Studies of Epidermal Growth Factors in Prostate Cancer Using the Cell Lines DU 145 & LNCaP

by

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ABSTRACT OF THESIS (Regulation 7.9)

Epidermal growth factor (EGF) receptor expression and modulation of growth rate by EGF were investigated in the androgen insensitive DU 145 and androgen sensitive LNCaP prostatic carcinoma cell lines.

Competition and saturation analysis of binding data revealed one high affinity binding site for both DU 145 (1nmol/l ±0.6) and LNCaP (2.8nmol/l±2.2), with DU 145 cells expressing high levels of receptor binding sites/cell ($2.5x10^5 \pm 1x10^5$) and LNCaP cells expressing 10 fold lower receptor binding sites/cell ($2x10^4 \pm 1x10^4$). Addition of exogenous EGF or TGFα to serum free cultures of DU 145 cells only minimally affected [^{3}H]- Thymidine incorporation and cell proliferation, whereas EGF was a potent mitogen for LNCaP cells.

Growth of LNCaP cells was also stimulated by the synthetic androgen Mibolerone, but no additive effect on growth was observed when EGF and Mibolerone were added together. Moreover, pre-incubation of LNCaP cells with Mibolerone did not affect either the number or affinity of the EGF receptor.

Further characterization of the EGF receptor demonstrated time and temperature dependence of EGF binding to its' receptor. The dissociation of EGF from the receptor was linear over the time period studied and binding sites on both cell lines were down regulated by pre-treatment with EGF. Specificity studies with ligands other than mEGF confirmed specificity, with only TGFα and hEGF competing with [^{125}I]-mEGF for binding to the receptor. The presence of the EGF receptor was also verified by Western blotting. Cell lyzates from DU 145 and LNCaP cells were separated by sodium dodecylsulphate polyacrylamide gel electrophoresis (SDS-PAGE) and subsequently analysed for the presence of the EGF receptor using monoclonal antibodies specific for the native external domain and the internal domain of the EGF receptor. A band of molecular weight 170 kDa (which is the molecular weight of the EGF receptor) was visualized with DU 145 cell lyzates, but not from LNCaP cell lyzates. This was probably due to the low numbers of receptor binding sites expressed by this cell line. The truncated, *v-erb* B gene product was not detected from either cell line.

The secretion of growth factors by some transformed cells is thought to enable these cells to proliferate in low serum concentrations as well as reducing the dependency upon exogenous growth factors. Since the growth of the DU 145 cell line was only minimally affected by exogenous EGF, serum free media conditioned by this cell line was analysed for growth factor production. EGF-like immunological activity as well as competitive activity in EGF-radio-receptor assays (RRA) was detected in the conditioned medium (CM) of these cells. Factors in the CM also inhibited and stimulated DNA synthesis of these cells and this effect was dose-responsive. Fractionation of the concentrated CM by gel filtration and reverse-phase high performance liquid chromatography (rHPLC) revealed several peaks of EGF-like competitive activity. One peak of rTGF-I immunological activity, which was separated by rHPLC, also demonstrated EGF-RRA competitive activity. None of the peaks demonstrating EGF-RRA competitive activity were related immunologically to hEGF.

DEDICATION

I dedicate this thesis to my mother Kathleen MacDonald and to the memory of my father James.

ACKNOWLEDGEMENTS

This research was carried out under the tenure of a University of Edinburgh studentship at the Department of Surgery, Western General Hospital; I am indebted to Professor Chisolm for the facilities provided.

I thank Dr Fouad Habib for all the help, support and advice that he has given me over the course of my postgraduate degree, Dr Peter Westbroek, University of Leiden for computing facilities and Matthew Collins for his contribution to the completion of the thesis.

DECLARATION

I, Anne Mac Donald, hereby declare that the work embodied in this thesis is the result of my own independent investigation. This is in accordance with rule 3.4.7 of Edinburgh University Postgraduate Study Programme 1987/88.

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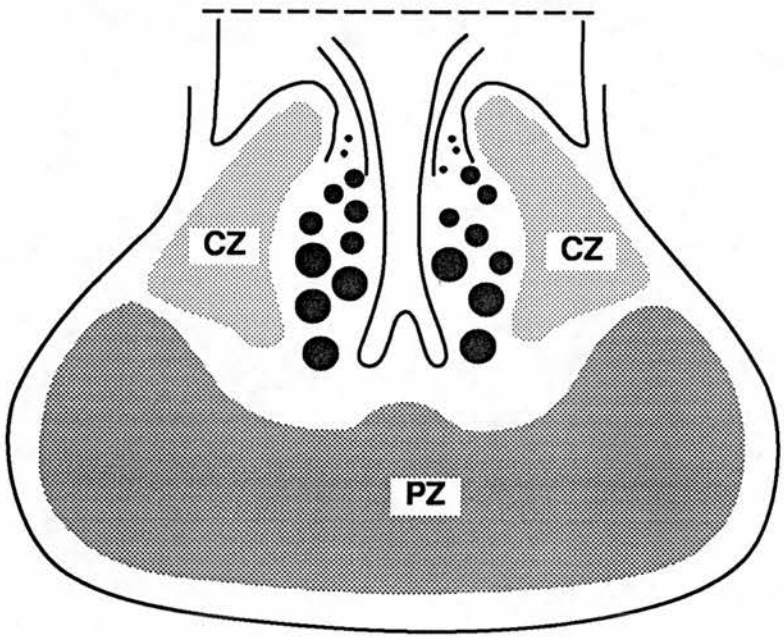
ABBREVIATIONS

MeCN	Acetonitrile
ACTH	Adrenocorticotrophic hormone
AEV	Avian erythroblastosis virus
BPH	Benign prostatic hyperplasia
BSA	Bovine serum albumin
CM	Conditioned medium
DHEA	Dehydroepiandrosterone
DHT	5α-dihydrotestosterone
EDTA	Ethylene diamino-tetra-acetic acid
EGF	Epidermal growth factor
EGF R	Epidermal growth factor receptor
FCS	Foetal calf serum
EGF R1	Monoclonal antibody to EGF R
F4	Monoclonal antibody to the internal domain of the EGFR
FCS	Foetal calf serum
FGF	Fibroblast growth factor
FSH	Follicle stimulating hormone
GH	Growth hormone
LH	Luteinizing hormone
Mab	Monoclonal antibody
Mibolerone	7α,7α-dimethyl-19-nortestosterone; DMNT
NGF	Nerve growth factor
PAP	Prostatic acid phosphatase
PDGF	Platelet-derived growth factor
PMSF	Phenylmethylsulphonylfluoride
PrDGF	Prostate-derived growth factor

PSA	Prostate-specific antigen
R1881	17α-methyl-17β-hydroxy-estra-4,9,11-triene-3-one
rHPLC	Reverse-phase high pressure liquid chromatography
RIA	Radioimmunoassay
RRA	Radio-receptor assay
SDS-PAGE	Sodium dodecyl-sulphate polyacrylamide gel electrophoresis
SFM	Serum-free medium
TFA	Trifluoroacetic acid
TGF	Transforming growth factor
Thr	Threonine
Tyr	Tyrosine


CZ
CZ
PZ

Chapter 1

INTRODUCTION

1.1 THE HUMAN PROSTATE GLAND - LOCATION, STRUCTURE AND FUNCTION

The human prostate gland is situated at the base of the bladder and surrounds the top of the urethra. The gland is a composite structure which includes glandular elements and a stroma of collagenous and muscle tissue. It consists of two sets of glands, an inner or central periurethral zone (CZ), and an outer peripheral zone (PZ) (Figure 1).

The normal physiological function of the prostate is unknown. The epithelial cells secrete a thin, milky alkaline fluid containing citric acid, calcium, acid phosphatase, a clotting enzyme and a profibrinolysin (Guyton, 1981). The prostate gland is also a rich source of growth factors, particularly epidermal growth factor (EGF) (Gregory *et al.*, 1986). During emission, the prostatic capsule contracts simultaneously with the vas deferens so that the prostatic fluid adds to the bulk of the semen. The acidic secretions of the vas deferens are subsequently neutralized by the alkaline prostatic fluid, hence achieving an optimal pH environment for the spermatozoa (pH 6.0-6.5). The secretions of the prostate may play an important role in male fertility, although this has not as yet been established.

FIGURE 1: Diagrammatic vertical coronal section through the human prostate. Showing the central zone (CZ) and peripheral zones (PZ); after Blacklock, (1982).

1.2 THE HYPERPLASTIC AND MALIGNANT PROSTATE

Disease of the prostate is relatively uncommon in young males but increases in incidence with age. The two main pathological processes which involve the gland are benign prostatic hyperplasia (BPH) and carcinoma. BPH, which affects a large percentage of men over the age of 50, is probably endocrinologically induced, although the aetiology of the disease is poorly understood. It is thought to begin in the inner or periurethral region of the prostate gland and is characterized by an increase in stromal proliferation in the periurethral zone (Franks, 1954).

In contrast to BPH, carcinoma of the prostate is associated with epithelial cells in the peripheral region of the prostate (McNeal, 1972; Robel, 1980) but like BPH it is age related. It is relatively uncommon in men before the age of 50, and has the highest incidence in men between 65 and 75 years of age. Prostate cancer is associated with an unusually and extremely high prevalence of latent or dormant cancer that is clearly identified on pathological examination but which in most cases will never grow further to become clinically manifested (Franks, 1954); for unknown reasons growth is held in check. About 10% of all men between the ages of 50-59 years and about 50% at 70-79 years have this latent form of prostate cancer. Those latent cancers which are activated to grow, account for a mortality rate which makes prostate cancer the second most common cause of male cancer deaths in the United Kingdom (CRC Factsheet 10.1, 1988). The aetiology of the disease is unknown, although several hypotheses including environmental, geographic, racial and dietary factors have been discussed (Griffiths, 1987). Since the finding in 1986 of the expression of the *ras* p21 oncogene in prostatic carcinoma patients (Viola *et al.*, 1986), a genetic factor is indicated, although as Bouffioux (1984) has commented, prostate cancer like most malignancies, does not depend upon a single aetiology but on a complex interaction of factors resulting in oncogenic transformation.

1.3 FACTORS AFFECTING PROSTATIC GROWTH

It is well accepted that the overall growth, maintenance and functional activity of the normal prostate is dependent upon androgens and to some extent upon peptide hormones (Ghanadian, 1982). However, this endocrinological view of the regulatory mechanisms affecting the growth of the prostate (or any target organ) largely ignores the interactions between different cell types and the function of growth factors derived from this "micro-environment" within the target tissue. In recent years attention has focussed on these intra-glandular growth control mechanisms and the functions of the diffusible factors derived from this "micro-environment" with a view to obtaining some insight into normal and malignant prostatic function.

In this section the endocrinology of those factors governing the growth and regulation of the normal and malignant prostate is discussed. This is followed by a discussion on the role of paracrine factors, such as polypeptide growth factors, and how they function within the environment of the prostate.

1.3.1 ANDROGENS

In 1786 John Hunter first established the relationship between testicular secretions and the growth of the prostate. However, it was the isolation of the active testicular hormone testosterone (David *et al.*, 1935), that established the basis for the study of the androgenic steroids in the control of prostatic growth and development. The pioneering work of Huggins and Hodges (1941) provided supportive evidence that prostatic cancer is also associated with hormones and, in particular, testicular androgens. Many attempts have since been made to understand the role of androgens in the growth and development of the prostate and their role in prostatic cancer (Griffiths, 1987).

Regulation of androgen secretion

In man the major serum androgen is testosterone, of which 90% is produced by the testes, with a small contribution from the adrenals. Testosterone release from the

testes is regulated by the hypothalamic-pituitary-gonadal axis. In the hypothalamus, luteinizing hormone-releasing hormone (LH-RH) is secreted in a pulsatile fashion into the hypophyseal portal circulation and reaches the anterior pituitary upon stimulus from the neurotransmitter norepinephrine. The pituitary is stimulated by the actions of LH-RH to release luteinizing hormone (LH) and follicle stimulating hormone (FSH). The LH is transported by the blood to the testes, where it binds to specific receptors located on the membranes of the Leydig cells causing the release of testosterone into the bloodstream. Plasma levels of testosterone, in turn, regulate the release of both LH-RH and gonadotrophins by a negative feedback mechanism, thought to operate both at the hypothalamic and the pituitary level (Gower, 1979). About 2% of the total plasma testosterone remains in the unbound state whilst the remainder is reversibly bound to either albumin or steroid hormone binding globulin. It is the free testosterone which is considered to be biologically active (Vermeulen, 1979). In the prostate testosterone is converted to the active metabolite, 5α-dihydrotestosterone (DHT), a conversion catalyzed by the enzyme 5α–reductase, which is thought to be a nuclear membrane bound protein (Houston *et al.*, 1985a). The conversion of testosterone to DHT is now believed to be an obligatory step in the mediation of androgen action in the prostate, with DHT the major androgen regulating the cellular events of growth, differentiation and function of the prostate (Ghanadian, 1982).

The adrenal cortex, which is also a source of androgen, has been shown to influence prostatic growth (Huggins, 1945; Sanford *et al.*, 1977). The main androgen secreted by the adrenals is dehydroepiandrosterone (DHEA) and its sulphate conjugate (DHEA-SO_4), which together account for about 10% of all circulating androgens. These steroids are released by the adrenal gland in response to adrenocorticotrophic hormone (ACTH) which in turn is under the influence of the hypothalamic corticotrophin releasing hormone (CRH). Adrenal androgens undergo metabolic conversion to testosterone and DHT, either in the plasma or in the prostate itself. Steroid hormones produced by the testes and adrenals are subsequently taken up by the

T
T
5 α reductase
D
D
?
Transcription
mRNA

prostate, but the contribution of adrenal androgens to the growth of the prostate, in particular to prostate cancer, is uncertain (Labrie *et al.*, 1986; Oesterling *et al.*, 1986).

The mechanism of action of androgens

The mechanism of action of androgens is continually being reviewed and adapted. The 'new' model which is a 'one-step' model for androgen receptor binding is illustrated in Figure 2. In this model extracellular testosterone diffuses into the cell through the cytoplasm to the nucleus. On the nuclear membrane testosterone is reduced to DHT by the enzyme 5α-reductase and DHT then binds, with high affinity, to a specific receptor site on the nuclear membrane. The resulting complex then undergoes a conformational change and interacts with specific receptor sites either located on DNA, chromatin associated proteins, matrix related components or distributed amongst all three. Binding of DHT to its receptor is then followed by DNA synthesis, the production of mRNA and ultimately cell division.

Recently the primary structure of the androgen receptor has been described (Chang *et al.*, 1988; Lubahn *et al.*, 1988). The receptor structure appears to be composed of several functional domains, which is also true of other steroid hormone receptors. Included are a ligand binding domain at the C-terminal end, a hinge region, a DNA binding domain and an N-terminal domain involved in transcriptional response. In general, steroid binding to the ligand binding domain results in an increased affinity of the receptor for DNA.

Steroid hormone regulated genes have short cis-acting enhancer sequences termed hormone-responsive elements, in or near their transcription units (Jantzen *et al.*, 1987; Strahle *et al.*, 1987). The DNA binding domain of steroid receptors contain highly conserved cysteine residues which may tetrahedrally coordinate zinc to form a "zinc

FIGURE 2: The mechanism of action of androgens: 'one-step' model where the receptor and the 5α-reductase are associated with the nuclear membrane. T= testosterone and D = DHT (after Griffiths *et al.*, 1987).

finger" (Green *et al.*, 1986b; Weinberger *et al.*, 1986). These zinc fingers are believed to become unmasked upon hormone binding and to interact with the hormone-responsive elements ultimately leading to the transcription of the gene.

Malignancy and endocrine status

The growth and function of the normal prostate is primarily dependent upon androgens (Coffey & Issacs, 1981). The diseased prostate also appears to respond to endocrine manipulation, as in a large proportion of patients with prostatic cancer the disease responds well to treatments which lower the plasma levels of testosterone or which are anti-androgenic (Griffiths *et al.*, 1987). Indeed, as early as 1941 endocrine therapy was a recognised form of treatment for prostatic carcinoma (Huggins & Hodges, 1941). Nevertheless, there are few lines of evidence to implicate the endocrine system in the aetiology of prostatic cancer even though the presence of the disease in the prepubertaly castrated male has never been reported. Rotkin (1979) suggested that the conditions which predispose towards the development of prostatic carcinoma may be imprinted on the gland at puberty. He suggested that early "oncogene transformation" of the gland may occur as a consequence of the diverse physiological and secretory patterns involved with the onset of puberty. Rotkin further suggested that men at risk of developing prostatic cancer would have a "strong overbalance of androgenic components". McNeal (1975) investigating the pathogenesis of prostatic cancer described areas of proliferating epithelial tissue within the prostate gland, which he described as premalignant changes closely associated with prostatic carcinoma. Perhaps prostatic cancer may develop from these proliferating epithelial cells induced by high levels of androgens as Rotkin (1979) suggested. However, there is little evidence to suggest that hormones are concerned in the initiation of prostatic cancer and it may be that the role of hormones are permissive rather than inciting.

It is generally accepted that the more malignant the cancer, the less hormone-dependent the cells are, and the less the degree of differentiation (Gleason, 1977). In advanced stages of prostatic cancer, removal of androgens by castration or anti-androgen treatment results in a marked regression of the cancer, although such regression is usually only transitory and in the majority of cases there is a recurrence of cancerous growth; the tumour now no longer responding to endocrine therapy, possibly as a result of a progression of androgen-independent cells. The processes concerned in the regulation of the maturation of the prostate gland and its growth must be complex, requiring a fine balance of growth stimulatory and inhibitory factors. Minor changes in this balance would allow the activation of cellular oncogenes and consequently prostatic cancer.

Despite intensive investigations there is at present little evidence to indicate that initiation of neoplasia, the progress of metastasis, or the aetiology of the disease is caused by excessive androgen stimulation, although the disease is generally androgen-dependent.

1.3.2 PROLACTIN, GROWTH HORMONE AND THE PROSTATE

Early experimental studies in animals showed that pituitary hormones influenced the growth of the prostate gland. Their actions were generally considered permissive in that they acted with steroids to promote their biological effects (Huggins & Russell, 1946; Lostroh & Li, 1957; Tullner, 1963). Since these early findings evidence has accumulated to suggest that prolactin has a specific role in the control of prostatic function. Several studies have indicated that the presence of prolactin increased the uptake of testosterone into prostatic tissue (Lasnitzki, 1972; Lloyd *et al.*, 1973; Jacobi *et al.*, 1978; Farnsworth 1981). Furthermore, Odoma *et al.* (1985) demonstrated a positive correlation between plasma prolactin levels and cytosolic androgen receptor content in patients with BPH, which complemented the earlier studies of Jacobi *et al.* (1978) and Farnsworth (1981). Synergistic effects of prolactin and androgens were

also observed on the growth of prostatic epithelial cells in culture (Syms *et al.*, 1985), further indicating a relationship between the roles of these hormones in regulating the biology of the prostate. The exact mechanism responsible for these modulatory effects remains far from being understood, but as Odoma *et al.* (1985) suggested, it is not inconceivable that prolactin receptors present on human prostate membranes (Leake *et al.*, 1983) might facilitate the entry of androgens into the prostate cell.

Equally interesting is the role played by growth hormone (GH) in the prostate. Immunocytochemical procedures have detected endogenous human GH in BPH and neoplastic lesions (El Etreby & Mahrous, 1979). Furthermore in a study by Sibley *et al.* (1984) they found that the gland demonstrated a capacity to bind exogenous levels of the peptide. The binding of GH was primarily confined to the stroma in both benign and malignant tissue, although evidence was obtained which suggested binding within the cytoplasm of epithelial cells. Although receptors for GH have been located in the prostate, the role of GH in BPH and prostatic cancer remains to be ascertained.

1.3.3 ANDROGENS, POLYPEPTIDE GROWTH FACTORS AND THE STROMAL, EPITHELIAL RELATIONSHIP OF THE PROSTATE

Since 1941, when Huggins and Hodges first demonstrated the regulatory role of the testes, and testicular androgens in the progression of prostate cancer, the principle goal in the treatment of the disease has been to suppress the androgenic stimulation of prostatic growth and function. However, this approach does not take into account the intra-glandular growth control mechanisms and the "growth factors" derived from this "micro-environment" within the prostate.

It has been known for several years that the prostate is composed of both epithelial and stromal elements. An understanding of the relationship between the two major prostatic cell types is crucial if we are to understand the biochemical factors involved in growth of the normal and malignant prostate. For many years the stroma was considered an inactive matrix and the functional activity attributed to the epithelium. However, several groups have shown the stroma to be important in the control of

prostatic growth and development. Franks *et al.* (1970) separated the epithelial and stromal components from BPH tissues and found that the growth of the epithelium in culture was dependent upon the presence of stromal elements. Moreover, Cunha (1973) demonstrated the interaction between these two cell types using embryonic mouse tissues, where he demonstrated that the normal development of the urogenital sinus epithelium was dependent upon the urogenital mesenchyme (stroma). These studies imply an influence of factors derived from connective tissue. Subsequent studies showed that the levels of androgen receptor (Lahtonen *et al.*, 1982), oestrogen receptor (Krieg *et al.*, 1981), steroid metabolizing enzymes (Orlowski *et al.*, 1983) and steroids (Bartsch *et al.*, 1982) are not the same in the two cell types. Several investigators have since suggested that the major target for androgen stimulation in the prostate may be the mesenchyme and not the epithelial cells (Shannon & Cunha, 1984; Cunha *et al.*, 1987). Furthermore, McKeehan *et al.* (1984) and Chaproniere & McKeehan (1986) observed that proliferation of rat and human prostatic epithelial cells in culture was not androgen dependent. These observations suggest that androgens are not directly involved in the proliferation and differentiation of prostatic epithelial cells and that prostatic stroma plays an important role both structurally and biochemically in maintaining the normal function of the organ. In addition to recognizing the differences between the stromal and epithelial cells in the prostate, it is important to recognize that the epithelial cells themselves cannot be treated as a homogeneous group of cells. Cunha and co-workers (1985) further demonstrated that after adult mice are castrated the regression of the prostate does not occur uniformally throughout the gland. The cells in the distal region of the ductal-acinar network are lost well before those in the more proximal portion of the gland. On injection of testosterone the number of branch points are restored to normal, demonstrating that the regeneration of the prostate occurs primarily through growth in the distal region of the gland (Cunha *et al.*, 1985).

These findings suggest that the mechanism of action of androgens on the prostate must be expanded to take into account the interactions between the stromal and epithelial cells which clearly act in concert to ensure the normal development and function of the prostate. The interaction of certain local tissue 'factors' or growth factors that are produced in an androgen-dependent manner must also be considered in the overall regulatory processes involved in prostatic function.

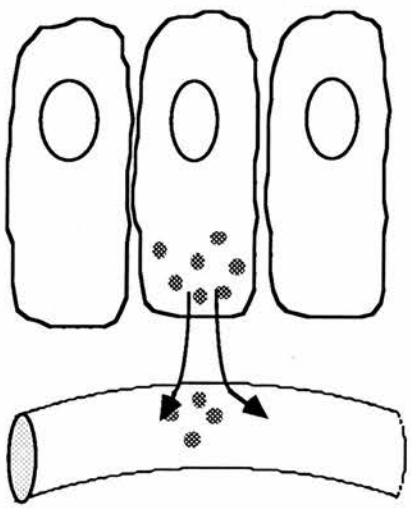
1.3.4. POLYPEPTIDE GROWTH FACTORS

Prostatic epithelial cells have been the focus of attention because not only are they the target for androgens, oestrogens and progestins, by virtue of their receptors but they may also be influenced by peptide growth factors and undefined factors secreted from the surrounding stroma. There is now growing evidence that chemical signalling in the form of soluble growth factors are involved in maintaining cell to cell communication and phenotypic expression in the prostate. In support of this hypothesis are observations of stromal regulation of epithelial differentiation, demonstrated during the development of the prostate and of other glandular structures in the foetus (Cunha, 1972; Cunha *et al.*, 1980). Many studies have sought to identify and characterize these diffusible factors derived from prostatic tissue (Jacobs & Lawson, 1980; Story *et al.*, 1983; Elson *et al.*,1984; Jinno *et al.*, 1986).

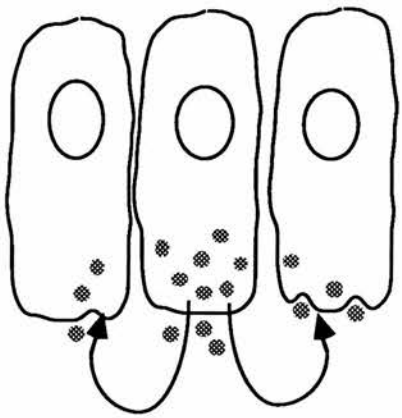
Mode of action of growth factors

Growth factors may be defined as polypeptides that stimulate cell proliferation through binding to specific high affinity cell membrane receptors. The receptors are located on the cell membrane and are usually glycoproteins which communicate with secondary messenger systems by conformational changes. This often involves the autophosphorylation of tyrosine residues located on the intracellular domain of the receptor. The secondary messenger systems utilized are diverse and include changes to intracellular calcium levels, cyclic AMP, cellular alkalization and phosphoinisitol

Endocrine



Paracrine



metabolites. For many, but not all growth factors this is followed by DNA synthesis and cell replication (Hill, 1989 for review).

Those peptide growth factors that have been best characterized are typically proteins of molecular weight < 30 000. Growth factors do not usually act in an endocrine manner, the concensus of opinion is that they diffuse over a short distance through intercellular spaces and act locally, in a paracrine manner (James & Bradshaw, 1984); both endocrine and paracrine mechanisms of action are illustrated in Figure 3. While classical hormones such as GH and ACTH can influence the overall rate of growth and maturation, unlike growth factors they do not contribute to the intracellular signalling within tissues. Although this form of signalling appears to be the primary role of polypeptide growth factors, growth factors have been identified in plasma and may act as hormones as well. Those present in serum are presumed to be derived from platelets and are released during the clotting process (Holley & Kiernan, 1974; Vogel *et al.*, 1978; Childs *et al.*, 1982; Oka & Orth, 1983). Unlike classical endocrine hormones each growth factor may be synthesized by a variety of tissues (both adult and embryonic), and are thought to be released by many, if not all cells in culture (Shields, 1978). Growth factors are not stored intracellularly in granules unlike classical hormones such as insulin, and release is largely dependent on de-novo synthesis.

Growth factors and the prostate

The first investigations with regard to the role of growth factors in the growth and regulation of the prostate gland was centred on the detection of EGF and its' receptor. Hirata & Orth (1979) were the first to demonstrate the presence of EGF in the human prostate using a radioimmunoassay, subsequently Elson *et al.* (1984) described an endogenous EGF-like activity in human prostate and seminal plasma, and high

FIGURE 3 : Schematic diagram of the endocrine and paracrine actions of hormones and growth factors.

concentrations of EGF have been reported in human prostatic fluid (Gregory *et al.*, 1986). Subsequent studies also suggest that EGF is associated with human BPH (Maddy *et al.*, 1987,1989) and prostatic cancer (Eaton *et al.*, 1988; Schuurmans *et al.*, 1988; Connelly & Rose, 1989; Maddy *et al.*, 1989; Wilding *et al.*, 1989).

In view of the involvement of growth factors in maintaining the functional integrity of the prostate gland, attempts have been made to identify the mechanisms by which these growth factors, in particular EGF, act on the prostate. Like other cell types (Carpenter & Cohen, 1979) the activities of EGF in the human prostate is mediated through receptors located on the cell surface membrane (Maddy *et al.*, 1987; Eaton *et al.*, 1988). The receptors are confined to the basal layers of epithelial cells with no apparent binding in the stromal component. Furthermore, Maddy *et al.* (1989) found that prostate cancer tissue had a reduced capacity to bind EGF when compared to benign tissue, with the depletion in the number of binding sites correlating with the loss of differentiation of the tumour. The depletion of EGF receptor binding sites may reflect a down regulation of receptors by the production of endogenous growth factor, although this remains to be ascertained.

Growth factors other than EGF are also associated with the prostate. Maehama *et al.* (1986) characterized a growth factor derived from rat ventral prostate. This rat prostate-derived growth factor (PrDGF) was distinct biologically from other known growth factors such as EGF, fibroblast growth factor (FGF), transforming growth factor α (TGFα) and transforming growth factor β (TGFβ) however, PrDGF has some biochemical similarities to TGFβ. Jacobs *et al.* (1979) and Jacobs & Lawson, 1980) reported on the presence of a fibroblast growth-promoting factor in crude extracts of human BPH, well differentiated carcinoma, and postpubertal normal prostate. This growth factor was distinct from EGF and TGFα in that it did not compete for the EGF receptor (Story *et al.*, 1983) and it was also distinct from FGF in that the components were acidic and not basic (Jinno *et al.*, 1986). Additionally, Nishi and co-workers (1985) purified a growth factor from cytosol preparations of human

benign adenoma which was capable of stimulating DNA synthesis of BALB/3T3 cells. This growth factor differed from bovine FGF and EGF.

Growth factors derived from the prostate might be associated with metastatic disease. A large percentage of patients with prostatic cancer develop bone metastasis and these osteoblastic lesions may be the result of growth factors produced by the tumour cells. Indeed several investigators have isolated factors from prostatic adenocarcinoma (Jacobs *et al.*, 1980) and from carcinoma cell lines (Simpson *et al.*, 1985) which stimulate osteoblast proliferation. Simpson *et al.*, (1985) identified a mRNA fraction from PC3 cells which encoded an osteoblast-stimulating activity.

The control of prostatic growth is not just determined by androgens alone but by a multiplicity of different factors. It is clear that not one, but several, growth factors may be involved in the development of BPH and prostatic cancer. This supports the hypothesis, forwarded by Sporn & Roberts (1988), that combinations of growth factors increases the amount of information that can be transmitted.

Growth factors and androgens

The relationship between steroids and growth factors in the prostate is intriguing and has prompted a number of investigations to elucidate the mechanisms by which growth factors and androgens might modulate growth of the normal and malignant prostate (Traish & Wotiz, 1987; St-Arnaud *et al.*, 1988; Schuurmans *et al.*, 1988a; Wilding *et al.*, 1989). In other model systems there is evidence for steroid-hormone regulation of growth factor content and activity (Byyny *et al.*, 1974; Hosoi *et al.*, 1981; Mukku & Stancel, 1985; Dickson *et al.*, 1986; Murphy *et al.*, 1986). In breast cancer Lippman *et al.* (1986; 1987) have studied in detail the actions of oestrogens and growth factors, and how they might act together to regulate cell proliferation. There are several ways in which androgens co-operating with growth factors could regulate cell proliferation in prostate cancer in a manner comparable to oestrogen action in breast cancer :-

(i) androgens may affect the production of growth factors; this mechanism has been well documented for oestrogen action in breast cancer, where oestrogens regulate the production of growth factors in hormone-responsive breast cancer cells (Dickson *et al.*, 1986).

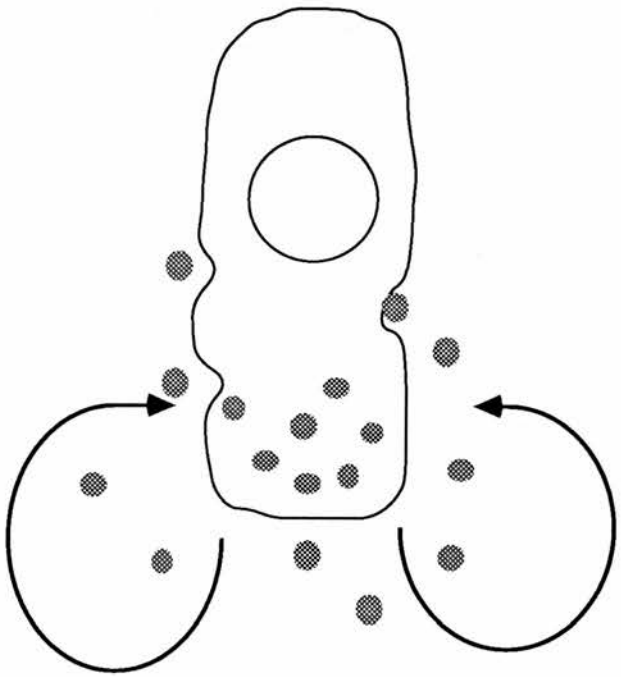
(ii) Steroid hormones might also suppress the secretion of inhibitory growth factors; oestrogens have been shown to suppress the secretion of TGFβ in MCF 7 breast cells thereby increasing the growth of these cells by oestrogen stimulation (Knabbe *et al.*, 1987).

(iii) Androgens might also modulate growth factor receptor expression, which in turn may increase the sensitivity to secretory growth factors; progestins have been shown to modulate EGF receptor levels in breast cancer cell lines (Murphy *et al.*, 1986).

Of the three mechanisms (for oestrogen modulation of growth factor action in breast cancer), recent evidence suggests that androgens regulate the production of the growth factor TGFα (Wilding *et al.*, 1989) and modulate the concentration of EGF receptors in the androgen-responsive prostate LNCaP cell line (Schuurmans *et al.*, 1988a; Wilding *et al.*, 1989).

These examples indicate the complex nature and the multiple pathways by which androgen-sensitive cells might be regulated by steroids. Equally complex is the progression from endocrine-responsive to -unresponsive prostate cancer. King (1990) suggested that altered growth factor activity might account for this transition in breast cancer. Evidence for this, is the clinical observation that steroid hormone-insensitive breast tumours grow faster and recur earlier than their endocrine-sensitive counterparts (Clark & McGuire, 1989), and that the EGF receptor is overexpressed in unresponsive relative to responsive tumours. Moreover, over-expression of the EGF receptor correlates with an increased rate of recurrence of breast tumours (Harris & Nicholson, 1988). Transition to the unresponsive state was visualised as, in part involving altered regulation of growth factors and/or their receptors (King, 1990). It was the discovery

Autocrine



that *ras* oncogene transfection into MCF-7 oestrogen-responsive breast cancer cells rendering them oestradiol-insensitive and that TGFα was overproduced (King, 1990) which confirmed this link. However, paradoxes exist (Liu *et al.*, 1987) and more than one pathway to insensitivity is not inconceivable. Moreover, the transition to the oestrogen-unresponsive state in breast cancer might not compare with the situation in prostate cancer. Nevertheless, there is little doubt that growth factors and their receptors are important in the progression of cancer, but how is unclear.

1.4 GROWTH FACTORS AND CANCER

Much of the impetus for study of growth factors has come through their probable role in cancer. Evidence for this role dates from early work showing a decreased serum requirement for growth of neoplastically transformed cells (Temin, 1966; Dulbecco, 1970; Paul *et al.*, 1971). Loss of requirement for specific growth factors is a common finding in many types of cancer cells (Moses *et al.*,1978; Kaplan *et al.*, 1982) and could be mediated by (a) the activation of autologous growth factor synthesis ("autocrine" activation), (b) synthesis of an altered growth factor receptor, or (c) activation of a post-receptor pathway that bypasses the growth factor requirement.

1.4.1 EVIDENCE FOR THE AUTOCRINE HYPOTHESIS

The concept of autocrine secretion of growth factors was first conceived by Sporn & Roberts (1985) in the search for the molecular and cellular basis of malignant transformation. There is now much circumstantial and direct evidence to support the original hypothesis of autocrine secretion. Many types of tumour cells release polypeptide growth factors into their conditioned medium (CM) when grown in cell culture and these same tumour cells often possess functional receptors for the secreted peptide (Figure 4).

FIGURE 4 : Schematic illustration of the autocrine mechanism of action of growth factors proposed by Sporn & Roberts (1985).

The peptide growth factors that function via an autocrine mechanism in cancer cells include TGFα, peptides related to platelet-derived growth factor (PDGF), bombesin and TGFβ. The action of each of these four peptides is mediated by a distinct membrane receptor, which in turn activates a post-receptor signalling mechanism and leads eventually to a primary alteration in the synthesis and release of a specific growth factor.

The autocrine action of a growth factor in a cancer cell was first described in rodent cells transformed by a Moloney murine sarcoma virus (De Larco & Todaro, 1978). The first peptides identified as participating in this transformation were structurally related to (but distinct from) EGF, and are now universally known as type α TGFs (Roberts *et al.*, 1983). The only known receptor for these peptides is the EGF receptor and all of their effects (such as growth stimulation) are thought to be mediated through this locus. Many data indicate that human cancer cells produce and release TGFα and have functional receptors for this peptide (De Larco & Todaro, 1978; Ozanne *et al.*, 1980; Todaro *et al.*, 1980; Kaplan *et al.*, 1982; Marquardt & Todaro, 1982; Halper & Moses, 1983; Roberts *et al.*, 1983; Moses, 1984; Salomon *et al.*, 1984a, 1986; Dickson *et al.*, 1986), which suggests that these cells will be continuously activated to grow. Other growth factors produced by transformed cells such as insulin-like growth factor (IGF) (Dulak & Temin, 1973; De Larco & Todaro, 1978) and fibroblast-derived growth factor (Burk, 1976) may also act as autocrine regulators of proliferation.

The experimental data providing the most evidence for the autocrine hypothesis comes from the relationship of the oncogene product of the simian sarcoma virus (SSV) and PDGF; homologies exist between the *c-sis* product and the B-chain of PDGF (Doolittle *et al.*, 1983; Waterfield *et al.*, 1983). SSV product can also function like PDGF as a growth factor for cells in culture (Deuel & Huang, 1983). The PDGF-like molecules produced by tumour cells seem to be encoded by cellular genes.

The signalling pathways activated by an autocrine peptide need not evoke a positive growth response, as seen most strikingly in the response of cells to TGFβ. Although this peptide was initially characterized by its ability to stimulate the growth of non-neoplastic fibroblasts as colonies in soft agar (Roberts *et al.*, 1983), it has been shown to be a potent inhibitor of the growth of many other types of cells (Holley *et al.*, 1980; Tucker *et al.*, 1984). These cells have receptors for TGFβ and respond with an inhibition of growth suggesting that in certain cells endogenous TGFβ plays a role in cell-cycle regulation, specifically in the maintenance of a resting state.

The autocrine hypothesis may be expanded to include the concept that malignant transformation may be the result not only of excessive production, expression and action of positive autocrine growth factors, but also of the inability of cells to synthesize, express or respond to specific negative growth factors they normally release to control their growth. This loss of negative growth control may result in a biochemical lesion in either the growth inhibitor, its receptor, or the post-receptor signalling pathway (Sporn & Roberts, 1985).

1.4.2 ONCOGENE PRODUCTS AS RECEPTORS

Some of the more convincing evidence linking growth factors and cancer has come from recent work linking oncogenes and growth factor receptors. The oncogenes *erb* B, *fms* and *neu* are known to encode cell-surface proteins. Cells transformed by these oncogenes seem to have obtained a growth advantage by mimicking mechanisms of normal cellular growth. However, in these transformed cells the normal system of regulation is evaded and the growth advantage is achieved by the cells' growth autonomy.

The *erb* B oncogene product bears close similarity to the EGF receptor (EGFR) (Downward *et al.*, 1984), (see also section 1.5.3). The *erb* B gene encodes the transforming protein of avian erythroblastosis virus (AEV) (Yamamoto *et al.*, 1983). Comparison of the v-*erb* B protein with the human EGFR shows a large degree of

homology, the v-*erb* B product representing a truncated form of the EGFR. v-*erb* B encodes the cytoplasmic and transmembrane domains of the EGFR, but lacks the greater part of the EGF-binding region by which normal growth is regulated (Downward *et al.*, 1984). The v-*erb* B protein has been found to induce tyrosine (Tyr) phosphorylation (the cytoplasmic region of EGFR contains a Tyr kinase domain; see section 1.5.2) *in vivo* and *in vitro*. In addition it contains sequences homologous to the kinase domain of the EGFR and corresponding regions of retroviral kinases (Gilmore *et al.*, 1985), suggesting that the unregulated kinase activity may be responsible for transformation. Thus, the structure of the v-*erb* B product, consisting of transmembrane and cytoplasmic, but not ligand binding domains, allows for an unregulated mitogenic signal.

Similarly, the product of the v-*fms* oncogene encodes a protein exhibiting receptor characteristics (Sherr *et al.*, 1985). The v-*fms* protein product is a transmembrane glycoprotein with the amino-terminal domain oriented outside the cells and the carboxy-terminal region in the cytoplasm (Rettenmier *et al.*, 1985). It has been shown that the v-*fms* products are expressed on the cell surface and are associated with clathrin, clathrin-coated pits, and endocytotic vesicles (Marger *et al.*, 1984); characteristics typical of receptor molecules. Common to growth-related receptors, v-*fms* displays *in vitro* phosphorylation of the glycoprotein at Tyr (Roussel *et al.*, 1984; Barbacid & Lauver, 1981). It was shown by Roussel and co-workers (1984) that cell surface expression of the Tyr kinase activity was necessary for v-*fms* transformation. The cellular counterpart of the transforming *fms* gene seems to be the receptor for the murine colony-stimulating factor, CSF-1 (Sherr *et al.*, 1985). The v-*fms* gene encodes an altered CSF-1R, however the mechanism of the subsequent transformation remains unclear.

In contrast to the two previously mentioned virus-induced oncogenes, v-*fms* and v-*erb* B, the *neu* oncogene results from a chemically induced mutagenesis. A phosphoprotein was found to be specifically associated with the *neu* transforming

sequence (Padhy *et al.*, 1982), which is required to maintain the *neu* -induced transformation (Drebin *et al.*, 1984). Homology between *erb* B and *neu* was found using Southern blot hybridization (Drebin *et al.*, 1985), although related, *erb* B and *neu* are distinct genes, mapping to different human chromosomes (Schechter *et al.*, 1985). Nucleotide sequence analysis strongly supports the possibility that *neu* encodes a receptor like molecule, the predicted product is a transmembrane protein with a structure similar to EGFR (Bargmann *et al.*, 1986). There is 50% overall homology between EGFR and *neu* products with greater than 80% amino acid identity in the Tyr kinase domain. The sequence shows a hydrophobic signal sequence at the amino terminus (which is associated with membrane bound molecules) followed by an extracellular domain. The extracellular domain has two cysteine-rich areas, which are highly conserved in relation to both the insulin receptor and EGFR. The cytoplasmic region shows a high degree of homology with EGFR (82%), with *neu* Tyr residues in the same positions as EGFR phosphotyrosine sites. Additionally, there is a threonine (Thr) residue that may serve as a site for negative regulation of receptor expression (Bargmann *et al.*, 1986).

The nontransforming *neu* gene (the protooncogene) encodes a receptor for a factor involved in growth regulation, however the ligand for the cell-surface protein is as yet unidentified.

The isolation and subsequent characterization of oncogenes have linked their mechanisms of transformation with various aspects of growth factor activation. It seems that some oncogenes obtain their growth advantage by conferring growth factor independence on the transformed cells. This is possible at a number of points in the normal mitogenic pathway; the growth factor, its transmembrane receptor and intracellular signalling mechanisms. Oncogenes affecting each level are known (Heldin & Westermark, 1984; Sporn & Roberts 1985). Activation (that is the alteration of DNA sequence resulting in RNA expression of proto-oncogenes to "cancer genes") can occur via point mutations, amplification at the gene level, over-

expression at the RNA or protein level or chromosomal translocations or rearrangements (Cooper, 1982; Bishop, 1983; Duesberg, 1985; Klein & Klein, 1985). Examples of each type have been found in diverse systems, for example the epidermoid carcinoma cell line A-431 cells possess an amplified EGF receptor DNA sequence whose transcription produces an abundant and aberrant message which codes for the external portion of the receptor but lacks the transmembrane and cytoplasmic portions (Ullrich *et al.*, 1984). A suggested mechanism of transformation is that the continual binding of EGF to the aberrant receptor, without the regulatory processes imposed by the transmembrane and cytoplasmic domains, would result in unregulated growth (Sporn & Roberts 1985). Characterization of oncogenic evasion of growth regulation and an understanding of the developmental conditions under which these ligands function may help in the clarification of both carcinogenic and normal receptor-mediated mitogenesis.

1.5. EPIDERMAL GROWTH FACTOR

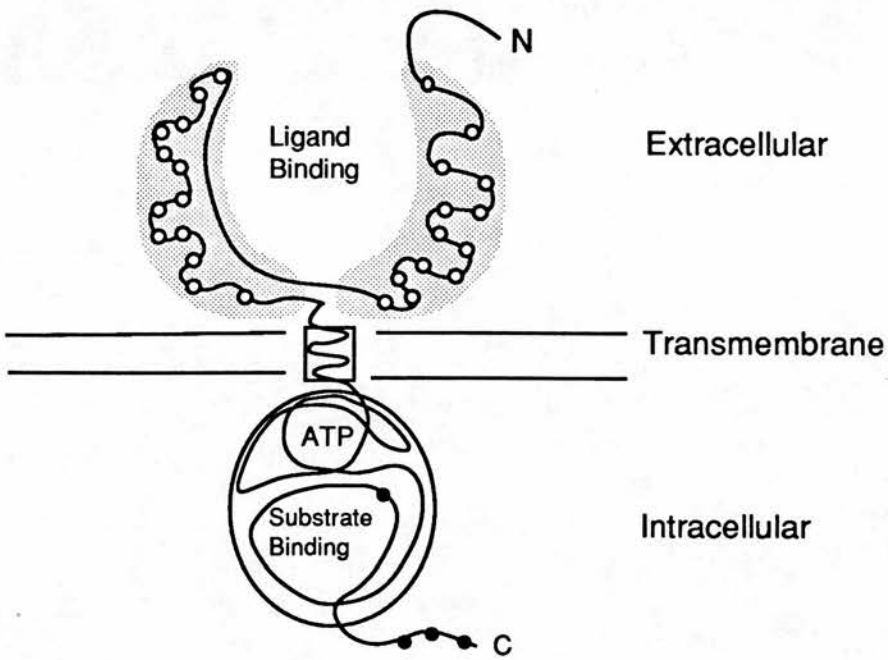
Since it became clear that the proliferation of cells *in vivo* and *in vitro* is controlled by hormones and growth factors, several growth factors have since been isolated and purified. One of the most characterized, EGF was first described by Cohen in 1962 as a peptide which would stimulate precocious eyelid opening and tooth eruption in newborn mice. Since then it has been realized that EGF may have a potential role in human disease (Carpenter *et al.*, 1978).

1.5.1 CHARACTERISTICS OF EGF

EGF was isolated and its amino acid sequence determined (Savage *et al.*, 1972) a decade after Cohen (1962) first described the extract from mouse submaxillary gland. Urogastrone (an inhibitor of gastric acid secretion) which was purified from human urine, was found to be almost identical to EGF in amino acid sequence and action (Cohen & Carpenter, 1975; Gregory, 1975). EGFs' ability to stimulate the growth of

cultured cells was soon recognised (Carpenter & Cohen, 1975; Hollenberg & Cuatrecasas, 1973). EGF is a single 6 kDa polypeptide chain of 53 amino acids, with 3 internal disulphide bonds (Taylor *et al.*, 1972). EGF is synthesized from a precursor (prepro EGF) which may be as large as 128 kDa (Gray *et al.*, 1983), and is composed of 7 EGF-like peptides (Scott *et al.*, 1983). Our understanding of the processing of the prepro EGF molecule is still very limited. In the mouse salivary gland the precursor molecule is broken down to the 53 amino acid mitogen, which is associated in the cell with several binding proteins in cytoplasmic granules (Kasselberg *et al.*, 1985). It is proposed that in other tissues (e.g. kidney) the EGF precursor is expressed as a transmembrane protein, but there is no indication of biological activity for the EGF precursor. EGF was originally thought to have a restricted range of tissue expression, but has now been described from a large variety of tissues (Waterfield, 1989).

EGF is mitogenic for a variety of cultured mesenchymal and epithelial cells. The mitogenic activity of EGF is strongly potentiated by other hormones such as insulin and IGF-1 (Rose *et al.*, 1975; Shipley *et al.*, 1984; Hiraki *et al.*, 1987), and it also acts in synergism with PDGF (Leof *et al.*, 1983). Aspects of differentiation are also induced following EGF treatment in certain *in vitro* cell lines and *in vivo* models (Oka *et al.*, 1983; Weissman & Aaronson, 1983). In addition to its mitogenic effect, EGF also induces various early and delayed responses in the cell. Early responses include stimulation of ion and nutrient transport (Hollenberg & Cuatrecasas, 1973), enhancement of the phosphorylation of endogenous membrane proteins (Carpenter *et al.*, 1979), induction of specific changes in the organization of the cytoskeleton (Schlessinger & Geiger, 1981) and changes in cell morphology (Chinkers *et al.*, 1979). Delayed responses include the activation of the enzyme ornithine decarboxylase (Stastny & Cohen, 1972) and enhancement of the biosynthesis of fibronectin (Chen *et al.*, 1977) and keratin (Rheinwald & Green,1977). Like other


N
Ligand
Binding
Extracellular
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ATP
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Binding
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growth factors, EGF induces a variety of cellular responses, the so called "pleiotropic response".

1.5.2 EGF RECEPTOR

Specific receptors in the cell membrane of target cells interact with EGF to initiate and maintain a complex series of biochemical and morphological events leading to cell growth (Carpenter *et al.*, 1975; Das *et al.*, 1977; Carpenter & Cohen, 1978). The EGF receptor was first purified from A-431 cells, a cell line which has an unusually high number of receptors for EGF (Fabricant *et al.*, 1977; Cohen *et al.*, 1982., Kawamoto *et al.*, 1983). In the initial studies, the purified receptor had an apparent molecular weight of 150 kDa; but subsequent purification methods that eliminated calcium from the assay buffers indicated that the actual size of the receptor was 175 kDa (Cohen *et al.*, 1982). Further characterization demonstrated that the receptor is an integral membrane protein exhibiting an extracellular binding domain that serves to bind the ligand EGF, and other EGF-like molecules, a transmembrane region and intracellular domain. A schematic diagram of the EGF receptor is shown in Figure 5.

The extracellular domain of the EGF receptor which accommodates high affinity binding is characterized by two structural features: a high cysteine content (approximately 10%) dispersed in two clusters and a relatively large number of canonical sequences for *N* -linked glycosylation. The extracellular cysteine residues are more likely to occur in the form of disulphide bonds. The ligand binding domain of the EGF receptor is also characterized by a relatively high level of *N*-linked carbohydrate (Soderquist & Carpenter, 1984).

FIGURE 5: A schematic illustration of the EGF receptor showing the extracellular, transmembrane and cytoplasmic domains. Indicated are amino (N) and carboxy (C) terminal ends of the receptor polypeptide chain, potential binding sites for the ligand ATP and endogenous substrates. Cysteine residues (open circles) that may play a role in forming the ligand binding pocket and tyrosine residues (filled circles) involved in receptor function are emphasized (after Yarden & Ullrich, 1988).

The cytoplasmic portion of the EGF receptor has been intensively studied since the Tyr kinase activity (that is encoded in this region of the molecule) was considered to be the primary effector system in the transmembrane signalling process. Tyr kinase activity is present in several other growth factor receptors and in several oncogene products (Anzano *et al.*, 1982). There is a large region in the cytoplasmic domain that shares sequence homology with other members of the Tyr kinase family (the src kinase of the Rous sarcoma virus). This region, considered to constitute the catalytic domain (Tyr kinases) of the receptor, contains the binding site for ATP.

Immediately after the membrane-spanning domain of the EGF receptor, at the cytoplasmic interface, is a 13-residue sequence which is highly enriched in basic amino acids. This sequence probably functions as a "stop transfer" sequence following the insertion of the amino-terminal portion of the EGF receptor polypeptide chain into the lumen of the endoplasmic reticulum. It is a common feature of other growth factor receptors and is probably important for the correct translation and membrane insertion of the receptor. This region of the EGF receptor also contains a Thr residue, which has been shown to be a target for protein kinase C phosphorylation (Hunter *et al.*, 1984; Davis & Czech, 1985) and may therefore play a role in signal modulation.

The catalytic portions of the EGF receptor contains an ATP binding site and a carboxy-terminal region that includes the Tyr kinase domain. Proteolysis experiments suggest that a hinge region separates the hydrophilic tail from the adjacent Tyr kinase domain (Gullick *et al.*, 1985; Ek & Heldin, 1986). In response to EGF the receptor is capable of autophosphorylation on Tyr residues (Dailey *et al.*, 1978; Gill & Lazar, 1981).

EGF receptor function and regulation

The EGF receptor can be viewed as a transmembrane allosteric enzyme, whose activity is regulated by noncatalytic domains. With the exception of haemopoietic cells, nearly all cell types have been reported to display some level of EGF binding

capacity (Carpenter & Cohen, 1979). Human fibroblasts have between 40 000 and 100 000 specific EGF receptors per cell, while the human epidermoid carcinoma cell line A-431 has over 3 million receptor sites per cell. It is not clear what role the expression of such a large number of EGF receptors plays, however over expression of the EGF receptor has been reported for a substantial number of carcinoma cell lines and tissues (Inman & Carpenter, 1986).

The initiation of cellular responses by EGF is a direct result of the growth factor binding to specific membrane receptors. Cells which are exposed to increasing concentrations of EGF gradually lose a substantial fraction of their receptors for the respective hormones. This down regulation depends upon ligand concentration, time and temperature. When the cells are returned to medium free of ligand, the number of receptors returns to normal within 8-16 hours (Carpenter & Cohen, 1976).

Quantitiative binding experiments with radiolabelled EGF indicate that the stoichiometry of ligand binding to the EGF receptor is 1:1 (Weber *et al.*, 1984). EGF binding sites appear to be functionally heterogeneous with different receptor classes, that have distinct affinities toward EGF. High affinity EGF receptors with an apparent K_d of 1-3 x 10^{-10}M comprise 5-10% of the total receptors, whilst the remaining low affinity receptors have an apparent K_d of 2-15 x 10^{-9}M (King & Cuatrecasas, 1982; Kawamoto *et al.*, 1983; Schlessinger, 1988). However, the occurrence of two classes of EGF binding site is not a uniform finding, and the interpretation of more than one binding site may well be due to difficulties in analysing binding data. Scatchard analysis, which is usually employed for the analysis of binding data is not ideal as it unevenly "weights" the lowest ligand concentrations. This makes interpretation of binding data difficult, and is prone to error when more than one binding site is predicted (Bennett & Yamamura, 1985).

After the growth factor binds to the receptor, the receptors aggregate and become localized into coated pit regions on the cell surface (Gorden *et al.*, 1978). It is not known whether the process of microaggregation may be a relevant mechanism for the

induction of the mitogenic activity of EGF (Schlessinger, 1980; Shechter *et al.*, 1978; Schlessinger, 1979), nor is it clear whether internalization of aggregated EGF-receptor complexes is required for the biological activities of the growth factor. Following internalization, the clustered EGF-receptor complexes pinch off by an energy-dependent process and form coated (and non-coated) vesicles. The endocytic vesicles are processed inside the cells and EGF is degraded by lysosomal enzymes, although in some cases the internalized receptors appear to be recycled to the cell surface with the gradual release of recycled ligand into the medium (Teslenko *et al.*, 1987).

The binding of the growth factor to its receptor induces a conformational change in the receptor molecule. This conformational change enhances the activity of the protein kinase, causing a rapid phosphorylation of several cellular proteins, including the receptor itself (Carpenter *et al.*, 1978; King *et al.*, 1980; Cooper *et al.*, 1982; Gates & King, 1982; Sawyer & Cohen, 1985). Both ATP and GTP can act as the phosphate donor of the phosphorylation reaction and either Mn^{2+} or Mg^{2+} is required for optimal activity of the protein kinase (King *et al.*, 1980). The majority of EGF receptor autophosphorylation sites are located at the carboxy-terminus, with the major site being just 13 residues from the end of the molecule (Downward *et al.*,1984). It is generally believed that Tyr phosphorylation is the major signalling event directed by the EGF receptor. The fact that other growth factor receptors and a large number of oncogene products demonstrate Tyr kinase activity strengthens the argument that Tyr kinases have a principal role in growth factor regulated activities. Activation of Tyr kinase activity and autophosphorylation are believed to represent the primary molecular events initiating the transmission of the mitogenic signal inside the cell, because receptor mutants devoid of kinase activity are defective in the transduction of the mitogenic effect of EGF (Livneh *et al.*, 1986). Phosphorylation of the EGF receptor by protein kinase C, specifically on Thr-654, results in a direct reduction of the EGF receptors' affinity for its' ligand and diminishes the autophosphorylation activity of the receptor

(Hunter & Cooper, 1985). The mechanism(s) by which these modulations occur is not clear.

EGF receptor transmodulation by heterologous ligands

The EGF receptor is envisaged as an "allosteric" protein where various ligands (allosteric regulators) can bind at various functional sites on the molecule and modulate the binding and response to EGF. The ligand binding affinity and the protein Tyr kinase activity of the EGF receptor can be regulated by the phorbol ester myristate acetate (PMA) or by growth factors such as bombesin or PDGF, which bind to specific receptors (Schlessinger, 1986). Both PMA and PDGF induce phosphorylation of Thr-654 of the EGF receptor by activating the Ca^{2+}-dependent protein kinase C. It was suggested that the phosphorylation state of this residue may play a role in receptor transmodulation (Hunter & Cooper, 1985; Schlessinger, 1986). Indeed, by the use of receptor mutants it was established that phosphorylation of Thr-654 by protein kinase C and protein kinase C-independent mechanisms (Friedman & Rosner, 1987) operates as a negative control mechanism for EGF induced mitogenesis, by reducing the affinity of the receptor for its ligand and diminishing the autophosphorylation activity of the receptor (Hunter & Cooper, 1985).

1.5.3 THE TRUNCATED EGF RECEPTOR

The v-*erb* B oncogene of AEV which encodes for the truncated EGF receptor is described in section 1.4.2. This truncated receptor, which is devoid of most of the extracellular ligand binding region and 13 amino acids from the C-terminus may act as a constitutively active EGF receptor (Downward *et al.*, 1984a; Ullrich *et al.*, 1984; Kris *et al.*, 1985). However, Riedel *et al.* (1987) have shown that a chimeric receptor composed of the extracellular and transmembrane regions of the human EGF receptor attached to the cytoplasmic region of avian v-*erb* B was still able to transform cultured rat-1 cells. Moreover, the binding of EGF to the chimeric receptor augmented its' transforming activity. This suggests that, in addition to the extracellular and C-

terminal deletions in v-*erb* B, structural differences between v-*erb* B and the cytoplasmic region of the EGF receptor may be major factors influencing the transforming potential of v-*erb* B (Riedel *et al.*, 1987).

1.5.4 TRANSFORMING GROWTH FACTOR α

The EGF receptor serves as the binding site not only for EGF but also for other polypeptide growth factors. The most interesting in terms of their association with the transformed state are the transforming growth factors, alpha and beta (TGFα & TGFβ). TGFs were first identified by Todaro and co workers in 1976 when they observed that cells transformed by murine sarcoma viruses had reduced or absent cell surface receptors, when compared to the untransformed counterparts or with cells transformed by other viruses. It was subsequently shown that sarcoma virus transformed cells produced a family of polypeptide growth factors which bound to the EGF receptor and induced cell proliferation. In addition to the mitogenic properties, the sarcoma growth factor (SGF) as it was then called, induced normal fibroblasts to grow in soft agar and to express phenotypic properties of transformed cells. SGF was subsequently found to consist of both TGFα and TGFβ, with the binding of the EGF receptor solely due to TGFα, and morphological changes of the fibroblasts being due to the co-operative effect of TGFα and β. As outlined in section 1.4.1, TGFs have been implicated in the autocrine growth of tumour cells (Sporn & Roberts, 1985) and were shown to be produced by different human tumour lines (DeLarco & Todaro, 1978; Ozanne *et al.*, 1980; Roberts *et al.*, 1980; Sporn & Todaro, 1980; Todaro *et al.*, 1980; Kaplan *et al.*, 1982; Marquardt & Todaro, 1982; Halper & Moses, 1983; Roberts *et al.*, 1983; Moses, 1984; Salomon *et al.*, 1984a, 1986; Dickson *et al.*, 1986). Moreover, similar TGFs were detected in mouse embryos (Proper & Moses, 1981) and foetal calf serum (Bjoroson & Moses, 1980). The TGFαs from these sources were isolated and were found to have the following properties; (i) their molecular weights range from 5 kDa to 24 kDa, (ii) they are heat stable polypeptides

containing disulphide bonds, (iii) they compete for binding with [^{125}I]-EGF for the EGF receptor, and (iv) they induce DNA synthesis and colony formation in soft agar.

TGFα is a Mr 5600 single chain polypeptide with 3 intrachain disulphide bonds. It shares 30-35% sequence homology with human and mouse EGF (Marquardt *et al.*, 1984) and competes with [^{125}I]-EGF for binding to the EGF receptor (Pike *et al.*, 1982; Massague, 1983). TGFαs and EGF show a variety of biological actions similar to EGF, indeed their biological activities when assayed in cell culture systems are almost identical (Bascom *et al.*, 1989), although in some cases TGFαs are more potent than EGF (Stern *et al.*, 1985; Ibbotson *et al.*, 1986).

The secretion of biologically active TGFα occurs via a membrane-associated intermediate (Bringman *et al.*, 1987; Teixido *et al.*, 1987). The 50 amino acid form of TGFα is derived from a 160 amino acid precursor which is anchored to the cell surface via its C-terminus. As well as the 50 amino acid TGFα mitogen, higher molecular weight forms are released from the N-glycosylated 160 amino acid precursor. No biological activity has been attributed to the surface associated TGFα precursor, but unprocessed membrane bound TGFα could still be available for interaction with the EGF receptor, thus permitting a role for TGFα in direct cell-to-cell signalling processes.

The presence of transforming growth factors in mouse embryos and in foetal calf serum suggest that both foetal development and neoplastic transformation may be affected by similar growth factors (Sporn & Todaro, 1980). Sporn & Todaro (1980) suggested that malignant transformation may result from the inappropriate expression of growth factors which are required in normal early embryogenesis. Nevertheless, the precise role of TGFα in neoplasia remains uncertain as recent evidence suggests that TGFα production occurs in normal adult epithelial cells that are responsive to the growth factor, suggesting the possibility of normal autocrine regulation of cell proliferation (Bascom *et al.*, 1989).

EGF itself is capable of inducing certain aspects of the transformed phenotype (Carpenter *et al.*, 1979). It has been shown to enhance both the incidence of transformation of granulosa cells by Kirsten sarcoma virus (Harrison & Auersperg, 1981) and the carcinogenic effect of skin tumours in mice, the latter by chemical induction (Rose *et al.*, 1976).

1.5.5 EGF RECEPTOR LEVELS IN CANCER CELLS

Many workers have sought to correlate the level of EGF receptors to cancer, although the results of such studies have failed to reveal a simple correlation between the two. In some cases EGF receptor levels are higher in malignant as compared to normal tissues; e.g. several different squamous cell lung carcinomas express high concentrations of EGF receptor levels compared to the normal lung. In contrast, other lung carcinomas have undetectable EGF receptor levels (Todaro *et al.*, 1976; Hirata *et al.*, 1983; Hendler & Ozanne, 1984). Similarly, increased levels of EGF receptors are found in some breast cancer cells as compared to normal cells, whilst in others the levels of EGF receptors are reduced (Fitzpatrick *et al.*, 1984).

EGF receptor concentration appears to vary according to the cell type and the stage of differentiation of the tumour. Poorly differentiated bladder and breast tumours express higher levels of EGF receptors (Neal *et al.*, 1985; Sainsbury *et al.*, 1985) than other histological grades of the cancer. In the case of prostatic tumours, EGF receptor levels are highest in well differentiated tumours and undetectable in those which are poorly differentiated (Maddy *et al.*, 1989).

It is clear from the above studies that EGF receptor levels may be decreased, increased, or not changed in cancer cells as compared to their normal counterparts. This demonstrates the complexity of the pathways involved resulting in transformation of cells.

1.6 MODEL SYSTEMS FOR THE STUDY OF PROSTATE CANCER

Our understanding of cancer has been enhanced by the use of both *in vivo* and *in vitro* model systems. The ideal model would reproduce the pathology seen in the human disease, demonstrate metastasis, be capable of reproducible replication, be sensitive to hormonal manipulation and be amenable to quantitative analysis. With the multiplicity of conditions to be met and the complexity of the natural disease it is easy to draw erroneous conclusions from an oversimplified model system. It is evident that some of the above parameters cannot be readily met with one model alone, and therefore a combined approach, using several model systems is more appropriate.

In several instances normally occurring disease in lower animals have provided model systems with which significant progress has been made in our understanding of cancer. However, no animal model system is presently available for the study of human prostatic neoplasia which satisfies the broad range of requirements listed above. Spontaneous adenocarcinoma of the prostate has rarely been reported amongst nonhuman mammals. Of the common laboratory animals, certain strains of aged rats appear to have the highest instance of spontaneous prostatic tumours, and the establishment of transplantable tumours offers a useful model system.

The Copenhagen rat Dunning tumour model, a spontaneous tumour found in a Copenhagen breeder rat (Dunning, 1963), is perhaps the most useful of such animal models. The tumour has been found to resemble human prostatic cancer in its histologic appearance, ultrastructure, and most significantly in its' metastatic capabilities (except that it does not metastasize to the bone). Although there are difficulties with this model due to variations in the embryology, histology and biochemistry of the animal prostate, it remains an unquestionably useful model and provides valuable information on prostatic tumour progression from the initiated cell(s) to the metastatic progeny. With the exception of the aged rat, spontaneously occurring

neoplasms occur with frequency only in man and the dog. Metastases occur amongst dogs with prostatic carcinoma, while bone metastases are not unusual.

1.6.1 HUMAN PROSTATE TUMOURS *IN VITRO* AND *IN VIVO* MODELS

There are several approaches taken for performing *in vitro* and *in vivo* studies on human prostatic carcinomas; namely organ explant cultures, short term primary cultures and cultures of established cell lines.

Organ culture of the prostate

Organ culture originated as a means for studying the interrelationships between different types of tissue in the same organ during growth and differentiation with the maintenance of normal cell interrelationships, but free of the more complex organ interactions or even tissue interactions common *in vivo*. There are many advantages to this model namely: (i) the maintenance of organ architecture plus tissue and cell relationships; (ii) long term maintenance of epithelial cells; (iii) the possibility to study mechanisms of growth control and phenotypic expression; (iv) the possibility to study the direct effects of exposure to hormones and other agents singly and in combination, in the absence of host homeostatic mechanisms and (v) interactions between epithelial and fibroblast cells from human prostatic tissues can be studied. However, there are also limitations to this procedure: (i) heterogeneity of tissue samples; (ii) alteration in phenotypic expression in one or more cell types; (iii) complications resulting from infected tissues; (iv) absence of systemic effects; (v) lack of systemic metabolism of a drug to its active form, and (vi) the tendency toward homogeniety of cell populations due to degeneration, necrosis or overgrowth (Merchant, 1976; Sandberg & Kadohama, 1980; Heatfield, 1987).

Primary cultures of the prostate

Short term primary culture comes some way between explant cultures and established cell lines. Primary culture is a culture started from cells, tissues or organs taken directly from organisms. The many advantages to this model include: (i) an

opportunity for studying prostatic cells in a state in which the cell populations are representative of the tissue of origin; (ii) the investigation of changes in nutritional, environmental or other factors (Merchant, 1987); (iii) the possibility to study stromal epithelial interactions and the signalling mechanisms involved between these two cell types, and (iv) the opportunity to study the direct effects of exposure to hormones and other agents singly and in combination. However, there are limitations to this procedure which are similar to those listed for organ culture, namely: (i) alteration in phenotypic expression in one or more cell types; (ii) complications resulting from infected tissues; (iii) absence of systemic effects, and (iv) lack of systemic metabolism of a drug to its active form. Other disadvantages exclusive to this model include: (i) the difficulty in separating and culturing homogeneous cell populations; (ii) lose of androgen sensitivity, and (iii) maintenance of viable cultures after sub-culturing (Peehl *et al.*, 1986).

Prostatic carcinoma cell lines

Human prostatic adenocarcinomas are notoriously difficult to culture *in vitro* or *in vivo* in immunodeficient animals (Lechner *et al.*, 1980; Reid *et al.*, 1981), which may be due to complex hormonal and nutritional requirements of prostatic cells. As a result few human prostatic tumour cell lines exist. Table 1 lists the characteristics of five established human prostatic cell lines reported in the literature (Kaighn *et al.*, 1979; Stone *et al.*, 1978; Horoszewicz *et al.*, 1983; Ito *et al.*, 1985). Out of the five lines, four originated from tumours recovered from metastatic sites rather than the primary prostatic tumour. Furthermore, only two out of five remain androgen-dependent, although the LNCaP line is androgen sensitive in culture.

TABLE 1: HUMAN PROSTATIC CARCINOMA CELL LINES

Cell line	Origin	Hormone-dependent[a]	Growth capacity *in vitro*	Growth capacity *in vivo* [b]	Metastatic capacity [c]
PC-3	metastasis to bone	no	yes	yes	poorly metastatic
DU 145	metastasis to brain	no	yes	yes	not metastatic
LNCaP	metastasis to lymph node	no	yes	yes	not metastatic
PC 82	primary	yes	no	yes	not metastatic
HONDA	metastasis to testicle	yes	no	yes	not metastatic

[a] Hormone-dependence indicates preferential growth in intact male nude mice, as compared with female or castrated nude mice. Preferential growth may be absolute or represent differences in growth rate.
[b] *In vivo* refers to growth in athymic nude mice.
[c] Metastatic capacity in athymic nude mice.

The apparent inability of these lines to metastasize in athymic mice eliminates the opportunity to study metastatic capacity. However, sublines of the PC-3 line which metastasize in nude mice are now available (Kozlowski *et al.*, 1984; Ware *et al.*, 1986; Ware, 1987). The DU 145 line has remained intractable to most manipulations of the host, although Kozlowski and co-workers (Kozlowski *et al.*, 1984) reported that implantation of Silastic tubes of β-estradiol into nude mice prior to DU 145-injection increased the incidence of spontaneous or experimental metastasis. Thus far no consistent metastasis in nude mice has been reported for the other human prostate tumour cell lines.

The PC-82 *in vivo* tumour model was the first transplantable human prostatic cancer in nude mice. It originated from a prostatectomy specimen, which was histologically classified as a moderately differentiated prostatic adenocarcinoma (Hoehn *et al.*, 1980). The tumour is androgen-dependent, secretes prostate acid phosphatase (PAP) and prostate-specific antigen (PSA) and has androgen receptors (Brinkmann *et al.*, 1987; van Steenbrugge *et al.*, 1988). However, attempts at establishing PC-82 *in vitro* have not been successful.

The PC-3 cell line was isolated from a bone metastasis of an adenocarcinoma of the prostate (Kaighn *et al.*, 1979). The characteristics such as growth rate, morphology, karyotype and ultrastructure are consistent with a poorly differentiated adenocarcinoma. It grows on soft agar and in suspension culture, it does not have any acid phosphatase activity, nor does it show 5α-reductase activity (Kaighn *et al.*, 1980). At the same time as the PC-3 was established, the inception of the DU 145 cell line was reported. This cell line, like PC-3, is also aneuploid and tumorigenic in nude mice and is similar to PC-3 ultrastructurally.

The DU 145 cell line

This thesis deals with the DU 145 cell line which was derived from a brain metastasis and was first described by Mickey *et al.* (1980). The metastasis was identified as a moderately differentiated adenocarcinoma, with foci of poorly differentiated cells. The cells are epithelial in appearance and form colonies in soft agar suspension culture. The cell line DU 145 also forms solid tumours in athymic nude mice which reveal a strong similarity to the original patient tumour, although in mice they do not metastasize. Cell line DU 145 is neither hormone-dependent nor hormone-sensitive as the cells grow equally well in media containing either foetal bovine serum or bull serum, which differ widely in their basic hormonal constitution. DU 145 reflects a weakly positive staining reaction for PAP by the method of Stonington *et al.* (1975) and had a modal chromosome number of 64 at passage 57. It also showed several marker chromosomes and a Y chromosome.

The cell lines DU 145 and PC-3 allowed for the first time studies on prostatic adenocarcinoma cell lines of proven prostatic origin that could be cultured indefinitely *in vitro*. Both of these cell lines are unresponsive to hormone treatment unlike the LNCaP cell line.

The LNCaP cell line

As a comparison with the DU 145 cell line this thesis also deals with the androgen sensitive LNCaP cell line whose properties are outlined below.

The LNCaP (Lymph Node Carcinoma of the Prostate) cell line was established from a metastatic supraclavicular lymph node of human prostatic adenocarcinoma (Horoszewicz *et al.*, 1983). LNCaP cells contain high affinity androgen receptors which are present in the cytosol and nuclear fractions of cells in culture and tumours. It is also the only prostatic cell line that shows androgen-responsive, but not dependent growth *in vitro*. Furthermore, *in vitro*, 5α-dihydrotestosterone and androgens modulate cell growth and stimulate PAP production (Horoszewicz *et al.*, 1983) and the cells express PSA. The malignant properties of LNCaP cells are maintained since athymic nude mice develop tumours at the injection site.

From the parental LNCaP line several sublines have developed either spontaneously or through clonal selection. Among these are the LNCaP-FGC (fast growing colony) which is similar to its parent line and differs only in growth rate. Lately from this subline at least two new lines have been developed, the FGC-GJ and FGC-JB lines, which show androgen responsiveness. The LNO subline which also originated from the parent cell line, contains high amounts of androgen receptors, but is nevertheless androgen-independent (van Steenbrugge *et al.*, 1988).

1.7 SERUM-FREE CELL CULTURE

Tumour cells generally have an altered response to growth factors that regulate the growth of normal cells. In cell culture, this change is reflected by a sharp decrease in the requirement for serum proteins (Holley & Kiernan, 1971). It is generally accepted that the growth of virtually all types of cells requires the presence of serum in the medium. However, as serum is a complex mixture of poorly characterized components the use of defined medium for cultured cells is advantageous. Several laboratories have now perfected the use of defined medium using either a stepwise

reduction of serum, by adjustment of low molecular weight constituents (Ham, 1981) or by replacement of serum by hormones and growth factors (Barnes & Sato, 1980). The observation that cell lines (which were clearly hormone-dependent *in vivo*) did not show hormone-dependent growth *in vitro* unless the serum added to the culture medium was first depleted of certain hormones, gave rise to the concept of serum providing a mixture of hormones which were stimulatory for cell growth (Sato *et al.*, 1975). In other words, hormones present in the untreated serum were stimulatory for cell growth and were masking the effects of any additional hormones which might be added. One of the main concerns in replacing serum in cell culture medium has been to find combinations of supplements which will support growth of the cells at a reasonable rate, without radically changing the properties of the cell lines. Although differences in the biochemical properties of some cell lines do exist in the two culture conditions (serum and serum-free medium), the differentiated functions expressed by lines in serum-supplemented media are in general also expressed in serum-free, hormone-supplemented media (Barnes & Sato, 1980).

It would follow by replacing serum in cell culture media by combinations of hormones, nutrients, binding proteins and attachment factors this would have several advantages for cell culture methodology. In most cases serum-containing medium is an unsuitable substitute for the *in vivo* environment, as it may be deficient quantitatively in one or more components essential for the survival and proliferation of some cell types. Serum might also be expected to be toxic at some concentration for most cell types, and it contains many substances which normally never come into contact with most cells of the body (Gospodarowicz *et al.*, 1979), since *in vivo* no cell types exist for extended periods in the presence of serum. It is however possible to replace serum in the culture medium for established cell lines (adapted to grow in serum-containing medium) by adding to the medium combinations of factors which can carry out the functions of serum. Several established cell lines representing widely

divergent cell types may be grown in medium supplemented with hormones, binding proteins and attachment factors (Barnes & Sato, 1980).

Serum-free systems also allow novel approaches to the recognition and treatment of disorders such as cancer. Defined media have been used to explore the hypothesis that some of the properties of neoplastic cells are related to a loss of requirements for stimulation by one or more hormones or growth factors which are necessary in order to stimulate growth of normal cells (Cherington *et al.*, 1979; Rockwell *et al.*, 1980).

1.8 SUMMARY

Since the realization that androgens probably play a permissive role in the initiation of prostatic cancer and that other "factors" may be involved, the interest in growth factors has blossomed. One of the first investigations of the role of growth factors in the growth and regulation of the prostate gland was centred on the detection of endogenous EGF (Hirata & Orth, 1979). Since then, EGF receptors have been located in human prostatic tissue (Maddy *et al.*, 1987, 1989; Eaton *et al.*, 1988). Maddy *et al.* (1989) has subsequently shown that prostatic cancer tissue has a reduced capacity to bind EGF when compared to benign tissue, with a depletion in the number of binding sites correlating with the loss of differentiation of the tumour.

Although the action of androgens in initiating prostatic cancer may be permissive rather than direct, androgens may act together with growth factors to regulate growth in the prostate. In other model systems there is evidence for steroid hormone regulation of growth factor content and activity and a similar relationship might exist in the prostate, as recent *in vivo* (Traish & Wotiz, 1987; St-Arnaud *et al.*, 1988) and *in vitro* (Schuurmans *et al.*, 1988; Wilding *et al.*, 1989) studies suggest.

There is little doubt that growth factors and their receptors are associated with prostatic cancer, but it is probable that not one but several growth factors may be involved in the development and progression of prostatic cancer and that growth factors are pluripotent in their response (Sporn & Roberts, 1988); how is unclear.

Several mechanisms have been proposed by which a cell could evade the normal cellular growth control: (a) the activation of autologous growth factor synthesis ('autocrine' activation); (b) synthesis of an altered growth factor receptor; or (c) activation of a post-receptor pathway that bypasses the growth factor requirement. There is now much circumstantial and direct evidence to support the hypothesis of autocrine secretion originally proposed by Sporn & Roberts (1985). Many types of tumour cells release polypeptides (such as TGFα) into their conditioned medium, and these same tumour cells often possess functional receptors for the secreted peptide. Autocrine secretion would allow tumour cells to evade normal cellular growth control by producing and responding to the secreted growth factor.

An unregulated mitogenic signal might also be initiated by oncogene products functioning as growth factor receptors. The expression of the *erb* B oncogene product which codes for a truncated version of the EGF receptor (devoid of the ligand binding domain), is thought to result in an uncontrolled proliferative response. Similarly, amplification of the EGF receptor DNA sequence, resulting in over-expression of the EGF receptor product would lead to unregulated growth. It has also been proposed that activation of a post-receptor pathway that bypasses the growth factor requirement might result in an unregulated mitogenic effect, leading to transformation. As yet there is no evidence for such a mechanism.

Clearly, an understanding of the developmental conditions under which the evasion of normal growth regulation takes place would help clarify carcinogenic receptor-mediated mitogenesis.

Our understanding of human neoplastic disease has been enhanced by the use of *in vivo* and *in vitro* model systems. In the case of prostatic cancer some valuable information has been obtained from animal models, but one must be cautious in extrapolating such results to the human system. Of the human models available, *in vitro* prostatic carcinoma cell lines are particularly useful as they permit the possibility of studying directly the effects of exposure to hormones and other agents, singly or in

combination. Unfortunately, very few prostatic carcinoma cell lines are available since they are notoriously difficult to culture. The LNCaP cell line is the only one which is androgen-responsive in culture, the remainder being insensitive to hormonal manipulation. Consequently, very little information has been obtained on prostatic neoplasia, in particular on the factors involved in the transition of prostatic tumours from a hormone-responsive to an unresponsive state.

Of those prostatic cell lines available, the carcinoma cell lines DU 145 and LNCaP provide an opportunity to study the growth responses to EGF-like factors and their receptors in an androgen-unresponsive (DU 145) and androgen-responsive (LNCaP) system. It is particularly useful to carry out such a comparative study since it is not inconceivable that the progression of tumours to an androgen-insensitive state might be due to altered regulation of growth factors and/or their receptors.

The use of serum-free cell cultures was an important consideration in this study since serum is a mixture of poorly characterized components which is normally stimulatory for cell growth. Thus, the effect of hormones or growth factors added to cell cultures may be masked by those present in the untreated serum. More importantly, serum-free medium has provided the opportunity to study the loss of requirement for a particular growth factor or hormone, thought to be an important property of neoplastic cells.

1.9 Objectives of the present investigation

Thus the objectives of this study were:

1. To investigate and compare the growth responses by EGF-like molecules on the androgen-insensitive DU 145 and androgen-sensitive LNCaP cell line.

2. To characterize the EGF receptor using radio ligand binding and investigate the expression of the truncated EGF receptor.

3. To investigate the relationship between androgens and EGF in relation to EGF receptor expression and activity.

4. To investigate and characterize EGF-like molecules produced by autologous growth factor synthesis.

Chapter 2

MATERIALS & METHODS

2.1 MATERIALS

2.1.1 CELL LINES

DU 145

The cell line DU 145 was obtained from Dr D. D. Mickey, Department of Urology, University of North Carolina, Chapel Hill, U.S.A. DU 145 is a long-term culture cell line derived from a human prostatic adenocarcinoma metastatic to brain (Mickey *et al.*, 1980). The cells arrived at the 50th passage and were immediately subcultured. Cells between passage numbers 60-70 were used for all experiments.

LNCaP

The LNCaP cell line (derived from a fast growing colony, FGC) was kindly donated by Dr C. Eaton, Tenovus Institute, Cardiff, Wales. The LNCaP cell line was originally derived from a lymph node carcinoma of the prostate. The cells used in this study were a subline of the original parent LNCaP (Horoszewicz *et al.*, 1983). This subline is similar to its parent line and differs only in growth rate. Cells from passage numbers 75-85 were used for all experiments in this investigation.

2.1.2 POLYPEPTIDE GROWTH FACTORS

Epidermal growth factor from mouse submaxillary gland (mEGF; receptor and tissue culture grade) was purchased from Collaborative Research (c/o Universal Biologicals Ltd., St. Ann's Rd., London). Rat transforming growth factor alpha (rTGFα) and human EGF (hEGF) or urogastrone were kindly donated by Dr H.

Gregory, ICI, Macclesfield, U.K. rat TGF-I (rTGF-I) was purchased from Peninsula Laboratories Europe Ltd., St. Helens, Merseyside, U.K. as part of a radioimmunoassay kit (RIA). Venom nerve growth factor (NGF) was purchased from Sigma as electrophoretically pure.

2.1.3 HORMONES

Human growth hormone (hGH) was obtained from NIADDK, Bethesda, MD, U.S.A. The synthetic androgens 7α,17α-dimethyl-19-nortestosterone (DMNT; Mibolerone) and 17α-methyl-17β-hydroxy-estra-4,9,11-triene-3-one (R1881) were purchased from Amersham International plc, Berks, U.K and Du Pont, New England Nuclear, Boston, U.S.A. respectively.

2.1.4 RADIOCHEMICALS

[Methyl-^{3}H] Thymidine; specific activity 74GBq/mmol.

[^{125}I]-mEGF; specific activity 3.7MBq/μg.

[^{125}I]-hEGF; specific activity 49TBq/mmol.

The above radiochemicals were supplied by Amersham International plc.

[^{125}I]-rTGF-I was obtained from Peninsula Laboratories Europe Ltd. as part of a RIA kit.

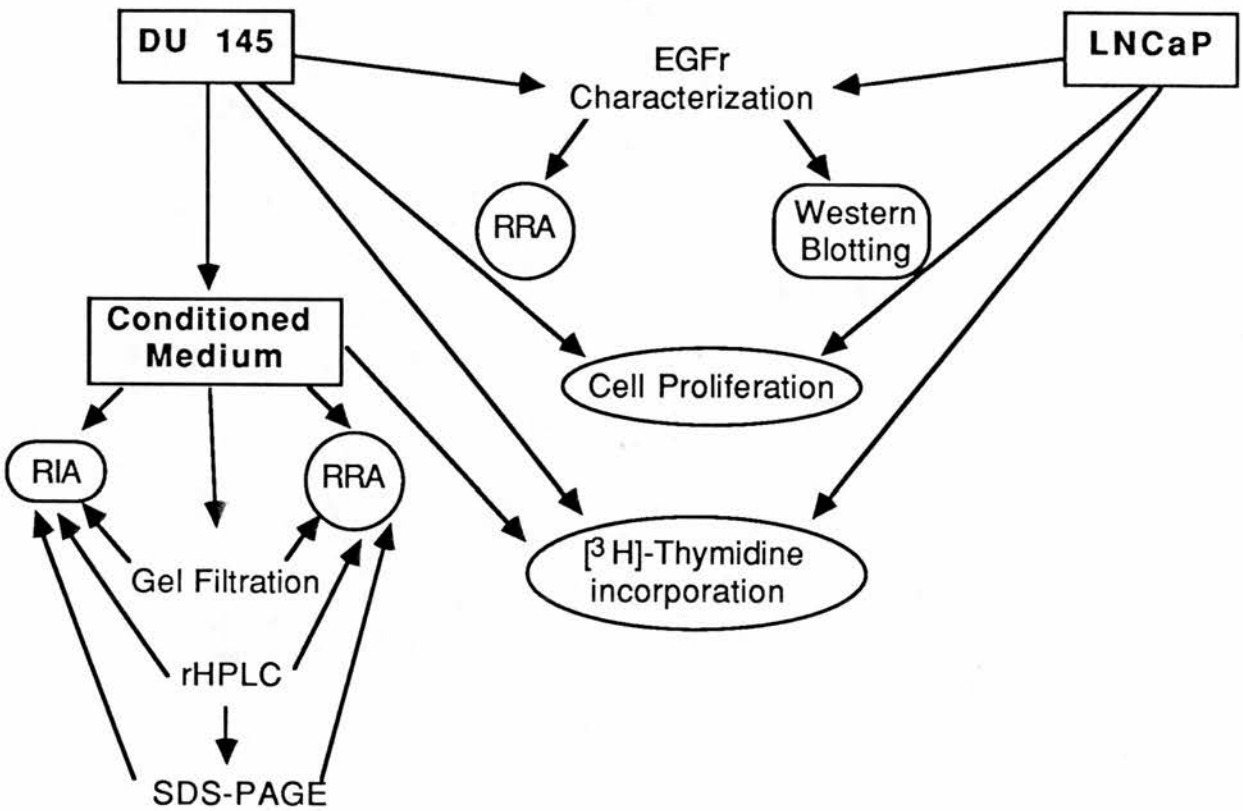
2.1.5 ANTIBODIES

The mouse monoclonal antibody (Mab) EGFR 1 of the IgG class was obtained from Amersham International plc. This Mab is directed against the native extracellular domain of the 175 kDa EGF receptor.

The mouse Mab F4 was kindly donated by Dr W. Gullick, Department of Oncology, Hammersmith Hospital, London, U.K. This Mab was produced to a synthetic peptide consisting of residues from the cytoplasmic domain of the EGF receptor.

Anti rabbit human EGF was used as part of a RIA for detection of hEGF and was kindly donated by Dr H. Gregory, ICI, Macclesfield, U.K.

Anti rTGF-I, a rabbit anti rat TGF-I antiserum was purchased from Peninsula Laboratories Europe Ltd. as part of a RIA kit.


DU 145
LNCaP
EGFr
Characterization
RRA
Western
Blotting
Conditioned
Medium
Cell Proliferation
RIA
RRA
[3 H]-Thymidine
incorporation
Gel Filtration
rHPLC
SDS-PAGE

METHODS

2.2.OUTLINE OF METHODS

Figure 6 gives an overview of the analytical techniques applied to the cell lines DU 145 and LNCaP. The first part of the investigation involved studying the effects of EGF-like growth factors on cell proliferation and DNA synthesis, using the techniques of cell enumeration and [^{3}H]-Thymidine incorporation. In the second part of the study the EGF receptor was characterized using a radio-ligand exchange assay with [^{125}I]-EGF (RRA) and by Western blotting. Thirdly, EGF-like bioactivity of medium conditioned by DU 145 cells (conditioned medium; CM) was analysed using an EGF radio-receptor competition assay (RRA) and [^{3}H]-Thymidine incorporation. EGF and TGFα immunological activity in the CM was analysed using RIAs for the detection of hEGF and rTGF-I. EGF-like components in the CM were partially purified using gel filtration, reverse-phase high pressure liquid chromatography (rHPLC) and sodium dodecyl sulphate - polyacrylamide gel electrophoresis (SDS-PAGE).

FIGURE 6: Outline of experimental techniques used in investigations with the cell lines DU 145 and LNCaP.

2.3 CELL CULTURE

2.3.1 CELL CULTURE MEDIA

DU 145 and LNCaP cells were cultured in complete media and serum-free media (SFM), as described below. RPMI-1640 (Flow Laboratories, Irvine, Scotland), which is a basic nutrient medium consisting of various amino acids, glucose, vitamins, inorganic salts, buffering salts and pH indicator, was used for both media.

2.3.1.1 Complete medium

To RPMI-1640 was added sterile 10% foetal calf serum (Gibco, Paisley, Scotland), 1% of the amino acid L-glutamine (Flow laboratories) and the antibiotics penicillin (100 units/ml; Gibco) and streptomycin (100 µg/ml; Gibco). The medium was aliquoted into volumes of 100 ml and stored at -20°C in sterile glass bottles which had previously been autoclaved at a pressure of 15 lb/in^2 for 20 mins.

2.3.1.2 Serum–free medium

The serum-free supplemented medium used in this study was a modification from that used by Kaighn *et al.* (1981) and Barnes & Sato (1980) for the culture of the prostatic cell line PC-3, as this cell line has similar characteristics to DU 145.

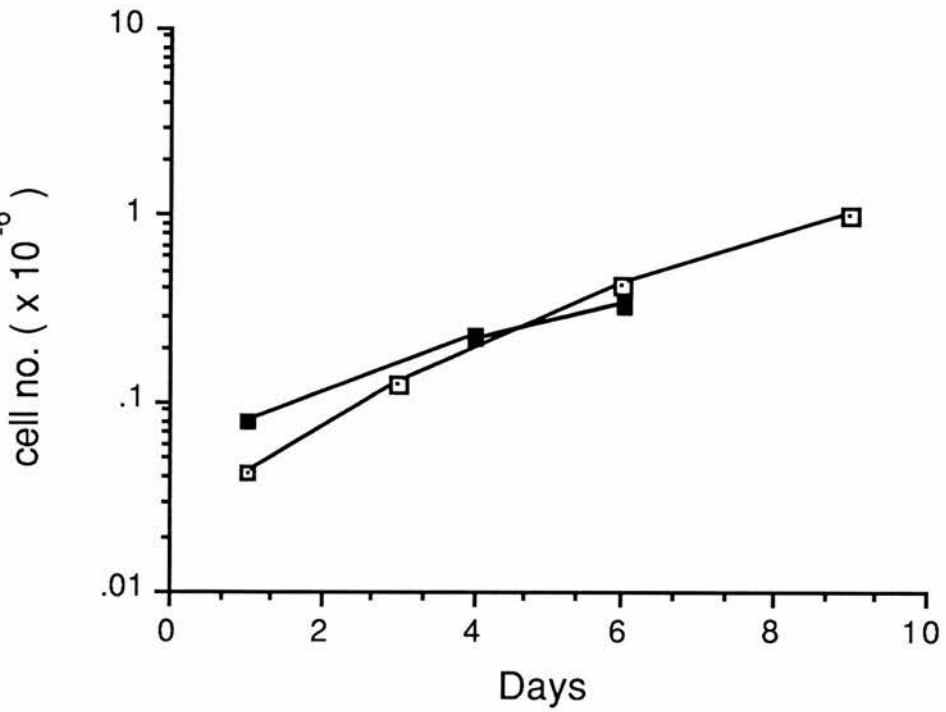
The basic nutrient medium, RPMI-1640 enriched with trace elements was used instead of PFMR-4 (Kaighn *et al.*, 1981). Stock solutions of the constituents of the SFM were made up in RPMI-1640 or ethanol and were filter sterilized (0.2 µm; Gelman Sciences Inc. Ann Arbor, Michigan, U. S. A) before addition to the medium. Insulin (Sigma Chemicals, Poole, Dorset), transferrin (Sigma) and phosphoethanolamine (Sigma) were made up in RPMI-1640 and stored in aliquots at -20°C. Both insulin and transferrin were added to RPMI-1640 at a concentration of 10 mg/l and phosphoethanolamine at a concentration of 50 µg/l. Hydrocortisone (100 x stock; Sigma) and 3,3',5-triiodo-thyronine (1000 x stock; Sigma) were dissolved in 100% ethanol and were added to the medium at concentrations of 1 mg/l and 2 ng/l

respectively. Trace element mix (Gibco) was reconstituted in 10 ml of sterile water and 0.1% of this solution added to the medium. RPMI-1640 was also supplemented with the amino acid L-glutamine (1%) along with the antibiotics penicillin (100 units/ml) and streptomycin (100 μg/ml). The medium was aliquoted into volumes of 100 ml and stored at -20°C in sterile glass bottles which had previously been autoclaved at a pressure of 15 lb/in^2 for 20 mins.

2.3.2 CULTURE OF CELL LINE DU 145

The cell line DU 145 was routinely maintained in complete medium, but for the purposes of this study a serum-free line was developed, by repeated subculture at high density, of DU 145 in RPMI-1640 SFM. The cell line DU 145 was maintained at 37°C in a humidified atmosphere of 95% air and 5% CO_2. All tissue culture preparations were performed under sterile conditions in a sterile air cabinet (Microbiological Class II; Howarth Air Engineering Ltd.). Cells were grown in monolayer culture in 75cm^2 tissue culture flasks (Corning, Staffordshire, England), and rapidly growing cultures were routinely harvested by trypsinization and passaged once per week 1: 3 (1.5 x10^6 cells/75cm^2 flask). This procedure was performed by disrupting the cells, which adhere firmly to the plastic tissue culture flasks by rinsing in sterile Dulbecco 'A' phosphate-buffered saline (Dulbecco 'A' PBS; Oxoid Ltd., England) and once with 0.25% trypsin and 0.02% EDTA (Gibco). The cells were then incubated for 5 mins at 37°C and subsequently resuspended in 45 ml of SFM supplemented with 0.5% foetal calf serum (0.5% FCS). FCS was added to the SFM to facilitate plating. The cells were dispersed by pipetting to obtain a single cell suspension and were then seeded 1: 3 into 75cm^2 flasks. The cells were subsequently maintained in SFM without FCS (15 ml/flask).

The culture of DU 145 in SFM yielded slow but sustained growth, the population doubling time for DU 145 was approximately 36 hours in 10% FCS, but cells cultured


10
1
.1
.01
cell no. (x 10^-6)
0
2
4
6
8
10
Days

in SFM reduced the growth rate to approximately 60 hours (Figure 7). Growth of this cell line in SFM was also population dependent (Kaighn *et al.*,1981), cell densities below 1 x 10^4 cell/cm^2 adhered to the culture flask, however the cells would lift off when SFM without FCS was added. Densities of (2 x 10^4- 4 x 10^4/cm^2) were chosen as optimal for growth experiments with EGF.

2.3.3 CULTURE OF CELL LINE LNCAP

The cell line LNCaP was routinely cultured in complete medium. For this study, attempts were made to culture in SFM by repeated subculture at high density. However, due primarily to lack of time and problems with culturing LNCaP (these cells adhere loosely to the culture vessels) this cell line was maintained in complete medium, but all growth and receptor binding experiments were carried out in SFM after plating in complete medium. LNCaP cells were maintained at 37°C in a humidified atmosphere of 95% air and 5% CO_2 in 75cm^2 plastic flasks (Corning). This cell line was passaged routinely once per week 1:3 (1.5 x10^6 cells/75cm^2 flask with 15 ml/flask). LNCaP cells were subcultured using a solution of sodium citrate (0.08M) instead of trypsin which is less harsh, as the cells are easily dislodged and re-attachment is slow. The flask of cells was rinsed with Dulbecco 'A' PBS, once with a 0.08M solution of sodium citrate and the cells subsequently resuspended in complete medium. For growth and receptor studies cells were plated in complete medium and after 3 days (in which maximum attachment to the plastic culture plates is achieved), the cells were maintained in SFM. In case of carry over from serum proteins, the

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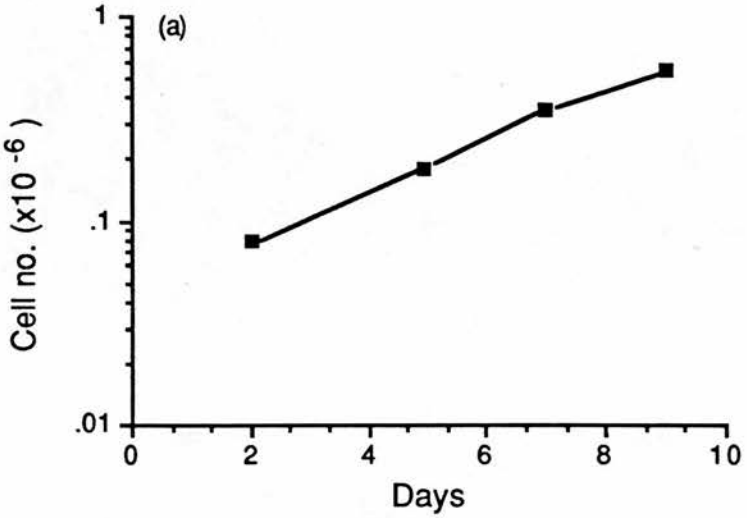
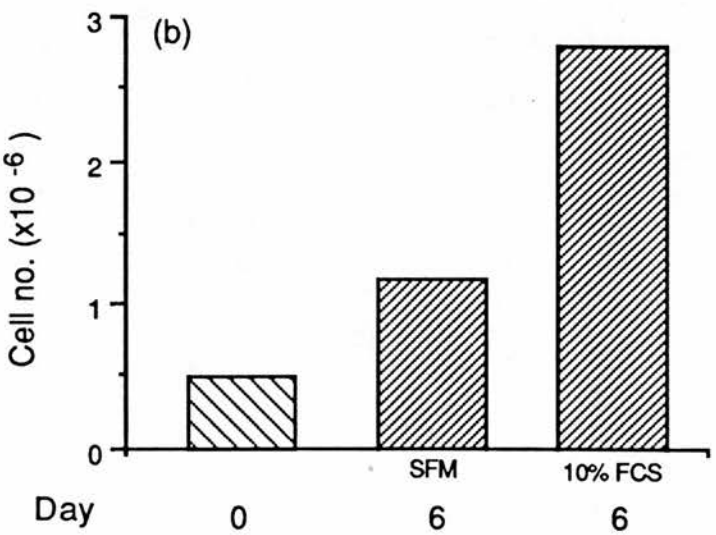
FIGURE 7: Growth curve for the DU 145 cell line in complete medium and SFM. A 6 well plate (9.6cm^2/well) was seeded with 0.5 x 10^5 cells/well or 2x10^5 cells/well and grown in complete medium (□) or SFM (■) for a period of 9 days. At 3 day intervals, the cells from triplicate wells were removed by trypsin-EDTA treatment and counted in a haemocytometer. The curve indicates a population doubling time of 36 hours for cells grown with serum, which is reduced to 60 hours in SFM.


(a)
1
.1
.01
Cell no. (x10 -6)
0
2
4
6
8
10
Days


(b)
3
2
1
0
Cell no. (x10 -6)
SFM
10% FCS
Day
0
6
6

monolayers were washed with SFM (once for eight hours and then rinsed in SFM) before the addition of test solutions (EGF and/or androgens). All growth experiments were carried out at a cell density of 2 x 10^4 cells/cm^2. The population doubling time for LNCaP-FGC is approximately 67 hours in the presence of 10% FCS (Figure 8a), but growth is reduced to 154 hours when cultured in SFM (Figure 8b).

FIGURE 8: (a): Growth curve of the cell line LNCaP. Cells were plated at a density of $2x10^4/cm^2$ in 6 well plates with 10% FCS. At particular time intervals triplicate wells of cells were harvested and counted. The curve indicates a population doubling time of 67 hours.

FIGURE 8: (b) Effect of 10% FCS and SFM on the proliferation of LNCaP cells in culture. LNCaP cells were counted following attachment at 3 days (Day 0). Cells were further incubated with 10% FCS or SFM for 6 days, harvested and counted. The growth rate of cells grown in 10% FCS was approximately 2.3 times that of cells growth in SFM. The doubling time of cells grown in SFM was reduced to 154 hours.

2.4. CELLULAR GROWTH AND MEASUREMENT OF DNA SYNTHESIS

Cell growth was measured by enumeration, and DNA synthesis by the incorporation of [^{3}H]-Thymidine. Cell viability was determined by Trypan Blue-exclusion; the stain is taken up into the membrane of dead cells leaving viable cells clearly distinguishable. Cells were counted using a Bürket type haemocytometer on an inverse phase-contrast light microscope.

In this section general methods used for cell enumeration and DNA synthesis are outlined, followed by sub sections detailing modifications.

2.4.1 CELL ENUMERATION OF DU 145 CELLS: GENERAL METHOD

Subconfluent DU 145 monolayers from 75cm^2 culture flasks were washed once with Dulbecco 'A' PBS and disrupted by trypsinization as described in section 2.3.3. The cells were resuspended in SFM/0.5% FCS, after pipetting several times to obtain a single cell suspension. The suspension of cells was subsequently pelleted at 1500 rpm for 10 mins, resuspended in 15 ml of SFM/0.5% FCS and an aliquot (100µl) of the cells taken and diluted 1:2 with trypan blue dye (0.03% final concentration) and counted on a haemocytometer with an inverse phase-contrast light microscope. The cells were then plated in either 6 well plates (9.6cm^2), at a density of 2x10^5 cells/well in 1ml of medium/well, or at a density of 50x10^3 cells/well in 24 well plates (2cm^2) in 1ml of medium/well. The test growth factor was added 24 hours later in SFM (2 ml/well to 6 well plates or 1 ml/well to 24 well plates) to the plated cells for up to nine days, with medium changes on alternate days. After this period the cells were harvested with trypsin and counted.

Modifications to general method

(a) The effect of EGF on cellular growth

The effect of mouse EGF (tissue-culture grade*) on the growth rate of DU 145 cells was measured by comparing changes in growth rate in treated cells with the growth rate in untreated cells.

EGF (which was previously made up in SFM or complete medium at a stock concentration of 100 nmol/l) was added to monolayer cultures at various concentrations (0.01-10nmol/l), for up to nine days. After this period the cells were harvested and counted.

(b) The effect of plating density on the growth of EGF treated cells.

The effect of EGF on the growth rate of the serum-free DU 145 cell line was investigated at various cell densities.

DU 145 cells were seeded in 9.6cm^2 wells in 6 well plates at innocula of 1x10^4, 2x10^4 and 4 x10^4 cells/cm^2. EGF (0.3nmol/l) was added in SFM 24 hours later, with changes of medium on alternate days. After six days the cells were harvested with trypsin and counted.

2.4.2 CELL ENUMERATION OF LNCAP CELLS: GENERAL METHOD

Subconfluent monolayers from 75cm^2 tissue culture flasks were washed once with Dulbecco 'A' PBS and disrupted using a solution of sodium citrate as described in section 2.3.4. The cells were subsequently resuspended in complete medium, after pipetting several times to obtain a single cell suspension. The suspension of cells was pelleted at 1500 rpm for 10 mins, resuspended in 15 ml of complete medium and an

*The tissue culture grade of EGF used for all the growth experiments had previously been tested by Collaborative Research Inc. for mitogenic activity. Human foreskin fibroblasts were grown for seven days in DME plus 10% foetal bovine serum. EGF (4ng/ml) in this culture medium caused a 1.54 fold increase in cell number over control cultures without EGF

aliquot (100µl) counted on a haemocytometer. In all experiments with LNCaP, cells were seeded in complete medium for 3 days, at a density of $2x10^4$ cells/cm^2 in 6 well culture plates. After three days the cells were washed with Dulbecco 'A' PBS before the addition of SFM, which was left in contact with the cells for approximately eight hours. After this time the test growth factor and/or androgen was added in SFM to each well, in a volume of 2ml/well, with control wells receiving no growth factor. The medium was changed after 3 days and after 6 days the cells were harvested with trypsin and counted.

Modifications to general method

(a) The effect of EGF on cellular growth

The proliferative response to EGF was investigated in the cell line LNCaP. EGF (diluted in SFM) was added to monolayer cultures of LNCaP cells over the concentration range 0.01-10nmol/l. After 6 days, the cells were harvested and counted. Cells without added growth factor were used as controls.

(b) The effect of EGF and androgens on cellular growth

The proliferative effects of the synthetic androgens Mibolerone and R1881 were investigated on the androgen-sensitive LNCaP cell line. These synthetic steroidal androgens have several times higher affinity than 5α-dihydrotestosterone to the androgen receptor (Liao *et al.*, 1973). Both ligands are metabolically stable, but as Mibolerone is more stable chemically, it was generally used in preference to R1881. Stock solutions of these androgens were made up in 100% ethanol (end concentration; < 0.1% ethanol). The androgens at a concentration of 0.1 nmol/l were added (singly or in combination with EGF) to plated LNCaP cells, and after 6 days the cells were harvested with trypsin and counted.

2.4.3 [^{3}H]-THYMIDINE INCORPORATION IN DU 145 CELLS: GENERAL METHOD

Subconfluent cells from 75cm^2 tissue culture flasks were rinsed with Dulbecco 'A' PBS, and trypsinized as previously described in section 2.3.3. The cells were then incubated at 37°C for 5 mins and subsequently resuspended in SFM. The suspension of cells was pelleted at 1500 rpm for 10 mins, resuspended in 15 ml of SFM with 0.5% FCS and an aliquot of cells (100µl) counted on a haemocytometer. Subsequently, 1 x 10^4 cells/well (in a volume of 100µl of suspended cells/well) were plated overnight in SFM (supplemented with 0.5% FCS to assist plating), in 96 well plates (0.32cm^2). After aspirating off the plating media, the test growth factor (100µl) was added in SFM for 8, 24, 48 and 72 hours. After the incubation period, [Methyl-^{3}H] Thymidine (specific activity 74GBq/mmol; 37KBq/well; 50µl/well) was added in RPMI for at least four hours. Each well was then carefully aspirated, the cells trypsinized (100µl solution/well) and resuspended by gently tapping the plate. Cellular material (DNA) was precipitated by the addition of 100µl of ice-cold trichloroacetic acid (TCA; 10%) for two hours. The precipitable cellular material was harvested (Skatron Combi Cell Harvester; Skatron, Norway) on to filter mats by washing the wells three times in water and then drying the filter mats at 60°C for 30 mins. Each disc of filter paper (containing the dried precipitable cellular material) was dispensed into plastic minivials (Skatron) with 3 ml of scintillation fluid (a Triton x 100 based cocktail; Vickers Laboratories, Burley-on-Wharfedale, West Yorks, U.K). The counts per minute (cpm) of each vial was determined in the tritium channel of a Hewlett Packard scintillation counter. All determinations were carried out with 6 to 12 samples per 'test growth factor'.

Modifications to general method

(a) The effect of EGF on [^{3}H]-Thymidine incorporation

The effect of EGF (0.001-10nmol/l) on DNA synthesis of DU 145 cells was measured by the incorporation of [^{3}H]-Thymidine as outlined in the general method; section 2.4.3. Growth factor activity was taken as any increase or decrease in incorporation of [^{3}H]-Thymidine relative to the untreated SFM control.

EGF was added to serum-free cultures of DU 145 cells for periods of 8, 24, 48 and 72 hours after the cells had plated. DNA synthesis was measured after each incubation time, by the addition of [^{3}H]-Thymidine as described in section 2.4.3.

(b) The effect of plating density on DNA synthesis of EGF treated DU 145 cells.

The effect of cell density on the mitogenic response to EGF was investigated using [^{3}H]-Thymidine incorporation.

DU 145 cells were seeded at innocula of $1x10^4$, $3x10^4$ and $6x10^4$ cells/cm^2 in 96 well plates (with 100µl/well of suspended cells). EGF (1nmol/l; 100µl/well) was added in SFM for 24 hours, with control wells receiving no growth factor. After 24 hours DNA synthesis was measured by the addition of [^{3}H]-Thymidine as previously described in section 2.4.3.

(c) The effect of TGFα on incorporation of [^{3}H]-Thymidine

The growth factor TGFα was tested for mitogenic response on the serum free DU 145 cell line.

TGFα (0.01-10 nmol/l) was added to plated cells (1×10^4 cells/well in 96 well plates) in SFM for 24 hours, with control wells receiving no growth factor. After 24 hours DNA synthesis was measured by the addition of [^{3}H]-Thymidine as previously described in section 2.4.3.

2.4.4 [^{3}H]-THYMIDINE INCORPORATION IN LNCAP CELLS: GENERAL METHOD

Subconfluent cells from 75cm^2 tissue culture flasks were rinsed in Dulbecco 'A' PBS, and once with 0.08M sodium citrate as previously described in section 2.3.4. The cells were incubated at 37°C for 5 mins and subsequently resuspended in complete medium. The suspension of cells was pelleted at 1500 rpm for 10 mins, resuspended in 15 ml of complete medium and pipetted several times to obtain a single cell suspension. An aliquot of cells (100µl) was counted on a haemocytometer and 2x10^4 cells/cm^2 (in a volume of 100µl/well) were then plated in 96 well plates (0.32cm^2) for a period of three days. After this time the 'test growth factor or androgen' was added in SFM to the LNCaP monolayers for a period of 24 hours. [^{3}H]-Thymidine incorporation was measured as previously described for DU 145 cells in section 2.4.3.

Modifications to general method

(a) The effect of EGF on [^{3}H]-Thymidine incorporation in LNCaP cells.

As a comparison with the growth response to EGF in DU 145 cells, [^{3}H]-Thymidine incorporation assays were carried out, to investigate the effect of EGF on DNA synthesis in LNCaP cells.

Increasing concentrations of EGF (0.01-10nmol/l) in SFM were added to LNCaP monolayers for a period of 24 hours, with control wells receiving no EGF. DNA synthesis was then measured by the addition of [^{3}H]-Thymidine as described in section 2.4.3.

(b) The effect of EGF and androgens on [^{3}H]-Thymidine incorporation

In some experiments Mibolerone and R1881 were tested on LNCaP cells either singly or in combination with EGF, to investigate the relationship between growth factors and androgens.

The synthetic androgens, at a concentration of (0.1nmol/l) were added to subconfluent monolayers in the presence or absence of EGF for a period of 24 hours.

After this time DNA synthesis was measured by the addition of [^{3}H]-Thymidine as described in section 2.4.3.

2.5 EGF RADIO-RECEPTOR BINDING ASSAYS

The characterization of the EGF receptor in LNCaP and DU 145 was investigated using a radio-ligand exchange assay with [^{125}I]-mouse EGF. The EGF binding assay used for DU 145 was adapted from Carpenter (1985). The EGF binding assay for LNCaP cells was performed without modification from that of Schuurmans *et al.* (1988a). For both cell lines, binding assays were performed with whole cells and not membrane preparations.

The general method used to characterize the EGF receptor (the ligand-exchange radio-receptor assay) is outlined in section (2.5.1) and is followed by sub-sections detailing modifications to this method.

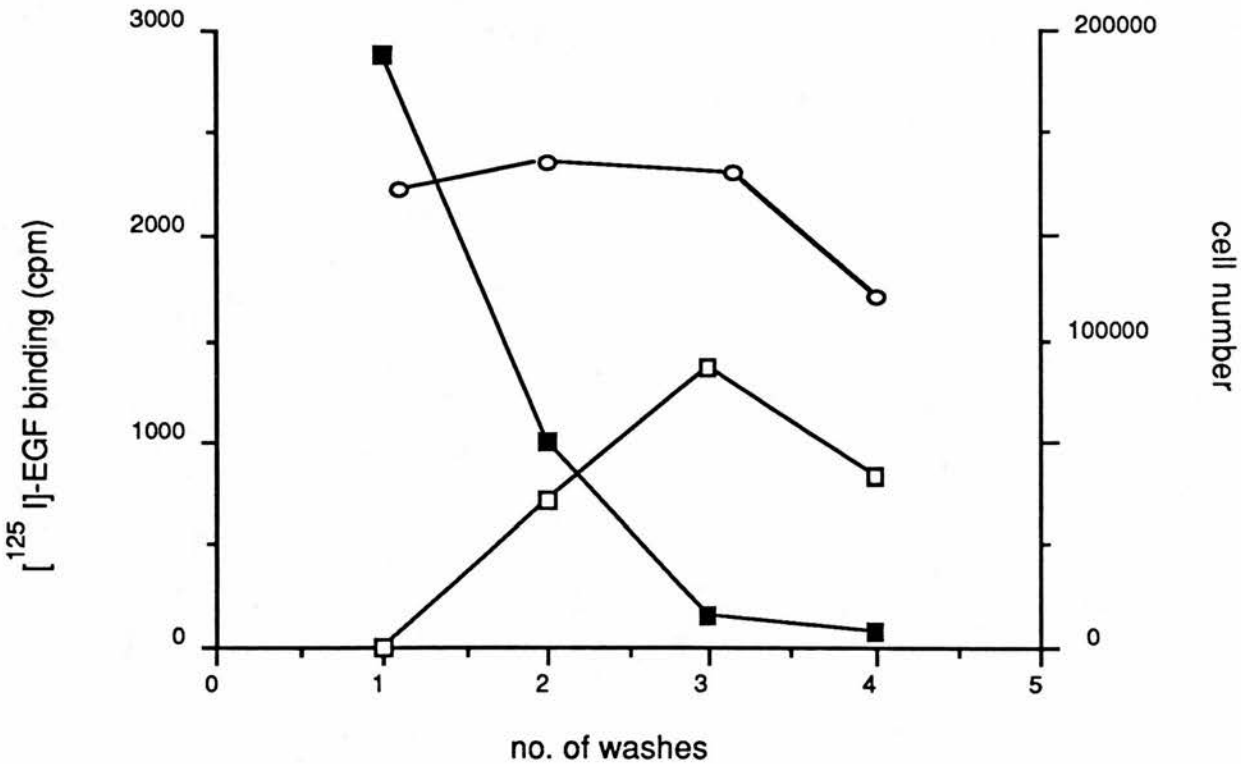
2.5.1 RADIO-RECEPTOR ASSAY FOR DU145 CELLS: GENERAL METHOD

DU 145 cells were prepared and plated for EGF binding assays as described for cell proliferation experiments (section 2.4.1). All binding assays were carried out on confluent monolayers (2 x10^5 cells/well), in 24 well plates (2cm^2/well). Before addition of the binding medium the SFM from each well was aspirated and the cells and binding media were cooled on ice to inhibit receptor internalization. The cell monolayers were then gently washed with 0.5 ml of Dulbecco 'A' PBS per well and binding was initiated upon the addition of the appropriate concentration of [^{125}I]-EGF (specific activity 4MBq/μg;100μl/well; Amersham International plc). To obtain the correct concentration of [^{125}I]-EGF, unlabelled EGF was added to the labelled material, with the highest concentration containing approximately 2 x 10^5 cpm. For each concentration of EGF, a measurement was made of the total [^{125}I]-EGF binding and the non-specific binding of radioactivity (that which is not displaced by an excess of non-radioactive EGF). Non-specific binding was determined in the presence of 100 fold excess unlabelled EGF (200 μl/well; receptor-grade). Each solution was made up beforehand in RPMI-1640 to a final volume of 0.5 ml, and then added to the

appropriate well. Each determination was carried out in triplicate (3 x total binding and 3 x non-specific binding). After incubating, unbound was separated from bound [^{125}I]-EGF by aspirating the contents of each well and then washing the cells three times with ice-cold Dulbecco 'A' PBS (0.5ml/well) by gently pipetting the wash solution against the side of the dish. The cells were subsequently solubilized with 1ml of 0.5N sodium hydroxide, for 15 minutes at room temperature and the dissolved cells transferred to plastic tubes for counting in a gamma counter. Specific binding was calculated as the difference between total binding and non-specific binding. For all binding experiments cell numbers were determined from control wells (usually in triplicate) in which only RPMI-1640 was added, without labelled or unlabelled EGF.

2.5.2 RADIO-RECEPTOR ASSAY FOR LNCAP CELLS: GENERAL METHOD

EGF binding assays were carried out on confluent monolayers (2 x 10^5 cells/well) in 24 well plates. The assay was essentially similar to that described for DU 145 cells in section 2.5.1. However, instead of terminating binding by washing the monolayers, separation of bound [^{125}I]-EGF from free [^{125}I]-EGF was carried out by firstly aspirating off the medium containing the radioligand and then adding 1ml of ice-cold Dulbecco 'A' PBS. The cells were resuspended by pipetting several times, transferred to disposable plastic centrifuge tubes and subsequently pelleted for 10 min at 1500 rpm. The supernatant was aspirated, and the radioactivity of the cell pellets measured in a gamma counter. Specific binding was calculated as the difference between total binding and non-specific binding and all determinations were carried out in triplicate. As for DU 145 cells, cells were counted in control wells which were processed with RPMI, without labelled EGF.


3000
2000
1000
0
200000
100000
0
0
1
2
3
4
5
[125 I]-EGF binding (cpm)
cell number
no. of washes

2.5.3 OPTIMIZATION OF EGF BINDING ASSAY FOR DU 145 CELLS

Before further ligand binding characterization of the EGF receptor was performed, preliminary experiments were carried out to determine the optimal conditions.

2.5.3.1 Separation of bound [^{125}I]-EGF from free [^{125}I]-EGF

Efficient separation of bound [^{125}I]-EGF from free [^{125}I]-EGF is crucial to the success of binding assays. The major constraint on separation techniques is the rate of dissociation of the ligand-receptor complex at the temperature of separation of bound from free ligand. Consequently, a separation procedure that is rapid must be utilized to avoid losing bound ligand. Washing of firmly bound cell monolayers offers just such a rapid and efficient way of separating bound from free [^{125}I]-EGF, and in the preliminary experiment depicted in Figure 9, the optimal number of washes was determined. To ensure that washing did not dislodge the cells from the tissue culture wells, after each wash the number of cells was counted. All other experimental details including the number of cells plated and the addition of radio-ligand are identical to that outlined under section 2.5.1 for DU 145 cells.

The results depicted in Figure 9 demonstrate that three washes are optimal for efficient separation of free from bound [^{125}I]-EGF. Greater than three washes decreased specific binding, which was probably due to the loss in cell numbers. With less than three washes, non-specific binding was unacceptably high while specific binding was low. All subsequent separations of bound [^{125}I]-EGF from free [^{125}I]-

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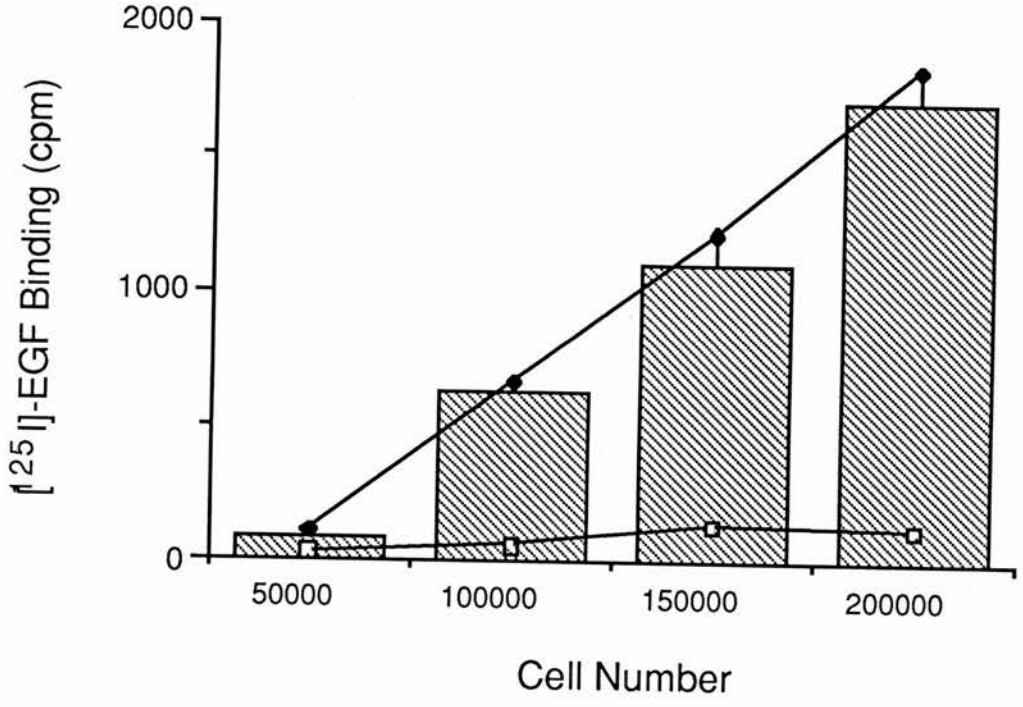
FIGURE 9: Optimization of separation of bound [^{125}I]-EGF from free [^{125}I]-EGF. DU 145 cells were seeded at a density of 2 x 10^5 cells/well in 24 well plates. Binding was initiated upon the addition of [^{125}I]-EGF (2nmol/l; 2 x 10^5 cpm) with or without 100x excess unlabelled EGF. After 4 hours at 4°C, binding was terminated by washing 1 to 4 times in ice-cold Dulbecco's. After each wash the cells in control wells were counted (o). The monolayers were subsequently solubilized in 0.5 N NaOH and the radioactivity counted. Specific binding (□) was determined by subtracting non-specific binding (■) from total binding.


2000
1000
0
[125I]-EGF Binding (cpm)
50000
100000
150000
200000
Cell Number

EGF in radio-receptor assays for DU 145 cells was carried out by washing the monolayers a maximum of three times.

2.5.3.2 Correlation between specific binding and cell number

If optimal conditions have been achieved, then increasing the number of cells exposed to [^{125}I]-EGF should increase the level of specific binding without notably increasing non-specific binding.

Increasing numbers of cells (50 x 10^3-200 x10^3) were incubated in 24 well plates with a constant amount of [^{125}I]-EGF (2nmol/l; 2 x 10^5 cpm) and 100x excess unlabelled EGF . The binding assay was then carried out as outlined in section 2.5.1 for DU 145 cells.

A positive correlation was found between cell number and specific binding (Figure 10) with no significant increase in non-specific binding as the number of cells increased. Non-specific binding was less than 10% of the total binding with 200, 000 cells/well.

Non-specific binding to the plastic tissue culture plates was also tested. [^{125}I]-mEGF was added to wells which had previously been coated with or without SFM; no significant non-specific binding was detected.

2.5.4 RADIO-RECEPTOR ASSAY CHARACTERIZATION OF THE EGF RECEPTOR

The EGF receptor for both DU 145 and LNCaP cell lines was characterized by a series of radio-ligand binding experiments. The number and affinity of receptors as well as the kinetics of association and dissociation of EGF binding to its receptor were

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FIGURE 10: Correlation between specific binding and cell number. DU 145 cells were seeded at the densities indicated in the figure; in 24 well plates. Binding was initiated upon the addition of [^{125}I]-EGF (2 nmol/l; 2 x 10^5 cpm) with or without unlabelled EGF. After 4 hours, at 4°C binding was terminated by washing 3x in Dulbecco A PBS and solubilizing the cells in NaOH. Specific binding (hatched bars) was determined by subtracting non-specific binding (□) from total binding (◆).

determined. In addition to down regulation and specificity studies, internalization of EGF binding sites was investigated.

2.5.4.1 Association & Dissociation Kinetics

Steady state binding of [^{125}I]-EGF to DU 145 and LNCaP cell monolayers was determined at 4°C and 37°C. Confluent monolayers (2 x 10^5 cells/well; 24 well plates) and solutions were either cooled on ice for a period of 15 mins, or incubated at 37°C. After this time the monolayers were incubated with sub-saturating concentrations of [^{125}I]-EGF (2nmol/l; 2 x 10^5 cpm) in the presence and absence of 100x excess unlabelled EGF. At regular time intervals, bound [^{125}I]-EGF was separated from unbound [^{125}I]-EGF in triplicate wells as described in sections 2.5.1 (DU 145) and 2.5.2 (LNCaP), and total and non-specific binding determined.

The time course of dissociation of [^{125}I]-EGF bound to DU 145 and LNCaP EGF binding sites was determined after a steady state had been achieved (this was determined by association kinetics; steady state was achieved after 4 hours and 6 hours with DU 145 and LNCaP cells respectively, at 4°C). Dissociation was then initiated by aspirating the binding medium in each well and adding 0.5 ml of SFM at 4°C. To determine monolayer bound [^{125}I]-EGF that had dissociated, bound [^{125}I]-EGF was separated from free [^{125}I]-EGF at various time intervals, in triplicate wells. This procedure was carried out as described in sections 2.5.1 (DU 145) and 2.5.2 (LNCaP).

2.5.4.2 Saturation & Competition Analysis

The affinity constant and the number of EGF binding sites present on DU 145 and LNCaP cells were determined using saturation and competition analysis.

Competition Studies

In competition analysis, the concentration of unlabelled ligand displacing 50% of specific radioligand binding (IC_{50}) and the number of binding sites is determined by competing labelled ligand with increasing concentrations of unlabelled ligand.

Confluent LNCaP and DU 145 monolayers (2 x10^5 cells/well, in 24 well plates) were incubated with [^{125}I]-EGF (2nmol/l; 2 x 10^5 cpm), with or without increasing concentrations of unlabelled EGF (0.01-300 nmol/l) at 4°C. After the incubation period (4 hours and 6 hours for DU 145 and LNCaP cells respectively) separation of bound [^{125}I]-EGF from free [^{125}I]-EGF was carried out as described in sections 2.5.1(DU 145) and 2.5.2 (LNCaP). For each concentration of EGF the amount of specific binding from triplicate determinations was calculated by subtracting total binding from non-specific binding. The data were subsequently analysed and the binding parameters evaluated by the computer program LIGAND (see section 2.8).

Saturation Studies

In saturation analysis, the amount of radioactive ligand that produces 50% occupancy (the dissociation constant; K_d), and the total receptor population (R_T) can be determined by using a range of radioligand concentrations. The labelled ligand concentrations should optimally cover a range from 10-20% of the estimated IC_{50} (as evaluated by competition analysis) to four to five times this value, with excess unlabelled ligand remaining constant.

Confluent monolayers (2 x10^5 cells/well) seeded in 24 well plates were incubated with increasing doses of [^{125}I]-EGF (0.02-10nmol/l) in triplicate wells (with or without a constant amount of unlabelled EGF; 200nmol/l) at 4°C, for either 4 hours (DU 145) or 6 hours (LNCaP). Separation of bound from unbound [^{125}I]-EGF was then carried out as described in sections 2.5.1 (DU 145) and 2.5.2 (LNCaP) and the amount of specific binding for each concentration of [^{125}I]-EGF determined as the difference between total binding and non-specific binding. The data were analysed and the binding parameters evaluated by the computer program LIGAND.

Saturation analysis of androgen treated LNCaP cells

Several recent studies have shown that EGF receptor levels are modulated by androgens (Traish & Wotiz, 1987; St-Arnaud *et al.*, 1988; Schuurmans *et al.*,1988a).

This relationship was investigated with the androgen-sensitive LNCaP cell line using the synthetic androgen Mibolerone.

LNCaP cells were plated at a density of $2x10^4$ cells/cm^2 in 24 well culture plates. After three days the cells were washed in Dulbecco 'A' PBS and SFM as described in section 2.3.4. Mibolerone (0.1nmol/l) was then added to the monolayers for a period of 6 days, with two changes of medium during this time. After this period, the culture medium was aspirated and increasing doses of [^{125}I]-EGF (0.05-10nmol/l) were added to triplicate wells with or without unlabelled EGF (200nmol/l) for 6 hours, at 4° C. Separation of bound from unbound [^{125}I]-EGF was carried out as described in section 2.5.2, and specific binding determined as the difference between total binding and non-specific binding for each radioligand concentration. The receptor number and receptor affinity were evaluated for androgen treated and untreated LNCaP cells using the computer program LIGAND.

2.5.4.3 Specificity Studies

The specificity of EGF binding to receptor sites on the DU 145 and LNCaP cell lines was determined by competing for EGF binding sites with [^{125}I]-mEGF and various ligands other than mEGF.

Confluent monolayers ($2x10^5$ cells/well in 24 well plates) were incubated with [^{125}I]-mEGF (2nmol/l; 2 x 10^5 cpm), with or without 100x excess unlabelled competing ligands (mEGF, TGFα, hEGF, NGF, GH, and Insulin) in triplicate wells. After the incubation period (at 4°C for 4 hours with DU 145 cells or for 6 hours with LNCaP cells) binding was terminated by washing or pelleting the monolayers as described in sections 2.5.1(DU 145) and 2.5.2 (LNCaP). Specific binding was determined by subtracting total binding from non-specific binding for each competitor.

2.5.4.4 Internalization of binding sites for EGF

To determine whether EGF was internalized in a temperature dependent fashion by human prostate cancer cells, the effect of brief exposure to saline/glycine, pH 3.0 on

monolayer-bound radioactivity was examined. Brief exposure to glycine at pH 3.0 extracts surface-bound ligand without damaging the cell, thus ensuring that only internalized material will be counted (Costlow & Hample, 1982).

Confluent cell monolayers (2 x10^5 cells/well) were incubated with [^{125}I]-EGF (2nmol/l; 2 x 10^5 cpm) in the presence or absence of unlabelled EGF (200 nmol/l), at 4°C and 37°C. At various time intervals, triplicate dishes were processed following washing with three changes of ice-cold Dulbecco 'A' PBS (0.5ml/well). Extraction of surface-bound EGF was carried out at 4°C with 0.5 ml/well of ice-cold NaCl/glycine, pH 3.0 (50mM Glycine, 0.15M NaCl; Costlow & Hample, 1982). Total binding, non-specific binding, and the level of specific [^{125}I]-EGF bound was subsequently determined as previously described in sections 2.5.1 (DU 145) and 2.5.2 (LNCaP).

2.5.4.5 Down-regulation studies

The ability of EGF to down regulate its receptor was investigated in both DU 145 and LNCaP prostate cell lines.

DU 145 and LNCaP cell monolayers (2 x10^5 cells/well in 24 well plates) were pre-incubated with increasing concentrations of EGF (0.01-10nmol/l) for 24 hours at 37°C; the control wells received no EGF. After incubating, the monolayers were washed to remove surface bound EGF with three changes of ice-cold Dulbecco 'A' PBS (0.5ml/well). Extraction of surface-bound EGF was carried out at 4°C with 0.5 ml/well of ice-cold NaCl/glycine, pH 3.0. Binding was initiated by the addition of [^{125}I]-EGF (2nmol/l; 2 x 10^5 cpm) with or without 100x excess unlabelled EGF, for 4 (DU 145 cells) or 6 hours (LNCaP) at 4°C and the amount of specific binding determined as previously described in section 2.5.1. Receptor down-regulation was seen as any decrease in specific binding relative to the untreated control.

2.6 DETECTION OF THE EGF RECEPTOR AND THE V-*erb* B GENE PRODUCT BY WESTERN BLOTTING

The presence of the EGF receptor in both cell lines was further verified by Western blotting (Harlow & Lane, 1988). Two Mabs were used in this analysis (EGFR I and F_4) which recognize and bind to specific domains on the EGF receptor. The EGFR1 Mab binds to the native extracellular domain of the 175 kDa receptor. The Mab F4 binds to the intracellular domain of the EGF receptor and will thus detect both the 175kDa receptor and the *v-erb* B gene product (which has a molecular weight of Mr 68 000).

2.6.1 PREPARATION OF CELL LYSATES

Cell lysates were prepared from confluent monolayers of LNCaP and DU 145 ($5x10^7$cells). The monolayers were washed with Dulbecco 'A' PBS and pelleted by gentle centrifugation (5 minutes at 1500 rpm). Lysis buffer (2 ml; 50mM Tris, 1% Triton X-100, 150mM NaCl, 25mM Benzamidine, 0.1% BSA, 0.3mM phenylmethylsulphonylfluoride; PMSF, 1mM dithiothreitol and 10% glycerol, pH 7.4) was added on ice to the pellet of cells and the lysate was centrifuged at 3000rpm for 30mins (PMSF was dissolved in 100% ethanol and was freshly prepared each time). The soluble material from the cell lysates was collected and was subjected to SDS-PAGE as described in section 2.6.2.

2.6.2 SDS-PAGE

The procedure of SDS-PAGE was based on the discontinuous Tris-Glycine buffer system described by Laemmli (1970) and was performed in a vertical slab gel electrophoresis tank (Bio-Rad Laboratories Ltd, Watford, Hertfordshire). Sample buffer (400µl; 0.063M Tris-HCl, pH 6.8, 10% (v/v) glycerol, 2% (w/v) SDS, 5% (v/v) 2β-mercaptoethanol and 0.0013% (w/v) bromophenol blue) was added to 100µl

of the solubilized cell lysates and (without bromophenol blue) to molecular weight standards ('Rainbow markers'; Amersham plc, which consisted of lysozyme, 14.3 kDa; trypsin inhibitor, 21.5 kDa; carbonic anhydrase, 30 kDa; ovalbumin, 46 kDa; bovine serum albumin, 69 kDa; phosphorylase b, 92.5 kDa and myosin, 200 kDa). Samples were boiled for 3 mins and were subsequently electrophoresed on a 7.5% separating gel with a 4% stacking gel in Tris/Glycine electrode running buffer (0.025M Tris, 0.19M Glycine and 0.1% (w/v) SDS, pH 8.3) for 5 hours at 35mA/gel, with continual cooling.

2.6.3 STAINING AND DESTAINING

After electrophoresis, a track of the gel containing the separated cellular material was stained for protein with Coomassie Brilliant Blue (0.1% Coomassie Blue R-250, 40% Methanol, 10% glacial acetic acid) for 30 minutes with continual shaking. The gel was destained overnight with continual shaking in a mixture of 40% methanol and 10% acetic acid.

2.6.4 IMMUNOBLOTTING

The protein bands on the gel were transferred on to a nitrocellulose membrane (30 min at 20V; transfer buffer; 48mM Tris, 39mM glycine, 20% methanol, 0.02% SDS, pH 9.2) using a Trans Blot SD Semi-dry electrophoresis transfer cell (Bio-Rad Labs). After blotting, part of the membrane corresponding to a track of cell lysate was stained with amido black (40% methanol, 10% acetic acid and 0.1% amido black) to confirm efficient transfer of proteins.

The remaining nitrocellulose strips were blocked with 5% (w/v) skimmed milk solution for 10 mins at room temperature. The strips were rinsed in washing buffer (50mM Tris, 150mM NaCl, 2mM EDTA at pH 7.5) and incubated with Mabs EGFR1 (5μg) and F4 (2μg), with 2% BSA in washing buffer, for 2 hours with continual

shaking at room temperature. A mouse Mab, raised against MHC IgG class 2 was used as a non specific control. The strips were rinsed in washing buffer and incubated with alkaline phosphatase antibody conjugate mouse IgG (Sigma) for 2 hours at room temperature. After this incubation period the strips were rinsed once more in washing buffer and developed by the addition of Naphthol-phosphate (15 mg Naphthol-AS-MX- phosphate (free acid) in 1.5ml dimethylformamide, added to 75ml saline/0.05M Tris, pH 8.8 with 75mg Fast Red TR salt; Sigma). The development of the colour product was terminated by rinsing in water.

Calculation of molecular weights

A calibration curve was constructed by plotting the relative mobilities (R_f) of the molecular weight markers (R_f = distance of protein migration/distance of tracking dye migration), against the $\log_{10}$ of the molecular weights. The molecular weights of unknowns were determined with reference to the standards.

2.7 PREPARATION AND ANALYSIS OF MEDIUM CONDITIONED BY DU 145 CELLS

Conditioned medium (CM) from DU 145 cells was collected from confluent monolayers over a period of 4 months and analysed for the presence of the growth factors TGFα and EGF, by RIA and a competitive EGF-radio receptor assay (EGF-RRA). CM was also assayed for bioactivity by the incorporation of [^{3}H]-Thymidine into DNA of DU 145 cells. The CM was further characterized by gel filtration, rHPLC and SDS-PAGE, with the fractions and eluted gel material assayed by RRA and RIA.

2.7.1 CONDITIONED MEDIUM PREPARATION

Cells were grown to confluency in 75cm^2 tissue culture flasks in SFM, the medium was changed once and discarded and the cells were grown for a further 48 hours in 20 ml of SFM. The CM was clarified by centrifugation (3 000 rpm for 15 minutes) and filtered through a 0.2μm filter (Gelman Sciences Inc.); at this stage medium was stored at -70°C until further use.

Dialysis & concentration of pooled CM

Pooled CM from 50 flasks (1 litre) was dialyzed against 50mM ammonium acetate (10 litres) (after the addition of freshly prepared PMSF) in Spectrapor 3 dialysis tubing at 4°C (molecular weight cut-off point of 3500; Pierce-Warriner, Chester, U.K). The dialysate was continually stirred over 24 hours, with 4 changes of buffer. The dialyzed CM was transferred into plastic universals, frozen at -70°C and subsequently lyophilized to dryness. The lyophilized material was reconstituted in 5 ml of PBS (0.02M $Na_2HPO_4.2H_20$, $NaH_2PO_4.2H_2O$, 0.9% NaCl, pH 7.4) and clarified by centrifugation (10 000 g, 30 mins).

2.7.2 PROTEIN ESTIMATION

The protein concentration of the concentrated CM from DU 145 cells was estimated by the method of Bradford (1976) using bovine serum albumin (BSA) as standard.

Bradford Reagent

Coomassie Brilliant Blue G (100mg) was dissolved in 50 ml of 95% (v/v) ethanolic solution, to which 100 ml of 85% phosphoric acid was added. The solution was made up to 1 litre with distilled water and filtered through Whatman paper.

Assay

Varying amounts of the BSA solution (0-80 μg; the assay is linear over this range for BSA) were dispensed into standard tubes and their volumes brought up to 80μl with 0.02 M PBS buffer, pH 7.4. Assay tubes contained the solution of CM in 0.02 M PBS, in a final volume of 80μl. Bradford reagent (5 ml) was added to each tube and the contents vortexed for 5 secs. The absorbance of the BSA standards and unknowns at 595nm were measured with a Pye Unicam spectrophotometer (SP6 550 uv/vis). A calibration curve constructed from the BSA standards was used to determine the concentration of protein in the unknowns.

2.7.3 EGF-RADIO RECEPTOR COMPETITION ASSAY

DU 145 cell monolayers were used as the source of EGF receptors in the EGF-RRA for analysis of competitive activity in the CM.

Confluent monolayers (2 x 10^5 cells/well) of DU 145 cells were incubated with [^{125}I]-EGF (2nmol/l; 2 x 10^5 cpm; 100μl) in the presence or absence of triplicate aliquots of CM samples (200μl/sample) for 4 hours at 4 °C. Each sample was made up in RPMI-1640 to a final volume of 0.5 ml; see section 2.5.1. After the incubation period bound was separated from unbound [^{125}I]-EGF as previously described in

section 2.5.1. The amount of specific binding for each sample of CM was determined by subtracting total binding from non-specific binding.

Usually two control wells were set up to test for components in the CM, such as proteolytic enzyme activity which might have resulted in a false interpretation of specific binding. Excess EGF was added to these cells (which had previously been incubated with CM) and specific binding was determined after a further hours incubation.

The amount of EGF equivalent units in the CM samples was calculated by comparison with the competition curves derived from unlabelled mEGF with [^{125}I]-mEGF competing for EGF binding sites on DU 145 cells; see section 2.5.4.2. In this assay the sensitivity for unlabelled mEGF, hEGF, or TGFα is approximately 0.12ng per assay with 1ng of [^{125}I]-mEGF; with 50% competition occurring at 0.6 ng per assay.

Experiments using the EGF-RRA for analysis of EGF competitive activity

(a) Concentrated media conditioned by DU 145 cells

Aliquots of concentrated CM were assayed for EGF-like competitive activity. [^{125}]-EGF was added to DU 145 cell monolayers with or without increasing concentrations of CM (0.05 - 2 mg/ml) for 4 hours at 4°C. After this time the cells were processed as previously described in section 2.5.1. Each concentration was assayed in duplicate and each experiment was repeated at least three times. The amount of EGF equivalents was calculated from competition curves of [^{125}I]-mEGF with unlabelled mEGF competing for EGF binding sites. In this assay hEGF and TGFα were assumed to be equivalent.

(b) Gel filtration and rHPLC fractions

CM was fractionated using gel filtration and rHPLC (see sections 2.7.6 and 2.7.7). Aliquots (200 µl) of every third fraction were analysed for competitive activity

and the amount of EGF equivalents was calculated from competition curves produced by unlabelled mEGF with [^{125}I]-mEGF as outlined above.

(c) Eluted material from SDS-fractions

Fractions from rHPLC demonstrating competitive activity were subjected to SDS-PAGE (see section 2.7.8). The supernatant of eluted material from SDS-PAGE gel slices were analysed for competitive EGF-like activity in radio receptor assays as outlined in section 2.7.3.

2.7.4 RAT TGF-I AND HUMAN EGF RADIOIMMUNOASSAYS

The levels of immunoreactive rTGF–I and hEGF in samples of CM, fractions from gel filtration, rHPLC and eluted material from SDS-PAGE were determined using liquid-phase competitive RIAs.

rTGF–I

A commercial kit (Peninsula Laboratories) with rTGF I as radioiodinated tracer and reference standard was used to detect levels of TGFα in the CM. Antisera to the synthetic rat TGF I were raised in rabbits. The rabbit anti-rat TGF I antiserum recognizes both rat and human TGFα, but the level of cross-reactivity with human TGFα is only 32% of the homologous antigen (rat TGF-I) (Moore, pers. comm. 1989). This correction was taken into consideration in all calculations. Rabbit anti-rat TGF I does not cross react with either mouse or human EGF. Half-maximal inhibition of binding of the [^{125}I]-peptide to the antibody occurred at 100pg/tube.

Briefly, the RIA was carried out over 3 days, on day 1 rat TGF I standards and samples were incubated at 4°C overnight with rabbit anti-rat TGF I serum. [^{125}]-rTGF- I was added the next day to the RIA assay tubes, followed by a further overnight incubation (4°C). On day 3 goat anti-rabbit IgG serum and normal rabbit serum as carrier protein was added to the contents of each tube and incubated at room temperature for 120 min. RIA buffer was subsequently added to each tube and the

samples were centrifuged at 3000 rpm for 20 min. The supernatant was aspirated and the radioactivity in the remaining pellets determined. The levels of human TGFα in each sample of CM were determined from a standard curve of %B/Bo, where Bo is equal to the amount of [^{125}]-rTGF I competing with anti-rTGF-I without added unlabelled TGF-I minus the NSB ([^{125}]-rTGF plus the second Ab).

hEGF

hEGF in the samples was detected by the competition of hEGF for [^{125}I]- hEGF binding to anti-hEGF serum. All dilutions of antibodies and standards were made in RIA buffer (0.04M phosphate, 150mM NaCl, 0.01M EDTA, 0.5% BSA). 100µl of a 1:40 000 dilution of anti-hEGF was incubated at 4°C with 100 µl of [^{125}I]-EGF (specific activity 49TBq/mmol; with approximately 10,000 cpm added per tube) and hEGF standards (0-20ng in triplicate) or samples. Each tube was vortexed for 5 seconds before incubating at 4°C. After three days, 500µl of donkey anti-rabbit (at a 1:20 dilution; 5µl/tube plus normal rabbit serum at a dilution of 1:200; 0.5µl/tube; Scottish Antibody Production Unit, Carluke, Scotland) was added, vortexed and incubated for a further 24 hours at 4°C. Distilled water (500µl) was then added and the contents of the tubes vortexed and subsequently centrifuged at 3000 rpm for 30 mins. The supernatant was aspirated and the radiolabel in the pellets was determined by counting in a gamma counter. Half-maximal competition was observed with 200-250 pg native hEGF. The anti-hEGF-urogastrone does not significantly cross react with either TGFα or mEGF (Gregory, per. comm. 1988).

2.7.5 BIOLOGICAL ACTIVITY OF CM: EFFECT ON DNA SYNTHESIS IN DU 145 CELLS

Medium conditioned by DU 145 cells was tested for bio-activity in a [^{3}H]-Thymidine incorporation assay. In the initial experiment spent medium at concentrations of 3-1000 µg/ml was added to DU 145 cell monolayers (1 x

10^4cells/well) for a period of 8, 24, 48 & 72 hours in 96 well plates; SFM was used as a control. This experiment was repeated with concentraions of 0.01-1000 μg/ml for 24 hours. After this time [^{3}H]-Thymidine was added to each well and the incorporation of [^{3}H]-Thymidine measured as previously described in section 2.4.3. Cell viability was > 90% after 24 hours as assessed by the trypan blue exclusion test.

2.7.6 GEL FILTRATION

The supernate of the concentrated CM (10 mg) was applied to a G-100 Sephadex (Superfine grade, with a fractionation range of 4 000 - 150 000 molecular weight) column (100 x 2.5 cm; Pharmacia (G.B) Ltd., Hounslow, Middlesex) equilibrated and eluted with 0.02M PBS, pH 7.4 at 4°C. 3 ml fractions were collected and the absorbance was monitored at 280 nm. Every third fraction was tested for competitive activity in EGF-RRA (as described in section 2.7.3.b) and immunological activity in hEGF and rTGF-I RIAs (as described in section 2.7.4).

Calculation of molecular weights

The molecular weights of the fractions from the separated CM were calculated from a calibration curve of molecular weight markers (thyroglobulin, 670 kDa; gamma globulin, 158 kDa; ovalbumin, 44 kDa; myoglobin, 17 kDa and vitamin B_{12}, 1.3 kDa; Bio-Rad Labs.). Thyroglobin is not retained with Sephadex G-100 and was used for calculating the void volume (Vo). The volume eluted (Ve) of each of the molecular weight markers and unknowns are related to their molecular weights.

2.7.7 REVERSE-PHASE HIGH PERFORMANCE LIQUID CHROMATOGRAPHY

Trifluroacetic acid (TFA; 0.1%) was added to the supernate of CM (15 mg), vortexed and spun at 15 000 rpm for 10 mins. The soluble material was loaded on to a Bio-Rad RP 304 C_4 column (250 mm x 10 mm), with a slurry packed guard column of

the same material. Before running the aliquot of CM, a blank run was carried out with buffer alone. The column was equilibrated with 95% Buffer A (0.2μm filtered double distilled water (DDW), 0.1% TFA; Rathburn Chemicals, Rathburn, Scotland, U.K) and 5% Buffer B (20% DDW, 80% acetonitrile (MeCN), 0.085% TFA; Rathburn Chemicals) and the sample eluted with a linear elution gradient of 5%-80% Buffer B, at a flow rate of 0.5 ml/min at room temperature. The absorbance of each fraction eluted was read at 280nm, with 1ml fractions collected. Acetonitrile was blown off each fraction under a stream of air, over 24 hours, and the remaining sample frozen to -70°C, lyophilized to dryness and reconstituted in 0.5 ml of 0.02M PBS with 1g/l BSA, pH 7.4. Every third fraction was analysed for competitive activity in an EGF-RRA (as described in section 2.7.3.b) and hEGF and rTGF immunological activity by RIA (as described in section 2.7.4).

2.7.8 NON-DENATURING SDS-PAGE.

Immunologically and biologically active fractions from rHPLC were subjected to SDS-PAGE on 12% polyacrylamide slab gels according to the method of Laemmli (1970). The method was essentially similar to that described in section 2.6.2, but the gels were run under non-reducing conditions (without 2β-mercaptoethanol in the sample buffer), with molecular weight markers (Sigma) ranging from 6.2kDa-66 kDa (apronitin, 6.2 kDa; cytochrome C, 12.4 kDa; carbonic anhydrase, 29 kDa and albumin, 66 kDa).

After electrophoresis a track of the gel was stained for protein with Coomassie Brilliant Blue (as described in section 2.6.3) while the remaining tracks were sliced into 5mm sections.

Detection of biological and immunological activity

For assay of biological and immunological activity, the method used to extract materials in individual SDS-PAGE gel slices was essentially similar to that used by

Stromberg *et al.* (1986). The material in the gel slices was extracted by adding 1 ml of 1 M acetic acid containing 100 µg of bovine serum albumin as carrier, crushing the gel slice in a conical tube with a glass rod, and incubating them for at least 24 hours at 4° C to permit polypeptides to leach out of the gel matrix. The supernatant was collected following a 5 minute centrifugation, frozen to -70°C, lyophilized and reconstituted in 0.5ml with 0.02M PBS prior to assay. A 100 µl aliquot of each eluate was assayed for rTGF-I immunological activity, and a 200 µl aliquot was assayed for EGF-RRA competitive activity.

2.8 DATA ANALYSIS

2.8.1 'LIGAND'

All competition and saturation analyses were performed using the weighted, nonlinear least-squares curve fitting program LIGAND (DeLean, *et al.*, 1978; Munson & Rodbard, 1980), run on an IBM-PC. Scatchard plot analysis of saturation curves was not employed due to the difficulty in estimating the lowest ligand concentrations, which are unevenly "weighted" in Scatchard analysis (Bennett & Yamamura 1985). The program makes a few assumptions in its analysis: (i) Multiple ligands can bind to multiple sites, (ii) the binding reaction being analysed is at equilibrium, (iii) the binding is bi-molecular and reversible and (iv) there is total and true separation of bound ligand from free ligand.

Statistical Curve Fitting

Curves can be fitted to a single - or multi-site model using the "extra sum of squares" *F*- test criterion. Thus a model for two binding sites is retained only when it fits the data significantly better ($P< 0.05$ partial *F* test) than a model for a single binding site.

Weighting Parameters

Weighting has also been incorporated to reduce the tendency for unreliable points to unduly influence the location of the curve. Thus, weights are assigned to each point as the reciprocal of the variance at that point, therefore points with the smaller variance (more precise) usually receive more weight (Rodbard *et al.*, 1976).

Correction Factors

Correction factors have also been included with this program. The factor (C) in the program is a fitted parameter which adjusts or scales the values of apparent receptor concentration for any particular experiment. Thus when comparing experiments the

scale factor for that curve is adjusted eliminating the variability in apparent receptor concentration between experiments.

2.8.2 SATURATION ANALYSES

Saturation curves were analysed using LIGAND. The curves were analysed according to a model for one or two binding sites. A model for two binding sites was retained only when it fitted the data significantly better ($P<0.05$ partial F test) than a model for a single binding site. This method represents a substantial improvement over the most common graphical Scatchard plot for estimation of affinity constants in ligand binding studies. The advantage of this method is that it fits raw experimental data in an untransformed coordinate system, where errors are more likely to be normally distributed and uncorrelated with the independent variable. The approximations used in many analyses of binding data are non-existent in this method. This method calculates the "free" values from the experimentally measured "total" radioactivity added minus the "bound" values and solving the equations which represent the mass-action law. Non-specific binding is also estimated as an independent parameter from the entire data set, rather than by the investigator. The parameters for the affinity constants and receptor densities are also provided with their standard errors permitting an assessment of the confidence limits for each parameter obtained. Curves from several experiments were analysed both separately and simultaneously.

2.8.3 COMPETITION ANALYSES

For each competition curve, estimates of the affinity of the radiolabelled ligand for the binding site was obtained from separate independent saturation curves. Statistical analysis comparing "goodness of fit" between one and two affinity state models was provided and used to determine the most appropriate model for the ligand being

examined. A model for two binding sites was retained only when it fitted the data significantly better ($P<0.05$ partial F test) than a model for a single binding site. Different curves were then analysed simultaneously to find an estimated value common to both curves, the parameters were constrained to be estimated as a common value. The effect of this constraint on the goodness of fit was tested and the shared parameters were considered statistically indistinguishable if the constraining process did not significantly worsen the simultaneous fit.

2.8.4 OTHER STATISTICAL ANALYSIS

In some experiments, statistical significance was determined using a two-tailed Student's t-test for comparison of means.

Chapter 3

RESULTS

In this chapter the results are presented as follows:-

1. Comparative study between DU 145 and LNCaP cell lines

   (a) Growth responses to EGF, TGFα and androgens

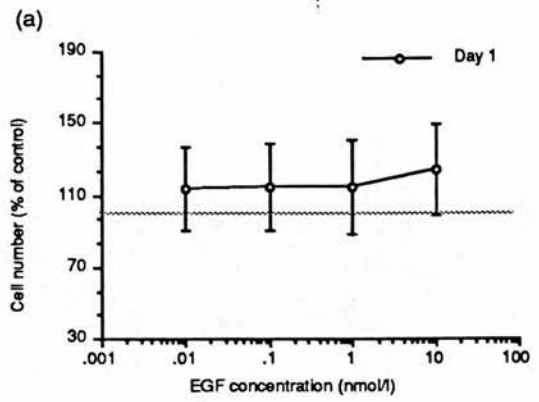
   (b) Characterization of the EGF receptor

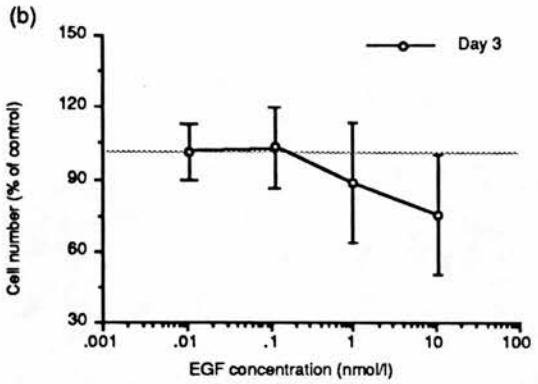
   (c) Analysis of the EGF and truncated receptor expression by Western blotting.

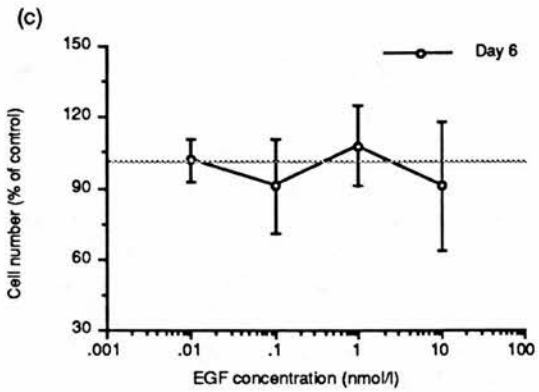
2. Analysis of medium conditioned by the DU 145 cell line for production of EGF-like molecules.

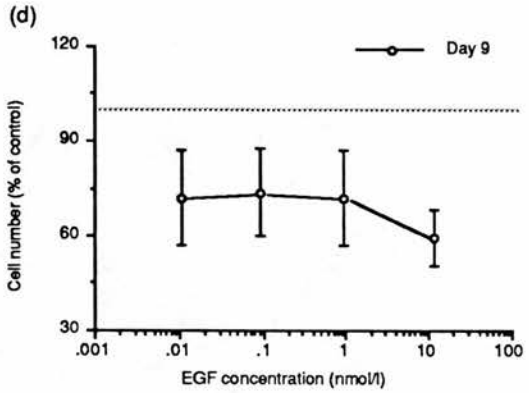
3.1 A COMPARATIVE STUDY OF THE GROWTH RESPONSES AND BINDING OF EGF BETWEEN THE CELL LINES DU 145 AND LNCAP

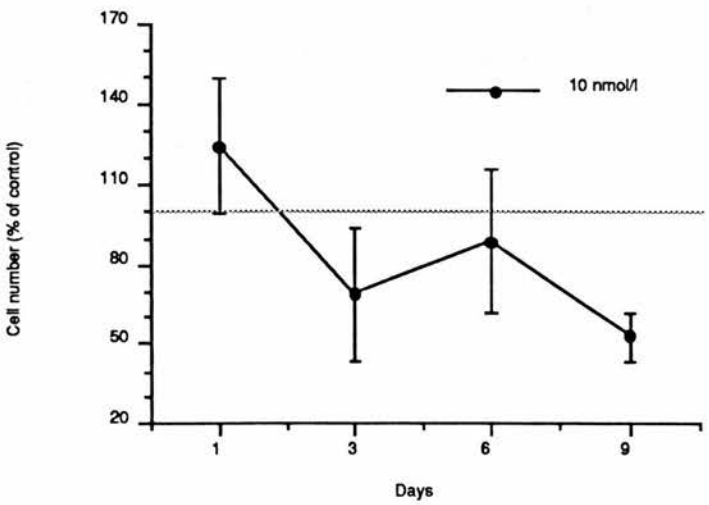
The relationship between EGF binding and the growth response of a cell line to EGF was investigated and compared in the androgen-insensitive (DU 145) and androgen-sensitive (LNCaP) prostatic carcinoma cell lines. Earlier studies have touched on the possibility of a synergistic relationship between EGF and androgens (Traish & Wotiz, 1987; St-Arnaud *et al.* 1988; Schuurmans *et al.* 1988a) and it was the aim of this investigation to shed further light on this complex relationship.


(a)
Day 1
Cell number (% of control)
190
150
110
70
30
.001
.01
.1
1
10
100
EGF concentration (nmol/l)


(b)
Day 3
Cell number (% of control)
150
120
90
60
30
.001
.01
.1
1
10
100
EGF concentration (nmol/l)


(c)
Day 6
Cell number (% of control)
150
120
90
60
30
.001
.01
.1
1
10
100
EGF concentration (nmol/l)


(d)
Day 9
Cell number (% of control)
120
90
60
30
.001
.01
.1
1
10
100
EGF concentration (nmol/l)


10 nmol/l
Cell number (% of control)
170
140
110
80
50
20
1
3
6
9
Days

3.1.1 THE EFFECTS OF EGF ON GROWTH AND DNA SYNTHESIS OF DU 145 AND LNCAP CELLS

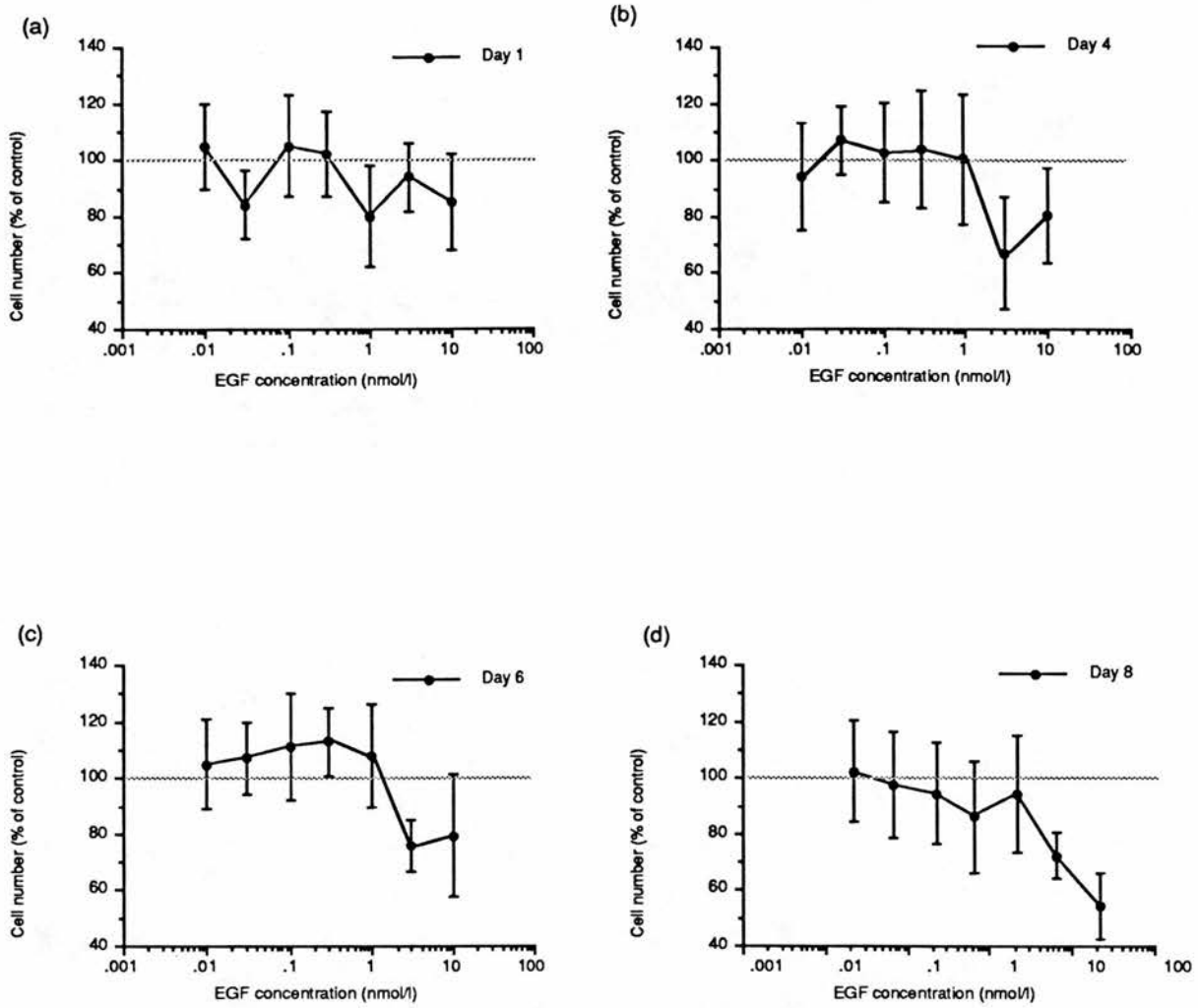
The effect of growth factors and/or androgens on the growth rate and DNA synthesis of the cell lines DU 145 and LNCaP are compared in the following section. The experiments with DU 145 and LNCaP were carried out with the cell lines cultured in SFM, with the exception of the experiment described in the following section (3.1.1.1).

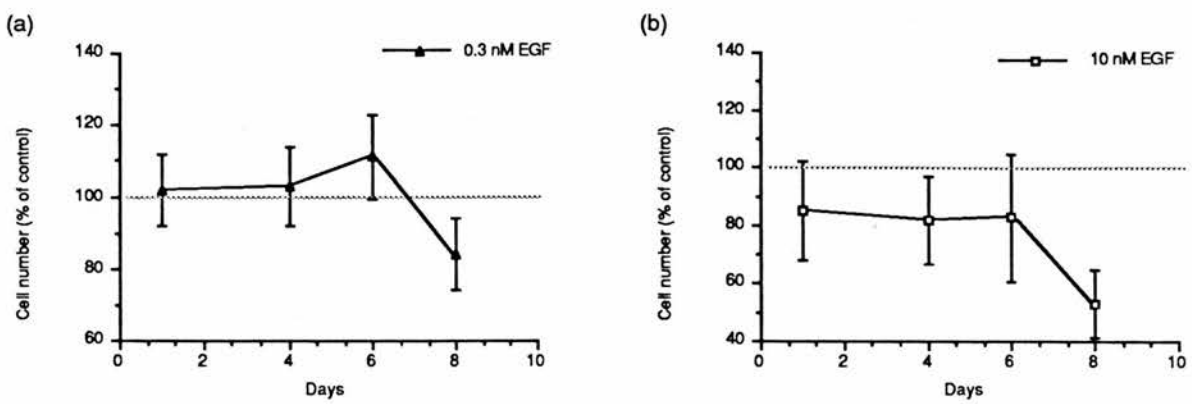
3.1.1.1 Growth response of DU 145 to EGF in serum-containing medium

The results depicted in Figure 11 demonstrate the impact of increasing concentrations of EGF on the growth of the cell line DU 145 over a period of 9 days. For the first 24 hours EGF had no discernable effect on cell growth, whereas after 3 days, EGF concentrations greater than 0.1 nmol/l inhibited growth in a dose-dependent fashion. This inhibitory effect on the growth of these cells was more pronounced after nine days, with EGF concentrations less than 0.1 nmol/l now significantly inhibiting proliferation ($P < 0.001$). EGF at a concentration of 10 nmol/l exerted the maximum inhibition on the growth of these cells, with this effect seen as early as 3 days after EGF was added (Figure 12). As the number of days exposure to EGF increased, a

FIGURE 11: *Dose-response effect of EGF on cell proliferation of DU 145 cells in the presence of foetal calf serum.* Cells in the exponential phase of growth were seeded in 9 well plates (5 000 cells/ cm^2) with 10% FCS. After plating (24 hours), EGF (0.01-10nmol/l) was added to triplicate wells for periods of 1 (Fig.11a), 3 (Fig.11b), 6 (Fig.11c) & 9 days (Fig.11d) and the cells counted after these times. Each data point represents the mean ± SD (n = 9) of three separate experiments normalized relative to the untreated control in 10% FCS. The control is represented by the dotted line at 100%.

FIGURE 12: *Time course of EGF inhibition of cell proliferation of DU 145 cells.* Cells were plated as described above, EGF (10 nmol/l) was added for up to 9 days and the cells subsequently counted. Each data point represents the mean ± SD (n = 9) of three separate experiments normalized relative to the untreated control in complete medium. The control is expressed as 100% (dotted line).


(a)
Day 1
Cell number (% of control)
EGF concentration (nmol/l)
(b)
Day 4
Cell number (% of control)
EGF concentration (nmol/l)
(c)
Day 6
Cell number (% of control)
EGF concentration (nmol/l)
(d)
Day 8
Cell number (% of control)
EGF concentration (nmol/l)


(a)
0.3 nM EGF
Cell number (% of control)
Days
(b)
10 nM EGF
Cell number (% of control)
Days

trend towards inhibition was apparent which after nine days was more pronounced, inhibiting cell proliferation by 40%± 9, relative to the untreated control ($P < 0.001$).

3.1.1.2 The effects of EGF on growth and DNA synthesis of DU 145 cells

The effects of EGF on the proliferation and DNA synthesis of a serum-free DU 145 line was investigated in a parallel study, the results of which are depicted in Figures 13, 14, 15 & 16.

No detectable effect in cell numbers was apparent after 24 hours exposure to EGF, whereas after 4 days EGF concentrations greater than 1 nmol/l significantly inhibited growth ($P< 0.05$). Similarly on day 6, EGF concentrations greater than 1 nmol/l inhibited growth ($P< 0.05$), with lower concentrations up to 0.3 nmol/l now stimulating proliferation. Eight days after exposure to EGF this biphasic effect on growth was abolished, with concentrations which had previously stimulated growth now inhibiting proliferation.

The data summarised in Figure 14 depicts concentrations which maximally stimulated (0.3 nmol/l; Fig 14a) and inhibited (10 nmol/l; Fig. 14b) proliferation expressed as a function of time. Only on day 6 did EGF (0.3 nmol/l) stimulate proliferation, with an increase of 12%± 12 in cell number, but this increase was not

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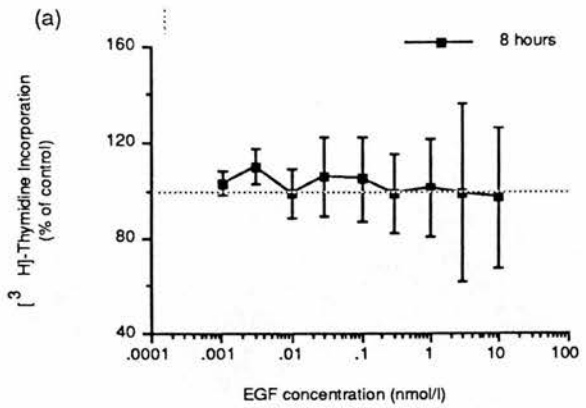
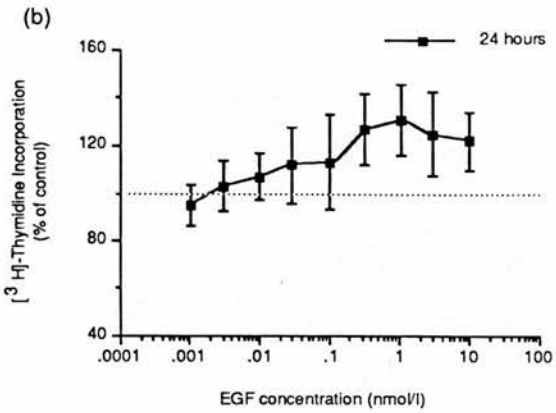
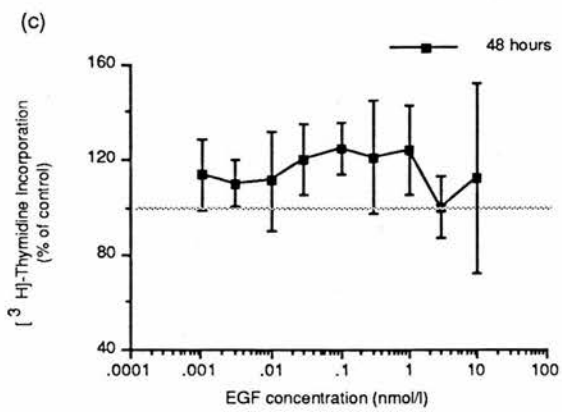
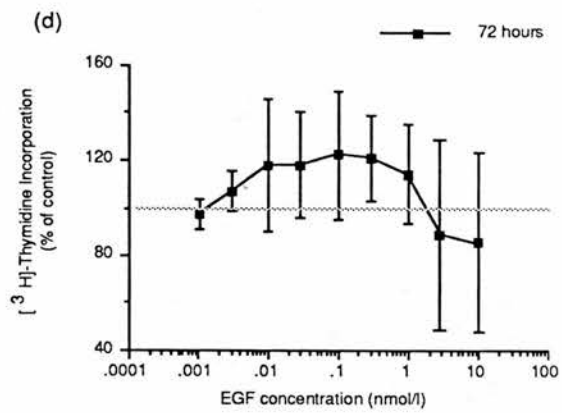
FIGURE 13: *Dose-response effect of EGF on cell proliferation of DU 145 cells in SFM.* Cells in the exponential phase of growth were seeded in 24 well plates (50 x 10^3 cells/well) or 9 well plates (2 x 10^5 cells/well). After 24 hours EGF (0.01-10nmol/l) was added in SFM to serum free cultures for 1 (Fig.13a), 4 (Fig.13b), 6 (Fig.13c) and 8 days (Fig.13d) and the cells counted after these times. The data are expressed as mean percentages ± SD (n = 12) of the untreated control, where the control is 100%. Four separate experiments were performed.

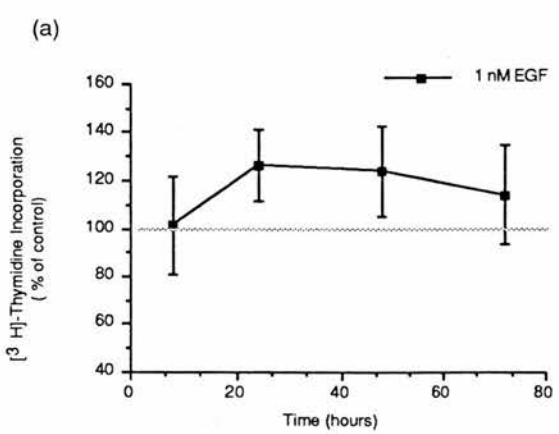
FIGURE 14:*Time course of EGF stimulation and inhibition of cell proliferation of DU 145 cells.* Cells were plated as described above, EGF (0.3 nmol/l, Fig. 14a; 10 nmol/l; Fig.14b) was added to SFM cultures for up to 8 days and the cells subsequently counted. Each data point represents the mean ± SD (n = 12) of four separate experiments and the data are normalized relative to the untreated SFM control (100%).

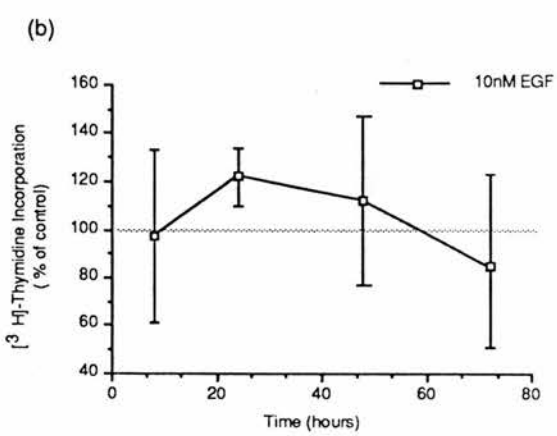

(a)
8 hours
[3 H]-Thymidine Incorporation (% of control)
160
120
80
40
.0001
.001
.01
.1
1
10
100
EGF concentration (nmol/l)


(b)
24 hours
[3 H]-Thymidine Incorporation (% of control)
160
120
80
40
.0001
.001
.01
.1
1
10
100
EGF concentration (nmol/l)


(c)
48 hours
[3 H]-Thymidine Incorporation (% of control)
160
120
80
40
.0001
.001
.01
.1
1
10
100
EGF concentration (nmol/l)


(d)
72 hours
[3 H]-Thymidine Incorporation (% of control)
160
120
80
40
.0001
.001
.01
.1
1
10
100
EGF concentration (nmol/l)


(a)
1 nM EGF
[3 H]-Thymidine Incorporation (% of control)
160
140
120
100
80
60
40
0
20
40
60
80
Time (hours)


(b)
10nM EGF
[3 H]-Thymidine Incorporation (% of control)
160
140
120
100
80
60
40
0
20
40
60
80
Time (hours)

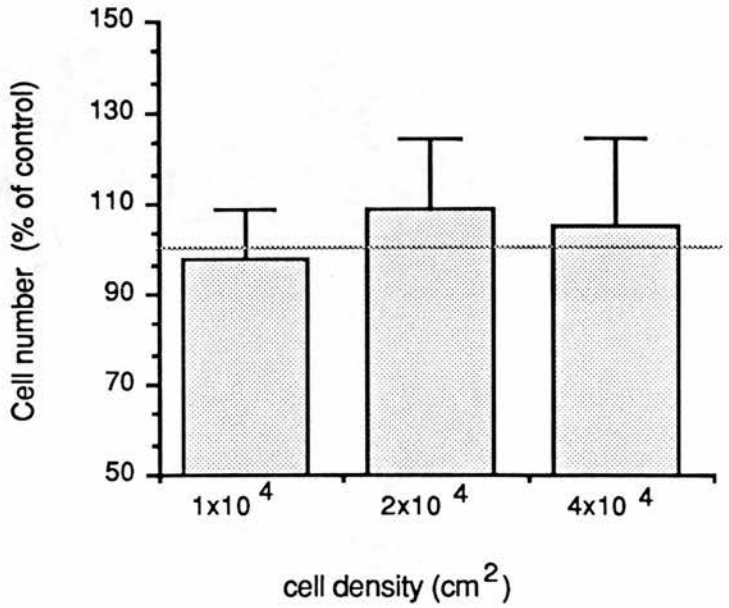
statistically significant ($P > 0.05$) and by day 8 inhibition rather than stimulation of growth was apparent. EGF at a concentration of 10 nmol/l inhibited growth as early as day 1, and after 8 days this inhibition was more pronounced, with a decrease of 47%±12 ($P < 0.001$) in cell number relative to the untreated control.

In a parallel study the impact of EGF on [^{3}H]-Thymidine incorporation was investigated (Figures 15 & 16). No effect was noted after 8 hours treatment with EGF, but at 24 hours EGF stimulated [^{3}H]-Thymidine incorporation in a dose-dependent manner. This stimulatory effect was still discernable at 48 hours and 72 hours with EGF concentrations up to 1 nmol/l. At 72 hours an inhibitory trend was apparent, with EGF concentrations greater than this value now inhibiting [^{3}H]-Thymidine incorporation. The maximum increase in [^{3}H]-Thymidine incorporation (26%±13; $P < 0.001$) was noted after 24 hours exposure to 1 nmol/l EGF (Figure 16a). Although still apparent after 72 hours, [^{3}H]-Thymidine incorporation decreased slightly and was not significantly different from the untreated control. Incorporation of [^{3}H]-Thymidine increased with 10 nmol/l EGF after 8 hours and reached a plateau at 24 hours. Thereafter, DNA synthesis decreased and was inhibited at 72 hours, although not significantly ($P > 0.05$; Figure 16b).

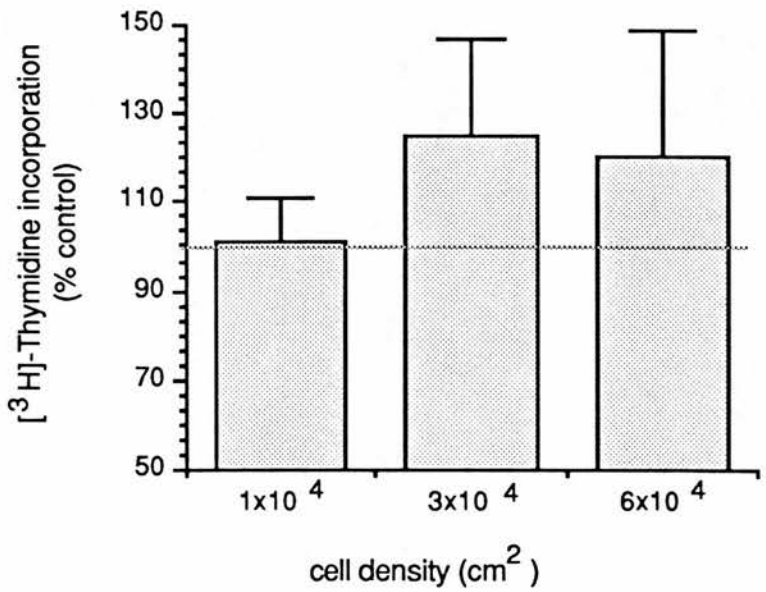
FIGURE 15: *Dose-response effect of EGF on [^{3}H]-Thymidine incorporation of DU 145 cells.* Cells (1 x 10^4 cells/well) were plated overnight in SFM/0.5% FCS in 96 well plates. EGF (0.001-10 nmol/l) was added in SFM (6 replicates/ EGF concentration) for 8 (Fig.15a), 24 (Fig.15b), 48 (Fig.15c) & 72 hours (Fig.15d). [^{3}H]-Thymidine (37Bq/well) was then added for 4 hours, the cells were trypsinized and 10% ice-cold TCA added for 2 hours. The cells were then harvested on to filter mats, dried and counted in scintillation fluid. Each data point represents the mean ± SD (n=60) of 10 separate experiments and the data are normalized relative to the untreated SFM control (100%).

FIGURE 16:*Time course of EGF stimulation of [^{3}H]-Thymidine incorporation of DU 145 cells.* The experiment was performed as described in Figure 15. EGF (1 nmol/l, Fig.16a; 10 nmol/l, Fig.16b) was added to serum free cultures for 8, 24, 48 & 72 hours. [^{3}H]-Thymidine incorporation was subsequently measured in TCA precipitable material. Each data point represents the mean ± SD (n = 60) of 10 separate experiments and the data are normalized relative to the untreated SFM control (100%).

(a)


150
130
110
90
70
50
Cell number (% of control)
1x10 4
2x10 4
4x10 4
cell density (cm 2)

(b)


150
130
110
90
70
50
[3H]-Thymidine incorporation (% control)
1x10 4
3x10 4
6x10 4
cell density (cm 2)

3.1.1.3 Cell density dependence of EGF response by DU 145 cells

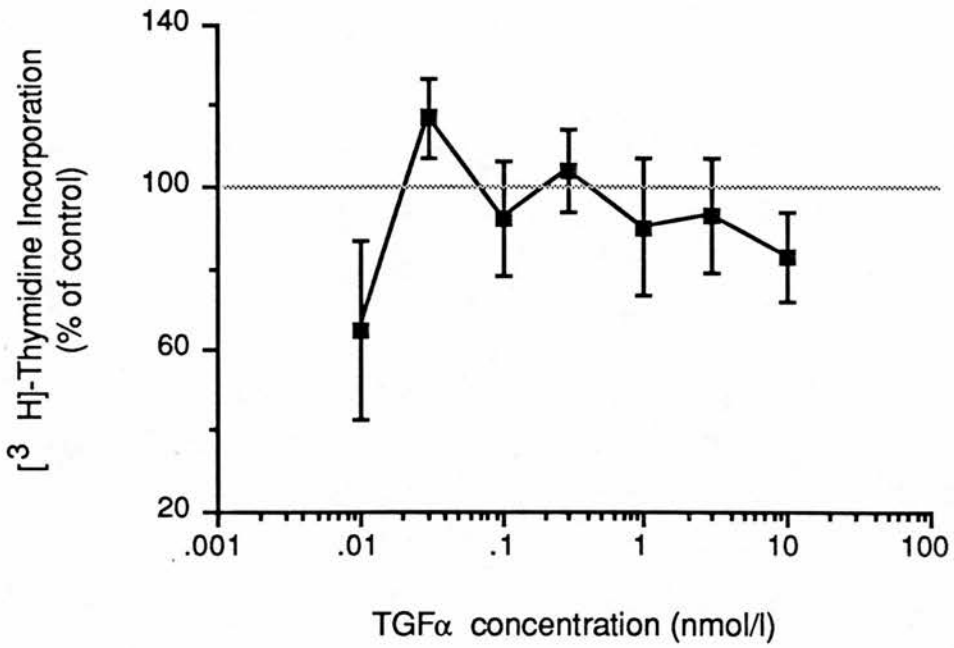
The proliferation of cell lines in a defined medium free of serum largely depends upon the interplay between population density, hormones and growth factors. Kaighn *et al.*, (1981) demonstrated that the growth of the DU 145 cell line in serum-free medium was population dependent and that these cells only responded to hormones and growth factors at high cell density. This was further confirmed by the present study where sustained growth of DU 145 cells in SFM was found to be dependent upon high plating densities (> 10^4 cells/cm^2). Cells would detach spontaneously if seeded at densities less than 10^4 cells/cm^2 and this effect was not overcome by the addition of growth factors. However, the weak stimulatory response to EGF observed in section 3.1.1.2 may have been due primarily to the population density. To test this hypothesis the growth response to EGF was studied at different cell densities.

DU 145 cells were seeded at innocula of $1x10^4$, $2x10^4$ and $4x10^4$ cells/cm^2 and the growth response to 0.3 nmol/l EGF was measured after 6 days. The results in Figure 17a indicate that EGF did not stimulate or inhibit cell growth significantly as the seeding density was increased from $1x10^4$ to 4 $x10^4$ cells/cm^2. Cell numbers were only enhanced by 12±18% at a seeding density of 2 x 10^4 cells/cm^2, although this effect was not significantly different from control values ($P > 0.05$).

In a parallel study DU 145 cells were seeded at innocula of 1 $x10^4$- $6x10^4$ cells/cm^2 and DNA synthesis was measured by [^{3}H]-Thymidine incorporation after treating the cells for 24 hours with 1 nmol/l EGF (Figure 17b). Overall, varying the cell density did not greatly enhance the mitogenic effect of EGF. The maximal stimulatory effect

---

FIGURE 17: *The effect of cell density on the response to EGF.* Cells were seeded at innocula of 1 x 10^4 - 6 $x10^4$ cells/cm^2 in 24 or 96 well plates. After plating (24 hours later) EGF (0.3 nmol/l or 1nmol/l) was added for 6 days and the cells subsequently counted or for 24 hours and [^{3}H]-Thymidine incorporation measured. The results are expressed as means ± SD relative to untreated controls in SFM. Three proliferation (n = 12) and four [^{3}H]-Thymidine incorporation (n =24) experiments were performed.


140
100
60
20
[3 H]-Thymidine Incorporation
(% of control)
.001
.01
.1
1
10
100
TGFα concentration (nmol/l)

with EGF was noted at a seeding innoculum of 3 x 10^4 cells/cm^2, where DNA synthesis was stimulated by 26%±20 ($P< 0.001$) relative to control values. Densities greater than 3 x 10^4 cells/cm^2 did not enhance this effect on [^{3}H]-Thymidine incorporation. However this stimulatory response by EGF was abolished when the density was reduced to 1 x 10^4 cells/cm^2.

3.1.1.4 Dose-response effect of TGFα on DNA synthesis of DU 145 cells

One class of growth factors which shares sequence and structural homology with EGF and shows a variety of biological actions similar to EGF are the alpha TGFs. TGFαs are potent mitogens for a number of cell types and bind to and interact with the EGF receptor (Massague, 1983). Consequently TGFα was assayed for its effect on the incorporation of [^{3}H]-Thymidine in DU 145 cells, as a comparison to EGF (Figure 18). TGFα had very little effect on DNA synthesis after 24 hours as measured by [^{3}H]-Thymidine incorporation. A weak, but significant stimulatory response was noted at a concentration of 0.03 nmol/l, with an increase in incorporation of 17%±10;$P < 0.05$ relative to control values. TGFα concentrations greater than 0.03 nmol/l showed a slight tendency towards inhibition, with 10 nmol/l inhibiting [^{3}H]-Thymidine incorporation by 17%±11 ($P < 0.05$).

FIGURE 18: *Dose-response effect of TGFα on DNA synthesis of DU 145 cells.* Cells were seeded in 96 well plates (1 x10^4 cells/well), and after plating TGFα (0.01-10 nmol/l) was added (8 replicates/ TGFα concentration) for 24 hours. [^{3}H]-Thymidine (37Bq/well) was then added for 4 hours, the cells were trypsinized and 10% ice-cold TCA added for 2 hours. The cells were subsequently harvested on to filter mats, dried and counted in scintillation fluid. Each data point represents the mean ± SD (n=24) of three separate experiments and the data are normalized relative to the untreated SFM control (100%).

Summary of effects of growth factors on proliferation and DNA synthesis of DU 145 cells

The growth of the DU 145 cell line was not stimulated by EGF when these cells were grown in the presence of foetal calf serum. However, growth was significantly inhibited with higher concentrations of EGF and this effect was dose-dependent. Prolonged treatment with EGF enhanced this inhibitory effect on these cells, with EGF concentrations that had previously shown no effect, inhibiting proliferation. Similarly, EGF inhibited the growth of DU 145 cells grown in SFM, but in SFM a slight stimulatory effect was noted with concentrations up to 0.3 nmol/l. Although this effect on growth was not significant, EGF (at a similar concentration) significantly enhanced DNA synthesis.

TABLE 2: DOSE-RESPONSE EFFECTS OF DU 145 CELLS TO EGF AND TGFα IN SFM

Addition to culture	Cell count (% of control)	[^{3}H]-Thymidine incorporation (% of control)
EGF		
0.03nM	107± 13 [a] (4) [b]	112 ±14 (10)
0.3nM	112± 12 (P >0.05) (4)	124±13 (P < 0.001) (10)
10nM	83 ± 20 (4)	122 ±12 (10)
TGFα		
0.03 nM	ND	117±10 (P <0.05) (3)
0.3 nM	ND	104±10 (3)
10 nM	ND	83±11 (3)

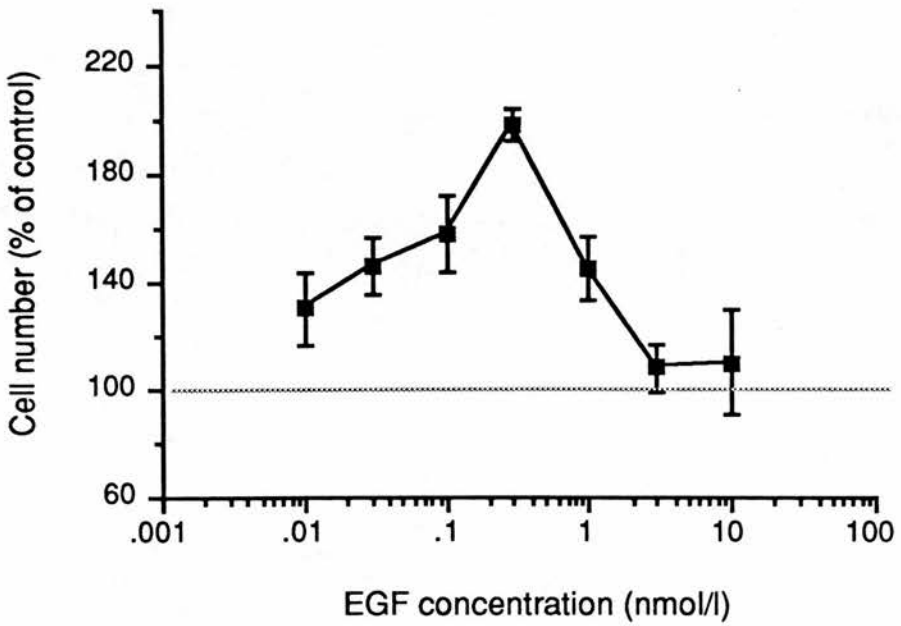
DU 145 cells were seeded at a density of 2 x 10^5 cells/well in 6 well plates or 1 x 10^4 cells/well in a 96 well plate. The indicated additions were made 24 hours after seeding. Cell counts were carried out 6 days after the initial addition of growth factors and DNA synthesis was measured by the incorporation of [^{3}H]-Thymidine after 24 hours. The results are expressed as a percentage of the untreated control (100%). ND = not determined.

[a] Mean ± SD.

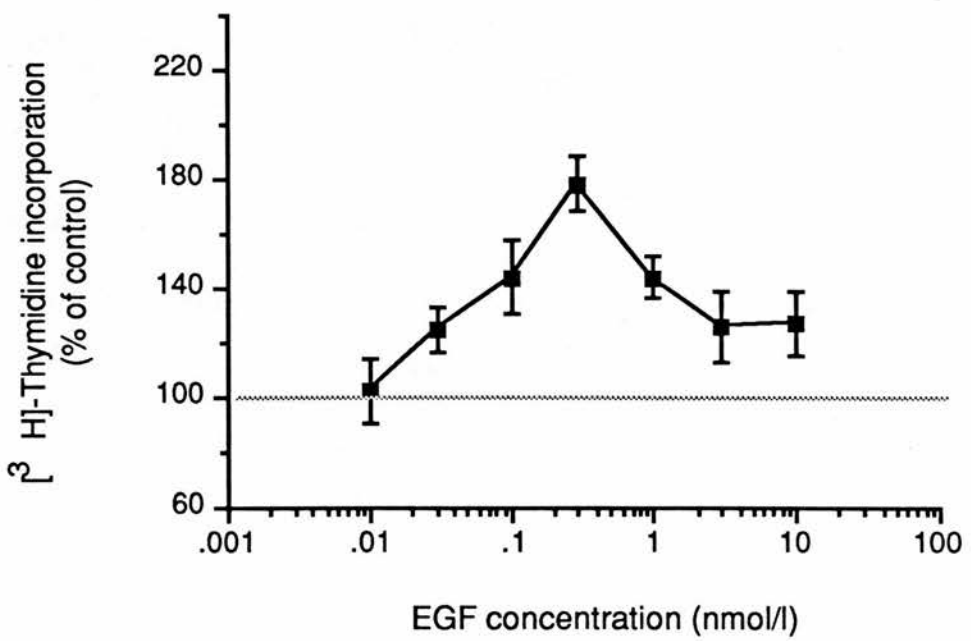
[b] Numbers in parenthesis, number of experiments.

The results summarised in Table 2 also depict the response of TGFα on DNA synthesis. Like EGF, TGFα is not a potent mitogen for DU 145 cells as DU 145 cells

(a)


Cell number (% of control)
220
180
140
100
60
.001
.01
.1
1
10
100
EGF concentration (nmol/l)

(b)


[3 H]-Thymidine incorporation
(% of control)
220
180
140
100
60
.001
.01
.1
1
10
100
EGF concentration (nmol/l)

were significantly but only minimally affected by TGFα. The concentration of TGFα which maximally stimulated DNA synthesis was 10 fold lower than that observed for EGF.

The mitogenic response of DU 145 cells to EGF was not enhanced significantly by varying the seeding densities of these cells, although the growth of these cells was density dependent.

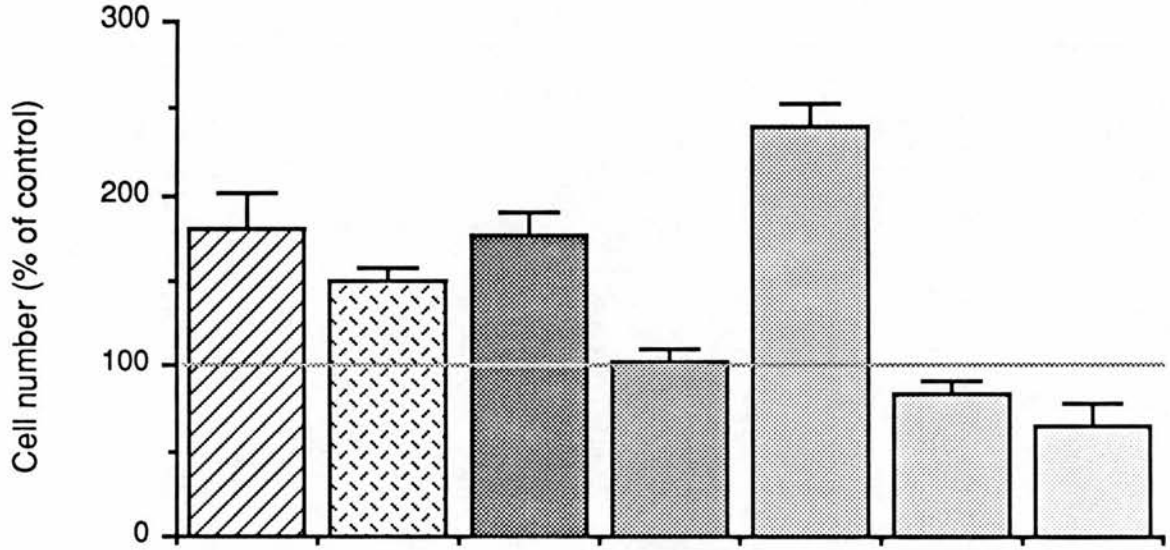
3.1.1.5 The effects of EGF on cell proliferation and DNA synthesis of LNCaP cells

After the initiation of this investigation a report was published by Schuurmans *et al.* (1988a), describing preliminary experiments on the growth response to EGF in LNCaP cells. Therefore, rather than determining the times at which the cells exhibited maximal stimulation to EGF, as carried out for DU 145 cells (section 3.1.1.2) the conditions determined by Schuurmans and co-workers were used.

Increasing concentrations of EGF were added to LNCaP monolayers for 6 days to determine the proliferative effects of EGF (Figure 19a) or alternatively for a period of 24 hours and the incorporation of [^{3}H]-Thymidine measured (Figure 19b). EGF exerted a biphasic effect on proliferation over the concentrations tested.

FIGURE 19: a) *Dose-response effect of EGF on cell proliferation of LNCaP cells.* Cells were seeded in 24 well plates (2 x 10^4 cells/cm^2) in complete medium. After 3 days the cells were washed once with Dulbecco 'A' PBS, SFM was added for several hours and was then replaced by fresh medium with EGF (0.01-10 nmol/l) and after 6 days the cells were counted. The results are expressed as cell number relative to the untreated control (100%; dotted line). Each data point is the mean of nine observations ± SD of three separate experiments.

b) *Dose-response effect of EGF on DNA synthesis of LNCaP cells.* Cells were seeded at innocula of 2 x 10^4 cells/cm^2 in 96 well plates in complete medium (as above). After three days EGF (0.01-10 nmol/l) was added in SFM for a period of 24 hours. [^{3} H]-Thymidine (37Bq/well) was then added for 4 hours, the cells were trypsinized and 10% ice-cold TCA was added for 2 hours. The cells were then harvested on to filter mats, dried and counted in scintillation fluid. Each data point represents the mean ± SD (n=24) of three separate experiments and the data are normalized relative to the untreated SFM control (100%).



[EGF]	0.3	-	0.3	-	-	-	-	nmol/l
[Androgen]	-	0.1	0.1	0.1	-	0.1	0.1	nmol/l

Concentrations up to 0.3 nmol/l EGF enhanced proliferation by 98% ± 6 relative to control values (P< 0.001), with higher concentrations abolishing this stimulatory effect.

EGF also stimulated DNA synthesis in a dose-dependent fashion, with the maximal effect observed with 0.3 nmol/l EGF, increasing [^{3}H]-Thymidine incorporation by 78±10% ($P < 0.001$). A dose-dependent decrease in [^{3}H]-Thymidine incorporation was noted with concentrations greater than 0.3 nmol/l (Figure 19b).

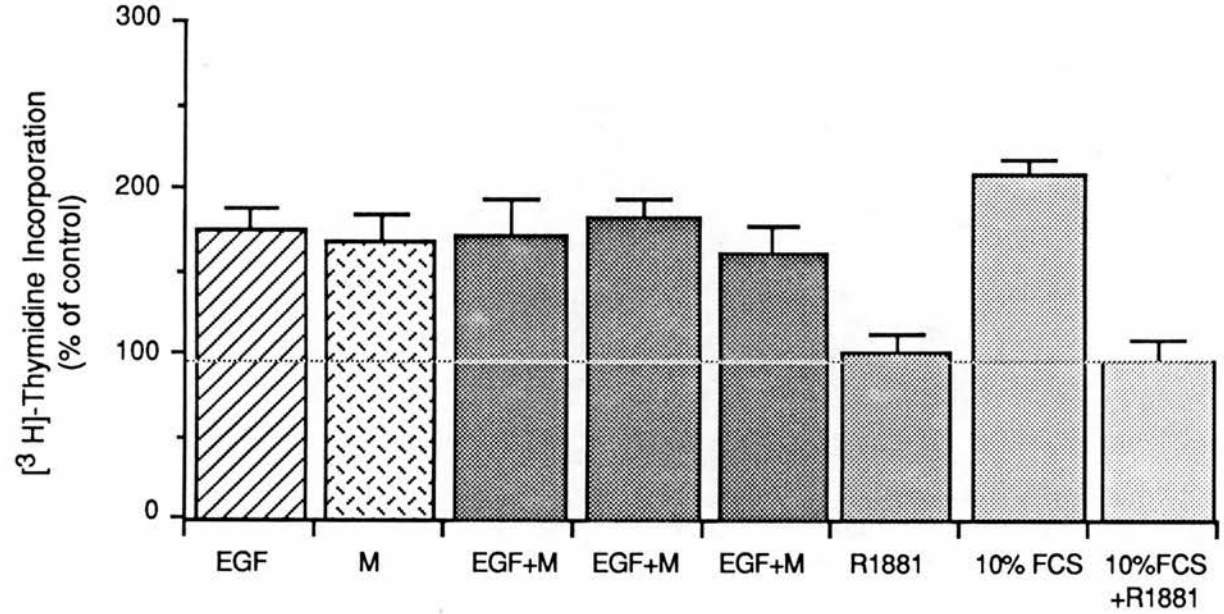
3.1.1.6 The effects of EGF and the androgens Mibolerone and R1881 on cell proliferation and DNA synthesis of LNCaP cells

The androgen sensitive LNCaP cell line was used to investigate the relationship between growth factors and androgens in prostate cancer.

The synthetic androgens Mibolerone and R1881 were added to SFM cultures of LNCaP cells with and without EGF, for a period of 6 days and the cells counted after this time (Figure 20) or for 24 hours and incorporation of [^{3}H]-Thymidine measured (Figure 21).

EGF and Mibolerone independently increased proliferation by 82%±18 ($P < 0.001$) and 51%±8.5 ($P < 0.02$) respectively. The addition of EGF and Mibolerone together to LNCaP cells did not produce a greater stimulatory effect than EGF alone (77%±12) (that is the effect on proliferation was not additive). Contrary to the stimulatory effect on proliferation with 0.1 nmol/l of Mibolerone, R1881 at the same concentration did not enhance proliferation. However, when R1881 was added to cells grown in 10% FCS, cell proliferation was inhibited by 74%±14 relative to the complete medium control ($P < 0.001$). Mibolerone, at the same concentration also

FIGURE 20: *The effect of EGF and Mibolerone/R1881 on cell proliferation of LNCaP cells.* Cells were seeded at innoculum of 2 x 10^4 cells/cm^2 in 6 well plates. After three days the factors to be tested were added in SFM as indicated, for 6 days and the cells subsequently counted. Each data point represents the mean ± SD (n=9) of three separate experiments and the data are normalized relative to the untreated SFM control (100%). M = Mibolerone.



[EGF]	0.3	-	0.1	1	10	-	-	-	nmol/l
[Androgen]	-	0.1	0.1	0.1	0.1	0.1	-	0.1	nmol/l

inhibited proliferation when added with 10% FCS, but not by the same extent (65%±7; $P < 0.05$).

EGF and Mibolerone independently stimulated DNA synthesis by 80%± 10 and 70±14% respectively ($P < 0.001$). However, this response was not additive as EGF and Mibolerone added together did not increase [^{3}H]-Thymidine incorporation above 79%±8 (Figure 21). Moreover, varying the concentration of EGF with Mibolerone did not enhance DNA synthesis above that already observed with EGF (0.3 nmol/l). Contrary to the growth response with Mibolerone, R1881 did not increase [^{3}H]-Thymidine incorporation when added to cultures grown in SFM, but DNA synthesis was significantly inhibited by 53%±8 (P <0.001; relative to the complete medium control) when R1881 was added to cultures grown in 10% FCS.

Summary of effects of EGF on proliferation and DNA synthesis in LNCaP and DU 145 cells

The data from proliferation and DNA synthesis studies with DU 145 and LNCaP are summarised in Table 3. The androgen insensitive DU 145 cell line was only minimally affected by EGF unlike the androgen sensitive LNCaP cell line. LNCaP cell numbers and [^{3}H]-Thymidine incorporation were increased by EGF; almost 2 fold, but this response with EGF was not affected by the androgen Mibolerone, although alone Mibolerone enhanced the growth of LNCaP cells. In contrast to Mibolerone, the synthetic androgen R1881 did not appear to affect cell proliferation or [^{3}H]-Thymidine incorporation in SFM. However, when LNCaP cells were cultured in complete

FIGURE 21: *The effect of EGF and Mibolerone/R1881 on DNA synthesis of LNCaP cells.* Cells were seeded at innoculum of 2 x 10^4 cells/cm^2 in 96 well plates. After three days the factors to be tested were added in SFM as indicated in the figure, for 24 hours and incorporation of [^{3}H]-Thymidine measured on TCA precipitable material. Each data point represents the mean ± SD (n=24) of three separate experiments and the data are normalized relative to the untreated SFM control (100%).

medium, the addition of R1881 to LNCaP cells inhibited both cell proliferation and DNA synthesis. A similar, but less dramatic effect was noted with Mibolerone.

TABLE 3: DOSE-RESPONSE EFFECTS OF EGF ON CELL PROLIFERATION AND [^{3}H]-THYMIDINE INCORPORATION OF DU 145 AND LNCAP CELLS

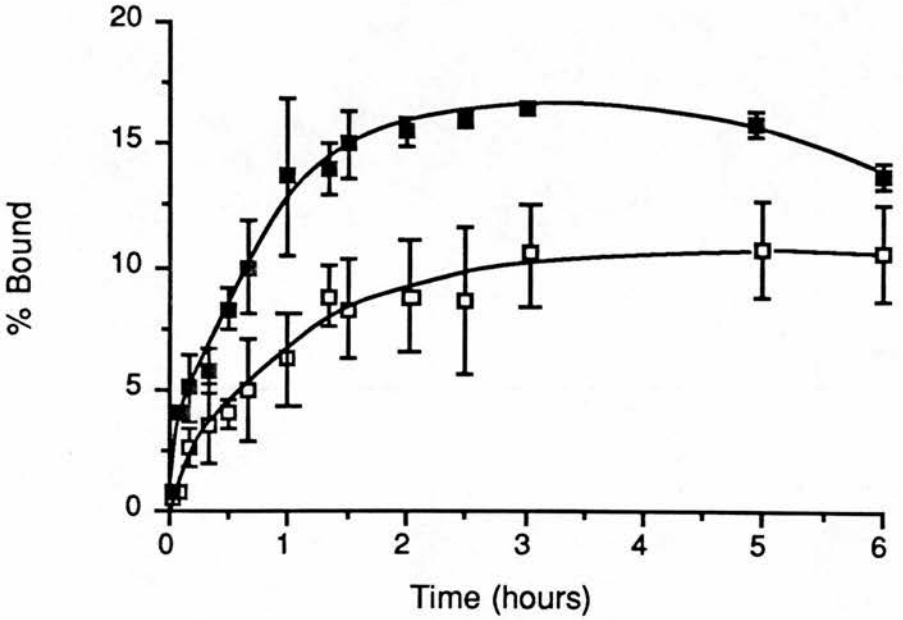
EGF nmol/l	Cell count (% of control)	[^{3}H]-Thymidine incorporation (% of control)
DU 145		
0.03nM	107± 13 [a] (4) [b]	112 ±14 (10)
0.3nM	112± 12 (P >0.05)(4)	124±13 (P < 0.001) (10)
10nM	83 ± 20 (4)	122 ±12 (10)
LNCaP		
0.03 nM	146±11(3)	125±8 (3)
0.3 nM	198±6% (P < 0.001)(3)	178±10 (P < 0.001) (3)
10 nM	110±19 (3)	127±12 (3)

DU 145 cells were seeded in 9 well plates (2 x 10^5 cells/well) for proliferation experiments and 96 well plates at a density of 1 x 10^4 cells/well for DNA synthesis experiments. LNCaP cells were seeded at a density of 2 x 10^4 cell/cm^2 in multiwell plates. The indicated additions were made 24 hours after seeding in SFM cultures. Cell counts were carried out 6 days after the initial addition of growth factors and DNA synthesis was measured by the incorporation of [^{3}H]-Thymidine after 24 hours. The results are expressed as a percentage of the untreated control (100%).

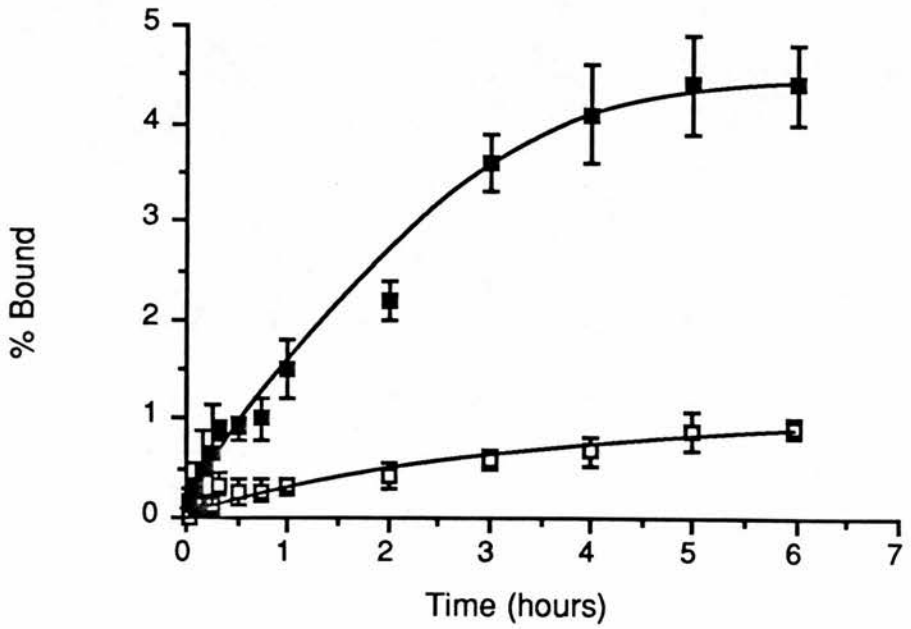
[a] Mean ± SD.

[b] Numbers in parenthesis, number of experiments.

(a) DU 145



(b) LNCaP



3.1.2 EGF RECEPTOR CHARACTERIZATION OF DU 145 & LNCAP CELL LINES

Since EGF affected the growth of both DU 145 and LNCaP cells the next step in this study was to characterize the EGF receptor of both cell lines. Using a ligand binding exchange assay the kinetics of association and dissociation were investigated, along with saturation, competition, internalization, specificity and down regulation of the receptor. The expression of the EGF receptor and the v-*erb* B gene product was also investigated by Western blotting and the results are presented in the following section.

3.1.2.1 Association kinetics: effect of temperature on the time course of [^{125}I]-EGF binding

The time course of [^{125}I]-EGF binding to prostatic carcinoma cells was examined at 37°C and 4°C (Figure 22 a & b). Binding of [^{125}I]-EGF to DU 145 and LNCaP cells was time and temperature dependent. Specific binding of [^{125}I]-EGF increased rapidly at 37°C, reaching a plateau after 3 hours with DU 145 cells and 5 hours with LNCaP cells, thereafter a decrease in specific binding was observed with DU 145 cells. At 4°C, binding of [^{125}I]-EGF to DU 145 monolayers also increased rapidly with time and a steady state was reached after 3 hours, although the percentage of total specific binding was substantially lower at 4°C than 37°C. Binding of [^{125}I]-EGF to LNCaP monolayers at 4°C increased at a much slower rate, a steady state was reached 6 hours

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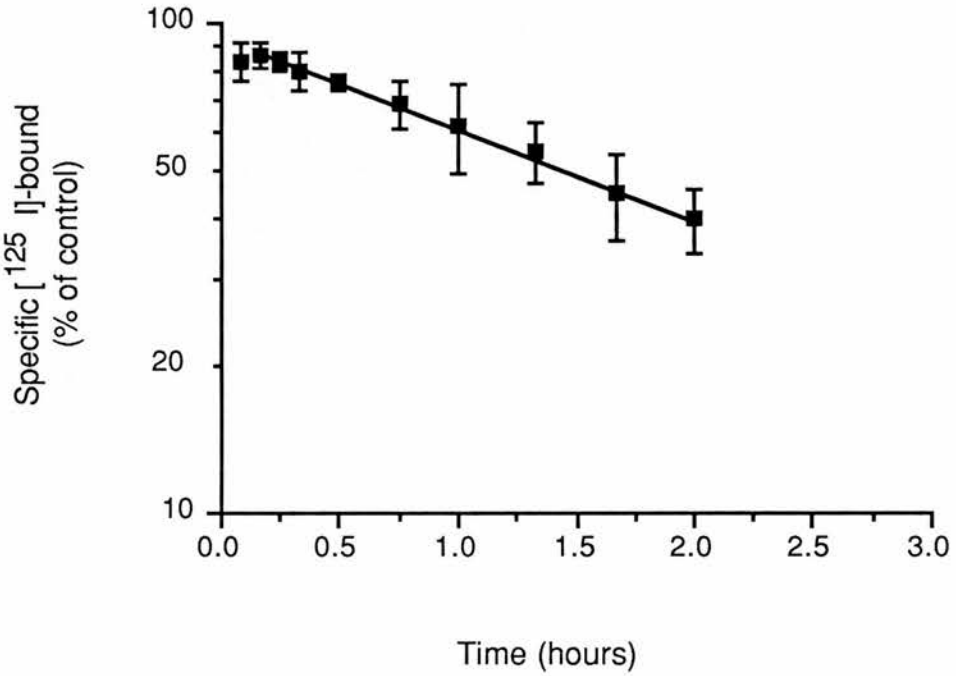
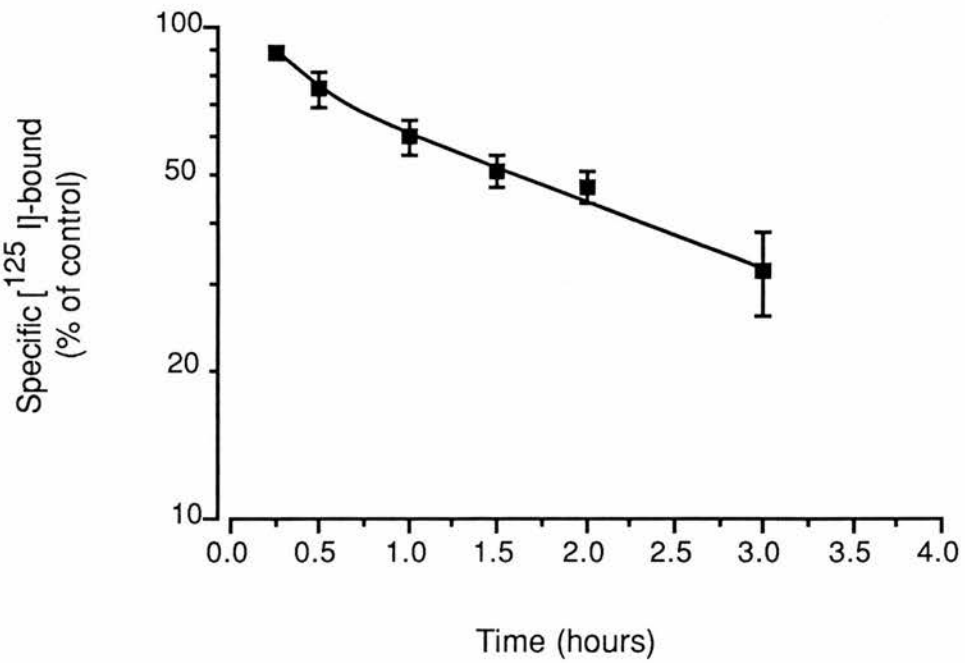
FIGURE 22: *Effect of time and temperature on specific binding of [^{125}I]-EGF in DU 145 & LNCaP cells.* Nearly confluent cultures of DU 145 and LNCaP monolayers (2 x10^5 cells/well) were plated in 24 well plates. Binding was carried out with sub-saturating levels of [^{125}I]-EGF (2nmol/l; 2 x 10^5 cpm) at 4°C (□) and 37°C (■) in the presence and absence of 100x excess unlabelled EGF. At the times indicated the monolayers were washed in Dulbecco'A' PBS (3x), the cells were dissolved in 0.5N NaOH and the radioactivity measured. Specific binding was measured by subtracting total binding from non-specific binding. The results are expressed as the percentage of [^{125}I]-EGF specifically bound. Each data point is the percentage mean ± S.D of three separate experiments (n = 9).

(a) DU 145



(b) LNCaP



after the addition of [^{125}I]-EGF and the percentage of specific binding was substantially lower at 4°C than 37°C. Specific binding of [^{125}I]-EGF to DU 145 monolayers at steady state (after 4 hours) was higher overall when compared with the percentage bound [^{125}I]-EGF to LNCaP monolayers at steady state (6 hours). Based on these results, subsequent binding studies for both cell lines were performed at 4°C for 4-6 hours, since at this temperature internalization of EGF is kept to a minimum.

3.1.2.2 Dissociation kinetics

The rate of [^{125}I]-EGF dissociating from the EGF receptor on DU 145 and LNCaP cells is shown in Figure 23. Dissociation was initiated after 4 hours (DU 145) and 6 hours (LNCaP) incubation at 4°C with [^{125}I]-EGF, at which time a steady state had been reached for both cell lines. For DU 145 cells, the rate of dissociation at 4°C was rapid and linear up to 2 hours after dissociation was initiated. The time taken for half of the EGF-EGF receptor complex to dissociate ($t_{1/2}$) at 4°C was 1.5 hours. Similarly, the rate of EGF-EGF receptor dissociation on LNCaP cells at 4°C was rapid and linear with a $t_{1/2}$ of 1.75 hours.

3.1.2.3 Competition & saturation analysis of DU 145 and LNCaP cell lines

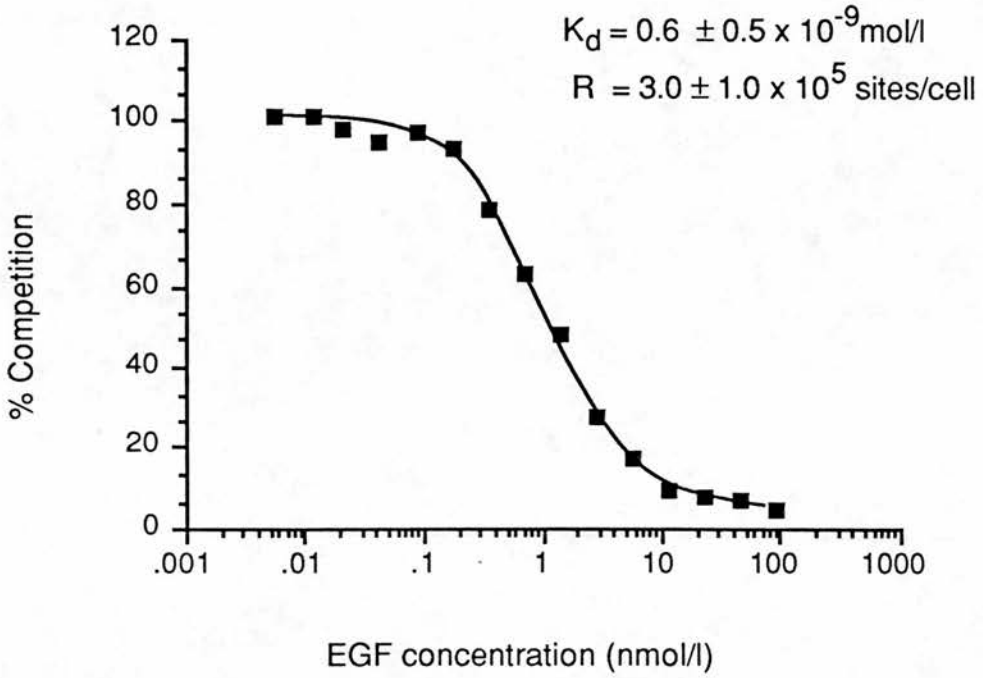
Saturation and competition analysis were used to determine whether EGF binding on DU 145 and LNCaP cells was of high affinity and limited capacity.

(a) Competition analysis:

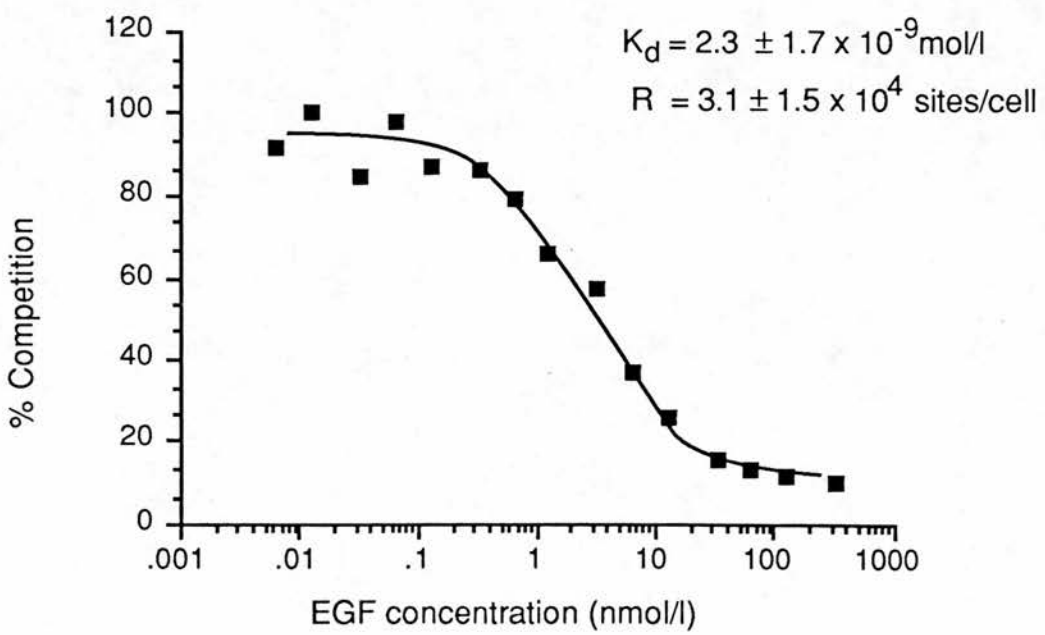
Increasing concentrations of unlabelled EGF successfully competed with [^{125}I]-EGF for binding sites on DU 145 and LNCaP monolayers (Figure 24). The binding

FIGURE 23: *Time course of dissociation of [^{125}I]-EGF bound to the EGF receptor of DU 145 and LNCaP cells.* Nearly confluent cells (2 x10^5 cells/well) were incubated with [^{125}I]-EGF (2nmol/l; 2 x 10^5 cpm) for 4 - 6 hours at 4°C. The binding medium was aspirated and the incubation was continued in SFM for the times indicated. At various time points triplicate dishes were processed to determine [^{125}I]-EGF bound, which is expressed as a percentage of [^{125}I]-EGF bound at time zero. Each data point is the percentage mean ± SD of three experiments (n = 9).

(a) DU 145



(b) LNCaP



parameters from both cell lines, calculated using the computer program LIGAND, fitted significantly to a one site receptor model ($P < 0.05$). The dissociation constants (K_d) were of high affinity and were of the same order of magnitude (0.6± 0.5 nmol/l; DU145 and 2.3 ±1.7 nmol/l; LNCaP). However, the number of binding sites (R) from DU 145 cells ($3.0 \pm 1.0 \times 10^5$ sites/cell) were 10 fold higher than those from LNCaP cells ($3.1 \pm 1.5 \times 10^4$ sites/cell).

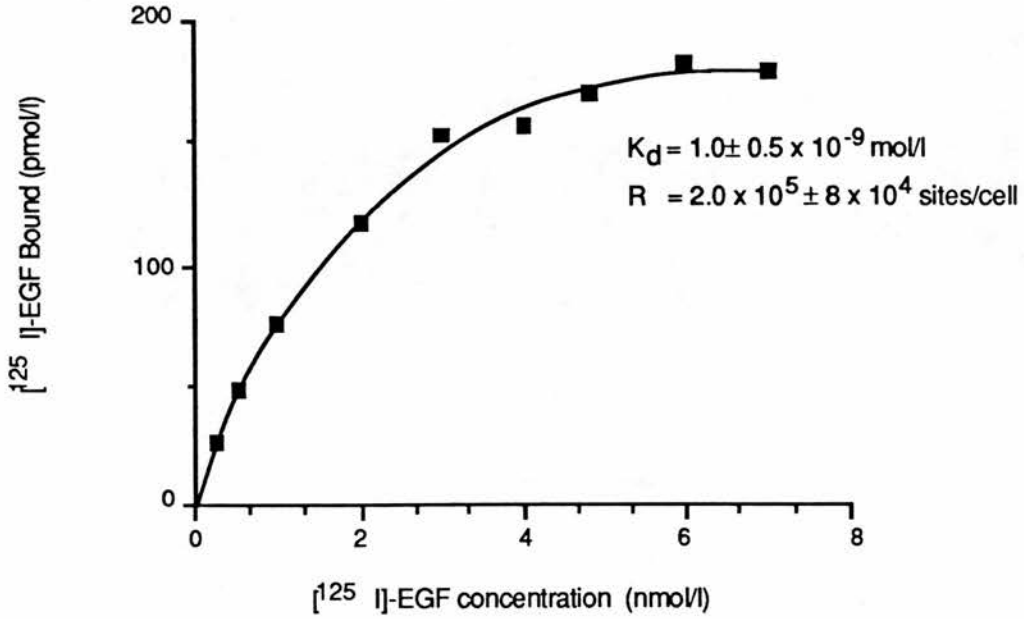
(b) Saturation Analysis:

Increasing concentrations of [^{125}I]-EGF saturated receptor binding sites on DU 145 and LNCaP cell monolayers, indicating specific binding of [^{125}I]-EGF (Figure 25). The data for DU 145 were analysed using the curve fitting program LIGAND and fitted significantly to one class of binding site ($P< 0.05$), with an estimated Kd value of 1.0 ± 0.5nmol/l. The number of binding sites/cell was calculated as $2.0 \times 10^5 \pm 8 \times 10^4$. The $\overset{\text{dissociation}}{\wedge}$ constant of the EGF receptor in LNCaP cells was calculated as 2.9 ± 2.2 nmol/l and the number of receptor binding sites/cell as $2.5 \pm 1.3 \times 10^4$; almost 10 fold lower than DU 145 cells.

The means of the binding parameters from saturation and competition analysis were compared and are tabulated in Table 4. Overall the binding affinity and capacity for DU 145 monolayers were 1.0 ± 0.6 nmol/l and $2.5 \pm 1 \times 10^5$ sites/cell respectively. The number of binding sites for LNCaP cells was considerably less ($2.0 \pm 1 \times 10^4$); a 10 fold difference in receptor number. The $\overset{\text{dissociation}}{\wedge}$ constant of 2.8 ± 2.2 nmol/l was lower overall, but was not significantly different from DU 145 cells.

FIGURE 24: *Competition of [^{125}I]-EGF with unlabelled EGF.* Confluent monolayers (2×10^5 cells/well) were incubated with [^{125}I]-EGF (2nmol/l; 2×10^5 cpm) and increasing concentrations of unlabelled EGF (0.01-300 nmol/l) for 4 hours (DU 145) and 6 hours (LNCaP) at 4°C. The cells were subsequently washed 3x in Dulbecco 'A' PBS, dissolved in 0.5N NaOH and the radioactivity measured. The affinity constant and the binding capacity of each cell line was determined using the binding program LIGAND. Four competition curves were analysed from the cell line DU 145 and two from the cell line LNCaP.

(a) DU 145



(b) LNCaP

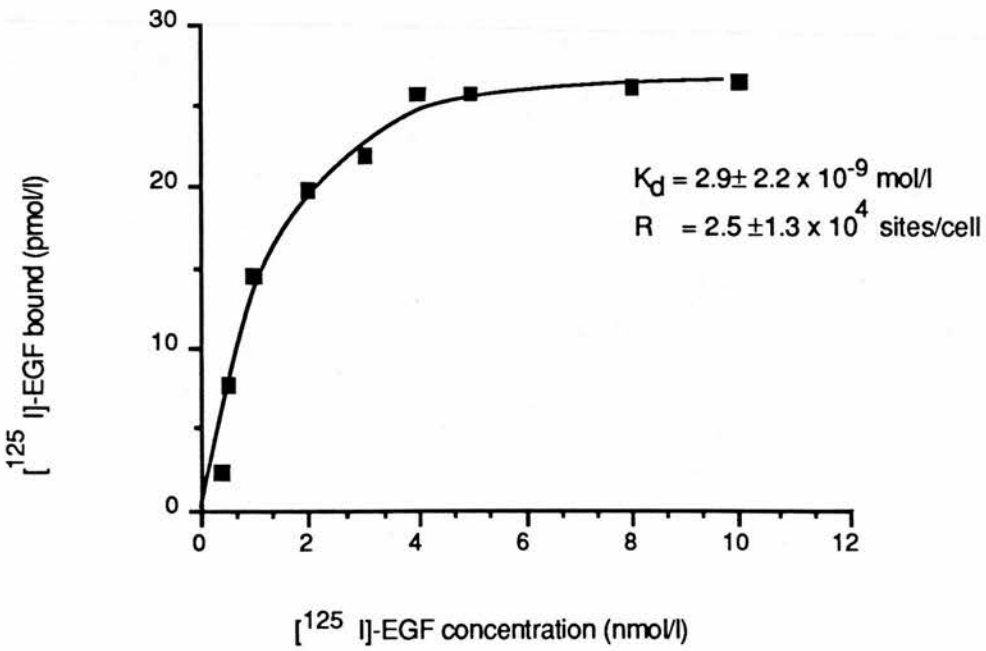


TABLE 4: THE AFFINITY AND BINDING CAPACITY OF EGF RECEPTORS OF DU 145 AND LNCAP CELLS

	DU 145	LNCaP
K_d mol/l	1 ± 0.6 nM[a]	2.8 ± 2.2 nM
R sites/cell	$2.5 \pm 1.0 \times 10^5$	$2.0 \pm 1.0 \times 10^4$

Saturation and competition analysis was performed on confluent DU 145 and LNCaP monolayers (2×10^5 cells/well) in multiwell plates. The affinity constant K_d and the number of binding sites/cell (R) were calculated using the binding program LIGAND. The means of the K_d and the binding capacity for both cell lines are shown.

[a] Mean ± SD.

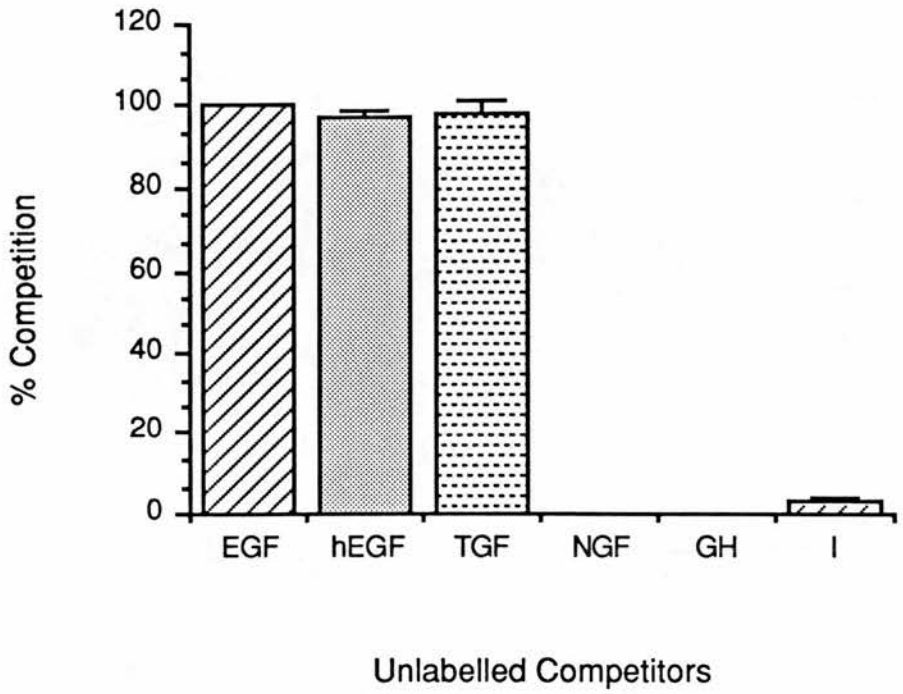
(c) Saturation analysis of Mibolerone treated LNCaP cells

Several workers have shown that the EGF receptor concentration is modulated by androgens in the prostate (Traish & Wotiz, 1987; St-Arnaud *et al.* 1988 and Schuurmans *et al.*, 1988a). This transmodulatory effect of androgens on the EGF receptor was further investigated using the androgen sensitive cell line LNCaP. Mibolerone was added to LNCaP monolayers for a period of 6 days, after which time the number and affinity of binding sites was determined by saturation analysis and analysed using the LIGAND binding program (Table 5).

After exposure to Mibolerone for 6 days neither the affinity constant nor the number of EGF binding sites were significantly altered ($P > 0.05$).

FIGURE 25: *Saturation Curves of DU 145 and LNCaP cell lines.* Increasing doses of [^{125}I]-EGF (0.01-10 nmol/l) were incubated in triplicate wells with or without a constant amount of unlabelled EGF (200 nmol/l) for 4 hours (DU 145) or 6 hours (LNCaP) at 4°C. The monolayers were washed 3x with Dulbecco 'A' PBS to separate bound [^{125}I]-EGF from free [^{125}I]-EGF, the cells were subsequently dissolved in 0.5 N NaOH and the radioactivity remaining was evaluated. The affinity constant and the number of EGF binding sites were determined by the binding program LIGAND. Ten saturation curves were analyzed from DU 145 and six from LNCaP cells.

(a) DU 145



(b) LNCaP

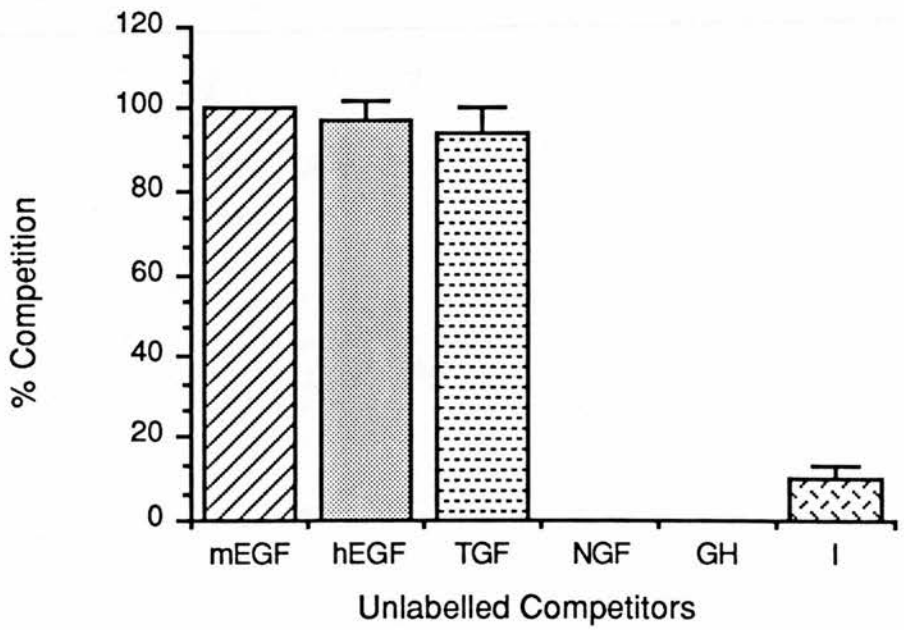


TABLE 5: EFFECT OF MIBOLERONE ON THE AFFINITY AND BINDING CAPACITY OF LNCAP EGF RECEPTORS

	Mibolerone	no Mibolerone
K_d mol/l	3.5 ± 2.6 nM[a] (3)[b]	2.8 ± 1.8 nM (3)
R sites/cell	$2.0 \pm 1.1 \times 10^4$	$2.5 \pm 1.3 \times 10^4$

Mibolerone (0.1 nmol/l) was added in SFM to LNCaP cell monolayers (2×10^4 cell/cm^2) in multiwell plates for a period of 6 days. Control wells were grown without Mibolerone. After 6 days, saturation analysis was performed with increasing concentrations of [^{125}I]-EGF (0.01-10nmol/l) and a constant amount of unlabelled excess ligand (200 nmol/lEGF). The affinity constant K_d and the number of binding sites/cell (R) were calculated using the binding program LIGAND.

[a] Mean ± SD.

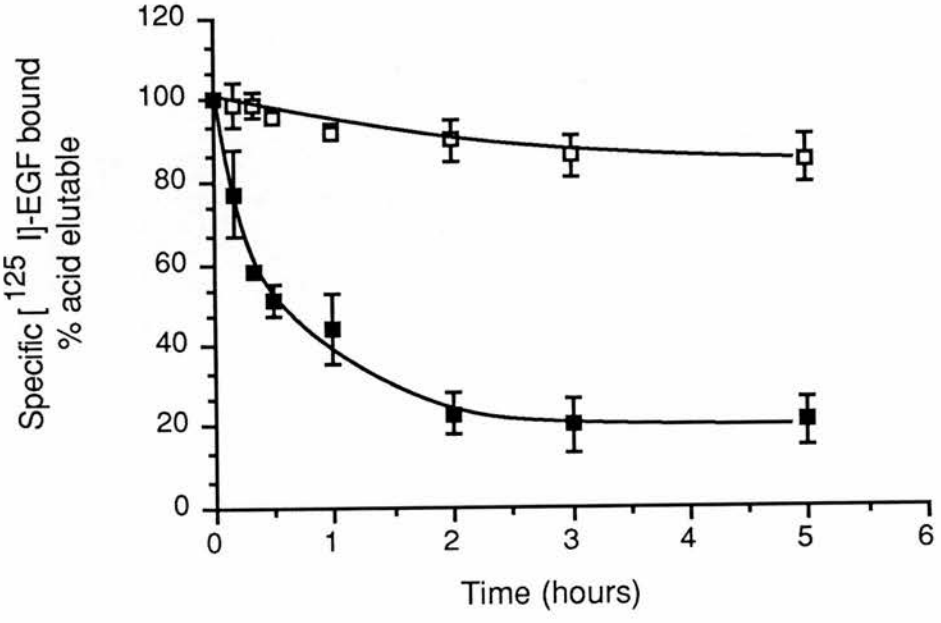
[b] Numbers in parenthesis, number of experiments.

3.1.2.4 Specificity of [^{125} I]-EGF binding to DU 145 and LNCaP EGF binding sites

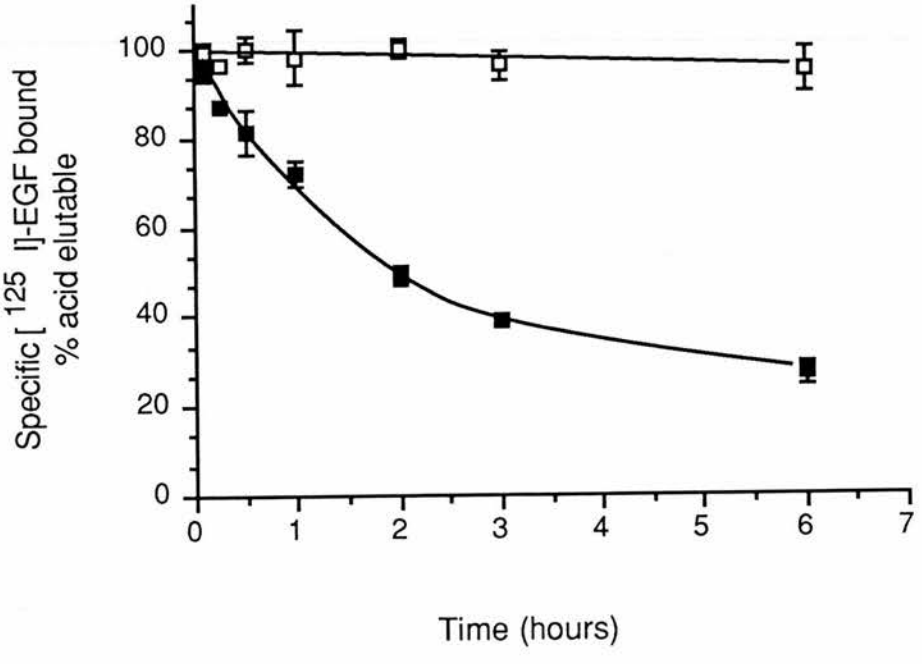
The specificity of [^{125}I]-EGF binding to specific binding sites on DU 145 and LNCaP cell monolayers was further established by competition experiments with various peptide hormones and growth factors (Figure 26). Mouse EGF, human EGF (urogastrone) and TGFα, which are of similar size and structure were equally effective in competing with [^{125}I]-mEGF for EGF binding sites on DU 145 and LNCaP cells (>95% competition). However, peptides unrelated structurally to EGF did not compete with [^{125}I]-mEGF for binding sites on either DU 145 or LNCaP monolayers.

FIGURE 26: *Specificity of [^{125}I]-EGF binding to its' receptor on DU 145 and LNCaP cells.* [^{125}I]-EGF (2nmol/l; 2×10^5 cpm) was added to confluent monolayers with or without 100x excess unlabelled competing ligands (mEGF, hEGF, TGFα, NGF, GH and Insulin which were added to triplicate wells for 4 hours (DU 145) or 6 hours (LNCaP) at 4°C. The monolayers were subsequently washed, the cells dissolved with 0.5 N NaOH and the radioactivity remaining, determined. Specific binding was determined by subtracting non-specific from total binding. Each data point represents the percentage mean ± SD of three experiments, n = 9. The data is expressed as the percentage of unlabelled ligand competing with [^{125}I]-EGF for EGF receptor sites.

(a) DU 145



(b) LNCaP



3.1.2.5 Internalization of EGF binding sites

To determine whether [^{125}I]-EGF is internalized by human prostate cancer cells, the effect of brief exposure to saline/glycine (pH 3.0) on monolayer-bound radioactivity was examined. The effect of acid extraction on specific [^{125}I]-EGF bound to DU 145 and LNCaP cells is shown in Figure 27. At 37°C the proportion of specifically bound [^{125}I]-EGF that was acid elutable decreased rapidly with time. When apparent steady state binding was achieved at 37°C, the majority of specifically bound [^{125}I]-EGF had been internalized. The rate of internalization was slower for LNCaP than for DU 145; after 2 hours the binding capacity of DU 145 monolayers was reduced by 80%, whereas for LNCaP cells, the binding capacity was reduced by 55%. At 4°C, almost no internalization of the EGF-receptor complex had occurred, since the majority of specifically bound [^{125}I]-EGF was acid elutable after 6 hours.

3.1.2.6 Down regulation of [^{125}I]-EGF binding sites

Cells which are exposed to increasing concentrations of EGF gradually lose a substantial fraction of their receptors for EGF (Carpenter & Cohen, 1979 and Schlessinger *et al.* 1986). This phenomenon called "down regulation" depends upon EGF concentration, time and temperature. Down regulation of EGF receptor sites from DU 145 and LNCaP cells was demonstrated by pre-incubating cell monolayers with increasing concentrations of EGF for 24 hours (Figure 28). Preincubation of DU 145 and LNCaP monolayers with EGF effectively reduced specific binding of radiolabelled growth factor. Half-maximal reduction in specific [^{125}I]-EGF binding

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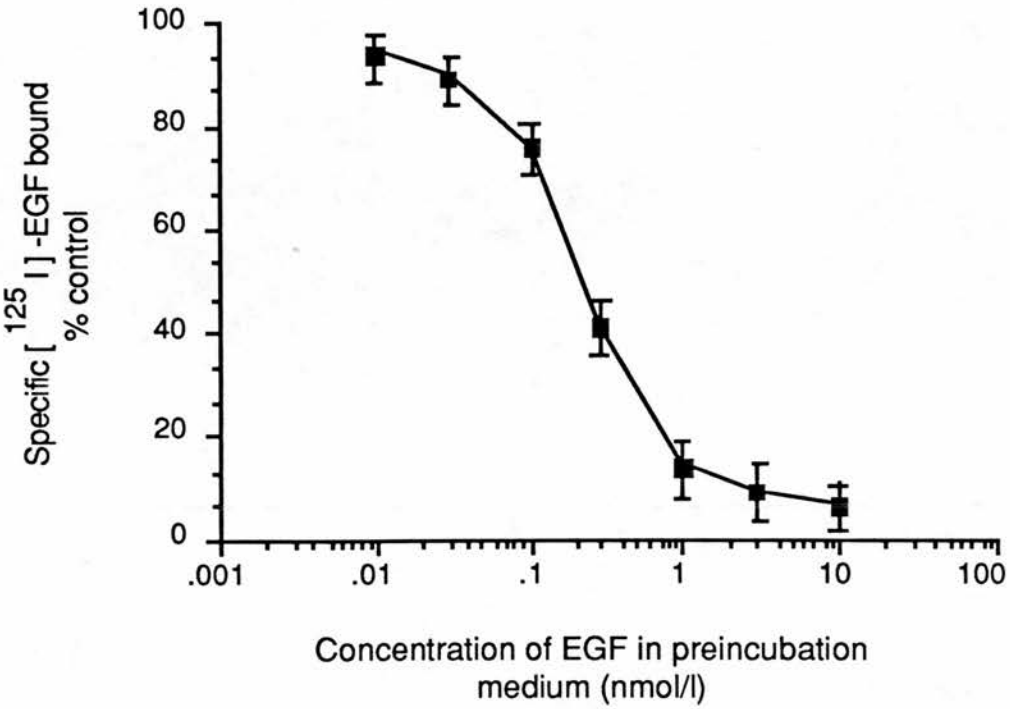
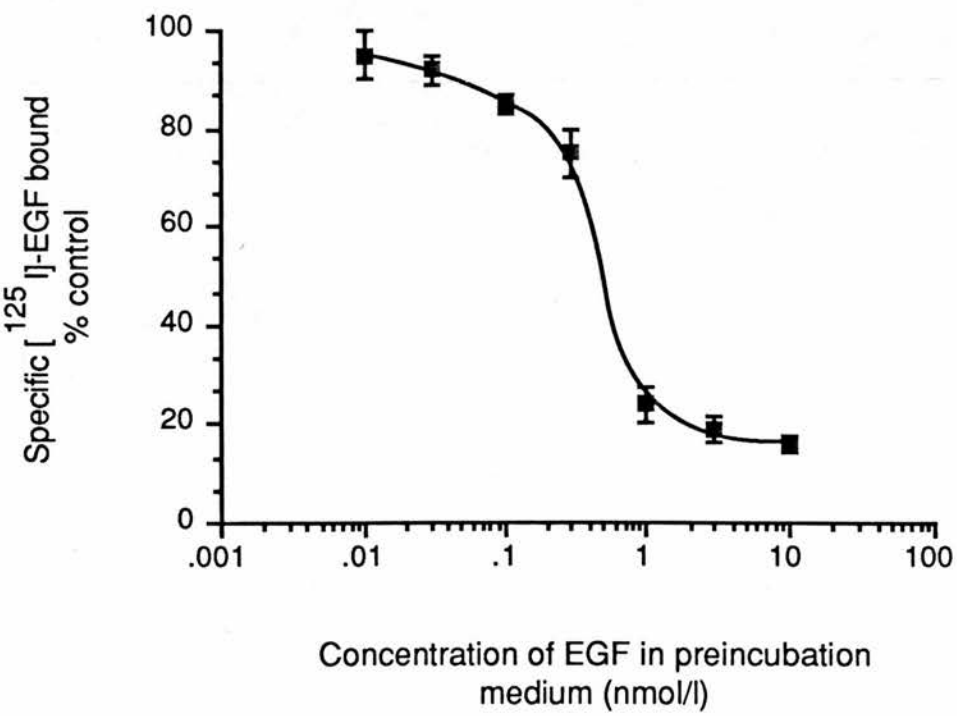
FIGURE 27: *Internalization of DU 145 and LNCaP EGF binding sites*: Confluent LNCaP and DU 145 monolayers (2 x 10^5 cells/well) were incubated with [^{125}I]-EGF (2 nmol/l; 2 x 10^5 cpm) in the presence and absence of unlabelled EGF (200 nmol/l) at 4°C (□) and 37°C (■). At various time points (as indicated), triplicate dishes were processed by washing 3x with ice-cold Dulbecco 'A' PBS and extracting surface bound EGF by washing with ice-cold NaCl/Glycine. Total binding, non-specific binding and acid elutable [^{125}I]-EGF binding to cell monolayers was then determined. The data is expressed as the percentage mean ± SD of [^{125}I]-EGF specifically bound which was acid elutable, n = 9. The experiment was carried out three times.

(a) DU 145



(b) LNCaP



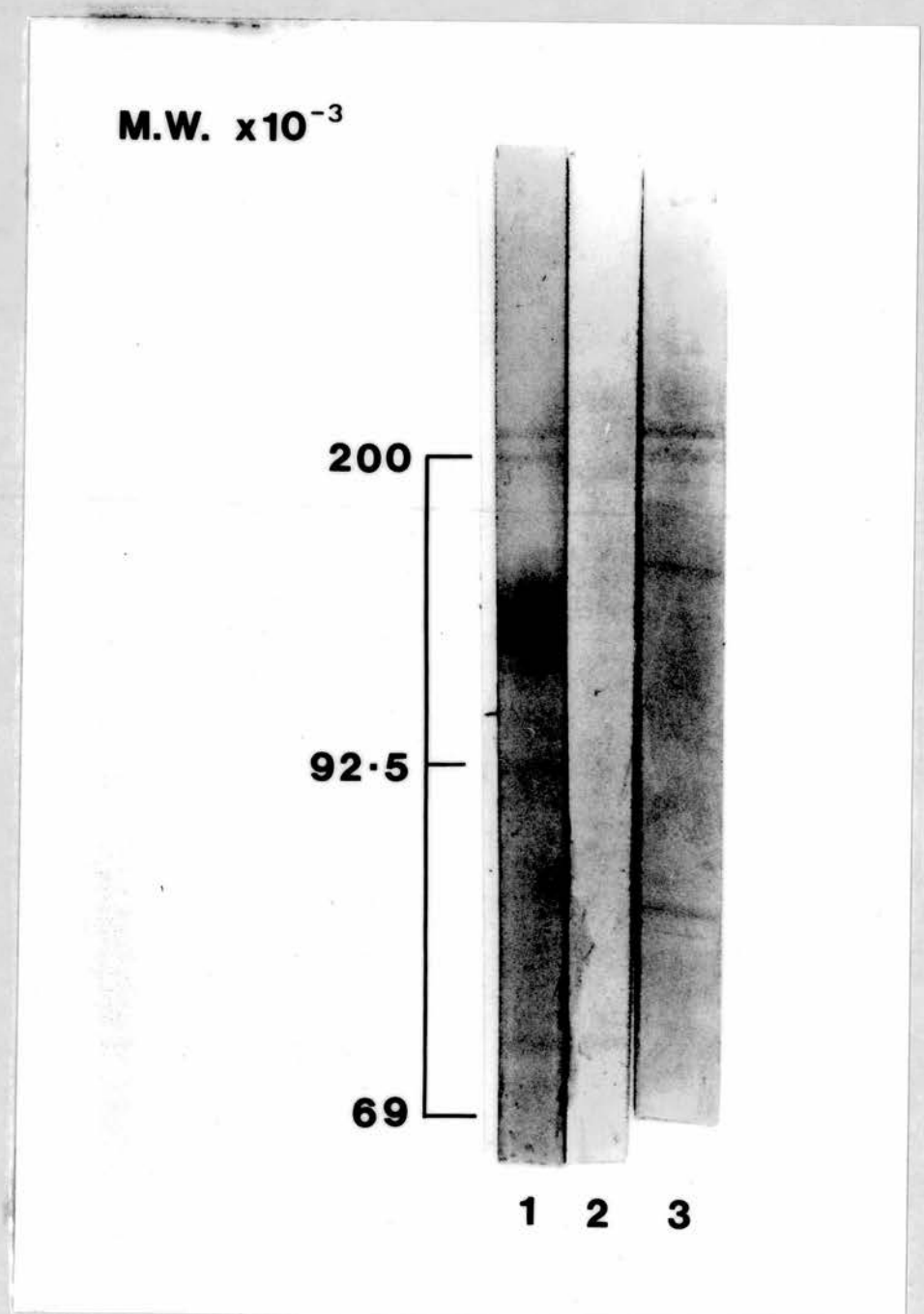
required a concentration of 0.2 nmol/l and 0.8 nmol/l in the preincubation medium of DU 145 and LNCaP cells respectively.

3.1.2.7 Expression of the EGF and truncated receptor on DU 145 and LNCaP cells by Western blotting

The expression of the EGF receptor from DU 145 and LNCaP cell lysates was demonstrated by Western blotting (Plates 1 & 2). DU 145 and LNCaP cell lysates were blotted onto nitrocellulose and incubated with two Mab against the EGF receptor and a non-specific MHC IgG class 2 antibody which served as a control.

Incubation of DU 145 cell lysates with the monoclonal F4, revealed a distinct band corresponding to a molecular weight of 170 000 on SDS-PAGE (Plate 1; track 1), but there was no expression of the truncated receptor ; i.e. there was no band at 68 kDa (track 1). EGFR1 (which has a higher affinity for the EGF receptor than F4) did not bind to the EGF receptor from DU 145 cell lysates as there was no expression of the native EGF receptor on the blotted gel (track 2). Neither the EGF receptor nor the truncated receptor were expressed in LNCaP cells using Western blotting (Plate 2).

FIGURE 28: *Down regulation of DU 145 and LNCaP EGF binding sites.* DU 145 and LNCaP monolayers (2 x 10^5 cells/well) were incubated with increased concentrations of EGF (0.01-10 nmol/l) for 24 hours at 37°C, with control wells receiving no EGF. Subsequently, the monolayers were washed extensively (by washing 3x in Dulbecco 'A' PBS and once with ice-cold NaCl/Glycine) to remove surface bound EGF. Binding was then initiated by the addition of [^{125}I]-EGF (2 nmol/l; 2 x 10^5 cpm) in the presence and absence of unlabelled EGF (200 nmol/l) for 4 hours (DU 145) or 6 hours (LNCaP) at 4°C. After the incubation period the monolayers were washed, the cells dissolved with 0.5 N NaOH and the radioactivity remaining determined. Receptor down regulation was seen as any decrease in specific binding relative to the untreated control. Each data point represents the mean percentage of specific [^{125}I]-EGF bound ± SD; n = 9 of three separate experiments.

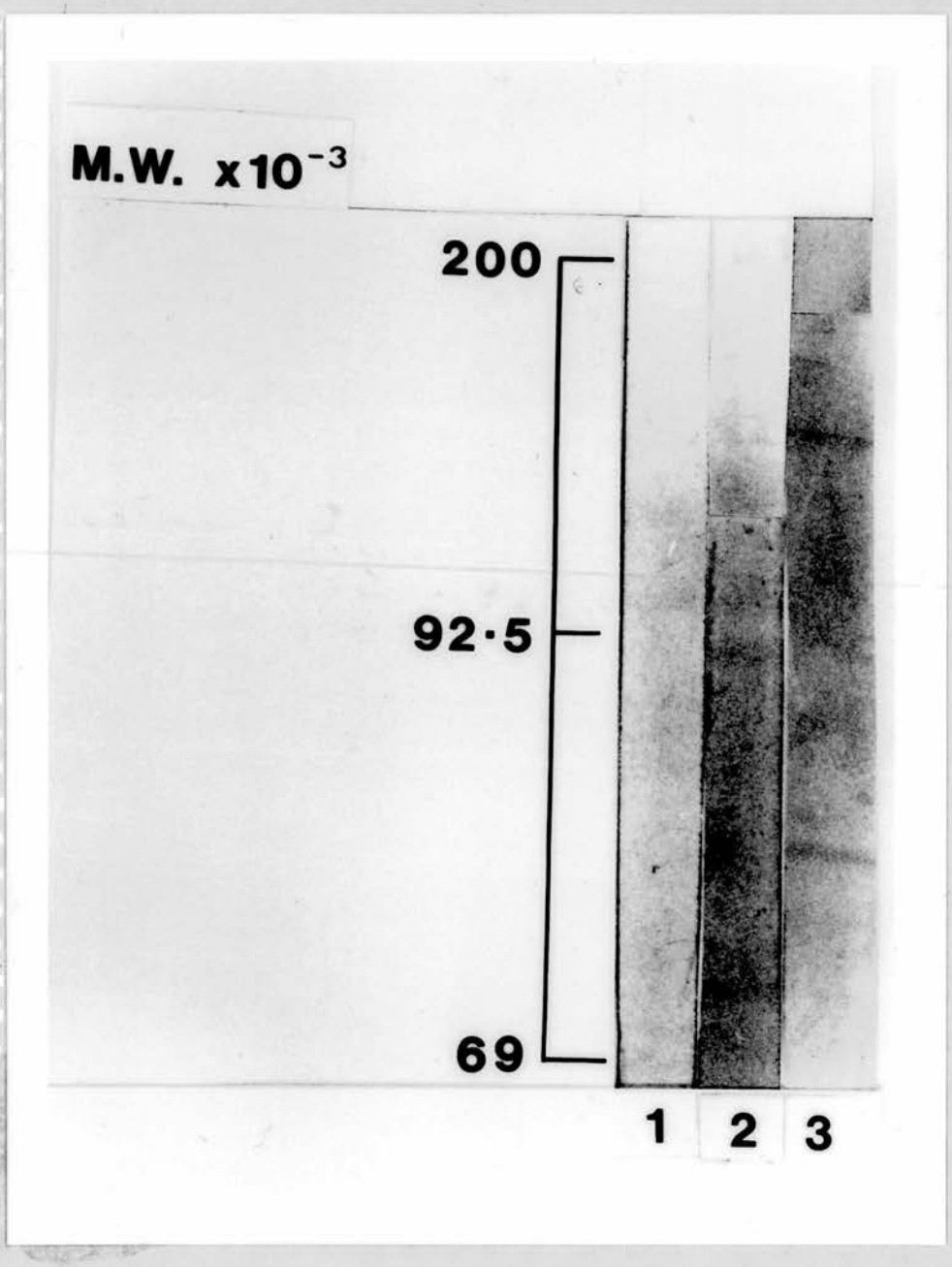

M.W. x10^{-3}
200
92·5
69
1
2
3

3.1.3 SUMMARY OF RESULTS FROM THE CHARACTERIZATION OF THE EGF RECEPTOR OF DU 145 AND LNCAP CELLS

The cell lines DU 145 and LNCaP express high affinity EGF receptors with similar dissociation constants (K_d = 1 ± 0.6 nmol/l and 2.8 ± 2.2 nmol/l respectively), but with dissimilar receptor numbers. The DU 145 cell line expresses high levels of EGF receptors (2.5 ± 1.0 x 10^5 sites/cell) whereas LNCaP cells have 10 fold lower receptor numbers (2.0 ± 1.0 x 10^4 sites/ cell).

Further characterization revealed that prostate cells specifically bound, internalized and down regulated their receptors. The expression of the EGF receptor (Mr 170 000) on DU 145 cells was further verified by Western blotting but LNCaP cells did not express the receptor using this technique. Neither DU 145 nor LNCaP cells expressed the truncated receptor.

Plate 1: *Expression of the EGF receptor and the truncated EGF receptor by Western blotting in DU 145 cells.* Cell lysates (5 x 10^7 cells) from DU 145 cells were electrophoresed, blotted onto nitrocellulose and incubated with the antibodies EGFR 1 (track 2), F4 (track 1) and a non-specific control (track 3)


M.W. x10⁻³
200
92·5
69
1
2
3

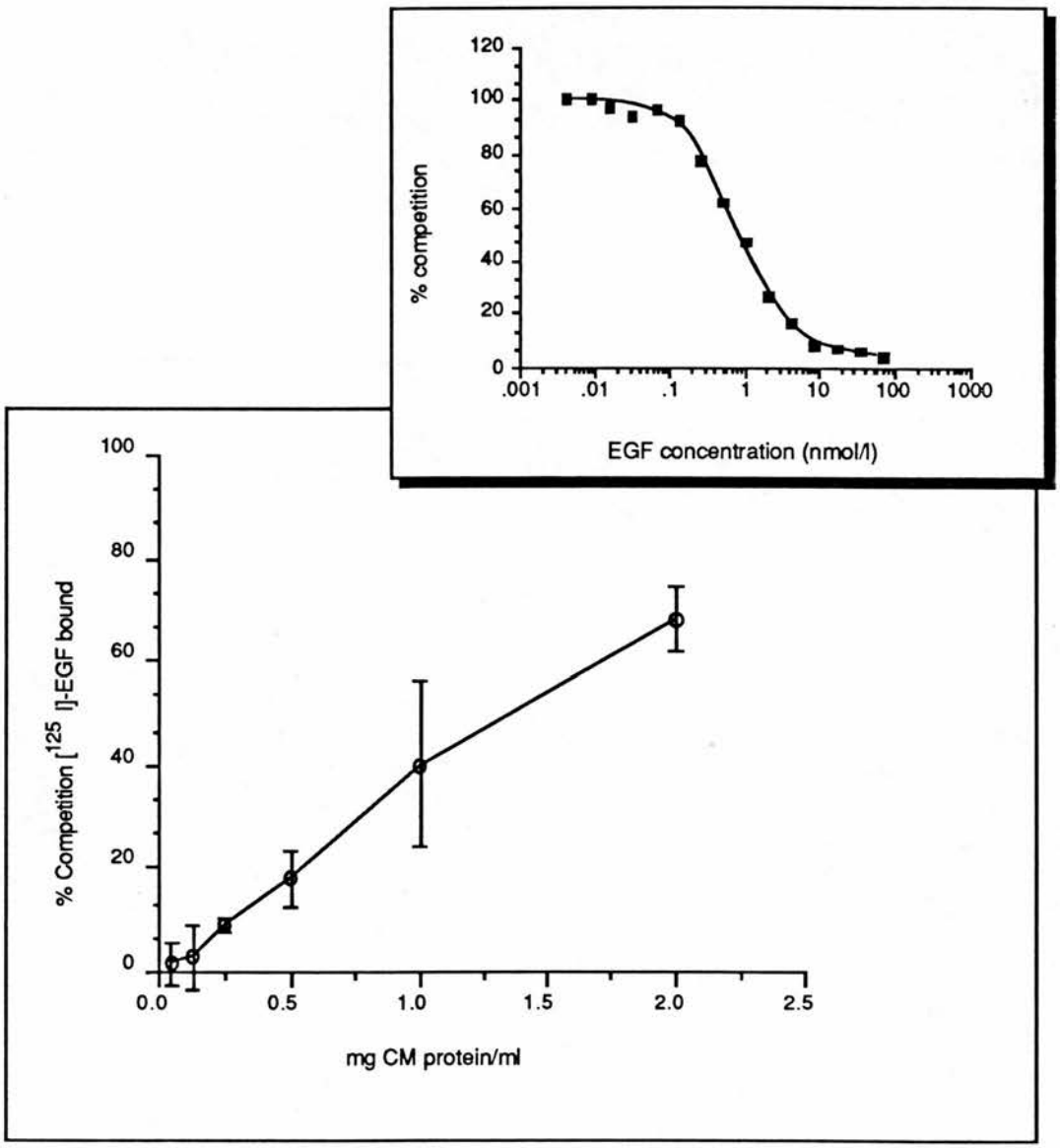
3.2 ANALYSIS OF CONDITIONED MEDIUM FROM DU 145 CELLS

The secretion of growth factors by some transformed cells is thought to enable these cells to proliferate in low serum concentrations, as well as reducing the dependency upon exogenous growth factors. One mechanism of transformation is thought to involve cells being able to produce and respond to growth factors (autocrine secretion), thus conferring a growth advantage over other cells (Sporn & Todaro, 1980). TGFαs (which bind to the EGF receptor) and other growth factors have been implicated in the autocrine and/or paracrine growth of tumour cells (Sporn & Roberts, 1985). Medium conditioned by DU 145 cells was therefore analysed for the production of the growth factors EGF and TGFα. The CM was also partially purified by chromatography and SDS-PAGE.

3.2.1 ANALYSIS OF CONCENTRATED CM FROM DU 145 CELLS

The production of EGF-like activity from medium conditioned (CM) by DU 145 was analysed for (a) EGF-like competitive activity using an EGF-radio receptor assay (RRA) (b) EGF and TGFα immunological activity using RIAs for rTGF-I and hEGF and (c) mitogenic activity by [^{3}H]-Thymidine incorporation into DU 145 cells.

Plate 2 : *Expression of the EGF receptor and the truncated receptor by Western blotting in LNCaP cells*: Cell lysates (5 x 10^7 cells) from the LNCaP cell line were electrophoresed, blotted onto nitrocellulose and incubated with the antibodies EGFR 1 (track 2), F4 (track 1) and a non-specific control (track 3).


120
100
80
60
40
20
0
% competition
.001
.01
.1
1
10
100
1000
EGF concentration (nmol/l)
100
80
60
40
20
0
% Competition [125 I]-EGF bound
0.0
0.5
1.0
1.5
2.0
2.5
mg CM protein/ml

3.2.1.1 Production of EGF-like competing factors

To examine for the presence of specific EGF-like molecules in the CM from DU 145 cells, increasing concentrations of CM were tested for the ability to compete with [^{125}I]-EGF for binding to DU 145 cells (Figure 29). Growth factors present in the CM inhibited [^{125}I]-EGF binding in a dose-dependent fashion. By comparison to the competition curves produced by unlabelled mEGF with [^{125}I]-EGF (inset of Figure 29), the amount of EGF equivalent units in the samples of CM was calculated. The 50% effective dose for DU 145 lyophilized CM in the EGF radioreceptor assay was approximately 6 ng/ml, since in this assay 50% competition was equivalent to 1nmol/l (0.6ng/assay) of EGF. Therefore, approximately 30 ng of EGF-like equivalent molecules were produced in 1 litre of media conditioned by DU 145 cells.

3.2.1.2 Production of immunoreactive EGF and TGFα

Equivalent amounts of concentrated CM were assessed for immunoreactivity using rTGF-I and hEGF RIAs. The relative amounts of immunoreactive species produced by DU 145 cells were compared with the levels of biologically active EGF-like molecules that competed with [^{125}I]-EGF for binding to DU 145 monolayers (Table 6). Approximately 20.3 ng/litre of immunoreactive TGFα was produced by DU 145 cells, compared with 30 ng/litre of EGF equivalent molecules (i.e. those which demonstrated competitive activity with [^{125}I]-EGF). As no EGF was detected when CM was assayed for hEGF by RIA, the remaining 32% of bioactive EGF-like molecules

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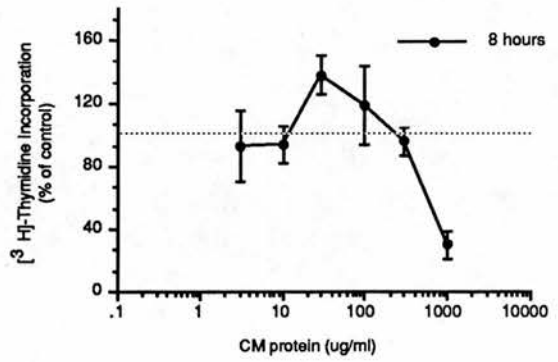
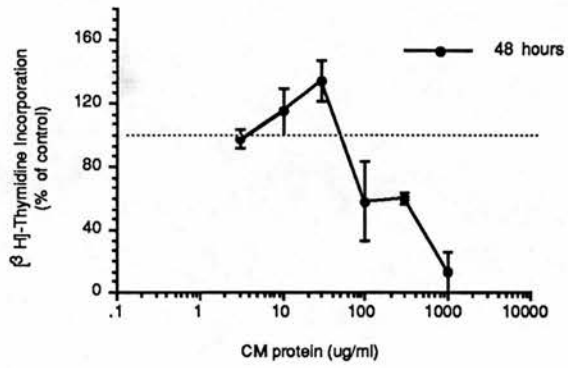
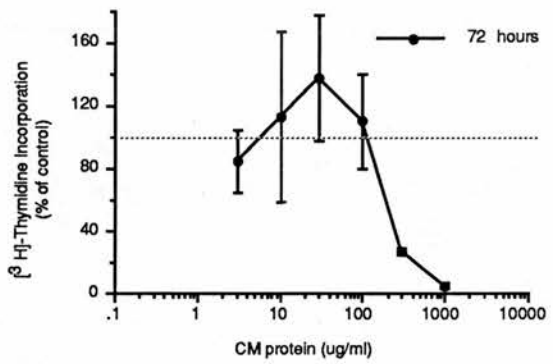
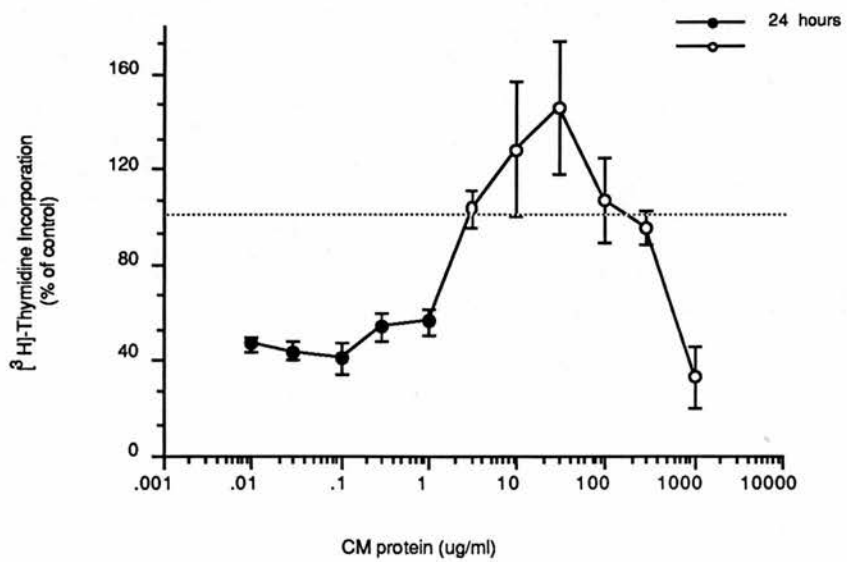
FIGURE 29: *Production of competitive EGF-RRA activity from medium conditioned by DU 145 cells.* Increasing concentrations of DU 145 conditioned medium (CM) were assayed for EGF-RRA competitive activity. [^{125}I]-EGF (2 nmol/l; 2 x 10^5 cpm) was incubated with or without increasing concentrations of CM for 4 hours at 4 °C. The cells were then processed by washing 3 x with Dulbecco 'A' PBS and the radioactivity remaining measured. The amount of EGF equivalent units in the CM was calculated by comparison to the competition curves produced by unlabelled EGF with [^{125}I]-EGF (inset).


8 hours
[3 H]-Thymidine Incorporation (% of control)
160
120
80
40
0
.1
1
10
100
1000
10000
CM protein (ug/ml)


48 hours
[3 H]-Thymidine Incorporation (% of control)
160
120
80
40
0
.1
1
10
100
1000
10000
CM protein (ug/ml)


72 hours
[3 H]-Thymidine Incorporation (% of control)
160
120
80
40
0
.1
1
10
100
1000
10000
CM protein (ug/ml)


24 hours
[3 H]-Thymidine Incorporation (% of control)
160
120
80
40
0
.001
.01
.1
1
10
100
1000
10000
CM protein (ug/ml)

produced by DU 145 cells (of which 68% were immunologically related to TGFα) could not be attributed to the production of immunoreactive EGF.

TABLE 6: PRESENCE OF EGF RECEPTOR COMPETING ACTIVITY AND IMMUNOREACTIVE TGFα S IN THE CM OF DU 145 CELLS.

Assay	Amount (ng/ litre CM)
RIA	
hEGF[a]	-
rTGF-I[b]	20.3 ± 2.4
RRA[c]	30

CM samples were assayed for hEGF, TGFα immunoreactivity by RIA and EGF-RRA competitive activity. Values are normalized to 1 litre of CM produced by DU 145 cells.

[a] hEGF-RIA in which half maximal inhibition of binding occurred at 200 pg/tube.

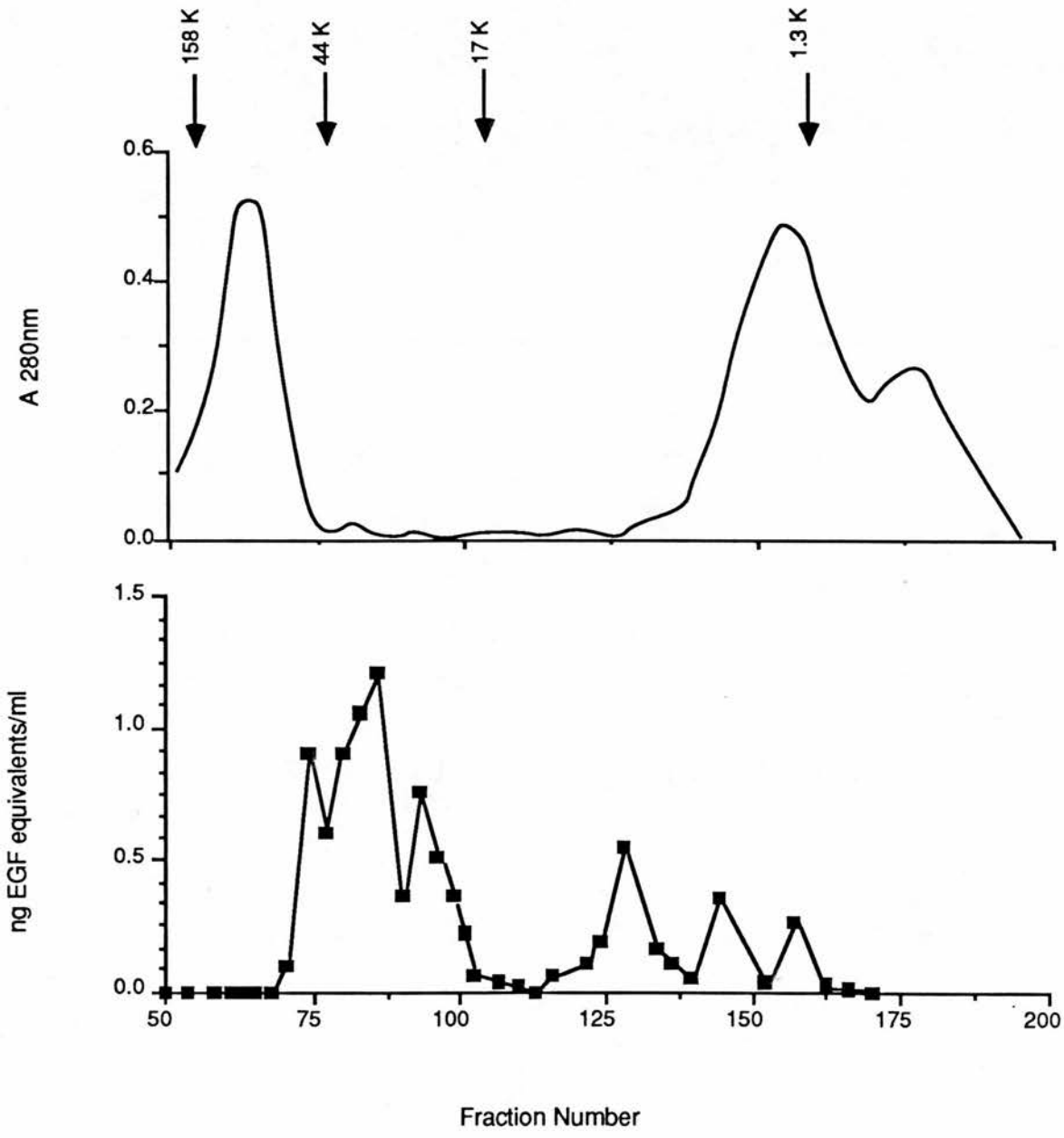
[b] rTGF-I. 50% competition occurred with 100 pg/tube. The value obtained is the mean ± SD of duplicate experiments.

[c] The level of EGF equivalent units was obtained by comparison to the competition curves produced by unlabelled mEGF with [^{125}I]-EGF

3.2.1.3 Effect of concentrated CM on DNA synthesis in DU 145 cells.

Increasing concentrations of pooled and concentrated CM from DU 145 cells were added to DU 145 cell monolayers for up to 72 hours and tested for its' effect on DNA synthesis (by incorporation of [^{3}H]-Thymidine; Figure 30). Initially concentrations between 3-1000 μg/ml of CM were tested over this time period and the maximum

FIGURE 30: *Dose-response effect of DU 145 CM on [^{3}H]-Thymidine incorporation of DU 145 cells.* DU 145 cells (1 x 10^4 cells/well) were seeded in 96 well plates and increasing concentrations of CM (3-1000 μg/ml) added for up to 72 hours and concentrations between 0.01-1000 μg/ml for 24 hours. After each incubation period the amount of [^{3}H]-Thymidine incorporated into DNA was measured by precipitating the cellular material with 10% ice-cold TCA; which was added for 2 hours. The TCA precipitable material was harvested, dried and the radioactivity measured. The data are expressed as the percentage of [^{3}H]-Thymidine incorporated relative to the untreated control with filled data points representing the mean ± SD of 6 observations and unfilled points as the mean ± SD of 12 observations. The experiment was carried out twice.


158 K
44 K
17 K
1.3 K
A 280nm
0.6
0.4
0.2
0.0
ng EGF equivalents/ml
1.5
1.0
0.5
0.0
50
75
100
125
150
175
200
Fraction Number

response was noted after 24 hours. The experiment was repeated for up to 24 hours, with concentrations between 0.01-1000 µg/ml.

Factors in the CM exerted a biphasic effect on DNA synthesis as early as 8 hours after the addition of CM and this effect was still prevalent up to 72 hours. The maximum stimulatory effect on DNA synthesis (after 24 hours) was observed with 30 µg/ml CM (46% ± 28; $P < 0.001$). Concentrations less than this value (from 0.01 to 1µg/ml) inhibited incorporation of [^{3}H]-Thymidine by approximately 50% ($P < 0.001$).

The decline in incorporation of [^{3}H]-Thymidine observed with concentrations greater than 100 µg/ml CM was probably due to dilution of nutrients in the serum free media.

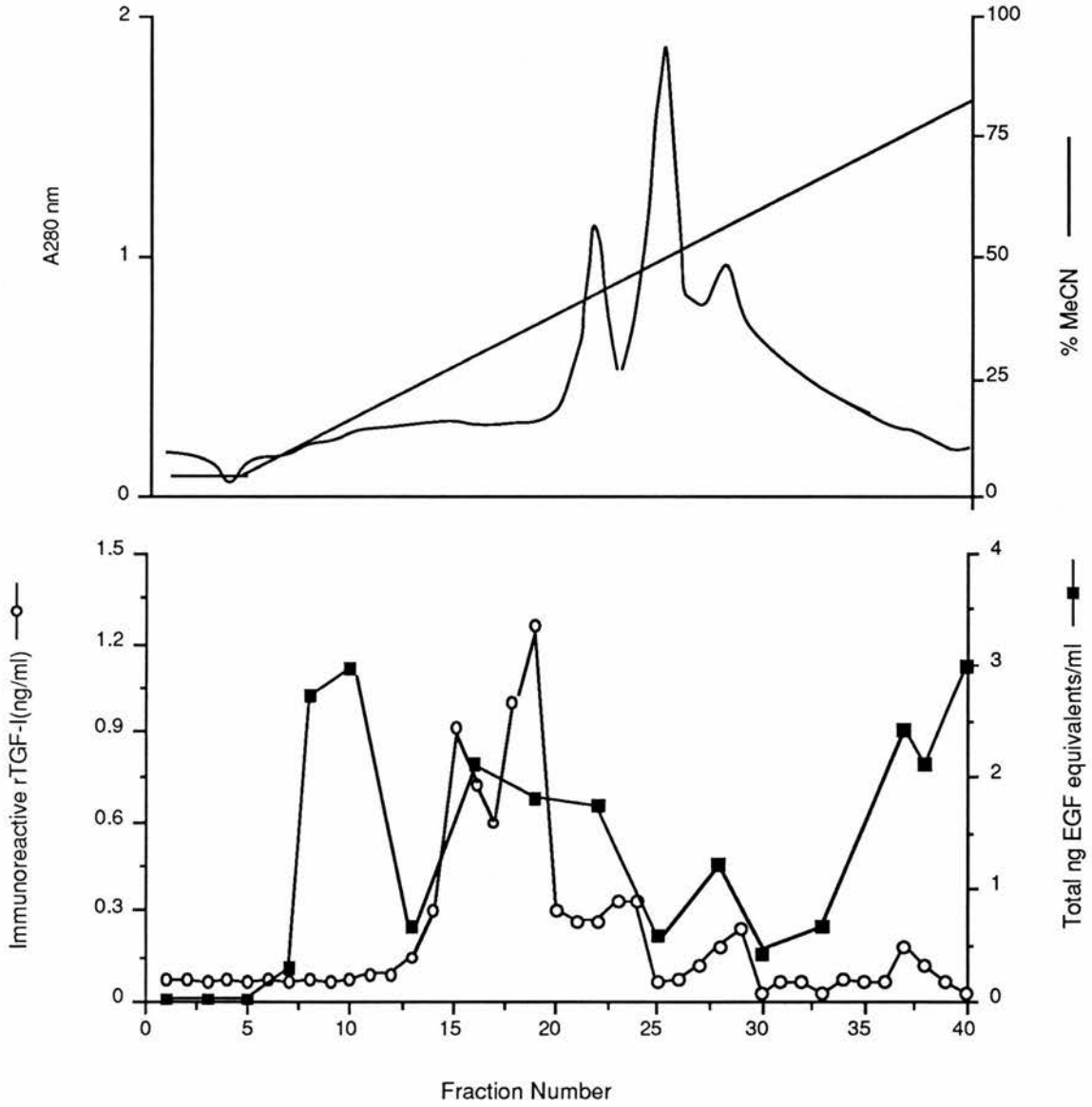
3.2.2 CHARACTERIZATION OF EGF-LIKE PRODUCTION BY DU 145 CELLS

The concentrated CM was fractionated by size exclusion chromatography and rHPLC and the fractions assayed for EGF-RRA competitive activity and rTGF-I and hEGF immunological activity. The results are presented in the following section.

3.2.2.1 Gel filtration profile of DU 145 conditioned medium

Concentrated DU 145 CM was subjected to size-exclusion chromatography on a Sephadex G-100 column, the profile of which is depicted in Figure 31. Absorbance at 280 nm is restricted mainly to the void volume, resulting from the transferrin (M_r 80

FIGURE 31: *Gel filtration chromatography of concentrated DU 145 CM.* Concentrated CM (10 mg) was applied to a Sephadex G-100 column (100 x 2.5 cm), equilibrated and eluted with 0.02M PBS, pH 7.4 in 3 ml fractions. Protein was determined by absorbance at 280 nm (–). A 200 µl aliquot of every third fraction was tested for competitive activity in an EGF RRA (–■–) and a 100µl aliquot tested for immunological activity in hEGF and rTGF-I RIAs (not detected). Molecular weight markers were thyroglobulin, 670 K; gamma globulin, 158 K; ovalbumin, 44 K; myoglobin, 17 K and vitamin B_{12}, 1.3 K.


A280 nm
% MeCN
Immunoreactive rTGF-I(ng/ml)
Total ng EGF equivalents/ml
Fraction Number

000) added to the SFM, and to the low molecular weight region (< 1350), due to coloured pH indicators in the culture media. Analysis of EGF-competing activity in the column eluants showed a broad major peak of activity between M_r 30 000 and 50 000 and a sharp minor peak of activity at an apparent size of M_r 5 000. However, the levels of EGF-like competitive activity detected in the fractions were such that only 23% competition was associated with the major peak and 12% activity with the minor peak. Moreover, neither hEGF nor TGFα immunological activity was detected in the fractions eluted from the column.

3.2.2.2 rHPLC profile of DU 145 conditioned medium

Medium conditioned by DU 145 cells was chromatographed using rHPLC, and the fractions examined for immunological rTGF I, hEGF activity and EGF radioreceptor competitive activity (Figure 32). Associated with the three major 280 nm absorbance peaks (> 1Au) in the middle of the gradient was a minor peak of competitive activity (20% competition), which was equivalent to approximately 1.4 ng of EGF/ml. Two other major peaks of competitive activity eluted at the beginning and end of the run. The peak of competitive activity eluting at the beginning of the run (fractions 7-12) was equivalent to approximately 2.9 ng EGF/ml (43% competition). The broad peak of competitive activity (fractions 36-40) inhibited [^{125}I]-EGF binding by 45%, (3 ng EGF equivalents/ml). Interestingly, the only peak of immunoreactive rTGF I (fractions 12-19, 1.250 ng/ml), also demonstrated EGF-like competitive activity (32% competition; 2.1ng EGF equivalents/ml). The fractions were also tested

FIGURE 32: *Reverse-phase HPLC of concentrated DU 145 CM.* Trifluro acetic acid (0.1%) was added to concentrated CM (15 mg) and the soluble material was loaded on to a Bio-Rad RP 304 C_4 column (250 mm x 10 mm) and subjected to HPLC. Every third fraction (200 µl aliquot) was assayed for competitive activity in an EGF-RRA (–■–) and 100 µl aliquots were assayed for immunological hEGF (not detected) and rTGF-I activity by RIA (–○–). Protein was determined by absorbance at 280 nm (–), and the linear gradient is marked by the solid line.

for immunoreactive hEGF, however hEGF was not detected in any of the rHPLC fractions.

3.2.2.3 Analysis of rHPLC fractions by SDS-PAGE

To elucidate the molecular weights of the fractions demonstrating EGF-like competitive activity and TGFα immunological activity, fractions 7-10 and 15-19 were pooled and subjected to SDS-PAGE under non-reducing conditions. The eluted material from 5mm gel slices were assayed for immunological rTGF-I and EGF-RRA activity. However, neither EGF-like competitive activity nor immunological activity was detected in any of the gel slices.

3.2.3 SUMMARY

The results from the analysis of CM from DU 145 cells clearly show that these cells produce EGF-like molecules which demonstrate competitive activity. Most of the competitive activity was immunologically related to TGFα and not EGF. When the CM was assayed for mitogenic activity, a biphasic effect on DNA synthesis was apparent, which was dependent upon the concentration of CM. In an attempt to further characterize the EGF-like factors produced by this cell line, CM was subjected to size-exclusion chromatography. Two peaks of EGF-RRA activity were detected (M_r 30 000 - 50 000 and M_r 5 000) in the fractions eluted from the column, however neither hTGFα nor hEGF immunological activity were detected. Several peaks of EGF-like competitive activity were detected by rHPLC, only one of which was related immunologically to TGFα. None of the peaks demonstrated any hEGF immunological activity.

Chapter 4

DISCUSSION

In well differentiated prostatic carcinoma androgen ablation therapy is often initially effective, but eventually the carcinoma progresses to a more aggressive form in which growth is androgen independent. This transition is a major impedance to successful treatment not only of carcinoma of the prostate but of other tissues, such as breast, under hormonal control. Since cell growth is no longer under the control of androgens some other factor(s) must be responsible for proliferation.

Many workers are coming to recognize growth factors as playing the key role in the progression of androgen-dependent to androgen-independent growth. This recognition has prompted more subtle considerations of the interplay between growth factors and androgens in the regulation of prostatic cell growth. A favoured mechanism is that the progression to endocrine independence may be due to an altered activity of the growth factor or its' receptor as suggested by King (1990). This hypothesis has been tested by comparison of androgen-insensitive (DU 145) and androgen-sensitive (LNCaP) cell lines. The comparison permits for the first time a study of the role of growth factors, in particular EGF, in the progression from androgen-dependent to androgen-independent prostate cancer.

To test the hypothesis that alteration in growth factor or receptor activity could be responsible for the progression to androgen independence, three questions were asked; (i) is the expression of the EGF receptor different in the two cell lines; (ii) what are the effects of exogenous EGF on cell proliferation; (iii) are these cells producing EGF-like molecules ?

4.1 EGF RECEPTOR

If alteration in growth factor or receptor activity is responsible for the progression to an androgen independent state then this may be reflected as differences in the expression of the receptor, before and after transition. By identifying differences in receptor expression of an androgen-insensitive and a sensitive cell line, it should be possible to gain insight into the changes occurring during the transition.

Characterization studies

The binding of EGF to receptors present on DU 145 and LNCaP cells was both time and temperature dependent and was in agreement with data on EGF binding to whole cells (Banks-Schlegel & Quintero, 1986; Häder *et al.*, 1987) including studies with LNCaP (Schuurmans *et al.*,1988a) and DU 145 (Connelly & Rose, 1989) cells. However, the rates of association were dissimilar and a higher percentage of specifically bound EGF to DU 145 cells was found relative to LNCaP cells. The higher level of [^{125}I]-EGF association to DU 145 cells, reflecting increased levels of receptors expressed, contrasted with the rate of dissociation which was almost identical with that of LNCaP cells.

Competition studies with ligands unrelated structurally to EGF revealed that mEGF binds specifically to the EGF receptor on both cell lines. Furthermore hEGF and TGFα competed effectively with [^{125}I]-mEGF for binding to the EGF receptor, although these ligands are immunologically unrelated to mEGF.

Several studies investigating the fate of EGF after binding to cell surface receptors have demonstrated that EGF becomes internalized by a process called "receptor mediated endocytosis" (Hollenberg, 1986; Schlessinger, 1986). In terms of hormone-triggered transmembrane signalling the concept of receptor mobility has evolved as an important property of receptor function. Thus, the binding of a ligand dramatically alters the ability of a receptor to migrate in the plane of the membrane and to interact

with other membrane components (Hollenberg, 1986; Schlessinger, 1986). Only at physiological temperatures can the binding of EGF lead to receptor clustering and internalization. Receptor internalization was demonstrated for both DU 145 and LNCaP cells; this process was both time and temperature dependent, with a slower rate of internalization for LNCaP than for DU 145 cells.

Cells are known to regulate their responsiveness to hormones through a variety of mechanisms. One such mechanism, down-regulation, decreases the number of receptors on the surface of the cell thereby reducing the capacity of the cell to respond to that particular hormone. Pre-incubation of DU 145 and LNCaP cells with increasing concentrations of EGF caused a significant loss of [^{125}I]-EGF-binding capacity, thereby demonstrating receptor modulation. This result is consistent with several reports demonstrating that cells exposed to increased concentrations of EGF gradually lose a substantial fraction of their receptors for their respective hormones by down-regulation (Bradshaw, 1978; Carpenter & Cohen, 1979; Schlessinger, 1986).

Competition and saturation analysis revealed that both cell lines possess EGF receptors with one high affinity binding site. The binding affinities were similar and were within the nanomolar range, comparable with the results of the growth experiments with EGF, where both cell lines were maximally stimulated with nanomolar concentrations of EGF. Maximal stimulation of DNA synthesis and cell proliferation was achieved at partial occupancy of the receptor. This finding is consistent with the majority of findings for EGF, where only a small percentage of receptor sites need to be occupied to induce the maximal proliferative response (Schlessinger, 1988).

One or two binding sites?

There is a lot of controversy as to whether cells possess one or two classes of EGF receptor. The results of this study demonstrate only one class of EGF binding site for DU 145 and LNCaP cells, with K_d values of $1.0 \pm 0.6 \times 10^{-9}$ M and $2.8 \pm 2.2 \times 10^{-9}$

M respectively. Consistent with these findings were those of Wilding *et al.* (1989), who found one high affinity EGF binding site for DU 145 (2.0×10^{-9} M) and LNCaP cells (0.5×10^{-9}M). Schuurmans *et al.* (1988a) also detected one high affinity binding site for LNCaP cells with a dissociation constant of 0.45×10^{-9} M. Contrasting with these findings Connolly and Rose (1989) observed two high affinity binding sites for DU 145 cells (1.8×10^{-10} M and 1.1×10^{-9} M).

In a recent review Schlessinger (1988) stated that EGF receptors are functionally heterogeneous with different classes of binding site, but how far was this conclusion influenced by an inappropriate analysis of the data?

In estimating the affinity of a ligand for its' receptor it is usual to employ a range of radioligand concentrations, from 10 to 20% of the estimated K_d to four to five times this value (Bennet & Yamamura, 1985). If radioligand concentrations are used which are outside this range then the data is difficult to interpret by Scatchard analysis. At the lowest ligand concentrations the differences observed in specific binding are relatively small and difficult to measure accurately. This often leads to a scatter of points which can easily be misinterpreted as conforming to a curvilinear relationship (ie. two binding sites).

The LIGAND method represents a substantial improvement over the graphical Scatchard plot for estimation of affinity constants in ligand binding studies. The advantage of this method is that it fits data in an untransformed coordinate system, where errors are more likely to be normally distributed. The approximations used in Scatchard analysis are not necessary in the LIGAND method which calculates the "free" values from the experimentally measured "total" radioactivity added minus the "bound" values. Non-specific binding is also estimated as an independent parameter, rather than by the investigator. Moreover, weights are assigned to each point, those with a smaller variance usually receiving more weight (Rodbard *et al.*, 1976). Curves are fitted to a single or multi site model and a model for two binding sites is only retained when it fits the data significantly better than a model for a single binding site.

The extent to which flaws in the Scatchard analysis have lead to serious errors in the interpretation of binding site data is currently difficult to judge, because it is rare for authors to present data in a form which can readily be re-analyzed. Although some cell types clearly do have more than one receptor (eg Boonstra *et al.*, 1985) it is clear that Schlessinger (1988) is imprudent in concluding that all EGF binding sites are functionally heterogeneous. In the present study one high affinity binding site for EGF was consistently found for DU 145 and LNCaP cells, lending further support to a number of studies (Banks-Schlegel & Quintero, 1986; Davidson *et al.*, 1987; Häder *et al.*, 1987; Traish & Wotiz, 1987; Carlin *et al.*, 1988; Schuurmans *et al.*1988a; Wilding *et al.*, 1989) which suggest that some target tissues and cell lines express only one class of EGF receptor.

EGF receptor levels

Although the binding constants from both cell lines were similar there were substantial differences in the number of receptors expressed by these cells. The number of binding sites for the DU 145 cell line was calculated as 2×10^5 sites/cell whereas the LNCaP cell line expresses only 2×10^4 sites/cell; an order of magnitude lower. Similar findings were recently reported by Wilding *et al.* (1989), where they calculated DU 145 cells as expressing 1×10^5 receptor sites per cell and LNCaP cells as expressing 4×10^4 receptor binding sites per cell.

Further characterization of the EGF receptor using the technique of Western blotting, with Mabs EGFR 1 and F4, further verified the presence of EGF receptors for DU 145 cells. This result confirmed that the EGF receptor of the DU 145 cell line was present and was structurally intact, at a molecular weight of approximately 170 kDa. However, the EGF receptor was not observed in LNCaP cell lysates using this technique, reflecting the much lower levels of receptors expressed by this cell line.

The affinity of the F4 Mab for the EGF receptor in solution is 5-10 fold lower than the EGFR1 Mab (Gullick *et al.*, 1986), but EGF receptor expression was only

observed with the Mab F4. The most likely interpretation is that the EGFR 1 epitope, which is situated in the extracellular domain of the receptor, must be more vulnerable to destruction by SDS-PAGE than the intracellular F4 epitope. Gullick *et al.* (1986), who produced both Mabs, also failed to detect the EGFR 1 epitope by Western blotting. The extracellular domain of the receptor has a high cysteine content, which may occur as cross-linked disulphide bonds, a common feature of many extracellular proteins, to maintain structural stability in extracellular environments. The reduction of disulphide bonds by β-mercaptoethanol will radically alter the tertiary structure of such a cysteine rich region and could therefore destroy the EGFR1 epitope. This possible role of β-mercaptoethanol in destroying antigenicity could readily be tested by non-denaturing SDS-PAGE.

The EGF receptor and androgens

Since the realisation that androgens are not alone in influencing the growth and regulation of the prostate, several investigations into the role of growth factors and their relationship with androgens have been undertaken. Davidson *et al.* (1987) reported that oestrogen-responsive breast cell lines express relatively low numbers of EGF receptors (< 70 000 per cell) whilst oestrogen-unresponsive cells express higher EGF receptor numbers (> 70 000 per cell). The finding of Davidson *et al.* (1987), that EGF receptor expression of breast cancer cell lines correlates inversely with the response of those cells to oestrogens, is repeated in the present comparative study of the androgen-responsive and unresponsive LNCaP and DU 145 cell lines.

In breast cancer the link between the over-expression of the EGF receptor and endocrine status was complemented by the observation that oestrogen receptor positive MCF-7 cells were converted (by drug selection) to an oestrogen receptor negative population that over-expressed the receptor. Over-expression of the EGF receptor is also associated with an increased recurrence of breast tumours (Harris & Nicholson, 1988). These findings strongly suggest that an alteration in growth factor expression,

such as over-expression of the receptor, is associated with the transition from an endocrine-responsive to an unresponsive state. Perhaps a comparable situation exists for the prostate since the results of this study suggest many parallels between both models.

The correlation between EGF receptor expression and endocrine status (from these two different tissues) implies that steroid hormones might influence the response to growth factors, by altering growth factor receptor expression. However, the nature of the interaction between androgens and EGF in prostatic cancer remains unclear since the results reported here demonstrate that androgens do not modulate EGF receptor expression. The steroidal androgen Mibolerone did not affect either the level of receptors expressed or the affinity of the receptor, contradicting the results of Schuurmans *et al.* (1988a) and Wilding *et al.* (1989). Both groups detected a two fold increase in the number of EGF binding sites upon treatment of LNCaP cells with androgens. Schuurmans *et al.* (1988a) reported a linear increase in the number of EGF receptors, after 6 hours exposure to androgens, until a plateau was reached at three days. However, the increases observed were relatively small, and there was no statistical treatment of the data. Similarly Wilding *et al.* (1989) not only failed to indicate how many times their experiment was repeated but also failed to report any statistical treatment of their data.

In the binding data reported here considerable inter-experimental variability of receptor concentration was noted. Correction factors for the variation have been included in the LIGAND program which adjusts or scales the values of apparent receptor concentrations for any particular experiment. There is also considerable variability in LNCaP receptor levels reported in the literature. Wilding *et al.* (1989) estimated 4×10^4 binding sites/cell for LNCaP cells, almost four times the number reported by Schuurmans *et al.* (1988a) and twice that reported here.

Considering that there is a large degree of variability when evaluating receptor concentration between experiments (Munson & Rodbard, 1980) it is perhaps

imprudent to claim that androgens up-regulate EGF receptors without showing evidence of statistical significance. In the study here receptor levels appeared to decrease when the cells were treated with the androgen Mibolerone, but upon analysis this decrease was shown to be not statistically significant.

It therefore remains questionable as to whether the EGF receptor is up-regulated by steroid hormones in LNCaP cells, as reported by Schuurmans *et al.* (1988a) and Wilding *et al.* (1989). Indeed, in the normal prostate both Traish & Wotiz (1987) and St-Arnaud *et al.* (1988) found that EGF receptor levels are down regulated by androgens, since castration of mature rats resulted in a significant increase in prostatic EGF binding, whilst treatment with DHT decreased the number of EGF binding sites. Although the findings for breast cell carcinomas may not be relevant to the role of androgens in the prostate it is worth noting that oestrogens do not appear to regulate the levels of EGF receptor (Murphy *et al.*, 1985; 1986; Headon & Allan, 1989). It is therefore not inconceivable that the interaction between androgens and the EGF receptor in the normal prostate may be lost, resulting in neoplastic growth.

Although there does not appear to be a link between the EGF receptor and steroid hormones in the androgen sensitive LNCaP cell line, it may be that over-expression of the EGF receptor (eg DU 145 cells) could have resulted in down regulation of the androgen receptor rendering these cells insensitive to androgen manipulation.

4.2 CELL GROWTH AND EGF

Since numerous studies with *in vitro* cell lines have reported EGF as a potent mitogen (Carpenter & Cohen, 1979 for review), it was surprising to discover that exogenous EGF only minimally stimulated proliferation of DU 145 cells in SFM.

Cell enumeration experiments with DU 145 cells grown in media containing foetal calf serum showed that these cells were not stimulated at any of the concentrations of EGF tested. In fact exposure of the cells for three days to higher concentrations of

EGF resulted in inhibition rather than stimulation of cell growth. Interestingly, EGF appeared to have a cumulative effect on these cells, for although the lower concentrations of EGF had no discernable effect after three days incubation, inhibition at these lower concentrations was seen after incubation for nine days. This was not a toxic effect since incubation with EGF never resulted in a net loss of cells. One possible influence was the presence of foetal calf serum in the cell culture medium. Consequently, the experiment was repeated with DU 145 cells which had been cultured in the absence of this supplement, for which it was necessary to develop a serum-free line of these cells; see section 2.3.1.2. Although the growth rate of these cells was slower in SFM than in complete medium, growth was sustained in the SFM and cells could be sub-cultured successfully.

Results obtained at the highest concentrations of EGF in SFM were essentially similar to those observed in the presence of foetal calf serum, but at the lower concentrations of EGF some differences were observed. EGF, up to a concentration of 0.3 nmol/l, increased both cell numbers and [^{3}H]-Thymidine incorporation into DNA, in a dose-dependent manner above which inhibition was observed. Although both measures of mitogenicity were comparable there were differences in the statistical significance of the increases observed in cell proliferation and [^{3}H]-Thymidine incorporation. There was an incremental trend in cell numbers with increasing concentrations of EGF, but the increases seen at each concentration of EGF were not significantly different from control values. However, [^{3}H]-Thymidine incorporation into DNA was increased by a statistically significant amount at similar concentrations of EGF.

It could be argued that the differences in statistical significance reflect the relative precisions of the two techniques, as cell numbers determined by haemocytometer are notoriously imprecise. EGF might also stimulate division of the cell nuclei without concomitant cell proliferation as has been reported for hepatocytes (Sargent *et al.*, 1988). Similar inconsistences between cell proliferation and [^{3}H]-Thymidine

incorporation, with respect to EGF, were reported by Connolly & Rose (1989) with the DU 145 cell line. They observed a three fold increase in incorporation of [^{3}H]-Thymidine with EGF when DU 145 cells were plated at low cell density (10^3 cells/cm^2), but cell proliferation was not affected at this density. The differences observed could be a function of cell plating density, or as Connolly & Rose (1989) have suggested, a deficiency of one or more nutrients in the SFM, with failure of the cells to proceed from interphase to the M-phase of the cell cycle.

It seems illogical that while DU 145 cells possess large numbers of receptors they are only minimally affected by exogenous EGF. However such findings are not unique, the endometrial carcinoma cell line RL95-2 is only marginally stimulated by EGF (Korc *et al.*, 1987) as are some human embryonal cell lines (Verbeek *et al.*, 1988), and yet all possess EGF receptors. The most complex pattern of proliferative response is seen with the *in vitro* epidermoid carcinoma cell line A-431 which is inhibited by nanomolar concentrations of EGF (Chinkers *et al.*, 1981; Barnes, 1982; Kawamoto *et al.*, 1983), whereas picomolar concentrations minimally stimulate proliferation (Kawamoto *et al.*, 1983). The biphasic effect observed with EGF on the proliferation of DU 145 most closely parallels that observed with A-431 cells, although the concentrations of EGF which inhibited proliferation of DU 145 cells are somewhat greater.

Other workers have noted no proliferative effect with EGF (Engström, 1986; Salomon *et al.*,1987). The embryonal carcinoma cell line Tera-2 has high affinity EGF receptors, but this cell line does not respond proliferatively to exogenous EGF (Engström, 1986). Similarly, ras-transformed mouse mammary epithelial cells express high affinity EGF receptors, but neither exogenous EGF nor TGFα have an effect on the anchorage-independent growth of these cells (Salomon *et al.*,1987).

It is possible to speculate on a number of possible mechanisms which could account for the apparent contradiction that DU 145 cells are only minimally stimulated by exogenous EGF, but possess 10^5 receptor sites/cell:-

(a) Tyrosine kinase activity

Activation of Tyr kinase activity and phosphorylation by ligand binding are believed to represent the primary molecular events initiating the transmission of the mitogenic signal inside the cell. Therefore a reduction in Tyr kinase activity, irrespective of the number of receptors, would cause a reduced mitogenic response by exogenous EGF. However, this is unlikely because exogenous EGF (at high concentrations) significantly inhibited cell proliferation of DU 145 cells.

(b) A non proliferative role for EGF

The embryonal carcinoma cell line Tera-2 is reported to have high affinity EGF receptors yet Engström (1986) found no detectable proliferative effect with exogenous EGF. It was suggested that EGF played an alternative (non proliferative) role in these cells (eg. cell locomotion). However, as was the case with the previous hypothesis, this explanation is unlikely because high concentrations of EGF significantly inhibited cell proliferation of DU 145 cells.

(c) The truncated receptor

Although there is no direct evidence that aberrant expression of the EGF receptor is involved in the transformation of human cells it has been suggested that the production of the truncated EGF receptor, which lacks the regulatory action of the ligand binding domain, may leave the tyrosine kinase of the v-*erb* B gene product in a constitutively active form (Gilmore *et al.*, 1985; Kris *et al.*, 1985). It is the activity of the Tyr kinase domain of the EGF receptor which is important in influencing the growth rate of cells, thus an abnormal EGF receptor with an uncontrolled Tyr kinase activity would cause rapid cell division. Aberrant expression of the EGF receptor of DU 145 cells would also explain why exogenous EGF appeared to have very little

effect on the growth rate of these cells. However, this is not true for DU 145 cells because Western blotting revealed only the 170kDa EGF receptor (and not the 68kDa truncated receptor).

(d) Plating density

Several workers have shown that the response of a cell line in serum-free defined medium to a particular hormone or growth factor is dependent to a large extent upon plating density (Holley *et al.*, 1977; Kaighn *et al.*, 1981; Korc *et al.*, 1987; Rizzino *et al.*, 1988). The effect of density was explored with the serum-free DU 145 cell line; densities between 1 x 10^4 cells/cm^2 and 6 x 10^4 cells/cm^2 did not greatly enhance the weak stimulatory response to EGF. Sustained growth of the serum-free DU 145 cell line was dependent upon high plating densities (> 10^4 cells/cm^2), with densities lower than this value causing cells to round up and detach, even in the presence of EGF.

In a recent study Connolly and Rose (1989) reported that DU 145 cells responded to exogenous EGF, contradicting the findings reported here. However, this response was only noted at low cell density and only DNA synthesis was affected and not cell numbers. They demonstrated a three fold increase in incorporation of [^{3}H]-Thymidine with EGF when DU 145 cells were plated at low cell density (10^3 cells/cm^2), but at high plating density (10^4 cells/cm^2) this stimulatory effect was abolished.

How can we account for these differences between the two studies? As previously mentioned, the serum-free DU 145 cell line would not proliferate at low density and all attempts to carry out studies at a low seeding density proved to be a fruitless exercise. Moreover, the DU 145 cell line used by Connolly and Rose was not a serum-free line, only the growth experiments were carried out with SFM, which suggests that the differences observed might be due to culturing conditions or to carry over from serum proteins. Nonetheless, cell density is obviously an important criterion to take into account with respect to the response to a particular hormone. Holley *et al.* (1977) noted that increasing the density of monkey kidney epithelial BSC-

1 cells decreased the response to EGF. This down regulatory effect was also observed by Rizzino *et al.* (1988) with non-transformed rat kidney fibroblasts (NRK cells). In contrast to these reports however, Korc *et al.* (1987) noted that the proliferative response to EGF of the RL95-2 human endometrial carcinoma cell line was increased at high plating densities.

In all of these cases the effect of density with respect to the proliferative response to EGF was observed in serum-supplemented medium. With regard to the serum-free DU 145 cell line, it would appear that the serum-free culture of these cells is density-dependent, but not the response to EGF.

(e) TGFα

The growth factor TGFα was tested for its' effect on DNA synthesis of DU 145 cells because DU 145 cells may respond proliferatively to TGFα in preference to EGF. TGFα has only 35% sequence homology with EGF (Derynck *et al.*, 1984; Marquardt *et al.*, 1984) yet it binds to the same receptor and appears to have similar biological properties (Bascom *et al.*, 1989).

TGFα stimulated [^{3}H]-Thymidine incorporation in DU 145 cells, but although this effect was minimal it was significantly different from the untreated control. However, there were differences between TGFα and EGF in the response elicited, as 10 fold lower concentrations of TGFα were required to maximally stimulate the growth of DU 145 cells relative to EGF. This suggests that TGFα has a higher affinity for the EGF receptor than EGF itself.

There is also evidence to suggest that EGF and TGFα bind to distinct sites on the receptor (Winkler, 1989). Winkler and co-workers (1989) described an antibody which blocked the binding of TGFα to the human EGF receptor, but had no effect on the affinity of EGF for binding to the EGF receptor. This evidence is consistent with the fact that TGFα has only 35% sequence homology with EGF and is immunologically distinct from EGF.

In a preliminary report, Wilding *et al.* (1988) noted that TGFα, at similar plating densities, had little effect on the growth of the DU 145 cell line, indeed the growth rate of DU 145 cells was not significantly altered by TGFα. In contrast, Fernandez-Pol *et al.* (1986) reported that TGFα significantly stimulated anchorage-independent growth of DU 145 cells.

From these studies it appears that there are inconsistencies with respect to the growth response to TGFα. What these studies do portray is that DU 145 cells are only minimally affected by exogenous TGFα. The differences in the response elicited might reflect assay and culture conditions. For example, Shipley and co-workers observed that TGFα did not stimulate DNA synthesis in serum-free cultures of AKR-2B mouse cells, but stimulated multiple rounds of DNA synthesis in serum-containing medium (Shipley *et al.*, 1984). However, unlike the DU 145 cell line, EGF induced a substantial mitogenic response on AKR-2B mouse cells both in the presence and absence of serum.

It was concluded from these findings that neither EGF nor TGFα, under serum-free conditions, are potent mitogens for DU 145 cells and that plating density did not affect this proliferative effect with EGF. Indeed EGFs' action upon these cells was found to be more inhibitory than proliferative. This inhibitory effect with high concentrations of EGF was probably due to down regulation of EGF receptors since this effect was enhanced with time, or as Barnes (1982) and Chinkers *et al.* (1981) suggested, to morphological changes caused by high concentrations of EGF. These workers observed that when inhibitory concentrations of EGF (in the range of 3 nM) were added to cultures of A431 cells, the cells rapidly round up, detach and cease to proliferate. Although inhibitory concentrations of EGF did not cause detachment of DU 145 cells, morphological changes may have resulted in cessation of proliferation.

(f) Autostimulation by endogenous growth factors

None of the previous examples could explain why DU 145 cells are only minimally stimulated by exogenous EGF. An alternative explanation is that DU 145 cells are auto-stimulated by endogenous EGF-like molecules. This is consistent with the observation that these cells possess receptors, yet they show a slight proliferative response to exogenous growth factors.

The loss of requirement for specific growth factors is a common finding in many types of neoplastic cells (Moses *et al.*, 1978; Kaplan *et al.*, 1982) and is also reflected in a reduced serum requirement for growth. Indeed DU 145 cells readily adapted to growth in chemically defined, serum-free medium containing no EGF.

EGF and LNCaP

In contrast to its' effect on the DU 145 cell line, EGF elicited a substantial mitogenic response on the androgen-sensitive cell line LNCaP. EGF, in a dose–dependent manner, induced a two fold increase in cell numbers and [^{3}H]-Thymidine incorporation into DNA. A similar mitogenic response by EGF was reported by Schuurmans *et al.* (1988a) who found that EGF, at similar concentrations to those reported here, almost doubled the DNA content of LNCaP cells under serum-free conditions. In a recent report Wilding *et al.* (1989) noted that exogenous EGF and TGFα stimulated LNCaP cell growth and that TGFα increased the uptake of [^{3}H]-Thymidine. They showed that EGF and TGFα, at a concentration of 5 ng/ml, were equipotent in stimulating cell proliferation, but the extent of the proliferative response with both ligands was not as pronounced as in the study reported here. Increased concentrations of TGFα (up to 20 ng/ml) stimulated proliferation 3 fold whereas in the study presented here, when EGF was used at similar concentrations, cell proliferation and DNA synthesis had returned to basal levels.

It is difficult to compare the results of the present study with those of Wilding *et al.* (1989) because they examined the proliferative response to TGFα over a range of

concentrations and the effect of EGF at one concentration. Moreover, the proliferation studies with exogenous growth factors were conducted with LNCaP cells cultured in the presence of serum and not SFM (as reported here). Nonetheless, both studies reveal that the LNCaP cell line is responsive to exogenous EGF and that this effect was substantial, which is not the case for EGFs' effect on DU 145 cells.

Androgens and EGF

As previously discussed the relationship between growth factors and androgens in the prostate remains unclear although similarities can be drawn between prostate and breast cancer cells with respect to the proliferative response to EGF, the expression of the EGF receptor and the mitogenic response to steroid hormones. In common with DU 145 and LNCaP prostate cancer cell lines, Davidson *et al.* (1987) reported that the presence of relatively low numbers of EGF receptors on breast cancer cell lines was associated with the ability of the cell to manifest a mitogenic response to EGF, whilst cells with higher EGF receptor numbers failed to respond. Those cells which were stimulated to grow with EGF were oestrogen-responsive whilst those which were only minimally affected by EGF were oestrogen-unresponsive (Davidson *et al.*,1987).

Could it be that the loss of steroid responsiveness of prostatic tumours may be linked with a loss of dependence or a reduced sensitivity to growth factors, possibly by autologous production of growth factors ?

The nature of the relationship between androgens and growth factors in the prostate has remained unclear despite intensive investigations. One such report demonstrated that androgens, together with EGF directly affected prostatic cancer cell proliferation (Schuurmans *et al.*, 1988b). This group observed a synergistic effect between the androgen R1881 and EGF on the proliferation of LNCaP cells. However, the results of the present study and of others (Eaton *et al.*, 1989; Somnenschien *et al.*, 1989) demonstrated no such synergistic effect between EGF and androgens on the proliferation of these cells. Indeed, the increase in cell numbers and DNA synthesis

reported here was less when EGF and Mibolerone were added together, than the combined effect of each of these factors added separately. To add to the confusion the group of Schuurmans published contradictory accounts of the relationship between androgens and EGF. In the first of these Schuurmans *et al.*(1988a) did not observe the synergism subsequently reported. In the second publication Schuurmans *et al.* (1988b) argued for a rapid metabolism of EGF which disguised the synergistic relationship with androgens unless EGF was administered daily.

Although a rapid metabolism of EGF is crucial to the argument of Schuurmans *et al.* (1988b) no experimental evidence for this rapid metabolism was given. Furthermore, in this study, when DNA synthesis was monitored 24 hours after the addition of growth factors and androgens (ie. using the same conditions as Schuurmans *et al.*, 1988b) no synergism was detected. Since the major difference between this study and that of Schuurmans *et al.* (1988b) is the use of serum-free medium (compared with a medium containing serum, but stripped of androgens) it is possible to argue that the 'synergistic effect' observed by the Dutch group was due to other factors in the serum. The difficulties of investigating the effect of a single factor using a medium containing serum were illustrated by the repetition of an experiment to monitor the effect of R1881 on the growth of LNCaP cells. Although Schuurmans *et al.* (1988) found that growth was stimulated when the cells were cultured in a medium stripped of androgens, R1881 did not affect the growth of LNCaP cells when cultured in the presence of SFM.

One other important difference between the two studies was the choice of androgens to demonstrate the synergistic effect with EGF. The synergistic effect observed by Schuurmans and co-workers was detected in the presence of the synthetic androgen R1881 and not Mibolerone (as used in the present study). Somnenschien *et al.* (1989) reported that both steroids had identical proliferative potencies for the LNCaP cell line. However, this does not appear to be the case because Mibolerone stimulated proliferation and [^{3}H]-Thymidine incorporation while R1881 (at the same

concentration) did not affect the growth of LNCaP cells. Although it would appear that Mibolerone is proliferatively more potent than R1881 in SFM, when the androgens were added to cells cultured in the presence of 10% FCS a greater inhibitory effect was demonstrated with R1881. Since the effect of androgens on growth is biphasic (increasing concentrations initially stimulating then inhibiting growth; Somnenschien *et al.*,1989), the results imply that the "effective concentration" of R1881 is greater than for Mibolerone. This finding is consistent with the higher binding affinity of R1881 for the androgen receptor (Schilling & Liao, 1984). 0.1 nM Mibolerone significantly stimulated both proliferation and DNA synthesis of LNCaP cells grown in SFM. Therefore the failure of 0.1 nM R1881 to stimulate growth in SFM studies presumably reflects its' use at a concentration greater than that which would affect maximum growth.

Differences in the proliferative potency of the two androgens will affect results if they are tested at the same concentration. However the failure to observe a synergistic effect with Mibolerone cannot be accounted for by such differences in proliferative response since Mibolerone significantly stimulated both proliferation and DNA synthesis, and there is no reason to believe that a genuine synergistic effect between Mibolerone and EGF would not be detected.

As the results reported here demonstrate, androgens together with EGF affect cell proliferation and DNA synthesis of LNCaP cells. However, the nature of this interaction is unclear since the combined effect of EGF and Mibolerone was not additive, neither was it synergistic.

4.3 AN AUTOCRINE MECHANISM

Since Sporn & Roberts (1980) first conceived the idea that cells could regulate their growth by the autologous production of growth factors, and that autocrine regulation may be an important mechanism involved in transformation, autocrine growth control

mechanisms have been postulated to be important for a variety of tumour systems. The existence of a TGFα auto-stimulatory loop for DU 145 cells is proposed based on findings that the cells produce endogenous EGF-like molecules and express high concentrations of EGF receptors, although neither EGF nor TGFα are potent mitogens.

Using a specific RIA for TGFα and an EGF-RRA competition assay, EGF-like molecules were detected in the medium conditioned by DU 145 cells. A number of previous studies have reported the production of EGF-related polypeptides by various cell lines from the colon (Coffey *et al.*, 1986; 1987; Watkins *et al.*, 1988), breast (Dickson *et al.*, 1986; Perroteau *et al.*, 1986; Lippman *et al.*, 1987), ovary (Bauknecht *et al.*, 1986) and prostate (Connolly & Rose, 1989; Wilding *et al.*, 1989). The detection of endogenous EGF-like molecules in the medium of these cells suggests an involvement in cell transformation since these cells are auto-regulated by virtue of possessing receptors for the secreted molecule. However, evidence has accumulated in recent years that the role of EGF/TGFα and their receptor in carcinogenesis is more complex than first thought.

Initial studies with cultured human breast cancer cell lines indicated that oestradiol up-regulated growth stimulators such as TGFα (Dickson *et al.*, 1986), and it was thought that transition to the unresponsive state involved alteration of this factor and/or its' receptor (King, 1990). This transition was thought to occur via a mechanism where an increase in growth factor production might occur irrespective of the presence of androgens. This link was confirmed by the observation that transfection of MCF-7 cells with the *ras* oncogene, which rendered the cells oestradiol insensitive, up-regulated TGFα production (Kasid *et al.*, 1985).

In addition to these finding Di Marco *et al.* (1989) recently demonstrated that the gene for TGFα was a potent oncogene for NIH 3T3 cells and those cells producing TGFα over-expressed the EGF receptor. These findings suggest that both ligand and receptor might induce the transformed state.

Could a similar mechanism be involved in prostate cancer? The results presented here demonstrate that DU 145 cells produce EGF-like molecules which may be autostimulatory. Furthermore DU 145 cells express increased levels of EGF receptor compared to the androgen sensitive LNCaP cell line.

LNCaP cells are also thought to produce EGF-like molecules, the production of this ligand is increased by androgen stimulation (Wilding *et al.*, 1989). If LNCaP cells produce EGF-like molecules why are they responsive to exogenous growth factors? One possible explanation is that those cells which are unresponsive to EGF/TGFα may have an increased capacity to secrete TGFα compared to those which are growth factor responsive.

Paradoxes do exist, however, as several workers have demonstrated a negative correlation between EGF receptor number and the production of TGFαs (Todaro *et al.*, 1980; Carpenter *et al.*, 1983; Coffey *et al.*, 1986; Sircar & Weber, 1988), contrary to the findings with breast and prostate cancer. These workers reported a decreased expression of the EGF receptor with the production of TGFαs, suggesting that the EGF receptor is down regulated by increased production of TGFα.

Recent findings with the breast carcinoma model also contradict earlier studies as two rodent models demonstrated a decreased TGFα expression in unresponsive tumours (Liu *et al.*, 1987), whilst oestradiol-responsive MCF-7 cells transfected with TGFα cDNA over-express TGFα but retain their sensitivity to steroids (Clarke *et al.*, 1989).

Whether the situation is as complex in the prostate remains to be determined, but it appears from the findings of this study that DU 145 cells have an increased expression of the EGF receptor, yet are in general, unresponsive to the mitogenic effects of EGF and TGFα because of an increased production of TGFα.

TGFαs- immunological and competitive activity

The bioactive EGF-like molecules produced by these cells (demonstrating EGF RRA competitive activity) are also immunologically related to TGFα, but not EGF. However, stoichiometrically the level of immunological TGFα activity detected was not equivalent to the amount of EGF-like competitive activity. The differences could be attributed to inaccurate assessments of the levels of EGF-like molecules in the CM, in particular the levels of TGFα since the rTGF-I antibody does not cross-react with human TGFα and rat TGF-I to the same extent. The amount of bioactive EGF equivalents in the CM was calculated by comparing the competitive activity of EGF binding to the EGF receptor, but the molecules detected in the CM are immunologically related to TGFα and not EGF. Although TGFα binds to the EGF receptor the affinity of binding may differ, and consequently the levels detected in the CM may be inaccurate. A second consideration is that the EGF-like molecules produced by DU 145 cells may be related to, but distinct from, TGFα. The secretion of heterogeneous EGF-like molecules, some identical with and others related to TGFα, might also account for this discrepancy. Indeed, DU 145 CM fractionated by rHPLC revealed several components of EGF-like competitive activity, only one of which proved to be related immunologically to TGFα.

Further evidence supporting the hypothesis that the prostatic cell line DU 145 is involved in an autostimulatory loop has come from two recent reports (Connolly & Rose, 1989; Wilding *et al.*, 1989) describing EGF-like molecules in the CM of these cells. The molecules showed EGF-like competitive activity and were immunologically related to TGFα, but Connolly and Rose (1989) also detected immunoreactive hEGF. This is a surprising result because EGF is not thought to be secreted by transformed cells. Although both ligands were detected by these workers it may be that the RIAs used to detect EGF cross reacted with TGFα in the CM, since TGFα has approximately 35% sequence homology with EGF (Derynck *et al.*, 1984; Marquardt *et al.*, 1984).

Mori *et al.* (1986) also claimed to have detected immunoreactive hEGF from MCF-7 breast cancer cells, but this finding was not confirmed when specific RIAs' were used in a follow-up study (Perroteau *et al.*, 1986). Perroteau and co-workers detected immunoreactive TGFα in the medium conditioned by MCF-7 cells and three other breast cancer cell lines, but no hEGF was detected (Perroteau *et al.*, 1986).

Stimulatory and inhibitory factors ?

Media conditioned by DU 145 cells had a significant stimulatory and inhibitory effect on DNA synthesis of DU 145 cells, which was dose-responsive. The inhibitory effect on the incorporation of [^{3}H]-Thymidine into DNA was demonstrated at low CM concentrations, whilst higher concentrations stimulated DNA synthesis. This biphasic effect on DNA synthesis of DU 145 cells could be due to the existence of at least two regulatory 'factors', one of which has an inhibitory effect on growth, the other stimulating proliferation or inhibiting the effect of the inhibitory factor. It would appear from the biphasic effect that the inhibitory factor(s) is either more abundant or more potent than those which stimulate DNA synthesis.

It is interesting to note that TGFβ which inhibits the growth of tumour derived epithelial cells (Tucker *et al.*, 1984; Knabbe *et al.*,1987) also inhibits the growth of the prostatic cancer cell line LNCaP (Schuurmans *et al.*, 1988). The stimulatory effects of EGF on the growth of the LNCaP cell line are also inhibited by TGFβ (Schuurmans *et al.*, 1988). Perhaps TGFβ may also play a role in the regulation of growth of DU 145 cells, negating the effects of EGF-like molecules. However, without biochemical analysis it is imprudent to speculate overmuch about the nature of these 'factors' produced by DU 145 cells.

TGFαs - heterogeneous molecules

Fractionation of CM from DU 145 cells by gel filtration chromatography revealed two molecular forms of EGF receptor binding polypeptides; a major and a minor peak of EGF-like competitive activity. The minor peak of EGF-like competitive activity had

an apparent molecular weight of 5 kDa which corresponds in its elution position to the low molecular weight form of TGFα. However this peak was not immunoreactive with either anti-hEGF or anti-rTGF-I antisera. The higher molecular weight component (apparent molecular weight 30 - 50 kDa) may comprise the precursor form of hEGF previously described in urine (Hirata & Orth, 1979) or the 160 amino acid precursor form of TGFα (Bringman *et al.*,1987; Waterfield, 1989). Consistent with this result were the findings of Connolly and Rose (1989) who described the size heterogeneity of EGF-like components secreted by the DU 145 cell line. They found several peaks of competitive activity from conditioned DU 145 medium, with apparent molecular weights of approximately 5 - 6 kDa, together with four additional peaks of considerably higher molecular weight.

High molecular weight EGF-like molecules have been described by other workers (Dickson *et al.*, 1986; Stromberg *et al.*, 1986; Culouscou *et al.*, 1987; Eaton *et al.*, 1988; Connolly & Rose, 1989). Dickson *et al.* (1986) described a 30kDa component secreted by the MCF7 breast cell line. Similarly, 48kDa forms have been observed in Western immunoblots of extracts of medium conditioned by human rhabdomyosarcoma A673 cells (Stromberg *et al.*, 1986).

Importantly, both bioactive regions from gel filtration analysis failed to compete in specific RIAs for hEGF or rTGF-I. This lack of immunoreactivity could mean (i) a lack of immunological relatedness of both components to either hEGF or rTGF-I or (ii) a problem of sensitivity, due to the dilution of active components by gel filtration. The latter is more likely because immunoreactive TGFα activity was demonstrated in the concentrated CM before it was subjected to gel filtration and immunoreactive TGFα activity was detected in components separated by rHPLC. The lack of hEGF immunoreactivity in any fraction is consistent with the result for the pooled CM, where only immunoreactive TGFα was detected.

It was not possible to subject either peak of EGF-like competitive activity separated by gel filtration to rHPLC directly, due to the low levels of EGF-like competitive

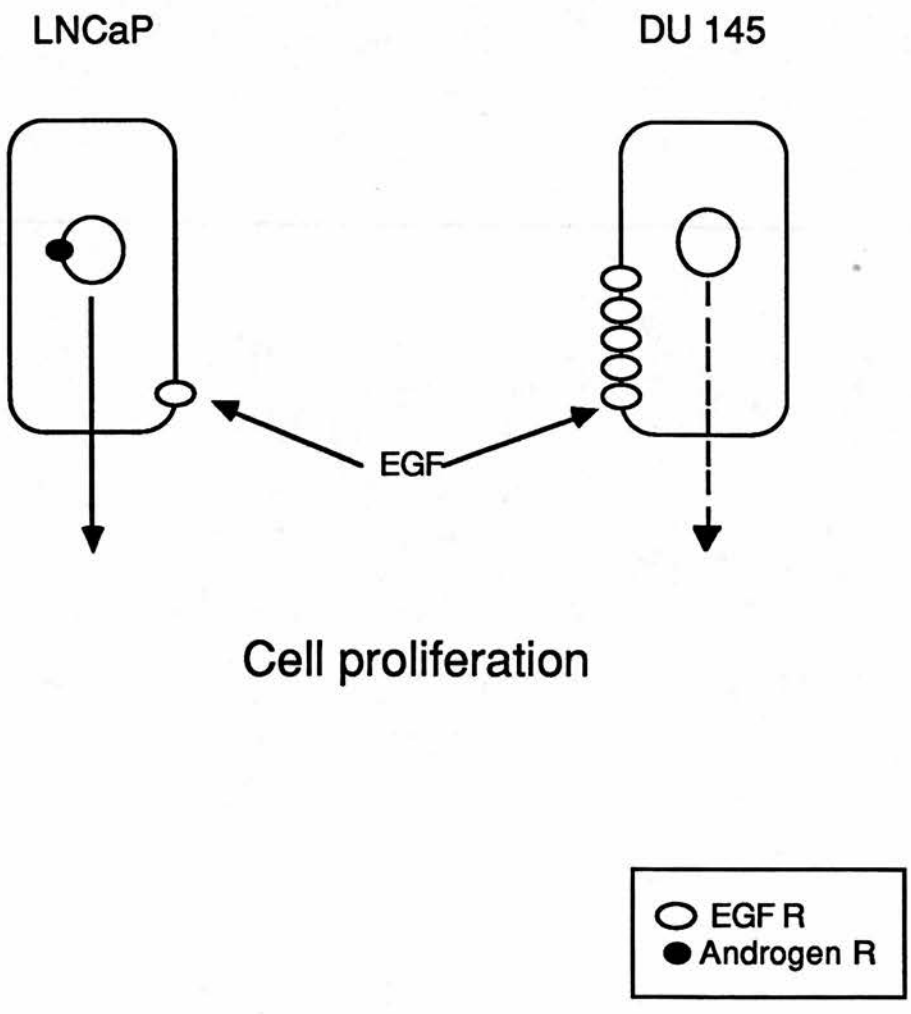
activity detected and the lack of TGFα immunological activity. Therefore, concentrated CM was separated using rHPLC (which fractionates components in smaller volumes thereby increasing sensitivity) and analysed for EGF-receptor binding activity as well as immunologic rTGF-I and hEGF activity. The results of the rHPLC revealed several components of EGF-like competitive activity and contrary to the results obtained with gel filtration, one of these major peaks demonstrated immunoreactive rTGF I activity. The other peaks of EGF-like competitive activity were immunologically distinct from both rTGF-I and hEGF.

The major EGF-like competitive activity which eluted at the end of the run is unusual in that bio-activity increased steadily with increasing acetonitrile concentration without any sign of peaking; suggesting that this activity is an artifact. Nonetheless, it appears that the DU 145 cell line secretes several different molecular species that are EGF-like biologically, only one of which is related immunologically to TGFα. Since immunological activity is more specific than competitive activity the inability of antisera to react with the bio-active EGF-like molecules produced by this cell line may be due to cryptic epitopes. Therefore a wider range of antibodies than is currently available will be required to detect all EGF-like growth factors of prostate origin.

The reasons why a cell may secrete several molecular species related biologically to EGF remains unclear, but it raises the possibility that a cell could make subtle changes in the response elicited by producing different molecular forms of EGF/TGFα.

The major peaks of EGF-like competitive and immunological activity were further subjected to non-reducing SDS-PAGE in an attempt to elucidate the molecular weights of the bio-active and immunologically active EGF-like molecules. However, the eluted material from the electrophoresed fractions did not demonstrate either EGF-like competitive activity or rTGF-I, hEGF immunological activity. The lack of biological and immunological activity from these fractions was probably due to the low concentration of EGF-like molecules eluted from the gel slices. In retrospect Western blotting would have probably succeeded in identifying the EGF-like molecules,

although biological activity could not have be elucidated by this method. Nonetheless, it is clear that the various different classes of TGFs secreted represent distinct molecular entities sharing both structural and functional determinants.


LNCaP
DU 145
EGF
Cell proliferation
EGF R
Androgen R

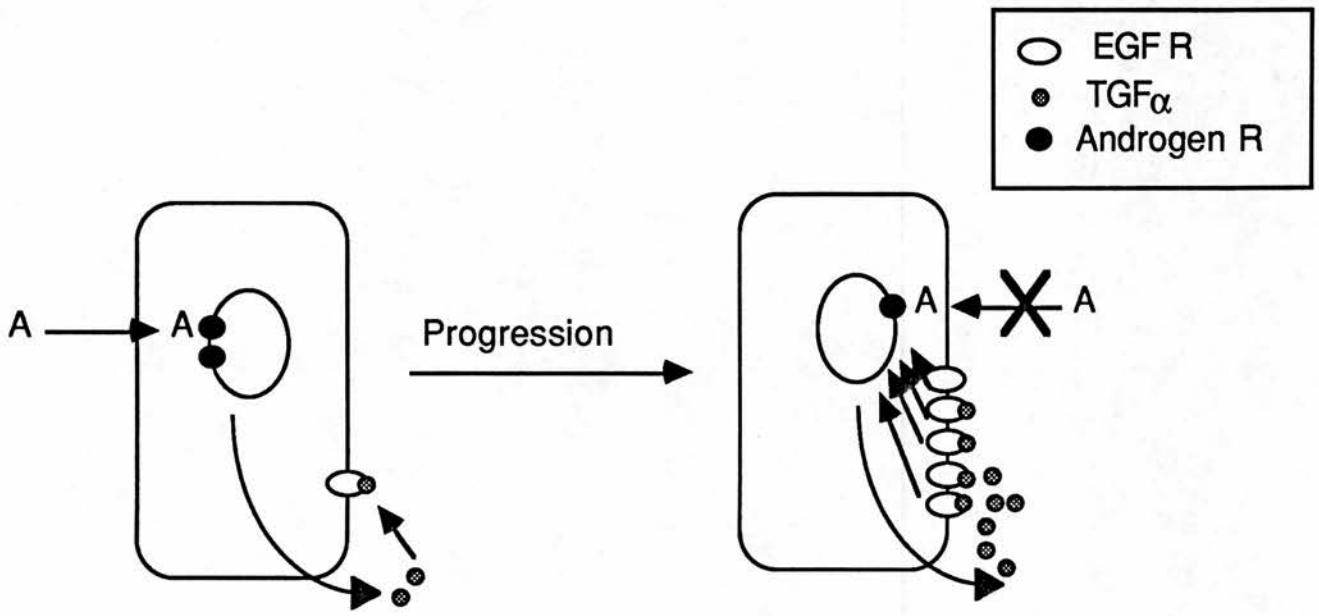
OVERVIEW

Using the *in vitro* epithelial cell lines DU 145 and LNCaP the role of growth factor involvement in prostate cancer was explored, in particular in the progression from an androgen-dependent to an androgen-independent state. By comparing the growth responses to exogenous EGF and EGF receptor expression it has been possible to gain some insight into this transition.

A relationship was demonstrated between the proliferative response to EGF, the expression of the EGF receptor and steroid responsiveness. The androgen-insensitive DU 145 cell line expresses high levels of EGF receptors yet growth is only minimally affected by exogenous EGF and TGFα. The inverse was found with the androgen-sensitive LNCaP cell line which has retained its' capacity to respond proliferatively to exogenous EGF. The relationship is similar to that found with breast cancer cell lines, where the presence of relatively low numbers of receptors is associated with the ability of the cell to manifest a mitogenic response to exogenous EGF, whilst cells with higher receptor numbers respond only minimally to exogenous EGF (Davidson *et al.* 1987). Moreover, those cell lines which failed to respond to exogenous EGF were endocrine-unresponsive, while those cells that retained their capacity to respond were endocrine-responsive (Figure 33).

The negative correlation between EGF receptor expression, the response to exogenous growth factor and endocrine status suggests that DU 145 cells, like endocrine-unresponsive breast cancer cell lines, are generally unresponsive to stimulation by exogenous growth factors. The available evidence presented here

FIGURE 33: Schematic diagram of the negative correlation between EGF receptor expression and the proliferative response to exogenous EGF of the androgen-insensitive DU 145 and androgen-sensitive LNCaP cell lines. The diagram shows androgen and EGF sensitivity of LNCaP cells which express low levels of EGF receptors compared to DU 145 cells, which express high numbers of receptors, are unresponsive to androgens and are affected minimally by EGF.


EGF R
TGFα
Androgen R
A
A
Progression
A
A

supports the view that the minimal response is caused by autologous production of EGF-like molecules, the levels of which are auto-stimulatory. In contrast to this the androgen-sensitive cell line LNCaP, like oestrogen-responsive breast cancer cells, appears to have retained its' capacity to respond to exogenous EGF, possibly because of lower secretion of bioactive EGF-like peptides. Indeed in a recent study Wilding *et al.* (1989) demonstrated an autocrine growth-stimulatory loop for LNCaP cells. Furthermore, the production of EGF-like molecules by LNCaP cells are thought to be stimulated by androgens (Wilding *et al.*, 1989).

We might envisage that progression to an androgen-independent status may, in part, be due to a loss of androgen regulation of growth factor production with cells producing growth factors irrespective of the presence of androgens. The increase in growth factor expression may in turn increase the expression of the EGF receptor resulting in down-regulation of the androgen receptor (Figure 34). Studies on the androgen-responsive (SC3) and androgen-independent (SC4) cell lines which are derived from a mouse mammary tumour (Nonomura *et al.*, 1988) suggest such mechanisms occur in the progression from androgen-dependent to independent cell status. Other studies with these cells lines imply that progression to androgen-independence may occur by a mechanism in which the cells obtain the ability to respond to growth factors (Nonomura *et al.*, 1988) and autonomously produce growth factor receptors.

Other authors have suggested that as well as up-regulating the production of growth factors, androgens might also up-regulate the receptor for EGF (Schuurmans *et al.*, 1988a; Wilding *et al.*, 1989), but this finding could not be substantiated in the

FIGURE 34 : Schematic diagram demonstrating the progression from an androgen-dependent to an androgen-independent state. The androgen-dependent state, where cells respond to androgens by an increase in growth factor production progress to a state where cells produce endogenous growth factors irrespective of the presence of androgens. Increased growth factor production may lead to increased expression of the EGF receptor which in turn may down regulate the androgen receptor.

study reported here. In the normal rat prostate the EGF receptor appears to be down regulated by androgens, and it is not inconceivable that this relationship may be lost, resulting in neoplastic growth.

The discovery that cancer cell lines secrete TGFα has spawned the idea that a cell could become independent of external growth control. Interestingly, the majority of findings with transformed cells and indeed the DU 145 cell line reveal that these cells secrete heterologous TGFαs. The EGF-like molecules are related biologically to EGF and TGFα by virtue of their competitiveness with EGF for binding to its' receptor. Immunological assays demonstrate that these molecules have a number of similar epitopes to TGFα. We might envisage a cell producing different molecular forms of TGFα (which differ in their biological activity) thereby regulating cellular response. Whether this is indeed the case remains unclear, but what is evident from this study is the important role of growth factors in prostate cancer. In recognising this a therapeutic use for growth factors may be imagined, particularly if growth factors and their receptors are involved in tumour progression, as they appear to be.

FUTURE STUDIES

There are several aspects of this study which merit further investigation:-

The DU 145 cell line, by autologous production of EGF-like molecules, appears to have little or no need for exogenous EGF or TGFα. The levels of TGFs produced may be autostimulatory, but this has not been established. It would be possible to prove or disprove this hypothesis by the use of antibodies, either against the EGF receptor or TGFα. Blocking the ligand binding site on the EGF receptor or "mopping-up" excess EGF-like molecules would result in a reduction of the growth rate if the levels secreted were indeed autostimulatory.

There are problems with this approach; firstly, antibodies raised against the receptor might not block the TGFα binding site or may act as an agonist. Secondly, the TGFαs produced by the DU 145 cell line and other cell lines are heterologous in nature, some appear to be immunologically related to but are not identical to TGFα, while others are quite distinct. Furthermore, antibodies raised against the EGF receptor might not necessarily block the binding site for TGFα as this ligand appears to bind to a site distinct from EGF (Winkler *et al.*, 1989).

The secretory products of DU 145 cells must be characterized further, notably the factors which stimulate and inhibit DNA synthesis. We know that TGFαs are produced by the DU 145 cell line, but other factors such as those which inhibit growth must be fully characterized. In a preliminary report by Wilding *et al.* (1988) DU 145 cells were found to transcribe TGFβ mRNA and when TGFβ was added to these cells, growth was inhibited. The presence of this growth factor might explain the inhibitory effect of the CM on the growth of DU 145 cells.

Although the components in the CM were partially characterized by gel filtration and rHPLC, further biochemical and biological characterization is needed. The approach taken here, namely to separate both immunological and biologically active components using gel filtration according to their molecular weights, is problematic because the samples are eluted in large volumes. The dilution effect could be limited

by using size exclusion HPLC. The next step would be to separate these active components of known molecular weight using either ion-exchange or rHPLC. Biological activity (such as transforming ability on fibroblasts and mitogenic activity) as well as biochemical characterization (heat and proteases stability) should then be ascertained.

The androgen-sensitive cell line LNCaP, like oestrogen-responsive breast cancer cells, appears to have retained the capacity to respond to exogenous EGF. This might be due to lower secretion of bioactive EGF-like peptides compared to the androgen-insensitive DU 145 cell line; a hypothesis which could be tested by a direct comparison of the levels produced.

The relationship between EGF receptor expression and TGFα production is not as simple as previously thought. Several studies have demonstrated an inverse correlation between the levels of EGF receptor expressed and the production of TGFαs. These studies have suggested that increased secretion of TGFs is accompanied by a down regulation in receptor number. Due to the finding with prostate cancer cell lines of a positive correlation between EGF receptor expression and the production of TGFαs it is clear that many more cell lines must be investigated before a clear picture emerges.

One of the disadvantages of *in vitro* studies is that they can never adequately simulate the *in vivo* environment. Therefore studies using *in vivo* models like PC-82 and the Dunning tumour model are necessary to accompany *in vitro* studies. Moreover, the limited number of *in vitro* prostate cell lines available for studying prostatic cancer severely limits the amount of information that can be accumulated. This is reflected in the picture that is evolving for breast cancer which suggests that there is no simple model for the transition to an endocrine-unresponsive state.

The use of primary culture of prostate cells is also necessary because the phenotype of *in vitro* cell lines might have changed over the time these cell lines have been in

culture. More importantly primary culture enables the study of paracrine interactions between different cell types.

Finally, the hypothesis that an alteration in the activity of the growth factor or its' receptor is responsible for progression of androgen-dependent to independent growth could be directly examined. This may be carried out by the use of a cell line, such as LNCaP "transformed" to an androgen-independent state by drug selection or oncogene transfection.

REFERENCES CITED

Anzano, M. A., Roberts, A. B., Smith, J. M., Sporn, M. B. & DeLarco, J. E. (1982). Sarcoma growth factor from conditioned media of virally transformed cells is composed of both type α and β transforming growth factors. *Proc. Natl. Acad. Sci. USA* **80**, 6264-6268.

Banks-Schlegel, S. P. & Quintero, J. (1986). Human oesophageal carcinoma cells have fewer, but higher affinity epidermal growth factor receptors. *J. Biol. Chem.* **261**, 4359-4362.

Barbacid, M. & Lauver, A. V. (1981). Gene products of McDonough feline sarcoma virus have *in vitro*-associated protein kinase that phosphorylates tyrosine residues: Lack of detection of this enzymatic activity *in vivo*. *J. Virol.* **40**, 812-821.

Bargmann, C. L., Hung, M-C. & Weinberg, R. A. (1986). The *neu* oncogene encodes an epidermal growth factor receptor-related protein. *Nature* **319**, 226-230.

Barnes, D. & Sato, G. (1980). Serum-free cell culture: a unifying approach. *Cell* **22**, 649-655.

Barnes, D. W. (1982). Epidermal growth factor inhibits growth of A-431 human epidermoid carcinoma in serum-free cell culture. *J. Cell Biol.* **93**, 1-4.

Bartsch, W., Krieg, M., Becker, H., Mohrmann, J. & Voigt, K. D. (1982). Endogenous androgen levels in epithelium and stroma of human benign prostatic hyperplasia and normal tissues. *Acta. Endocrinol.* **100**, 634-640.

Bascom, C. C., Sipes, N. J., Coffey, R. J. & Moses, H. L. (1989). Regulation of epithelial cell proliferation by transforming growth factors. *J. Cell. Biochem.* **39**, 25-33.

Bauknecht, T., Kiechle, M., Bauer, G. & Siebers, J. W. (1986). Characterization of growth factors in human ovarian carcinomas. *Cancer Res.* **46**, 2614-2618.

Bennett, J. P., Jr. & Yamamura, H. I. (1985). Neurotransmitter, hormone, or drug receptor binding methods. Neurotransmitter receptor binding. 2nd Ed., pp. 61-89. Raven Press.

Bishop, J. M (1983). Cellular oncogenes and retroviruses. *A. Rev. Biochem.* **52**, 301-354.

Bjoroson, C. L. & Moses, H. L. (1980). Fetal bovine serum contains "Transforming growth factor". *Proc. of ICN-UCLA Meeting on Mechanism of Chemical Carcinogenesis*. Abst. **648**, 235.

Blacklock, N. J. (1982). The development and morphology of the prostate. In: *The Endocrinology of Prostate Tumours* (Ed. R. Ghanadian). pp. 1-15, MTP Press Ltd., London.

Boonstra, J., De Laat, S. W. & Ponec, M. (1985). Epidermal growth factor receptor expression related to differentiation capacity in normal and transformed keratinocytes. *Exp. Cell Res.* **161**, 421-433.

Bouffioux, C. (1984). Endocrinological aspects of aetiology and epidemiology in prostatic tumours. Advances in Urological Oncology and Endocrinology. Aeta Medica. Edizioni e Congressi, 122-129.

Bradford, M. M. (1976). A rapid and sensitive method for the quantitation of microgram amounts of protein utilising the principle of protein dye binding. *Analytical Chemistry* **72**, 248-254.

Bradshaw, R. A. (1978). Nerve growth factor. *Ann. Rev. Biochem.* **47**, 191.

Bringman, T. S., Lindquist, P. B. & Derynck, R. (1987). Different transforming growth factor-alpha species are derived from a glycosylated and palmitoylated transmembrane precursor. *Cell* **48**, 429-440.

Brinkmann, A. O., Bolt, J., van Steenbrugge, G. J., Kuiper, G. G. J. M., de Boer, W. & Mulder, E. (1987). Characterization of androgen receptors in a transplantable human prostatic adenocarcinoma (PC-82). *The Prostate* **10**, 133-143.

Burk, R. R. (1976). Induction of cell proliferation by a migration factor released from a transformed cell line. *Expt. Cell. Res.* **101**, 293-298.

Buss, J. E., Kudlow, J. E., Lazar, C. S. & Gill, G. N. (1982). Altered epidermal growth factor (EGF)-stimulated protein kinase activity in variant A431 cells with altered growth responses to EGF. *Proc. Natl. Acad. Sci. USA* **79**, 2574-2578.

Byyny, R. L., Orth, D. N., Cohen, S. & Doyne, E. S. (1974). Epidermal growth factor; effects of androgens and andrenergic agents. *Endocrinology* **95**, 776-782.

Carlin, C. R., Simon, D., Mattison, J. & Knowles, B. B. (1988). Expression and biosynthetic variation of the epidermal growth factor receptor in human hepatocellular carcinoma-derived cell lines. *Mol. & Cell. Biology* **8**, 25-34.

Carpenter, G. & Cohen, S. (1975). Human epidermal growth factor and the proliferation of human fibroblasts. *J. Cell. Physiol.* **88**, 227-238.

Carpenter, G., Lembach, K, J., Morrison, M. M. & Cohen, S. (1975). Characterization of the binding of ^{125}I-labelled epidermal growth factor to human fibroblasts. *J. Biol. Chem.* **250**, 4297-4304.

Carpenter, G. & Cohen, S. (1976). ^{125}I-labelled human epidermal growth factor: Binding and internalization of epidermal growth factor in human carcinoma cells A-431. *J. Cell. Biol.* **71**, 159-171.

Carpenter. G. & Cohen, S. (1978). In: *Biochemical actions of hormones.* (Ed. G. Litwack). **5**, pp. 203-247. Academic, N. Y.

Carpenter, G., King, L. & Cohen, S. (1978). Epidermal growth factor stimulates phosphorylation in membrane preparations *in vitro*. *Nature* **276**, 409-410.

Carpenter, G. & Cohen, S. (1979). Epidermal growth factor. *Annu. Rev. Biochem.* **48**, 193-216.

Carpenter, G., King, L. & Cohen, S. (1979). Rapid enhancement of protein phosphorylation in A-431 cell membrane preparations by epidermal growth factor. *J. Biol. Chem.* **254**, 4884-4891.

Carpenter, G., Stoscheck, C. M., Preston, Y. A. & DeLarco, J. E. (1983). Antibodies to the epidermal growth factor receptor block the biological activities of sarcoma growth factor. *Proc. Natl. Acad. Sci. USA* **80**, 5627-5630.

Carpenter, G. (1985). Binding assays for epidermal growth factor. In: *Methods in Enzymology*. **109**, pp. 101-109. Academic Press, Inc.

Chang, C., Kokontis, J. & Liao, S. (1988). Molecular cloning of human and rat complementary DNA encoding androgen receptors. *Science* **240**, 324.

Chaproniere, D. M. & McKeehan, W. L. (1986). Serial culture of single adult human prostatic epithelial cells in serum-free medium containing low calcium and a new growth factor from bovine brain. *Cancer Res.* **46**, 819-824.

Chen, L. B., Gudor, R. C., Sur, T. T., Chen, A. B. & Mosesson, M. W. (1977). Control of a cell surface major glycoprotein by epidermal growth factor. *Science* **197**, 776-778.

Cherington, P. V., Smith, B. L. & Pardee, A. (1979). Loss of epidermal growth factor requirement and malignant transformation. *Proc. Natl. Acad. Sci. USA* **76**, 3937-3941.

Childs, C. B., Proper, J. A., Tucker, R. F. & Moses, H. L. (1982). Serum contains a platelet-derived transforming growth factor. *Proc. Natl. Acad. Sci. USA* **79**, 5312-5316.

Chinkers, M. J., McKanna, J. A. & Cohen, S. (1979). Rapid induction of morphological changes in human carcinoma cell line A-431 by epidermal growth factor. *J. Cell. Biol.* **83**, 260-265.

Chinkers, M. J., McKanna, J. A. & Cohen, S. (1981). Rapid rounding of human epidermoid carcinoma cells A-431 induced by epidermal growth factor. *J. Cell. Biol.* **88**, 422-429.

Clark, G. M. & McGuire, W. L. (1989). The clinical usefulness of estrogen-receptor and other markers of hormone dependence. *Proc. Roy. Soc. Edin.* **95B**, 145-150.

Clarke, R., Brünner, N., Katz, D., Glanz, P., Dickson, R. B., Lippman, M. E. & Kern, F. G. (1989). The effects of a constitutive expression of transforming growth factor-alpha on the growth of MCF-7 human breast cancer cells *in vitro* and *in vivo*. *Mol. Endocrin.* **3**, 372-380.

Coffey, D. S. & Issacs, J. T. (1981). Control of prostate growth. *Urology* **17**, 17-24.

Coffey, R. J, Jr., Shipley, G. D. & Moses, H. L. (1986). Production of transforming growth factors by human colon cancer lines. *Cancer Res.* **46**, 1164-1169.

Coffey, R. J, Jr., Goustin, A. S., Soderquist, A. M., Shipley, G. D., Wolfshohl, J., Carpenter, G. & Moses, H. L. (1987). Transforming growth factor α and β expression in human colon cancer lines: Implications for an autocrine model. *Cancer Res.* **47**, 4590-4594.

Cohen, S. (1962). Isolation of a mouse submaxillary gland protein accelerating incisor eruption and eyelid opening in the newborn animal. *J. Biol. Chem.* **237**, 1555-1562.

Cohen, S. & Carpenter, G. (1975). Human epidermal growth factor: isolation and chemical and biological properties. *Proc. Natl. Acad. Sci. USA* **72**, 1317-1321.

Cohen, S., Ushiro, H., Stoscheck, C. & Chinkers, M. (1982). A native 170,000 epidermal growth factor receptor kinase complex from plasma membrane vesicles. *J. Biol. Chem.* **257**, 1523-1531.

Connolly, J. M. & Rose, D. P. (1989). Secretion of epidermal growth factor and related polypeptides by the DU 145 human prostate cancer cell line. *The Prostate* **15**, 177-186.

Cooper, G. M. (1982). Cellular transforming genes and oncogenes. *Science* **217**, 801-806.

Cooper, J. A., Bowen-Pope, D., Raines, E., Ross, R. & Hunter, T. (1982). Similar effects of platelet-derived growth factor and epidermal growth factor on the phosphorylation of tyrosine in cellular proteins. *Cell* **31**, 263-275.

Costlow, M. E. & Hample, A. (1982). Prolactin receptors in cultured rat mammary tumor cells: unmasking of cell surface receptors by energy depletion. *J. Biol. Chem.* **257**, 6971-6977.

Culouscou, J-M., Remacle-Bonnet, M., Garrouste, F., Marvaldi, J. & Pommier, G. (1987). Simultaneous production of IGF-I and EGF competing growth factors by HT-29 human colon cancer line. *Int. J. Cancer* **40**, 646-652.

Cunha, G. R. (1972). Tissue interactions between epithelium and mesenchyme of urogenital and integumental origin. *Anat. Rec.* **172**, 529-542.

Cunha, G. R. (1973). The role of androgens in the epithelio-mesenchymal interactions involved in prostatic morphogenesis in embryonic mice. *Anat. Rec.* **175**, 87-96.

Cunha, G. R., Chung, L. W. K., Shannon, J. M. & Reese, B. A. (1980). Stromal-epithelial interactions in sex differentiation. *Biol. Reprod.* **22**, 19-42.

Cunha, G. R., Sugimura, Y. & Bigsby, R. M. (1985). Androgenic response in the prostate: role of stromal-epithelial interactions. In *Regulation of Androgen Action.* (Eds N. Bruchovsky, A. Chapdelaine, F. Neumann). pp. 191-197. Congressdruck R. Bruckner, Berlin.

Cunha, G. R., Donjacour, A. A., Cooke, P. S., Mee, S., Bigsby, R. M., Higgins, S. J. & Sugimura, Y. (1987). The endocrinology and developmental biology of the prostate. Endocrine Factors. *Endocrinol. Rev.* **8**, 338.

Dailey, G. E., Krause, J. W. & Orth, D. N. (1978). Homologous radioimmunoassay for human epidermal growth factor (urogastrone). *J. Clin. Endocrinol. Metab.* **48**, 929-936.

Das, M., Miyakawa, T., Fox, C. F., Pruss, R. M., Aharonov, A. & Herschman, H. R. (1977). Specific radiolabelling of a cell surface receptor for epidermal growth factor. *Proc. Natl. Acad. Sci. USA* **74**, 2790-2794.

David, K., Dingemanse, E., Freud, J. & Laquer, E. (1935). Uber Krystallinisches mannliches hormon aus hoden (testosteron), Wirksamer als aus harn oder aus cholesterin bereittes androsteron. *Hoppe-Syel, Z. Physiol. Chem. Phys* **233**, 281.

Davidson, N. E., Gelmann, E. P., Lippman, M. E. & Dickson, R. B. (1987). Epidermal growth factor gene expression in estrogen receptor-positive and negative human breast cancer cell lines. *Mol. Endocrinol.* **1**, 216-223.

Davis, R. J. & Czech, M. P. (1985). Tumor promoting phorbol diesters cause the phosphorylation of epidermal growth factor receptors in normal human fibroblasts at threonine-654. *Proc. Natl. Acad. Sci. USA* **82**, 1974-1978.

DeLarco, J. E. & Todaro, G. J. (1978). Growth factors from murine sarcoma virus-transformed cells. *Proc. Natl. Acad. Sci. USA* **75**, 4001-4005.

DeLean, A., Munson, P. J. & Rodbard, D. (1978). Simultaneous analysis of families of sigmoidal curves: Application to bioassay, radioligand assay, and physiological dose-response curves. *Amer. J. Physiol.* **235**, E97-E102.

De Marco, E., Pierce, J. H., Fleming, T. P., Kraus, M. H., Molloy, C. J., Aaronson, S. A. & Di Fiore, P. P. (1989). Autocrine interaction between TGFα and the EGF-receptor: quantitative requirements for induction of the malignant phenotype. *Oncogene* **4**, 831-838.

Derynck, R., Roberts, A. B., Winkler, M. E., Chen, E. Y. & Goedeel, D. V. (1984). Human transforming growth factor-α: Precursor structure and expression in E. *coli*. *Cell* **38**, 287-297.

Deuel, T. F. & Huang, J. S. (1983). Platelet-derived growth factor: purification, properties and biological activities. *Progress in Hematology* **13**, 201-221.

Dickson, R. B., Huff, K.K., Spencer, E. M. & Lippman, M. E. (1986). Induction of epidermal growth factor-related polypeptides by 17β-estradiol in MCF-7 human breast cancer cells. *Endocrinology* **118**, 138-142.

Doolittle, R. F., Hunkapillar, M. W., Hood, L. E., Devare, S. G., Robbins, K. C., Aaronson, S. A. & Antoniades, H. N. (1983). Simian sarcoma virus *onc* gene, *v-sis*, is derived from the gene (or genes), encoding a platelet-derived growth factor. *Science (Washington)* **221**, 275-277.

Downward, J., Parker, P. & Waterfield, M. D. (1984). Autophosphorylation sites on the epidermal growth factor receptor. *Nature* **311**, 483-485.

Downward, J., Yarden, Y., Mayes, E., Scrace, G., Totty, N., Stockwell, P., Ullrich, A., Schlessinger, J. & Waterfield, M. D. (1984). Close similarity of epidermal growth factor receptor and v-erb B oncogene protein sequences. *Nature* **307**, 521-527.

Drebin, J. A., Stern, D. F., Link, V. C., Weinberg, R. A. & Greene, M. I. (1984). Monoclonal antibodies identify a cell-surface antigen associated with an activated cellular oncogene. *Nature* **312**, 545-548.

Drebin, J. A., Link, V. C., Stern, D. F., Weinberg, R. A. & Greene, M. I. (1985). Down-modulation of an oncogene protein product and reversion of the transformed phenotype by monoclonal antibodies. *Cell* **41**, 695-706.

Duesberg, P. H. (1985). Activated proto-oncogenes: Sufficient or necessary for cancer? *Science* **228**, 665-677.

Dulak, N. C. & Temin, H. M. (1973). A partially purified polypeptide fraction from rat liver cell conditioned medium with multiplication-stimulating activity for embryo fibroblasts. *J. Cell. Physiol.* **81**, 153-160.

Dulbecco, R. (1970). Topoinhibiton and serum requirement of transformed and untransformed cells. *Nature* **227**, 802-806.

Dunning, W. F. (1963). Prostate cancer in the rat. *Natl. Cancer. Inst. Monograph* **12**, 351-369.

Eaton, C. L., Davies, P. & Phillips, M. E. A. (1988). Growth factor involvement and oncogene expression in prostate tumours. *J. Ster. Biochem.* **30**, 341-345.

Eaton, C. L., France, T. D., Harper, M. E. & Davies, P. (1989). Intermodulation of growth factor and steroid hormone actions in normal and neoplastic prostatic cell lines. *Urological Res.* **17**, 347.

Ek, B. & Heldin, C-H. (1986). Specific cleavage of the fibroblast receptor for platelet-derived growth factor by an endogenous Ca^{2+} dependent thiol protease. *Eur. J. Biochem.* **155**, 409-413.

El Etreby, M. P. & Mahrous, A. T. (1979). Immunocytochemical technique for detection of prolactin (PRL) and growth hormone (GH) in hyperplastic and neoplastic lesions of dog prostate and mammary gland. *Histochemistry* **64**, 279-286.

Elson, S. D., Browne, C. A. & Thorburn, G. D. (1984). Identification of epidermal growth factor-like activity in human male reproductive tissues and fluids. *J. Clin. Endocrinol. Metab.* **58**, 589-594.

Engström, W. (1986). Differential effects of epidermal growth factor (EGF) on cell locomotion and cell proliferation in a cloned human embryonal carcinoma-derived cell line *in vitro*. *J. Cell. Sci.* **86**, 47-55.

Fabricant, R. N., DeLarco, J. E. & Todaro, G. J. (1977). Nerve growth factor receptors on human melanoma cells in culture. *Proc. Natl. Acad. Sci. USA.* **75**, 565-569.

Farnsworth. W. E., Slaunwhite, W. R., Jr., Sharma, M., Oseko, F., Brown, J. R., Gonder, M. J. & Cartagena, R. (1981). Interaction of prolactin and testosterone in human prostate. *Urol. Res.* **9**, 79.

Fernandez-Pol, J. A., Klos, D. J. & Grant, G. A. (1986). Purification and biological properties of type α transforming growth factor from mouse transformed cells. *Cancer Res.* **46**, 5153-5161.

Fitzpatrick, S. L., LaChance, M. P. & Schultz, G. S. (1984). Characterization of epidermal growth factor receptor and action on human breast cancer cells in culture. *Cancer Res.* **44**, 3442-3447.

Franks, L. M. (1954). Latent carcinoma of the prostate. *J. Pathol. Bact* **68**, 603.

Franks, L. M., Riddle, P. N., Carbonell, A. W. & Gey, G. O. (1970). A comparative study of the ultrastructure and lack of growth capacity of adult human prostate epithelium mechanically separated from its stroma. *J. Pathol.* **100**, 113-119.

Friedman, B-A. & Rosner, M. R. (1987). Growth factors modify the epidermal growth factor receptor through multiple pathways *J. Cell. Biochem.* **34**, 1-11.

Gates, R. E. & King, L. E., Jr. (1982). The EGF receptor-kinase has multiple phosphorylation sites. *Biochem. Biophys. Res. Commun.* **105**, 57-66.

Ghanadian, R. (1982). Hormonal environment of the normal prostate. In *The Endocrinology of Prostate Tumours*. (Ed. R Ghanadian). p. 35-57. Lancaster: M. T. P. Press.

Gill, G. N. & Lazar, C. S. (1981). Increased phosphotyrosine content and inhibition of proliferation in EGF-treated A-431 cells. *Nature* **293**, 305-307.

Gilmore, T., DeClue, J. E. & Martin, G. S. (1985). Protein phosphorylation at tyrosine is induced by the *v-erb* B gene product in vivo and in vitro. *Cell* **40**, 609-618.

Gleason, D. F. (1977). Histological grading and clinical staging of prostatic carcinoma. In *Urologic Pathology: The prostate*. (Ed. M. Tannenbaum). pp. 171-197. Lea & Febiger, Philadelphia.

Gorden, P., Carpenti, J. L., Cohen, S. & Orci, L. (1978). Epidermal growth factor: Morphological demonstration of binding internalization and lysosomal association in human fibroblasts. *Proc. Natl. Acad. Sci. USA* **75**, 5025-5029.

Gospodarowicz, D., Vlodavsky, I., Greenberg, G. & Johnson, L. K. (1979). Cellular shape is determined by the extracellular matrix and is responsible for the control of cellular growth and function. In: *Hormones and Cell Culture*. (Eds R. Rose & G. Sato). pp. 561-592. New York: Cold Spring Harbor.

Gower, D. B. (1979). *Steroid Hormones*, p. 69. London: Cromm Helm.

Gray, A., Dull, T. J. & Ullrich, A. (1983). Nucleotide sequence of epidermal growth factor cDNA predicts a 128,000-molecular weight protein precursor. *Nature* **303**, 722-725.

Green, S., Walter, P., Kumar, V., Krust, A., Bornert, J. M., Argos, P. & Chambon, P. (1986b). Human oestrogen receptor cDNA: Sequence, expression and homology to *v-erb*-A. *Nature* **320**, 134-139.

Gregory, H. (1975). Isolation and structure of urogastrone and its relationship to epidermal growth factor. *Annual Review of Biochemistry* **48**, 193-216.

Gregory, H. Willshire, I. R., Kavanagh, J. P., Blacklock, N. J., Chowdury, S. & Richards, R. C., (1986). Urogastrone-epidermal growth factor concentrations in prostatic fluid of normal individuals and patients with benign prostatic hypertrophy. *Clinical Science* **70**, 359-363.

Griffiths, K., Davies, P., Eaton, C. L., Harper, M. E., Peeling, W. B., Turkes, A. O., Turkes, A., Wilson, D. W. & Pierrepoint, C. G. (1987). Cancer of the prostate: endocrine factors. In: *Oxford Reviews of Reproductive Biology* (Ed. J. R. Clarke). **9**, pp. 192-259, Oxford Publ.

Gullick, W. J., Downward, J. & Waterfield, M. D. (1985). Antibodies to the autophosphorylation sites of the epidermal growth factor protein-kinase as probes of structure and function. *EMBO J.* **4**, 2869-2877.

Gullick, W. J., Marsden, J. J., Whittle, N., Ward, B., Bobrow, L. Waterfield, M. D. (1986). Expression of epidermal growth factor receptors on human cervical, ovarian and vulval carcinomas. *Cancer Res.* **46**, 285-292.

Guyton, A. C. (1981). In: *Textbook of Medical Physiology*, 6th Edition (Ed. A. Guyton). pp. 994. Saunders & Co., London.

Halper, J. & Moses, H. L. (1983). Epithelial tissue-derived growth factor-like polypeptides. *Cancer Res.* **43**, 1972-1979.

Ham, R. G. (1981). *Handbook of Experimental Pharmacology* . pp. 13-88. **57**, Springer, Berlin.

Harlow, E. & Lane, D. (1988). Antibodies a Laboratory Manual. Cold Spring Harbor Laboratory.

Harris, A. L. & Nicholson, S. (1988). In: *Endocrine Relationships in Breast Cancer*, (Ed. B. A. Stoll) pp. 104-143. Heinemann: London.

Harrison, J. & Auersperg, N. (1981). Epidermal growth factor enhances viral transformation of granulosa cells. *Science* **213**, 218-219.

Häder, M., Stach-Machado, D., Plüger, K-H., Rotsch, M., Heimann, B., Moelling, K. & Havemann, K. (1987). Epidermal growth factor receptor expression, proliferation, and colony stimulating activity production in the urinary bladder carcinoma cell line 5637. *J. Cancer Res. Clin. Oncol.* **113**, 579-585.

Headon, D. R. & Allan, G. (1989). Steroid hormones and growth factor receptors. *Biochem Soc. Trans.* **17**, 598-599.

Heatfield, B. M. (1987). *In vitro* models: Organ explant culture of normal and neoplastic human prostate. In: *Current concepts and approaches to the study of prostate cancer*. (Eds D. S. Coffey, N. Bruchovsky, W. A. Jr. Gardner, M. I. Resnick, J. P. Karr). p. 433. Alan R. Liss, NY.

Heldin, C. H. & Westermark, B. (1984). Growth factors: mechanism of action and relation to oncogenes. *Cell* **37**, 9-20.

Hendler, F. J. & Ozanne, B. W. (1984). Human squamous cell lung cancers express increased epidermal growth factor receptors. *J. Clin. Invest.* **74**, 647-651.

Hill, D. J. (1989). Growth factors and their cellular actions. *J. Repro. Fertil.* **85**, 723-734.

Hiraki, V., Inoue, H., Kato, Y., Fukuya, M. & Susuki, F. (1987). Combined effects of somatomedin-like growth factors with fibroblast growth factor or epidermal growth factor in DNA synthesis in rabbit chondrocytes. *Mol. Cell. Biochem.* **76**, 185.

Hirata, Y. & Orth, D. N. (1979). Epidermal growth factor (urogastrone) in human fluids: size heterogeneity. *J. Clin. Endocrinol. Metab.* **48**, 673-679.

Hirata, Y., Uchihashi, M., Fujuta, T., Matsukura, S., Motoyama, T., Kaku, M. & Koshimizu, K. (1983). Characteristics of specific binding of epidermal growth factor (EGF) on human tumor cell lines. *Endocrinol. Jpn.* **30**, 601-607.

Hoehn, W., Schroeder, F. H., Riemann, J. F., Joebsis, A. C. Hermanek, P. (1980). Human prostatic adenocarcinoma: some characteristics of a serially transplantable line in nude mice (PC 82). *Prostate* **1**, 95-104.

Hollenberg, M. D. & Cuatrecasas, P. (1973). Epidermal growth factor receptors in human fibroblasts and modulation of action by cholera toxin. *Proc. Natl. Acad. Sci. USA* **70**, 2964-2968.

Hollenberg, M. D. (1986). Mechanisms of receptor-mediated transmembrane signalling. *Experientia* **42**, 718-727.

Holley, R. W. & Kiernan, J. A. (1971). Studies of serum factors required by 3T3 cells and SV3T3 cells. In: *Growth Control in Cell Cultures*. (Eds G. E. W. Wolsenholme & J. Knight). pp. 3-16. London: Churchill Livingston.

Holley, R. W. & Kiernan, J. A. (1974). Control of the initiation of DNA synthesis in 3T3 cells: serum factors. *Proc. Natl. Acad. Sci. USA.* **71**, 2908-2911.

Holley, R. W., Armour, R., Baldwin, J. H., Brown, K. D. & Yeh, Y-C. (1977). Density-dependent regulation of growth of BSC-1 cells in cell culture: control of growth by serum factors. *Proc. Natl. Acad. Sci. USA* **74**, 5046-5050.

Holley, R. W., Böhlen, P., Fava, R., Baldwin, J. H., Kleeman, G. & Armour, R. (1980). Purification of kidney epithelial cell growth inhibitors. *Proc. Natl. Acad. Sci. USA* **77**, 5989-5992.

Horoszewicz, J. S., Leong, S. S., Kawinski, E., Karr, J. P., Rosenthal, H., Chu, T. M., Mirand, E. A. & Murphy, G. P. (1983). LNCaP model of human prostatic carcinoma. *Cancer Res.* **43**, 1809-1818.

Hosoi, K., Tanaka, I. & Ueha, T. (1981). Induction of epidermal growth factor by tri-iodo-L-thyronine in the submandibular glands of mice with testicular feminization. *J. Biochem* **90**, 267-272.

Houston, B., Chisholm, G. D. & Habib, F. K. (1985). Evidence that human prostatic 5α-reductase is located exclusively in the nucleus. *Febs Letters* **185**, 231-234.

Huggins, C. & Hodges, C. V. (1941). Studies on prostatic cancer. The effects of castration, of estrogen and of androgen injection on serum phosphatases in metastatic carcinoma of the prostate. *Cancer Res.* **1**, 293-297.

Huggins, C. (1945). The physiology of the prostate gland. *Physiol. Rev.* **25**, 281.

Huggins, C. & Russell, P. S. (1946). Quantitiative effects of hypophysectomy on testis and prostate of dogs. *Endocrinology* **39**, 1-7.

Hunter, J. (1786). Observations on certain parts of the animal oeconomy, 1st ed., Bibliotheca Osteriana. pp. 38-39. London.

Hunter, T., Ling, N. & Cooper, J. A. (1984). Protein kinase C phosphorylation of the EGF receptor at a threonine residue close to the cytoplasmic face of the plasma membrane. *Nature* **311**, 480-483.

Hunter, T. & Cooper, J. H. (1985). Protein-tyrosine kinases. *Annu. Rev. Biochem.* **54**, 897-930.

Ibbotson, K. J., Harrod, J., Gowen, M., D'Souza, S., Smith, D. D., Winkler, M. E., Derynck, R. & Mundy, G. R. (1986). Human recombinant transforming growth factor stimulates bone resorption and inhibits formation *in vitro*. *Proc. Natl. Acad. Sci. USA* **83**, 2228-2232.

Inman, W. H. & Carpenter, G. (1986). The receptor for epidermal growth factor. In: *Development and Receptors of the transformed cell.* (Eds M. O. Greene & T. Hamaoka). pp. 111-123. Plenum Pub. Corp. (N.Y.).

Ito, Y. Z., Mashimo, S., Nakazato, Y. & Takikawa, H. (1985). Hormone dependency of a serially transplantable human prostatic cancer (Honda) in nude mice. *Cancer Res.* **45**, 5058-5063.

Jacobi, G. H., Sinterhauf, K., Kurth, K. H. & Altwein, J. E. (1978). Bromocriptine and prostatic carcinoma: plasma kinetics, production and tissue uptake of ^{3}H-testosterone in vivo. *J. Urol.* **119**, 240.

Jacobs, S.C., Pinka, D. & Lawson, R. K. (1979). Prostatic osteoblastic factor. *Invest. Urol.* **17**, 195-199.

Jacobs, S. C. & Lawson, R. K. (1980). Mitogenic factors in human prostate extracts. *Urology* **16**, 488-493.

James, R. & Bradshaw, R. A. (1984). Polypeptide growth factors. *Ann. Rev. Biochem.* **53**, 259-292.

Jantzen, H-M., Strahle, U., Gloss, B., Stewart, F., Schmid, W., Boshart, M., Miksicek, R. & Schutz, G. (1987). Cooperativity of glucocorticoid response elements located far upstream of the tyrosine aminotransferase gene. *Cell* **49**, 29-38.

Jinno, H., Ueda, K., Otazuro, K., Kato, T. & Ito, J. (1986). Prostate growth factor in extracts of benign prostatic hypertrophy. *Eur. Urol.* **12**, 41-48.

Kaighn, M. E., Narayan, K. S., Ohnuki, Y., Lechner, J. F. & Jones, L.W. (1979). Establishment and characterization of a human prostate carcinoma cell line (PC-3). *Invest. Urol.* **17**, 16-23.

Kaighn, M. E., Lechner, J. F., Babcock, M. S., Marnell, M., Ohnuki, Y. & Narayan, K. S. (1980). The Pasadena cell lines. In: *Models for Prostate Cancer.* (Ed G. P. Murphy). pp. 85-109. Alan R. Liss, N. Y, Inc.

Kaighn, M. D., Kirk, D., Szalay, M. & Lechner, J. F. (1981). Growth control of prostatic carcinoma cells in serum-free media: Interrelationship of hormone response, cell density, and nutrient media. *Proc. Natl. Acad. Sci. USA* **78**, 5673-5676.

Kaplan, P. L., Anderson, M. & Ozanne, B. (1982). Transforming growth factor production enables cells to grow in the absence of serum: an autocrine system. *Proc. Natl. Acad. Sci. USA* **79**, 485-489.

Kasid, A., Lippman, M. E., Papageorge, A. G., Lowy, D. R. & Gelmann, E. P. (1985). Transfection of v-ras H DNA into MCF-7 human breast cancer cells bypasses dependence on estrogen for tumorigenicity. *Science* **228**, 725-728.

Kasselberg, A. G., Orth, D. N., Gray, M. E. & Stahlman, M. T. (1985). Immunocytochemical localization of human epidermal growth factor/urogastrone in several human tissues. *J. Histochem. Cytochem.* **33**, 315-322.

Kawamoto, T., Sato, J. D., Le, A, Polikoff, J., Sato, G. M. & Mendelsohn, J. (1983). Growth stimulation of A-431 cells by epidermal growth factor: identification of high-affinity receptors for epidermal growth factor by an anti-receptor monoclonal antibody. *Proc. Natl. Acad. Sci. USA* **80**, 1337-1341.

King, A. C. & Cuatrecasas, P. (1982). Resolution of high and low affinity growth factor receptors. *J. Biol. Chem.* **257**, 3053-3060.

King, L. E., Jr., Carpenter, G. & Cohen, S. (1980). Characterization by electrophoresis of epidermal growth factor stimulated phosphorylation using A-431 membranes. *Biochemistry* **19**, 1524-1528.

King, R. J. B. (1990). Receptors, growth factors and steroid insensitivity of tumours. *J. Endocrin.* **124**, 179-181.

Klein, G. & Klein, E. (1985). Evolution of tumours and the impact of molecular oncology. *Nature* **315**, 190-195.

Knabbe, C., Lippman, M. E., Wakefield, L. M., Flanders, K. C., Kasid, A., Derynck, R. & Dickson, R. B. (1987). Evidence that transforming growth factor-β is a hormonally regulated negative growth factor in human breast cancer cells. *Cell* **48**, 417-428.

Korc, M., Haussler, C. A. & Trookman, N. S. (1987). Divergent effects of epidermal growth factor and transforming growth factors on a human endometrial carcinoma cell line. *Cancer Res.* **47**, 4909-4914.

Kozlowski, J. M., Fidler, I. J., Campbell, D., Xu, Z., Kaighn, M. E. & Hart, I. R. (1984). *Cancer Res.* **44**, 3522-3529.

Krieg, M., Klotzl, J., Kaufmann, J. & Voigt, K. D. (1981). Stroma of human prostatic hyperplasia: preferential tissue for androgen metabolism and oestrogen binding. *Acta Endocrinol.* **96**, 422-432.

Kris, R. M., Lax, I., Gullick, W., Waterfield, M. D., Ullrich, A., Fridkin, M. & Schlessi, J. (1985). Antibodies against a synthetic peptide as a probe for the kinase activity of the avian EGF receptor and *v-erb* B protein. *Cell* **40**, 619-625.

Labrie, F., DuPont, A., Belanger, A., St-Arnaud, R., Giguere, M., Lacourciere, Y., Emond, J. & Monfette, G. (1986). Treatment of prostatic cancer with gonadotrophin-releasing hormone agonist. *Endocr. Rev.* **7**, 67-74.

Laemmli, U. K. (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **227**, 680-685.

Lahtonen, R., Bolton, N. J., Konturri, M. & Vihko, K. D. (1982). Nucleur androgen receptors in the epithelium and stroma of human benign prostatic hypertrophic glands. *The Prostate* **4**, 129-139.

Lasnitzki, I. (1972). The effect of prolactin on the prostate and in organ culture. In *Prolactin and Carcinogenesis, Fourth Tenovus Workshop* (Eds A. R. Boyns and K Griffiths). pp. 200-206. Alpha Omega Publ.

Leake, A., Chisholm, G. D. & Habib, F. K. (1983). Characterization of the prolactin receptor in human prostate. *J. Endocrinol.* **99**, 321-328.

Lechner, J. F., Babcock, M. S., Marnell, M., Shankar, N. K. & Kaighn, M. E. (1980). In: *Methods in Cell Biology.* (Eds C. C. Harris., B. F. Trump. & G. D. Stoner). pp. 193-223. **21B**.

Leof, E. B., Van Wyk, J. J., O'Keefe, E. J. & Pledger, W. J. (1983). Epidermal growth factor (EGF) is required only during the traverse of early G_1 in PDGF stimulated density-arrested BALB/c-3T3 cells. *Exp. Cell. Res.* **147**, 202-208.

Liao, S., Liang, T., Fang, S., Castaneda, E. & Shao, T. C. (1973). Steroid structure and androgenic activity: Specificities involved in the receptor binding and nucleur retention of various androgens. *J. Biol. Chem.* **248**, 6154-6162.

Lippman, M. E., Dickson, R. B., Bates, S., Knabbe, C., Huff, K., Swain, S., McManaway, M., Bronzert, D., Kasid, A. & Gelmann, E. P. (1986). Autocrine and paracrine growth regulation of human breast cancer. *Breast Cancer Res. Treat.* **7**, 59-70.

Lippman, M. E., Dickson, R. B., Gelmann, E. P., Rosen, N., Knabbe, C., Bates, S., Brozert, D., Huff, K. & Kasid, A. (1987). Growth regulation of human breast carcinoma occurs through regulated growth factor secretion. *J. Cell. Biochem.* **35**, 1-16.

Liu, S. C., Sanfilippo, B., Perroteau, I., Derynck, R., Salomon, D.S. & Kidwell, W. R. (1987). Expression of transforming growth factor-alpha (TGF-alpha) in differentiated rat mammary tumors. Estrogen induction of TGF-alpha production. *Mol. Endocrin.* **1**, 683-692.

Livneh, E., Prywes, R., Kashles, O., Reiss, N., Sasson, I., Mory, Y., Ullrich, A. & Schlessinger, J. (1986). Reconstitution of human epidermal growth factor receptors and its deletion mutants in cultured hamster cells. *J. Biol. Chem.* **261**, 12490-12497.

Lloyd, J. W., Thoman, J. A. & Mawhinney, M. G. (1973). A difference in the *in vitro* accumulation and metabolism of testosterone-1, 2-^{3}H by the rat prostate gland following incubation with ovine or bovine prolactin. *Steroids* **22**, 473-483.

Lostroh, A. J. & Li, C. H. (1957). Stimulation of the sex accessories of hypophysectomised male rat by non-gonadotrophin hormones of the pituitary gland. *Acta endocr. Copenh.* **25**, 1-16.

Lubahn, D. B., Joseph, D. R., Sullivan, P. M., Willard, H. F., French, F. S. & Wilson, E. M. (1988). Cloning of human androgen receptor complementary DNA and localisation to the X-chromosome. *Science* **240**, 327-330.

Maddy, S. Q., Chisholm, G. D., Hawkins, R. A. Habib, F. K. (1987). The localization of epidermal growth factor receptor in the human prostate by biochemical and immunological methods. *J. Endocrinol.* **112**, 147.

Maddy, S. Q., Chisholm, G. D. & Habib, F. K. (1989). Epidermal growth factor receptors in human prostate cancer: correlation with histological differentiation of the tumour. *Br. J. Cancer.* **60**, 41-41.

Maehama, S., Li, D., Nanri, H., Leykam, J. F. & Deuel, T. F. (1986). Purification and partial characterization of prostate-derived growth factor. *Proc. Natl. Acad. Sci. USA.* **83**, 8162-8166.

Marger, R., Najita, L., Nichols, E. J., Hakomori, S. & Rohrschneider, L. (1984). Cell surface expression of the McDonough strain of feline sarcoma virus *fms* gene product (gp140 ^{fms}), *Cell* **39**, 227-337.

Marquardt, H. & Todaro, G. J. (1982). Human transforming growth factor production by a melanoma cell line, purification and initial characterization. *J. Biol.Chem.* **257**, 5220-5225.

Marquardt, H., Hunkapiller, M. W., Hood, L. E. & Todaro, G. J. (1984). Rat transforming growth factor type 1: Structure and relation to epidermal growth factor. *Science* **223**, 1079-1081.

Massague, J. (1983). Epidermal growth factor-like transforming growth factor. *J. Biol. Chem.* **258**, 13614-13620.

McKeehan, W. L., Adams, P. S. & Rossner, M. P. (1984). Direct mitogenic effects of insulin, epidermal growth factor, glucocorticoid, cholera toxin, unknown pituitary factors and possibly prolactin, but not androgen, on normal rat prostate epithelial cells in serum-free primary culture. *Cancer Res.* **44**, 1998-2010.

McNeal, J. E. (1972). The prostate and prostatic urethra: A morphologic synthesis. *Journal of Urology* **107**, 1008-1016.

McNeal, J. E. (1975). Structure and pathology of the prostate. In *Normal and Abnormal Growth of the Prostate.* (Ed. M. Goland). pp. 56-65. Charles C. Thoman, Springfield, Illinois.

Merchant, D. J. (1976). Prostatic tissue cell growth and assesement. *Seminars in Oncology.* **3**.

Merchant, D. J. (1987). Primary culture as *in vitro* model. In: *Current concepts and approaches to the study of prostate cancer.* (Eds D. S. Coffey, N. Bruchovsky, W. A. Jr. Gardner, M. I. Resnick, J. P. Karr). pp. 473-495. Alan R. Liss, NY.

Mickey, D. D., Stone, K. R., Wunderli, H., Mickey, G. & Paulson, D. F. (1980). Characterization of a human prostate adenocarcinoma cell line (DU 145) as a monolayer culture and as a solid tumour in athymic mice. In: *Models for Prostate Cancer.* (Ed. G. P. Murphy). pp. 67-84. 37. Alan R. Liss, Inc., N. Y.

Mori, K., Kurobe, M., Furukawa, S., Kubo, K. & Hayashi, K. (1986). Human breast cancer cells synthesize and secrete an EGF-like immunoreactive fraction in culture. *Biochem. Biophys. Res. Commun.* **136**, 300-305.

Moses, H. L., Proper, J. A., Volkenant, M. E., Wells, D. J. & Getz, M. J. (1978). Mechanism of growth arrest of chemically transformed cells in culture. *Cancer Res.* **38**, 2807-2812.

Moses, H. L. (1984). Growth factors and transforming growth factors: potential roles in cancer. *Proc. Am. Ass. Cancer Res.* 412-413.

Mukku, V. R. & Stancel, G. M. (1985). Regulation of epidermal growth factor receptor by estrogen. *J. Biol. Chem.* **260**, 9820-9824.

Munson, P. J. & Rodbard, D. (1980). LIGAND: A versatile computerised approach for characterisation of all ligand binding systems. *Analytical Biochem.* **107**, 220-239.

Murphy, L. J., Sutherland, R. L. & Lazarus, L. (1985). Regulation of growth hormone and epidermal growth factor receptors by progestins in breast cancer cells. *Biochem. Biophys. Res. Commun.* **131**, 767-773.

Murphy, L. J., Sutherland, R. L., Stead, B., Murphy, L. C. & Lazarus, L. (1986). Progestin regulation of epidermal growth factor receptor in human mammary carcinoma cells. *Cancer Res.* **46**, 728-734.

Neal, D. E., Marsh, C., Bennet, M. K., Hall, R. R., Abel, P. D. (1985). Epidermal-growth factor receptors in human bladder cancer: comparison of invasive and superficial tumours. *The Lancet* **i**, 16.

Nishi, N., Matuo, Y., Mugurama, Y., Yoshitake, Y., Nishikawa, K. & Wada, F. (1985). A human prostatic growth factor (hPGF): partial purification and characterization. *Biochem. Biophys. Res. Commun.* **132**, 1103-1109.

Nonomura, N., Nakamura, N., Uchida, N., Noguchi, S., Sato, B., Sonoda, T. & Matsumoto, K. (1988). Growth-stimulatory effect of androgen-induced autocrine growth factor(s) secreted from Shionogi carcinoma 115 cells on androgen-unresponsive cancer cells in a paracrine mechanism. *Cancer Res.* **48**, 4904-4908.

Odoma, S., Chisholm, D., Nicol, K. & Habib, F. K. (1985). Evidence for the association between blood prolactin and androgen receptors in BPH. *J. Urol.* **133**, 717-719.

Oesterling, J. E., Epstein, J. L. & Walsh, P. C. (1986). The inability of adrenal androgens to stimulate the adult human prostate: an autopsy evaluation of men with hypogonadotrophic hypogonadism and panhypopituitarism. *J. Urol.* **136**, 1030-1037.

Oka, Y. & Orth, D. N. (1983). Human plasma epidermal growth factor/ β-urogastrone is associated with blood platelets. *J. Clin. Invest.* **72**, 249-259.

Oka, Y., Chishan, F. K., Greene, H. L. & Orth, D. N. (1983). Effect of mouse epidermal growth factor/urogastrone on the functional maturation of rat intestine. *Endocrinol.* **112**, 940-944.

Orlowski, J., Bird, C. E. & Clark, A. F. (1983). Androgen 5α-reductase and 3α-hydroxysteroid dehydrogenase activities in the ventral prostate epithelial and stromal cells from immature and mature rats. *J. Endocrinol* **99**, 131-139.

Ozanne, B., Fulton, R. J. & Kaplan, P. L. (1980). Kirsten murine sarcoma virus transformed cell lines and a spontaneously transformed rat cell-line produce transforming factors. *J. Cell. Physiol.* **105**, 163.

Padhy, L. C., Shih, C., Cowing, D., Finkelstein, R. & Weinberg, R. A. (1982). Identification of a phosphoprotein specifically induced by the transforming DNA of rat neuroblastomas. *Cell* **28**, 865-871.

Paul, D., Lipton, A. & Klinger, I. (1971). Serum factor requirements of normal and simian virus-40 transformed 3T3 mouse fibroblasts. *Proc. Natl. Acad. Sci. USA* **68**, 645-648.

Peehl, D. M. & Stamey, T. A. (1986). Serum-free growth of adult human prostatic epithelial cells. *In Vitro Cell. Dev. Biol.* **22**, 82-89.

Perroteau, I., Salomon, D., DeBortolo, M., Kidwell, W., Hazarika, P., Pardue, R., Dedman, J. & Tam, J. (1986). Immunological detection and quantitation of alpha transforming growth factors in human breast carcinoma cells. *Breast Cancer Res. Treat.* **7**, 201-210.

Pike, L. J., Gallis, B., Casnellie, J. E., Bornstein, P. & Krebs, E. G. (1982). Epidermal growth factor stimulates the phosphorylation of synthetic tyrosine containing peptides by A-431 cell membrane. *Proc. Natl. Acad. Sci. USA* **79**, 1443-1447.

Proper, J. A. & Moses, H. L. (1981). Detection of "Transforming growth factor" in mouse embryos. *Proc. of ICN-UCLA Conference on Mechanism of Chemical Carcinogenesis*, Abst. **647**, 235.

Reid, L. M., Minato, N., Gresser, I., Holland, J., Kadish, A. & Bloom, B. R. (1981). Influence of anti-mouse interferon serum on the growth and metastasis of tumor cells persistently infected with virus and of human prostatic tumors in athymic nude mice. *Proc. Natl. Acad. Sci. USA* **78**, 1171-1175.

Rettenmier, C. W., Chen, J. H., Roussel, M. F. & Sherr, C. J. (1985). The product of the *c-fms* proto-oncogene: A glycoprotein with associated tyrosine kinase activity. *Science* **228**, 320-322.

Rheinwald, J. G. & Green, H. (1977). Epidermal growth factor and the multiplication of cultured human epidermal keratinocytes. *Nature* **265**, 421-424.

Riedel, H., Schlessinger, J. & Ullrich, A. (1987). A chimeric, ligand binding v-erb B/EGF receptor retains transforming activity. *Science* **236**, 197-200.

Rizzino, A., Kazakoff, P., Ruff, E., Kuszynski, C. & Nebelsick, J. (1988). Regulatory effects of cell density on the binding of transforming growth factor β, epidermal growth factor, platelet-derived growth factor, and fibroblast growth factor. *Cancer Res.* **48**, 4266-4271.

Robel, P. (1980). *Annals Clin. Res.* **12**, 216-222.

Roberts, A. B., Lamb, L. C., Newton, D. L., Sporn, M. B., DeLarco, J. E., *et al.* (1980). Transforming growth factors: Isolation of polypeptides from virally and chemically transformed cells by acids/ethanol extraction. *Proc. Natl. Acad. Sci. USA* **77**, 3494-3498.

Roberts, A. B., Frolik, C. A., Anzano, M. A. & Sporn, M. B. (1983). Transforming growth factors from neoplastic and nonneoplastic tissues. *Fed. Proc. Red. Am. Soc. Exp. Biol.* **42**, 2621-2626.

Rockwell, G. A., McClure, D. Sato, G. H. (1980). The growth requirements of SV40 virus-transformed Balb/c-3T3 cells in serum-free monolayer culture. *J. Cell. Physiol.* **103**, 323-331.

Rodbard, D., Lenox, R. H., Wray, H. L. & Ramseth, D. (1976). Statistical characterization of the random errors in the radioimmunoassay dose-response. *Clin. Chem.* **22**, 350-358.

Rose, S. P., Pruss, R. M. & Herschmann, H. R. (1975). Initiation of 3T3 fibroblast cell division by epidermal growth factor. *J. Cell. Physiol.* **86**, 593-598.

Rose, S. P., Stahn, R., Passovoy, D. S. Herschman, H. (1976). Epidermal growth factor enhancement of skin tumor induction in mice. *Experientia (Basel)* **32**, 913-915.

Rotkin, I. D. (1979). Epidemiological factors associated with prostatic cancer. In *Prostatic cancer, UICC Technical Report Series* Vol **48** (Eds D. S Coffey & J. T Isaacs) pp. 56-80. Geneva.

Roussel, M. F., Rettenmier, C. W. & Sherr, C. J. (1984). Cell surface expression of *v-fms*-coded glycoproteins is required for transformation. *Mol. Cell. Biol.* **4**, 1999-2009.

Sainsbury, J. R. C., Sherbet, G. V., Farndon, J. R. & Harris, A. L. (1985). Epidermal-growth factor receptors and oestrogen receptors in human breast cancer. *The Lancet* **i**, 660.

Salomon, D. S., Zwiebel, J. A., Bano, M., Losonczy, I., Fehnel, P. & Kidwell, W. R. (1984). Presence of transforming growth factors in human breast cancer cells. *Cancer Res.* **44**, 4069-4077.

Salomon, D. S., Bano, M. & Kidwell, W. R. (1986). Polypeptide growth factors and the growth of mammary epithelial cells. In: *Breast Cancer: Origins, Detections and Treatment.* (Eds M. A. Rich., J. C. Hayer & J. T. Papadimitriou). pp. 42-56 Martinus Nijhoff, Inc., Boston, MA.

Salomon, D. S., Perroteau, I., Ridwell, W. R., Tam, J. & Derynck, R. (1987). Loss of growth responsiveness to epidermal growth factor and enhanced production of alpha-transforming growth factors in ras-transformed mouse mammary epithelial cells. *J. Cell. Phys.* **130**, 397-409.

Sandberg, A. A. & Kadohama, N. (1980). Regulation of prostate growth in organ culture. In: *Progress in Clinical and Biological Research.* (Ed. G. P. Murphy). pp. 9-29. **37**. Alan R. Liss

Sanford, E. J., Paulson, D. F., Rohner, T. J., Drago, J. R., Santen, R. J. & Bardin, C. W. (1977). The effects of castration on adrenal testosterone secretion in men with prostatic cancer. *J. Urol.* **118**, 1019-1021.

Sargent, N. S. E., Oestreicher, M., Haidvogl, H. M. & Burger, M. M. (1988). Growth regulation of cancer metastases by their host organ. Proc. Natl. Acad. Sci. USA **85**, 7251-7255.

Sato, G., Desmond, W. & Kelly, F. (1975). Human prostatic tumors in conditioned animals and culture. *Cancer Chemother. Rep.* **59**, 47-49.

Savage, C. R., Inagami, T. & Cohen, S. (1972). The primary structure of epidermal growth factor. *J. Biol. Chem.* **247**, 7612-7621.

Sawyer, S. T. & Cohen, S. (1985). Epidermal growth factor stimulates the phosphorylation of the calcium-dependent 35,000-dalton substrate in intact A-431 cells. *J. Biol. Chem.* **260**, 8233-8236

Scatchard, G. (1949). The attraction of proteins for small molecules and ions. *Ann. N.Y. Acad. Sci.* **51**, 660-672.

Schechter, A. L., Hung, M-C., Vaidyanathan, L., Weinberg, R. A., Yang-Feng, T. L., Francke, U., Ullrich, A. & Coussens, L. (1985). The *neu* gene: an erb B-

homologous gene distinct from and unlinked to the gene encoding the EGF receptor. *Science, N. Y.* **229**, 976-978.

Schilling, K. & Liao, S. (1984). The use of radioactive 7α, 17α-Dimethyl-19-Nortestosterone (Mibolerone) in the assay of androgen receptors. *The Prostate* **5**, 581-588.

Schlessinger, J. (1979). Receptor aggregation as a mechanism for transmembrane signalling models for hormone action. *In Physical Chemical Aspects of Cell Surface Events in Cellular Regulation.* (Eds C. De Lisi & R. Blunmenthal). p. 89. Elsevier Press, N. Y.

Schlessinger, J. (1980). The mechanism and role of hormone-induced clustering of membrane receptors. *Trends in Biochem. Sci.* **5**, 210.

Schlessinger. J. & Geiger, B. (1981). Epidermal growth factor induces redistribution of actin and α-actinin in human epidermal carcinoma cells. *Exp. Cell. Res.* **134**, 273.

Schlessinger, J. (1986). Allosteric regulation of the epidermal growth factor receptor kinase. *J. Cell. Biol.* **103**, 2067-2073.

Schlessinger, J., Schreiber, A. B., Levi, A., Lax, I., Libermann, T. & Yarden Y. (1986). Regulation of cell proliferation by epidermal growth factor. *CRC Crit. Rev. Biochem.* **14**, 93-110.

Schlessinger, J. (1988). The epidermal growth factor receptor as a multifunctional allosteric protein. *Biochemistry* **27**, 3119-3123.

Schuurmans, A. L. G., Bolt, J. & Mulder, E. (1988a). Androgens stimulate both growth rate and epidermal growth factor receptor activity of the human prostate tumor cell line LNCaP. *Prostate* **12**, 55-63.

Schuurmans, A. L. G., Bolt, J. & Mulder, E. (1988b). Androgens and transforming growth factor β modulate the growth response to epidermal growth factor in human prostatic tumor cells (LNCaP). *Mol. Cell. Endocrinol.* **60**, 101-104.

Scott, J., Urdea, M., Quiroga, M., Sanchez-Prescador, R., Fong, N., Selby, M., Rutter, W. J. & Bell, G. I. (1983). Structure of a mouse submaxillary messenger RNA encoding growth factor and seven related proteins. *Science* **226**, 236-240.

Shannon, J. M. & Cunha, G. R. (1984). Characterization of androgen binding and deoxyribonucleic acid synthesis in prostate-like structures induced in the urothelium of testicular feminized (Tfm/Y) mice. *Biol. Reprod.* **31**, 175-183.

Shechter, Y., Hernaez, L., Schlessinger, J. & Cuatrecases, P. (1978). Local aggregation of hormone receptor complexes is required for activation by epidermal growth factor. *Nature* **278**, 835-838.

Sherr, C. J., Rettenmier, C. W., Sacca, R., Roussel, M. F., Look, A. T. & Stanley, E. R. (1985). The *c-fms* proto-oncogene product is related to the receptor for the mononucleur phagocyte growth factor, CSF-1. *Cell* **41**, 665-676.

Shields, R. (1978). Growth factors from tumours. *Nature* **272**, 670-671.

Shipley, G. D., Childs, C. B., Volkenant, M. E. & Moses, H. L. (1984). Differential effects of epidermal growth factor, transforming growth factor, and insulin on DNA and protein synthesis and morphology in serum-free cultures of AKR2B cells. *Cancer Res.* **44**, 710-716.

Sibley, P. E. C., Harper, M. E., Peeling, W. B. & Griffiths, K. (1984). Growth hormone and prostatic tumours: localization using a monoclonal human growth hormone antibody. *J. Endocr.* **103**, 311-315.

Simpson, E., Harrod, J., Eilon, G., Jacobs, J. W. & Mundy, G. R. (1985). Identification of a messenger ribonucleic acid fraction in human prostatic cancer cells coding for a novel osteoblast-stimulating factor. *Endocrinology* **117**, 1615-1620.

Sircar, S. & Weber, J. M. (1988). Normalization of epidermal growth factor receptor and transforming growth factor production in drug resistant variants derived from adenovirus transformed cells. *J. Cell. Physiol.* **134**, 467-472.

Soderquist, A. M. & Carpenter, G. (1984). Glycosylation of the epidermal growth factor receptor in A-431 cells. *J. Biol. Chem.* **259**, 12586-12594.

Somnenschien, C., Olea, N., Pasanen, M. E. & Soto, A. M. (1989). Negative controls of cell proliferation: Human prostate cancer cells and androgens. *Cancer Res.* **49**, 3474-3481.

Sporn, M. D. & Todaro, G. J. (1980). Autocrine secretion and malignant transformation of cells. *N. Engl. J. Med.* **303**, 878-880.

Sporn, M. B. & Roberts, A. B. (1985). Autocrine growth factors and cancer. *Nature* **313**, 745-747.

Sporn, M. B. & Roberts, A. B. (1988). Peptide growth factors are multifunctional. *Nature* **332**, 217-218.

St-Arnaud, R., Poyet, P., Walker, P. & Labrie, F. (1988). Androgens modulate epidermal growth factor receptor levels in the rat ventral prostate. *Mol. Cell. Endocrinol.* **56**, 21-29.

Stastny, M. & Cohen, S. (1972). The stimulation of ornithine decarboxylase activity in tests of the neonatal mouse. *Biochem. Biophys. Acta.* **261**, 177-180.

Stern, P. H., Krieger, N. S., Nissenson, R. A., Williams, R. D., Winkler, M. E., Derynck, R. & Strewler, G. J. (1985). Human transforming growth factor-alpha stimulates bone resorption in vitro. *J. Clin. Invest.* **76**, 2016-2019.

Stone, K. R., Mickey, D. D., Wunderli, H., Mickey, G. H. & Paulson, D. F. (1978). Isolation of human prostatic carcinoma cell line (DU 145). *Int. J. Cancer* **21**, 274-281.

Stonington, O. G., Szwec, N. & Webber, M. (1975). Isolation and identification of the human malignant prostatic epithelial cell in pure monolayer culture. *J. Urol.* **114**, 903.

Story, M. T., Jacobs, S. C. & Lawson, R. K. (1983). Epidermal growth factor is not the major growth-promoting agent in extracts of prostatic tissue. *J. Urol.* **130.** 175-179.

Strahle, U., Kloch, G. & Schutz, G. (1987). *Proc. Natl. Acad. Sci. USA* **84**, 78718.

Stromberg, K., Hudgins, W. R., Fryling, C. M., Hazarika, P., Dedman, J. R., Pardue, R. L., Hargreaves, W. R. & Orth, D. N. (1986). Human A673 cells secrete high molecular weight EGF-receptor binding growth factors that appear to be immunologically unrelated to EGF or TGF-α. *J. Cell. Biochem.* **32**, 247-259.

Syms, A. J., Harper, M. E. & Griffiths, K. (1985). The effect of prolactin on human BPH epithelial cell proliferation. *The Prostate* **6**, 145-153.

Taylor, J. M., Mitchell, W. M. & Cohen, S. C. (1972). Epidermal growth factor. Physical and chemical properties. *J. Biol. Chem.* **247**, 5928-5934.

Teixido, J., Gilmore, R., Lee, D. C. & Massague, J. (1987). Integral membrane glycoprotein properties of the prohormone pro-transforming growth factor-α. *Nature* **326**, 883-885.

Temin, H. M. (1966). Studies on carcinogenesis by avian sarcoma viruses, VI. The differential effect of serum and polyanions on multiplication of uninfected and converted cells. *J. Natl. Cancer. Inst.* **37**, 167-175.

Teslenko, L. V., Kornilova, E. S., Sorkin, A. D. & Nikolsky, N. N. (1987). Recycling of epidermal growth factor in A-431 cells. *FEBS (Letters)* **221**, 105-110.

Todaro, G. J., DeLarco, J. E. & Cohen, S. (1976). Transformation by murine and feline sarcoma viruses specifically blocks binding of epidermal growth factor to cells. *Nature*, **264**, 26-31.

Todaro, G. J., Fryling, C. & DeLarco, J. E. (1980). Transforming growth factors produced by certain tumor cells: polypeptides that interact with epidermal growth factor receptor. *Proc. Natl. Acad. Sci USA* **77**, 5258-5262.

Traish A. M. & Wotiz, H. H. (1987). Prostatic epidermal growth factor receptors and their regulation by androgens. *Endocrinology* **121**, 1461-1467.

Tucker, R. F., Shipley, G. D., Moses, H. L. & Holly, R. W. (1984). Growth inhibitor from BSC-1 cells closely related to platelet type beta transforming growth factor. *Science* **226**, 705-707.

Tullner, W. W. (1963). Hormonal factors in the adrenal-dependent growth of the rat ventral prostate. *Natn. Cancer. Inst. Monogr.* **12**, 211-223.

Ullrich, A., Coussens, L., Hayflick, J. S., Dull, T, J., Gray, A., Tarn, A. W., Lee, J., Yarden, Y., Libermann, T. A., Schlessinger, J., Downward, J., Mayes, E. L. V., Whittle, N., Waterfield, M. D. & Seeburg, P. H. (1984). Human epidermal growth factor receptor cDNA sequences and aberrant expression on the amplified gene in A-431 epidermoid carcinoma cells. *Nature* **309**, 418-425.

van Steenbrugge, G. J., Bolt-de Vries, J., Blankenstein, M. A., Brinkmann, A. O. & Schröder, F. H. (1988). Transplantable human prostatic carcinoma (PC-82) in athymic nude mice. II. Tumor growth and androgen-receptors. *The Prostate* **12**, 145-156.

van Steenbrugge, G. J., Groen M., Bolt, J., van der Korput, H., Trapman, J., Hasenson, M. & Horoszewicz, J. S. (1988). The human prostatic carcinoma cell line LNCaP and its derivatives: an overview. In: *Abstracts of The 6th Congress of the European Society for Urological Oncology and Endocrinology*. p. 44.

Verbeek, W., Bokemeyer, C., Falk, H. & Schmoll, H-J. (1988). Growth requirements, growth factor responsiveness, and growth factor secretion of three human embryonal carcinoma cell lines. *J. Cancer. Res. & Clin. Onc.* **114**, 553-559.

Vermeulen, A. (1979). The androgens. *In Hormones in Blood*, (3). p. 355. (Eds C. H. Grey and V. H. T. James). London: Academic Press.

Viola, M. V., Fromowitz, F., Oravez, S., Deb, S., Finkel, G., Lundy, J., Hand, P., Thor, A. & Sclom, J. (1986). Expression of *ras* oncogene p21 in prostate cancer. *New Engl. J. Med* **314**, 133-137.

Vogel, A., Raines, E., Kariya, B., Rivest, M. J. & Ross, R. (1978). Coordinate control of 3T3 cell proliferation by platelet-derived growth factor and plasma components. *Proc. Natl. Acad. Sci. USA* **75**, 2810-2814.

Ware, J. L., Lieberman, A. P. & Webb, K. S. (1986). Metastatic phenotype of human prostatic tumor cells in athymic nude mice: alteration by exposure to ethyl methanesulfonate and "reversion" by 5-azacytidine. *Cancer Immunol. Immunother.* **21**, 58-62.

Ware, J. L. (1987). Prostate tumor progression and metastasis. *Biochemica et Biophysica Acta* **907**, 279-298.

Waterfield, M. D., Scrace, G. T., Whittle, N., Stroobant, P., Johnsson, A., Wasteron, A., Westermark, B., Heldin, C. H., Huang, J. S. & Deuel, T. F. (1983). Platelet-derived growth factor is structurally related to the putative transforming protein p28 *sis* of simian sarcoma virus. *Nature* **304**, 35-39.

Waterfield, M. D. (1989). Structure-function relationships of growth factors and their receptors. *Brit. Med. Bull.* **45**, 541-554.

Watkins, L. F., Brattain, M. G. & Levin, A. E., (1988). Modulation of a high molecular weight form of transforming growth factor-α in human colon carcinoma cell lines. *Cancer Lett.* **40**, 59-70.

Weber, W., Gill, G. N. & Spiess, J. (1984). Production of an epidermal growth factor-related protein. *Science* **224**, 294-297.

Weinberger, C., Giguere, V., Hollenberg, S., Rosenfeld, M. G. & Evans, R. M (1986). Cold Spring Harbor Symposia on Quantitiative Biology. 51, 759.

Weissman, B. E. & Aaronson, S. A. (1983). BALB and Kirsten murine sarcoma viruses after growth and differentiation of EGF-dependent BALB/c mouse epidermal keratinocyte lines. *Cell* **32**, 599-606.

Wilding, G., Zugmeier, G., Knabbe, C., Valverius, E., Flanders, K. & Gelmann, E. P. (1988). The role of transforming growth factor α and β in human prostate cancer cell growth. *Proc. Am. Assoc. Cancer. Res.* **29**, 241.

Wilding, G., Valverius, E., Knabbe, C. & Gelmann, E. P. (1989). Role of transforming growth factor-α in human prostate cancer cell growth. *The Prostate* **15**, 1-12.

Winkler, M. E., O'Connor, L., Winget, M. & Fendly, B. (1989). Epidermal growth factor and transforming growth factor α bind differently to the epidermal growth factor receptor. *Biochem.* **28**, 6373-6378.

Yamamoto, T., Nishida, T., Miyajima, N., Kawai, S., Ooi, T. & Toyoshima, K. (1983). The erb B gene of avian erythroblastosis virus is a member of the src gene family. *Cell* **35**, 71-78.

Yarden, Y. & Ullrich, A. (1988). Growth factor receptor tyrosine kinases. *Ann Rev. Biochem.* **57**, 443-478.