptor sequence and, if they were to give rise to any amplification product from that sequence, it should not be of the observed length.

Immunocytochemical studies reveal intensive staining of LNCaP cells for both ER and PgR (see Figure 25). The latter is commonly thought to be an index of a functional oestrogen receptor machinery in human breast and endometrial cells, so that clinicians have been using it as a helpful discriminant in the management of breast cancer patients. On the other hand, ER staining was much lower, though appreciable, in androgen non responsive PC3 cells and absent in DU145 cells. These results seem at variance with a previous study by Brolin and colleagues [Brolin *et al.*, 1992], where, using the same immunocytochemical assay, both LNCaP and PC3 cells were found to be ER and PgR-negative. However, this apparent discrepancy could simply be ascribed to the different conditions used for exposure to primary antibodies (1 h at 37°C as opposed to 24 h at 4°C in the present study).

Presence of functional ER in human prostate cancer cells was also inspected through immunofluorescence of the 27 kDa hsp27, which has been reported to be a marker of oestrogen sensitivity in both breast and endometrial epithelial cells [King *et al.*, 1987]. While LNCaP cells show an intensive hsp27 staining (see Figure 28, top), DU145 cells appear to be hsp27-negative. A positive staining for hsp27, though less intense than in LNCaP cells, is documented in PC3 cells (see Figure 28, bottom). This seemingly conflicts with the very low expression of both ER protein and transcript in this cell line. Hsp27 appears to be qualitatively and quantitatively related to ER in several human tissues [King *et al.*, 1987]. A linear correlation between ER content and hsp27 levels has been reported in human breast tumours, although ER-poor tissues too exhibited a variable degree of hsp27 staining [Cano *et al.*, 1986]. In addition, although the relationship between ER and hsp27 has been established within individual target tissues, it may be different in prostate.

2.3. Mechanisms of oestrogen action

To ascertain whether or not the E_2-induced increase of growth seen in LNCaP cells is mediated via ER, cells were exposed to various E_2 concentrations in presence or absence of the pure antioestrogen ICI-182,780. The evidence that the growth response of LNCaP to oestradiol is completely abolished by addition of ICI-182 (see Figure 32) strongly supports the view that oestradiol acts via its own receptor. This finding is in accord to previous observation by Labrie's group, where the pure

antioestrogen EM-139 not only reversed the androgen-induced increase of LNCaP proliferative activity, but also significantly inhibited basal cell growth [De Launoit *et al.*, 1991]. There is an apparent discrepancy between growth data from DNA and thymidine uptake assays, in that, using 10% DHICT-FCS, only 1 nM E_2 noticeably increased growth of LNCaP cells, whilst cells grown in the presence of 5% CT-FCS were consistently stimulated by any E_2 dose (from 10^{-11} up to 10^{-7}M). This uneven result is bound to the different experimental conditions used and could be ascribed to the presence in stripped serum of as yet unidentified factor(s) which is dyalized off in DHICT-FCS.

The possibility that the E_2-induced growth inhibition of PC3 cells is mediated by TGFβ has been investigated. Addition of neutralising anti-$TGF\beta_1$ antibody in stringent experimental conditions provokes a remarkable increase (close to 300% of control: see Figure 31B) of cell proliferation, revealing the presence of high levels of endogenous $TGF\beta_1$ in PC3 cells; oestradiol produces a reversal of this effect, which is almost complete at the 10^{-7}M dose (Figure 31B). However, evaluation of possible regulation of $TGF\beta_1$ mRNA by E_2 indicates that its expression in PC3 cells is unaffected by different doses (10^{-11} to 10^{-7}M) of E_2 (see Figure 33); this is true at any incubation time (6, 24 or 72 h) used. Previous work [Knabbe *et al.*, 1987] revealed that antioestrogens, such as TAM, do not affect $TGF\beta_1$ mRNA levels in the oestrogen-dependent MCF7 breast cancer cells, even though $TGF\beta_1$ production is increased (from 8 up to 27 fold) in this cell line after TAM treatment. Recent experimental

evidence has also indicated that E_2 does not modify levels of $TGF\beta_1$ mRNA either in ER positive breast cancer cell lines (MCF7, ZR75-1), or in ER negative cancer cells, like mammary MDA-MB231 [Arrick *et al.*, 1990] or endometrial HEC-50 [Anzai *et al.*, 1992]. It is of interest to note that these ER negative human cancer cells have been reported to be exquisitely sensitive to $TGF\beta_1$ [Arteaga *et al.*, 1988].

3. Growth Factor Studies

Recent experimental evidence has proposed a crucial role for both TGFα and TGFβ in the onset of a transformed phenotype [Goustin *et al.*, 1986]. The balance between these and other growth factors, as well as the presence of adequate functional growth factor receptors, may in turn direct the cancer cell to a quiescent or a progressive state.

Unfortunately, little is known about interaction between steroids and peptide growth factors in growth regulation of target cancer cells from human breast or prostate. In vitro studies have demonstrated that non responsive, ER negative breast cancer cells strongly differ from ER positive cells in terms of both response to and production of various growth factors, such as IGF-I, TGFα and TGFβ [Dickson *et al.*, 1986; De Leon *et al.*, 1989]. Similarly, it has been recently reported that $TGF\beta_1$ differentially affects growth of human prostate cancer cells, also depending on their steroid sensitivity [Wilding *et al.*, 1989a].

Furthermore, experimental evidence indicates that androgens and oestrogens may regulate production of peptide growth factors and expression of their receptors respectively in breast and prostate tumour cell lines [Traish & Wotiz, 1987; Lippman & Dickson, 1989].

3.1. Growth factor and growth factor receptor content

Presence of high affinity sites of EGF binding in all three cell lines studied is here documented using the radioreceptor assay (see Table 5). Overall, K_d and concentration values are in the order of those previously reported [Schuurmans *et al.*, 1988a; MacDonald & Habib, 1992]. In particular, DU145 cells exhibit EGFR concentrations higher than those found in PC3 and LNCaP cells. This is in agreement with previous report indicating that DU145 cells contain a significantly higher number of EGFR binding sites than that expressed in LNCaP cells [MacDonald & Habib, 1992]. This evidence is further supported by results of Western blot analysis, indicating that DU145 cells have the highest levels of EGFR expression, while PC3 and LNCaP cells respectively display intermediate and lower expression levels (see Figure 37).

Immunofluorescent staining revealed remarkable amounts of EGFR, TGFα and, to a lesser extent, of EGF in DU145 and LNCaP cells, whilst their levels in PC3 cells appeared to be much lower (see Table 6). On the other hand, appreciable $TGF\beta_1$ was detected in PC3 cells and, in far lower amounts, in LNCaP cells (Table 6). Recent observation has shown that DU145 cell line secretes much

greater amounts of EGF and EGF-related polypeptides into culture medium than do LNCaP cells [Connolly & Rose, 1990].

3.2. Growth factor effects on cell proliferation

TGFα and TGFβ$_1$ growth effects on prostate cancer cells were firstly assessed in routine medium after either 24 or 48 h incubation. The results presented here (see Figure 38A and B) indicate that there is a significantly different response by the different cells to both growth factors, even in short-term experiments. The PC3 cells appear to be remarkably sensitive to the action of TGFα and TGFβ$_1$, whilst growth of LNCaP cells is minimally affected by either factor. Although some studies have previously reported that TGFα stimulates growth of LNCaP cells after 6-10 days exposure [Wilding *et al.*, 1989b; Schuurmans *et al.*, 1991], no evidence of an early response of this cell line to TGFα has been thus far provided; in particular, Wilding and colleagues showed that addition of 10 or 20 ng/ml TGFα did not significantly increase the proliferative activity of LNCaP cells up to 3 days exposure [Wilding *et al.*, 1989b].

DU145 cells seem to have a peculiar behaviour. These cells respond to TGFβ$_1$, although less than PC3 cells; this confirms previous observation by Wilding and colleagues [Wilding *et al.*, 1989a]. TGFβ$_1$ effects are known to be dependent on culture conditions; however, the TGFβ data from Wilding appear comparable to those reported here in spite of the different experimental conditions used (respectively, routine IMEM

medium plus 10% charcoal-stripped, sulphatase-treated FCS vs RPMI medium plus 10% FCS in the present work).

Conflicting reports on the effects of both TGFα and EGF on growth of DU145 cells exist. Anchorage-independent assays have shown that this cell line is stimulated by TGFα [Fernandez-Pol *et al.*, 1986]; further, a mitogenic effect of EGF on DU145 cells has been recently observed [Connolly & Rose, 1991]. In contrast, other groups have found that both EGF and TGFα have very little effect on either DNA synthesis or tritiated thymidine incorporation by DU145 cells [MacDonald *et al.*, 1990]. In the present study, after short-term incubation with increasing TGFα concentrations in routine culture medium, DU145 cells do not exhibit any significant growth stimulation (see Figure 38A).

In a separate set of experiments the growth effects of both TGFα (10 and 50 ng/ml) and TGFβ$_1$ (1 and 5 ng/ml) were investigated after 48 h and 6 days exposure using DHICT-FCS supplemented RPMI medium (see Experimental, Section 5.1.; Figures 39 and 40).

Surprisingly, while neither growth factor changes the proliferative activity of LNCaP cells, TGFα (50 ng/ml) produces a decrease (25%) of growth of DU145 cells after 6 days. More, in PC3 cells, comparison of results from both 48 h and 6 days experiments with those obtained using routine medium shows that the effects of TGFα are completely abolished and that those of TGFβ$_1$ are markedly (about 50%) lessened.

This unexpected evidence may be ascribed to the use of DHICT-FCS in this second set of experiments; since the molecular

weight cut-off used for dialysis is of 12 kDa, it is reasonable to assume that presence of some as yet unidentified factor, which is lost during dialysis, is required for a complete accomplishment of both TGFα and $TGF\beta_1$ action. In particular, EGF and IGF-I appear to be likely candidates, since both would be readily removed from FCS by dialysis. Recent studies have shown that removal of EGF from the culture medium markedly reduces the growth inhibition induced by administration of $TGF\beta_1$ to cultured benign human prostatic epithelial cells [Sutkowski *et al.*, 1992]. On the other hand, it is recognised that both EGF and TGFα can act in synergy with other growth factors [Rozengurt, 1986]; in this respect, the absence of IGF-I, which is known to behave as a "progression factor" during the cell cycle, may well impede completion of the growth stimulatory effects induced by TGFα or EGF. This assumption is also supported by the recent evidence that IGF-I significantly stimulates DNA sytnthesis in both DU145 and PC3 cells, while growth of LNCaP cells appears to be unchanged [Iwamura *et al.*, 1993]. Therefore, the lack of IGF-I from the culture medium could result in a noticeable decrease of basic proliferative activity of the above androgen-independent cells and/or seriously affect growth response of these cells to either TGFα or $TGF\beta_1$.

4. Steroid Metabolism

4.1. Androgens

Multiple sequential analysis of enzymes of testosterone metabolism in LNCaP, DU145 and PC3 human prostate cancer cells has been carried out using a novel chromatographic approach [Castagnetta *et al.*, 1991a]. The latter allows measurement of both precursor degradation and rates of produtc formation in intact cultured cells, using physiological concentrations of radioactive steroids. Two main distinct patterns of testosterone metabolism are observed. Androgen receptor positive LNCaP and DU145 cells exhibit little oxidation of T to Δ^4Ad through the 17βHSD enzyme and a relatively high 5α-reductase (T--->DHT) activity; by contrast, androgen receptor negative, hormone unresponsive PC3 cells display extensive 17βHSD oxidation to yield Δ^4Ad and subsequent production of the 5α-reduced derivatives of the 17-keto series, while no 5α-reduction of T to DHT can be detected (see Tables 7 and 8). The latter finding is noteworthy. Conversion of T by the 17βHSD activity in PC3 cells is remarkably shifted in favour of Δ^4Ad production (oxidative way), with a consequent formation of 5α-Adione. Although the possibility that DHT is quickly oxidised to 5α-Adione via 17βHSD can not be excluded, this seems unlikely since that neither short nor longer incubation times reveal measurable DHT formation in this cell line (see Figure 45). Additionally, any production of DHT from T is also doubtful

because no evidence of DHT derivatives, such as 3α/3β-diols, can be obtained at any incubation time.

There is overall consensus that 5α-reductase activity is distinctly decreased in human prostatic carcinoma with respect to both normal and hyperplastic tissues [Bartsch *et al.*, 1990; Klein *et al.*, 1991]. Surprisingly, in spite of the decrease of 5α-reductase, these studies have found unchanged or even higher DHT concentrations in malignant with respect to benign prostate tissues, together with significantly higher intratumour testosterone levels than in nonmalignant prostate. This finding has been interpreted as a consequence of DHT "trapping" in nuclei of prostate cancer cells through binding to intact androgen receptors [Klein *et al.*, 1991]. This assumption, however, is rather unlikely since both the detected DHT concentrations (in the order of pmol/mg DNA) and the assessed 5α-reductase levels (in the order of nmol/h/mg DNA) are far exceeding the AR binding capacity (in the order of fmol/mg DNA).

It ought to be emphasized that these classical enzymology studies use artificial environments (cell lysates, pH and temperature, excess of substrate concentration, cofactors addition) and are optimized to yield the maximum conversion rates for quantitation of a single enzyme activity. Therefore, this approach does not allow inspection of enzyme activity in intact cells and hence enzymology data are highly unlikely to resemble the *in vivo* condition. The present "live analysis" HPLC system, which allows quantitation of both undegraded substrate and formation of several metabolic products, may encompass the

artifacts of classically measured enzymic reactions but, above all, may yield direct information about the metabolic direction in individual tissues and cells. The present data provide a likely explanation of the previously reported accumulation of both T and DHT in hormone-responsive prostate tumours [Geller & Albert, 1985]. In fact, in androgen-responsive LNCaP cells, activities of either 17βHSD or 5α-reductase enzymes are directed in such a way that both T and DHT accumulate. Conversely, unresponsive PC3 cells favour oxidative degradation of T to Δ^4Ad and its 5α-reduced derivative 5α-Adione, consequently removing the proper 5α-reductase substrate for DHT production. DU145 cells behave peculiarly. Like LNCaP, DU145 cells metabolise poorly T and form appreciable DHT but, conversely, they do not produce conjugate androgens, as occurs in PC3 cells. The different amount of DHT produced by LNCaP and DU145 cells may be accounted for by the remarkable levels of androgen conjugates (mostly testosterone) present in LNCaP cells, which eventually take testosterone away from any further metabolism.

The situation is further complicated by the fact that human prostate might contain several types of 5α-reductase enzyme [Rennie *et al.*, 1983; Hudson, 1987]. More importantly, Martini and associates [Martini *et al.*, 1990] demonstrated that the rat prostate contains two distinct 5α-reductase isoenzymes, having differential sensitivity to both ageing and 4-hydroxy-4-androstene-3,17-dione (4OH-A), an inhibitor of both 5α-reductase and aromatase prostatic enzymes. The two 5α-reductases individually preside over formation of the 5α-reduced

metabolites of the 17-OH series (DHT and 3α/3β-diols) and the 17-keto series. This evidence comes in support of the present data suggesting that these two activities may be differently expressed and/or regulated in human prostate cancer cell lines.

4.2. Oestrogens

Rates and direction of oestradiol metabolism in LNCaP, DU145 and PC3 human prostate cancer cells have been investigated using the same RP-HPLC approach employed to analyse enzyme activities of testosterone metabolism in these systems.

Two opposite patterns of oestradiol metabolism are encountered. Androgen receptor positive, hormone responsive LNCaP and unresponsive DU145 cells display little degradation of labelled E_2 and a relatively limited oxidation to E_1; by contrast, androgen receptor negative, androgen unresponsive PC3 cells show extensive conversion of E_2 to yield E_1 and an appreciable formation of 16αOHE_1. This is true at either 24 or 72 h incubation (see Tables 9 and 10). Overall, the reductive metabolism appears to be highly prevalent in both LNCaP and DU145 cells and eventually leads to E_2 accumulation; in contrast, the oxidative pathway, represented by E_1 production, is clearly predominant in PC3 cells. This picture is peculiarly cognate to that observed with androgens, where oxidation of T to yield Δ^4Ad and its 17keto derivatives is particularly evident in PC3 cells, while LNCaP and DU145 cells metabolise T only slowly, producing a relatively high

proportion of metabolites (DHT and 3α-diol) through the reductive pathway.

Androgen metabolism and content of both benign and malignant human prostate tissues have been extensively compared by others. However, data in the literature concerning metabolic patterns of oestrogens in prostate tissues and cells are surprisingly rare [Robinette *et al.*, 1978]. This is the first report describing the ability of cultured human prostate tumour cells to actively metabolise oestrogens in physiological environments. Previous studies indicated that prostatic tissues are endowed with enzymes of oestrogen metabolism. Early investigations revealed that BPH tissues may readily hydrolyse E_1S to E_1 [Carlström *et al.*, 1980]; in addition, presence of 17βHSD activity was documented in both normal [Acevedo & Goldzieher, 1965a] and diseased [Acevedo & Goldzieher, 1965b] human prostate gland. Therefore, regulation of the oestrogen milieu in the prostate may well involve cleavage of E_1S to E_1 and the subsequent 17β-reduction to yield E_2. In a more recent report, Orlowski and Clark [Orlowski & Clark, 1988] examined oestrogen metabolism in primary cultures of rat ventral epithelial and stromal cells using HPLC approach. The authors found that epithelial cells exhibit an almost equivalent reductive and oxidative 17βHSD activity, whilst stromal cells preferentially form and maintain high levels of biologically active E_2.

The present results are noteworthy in that they clearly suggest that pathways of oestrogen and androgen metabolism are consistently and differently regulated in human prostate

carcinoma cell lines according to whether they are hormone-sensitive.

5. Effects of Transforming Growth Factors on Metabolism of Steroid Hormones

5.1. Testosterone metabolism

The influence of transforming growth factors on both rates and direction of testosterone metabolism in human prostate cancer cells has been investigated.

Effects of either TGFα or $TGF\beta_1$ vary markedly depending on the cell line studied (see Figures 49 to 51). While TGFα enhances the oxidative pathways in both LNCaP and DU145 cells, it has opposite effects on the reductive metabolism of testosterone in these two cell lines. Therefore, in LNCaP cells TGFα seems to act as a stimulator of the overall testosterone metabolism, whilst in DU145 cells it selectively favours the oxidative direction, also to the detriment of the reductive way. The effects of $TGF\beta_1$ on LNCaP and DU145 cells, although less pronounced, appear to be similar to those of TGFα, apart from a limited reduction of the oxidative pathways seen in LNCaP cells. In contrast, neither growth factor significantly changed patterns of androgen metabolism in PC3 cells, where the metabolic fate of testosterone remained dramatically oriented towards oxidation.

It has been previously reported that androgens induce both the secretion of a TGFα immunoreactive protein [Wilding *et al.*, 1989b] and the overexpression of EGFR in LNCaP cells

[Schuurmans *et al.*, 1988b]; on the other hand, levels of both $TGF\beta_1$ mRNA and TGFβ receptors are known to be down-modulated by androgens in prostatic tissues [Kyprianou & Isaacs, 1988; Kyprianou & Isaacs, 1989a]. Therefore, it is conceivable that androgen action in LNCaP cells may result in an enhancement of TGFα and a reduction of $TGF\beta_1$ actions. This, on a metabolic standpoint, would imply an extension of either way of testosterone metabolism, leading mainly to higher amounts of both DHT and Δ^4Ad available to the cells. The latter metabolite could in turn be converted to 5α-Adione and to further 17keto androgens (A and EpiA) or, alternatively, serve as substrate for aromatase to yield E_1 and E_2. Although merely speculative, this last possibility is noteworthy because oestrogen effects on growth of LNCaP cells are at least equally important to those induced by androgens.

The DU145 cell line represents a peculiar case. Although the presence of andogen binding sites is herein supported by both ligand binding assay and RT-PCR approaches, these cells fail to respond to either androgens (T and DHT) or oestradiol. In this context, it has been proposed that growth of DU145 cells may be regulated via an autocrine mechanism, involving also TGFα [Connolly & Rose, 1991]. In this cell line both TGFα and $TGF\beta_1$ produce an increase of the oxidative pathways of testosterone metabolism, with a correspondent decrease of the reductive way. The potential value of this variation in such a hormone-unresponsive cell line, however, remains to be elucidated.

5.2. Oestradiol metabolism

The effects of both TGFα and TGFβ$_1$ on oestradiol metabolism in human prostate cancer cells have been assessed.

In LNCaP cells (see Figure 52), both TGFα and TGFβ$_1$ produce a reduction of the E_2 oxidation to E_1 and a consequent additional increase of accumulating E_2; conversely, PC3 cells show a further E_2 degradation and E_1 formation in response to both growth factors (see Figure 54). In DU145 cells, TGFα and TGFβ$_1$ show a tendency to act oppositely, in that the former enhances while the latter reduces the oxidative metabolism of E_2 (see Figure 53).

Although no data are currently available on paracrine regulation of oestrogen metabolism in human prostate tissues or cells, recent studies have investigated possible influence of several factors on 17βHSD activity in human breast tissues and mammary carcinoma cells. Firstly, McNeill and colleagues [McNeill *et al.*, 1986b] showed that EGF, TGFs and breast tumour homogenates affect 17βHSD activity in human breast adipose tissues. Further studies revealed that either breast tumour cytosol or human breast fibroblast conditioned medium increase the reductive 17βHSD activity in MCF7 mammary carcinoma cells [Adams *et al.*, 1988a; Singh *et al.*, 1989], suggesting that some diffusible factor is responsible for this effect. More recently, Singh and associates have found that IGF-I stimulates the 17βHSD conversion of E_1 to E_2 in MCF7 cells and that this effect is strikingly enhanced by the combination of IGF-I and albumin [Singh *et al.*, 1992]. Furthermore, it has been reported that

addition of TGFα to nonmalignant human breast tissue *in vitro* significantly increases the reductive pathway of 17βHSD activity [Metha & Graves, 1992].

In the present work, both TGFα and TGFβ$_1$ appear to stress the preferential direction of E_2 metabolism in either LNCaP (reductive) or PC3 (oxidative) cells. This activity is more pronounced for TGFα in LNCaP cells and almost equivalent in PC3 cells. This potentially leads to an increase of E_2 accumulation in LNCaP cells and a consequent enhancement of the E_2-induced growth stimulation. On the other hand, while PC3 cells are sensitive to and possess specific cell surface receptor for either growth factor, their growth is unaffected by androgens but significantly inhibited by E_2; therefore both TGFα and TGFβ$_1$ would at the same time reinforce the prevalence of E_1 formation (presumably via autocrine loops) and eventually remove the inhibitory influence of E_2. However, the biological value of such a noticeable E_1 production in PC3 cells remains unclear.

CONCLUSIONS.

CONCLUSIONS

Although some results of the present work are preliminary and others may be difficult to explain, a few major inferences can be drawn. They primarily concern the potential role of both steroids and transforming growth factors and their receptors in the growth regulation and metabolic aptitudes of the human prostate cancer cell lines studied.

1. Cell Growth Regulation

The stimulatory effects exerted by both androgens (T and DHT) and oestradiol on the proliferation of LNCaP cells clearly indicate, as expected, that this cell line is under an endocrine type of growth control.

However, neither T nor DHT induce proliferative activity in DU145 cells, despite the presence of apparently intact androgen receptors in this cell line (here supported by both ligand binding assay and RT-PCR approaches). This seemingly conflicting evidence provides relevant, interesting clues to the human androgen resistance syndromes. These clinical entities, phenotypically ranging from a complete testicular feminization to undervirilized males, are associated with abnormalities of the androgen receptors [Griffin, 1991]. The latter include partial or complete gene deletions and splicing defects, leading to large-scale alterations of the primary structure of receptor, or single amino acid substitutions in both the hormone-binding and the DNA-binding domains (reviewed by [McPhaul, 1993]). In some

patients having endocrine profiles and family history of androgen resistance, receptor binding can be qualitatively and quantitatively normal; in these patients analysis of the androgen receptor gene shows an amino acid substitution in the DNA-binding domain, yielding a mutant receptor with minimal (if any) alteration of ligand binding but unable to activate a target reporter gene [Zoppi *et al.*, 1992]. Although partial deletions or larger rearrangements of the X chromosome, where the androgen receptor gene is located, occur rarely in human prostate cancer as compared to changes in other chromosomes, presence of more subtle defects, such as point mutations, may well cause structural and functional abnormalities of the receptor. In DU145 cells, where the androgen receptor gene has been found to be normally present [Tilley *et al.*, 1990], amino acid substitutions in the DNA-binding domain may lead to an impaired receptor function, without any apparent change of ligand binding characteristics.

Interestingly, the E_2-induced growth inhibition of PC3 cells, which may provide a meaningful interpretation on the efficacy of oestrogen treatment in androgen-refractory human prostate cancer, raises the question as to how E_2 action is mediated. In this respect, further studies are needed to ascertain whether or not the growth inhibition induced by E_2 involves different TGFß species; thus it is appropriate to determine whether E_2 acts through a direct, receptor-mediated pathway or by alternative paracrine/autocrine mechanisms. Overall, when comparing responsive LNCaP to non responsive PC3 cells, transcript levels

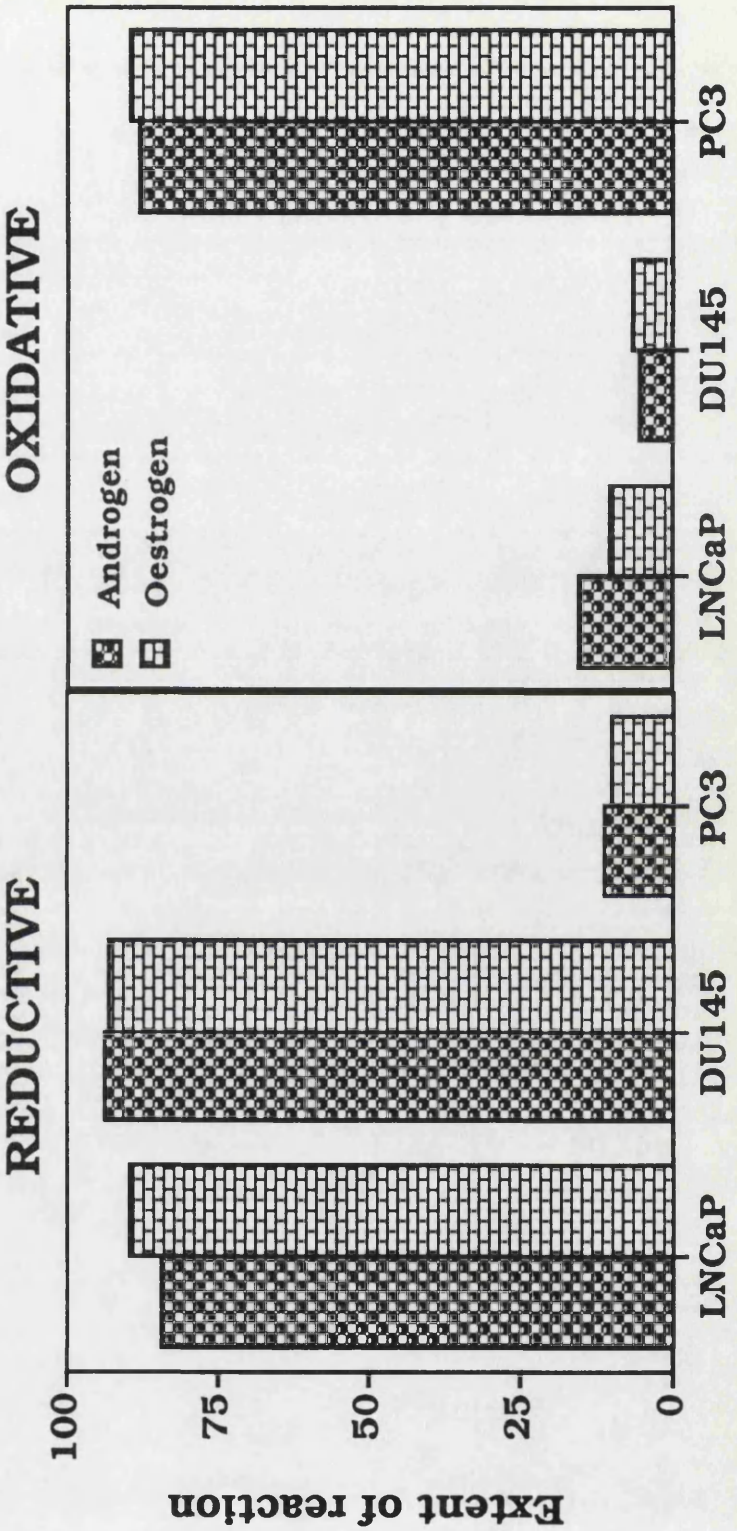
and intensity of staining and both quantitative and qualitative status of ER are significantly and consistently different.

The present data also indicate that the three prostate cancer cell lines respond differently to the same transforming growth factor and that, as far as TGFß is concerned, this response appears to be inversely related to individual characteristics of androgen sensitivity. Concerning TGFα, the growth effects seem to be not proportional to the EGF receptor content of cells; this apparent lack of correlation may suggest different levels of either endogenous growth factors or growth factor-binding proteins, and/or a diverse activity of membrane-bound protein kinases.

2. Steroid Metabolism

Overall, the present work consistently indicates that human prostate cancer cell lines, having different biochemical and biological features, are endowed with distinct aptitudes to alternatively favour reductive or oxidative patterns of steroid metabolism. This evidence is highly reproducible, being found using different incubation intervals (24-72 h) and varying concentrations of the labelled precursor ($0.5\text{-}10\times10^{-9}$M). There is a striking similarity between androgen and oestrogen metabolic patterns in human prostate cancer cells. This is reflected in a surprising correspondence between the respective extents of both oxidative and reductive reactions in androgen and oestrogen metabolisms for all three cell lines (see Figure 55).

Figure 55. Reductive and oxidative pathways of steroid metabolism in prostate cancer cells. The diagram compares the relative extent of reductive and oxidative pathways of both androgen and oestrogen metabolism in LNCaP, DU145 and PC3 cells. For details see text.


REDUCTIVE
OXIDATIVE
Androgen
Oestrogen
Extent of reaction
100
75
50
25
0
LNCaP
DU145
PC3
LNCaP
DU145
PC3

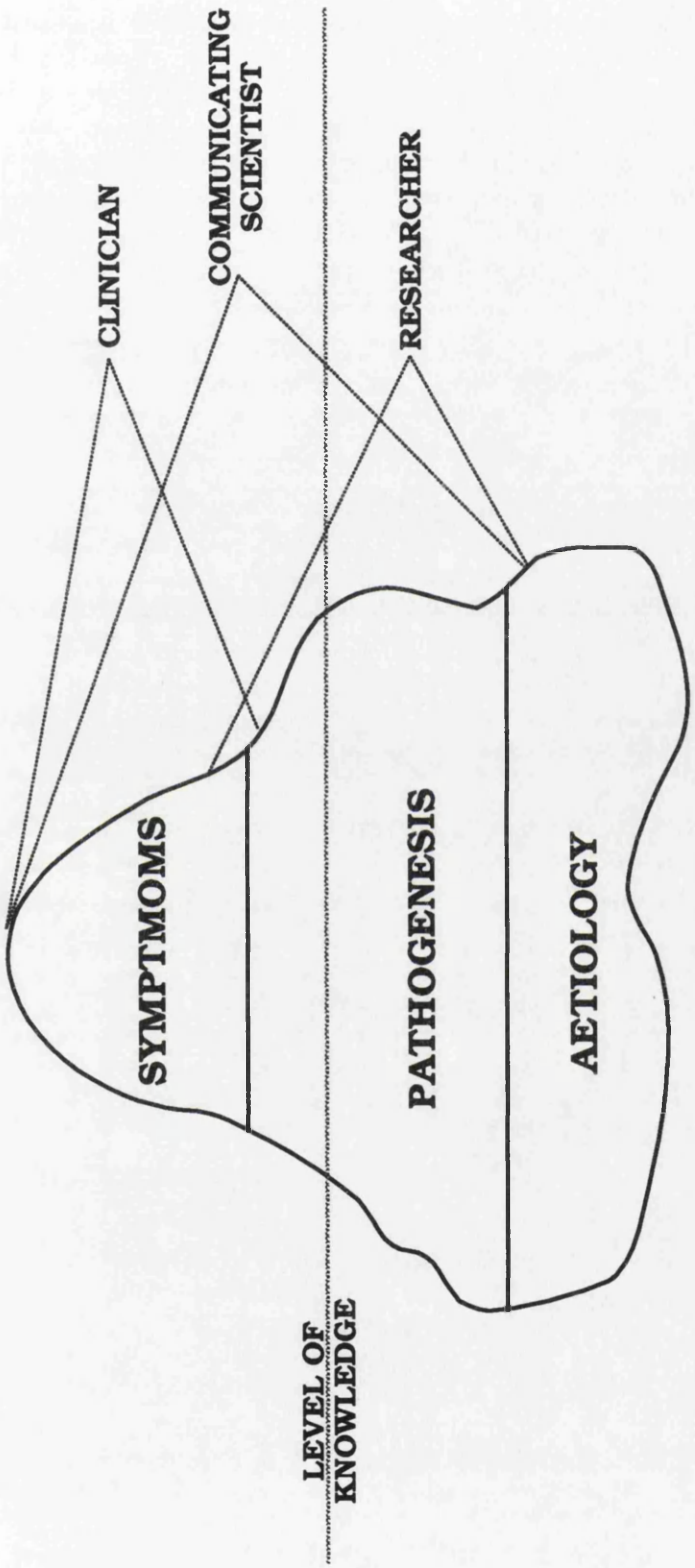
The finding that overall metabolism is remarkably shifted in favour of the reductive pathways in both LNCaP and DU145 cells, and of the oxidative pathways in PC3 cells, suggests that this uneven condition is strictly controlled in either hormone-dependent or independent cells. Therefore, the variations induced by both TGFα and $TGF\beta_1$ on the extent of individual reactions and, especially, on the overall direction of steroid metabolism in prostate tumour cells, although apparently moderate, appear worth noting. This could be significant in determining the actual concentration of biologically active metabolites, which will also be influenced by the microenvironment composition and the hormonal state of the cell.

Further studies are needed to clarify the potential contribution of the stromal compartment and of other local paracrine factors in the regulation of enzyme activities that ultimately direct the metabolic fate of biologically active steroids in these systems.

3. The Cancer Iceberg

Many reasons may contribute to the lack of progress in cancer research. Differences in both methodologies and model systems used very often make difficult data comparison and combination. However, one major aspect is to my mind critical: the shortage of communication in science. I always thought that if all the existing literature could be united in a single (capable

Figure 56. The cancer iceberg. The cancer phenomenon could be depicted as an iceberg, whereby clinicians mainly contemplate the visible part, while researchers look mostly at the submerged mass. Only communication would provide each with a deeper knowledge.



THE CANCER ICEBERG

and capacious) uncorrupted intellect, many of the current enigmas in the cancer phenomenon could be, for sure, deciphered or, at least, may become more transparent.

Unfortunately, scientists throughout the world tend to communicate poorly. Let me say that some of them are often inclined to think they hold the truth while the rest of the world is wrong! An example in point is given by the problematic relationship which frequently isolates clinicians from basic researchers. Both of them commonly approach the cancer problem from rather separate standpoints; in this respect, as sketched in Figure 56, cancer could be depicted as an iceberg, where clinical views are mainly directed towards the visible part, while researchers mostly look at the submerged mass. Intuitively, none of them would attain any decisive inference without the other's knowledge.

Although the recent progresses in computer science have markedly eased and expanded the opportunities to gain a rapid access to large data collections, a critical problem remains bound to humans and to their way of thinking. Sadly, barriers of prejudice, also in science, are at times even more impenetrable than the economic and social ones.

In a world where the evergrowing fragmentation of interests, competencies, responsibilities, or even ethnic realities, fatally leads to the seclusion of individuals, communication appears the only means to survive.

CITATIONS.

CITATIONS

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