Localisation and characterisation of epidermal growth factor (EGF) receptors on normal and cancerous human testicular tissue.

The possible role which EGF and transforming growth factor-α (TGF-α) may play in testicular cell-cell communication.

UNIVERSITY OF EDINBURGH

ABSTRACT OF THESIS (Regulation 3.5.10)

EGF receptors were identified on peritubular cells and Leydig cells of normal human testis using immunohistochemistry with monoclonal antibodies EGF-R1, EGF-R and F4. In a radioligand exchange assay the binding site for EGF on human testicular tissue was characterised as having a Kd of 1.18 ± 0.32 nM with approximately 528 ± 116 fmoles binding sites per mg of protein. The molecular weight of the receptor/ligand complex, identified by crosslinking and SDS-page gel electrophoresis was approximately 125 kDa. This may represent a proteolysed form of the receptor complex. Competition studies illustrated that of the peptides tested, only EGF and TGF-α were specific for the EGF binding site.

In cancerous testicular tissue EGF receptors were not identified on seminoma and Leydig cell tumours but that they were located, by both immunohistochemical and biochemical techniques on teratoma tumours. The EGF receptor on teratoma cells was characterised using the Tera-2 cell line. The EGF receptor was a high affinity site with a Kd of 0.21 ± 0.08 nM, with approximately $6.73 \pm 0.81 \times 10^4$ binding sites per cell. Molecular characterisation was performed by Western Blot Analysis employing the monoclonal antibodies EGF-R1 and F4. The receptor was identified in both the 170 and 125 kDa form. Competition studies with other peptides clarified once again the specificity which EGF and TGF-α have for the receptor site.

EGF and TGF-α were both present in normal testicular tissue at 5.16 ± 0.97 ng and 2.76 ± 0.15 ng per gram of wet human testicular tissue respectively. The concentrations of androstenedione, DHT and testosterone in human testicular tissue were 0.46 ± 0.13 nmoles, 0.29 ± 0.06 nmoles and 15.58 ± 2.55 nmoles per gram of dry human testicular tissue, respectively.

EGF and TGF-α were also secreted by Tera-2 cells at concentrations of 2.40 ± 0.33 pg and TGF-α at 2.55 ± 0.78 pg per ml of culture media. TGF-α and EGF both competed for the EGF binding site previously identified on the Tera-2 cells. EGF did not alter thymidine incorporation by the cells when incubated over 24 hours at concentrations ranging from 0.3 to 100 nM. TGF-α however, increased thymidine incorporation by approximately 2-fold when employed at concentrations greater than 3 nM over 7 hours in culture. The maximum increase in thymidine incorporation was apparent after 24 hours employing a concentration of 100 nM TGF-α. Mybolerone was found to have no effect on thymidine incorporation or EGF receptor expression when Tera-2 cells were incubated in concentrations from 0.3 to 100 nM for 24 hours. It was therefore postulated that Tera-2 cells are not androgen sensitive.

The results of these studies indicate that EGF and TGF-α may be involved in testicular cell-cell communication and in the development of testicular cancer.

Dedication

I dedicate this thesis to my late grandmother Mrs Ida Lois Hufton, my thoughts will always be with her.

Acknowledgements

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I would also like to thank the following people who have helped and encouraged me during the course of my studies; Mr Lawrence Brett, M.L.S.O., Department of Pathology, Western General Hospital who was of great assistance in helping me establish the optimum immunohistochemical technique; Dr Anne MacDonald, Fellow Researcher, for her help and support; Dr Frances Hay for the kind donation of the EGF-R monoclonal antibody which I would not otherwise have been able to obtain; Mr Chris Goodman, Mr Alistair Ritchie, Mr John Cummings, Mr Gordon Smith, Mr Steven Prescott and Dr Kenneth Grigor for their good humour and cooperation in obtaining the necessary tissue; Mrs Susan McHugh for her assistance with graphs and illustrations, and for her patience and friendship; and finally Professor Graham who kindly donated the Tera-2 cell line.

Declaration

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

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Abbreviations

EGF	Epidermal Growth Factor
TGF-α	Transforming growth factor-α
TGF-β	Transforming growth factor-β
NGF	Nerve growth factor
PDGF	Platelet derived growth factor
FGF	Fibroblast growth factor
IGF	Insulin growth factor
GnRH	Gonadotropin releasing hormone
FSH	Follicle stimulating hormone
LH	Luteinising hormone
GH	Growth hormone
Prl	Prolactin
DHT	5α-dihydrotestosterone
RIA	Radioimmunoassay
SDS-PAGE	Sodium dodecyl-sulphate polyacrylamide gel
SFM	Serum-free medium
BSA	Bovine Serum Albumin
FCS	Foetal calf serum
PMSF	Phenylmethylsulphonylfluoride
EDTA	Ethylenediamino-tetra-acetic acid
EGTA	Ethyleneglycol-tetra-acetic acid
min	minutes
TEMED	Tetra-methylethylethylenediamine

INTRODUCTION Chapter 1

1.1 Introduction

Increasing awareness that growth factors are regulators of cell growth (Rose *et al,* 1975; Tonelli and Sorof, 1980; Zetterberg *et al,* 1984; Schlessinger *et al,* 1988) has prompted this investigation to study the involvement of specific growth factors in the control of normal testicular function and in the development of testicular cancer. Spermatogenesis, one of the main functions of the testis, involves multiple germ cell division. The possibility of the involvement of growth factors in the uncontrolled division of such germ cells (which is characteristic of some germ cell tumours) must therefore be considered. As yet very little work has been performed to evaluate the contribution of growth factors to the proliferation and differentiation of testicular cancer. However numerous papers have been published on the involvement of certain growth factors and their receptors in other types of cancer such as breast (Osborne *et al,* 1980; Imai *et al,* 1982; Fitzpatrick *et al,* 1984a; Sainsbury *et al,* 1985a; Harris *et al,* 1987), lung (Berger *et al,* 1987a), prostate (Maddy *et al,* 1989) and bladder (Neal *et al,* 1985; Berger *et al,* 1987b; Harris *et al,* 1987). It was therefore with similar objectives in mind that this study was undertaken to investigate the possible role of epidermal growth factor (EGF) and its receptor in the normal functioning of the testis and in the proliferation and differentiation of testicular cancer.

It has already been postulated, and to some extent established, that certain growth factors do play a role in testicular function (Ritzen, 1983; Tres *et al,* 1986; Radford *et al,* 1987; Skinner and Moses, 1989, Skinner *et al,* 1989a; Skinner, 1990; Bartlett *et al,* 1990). EGF is thought to be specifically involved in spermatogenesis (Bartlett *et al,* 1990), the process which describes differentiation of germ cells to spermatozoa (described in section 1.4.7). Similarly EGF has also

recently been shown by Teerds *et al* (1990) to be involved in differentiation of germinal cells during which transforming growth factor-α (TGF-α) was found to be secreted.

Stem germ cells as well as differentiating to become spermatozoa also proliferate to maintain renewal of germ cells. The process of cell proliferation involves the development of all cell components leading eventually to division of the cell into two new cells. Four phases are usually recognised: G1- S- G2- M; G1 is a gap or pause after stimulation where the cell appears relatively inactive although there is some biochemical activity; S is the phase of synthesis, particularly of deoxyribonucleic acid (DNA), to double the normal amount, although other components also increase; G2 is a second gap period; and M is the stage of mitosis in which the nucleus breaks down and chromosomes separate into two individual groups, the nuclear membranes then reform about each group, and the whole cell divides into two identical cells. Cells may also move out of the division cycle into a resting phase known as G0.

In cancer the control of cell proliferation is deranged. Comparative studies of growth of both normal and cancerous cells have helped to identify significant regulatory events which are required for cell division. Such studies have also enabled scientists to identify growth factors, hormones, nutrients *e t c.*. which the cells require for growth either in the normal or cancerous state, or both. It is to be hoped that an increased understanding of the mechanisms responsible for and the factors involved in the control of normal proliferation and differentiation of cells will aid in the treatment and cure of cancer. At the moment particular attention has been focused on the role of growth factor involvement in both abnormal and normal growth control (Deuel, 1987; Tauber and Tauber 1987; Waterfield, 1989), whereas previously particular attention had been paid to hormone involvement in cancer (Leake *et al,* 1979; 1981). It now appears that both hormones and growth factors are of importance

in the growth of certain types of cancer (Fitzpatrick *et al,* 1984b; Sainsbury *et al,* 1985b; 1985c; Goustin *et al,* 1986; Lippman *et al,* 1986; 1987; Boluffer *et al,* 1990). As spermatogenesis is dependent on testosterone (Sharpe *et al,* 1988a) and EGF is also linked to germ cell development (Bartlett *et al,* 1990) both the concentration of androgens and EGF within normal and cancerous testicular tissue may be of relevance in the understanding of testicular function.

Other findings regarding the involvement of EGF and its receptor in cancer have been reported. In 1984 Downward reported having discovered an oncogene (the name given to delineate a gene capable of causing cancer) which showed a resemblance to the gene for the EGF receptor. Since then numerous studies have been performed with the aim of evaluating the possible role of EGF and its receptor in various types of cancer. Transforming growth factor-α has also been shown to activate the EGF receptor (Massague, 1983; Pike *et al,* 1983; Derynck, 1986) and therefore its presence in various types of cancer which express EGF receptors should also be evaluated. As mentioned previously the identification of TGF-α in germ cell division by Treeds *et al* (1990) must also raise the question of TGF-α involvement in the development of germ cell tumours.

1.2 The Testes

1.2.1 The Function of the Testes.

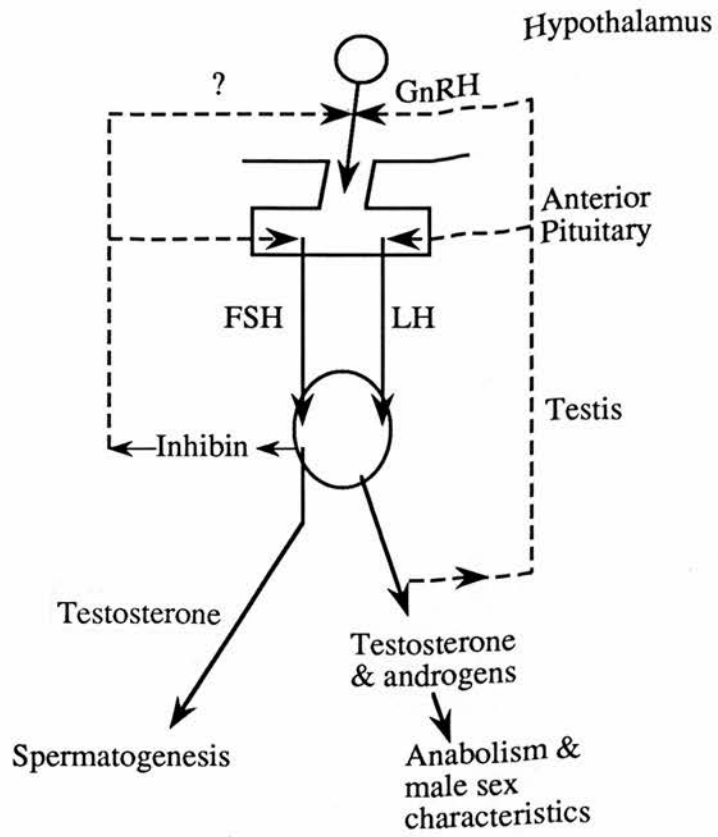
There are two main functions performed by the testis. Firstly it is the site of gamete production in the male. Each day millions of gametes are produced by the testes and stored in the epididymis until ejaculation takes place. Each gamete is produced for the sole function of fertilising an oocyte, subsequently leading to the propagation of human life (reproduction).

Secondly the testes is the principle organ producing testosterone and other androgens which are required for anabolism and the development of male

sexual characteristics. Such characteristics include development of the reproductive tract and the genital organs as well as secondary sexual characteristics such as hair and bone growth, haemoglobin production, nitrogen balance, libido and erectile potency, muscle development, vocal register and psyche.

1.2.2 Hypothalamic Control of the Testes via the Pituitary Gland.

Testicular function is controlled by the hypothalamus via gonadotropin releasing hormone (GnRH) which in turn stimulates the anterior pituitary to release the luteinising hormone (LH) and follicle stimulating hormone (FSH). GnRH is a decapeptide which is released from axons of hypothalamic neurones in pulses (Steinberger and Steinberger, 1972). GnRH stimulates the production of LH and FSH which are glycoproteins of molecular weights 38,000 and 29,000 respectively, and consist of two dissimilar sub-units α and β (Steinberger and Steinberger, 1972). The α subunit is common to both FSH and LH, while the β subunit specifies the biological activity of the hormone (Pierce and Parsins, 1981). Both of these gonadotrophins have the testis as their target organ, but their functions differ in that FSH acts almost exclusively on the tubules to control the process of spermatogenesis, whereas LH acts on the Leydig cells to stimulate androgen biosynthesis (Means *et al*, 1976; Ewing and Brown, 1977). Negative feedback loops exist for the regulation of both hormones (Figure 1). Inhibin is a peptide hormone synthesized by the Sertoli cells which is thought to feed back to the anterior pituitary to regulate FSH production. Evidence however, is now available to suggest that this concept of inhibin as a testicular negative feedback regulator, is overly simplified (Sharpe *et al*, 1988b). LH secretion in contrast is controlled by the feed-back action of testosterone on both the hypothalamus and pituitary (Naess *et al*, 1977). Both FSH and LH are required for



GnRH - Gonadotrophin Releasing Hormone
FSH - Follicle Stimulating Hormone
LH - Luteinising Hormone

Figure 1
Hypothalamic control of the testis is via GnRH which stimulates the anterior pituitary to secrete LH and FSH. LH and FSH act on the testis; LH to stimulate steroidogenesis and FSH to stimulate spermatogenesis. Feedback from the testis to the hypothalamus and the anterior pituitary occurs via the secretion of inhibin and testosterone into the systemic circulation.

the successful initiation and maintenance of spermatogenesis.

The FSH receptor is located on the basal membrane of the Sertoli cell. FSH binds to the receptor to activate a membrane bound adenylate cyclase system which then rapidly activates protein kinase (Davies, 1981). FSH has subsequently been shown to have multiple effects on Sertoli cell function. For example FSH has been shown to stimulate the production of androgen binding protein (ABP) in the rat testes (Bardin *et al,* 1981, Ritzen *et al,* 1982) as well as stimulating lactate, pyruvate (Jutte *et al,* 1982) and transferrin (Skinner and Griswold, 1982) production.

LH is synthesized in the anterior pituitary gland and acts upon the Leydig cell. It is the primary regulator of testosterone secretion and acts by stimulating testosterone synthesis and secretion through specific LH receptors located on the surface of Leydig cells. It is therefore evident that testosterone secretion is also regulated by the number of LH receptors available for LH to activate (for review see Sharpe, 1982). It has been shown that LH (or hCG another peptide hormone which acts on the LH receptor) negatively controls the number of LH receptors expressed by the Leydig cells (Sharpe, 1984a).

Prolactin is also involved in the normal endocrine regulation of the testis although its role is still poorly understood. Its target cell in the testis is the Leydig cell where it regulates the number of LH receptors. Induction of hyperprolactinaemia in the rat has been shown to produce an increase in the number of LH receptors expressed with a concomitant decrease in circulating LH, producing overall no change in testosterone secretion (for review see Sharpe, 1982). However in the human hyperprolactinaemia is associated with impotence and infertility. It appears therefore that prolactin plays an important role in male reproduction (for review see Sharpe, 1982).

1.3 The Gross Anatomy of the Testes.

The mammalian testes are bilateral organs which are suspended in the scrotal sac, the scrotal sac being situated at the base of the penis (Figure 2). The parenchymal tissue of the testis is enclosed by the tunica albuginea and can be conveniently divided into two functional compartments, the seminiferous tubules and the vascularized interstitium (see section 1.4). Spermatogenesis, the process of gamete formation, takes place within the seminiferous tubules. The tubules are long convoluted structures connected at both ends to the rete testis. The rete testis merges into the efferent ducts near the cranial pole of the testis. The efferent ducts then connect with the epididymides which in turn connect with the ductus deferens which finally merge with the urethra (Figure 3). At the point at which the ductus deferens merge with the urethra an ampulla is formed in which secretions from the prostate gland and the seminal vesicles have been identified and are believed to be stored. As shall become evident later (section 1.4.7) such secretory products along with those from the epididymis are necessary for the maturation and motility of normal sperm (Cooper, 1986).

In the young adult human the testes have been found to weigh between 10 and 16 g each. In this study it was found that the testes obtained from men aged 55-78 weighed between 4 g and 10 g each. Harman and Tsitouras (1980) and Tsitouras (1987) reported having found a decrease in testicular mass with advancing age, whereas in other studies performed by Johnson *et al* (1986) and Neaves *et al* (1984) in which the data from men with concomitant chronic illness were excluded, it was concluded that no relationship was apparent between decrease in testicular mass and increasing age. Although there is no general consensus in the relationship between advancing age and testicular mass, it does appear that in this study the weights of the testis collected were in the lower normal range.

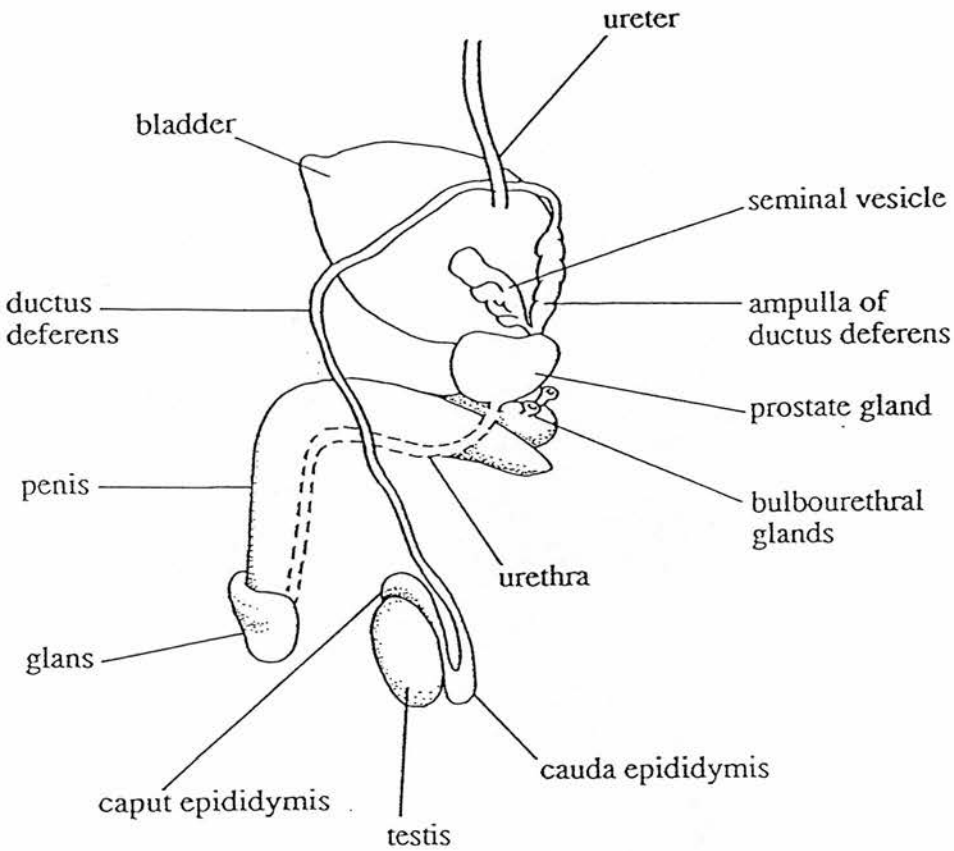


Figure 2 Overview of the human male genitalia.

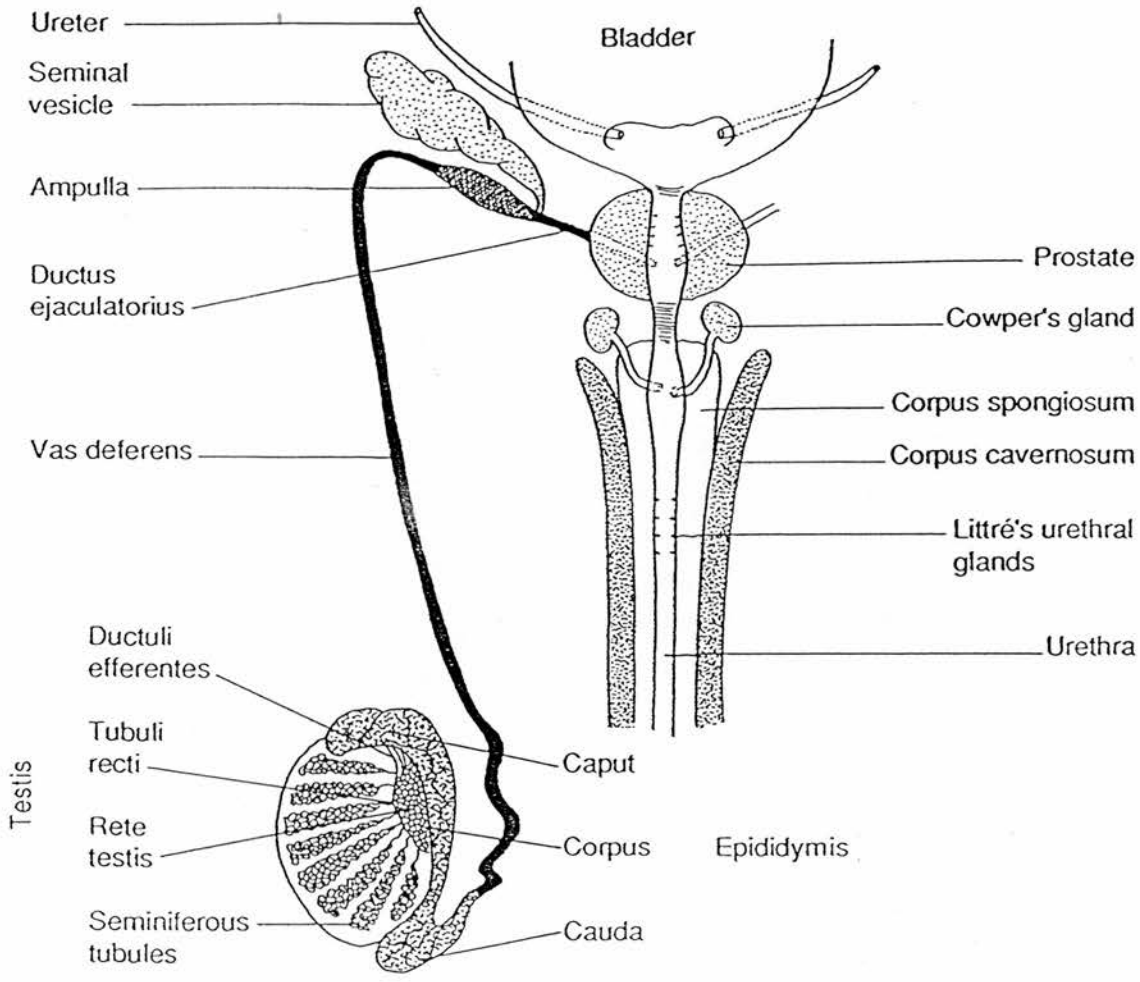


Figure 3 Cross section of the human male genitalia.

1.3.1 The Supporting Structures and the Vascular Supply to the Testes.

As described in section 1.3 the parenchymal tissue of the human testes is enclosed by the tunica albuginea. The scrotal covering layers external to the albuginea are the skin, dartos muscle and Colles' fascia, external spermatic fascia, and the parietal layer of the tunica vaginalis (Figure 4). A small fluid filled space exists between the parietal tunica vaginalis and the visceral tunica vaginalis which is adjacent to the tunica albuginea. The parenchymal tissue consists of the tunica albuginea which in turn merges with the tunica vascularis and the numerous fibrous septa that divide the testis into the two compartments ie. the avascular seminiferous tubules and the vascular interstitium (Figure 4). A posterior thickened portion of the capsule (the mediastinum) contains the blood vessels, lymph vessels and the intratesticular portion of the rete testis from which 15-20 efferent ductules connect with the epididymis. The blood supply to the testis is through the internal spermatic arteries. These arise from the abdominal aorta on the right and the renal artery on the left. There is also a secondary blood supply via the vas deferens artery. The blood supply is in indirect contact with the seminiferous tubules through a capillary plexus. A number of veins/venules emerge from the testis and together with the venous branches from the epididymis unite to form the plexus pampiniformis, which ascends along the spermatic cord. The vascular supply to the testes plays an important role in regulating of the temperature of the testes as a counter current exchange mechanism of heat between in-coming arterial blood and the out-going venous blood is apparent. It is thought that the testis only function properly at a temperature which is 2-3^{o}C lower than body temperature as increased temperature, has been shown to produce disruption of spermatogenesis (Moore, 1974). Hence the cooling process of the testes is of fundamental importance.

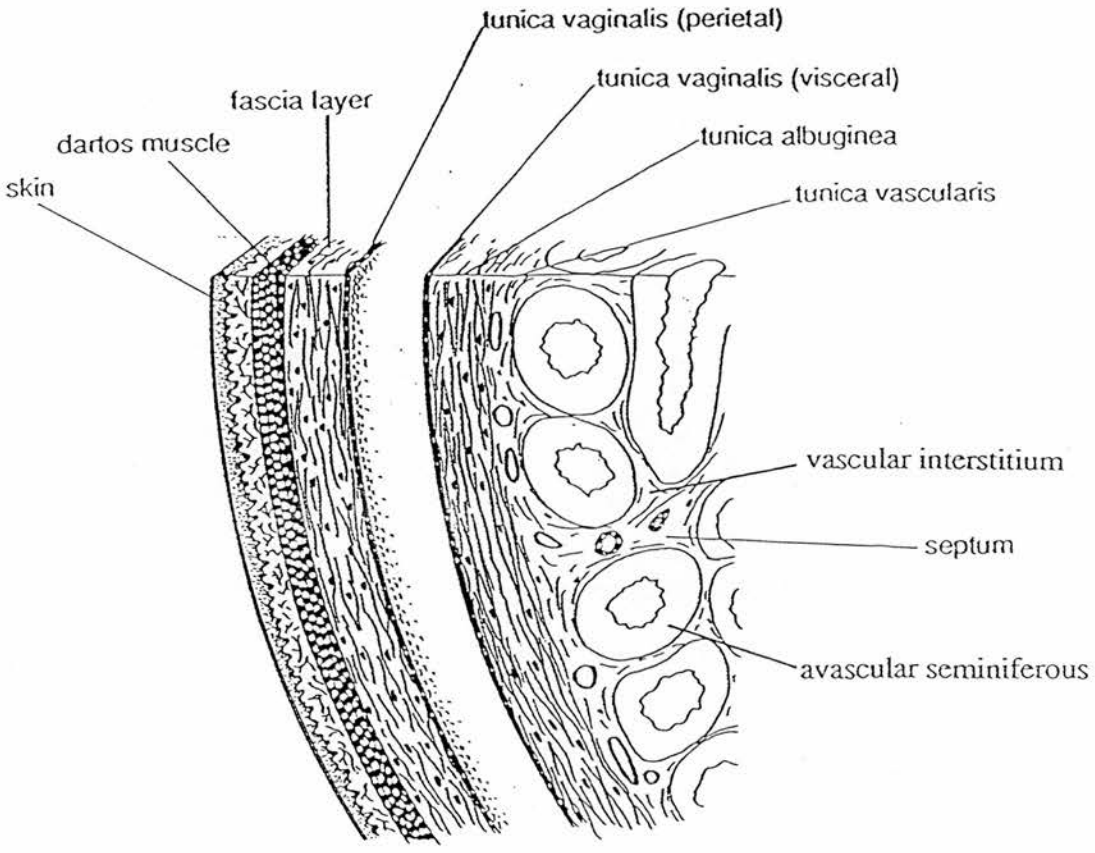


Figure 4 Cross section of the scrotal sac and testicular capsule. Compartmentation of the parenchymal tissue of the human testes into the vascular interstitium and the avascular seminiferous tubules is also illustrated.

1.4 The Intrinsic Anatomy of the Testes.

The parenchymal tissue of the testis which in this study is referred to as the "testicular tissue" is comprised of various different cells and structures. As discussed previously it is divided into two compartments; the avascular seminiferous tubules and the vascular interstitium (for review see Sharpe, 1982).

The seminiferous tubules are long convoluted cylindrical structures which contain Sertoli cells and several generations of differentiating germ cells which together comprise the seminiferous epithelium. Within the seminiferous tubules is a fluid filled lumen. The tubules are separated from the interstitium by multiple layers of peritubular cells.

1.4.1 The Interstitium

The interstitium of the testis is the area surrounding the seminiferous tubules. It contains Leydig cells, macrophages, fibroblasts, capillaries and lymphatic vessels amongst sparse loose connective tissue with interstitial fluid (Christensen, 1975; Setchell and Sharpe, 1981). In close contact with the interstitium are the peritubular cells which surround the seminiferous tubule (Figure 5). The cells appear as spindle shaped and form multiple layers around the outer edge of the basement membrane of the seminiferous tubules. The peritubular cells shall therefore be classified as part of the interstitium since they are exterior to the basement membrane .

1.4.2 Leydig Cells

Leydig cells differentiate from the mesenchymal elements of the testicular stroma. After 15 weeks of gestation, abundant well defined Leydig cells are clearly evident in the foetal testis and fill the interstitium. In this early stage of foetal growth the Leydig cells increase rapidly in number and display marked metabolic activity with the production of foetal sex hormones (Rommerts and van der Molen, 1989). The Leydig

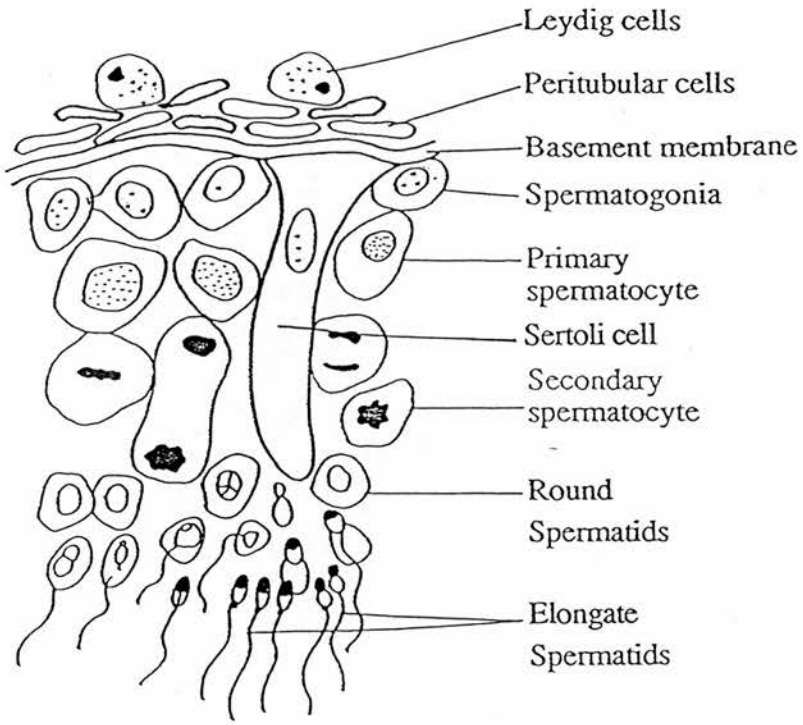


Figure 5 Cross section of the cellular composition of the spermatogenic cycle in the seminiferous tubules in man.

cell is the most widely investigated cell of the interstitium because of the major role which it plays via the production of testosterone. Studies have shown that of the intracellular organelles of the Leydig cell, the smooth endoplasmic reticulum, is the site of many of the enzymes required for steroidogenesis (Mori and Christensen, 1980). The cells themselves have a large distinguishable spherical nucleus and often occur in clusters. According to Neaves *et al* (1984) the Leydig cell population declines by approximately 44% in older individuals, although this is in conflict with the work of Kothari and Gupta (1974) and Deslypere and Vermeulen (1984) who reported an increase in Leydig cell number in men of 55-65 years of age when compared to a similar population of younger men. As yet no consensus regarding the relationship between the change in Leydig cell number and advancing age has been reached. In relation to this study however the possibility of a change in testicular structure with age must be considered. It may be for instance that certain factors are present at different concentrations in younger testicular tissue than in older testicular tissue.

As discussed earlier the Leydig cell is the main site of steroidogenesis (Purvis *et al,* 1981). Testicular steroidogenesis is stimulated by LH which is released from the pituitary gland, which in turn acts upon the LH receptor located on the surface of the Leydig cell (Hall, 1970; Catt and Dufau, 1973) (for review see Tahka, 1986)). The stimulation of steroidogenesis by LH is therefore dependent on the number of LH receptors expressed by the Leydig cell (see review by Sharpe, 1982). There is evidence to suggest that LH negatively regulates the number of its own receptors expressed by the Leydig cell (Sharpe, 1984a). Binding of LH to its receptor is followed by an increase in cAMP and the secretion of testosterone (Hansson *et al,* 1975; 1986).

Numerous other agonists have also been found to interact with the Leydig cell. These include IGF-I (Bernier *et al,* 1986; Lin *et al,* 1986; DeMellow *et*

al, 1987), LHRH-like peptide (Sharpe *et al*, 1981; Sharpe 1984a; 1984b), arginine vasopressin (Sharpe and Cooper, 1987) EGF (Welsh and Hsueh, 1982; Lloyd and Ascoli, 1983; Ascoli *et al*, 1987; Verhoeven and Cailleau, 1986) and TGF-β (Lin *et al*, 1987; Benahmed *et al*, 1988). Of these growth factors LHRH-like peptide (Sharpe *et al*, 1981) and IGF-I (Hall *et al*, 1983) are produced by the Sertoli cell.

Leydig cells also produce a number of other factors besides androgens. Opiates (β-endorphin in particular) are known to be produced by the rat Leydig cell and are thought to act on the Sertoli cell (Fabri *et al*, 1988). It has been illustrated by Fabri *et al* (1988) that hCG and testosterone may increase the synthesis and release of β-endorphin from rat Leydig cells. Oxytocin is also produced by the Leydig cells in both the rat and the human. It is known to act on the peritubular myoid cells that surround the seminiferous tubules to bring about their contraction and thus is thought to aid in the transport of shed spermatids to the rete testis (Wathes, 1984). Vasopressin or vasopressin-like peptide is also present in the testis and in cultures of mixed testicular cells and has been shown to produce inhibition of Leydig cell testosterone secretion (Sharpe and Cooper, 1987).

1.4.3 Peritubular Cells

The peritubular cells are arranged in layers around the seminiferous tubules and gradually increase in number with age. They differentiate from the mesenchymal elements of the testicular stroma as do the Leydig cells (see section 1.4.2). Although currently relatively little is known about the function of the peritubular cells it is apparent that they too play a fundamental role in testicular function. The peritubular cells are also known to secrete a factor called P-Mod-S while under the control of androgens and this is known to modify Sertoli cell function (Skinner and Fritz, 1985; 1986; Verhoeven and Cailleau, 1988a; 1988b). The P-Mod-S factor has been shown

to stimulate transferrin, ABP and cAMP production by rat Sertoli cells *in vitro* to the same extent as did maximal hormonal stimulation. P-Mod-S was then further characterised by Skinner *et al* (1988) who identified two forms of different apparent molecular weight of 54 - 56 kDa and 59 kDa respectively. The physiological significance of P-Mod-S as a possible paracrine regulator has yet to be elucidated and shall be discussed later in paracrine regulation of testicular function (section 1.6).

1.4.4 Organization within the Seminiferous Tubules.

The human seminiferous tubules are comprised of multiple cell types of which the Sertoli cells are of major importance (Setchell, 1978). Sertoli cells are columnar in shape with extensive cytoplasm extending from the basement membrane to the lumen of the seminiferous tubule between developing germ cells (Figure 5). The nuclei of the Sertoli cells are located toward the basal aspect of the tubule adjacent to the nuclei of spermatogonia. The spermatogonia differentiate to become gametes by the process termed spermatogenesis. (This will be discussed in more detail in section 1.4.7). In any one section of the human seminiferous tubule the seminiferous epithelium consists of four or five distinct generations of germ cells at different stages of the spermatogenic cycle (Clermont, 1963). It has been shown using cytological examinations that at any point in the seminiferous tubule the specific cells formed during the developmental stages of spermatogenesis are not randomly arranged but form cellular associations which are repeated (Schulze and Rehder, 1984). The activities of the several generations of germ cells are cyclical, the duration of which cycle and the number of stages involved vary in different species (Clermont, 1972). In man each cycle occupies about sixteen days (Heller and Clermont, 1964) and comprises six different cell associations or stages (Clermont, 1963)(Figure 5). In the rat, the cycle comprises fourteen different stages lasting approximately twelve days (Le Blond and Clermont, 1952).

Despite the stages of spermatogenesis being different in the human in comparison to the rat there is evidence to suggest that some hormonal requirements for spermatogenesis in the human are similar to those in the rat (Sharpe, 1986). However while the rat testes is a well established model for investigating testicular function, human testicular tissue should be employed wherever possible as animal models do not always represent accurately the human situation.

1.4.5 The Blood-Testis Barrier.

Evidence for a blood-testis barrier first came from the realization that seminiferous tubule fluid and rete testis fluid were very different in composition from blood plasma and testicular interstitial fluid (Setchell, 1969). The blood-testis barrier exists between adjacent Sertoli cells in the seminiferous tubules (Setchell, 1980). It plays an important part both in the maintenance of the intratesticular environment and in controlling the entry of peripheral hormones and the exit of endogenous hormones. The barrier is impermeable to many large molecules, and at the level of the basement membrane it functionally separates the tubules from the interstitium. The main function of the barrier, however, is thought to be to maintain a specific ionic and hormonal environment which enables the complex process of spermatogenesis to be supported (Waites and Gladwell, 1982).

The cell-cell barrier is made up of tight junctions (Bawa, 1963; Ross, 1976) between adjacent Sertoli cells which separate the Sertoli cells from the differentiating spermatogonia, dividing the tubule into basal and adluminal compartments. The former compartment consists of the spermatogonia and the preleptotene

spermatocytes; the latter containing the remaining primary spermatocytes, secondary spermatocytes and spermatids. A third and transient chamber is formed by adjacent Sertoli cells as germ cells move from the basal compartment to the adluminal compartment. (Bellve, 1979; McGinley *et al,* 1979). The tight junction complex then forms once again behind the germ cells and between the adjacent Sertoli cells, sealing off the intracellular space and thus ensuring the integrity of the blood-testis barrier (Fawcett, 1975; Russell, 1978). Substances required by the maturing germ cells must therefore pass through the Sertoli cell cytoplasm before entering the luminal compartment. Intracellular junction-like structures have also been described between Sertoli cells and germ cells (McGinley *et al,* 1979; Russell, 1980). Their function is as yet unknown but it is presumed that information is transmitted from the Sertoli cell to the germ cell, thereby regulating spermatogenesis. Factors produced by the seminiferous tubules in turn are transmitted to the interstitium via or from the Sertoli cell cytoplasm. Certain factors produced by the Sertoli cells have been shown to regulate Leydig cell function (example LHRH-like agonist) (Sharpe *et al,* 1981). This factor although distinct from LHRH acts upon specific receptors present on the Leydig cell via which it has been found to exert both an inhibitory and stimulatory effect on Leydig cell steroidogenesis (Sharpe, 1986). Similarly certain factors secreted by the seminiferous tubules have also been shown to effect Leydig cell function and *vice versa* (Bergh, 1982; Bergh, 1983; Sharpe et al, 1988c; Vihko and Huhtaniemi, 1989).

1.4.6 Sertoli Cells

As discussed in the previous section the Sertoli cells form part of the blood-testis barrier and thus provide a means through which messages from the interstitium can be passed to the differentiating germ cells (Ritzen, 1983). However evidence is now available which suggests that certain factors produced by the Sertoli cells are in fact

secreted in a bidirectional manner (Sharpe, 1988). That is to say that secretions occur via the apical and basal aspects of the Sertoli cells into the seminiferous tubule fluid and interstitial fluid. Such factors include inhibin (Maddocks and Sharpe, 1989) and testibumin (Cheng and Bardin, 1986).

Sertoli cells secrete a whole range of factors which include various growth factors, endocrine hormones, enzymes and matrix components as well as certain materials whose functions are as yet unidentified (for review see Grootegoed, 1987 and Ritzen *et al,* 1989). Some of the substances with particular reference to this project include seminiferous growth factors (Feig *et al,* 1980; 1983; Bellve and Feig, 1984; Brown *et al,* 1982; Holmes *et al,*1984; 1986; Lamb *et al,* 1987), insulin like growth factor -I (or somatomedin- C, Hall *et al,* 1983; Benhamed *et al,* 1987), TGF-β (Benhamed *et al,* 1988), inhibin (de Jong and Robertson, 1985), ABP (Hsu and Troen, 1978; Lee *et al,* 1980) and transferrin (Holmes *et al,* 1984).Furthermore factors produced by the peritubular myoid cells and germ cells may also act on the Sertoli cell.

With respect to testosterone action on the Sertoli cell the picture is still very unclear. Little is known about the effect of testosterone on the expression of specific genes in Sertoli cells although the Sertoli cell is known to contain receptors for testosterone (Sharpe, 1982; 1983; 1986). It has been suggested that the action of testosterone on the rat Sertoli cells involves the androgen-dependent production of regulatory proteins by the peritubular myoid cells (P-Mod-S) (Skinner and Fritz, 1985). There is still no general consensus about the intratesticular level of testosterone that is required to maintain spermatogenesis, although there is general agreement that testosterone maintains spermatogenesis in co-operation with other regulatory factors (Huang and Nieschlag, 1986; Sharpe *et al,* 1988a). Several authors have indicated that there is remarkable specificity with respect to the action of

hormones on Sertoli cells and hence on germ cells at different stages of the cycle of the seminiferous epithelium (Russell and Clermont, 1977; Parvinen, 1982; Rommerts *et al,* 1982)

A Sertoli cell mitogen referred to as the seminiferous growth factor (SGF) has also been identified in both human and rat testes and is thought to play a role in the paracrine regulation of germ cell mitosis (Bellve and Feig, 1984). However as yet little is known of the mechanisms of SGF secretion and particularly whether it is produced at only specific stages of spermatogenesis.

Characterization of a growth factor secreted by rat Sertoli cells in culture was also reported by Holmes *et al,* (1986). He found that conditioned medium from rat Sertoli cells had the ability to displace ^{125}I EGF binding to its receptor. The amount of EGF competing activity was also positively correlated with mitogenic activity. Feig and Bellve (1980) have also characterised a Sertoli cell growth factor which is thought to possibly act in an autocrine manner upon the Sertoli cell itself.

Grinsted and Byskov (1981) have also reported having identified a meiosis stimulating and a separate meiosis-inhibiting factor which are produced by the Sertoli cells at certain stages of the spermatogenic cycle. This was confirmed by Parvinen (1982) and Parvinen *et al* (1986).

IGF-I has also been found to be secreted by immature rat Sertoli cells in culture and is thought to act in a paracrine manner. It has also been found that the Leydig cells of rat testis express IGF-I receptors (Handelsman *et al,* 1985) and that both IGF-I and IGF-II can stimulate gonadotropin-induced steroidogenesis *in vitro* (Bernier *et al,* 1986; DeMellow *et al,* 1987; Kasson and Hsueh, 1987). Similar results have also been reported using porcine interstitial cell cultures (Benhamed *et al,* 1987; Perrard-Sapori *et al,* 1987). Hence these growth factors may be involved in the paracrine regulation of Leydig and Sertoli cell functions.

TGF-β secretion by Sertoli cells and testicular somatic cells was first discovered by the identification of TGF-β like activity in the conditioned medium from these cells and by the presence of TGF-β mRNA in rat Sertoli cells (Sporn *et al*, 1986). TGF-β has also been found to have a significant degree of homology with inhibin. With reference to other cells in the testes TGF-β has also been found to inhibit steroidogenesis by the Leydig cell (Lin *et al,* 1987).

The glycoprotein hormone inhibin, which is part of the negative feedback mechanism identified in regulating the hormone FSH and is secreted by the Sertoli cell, is present in two forms. Inhibins A and B exist, consisting of a common alpha-subunit and a similar but distinguishable beta-subunit, both of which act to suppress the secretion and cell content of FSH (see review by Ying, 1989). However the possibility of dual regulation of the pituitary by inhibin has been raised by the observation that the heterodimer (formed by the combination of the α and β subunits of inhibin) inhibits FSH production by the pituitary whereas the homodimer (formed from the combination of the β subunits of inhibin *i.e.*activin) stimulates FSH production. Activin was indeed found to inhibit Leydig cell steroidogenesis as did TGF-β *in vitro*, whereas inhibin produced a dose dependent stimulation (Hsueh *et al*, 1987). Inhibin may also regulate germ cell division according to the work reported by Hsueh *et al,* (1987).

Testicular interstitial fluid (IF) has also been shown to be a potent stimulant of steroidogenesis but as yet the active material in IF has not been identified, purified or fully characterised (Sharpe and Cooper, 1984; Sharpe *et al,* 1986).

In contrast oestradiol has been shown to have an inhibitory effect on Leydig cell steroidogenesis and is produced by the Sertoli cells of the immature rat testis. However in the adult rat oestradiol has been shown to be produced by the Leydig cells. Furthermore the conversion of androgens to oestrogens is stimulated by

FSH.

An LHRH-like factor has also been identified within the rat testis, and is thought to be secreted by the Sertoli cells and has been found to exclusively act on the Leydig cells as these are the only cell type within the testes known to possess LHRH-like receptors (Sharpe 1984a; 1984b; Rommerts and Themmen, 1986). It appears that the LHRH-like substance is distinct from that known to be secreted by the hypothalamus but as yet attempts to purify it have failed (see review by Sharpe, 1986). However using LHRH agonists it has been found that in the short term (24 hours) LHRH agonists stimulate Leydig cell testosterone secretion *in vivo* where as in the longer term (greater than 3 days) an inhibitory effect on testosterone secretion is apparent which if treatment is continued can lead to severe atrophy of the testis (see review by Sharpe, 1986).

As with the Leydig cells, Sertoli cell function and number are also thought to be influenced by age. According to studies by Rowley and Heller (1971) the Sertoli cell population remains stable throughout the postpubertal years. However Johnson *et al* (1984; 1986) found that men aged 20-48 had significantly more Sertoli cells than did men aged 50-85 years. Furthermore these authors also suggested that the decline in spermatozoa production identified in the elderly may have been caused by the decrease in Sertoli cell number as a relatively constant relationship between Sertoli and germ cells was reported in studies upon the rat (Johnson *et al,* 1984; 1986). As well as a decrease in Sertoli cell number with advanced age, a decrease in sperm production and in the number of spermatid-containing tubules was also noted, from 90% in the third decade to 50% in the 5-7 th decades. As suggested earlier this may indicate that Sertoli secreted products are present at different concentrations in the older human testis in comparison to the younger human testis. As yet however no general consensus between Sertoli cell number and age has been reached.

1.4.7 Germ Cells and Spermatogenesis

For normal spermatogenesis to take place the environment in which it occurs must have a functional endocrine system. The central nervous system influences the hypothalamic secretion of GnRH which in turn stimulates the secretion of LH and FSH from the pituitary, the two major hormones which act on the testis. The hypothalamic control of spermatogenesis in mammalian testis appears to be via hormonal action on gonadal somatic cells without direct actions on germ cells. For instance in the rat and the human, LH acts directly on Leydig cells and FSH on Sertoli cells. Similarly although testosterone produced by Leydig cells is essential for spermatogenesis and fertility it does not directly act on germ cells but on the peritubular and Sertoli cells. The exact mechanism by which the hormonally regulated gonadal somatic cells influence spermatogenesis remains to be elucidated but it is likely that multiple interactions between somatic cells are required to create an environment for the propagation of spermatogenesis.

As mentioned previously in section 1.4 the type A spermatogonia are the stem cells from which all germ cells are derived, the spermatogonia themselves being descended from the primordial germ cells which reach and multiply in the genital cords in the developing testis. In the fully differentiated testis the spermatogonia are situated along the basement membrane of the seminiferous tubule. In man, three basic types of spermatogonia can be distinguished; dark type A, pale type A and type B (Clermont, 1963). Dark type A spermatogonia divide to maintain the basic store of spermatogonia and also give rise to some pale type A cells which divide and differentiate into type B spermatogonia. Type B spermatogonia divide to produce the primary spermatocytes. The spermatocytes then proceed through a long prophase which shows characteristic configurations of the chromosomes (leptotene, zygotene, pachytene). This prophase is then followed by the subsequent steps of the first reduction division that yields the secondary spermatocytes containing diploid

number of chromosomes. Each secondary spermatocyte then undergoes the second reduction division to produce two haploid round spermatids. These spermatids then go through a series of nuclear and cytoplasmic modifications to produce spermatozoa (Figure 6). The spermatozoa then pass via the rete testis to the epididymis where they mature and gain motility (Cooper, 1986).

1.5 Steroids and Steroidogenesis

LH is the major hormone secreted by the anterior pituitary to regulate Leydig cell steroidogenesis. The original precursor for the production of steroids is cholesterol (for review see Rommerts and van der Molen, 1989). Cholesterol is converted to pregnenolone (the parent compound for androgen biosynthesis) in the mitochondria and microsomes of the Leydig cell by two hydroxylases and a lyase enzyme. There are two pathways through which androgen biosynthesis can be achieved from the parent compound. One of these involves 5α-ene-3β-hydroxysteroid metabolites such as 17α-hydroxypregnenolone the other 4α-ene-3β-oxosteroid metabolites such as progesterone (Figure 7). The final conversion in either pathway is to the potent androgen testosterone. However it is now known that in some tissues 5α–dihydrotestosterone (5α-DHT) is the active form of testosterone which binds to the nuclear receptor in the androgen dependent cell to achieve the physiological and biochemical reactions. One example of an organ which contains such cells is the skin. 5α-DHT is then metabolised in the cells cytoplasm to 5α-androstane-3α,17β-diols and 5α-androstane-3β,17β-diols. However, whether 5α-DHT is the active reagent in the testis is as yet unknown. Androstenedione is also produced during the biosynthesis of testosterone but is a less potent androgen than testosterone. The steroids then leave the testis via the blood supply to be circulated around the body. In a normal male of age 18-31 years the average concentration for testosterone in plasma is in the range 10-31 nmol/l.

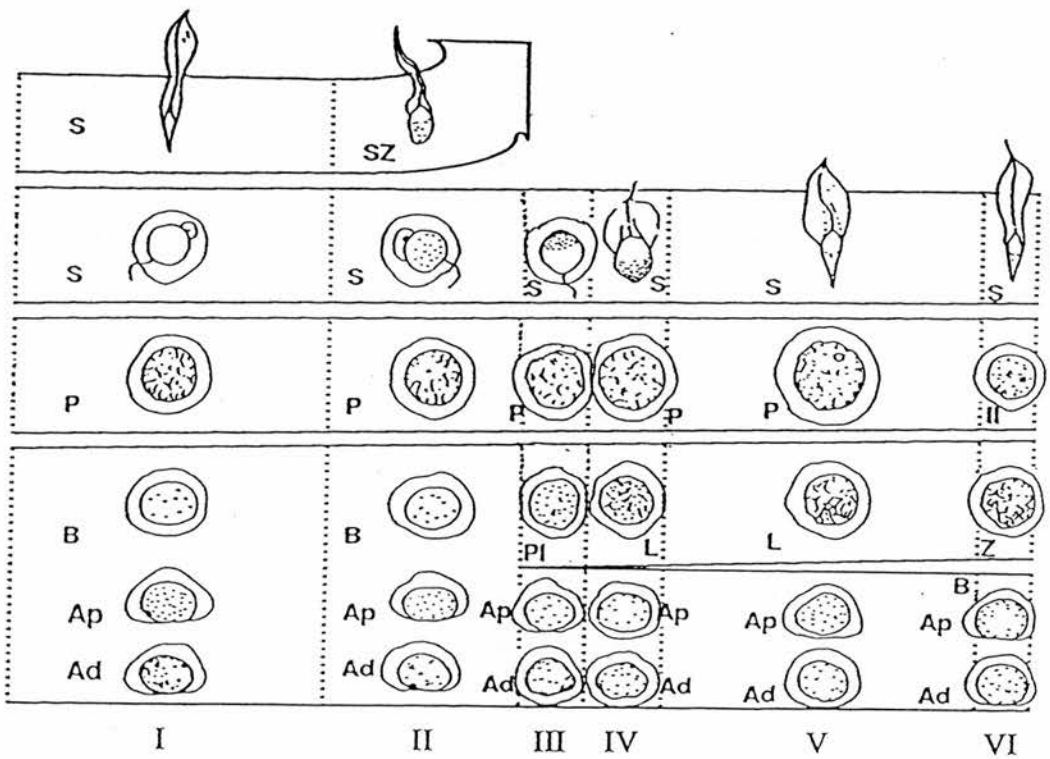


Figure 6 Diagramatic illustration of the cellular composition of six stages (I-VI) of the cycle of the seminiferous epithelium in man. The stages correspond to cell associations which succeed one another in time in any given area of the seminiferous tubule according to the sequence I-VI. after which the sequence starts again.

Ad, Ap, B - dark type A, pale type A, type B spermatogonia; Pl, L, Z, P preleptotene, leptotene, zygotene, pachytene primary spermatocytes; II - secondary spermatocytes; S - spermatids; SZ - spermatozoa.

From: Y. Clermont (1970), In: The Human Testis. Eds. E. Rosemberg and C.A. Paulsen, Plenum Press.

Figure 7

The biosynthesis of testosterone from pregnenolone in the mammalian testis can occur via several routes. The testicular rate of conversion of pregnenolone through the Δ4-pathway (involving progesterone, 17α-hydroxy-progesterone and 4-androstene-3,17 dione) and Δ5-pathway (involving pregnenolone, 17α-hydroxy-pregnenolone, dehydroepiandrosterone and 5-androstene-3β, 17β-diol) is different in different animal species. In the human testis the 5-pathway is the most significant.

Key

1 = cholesterol side-chain cleavage complex

2 = 3β-hydroxysteroid dehydrogenase

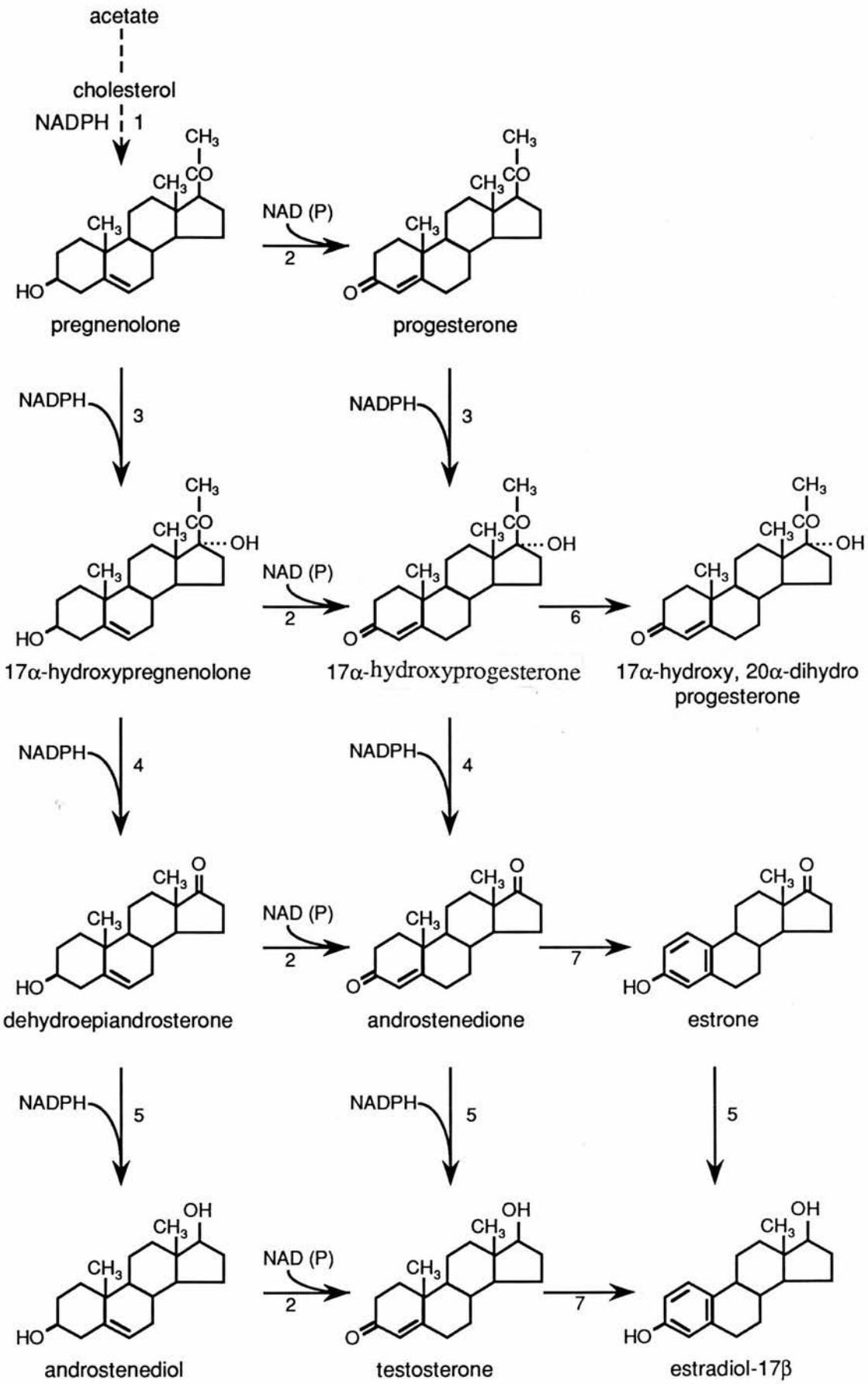
3 = 17α-hydroxylase

4 = steroid C_{17-20}-lyase

5 = 17β-hydroxysteroid dehydrogenase

6 = 20α-hydroxysteroid dehydrogenase

7 = aromatizing enzyme complex


acetate
cholesterol
NADPH
1
pregnenolone
NAD (P)
2
progesterone
NADPH
3
NADPH
3
17α-hydroxypregnenolone
NAD (P)
2
17α-hydroxyprogesterone
6
17α-hydroxy, 20α-dihydro progesterone
NADPH
4
NADPH
4
dehydroepiandrosterone
NAD (P)
2
androstenedione
7
estrone
NADPH
5
NADPH
5
5
androstenediol
NAD (P)
2
testosterone
7
estradiol-17β

Although the production of testosterone by the body is well understood the influence which it has on the testes themselves is somewhat complex and less well understood. Testosterone is a hormone native to the testis and one on which spermatogenesis completely depends; in its absence spermatogenesis can not be maintained (Sharpe *et al,* 1988a). It has been shown that testosterone can stimulate the Sertoli cells to secrete ABP and seminiferous tubule fluid, but neither of these are believed to be involved in the actual process of spermatogenesis (Sharpe, 1983; 1986). Skinner and Fritz (1986) have also reported that testosterone acts on the peritubular myoid cells to stimulate the production of macromolecules which then act on the Sertoli cells to modulate ABP and transferrin secretion. It has since been established that some of the actions of testosterone on the testis may be mediated by the peritubular cells. The effect of testosterone on the Leydig cell (the cell from which testosterone is secreted) is unclear, although numerous other factors have been reported to effect steroidogenesis and subsequently spermatogenesis. Verhoeven and Cailleau (1985) demonstrated that spent media derived from Sertoli cell-enriched cultures contains a protein that stimulates androgen production in adult and immature rat Leydig cells. Transforming growth factor-β like activity is also present in Sertoli cell-conditioned media and has been found to inhibit steroidogenesis (Benhamed *et al,* 1988). Inhibin whose β-subunit gene displays homology with TGF-β and mullerian duct inhibiting substance is secreted by the Sertoli cell (Steinberger and Steinberger, 1976a; 1976b) and has been shown to inhibit the LH stimulated androgen production by cultured testicular cells (Hsueh *et al,*1987). Fibroblast growth factor has been shown to inhibit 5α-reductase activity in cultured immature rat Leydig cells thus inhibiting the conversion of testosterone to 5α-DHT .

1.6 Paracrine Regulation of the Testis

As previously mentioned the two main testicular functions, androgen secretion and spermatogenesis are predominantly controlled by the pituitary hormones, FSH and LH. However, increasing evidence indicates that subtle regulation of testicular function can be locally modulated. Thus pituitary hormones provide the basic stimuli but the time and the intensity of the response itself appears to be modulated by complex interactions between the cells within the testis. Such interactions are mediated by the release of soluble factors and occur between various cell types within the testis including Sertoli cells, germ cells, peritubular cells, Leydig cells and vascular endothelial cells.

1.6.1 Interactions Between Sertoli and Germ Cells.

Under physiological conditions the initiation and maintenance of spermatogenesis is under the control of FSH and testosterone. Because androgen receptors are present in Sertoli cells (Tindall *et al,* 1977) but have not been identified in germ cells (Grootegoed *et al,* 1977) it has been proposed that the effect of testosterone on spermatogenesis is mediated by Sertoli cells (Saez *et al,* 1985). The functional activity of Sertoli cells is also modulated by the stage which the germ cells are at in their spermatogenic cycle. For example towards the end of the spermatogenic cycle Sertoli cells have the highest secretion rates of androgen binding proteins (Ritzen *et al,* 1982) and minimal binding of ^{125}I FSH (Parvinen, 1982). These multiple and bidirectional interactions which exist between Sertoli and germ cells can be mediated either by direct cell to cell contact which is very developed between these cells (Russell, 1980; Russell and Peterson, 1985) and/or through diffusible paracrine factors. Nerve growth factor (NGF) acts upon the Sertoli cell and has been found to be synthesized in germ cells by Persson *et al* (1990). NGF receptor mRNA has been identified in Sertoli cells and the receptor is

modulated by androgens. Hence it has been suggested that NGF produced by the male germ cell could regulate testicular function via an interaction between germ cells and Sertoli cells (Persson *et al,* 1990).

Although there has been no specific characterization of factors produced by the Sertoli cells which act on the germ cells and *vice versa* there is evidence supporting the existence of communication between the two groups of cells. As detailed above NGF is produced by the germ cells and acts on the Sertoli cells. It has been further demonstrated both *in vivo* and *in vitro* that when germ cells are destroyed there is a concomitant decrease in Sertoli cell specific functions (Jegou *et al,* 1984; Fritz and Tung, 1987). Variations in Sertoli cell secretions have also been identified on the addition of certain stages of differentiating germ cells to Sertoli cell monocultures. For example in rat Sertoli cell monocultures FSH stimulates ABP production by Sertoli cells, which is enhanced in the presence of pachytene spermatocytes (Le Magueresse *et al,* 1986) but not by co-culture with round spermatids (Galdieri *et al,* 1984). A Sertoli cell secreted growth factor has also been characterised by Holmes *et al,* (1986) which is thought to be secreted by Sertoli cells and thought to have an effect on germ cells.

1.6.2 Interactions Between Sertoli and Leydig Cells

These two cell types are located in two different compartments of the testes, without direct cell to cell contact and therefore all interactions between Leydig and Sertoli cells must be mediated by diffusible factors.

Papadopoulos *et al* (1986; 1987) reported that a Sertoli secreted factor or factors were shown to modulate Leydig cell function in the rat. Saez *et al* (1987) also describes in detail possible interactions between Sertoli and Leydig cells by looking at the experimental models used and the results which were obtained. For example a co-culture of Leydig and Sertoli cells was established and from this it was

found that the stimulatory effect of Sertoli cells on Leydig cell function depended on the Sertoli/Leydig cell ratio in the coculture (Benhamed *et al,* 1985). A body of evidence now points to a growth factor secreted by Sertoli cells although it is not yet clear on which cell or cells this growth factor acts.

Several investigators have described the presence of a factor from Sertoli cell spent media distinct from the LHRH-like factor which increases testosterone secretion by Leydig cells *in vitro* (Grotjan and Heindel, 1982, Janecki *et al,* 1985; Papadopoulos *et al, 1985;* 1987; Verhoeven and Cailleau, 1985) These factor(s) modulate LH action on Leydig cells and are thermolabile, FSH-dependent and have molecular weights between 10 kDa and 50 kDa.

As previously indicated testosterone is the most prevalent factor mediating the effects of Leydig cells on Sertoli cells. However pro-opiomelanocortin (POMC) derived proteins have also been investigated in the testis as β–endorphin was postulated to be secreted by the Leydig cells (Bardin *et al,* 1987) and act as a paracrine modulator of pubertal Sertoli cell function.

A number of studies have also reported the presence of the renin-angiotensin system in Leydig cells. Leydig cells have specific angiotensin-II receptors and angiotensin-II binding activates the subunit of adenylate cyclase, thus inhibiting gonadotropin stimulation of cAMP, resulting in decreased testosterone production (Khanun and Dufau, 1988). The significance of this is as yet unknown but if it were to produce a decrease in the level of testosterone then this could in turn affect Sertoli cell and peritubular cell function.

1.6.3 Interactions Between Sertoli, Peritubular and Leydig Cells.

Because of the close proximity between Sertoli and peritubular cells the peritubular cells are capable of influencing the morphology and organization of rat Sertoli cells in vitro as well as stimulating the secretion of ABP (Tung *et al,* 1980; Hutson and Stocco, 1981; Skinner *et al,* 1989b).

Skinner and Fritz (1985) discovered that in addition to a Sertoli secreting growth factor influencing peritubular cells in the rat testes there was a peritubular secreted paracrine factor termed P-Mod-S which modifies Sertoli cell function. It could therefore be concluded from this that androgens act at two levels on Sertoli cells via a direct action on the Sertoli cell androgen receptors and via an indirect action on the peritubular cell androgen receptors via P-Mod-S. Furthermore TGF-α which is produced by both the Sertoli and peritubular cells (Skinner 1989; Skinner *et al,* 1989a,) may possibly act as an important paracrine growth stimulant for almost any of the cells previously mentioned. It may also act in an autocrine manner. Transforming growth factor-β is also produced by Sertoli and peritubular cells and it too may act as an important paracrine/ autocrine inhibitor (Skinner and Moses, 1989).

The evidence available on Leydig/peritubular cell interactions is somewhat limited. However Skinner and Fritz (1985; 1986) have shown that androgens secreted by Leydig cells exert an effect on peritubular cells by increasing the apparent production of P-Mod-S.

The significance of Sertoli/peritubular/Leydig cell communication is not yet clear but as peritubular cells are positioned between the Leydig and Sertoli cells, it is possible that these cells may mediate some of the effects of Leydig cell secretory products which act upon the Sertoli cells and *vice versa*. Therefore such factors as β-endorphin, angiotensin, prostaglandins and oestrogens which are

produced by the Leydig cell may have an effect on peritubular cells as well as possibly on the Sertoli cells.

1.6.4 Summary of Paracrine Regulation of the Testes.

In summary, there are complex inter-relationships between the various components of the testes, the nature of which we are only just beginning to understand. It appears that through endocrine, paracrine and autocrine mechanisms, the function of each cell is modulated to integrate its activity with that of other cells. However without endocrine support, on which the whole system depends, spermatogenesis would break down. It appears that the interactions between different cell types within the testes have an important role in the maintenance and control of tissue function and growth. The fact that some of the interactions which are initiated by the endocrine hormones LH and FSH, secreted by the pituitary, are under the fine control of the peptide growth factors has prompted this investigation to look at EGF and its receptors in normal human testicular tissue.

1.7 Growth Factors

1.7.1 Introduction

The role of polypeptide growth factors in differentiation, development, chemotaxis and activation of inflammatory cells, tissue repair and disease has been increasingly appreciated. Subsequently the gene structure, cDNA sequence and complete amino acid sequence of several growth factors have been reported. The receptors for several growth factors have also been isolated and characterised. Activation of these receptors by growth factors has been shown to lead to gene activation, transcription and ultimately cell division. It is with this in mind that several investigators have looked towards growth factor research as a possible way of gaining insight into the development and maintenance of certain types of cell growth including cancer. At the

same time it is also recognized that growth factors play an important role in the development of normal tissue growth and function.

Many peptides with potent stimulatory effects on the proliferation of either epithelial or mesenchymal cells have been identified in the past ten years. Because of their regulatory action on tissue growth these peptides have been termed growth factors. Growth factor classification is complex, since in most cases their denomination is linked either to the target cell which revealed the mitogenic activity of the factor, or to the producing cell. For example platelet derived growth factor was isolated by Ross and Vogel (1978). It is stored in alpha granules of platelets and is released during blood clotting and is a 30-32 kDa glycoprotein composed of two peptide chains. Similarly the fibroblast growth factor obtained its name because its mitogenic activity was first demonstrated on a Balb/c-3T3 fibroblastic cell line. It is a 16.5 kDa single polypeptide chain isolated for the first time in 1974 from the bovine pituitary (Gospodarowicz, 1975; Gospodarowicz *et al,* 1986a; 1986b) and is synthesized by most tissues of mesodermic origin and is active on many cell types of both mesodermic and neuroectodermic origin.

This study was therefore undertaken to establish if EGF receptors and indeed EGF were present in normal and cancerous human testicular tissue. EGF was chosen because of its involvement in both cancer of the breast and of the prostate.

1.7.2 Epidermal Growth Factor

Epidermal growth factor (EGF) was first discovered by Stanley Cohen in 1962. EGF was recognized by its ability to accelerate the eruption of mouse teeth and the opening of eyelids in new-born mice. Later EGF was purified and its amino acid sequence determined in 1972 by Savage *et al* (1972, 1973). Although no human equivalent had been identified at that time it was known that a similar concentrate was present in human urine and in 1975 Gregory purified and analysed this compound giving it the

name urogastrone. He later concluded that "urogastrone and human epidermal growth factor are one and the same". EGF is a 6 kDa single polypeptide chain of 53 amino acids (Taylor *et al,* 1972; Cohen and Carpenter, 1975). It is synthesized in the form of a macromoleculer 128 kDa precursor (Gray *et al,* 1985). The development of radioreceptor (Carpenter *et al,* 1975; 1985) and radioimmunoassays (Dailey *et al,* 1978) quickly made it possible to associate urogastrone, found in human urine, to EGF. Subsequently the two peptides were found to elicit identical biological effects *in vivo* and *in vitro.* EGF is strongly mitogenic for many cell types of mesodermic and ectodermic origin. It has been shown to act in synergism with other growth factors such as platelet derived growth factor (PDGF) and IGF-I (Rose *et al,* 1975). EGF is also a powerful mitogen for embryonic and adult cells. Cloning of the EGF gene (Scott *et al,* 1983) and the use of messenger RNA probes (Scott *et al,* 1983) have shown that this factor is present in many tissues. EGF has also been found to inhibit steroidogenesis in cultured rat Leydig cells (Hsueh *et al,* 1981).

EGF stimulates the proliferation of various cultured cells from many different species (Carpenter and Cohen, 1979) and in addition to its mitogenic response EGF induces both early and delayed responses. Early responses include stimulation of ion and nutrient transport (Hollenberg and Cuatrecasas, 1973) and the enhancement of the phosphorylation of endogenous membrane proteins (Carpenter *et al,* 1979). Delayed responses include the activation of the enzyme ornithine decarboxylase (Stastny and Cohen, 1970; Statsbury and Cohen, 1972) and the enhancement of the biosynthesis of fibronectin (Chen *et al,* 1977) and keratin (Rheinwald and Green, 1977). As with other growth factors EGF acts on target cells to produce an effect by means of specific receptors.

1.7.3 Transforming Growth Factors

Joseph De Larco and George Todaro discovered that the retroviral transformation of murine fibroblastic cells was associated with the secretion of a molecule they termed sarcoma growth factor [SGF] (De Larco and Todaro, 1978; Sporn and Todaro, 1980). Subsequently purification procedures have revealed that SGF consists of two active components termed transforming growth factors, TGF-α and TGF-β (Roberts *et al,* 1980). TGF-α was characterised by its ability to bind to the EGF receptor (Carpenter *et al,* 1983). Later its amino acid sequence was also determined and found to be very similar to that of EGF (Sporn *et al,* 1986). Twenty five of the 50 residues of human TGF-α, including all six cysteines, were found in corresponding positions to that found in the human EGF sequence. Recently various forms of TGF-α have been identified (Bringman, 1987; MacDonald pers. commun. 1990). TGF-β however was found to have no structural relationship to TGF-α, but was found to modulate the availability of the EGF/TGF-α receptor. Transforming growth factor-β was first identified by its ability to cause phenotype transformation of rat fibroblasts (Roberts *et al,* 1981a; 1981b; 1985). It is a homodimer consisting of two identical 12.5 kDa subunits held together by disulphide bonds with 112 amino acids in each (Assoian *et al,* 1983;1986; 1987; Derynck, 1986). Subsequently it has been shown to have both growth-inhibitory and growth-stimulatory properties (Anzano *et al,* 1985; Massague, 1987; Moses *et al,* 1988; Sporn and Roberts, 1988). It has also recently been established that in fact three forms of TGF-β exist; β_1, β_2 and β_3 (Chiefetz *et al,* 1989). The potential role of TGF-β on gonadal function has been reported in publications by Avallet *et al* (1987) where TGF-β was found to inhibit primary cultured pig Leydig cell function. This was further supported by Lin *et al* (1987) who discovered that TGF-β actually inhibited Leydig cell steroidogenesis in primary rat culture. TGF-β cDNA has also been found in both normal and transformed cells (Derynck et al, 1985) as have TGF-β receptors (Massague, 1985a; 1985b; Massague

and Like, 1985).

1.7.4 Insulin-Like Growth Factors

First described by Salmon and Daughaday (1957) as a "sulfation factor" , somatomedin C (IGF-I) is the best known member of the family of insulin-like peptides; other members include insulin and IGF-II. IGF-I is human somatomedin C and IGF-II is human somatomedin A and rat multiplication-stimulating activity. IGF-I is the most active form in the adult where as IGF-II is thought to be the most active somatomedin during embryogenesis. *In vitro* IGFs have been found to be potent stimulants of mesenchymal cells proliferation. IGF-I is a progression growth factor which allows DNA synthesis in cells previously exposed to other growth factors termed commitment growth factors, for example PDGF (Van Wyk *et al,* 1981). It is produced in response to circulating growth hormone and can be found in serum and plasma (Svoboda *et al,* 1980) and is active in stimulating a large number of cultured cells (Van Wyk *et al,* 1981). IGF-I's effect on cell growth has been explored employing membrane assays (Van Wyk *et al,* 1975) and it has been purified from human serum and sequenced (Rinderknecht and Humbel, 1978). It is a single chain of 70 amino acids with three internal disulphide bonds.

IGF-I and IGF-II bind to two distinct receptors. IGF-I has a higher affinity for the IGF-I receptor in comparison to the IGF-II receptor and similarly IGF-II a greater affinity for the IGF-II receptor. However cross reaction can be seen at high concentrations of the two growth factors (Massague and Czech, 1983). The cellular receptor for IGF-I shows homology to the insulin receptor, a 450 kDa complex consisting of two transmembrane β–subunits (98 kDa each), each disulphide bonded to one α-subunit (130 kDa) (Pilch and Czech, 1980). The α-subunits provide the insulin or IGF binding domains (Pilch and Czech, 1979) whereas the β– subunit possesses ATPase and tyrosine kinase activities (Van

Oberghen *et al,* 1983). The α-subunit (the extracellular region) shows homology to the extracellular domain of the human EGF receptor. The β–domain shows homology with the *src* family of tyrosine kinases, although homology is highest with the *ras* oncogene (Ullrich *et al,* 1985). These homologies suggest that that one or more of these oncogenes may encode growth factor receptors. The IGF-II receptors (preferential for IGF-II) are simpler, exhibiting only a 250 kDa component which may be a single chain (Kasuga, 1981). Type-II IGF receptors may not undergo ligand induced down regulation (Massague, 1985).

IGFs are thought to stimulate growth in an autocrine fashion (Temin *et al,* 1972). However BRL-3A cells (derived from buffalo rat liver) secrete large amounts of IGF-II into the medium (Dulak and Temin, 1973) yet do not require the IGF-II for proliferation and therefore do not satisfy the autocrine hypothesis (Nissley *et al,* 1977). IGFs are also thought to play a role in testicular cell-cell communication as immunoreactive sites and IGF-I have been identified in rat Sertoli-spermatogenic cell co-cultures (Tres *et al,* 1986).

1.7.5 Nerve Growth Factor.

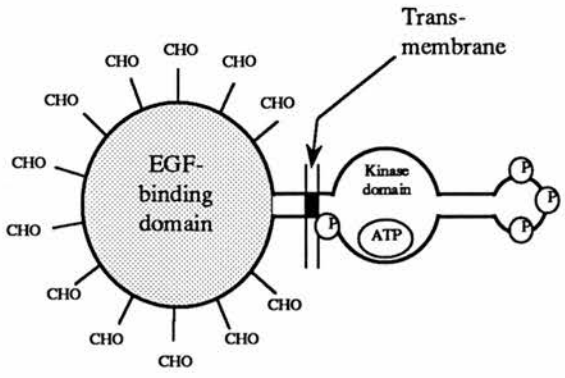
NGF is involved primarily in the maintenance and differentiation of sensory and sympathetic neurons and because of this its inclusion in a strict list of growth factors does not always appear correct. Nevertheless, NGF fits into the general scheme of growth factors in many ways. Indeed recent evidence points to NGF playing a mitogenic role for example in cultured rat adrenal chromaffin cells (Folkman, 1983). NGF was first detected as a substance released from transplanted tumours (Levi-Montalcini and Hamburger, 1951). It was then purified from snake venom and then subsequently from mouse submaxillary gland. NGF isolated from submaxillary gland is found in a 7S complex , containing three protein sub species labelled α, β, γ

(Bradshaw, 1978). NGF activity resides in the β-chain, which comprises of a 26 kDa dimer of two identical NGF chains (118 amino acids per chain) which has been sequenced (Angeletti and Bradshaw, 1971).

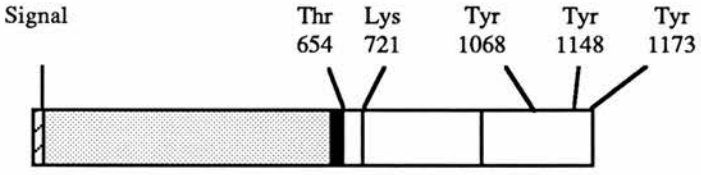
Receptors for NGF are present on a variety of normal sympathetic and sensory neurons as well as normal and neoplastic chromaffin cells. The rat pheochromocytoma cell line PC12 has been used extensively in studies concerning NGF. Proliferation of PC12 cells is inhibited by NGF whereas the differentiation of the cells is stimulated (Greene and Tischler, 1976) the mechanism controlling this response is presently unknown. The PC12 receptor has been defined as a single chain protein of 130 kDa , although a smaller receptor of 100 kDa is also present which is possibly a degraded form of the receptor. On the A875 melanoma cell line however two receptors with different affinities and molecular weights for NGF have been detected. The affinity constants of the two receptors are 2.0 pM and 2.0 nM and the molecular weights 98 kDa and 138-190 kDa respectively (Puma *et al,* 1983; Landreth and Shooter, 1980; Costrini and Bradshaw, 1979).

1.8 The EGF Receptor and Characteristics of EGF Binding.

The EGF receptor is a 170 KDa protein. It comprises of three major binding domains; an EGF-binding domain which lies external to the plasma membrane, a transmembrane domain and a cytoplasmic domain (Figure 8). Epidermal growth factor receptors have been found on various tissues both normal and cancerous (Hollenberg and Cuatrecasas, 1973; O'Keefe *et al,* 1974; Taketani and Oka, 1982; Lai *et al,* 1984; 1986). However the reports on the affinity of the ligand for its receptor are not always consistent. Furthermore it has often been reported that two binding sites exist rather than simply one. For example in human prostate tissue Maddy *et al* (1987) reported finding two binding sites, one with a high affinity of 0.8 nM and one with a low affinity of 7.6 nM. In contrast was the single high affinity



Domain structure
of EGFR



EGFR
polypeptide

Figure 8
The EGF receptor: the structure and the polypeptide. EGF binds to the external domain of the receptorto initiate a signal which is transmitted via the transmembrane domain to the internal domain of the EGF receptor. In progressing to the internal domain, tyrosine kinase is stimulated.

binding site reported by Traish and Wotiz (1987) on rat prostate tissue which expressed a Kd of 1.16 nM. In this case the difference in binding sites observed could be accounted for by the difference in species but there are numerous other examples which could be referenced. For example in characterization of the EGF receptor in human meningioma two binding sites with dissociation constants of 0.9 nM and 5.0 nM were detected (Weisman *et al,* 1987). Similarly in leiomyomas two binding sites of 0.1 and 3.7 nM were detected (Hofmann *et al,* 1984) whereas on normal uterine tissue (myometrium) only one EGF receptor was characterised with a Kd of 0.7 nM. From the aforementioned data there appears to be two groups of EGF receptor populations. The first population consists of two receptors with affinity constants in the range 0.1-0.9 nM and 3.7-7.6 nM and the second population just one receptor with an affinity constant in the range 0.7 nM to 1.16 nM.

1.8.1 Activation of the EGF Receptor and Regulation

On binding of EGF to its receptor it is known that a signal is passed through the three domained binding site to initiate a reaction at the second messenger. The human epidermal carcinoma cell line A431 which has $>10^6$ EGF receptor sites per cell (Fablicant *et al,* 1977; Haigler *et al,* 1978; 1979; Stoscheck and Carpenter, 1984) has been used to identify phosphoproteins of 150 and 170 kDa which are believed to represent the EGF receptor. EGF has also been found to stimulate tyrosine phosphorylation of these proteins (Carpenter *et al,* 1978; 1979; King *et al,* 1980).

The EGF receptor was purified from A431 cells and shown to be a tyrosine-specific protein kinase with the capacity for autophosphorylation and activity for exogenous substrates (Cohen *et al,* 1980; 1982; Erhart *et al,* 1981). It is an integral membrane protein of 170 kDa (Cohen *et al,* 1982), the non-glycosylated precursor polypeptide is approximately 130 kDa. In addition to glycosylation, other modifications include serine, threonine and tyrosine phosphorylation (Hunter and

Cooper, 1981;1985; Pike and Krebs, 1986). The endogenous kinase mediates tyrosine phosphorylation, whereas other kinases are responsible for phosphorylation of serine and threonine residues, including protein kinase C (Cochet *et al,* 1984) which preferentially phosphorylates threonine 654 (Hunter *et al,* 1984; Davis and Czech, 1985). Phosphorylation of the EGF receptor by protein kinase C inhibits the intrinsic tyrosine kinase activity, reduces the apparent affinity of the receptor for EGF and stimulates internalisation of the receptor (Lee and Weinstein, 1978; Fox *et al,* 1979; Downward *et al,* 1984a; Davis and Czech, 1985; Whitley and Glaser, 1986). Thus protein kinase C has a negative influence on the EGF receptor. The EGF receptor is internalised when activated by EGF and is subsequently degraded (Carpenter and Cohen, 1976). In the absence of ligand the EGF receptor is distributed randomly over the cell surface. Following binding of EGF at 37^{o}C, the receptor becomes localised in coated pits, internalised in endosomes and delivered, possibly via the Golgi bodies to lysosomes, where it is rapidly degraded (Stoscheck and Carpenter, 1984a; 1984b). As well as stimulating protein kinase C the intrinsic kinase activity is also able to phosphorylate other proteins such as phosphatidylinositol kinase (Walker and Pike, 1987) which in turn effects transmission of the second messenger signal. The EGF receptor has a half-life (T 1/2) of approximately 10 hours in human fibroblasts, but in the presence of EGF this value is reduced to approximately 1 hour (Stoscheck and Carpenter, 1984a; 1984b). The recycling of the EGF receptor has been found to be insignificant by Stoscheck and Carpenter (1984a; 1984b) and Teslenko *et al* (1987), consequently the interaction of EGF with its receptor provides a negative feedback loop that down regulates the potential for additional signalling by EGF.

1.8.2 The *v-erb* B Oncogene.

Structural studies carried out by Downward *et a*l (1984a) have demonstrated that 74 of the 83 EGF receptor amino acid residues sequenced are identical to those of the transforming protein encoded by the *v-erb* B oncogene of avian erythroblastosis virus. Several lines of evidence suggest that the *v-erb* B oncogene encodes the transmembrane region of the EGF receptor and the domain associated with the tyrosine kinase activity (Hayman *et al,* 1983; Hayman and Beug, 1984; Lax *et al,* 1985; Nilsen *et al,* 1985; Akiyama *et al,* 1986; Hayman *et al,* 1986).

Viral oncogenes are known to be directly responsible for the transformation of some cell types (Slamon, 1987) and therefore it is plausible that the proto-oncogene as well as the EGF receptor may encode information with the potential to induce cancer. For example in the literature it has been suggested that the protein kinase activity of the *v-erb* B protein is constantly active and therefore presumably provides a permanent proliferative signal (Hayman *et al,* 1986). As well as the *v-erb* B oncogene the *neu* oncogene (Coussens *et al,* 1985; Schechter *et al,* 1985) and the Her-2 oncogene also show extensive homology to the EGF receptor although the*neu* gene is distinct from and unlinked to the gene encoding the EGF receptor, and yet it is homologous to the *erb* B gene (Schechter *et al,* 1985). Oncogenes have also been identified in germ cell tumours (Sikora *et al,* 1987) although the oncogenes found were not of the *src* family of which *erb* B is a member (Yamammoto *et al,* 1983). The Her-2 and neu oncogenes have however been shown to be amplified in some human breast cancers (Slamon *et al,* 1987; Zeillinger *et al,* 1989).

1.9 Autocrine and Paracrine Modes of Action of Growth Factors

There is now much circumstantial and direct evidence to support an autocrine method of action by certain growth factors. For example, many types of tumour cells release

polypeptide growth factors into their medium when grown *in vitro* and these same tumour cells often possess functional receptors for the released peptide. The peptide growth factors which are known to function via an autocrine mechanism in cancer cells include TGF-α and PDGF. Similarly evidence is also now available to support the paracrine hypothesis whereby a growth factor secreted by one cell can act on a neighbouring cell. Growth factors which act in this way include both EGF and TGF-α. In contrast growth factors that act on distant tissues accessed via the blood stream are said to act in an endocrine fashion (Figure 9).

The term paracrine was proposed by Feyrter in 1938 to describe the action of a network of epithelial clear cells throughout the gut that he thought might be peripheral endocrine glands (Feyrter, 1946). He speculated that they exercised local paracrine effects on gut function in addition to their endocrine effects on distant tissues.

The concept of autocrine secretion was introduced by Sporn and Todaro (1980) to explain the endogenous production of autostimulatory growth factors by transformed cells. Since then autocrine control mechanisms have also been observed in normal cells.

More recently greater consideration has been given to the possibility of intracrine regulation of the nucleus by growth factors. Logan (1990) recently published a report suggesting another category for the mode of action of growth factors. Growth factors which act in this way apparently need not be secreted, nor do they require receptors located on the cell surface to mediate their activity. Rather they remain within the cell of origin and act directly themselves as intracellular messengers to regulate cellular function. A number of cytoplasmic growth factors have now been characterised which lack classical consensus signal peptide sequences to direct their secretion. These include the precursors of Interleukin-1α and Interleukin-1β (Auron *et al,* 1984; March *et al,* 1985), ciliary neurotrophic factor

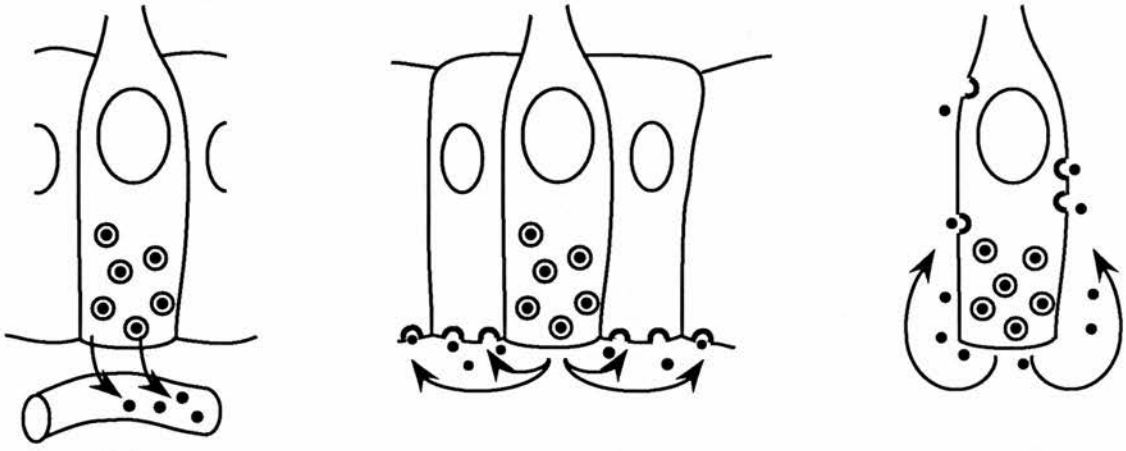


Figure 9
Endocrine, Paracrine and Autocrine mechanisms of action for growth factors. In the endocrine system growth factors are secreted into the blood stream to act on distant targets. In the paracrine system growth factors are secreted to act on neighbouring cells and in the autocrine system growth factors are secreted by the cells on which they are going to act.

(Stockli *et al,* 1989) and the newly discovered platelet derived endothelial cell growth factor (Ishiwaka *et al,* 1989). Other members of this group include some of the fibroblast growth factors. Secretion has conventionally been considered to be a prerequisite of growth factor bioactivity but in light of the above evidence this now appears to be open to question.

1.9.1 Interaction between Growth Factors

In any organ or tissue containing multiple cell types, local co-ordination of the functions of the different cell types is fundamental to the efficient working of the organ in question. Such local or paracrine regulation must by definition involve agents which are produced by one cell type and act on another. Even in a single cell the action produced by the growth factor may depend on the context set by other substances which are present. For example TGF-β stimulates growth of fibroblasts in the presence of PDGF, but inhibits their growth in the presence of EGF (Roberts *et al,* 1985). The action of growth factors can also depend on the state of the differentiation of the target cell. For example TGF-β stimulates or inhibits the expression of cartilaginous phenotype in embryonic mesenchyme according to the development stage of the cell (Seyedin *et al,* 1986; Rosen *et al,* 1988).

1.9.2 Interaction between Receptors

The ability of a receptor for a specific peptide to alter either the cellular distribution or the binding affinity of a neighbouring receptor for a second peptide growth factor, independent of any direct crossreactivity of the peptide themselves upon the two receptors, is now also apparent. For example PDGF can decrease the affinity of EGF receptor for its ligand (Bowen-Pope *et al,* 1983, and Zachary and Rosengurt, 1985). This phenomenon is termed transmodulation ("receptor-cross- talk") to distinguish it

from the reduction in receptor numbers caused by homologous ligands, known as down regulation or down modulation. In addition EGF receptors on some tissues have been shown to be controlled by other substances apart from growth factors. For example Mukku and Stancel (1985a) reported finding that the EGF receptor on uterine membranes could be regulated by estrogen. Similarly Murphy *et al* (1986) reported that progestin regulated EGF receptor in human mammary carcinoma cell lines. In the case of steroids it is likely that regulation of the EGF receptor occurs via stimulation of the internal steroid receptor present within the same cell. In the study of Murphy *et al* (1986) this was indeed the case for it was found that the expression of the EGF receptor in some breast cancer cells was regulated in part by mechanisms mediated via the progesterone receptor. The effect was correlated with the affinities of a series of progestins for the progesterone receptor.

1.10 Cancer and Growth Factors

Over the past fifteen years various hypotheses for the involvement of growth factors in the development and progression of certain types of cancer have been put forward. A number of these hypothesis have in turn been substantiated. Growth factors are involved in multiple types of cancer (Kawamoto *et al,* 1983; Sainsbury *et al,* 1985a; Bepler *et al,* 1988). In MCF-7 human breast cancer cells IGF-I and EGF are released into serum-free culture medium (Dickson *et al,* 1986a; 1986b; 1986c). Treatment of MCF-7 cells with 17β oestradiol, which is required *in vivo* for MCF-7 tumour growth in the nude mouse and stimulates MCF-7 growth rate *in vitro*, resulted in selectively increasing growth factor concentrations in serum-free medium (Dickson *et al,* 1986b). Autostimulatory growth promoting activity was elevated at least two-fold, and concentrations of EGF-like polypeptides were elevated 5-fold while IGF-I immunoreactivity was not elevated. Lippman *et al* (1986) hypothesised that oestrogen control of hormone dependent breast cancer was mediated by autocrine and

paracrine growth factors secreted by breast cancer cells. Human breast cancer cells were found to secrete a collection of growth factors (IGF-I, TGF-α , TGF-β, a PDGF-like competency factor, and at least one new epithelial colony stimulating factor) (Lippman *et al,*1986). However not all of these factors were found to be regulated by œstrogen although they are constitutively increased in cells which acquire hormone independence either spontaneously or by *ras* transfection. Collectively the secreted growth factors were found to to be capable of promoting tumour formation by MCF-7 cells in nude mice, though not to the same extent as oestrogens. There would therefore seem to be some potential in the autocrine and paracrine control of breast cancer cells, including some cells which are no longer dependent on oestrogens.

In primary breast cancers it has been established that there is an inverse relationship between EGF receptor and oestrogen receptor (ER) status. For example the relapse-free survival and overall survival times for patients with EGF receptor positive tumours were found to be significantly worse. The tumours of patients with positive EGF receptor identification also illustrated a significant association between tumour size and differentiation (Sainsbury *et al,* 1985d; 1987). Relapse-free and overall survival times were also worse for ER negative tumours. EGF receptor positive and ER negative in fact indicated the poorest overall survival and relapse-free times. From this it was suggested that EGF receptor status was the most important variable in primary tumours for predicting overall survival time. Similarly EGF receptor expression has been of prognostic significance in oesophageal squamous cell carcinomas (Ozawa *et al,* 1989). In the study by Ozawa *et al* (1989) it was found that the survival rate of the high EGF binding group was significantly lower than that of the low EGF binding group. In carcinoma of the prostate EGF receptors have been identified and are expressed in much higher numbers in the tissues which are poorly differentiated (which are also possibly more aggressive) than

in tissues which are well differentiated. In a report published by Traish and Wotiz (1987) it was found using prostatic membranes from rats that EGF receptor expression could be modulated by androgens. Similarly in the study performed by Ewing *et al* (1989) in which breast cancer cell lines were used it was found that EGF receptors could be regulated by both progestins and glucocorticoids acting via their respective receptors to induce increases in EGF receptor mRNA levels. One principle aim of this study would therefore be to investigate the presence and possible role of EGF and its receptor in cancerous human testicular tissue.

1.11 Cancer of the Testes.

Testicular tumours are infrequent, but most often malignant. They are the commonest type of cancer in men under 40 years of age and there appears to be an increase in incidence (pers. commun. Mr T.B. Hargreave). They account for approximately 0.5 to 1.0% of malignant tumours in the human male. The large majority of primary testicular tumours, the so called germ cell tumours, originate from testicular germ cells. Rare testicular neoplasms like Leydig cell tumours originate from testicular stroma and are therefore classified as gonadal stromal tumours. To this day there is still controversy concerning classification of testicular germ cell tumours. Consequently for the purposes of this study the tumours have been classified as seminoma, teratoma and Leydig cell as shown in Figure 10 and in accordance with the classification of Mostofi (1974) as slightly modified by the World Health organisation (Bergami *et al,* 1977).

Both benign and malignant tumours are less common in children than in adults. Only 2 to 5% of all testicular tumours occur in childhood (Collins and Pugh, 1964).

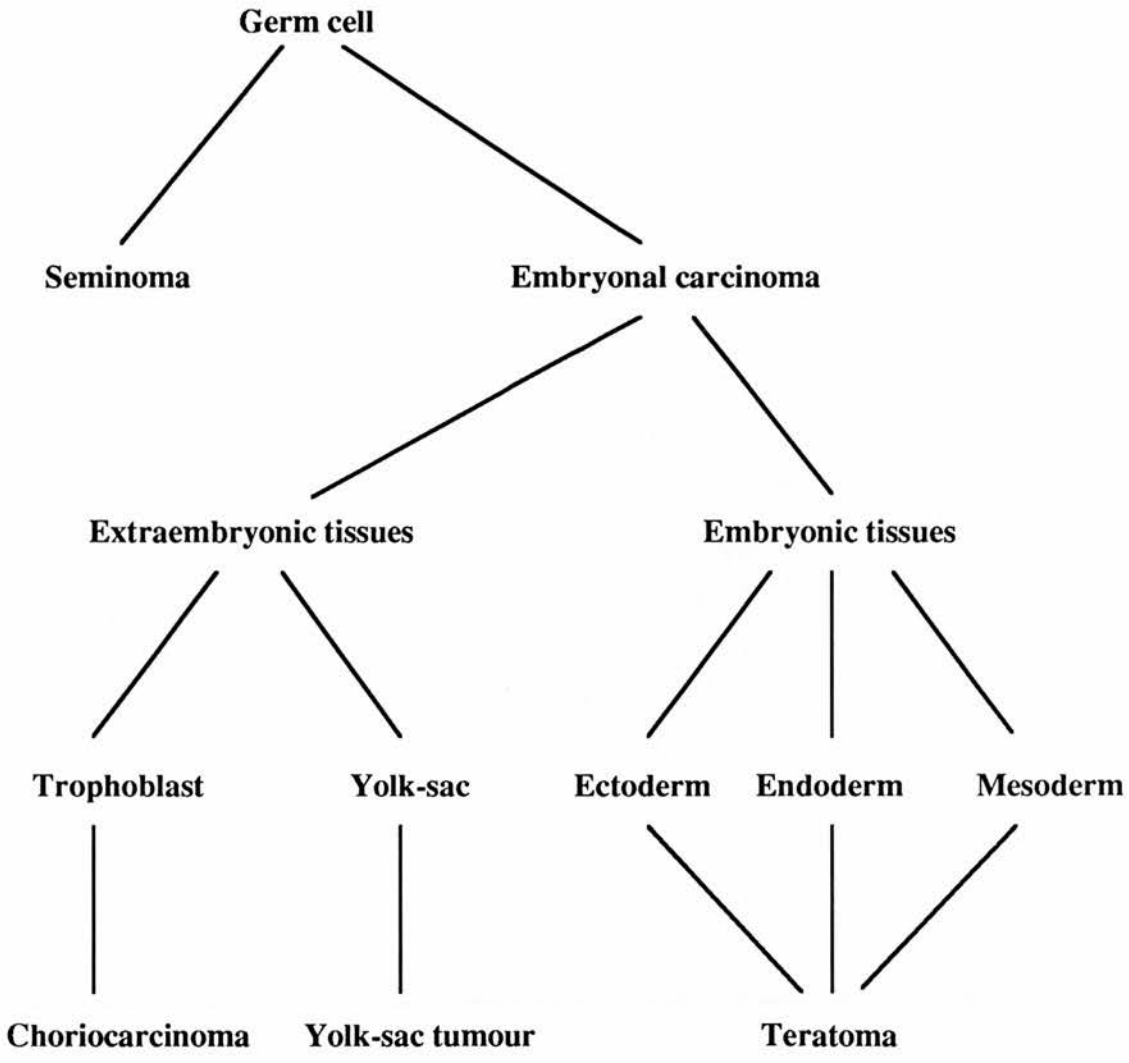


Figure 10 - Classification of Germ Cell Tumours.
Germ cell tumours arise from the germ cells of the testis. A germ cell tumour may be classified primarily as a seminoma or an embryonal carcinoma. In the latter case the embryonal carcinomas can be subdivided due to them having the ability to differentiate in multiple directions, primarily to resemble extraembryonic or embryonic tissue. An embryonal carcinoma which takes on the appearance of embryonic tissue is classified as a teratoma.

1.11.1 Embryonal Carcinoma

Embryonal carcinoma or yolk sac tumour forms a network of undifferentiated embryonal tubular structure (Karamehmedovic *et al,* 1975). It is thought by some to belong to the germ cell tumour category and by others to represent a teratoma (Sabio *et al,* 1974; Brown, 1976).

1.11.2 Seminoma

Seminomas are the most common tumours of the testis. They generally occur in men of between 30 and 50 years of age. They occur more frequently in maldescended testes than in normal descended testes. During adulthood malignant tumours are approximately ten times more common in undescended testes than in scrotal testes (Jones and Campbell, 1976; Davies, 1980; Depue *et al,* 1983; 1984). However even after an undescended testis has been transferred into the scrotum it still has a predisposition to tumour growth. Histologically seminomas consist of clusters of cells. The cells are large possessing ill defined clear cytoplasmic edges and rather large round nuclei, giving a similar appearance to the germ cell from which they arose.

1.11.3 Teratoma

The term teratoma in this study is used to classify the tumours composed of tissues representing different germinal layers. They occur somewhat less frequently than seminomas and in a much younger age group. In this study the age group of the teratoma tissues investigated ranged from 14 to 33 years of age. In the past teratomas have been shown to be capable of producing hCG as well as progesterone and oestrogens. If the levels of hCG and oestrogen are high enough then this can manifest itself in the patient as gynaecomastia. Teratomas histologically can be seen to contain

keratinising tissue and nerves (Mostofi and Price, 1973). The usual microscopic pattern however is representative of a wide variety of tissues derived from all three germ cell layers; glial and retinal tissues are not uncommon. Mesodermal structures include muscle cartilage and bone. Numerous mitoses may be observed, but areas resembling embryonal carcinoma are rarely present. In adults, teratomas are almost always malignant whereas in adolescent males malignancy is not as common as they represent a different subgroup of teratoma tissues (pers. commun. Mr T. B. Hargreave). The group of testicular tumours represented in this study by teratoma consisted of both mature and immature teratomas as well as those with malignant transformation and those identified as illustrating embryonal carcinoma. Adolescent teratomas however can often behave like adulthood teratomas and consequently may often be malignant.

1.11.4 Leydig Cell Tumour

Leydig cell tumours are very rare and as the name suggests are composed of cells resembling Leydig cells. The tumours vary in size and are usually well separated from the rest of the testicular tissue. They have a firmer consistency than the testis and are usually unilateral and benign. Hormone production is often increased; high levels of plasma testosterone with low levels of gonadotropins (Wegienka and Kolb, 1967). In some cases other androgenic steroids such as androstenedione may be increased (Root *et al,* 1972; Wegienka and Kolb, 1967). One clinical indicator of a Leydig cell tumour is the level of 17α-ketosteroid excretion. Increased amounts of oestrogens may also be excreted in the presence of these tumours which in turn may become clinically manifest in the development of gynaecomastia or infertility which is represented in a low sperm count.

1.11.5 Carcinoma-in-situ

In 1972 a possible link between an abnormal germ cell pattern in seminiferous tubules and the subsequent development of germ cell tumours was described in the literature (Holstein *et al,* 1987; Skakkebaek, 1972; 1975). This finding led to the suggestion that the abnormal germ cells did in fact represent a carcinoma in situ (CIS). CIS cells are characteristically located in a single row inside seminiferous tubules of decreased diameter with thickened basal membranes. Usually Sertoli cells are the only other cells present in the tubules. CIS cells have a distinct appearance as they are much larger than normal spermatogonia.

In the last 18 years a considerable weight of evidence has confirmed the theory that CIS germ cells are precursors of all types of testicular germ cell tumours except the spermatocytic seminoma (Skakkebaek *et al,* 1987). It has also been found that in a study of men with CIS, 50% developed invasive tumours within 5 years of diagnosis. An identical figure was also found for CIS in the contralateral testes of men with unilateral testicular cancer who had not been given chemotherapy (von der Maase *et al,* 1986; von der Maase *et al,* 1987). Subsequently high risk groups have now been identified for CIS. These include men with unilateral testicular cancer patients with a history of cryptorchidism (Waxman, 1976), infertile men (Pryor *et al,* 1983), intersex patients (Muller and Skakkebaek, 1988) and patients with extragonadal germ cell tumours (Daugaard *et al,* 1987).

1.11.6 *In vitro* Models for Testicular Cancer

Relatively few human teratoma cell lines have been established *in vitro* and the characteristics of these are poorly understood. SuSa (Hogan *et al,* 1977) , Tera-1 and Tera-2 (Holden *et al,* 1977; Fogh and Trempe, 1975) and PA1 (Zeuthen *et al,* 1980) are four human teratoma cell lines which were established in the late 1970s. Human embryonal carcinoma cell lines GCT 27, GCT 35 and GCT 48 have more recently

been established by Pera *et al* (1987). The Tera-2 cell line was chosen particularly for this study because it expresses the EGF receptor (Engstrom *et al,* 1985).
It is a human embryonal carcinoma cell line (Tera-2) and was originally established in 1971 by Fogh and Trempe (1975). It was cultured from a lung metastasis from a primary testicular embryonal carcinoma. Engstrom (1986) not only performed ligand binding experiments but also cell proliferation studies performed under serum-free conditions. No effect was seen on proliferation of the cells although cell locomotion was found to be stimulated by EGF (Engstrom, 1986). When culturing in serum-free medium the cells had to be plated at a high density to survive because of a low plating efficacy while under the serum-free conditions. This could have in fact hindered any proliferative effect which EGF may have had on the Tera-2 cells because of the lack of space available for growth. The cells must be cultured in serum-free medium due to the multiple factors present in serum which are as yet unidentified (Kaplan *et al,* 1982).

The cell types present in the teratoma tumours vary greatly. Frequently they contain embryonal carcinoma, seminoma and cells that resemble those found in extra-embryonic foetal tissues, for example placental trophoblast and yolk sack. Less frequently, there are groups of cells that look like embryonic and adult tissues, such as muscle, cartilage and nerve (Mostofi and Price, 1973; Pugh, 1976). In the mouse it has been established that these multiple cell types arise from a single malignant pluripotential embryonal carcinoma cell (Kleinsmith and Pierce, 1964). This may also occur in human germ-cell tumours since cloned cell lines derived *in vitro* from such tumours can form a variety of cell types *in vivo* and *in vitro* (Andrews *et al,* 1983; Thompson *et al,* 1984). It is known that the Tera-2 cells possess an aneuploid karyotype with a near triploid number of chromosomes (Thompson *et al,* 1984). The cell line has also been found to differentiate into neuron-like cells in the presence of

retinoic acid (Thompson *et al,* 1984).

1.12 Summary

Interest in growth factors was greatly stimulated by the discovery that transformed cells generally have lower serum requirements than their normal counterparts. Subsequently the possibility of growth factor involvement in the growth of normal and transformed cells *in vitro* was investigated in the hope that not only would this shed some light on the control of normal cell growth but also cancerous cell growth. As previously stated EGF does indeed stimulate the proliferation of cultured cells, obtained from many different species (Carpenter and Cohen, 1979). It was therefore in this context that the main aims and objectives of this study were defined.

The aims of this study were therefore to :-

a) characterise and localise the EGF receptor on normal and cancerous human testicular tissue by biochemical and immunohistochemical techniques.

b) ascertain whether there was any relationship between hEGF, hTGF-α and androgen concentrations in normal and cancerous human testicular tissue.

c) characterise the EGF receptor on Tera-2 cells.

d) establish if the Tera-2 cells secreted EGF or TGF-α.

Materials and Methods Chapter 2

2.1 Materials

2.1.1 Radioactive Materials

i) $Na^{125}I$ (Specific Activity [S.A] 15-20 GBq/mmol)

ii) ^{125}I mEGF (S.A. 20-25 TBq/mmol)

iii) ^{125}I hEGF (S.A. 45-50 TBq/mmol)

iv) (1,2,6,7-^{3}H) Androstenedione (S.A. 3.0-4.1 TBq/mmol)

v) (1,2,6,7- ^{3}H) Dihydrotestosterone (S.A. 1.8-2.2 TBq/mmol)

vi) (1,2,6,7- ^{3}H) Testosterone (S.A. 3.0-3.9 TBq/mmol).

vii) [methyl ^{3}H] Thymidine (S.A. 74 GBq/mmol)

were purchased from Amersham International plc. (Berks, U.K.)

2.1.2 Growth Factors

Mouse Epidermal Growth Factor (mEGF)

Rat Transforming Growth Factor alpha (rTGF-α)

Snake Venom Nerve Growth Factor (vNGF)

Bovine basic Fibroblast Growth Factor (bFGF)

were purchased from Sigma Ltd (Dorset, U.K.)

Human Insulin-Like Growth Factor- I (hIGF-I) was purchased from Sera Labarotories, Sussex, U.K. and urogastrone (hEGF) was kindly donated by Dr H. Gregory, ICI, Macclesfield, U.K.

2.1.3 Hormones

Human Prolactin (hPrl)

Human Insulin (hI)

Human Luteinising Hormone (hLH)

Human Follicle Stimulating Hormone (hFSH)

Human Growth Hormone (hGH)

were generously donated by NIADDK, Bethesda, MD, USA.

Androstenedione (4-Androsten-3,17-dione)

5α-Dihydrotestosterone (17β-Hydroxy-5α-androstan-ol-3-one)

Testosterone (4-Androsten-17β-ol-3one)

were purchased from Sigma. Methenolone

(7,17β-dimethyl-19-nortestosterone) was purchased from Amersham.

2.1.4 Monoclonal Antibodies

The monoclonal antibody for the external domain of the human EGF receptor (EGF-R_1) was kindly donated by Dr M.D.Waterfield, Imperial Cancer Research Fund Laboratory, London (Waterfield *et al,* 1982). The anti-mouse EGF receptor monoclonal antibody (EGF-R) for the external domain of the receptor for use on paraffin sections was kindly donated by Dr F. Hay, Imperial Cancer Research Fund Laboratory, Oncology Group, Western General Hospital, Edinburgh who had previously purchased it from Oncor, MD, USA (Starkey *et al,* 1975). The third monoclonal antibody recognising the internal domain of the receptor (F4) was a gift from Dr W.J. Gullick of the Imperial Cancer Research Fund Laboratory Oncology Group, Hammersmith Hospital, London (Gullick *et al,* 1986).

2.1.5 Enzymes and Enzyme Inhibitors

i) Trypsin - Type 1 (bovine pancreas) 10,000 BAEE units per mg protein. One BAEE unit produces an A_{253} of 0.001 per min with BAEE as substrate at pH 7.6

ii) Hyaluronidase - Type 1-S (bovine testes) 300 units per mg solid. One unit produces color equivalent to 1μg glucuronic acid per hour from hyaluronic acid using 3,5-dinitrosalicylic acid to develop color. The enzyme randomly cleaves β-N-acetyl-hexosamine-[1- 4] glycosidic bonds in hyaluronic acid, chondroitin and chondroitin sulphates.

iii) Collagenase - Type 1A (Clostridium Histolyticum) >125 collagen digestion units per mg solid.

One unit liberates peptides from collagen equivalent in ninhydrin colour to 1.0 μmole of leucine in 5 hours at pH 7.4 at 37°C in the presence of calcium ions.

iv) Trypsin Inhibitor - Type 1-S (soybean)

1 mg of trypsin inhibitor will inhibit 1-3 mg of trypsin with activity of approximately 10,000 BAEE units per mg protein.

The above were all purchased from Sigma. All other reagents which were used were of analytical standard unless otherwise stated.

2.2.1 Specific Reagents

a) Iodination

i) Iodogen reagent was made by dissolving 1,3,4,6 Tetra chloro-3,6 diphenyl glycouril in methylene chloride to give a final concentration of 150 μg/ml.

ii) 0.05 M Phosphate buffered saline (PBS) [0.05 M $Na_2HPO_4.2H_2O$,

0.05 M NaH_2 $PO_4.H_2O$ and 0.9% NaCl (w/v) in distilled water] pH 7.4 and 0.1% BSA /PBS (w/v) were also prepared. Bovine serum albumin (BSA) (fraction V) was purchased from Sigma.

iii) Sephadex G50 was purchased from Pharmacia, Milton Keynes, U.K.

2.2.2 Homogenisation, Protein Estimation and Ligand Exchange Assay

i) Buffer A - 10 mM Tris-HCl, 1mM Ethylenediaminetetra-acetic acid (EDTA), 1 mM Ethyleneglycol-bis-(β-aminoethyl ether) tetra-acetic acid (EGTA), 0.25 M sucrose and 0.05 mM Phenylmethylsulfonyl fluoride (PMSF) in distilled water (pH 7.4). Note PMSF (0.1 mM) was prepared fresh each week in ethanol and the appropriate volume added to the required volume of buffer A to achieve a concentration of 0.05 mM.

ii) Buffer B - 10 mM Tris-HCl, 0.9% (w/v) sodium chloride and 0.1% (w/v) BSA in distilled water (pH 7.4).

iii) Bradford Reagent - 100 mg Coomassie Brilliant Blue G was dissolved in 50 ml of 95% (v/v) ethanol in distilled water. To this 100 ml of 85% (v/v) phosphoric acid in distilled water, was added. The solution was then made up to 1 litre with distilled water and thoroughly mixed before being filtered through Whatman filter paper.

iv) 10% and 20% Polyethylene Glycol (PEG) (w/v) [mw 8000, Sigma] in buffer B.

2.2.3 Crosslinking and Electrophoresis

The procedure of sodium dodecyl sulphate-polyacrylamide gel electrophoresis (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 (Protean-II slab electrophoresis cell, Bio-Rad Laboratories Ltd, Watford, U.K.). A molecular weight standard kit with molecular weight markers of 45, 66, 97.4, 116 and 205 kDaltons was purchased from Sigma.

Buffers:

i) 1 mM Disuccinimidyl suberate (DSS) [Pierce and Warriner, Chester, U.K.] dissolved in 5% (v/v) Dimethyl sulphoxide (DMSO) in H_2O.

ii) Sample Buffer - 4% (w/v) Sodium dodecyl sulphate (SDS), 10% (v/v) glycerol, 5% (v/v) 2β-mercaptoethanol, 0.25% (w/v) Bromophenol Blue R in 0.06 M Tris-HCl Buffer (pH 6.8).

iii) 30% (w/v) Acrylamide- 29.2 g acrylamide and
0.8 g N'N'-Bis-methylene-acrylamide dissolved in 100 ml distilled water.

iv) Running Buffer - 0.5% SDS (w/v) in 25 mM Tris-HCl and 0.3 M glycine (pH 8.3).

v) Preparation of Gels

4% Stacking Gel (20 ml)

30% Acrylamide/Bis (2.6 ml)

0.5 M Tris (pH 6.8) (5 ml)

10% (w/v) SDS (200 µl)

Distilled water (12.2 ml)

TEMED (20 µl)

10% Ammonium persulfate (freshly prepared) (100 µl)

7.5% Seperating Gel (100 ml)

30% stock Acrylamide/Bis (25 ml)

1.5 M Tris Buffer (pH 8.8) (25 ml)

10% (w/v) SDS (1 ml)

Distilled water (48.5 ml)

TEMED (50 µl)

10% Ammonium persulfate (freshly prepared) (500 µl)

v) Staining solution - 0.1% Coomassie Brilliant Blue R 250 (w/v) in 20% (v/v) methanol and 10% (v/v) acetic acid in distilled water.

vi) Destaining solution - 20% (v/v) methanol and 10% (v/v) acetic acid in distilled water.

2.2.4 Immunohistochemistry

The avidin and biotin blocking reagents were purchased as a kit from Vector (Peterbrough, U.K.).

i) Tris Buffered Saline (TBS) - 0.9% (w/v) sodium chloride in 50 mM Tris-HCl (pH 7.6).

ii) Michaelis Veronal Buffer - 0.14 M sodium acetate trihydrate, 0.14 M sodium barbitone, 0.04% (v/v) formaldehyde in distilled water.

iii) Streptavidin alkaline phosphatase substrate (ICN Biomedicals, High Wycombe, U.K.) /chromagen in Michaelis Veronal Buffer - 1.3 mM Fast Red ITR (Sigma), 1.0 mM levamisole, 0.75 mM Napthol ASBI phosphate (Sigma) in Michaelis Veronal Buffer.

iv) Glycerine jelly

13% (w/v) gelatine in distilled water, diluted 1:1 (v/v) in glycerol with the addition of six crystals of thymol.

2.2.5 Epidermal Growth Factor Radioimmunoassay.

Normal rabbit serum (NRS) and donkey anti-rabbit antiserum (DARS) were obtained from the Scottish Antibody Production Unit, (Carluke, Lanarkshire, U.K.).

Rabbit anti human EGF was kindly donated by Dr H. Gregory, ICI, Macclesfield, U.K.

i) TEP Buffer - 50 mM Tris-HCl, 10 mM EDTA, 5 µM PMSF (pH 7.2)

(A concentrated solution of PMSF was prepared fresh each week in ethanol and the appropriate amount added accordingly).

ii) RIA Buffer - 0.5% (w/v) BSA in 0.04 M Phosphate Buffer, 0.15M sodium chloride, 0.01 M EDTA (pH7.2).

iii) Digestion Buffer - 1 mg trypsin inhibitor, 225 x 10^3 units collagenase, 125 x 10^3 units hyaluronidase and 1.2 g bicarbonate in 100 ml RIA buffer.

2.2.6 Transforming Growth Factor-α Radioimmunoassay.

Rat Transforming Growth Factor-α Radioimmunoassay kit (rTGF-α RIA) was obtained from Peninsula Laboratories (Merseyside, U.K.). In using the rTGF-α RIA kit to detect hTGF-α an assumption was made regarding the crossreactivity of the rTGF-α antibody for hTGF-α. Experiments by Peninsula to determine the IC50 values for rTGF-α and hTGF-α had shown that the antibody cross reacted 32% with hTGF-α compared to 100% with rTGF-α (Moores pers. comm. 1990). However no data was presented which showed that the antibody bound to hTGF-α with the same affinity as it did to rTGF-α. The assumption therefore made in this study is that the the antibody binds with the same affinity to hTGF-α as it does to rTGF-α. In multiplying the concentration of TGF-α detected in tissue samples by approximately 3.3 the amount of hTGF-α present in the sample was calculated. Values which are quoted are therefore not absolute values until this assumption has been clarified. The experiment was not performed as part of this study due to the lack of finance available.

2.2.7 Steroid Radioimmunoassays.

Lyophilised polyclonal sheep anti-human androstenedione and dihydrotestosterone (DHT) antiserum were purchased from Guildhay antisera Ltd., Surrey, U.K. The lyophilised material was dissolved in 10 ml PBS Gel Buffer to give a nominal titre of 1:100. The solution was then stored in 1 ml aliquots at -20°C until required. The DHT antibody was used to detect both DHT and testosterone by employing the antibody at two different titres.

i) PBS-Gel Buffer - 0.1% (w/v) Gelatine in 0.01 M Phosphate buffered saline (PBS) [0.9% (w/v) NaCl and 0.1% (w/v) NaN3 in 0.01 M Na_2HPO_4 and 0.01M KH_2PO4] (pH 7.4).

ii) Dextran Coated Charcoal Buffer - 0.025% (w/v) Dextran and 0.25% (w/v) charcoal in PBS-Gel Buffer.

iii) A Triton X-14 scintillation cocktail was purchased from A.M.Vickers Laboratories Ltd. (Burley-in-Wharfedale,U.K)

2.2.8 Western Blot Analysis

i) Lysis Buffer- 10% (v/v) Triton-X100 in distilled water with the addition of 0.5mM PMSF in ethanol.

ii) Sample Buffer-10% (w/v) glycerol, 2% (w/v) SDS, 5% (v/v) 2β-mercaptoethanol and 0.0013% (w/v) bromophenol blue in 0.06 M Tris-HCl, pH 6.8.

iii) Transfer Buffer - 20% (v/v) methanol in 48 mM Tris-HCl, 39 mM glycine, pH 9.2. (Note- the buffer pH ranged from 9.0 to 9.4. If the pH did not fall in this range then the buffer was discarded as acid or base could not be added to the buffer. This was because the addition of ions present in the acid or base would aid the flow of the electric current in the transblotting apparatus and could possibly lead to scorching of the membrane).

2.2.9 Tissue Culture

Hydrocortisone-21-hemisuccinate, human insulin (tissue culture grade), human transferrin, O-phosphylanolamine, tri 3,3,5, iodo-L-thyronine, penicillin (10,000 IU/ml) and streptomycin (10,000 µg/ml) were all obtained from Sigma. Glutamine, nutrient broth, foetal-calf serum, RPMI 1640 media, Dulbeccos solution and trypsin/EDTA (5.0 g trypsin and 2.0 g EDTA in 1 litre 0.9% (w/v) sodium chloride in distilled water)were purchased sterile from Gibco, Paisley, Scotland. Mibolerone was purchased from Amersham International p.l.c. (Berks. U.K.)

i) 10% FCS RPMI media - 0.2% (v/v) Penicillin/Streptomycin solution, 10% (v/v) Foetal-Calf Serum and 1% (v/v) Glutamine solution in RPMI 1640.

ii) Serum-Free Medium (SFM) -

bovine insulin (10 µg/ml)

human transferrin (10 µg/ml)

hydrocortisone (1 µg/ml)

O-phosphylanolamine (50 ng/ml)

tri 3,3',5, iodo-L-thyronine (26 ng/ml)

glutamine solution 1.0% (v/v)

penicillin/streptomycin solution 0.2% (v/v)

in RPMI 1640.

iii) RPMI media -0.2% (v/v) Penicillin/Streptomycin solution and 1% (v/v) Glutamine solution in RPMI 1640.

iv) 0.5% (v/v) Foetal-Calf Serum (FCS) in RPMI media and 5% (v/v) FCS in RPMI media.

v) Trypan Blue solution - 5% (w/v) of trypan blue (Direct blue 14 - dye content 40%) in distilled water.

vi) Freezing Medium - 10% (v/v) Dimethylsulphoxide (DMSO) in RPMI 1640, sterilised by passing it through an Acrodisc 25 µm filter (Gelman Science Northampton, U.K.)

2.3 METHODS

2.3.1 Iodination of Mouse EGF (mEGF).

The iodogen method first described by Fraker and Speck (1978) was used to radiolabel mEGF with ^{125}I. Briefly, iodogen mixture (200µl) was placed in an eppendorf tube in order to coat the inside of the vessel. The excess iodogen was then evaporated off by placing the tube under a fine stream of nitrogen gas. A number of tubes were treated in this way, then wrapped in foil and stored at -20°C for later use.

Mouse EGF [10µg in buffer B (100µl)] was placed in the eppendorf tube along with $Na^{125}I$ (5 µl, 18.5 GBq/mmol). The mixture was gently agitated and the reaction allowed to proceed for at least 20 min. The solution was then purified on a Sephadex G-50 column chromatogram (1 x 46 cm). The Sephadex was pre-soaked in 0.05 M PBS overnight prior to packing the column and once packed the column was equilibrated with 0.1% (w/v) BSA in 0.05 M PBS immediately prior to iodination. 1 ml fractions were collected over a 2-3 hour period every 2 min using a Pharmacia Fraction Collector Frac 300 (Figure 11) and were counted in a Packard Crystal 2 Multidetector Gamma System Model 5412, along with the radioactivity still bound to the reaction vessel, the administering pipette tip and the column. The experiment was performed at room temperature. The specific activity of the compound could then be calculated as follows:-

2.3.2 Determination of the Specific Activity of ^{125}I mEGF and the Percentage of ^{125}I Bound to mEGF.

The formula used in calculating the specific activity of the radiolabelled peptide was as follows:-

The amount of radioactivity used to combine with 10µg mEGF was

(the decay factor) D x T (the total radioactivity) = M (the maximum theoretical activity of mEGF).

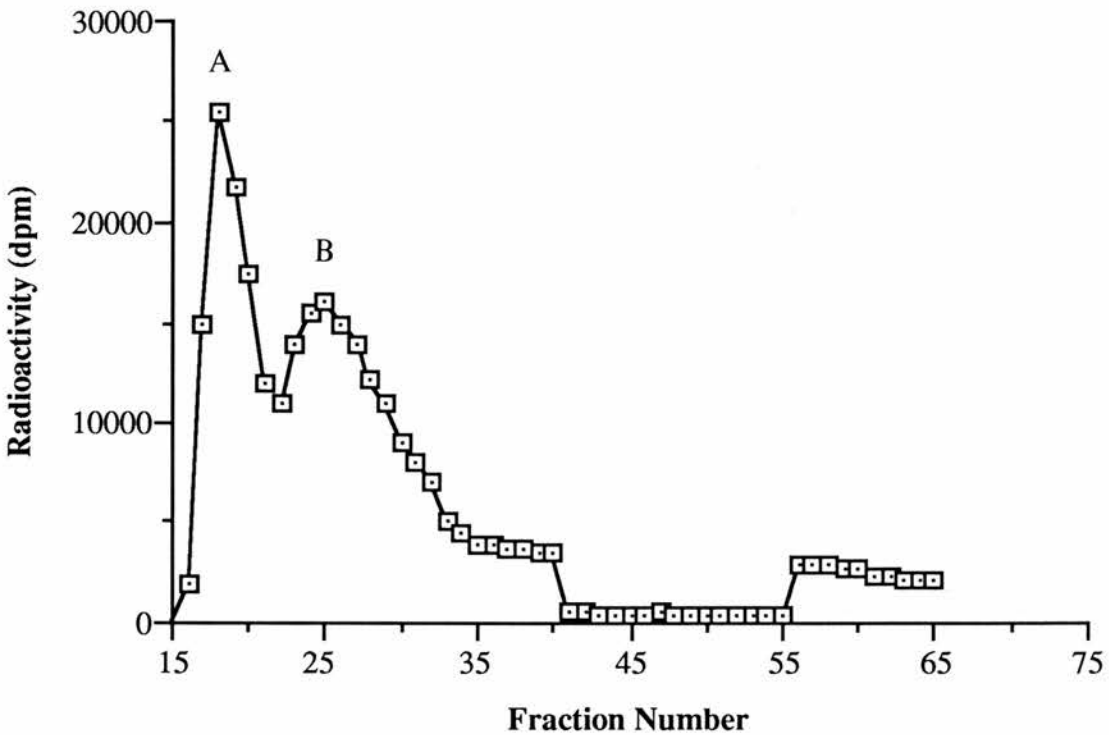


Figure 11 - Chromatography labelled EGF.
Mouse EGF was radiolabelled with Na 125 I, using the iodogen method described by Fraker and Speck (1978). Fractions of eluted product from the G50 Sephadex packed column (1 x 46 cm) were collected after an intitial run out period of 45 min, at 3 min intervals over a 150 min period. Two peaks of radioactivity were eluted at approximately fraction 18 (peak A) and fraction 25 (peak B).

The actual amount of radioactivity used in the experiment was calculated by measuring the radioactivity of:-

a) the reaction vessel = a

b) the pipette tip = b

c) the column = c

d) the eluted fractions = d [The radioactivity eluted from the column appeared in two peaks A (d1) and B (d2)]

The total amount of radioactivity (e) used in this experiment was therefore a+b+c+d= e

Therefore the specific activity of Peak B which was shown by Maddy *et al* (1987) to produce the optimum specific binding of ^{125}I mEGF to its receptor was

$$\frac{d2}{e} \times M = V \text{ Bq}/\mu g$$

It is necessary then to convert Bq to cpm (0.84 was the efficiency of the gamma counter used and there are 60 dpm in 1 Bq), therefore,

S.A.= V x 60 x 0.84 = W cpm/μg mEGF

Therefore in 200,000 cpm (100μl) there must be $\frac{1}{W} \times 200{,}000 = Y$ μg of mEGF

200,000 cpm was shown by Maddy *et al* (1987) to produce sufficient specific binding for counting.

The molarity of Peak B therefore = $\frac{Y \times 10{,}000}{6045} = Z$ nM MW of mEGF = 6045

The percentage of ligand bound was also measured using the TCA method described by

Fraker and Speck (1978). Briefly, labelled mEGF (200,000 cpm in 100 µl buffer B) was mixed with TCA [20% (w/v) in distilled water, 1 ml] in a test tube previously soaked in 0.1% (w/v) BSA in distilled water and spun down at 1,780 g for 20 min. This was followed by aspiration of the supernatant. TCA [10% (w/v) in distilled water, 1 ml)] was then added and the tube vortexed and spun at 1,780 g for 20 min before aspirating off the supernatant and the remaining pellet counted in the Gamma counter. The percentage of mEGF bound to ^{125}I was then calculated as described below :-

$$\frac{\text{final cpm}}{200{,}000 \text{ cpm}} \times 100 = \text{approximately } 50\% \text{ (n=6)}$$

In using the method described in section 2.3.1 for radiolabelling mEGF an adequate supply of radioactive mEGF was never produced and consequently ^{125}I mEGF was purchased from Amersham Ltd as well. Eventually it was decided to purchase all ^{125}I mEGF from Amersham with its specific activity pre-evaluated.

2.4 Characterisation of the EGF Receptor on Human Testicular Tissue

2.4.1 Tissue Collection and Storage

Tissue was collected from theatre immediately after surgical removal from the patient. It was transported to the laboratory on ice and washed in ice cold 0.9% saline (w/v) in distilled water before being cut into 1 cm^3 pieces. The tissue was then used in either biochemical studies or in immunohistochemical assessment. In the former case the tissue was either used fresh (within 30 mins of its arrival in the laboratory), or snap frozen in liquid nitrogen and stored at -70^{o}C until required. Two cross sections of the tissue were always placed in formalin for obtaining a pathology report or on some occasions for immunohistochemical studies. On obtaining the pathology report only tissues which did not show gross atrophic changes or excessive inter-tubular fibrosis were used.

2.4.2 Homogenisation of Human Testicular Tissue

The procedure adopted in this study was similar to that employed by Maddy *et al* (1987) and was performed at 4^{o}C to ensure minimum degradation of the receptor. Approximately 3-5 g of tissue was taken and finely chopped until it resembled a pulp. This was then homogenised in buffer A (3 equal volumes) using a Ystral homogeniser (Scottish Scientific Instruments Centre Ltd, Edinburgh, U.K.) for two periods of 20 sec and 15 sec at position 6 with a 2 min cooling interval. The crude homogenate was then filtered through a metal strainer and the filtrate placed in a 10 ml capacity polycarbonate centrifuge tube (Sorvall, Du Pont, Stevenage, U.K.). Ultracentrifugation was carried out at 105,000 g for 40 min and the pellet obtained was resuspended in buffer A (3 equal volumes) and homogenised in a glass dounce homogeniser using 15 strokes of a loose fitting pestle. A second ultracentrifugation was then performed at 105,000 g for 20 min and the resultant pellet (the particulate fraction) was resuspended in buffer B (2 vols) and homogenised using a tight fitting pestle. The protein concentration of the particulate fraction was then either adjusted to 2 mg/ml before freezing in liquid nitrogen and stored at -70^{o}C or alternatively used immediately and the protein concentration determined later. All preparations of particulate fractions were treated in this way unless stated otherwise.

Note- In studies in which human testicular tissue was used experiments were performed in duplicate, employing three tissue samples obtained from three different patients unless otherwise stated.

2.4.3 Subcellular Fractionation

Subcellular fractions were prepared by the method described by Leake *et al* (1983) with some modifications. The crude homogenate was prepared according to the method in section 2.4.3 and then spun at 800 g for 20 min to obtain a crude heavy pellet. The supernatant from this spin was then recentrifuged at 15,000 g to obtain the mitochondrial

pellet. The resultant supernatant was then respun at 105,000 g to yield a microsomal pellet and the final supernatant which corresponded to the cytosolic fraction. All pellet fractions were subsequently reconstituted in buffer B and where necessary the protein concentration adjusted to 1 mg/ml. The aliquots were then frozen in liquid nitrogen and stored at -70^{o}C for future use.

2.4.4 Protein Determination.

The protein concentration of the particulate fraction was measured by the Bradford (1979) method. Concentrations of BSA over a range of 0.25-1.0 mg/ml in PBS (80µl) were employed and mixed with Bradford Reagent (5 ml). The mixtures were first vortexed and allowed to sit for 10 min before measuring their absorbance at 595 nM with a Pye Unicam spectrophotometer (model 5P6-550) against a PBS blank. The absorbance of the particulate fraction was measured at the same time in the same way except that prior to being added to the Bradford reagent the particulate fraction was boiled for 5 min with an equal volume of sodium hydroxide (2.0 M) in order to disrupt the proteins. A standard curve of absorbance of the Bradford Reagent at 595 nm against the concentration of BSA was constructed (Figure 12). The protein concentration of the particulate fraction was then estimated by reading form the standard curve the concentration of protein which was equivalent to the absorbance measured.

2.4.5 EGF Binding Studies Employing Human Testicular Tissue.

Initially a saturation study was performed to calculate the concentration of labelled mEGF required to saturate the EGF binding sites on human testicular tissue. 100µl aliquots of the particulate fraction were incubated with equal volumes of labelled mEGF (100µl) of concentrations ranging between 0.2-12.0 nM (3333-200,000 cpm) both in the presence and absence of 100-fold excess unlabelled mEGF (200 µl). In the absence of unlabelled mEGF

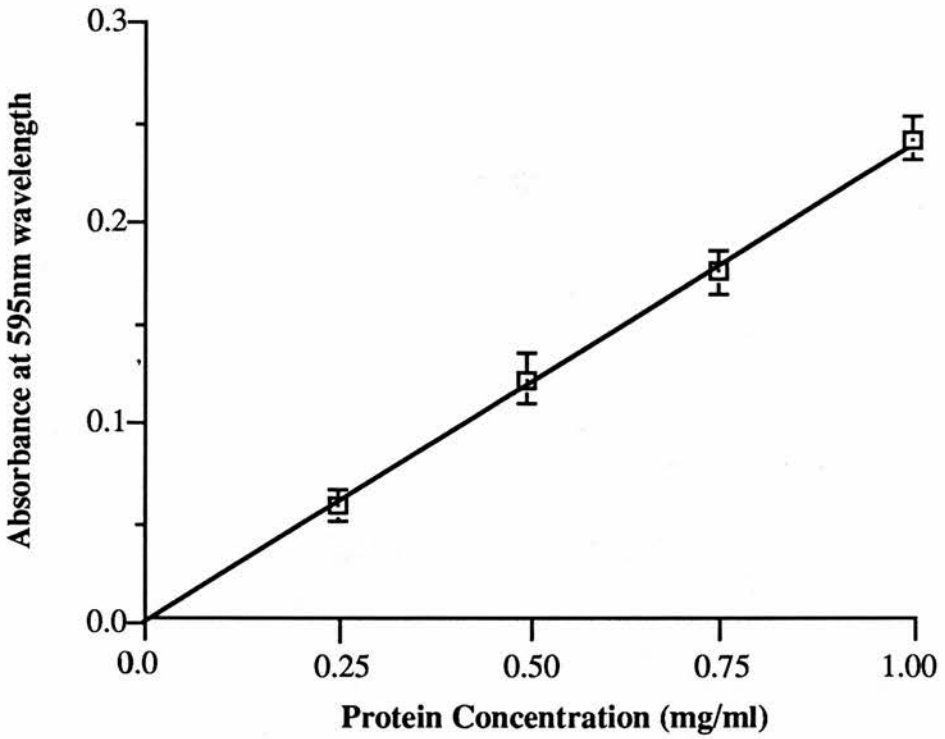


Figure 12
A standard curve of absorbance at 595 nM wavelength against protein concentration of BSA. Values represent mean data (n=3, ± SEM).

buffer B (200 µl) was added. The mEGF radiolabelled ligand which was used varied in its molarity (between 1.7 and 2.5 nM). Consequently, in order to achieve a higher molarity (for example 12.0 nM which was the maximum concentration employed in this study), unlabelled mEGF was added to the radiolabelled material. In doing this the specific activity of the radiolabelled material was reduced by a factor related to the concentration of unlabelled mEGF which was added. From this experiment the concentration of labelled mEGF required to saturate the EGF binding sites was calculated to be 8 ± 0.76 nM.

All the gamma emitting radioactive samples which were employed in this study were counted in the Gamma counter which was found to have 84 % efficiency. It was therefore necessary to convert the level of cpms measured by the counter to dpms by using the following equation.

$$\text{Efficiency (\%)} = \frac{\text{cpm}}{\text{dpm}} \times 100$$

2.4.6 Time and Temperature Studies

Binding of mEGF to testicular particulate fractions was performed by employing a modified version of the methods first described by Carpenter and Cohen (1975), Sainsbury *et al* (1985) and Mukku and Stancel (1985b).

Aliquots of the particulate fraction (100 µl) were incubated in BSA coated tubes with equal volumes of labelled mEGF (8 nM, 200,000 cpm) both in the presence and the absence of 50-fold excess unlabelled mEGF (200 µl). In the latter case buffer B (200µl) was added to make up the desired volume of 400µl. It was also used for all other dilutions.

Incubation of the particulate fraction was performed at 4°C, 25°C, 32°C and 37°C and over a time range of 0-3 hours to assess respectively the optimum temperature and incubation period for binding. The reaction was terminated by adding ice cold PEG (20% w/v in distilled water, 1 ml), vortexing the solution and leaving it to stand

for 10 min on ice before centrifuging at 1,780 g for 20 min. The supernatant was then aspirated off and PEG [10% (w/v) in distilled water, 1 ml] added to each tube. Again the mixture was vortexed and allowed to stand for 10 min before centrifuging at 1,780 g for 20 min. Finally the supernatant was aspirated off and the remaining pellet counted in a Gamma Counter.

2.4.7 The Effect of Protein Concentration on EGF Binding

The effect of protein concentration on EGF binding was investigated by incubating the particulate fraction (100 µl, at concentrations ranging from 0.5-8.0 mg/ml) with labelled mEGF (100 µl) in the presence and the absence of unlabelled mEGF (200 µl) as detailed in section 2.4.6 at 32^{o}C.

2.4.8 Distribution of EGF Binding in Subcellular Fractions

Subcellular fractions were obtained as described in section 2.4.3. Each fraction was taken out of storage and defrosted at 4^{o}C . A ligand exchange assay as described in section 2.4.6 was then performed on a 100 µl aliquot of each fraction to assess the EGF binding.

2.4.9 Displacement Studies with Unlabelled mEGF

The competition between labelled mEGF and unlabelled mEGF for the EGF receptor site on the particulate fraction was assessed. Unlabelled mEGF (200 µl) at concentrations ranging from 10 to 400 nM was incubated with labelled mEGF (100 µl, 8 nM, 200,000 cpm) and with the particulate fraction (100 µl) as described in the ligand exchange assay in section 2.4.6.

2.4.10 Saturation Analysis and Scatchard Plot

Saturation of the EGF binding site with labelled mEGF was performed by incubating the particulate fraction (100 µl) with increasing concentrations of labelled mEGF from 1.0-10.0 nM(100 µl, 20,000 cpm - 200,000 cpm). Binding was carried out at 32°C for 40 min. Non-specific binding at each concentration of radiolabel was determined in the presence of 50-fold excess unlabelled mEGF (200 µl). Total binding at each concentration of labelled mEGF was determined in the presence of buffer B (200 µl). Binding was carried out at 32°C for 40 min and the specific binding data analysed by a computer programme (Munson and Rodbard, 1980) to yield the dissociation constant (Kd) and the number of specific binding sites.

For the Rodbard and Munson (1980) computer programme the S.A. of the radioactive ligand used had to be calculated in dpm/mole, therefore,

the specific activity A Bq/µg = A x 60 dpm/ µg

$$\text{Number of moles in 1 µg mEGF} = \frac{1 \times 10^{-6}\text{g}}{6045} = 1.65 \times 10^{-10} \text{ moles}$$

$$\text{Therefore dpm/mole mEGF} = \frac{A \times 60}{1.65 \times 10^{-10}}$$

If unlabelled mEGF is added to Amersham ^{125}I EGF to achieve a certain molarity, the S.A. of the radioligand will be reduced as previously discussed in section 2.3.2.

The application of Rodbard and Munsons computer programme 'Ligand' is discussed in more detail in section 2.20.2.

2.4.11 Competition Studies

The specificity of the receptor for EGF was examined in the presence of the following competitors: rTGF-α, hIGF-I, bFGF, vNGF, hI ,hPrl, hLH and hFSH at a concentration

50-fold in excess of the ^{125}I mEGF employed. The methodology of the radioligand exchange assay in section 2.4.6 was used substituting the aforementioned peptides for unlabelled mEGF.

2.4.12 Effect of pH on Specific EGF Binding

Buffer B was used at a pH of 7.4 to reconstitute the particulate fraction as well as for diluting all the other ingredients used in the ligand exchange assay. In order to study the effect of pH on the specific binding of radiolabelled mEGF to the particulate fraction, the pH of buffer B was varied over the range 6.8-8.0 and the ligand exchange assay described in section 2.4.6 performed on three different particulate fractions at 32°C for 40 min.

2.4.13 Effect of Heat and Trypsinisation on Specific EGF Binding

Particulate fraction preparations were either preheated at 4°C, 45°C, 60°C and 80°C for 15 min or preincubated with and without 0.05% (w/v) trypsin in buffer B for 30 min at 32°C, prior to use in the ligand exchange assay. In the case of treatment with trypsin the particulate fraction mixture had to be washed once in buffer B (1 ml) to remove the excess trypsin and then spun down at 1,780 g before aspirating off the supernatant. The resultant pellet was then reconstituted in buffer B to form a homogenate and the protein concentration adjusted to 1mg/ml before being used in a radioligand exchange assay as described in section 2.4.6.

2.4.14 Effect of Storage at -70°C on Specific EGF Binding

The possible effect of storage at -70°C on EGF binding in tissue and in the particulate fraction was investigated. The specific binding of EGF on 3 different samples obtained from three different tissues was assessed when they were fresh and then at various intervals of storage at -70°C by homogenising them and performing a ligand exchange assay on the resultant particulate fractions. The time of storage of the tissue prior to

homogenisation varied between 0 and 6 months. The effect of storage at -70°C on the particulate fraction was also investigated over a two week period by similarly performing a radioligand exchange assay on aliquots of the particulate fraction to measure the specific binding of EGF.

2.5 Molecular Characterisation of the EGF Receptor: Affinity Labelling and Crosslinking of the EGF Receptor followed by Electrophoresis (SDS-PAGE) and Autoradiography.

Particulate fraction preparations containing 4-6 mg protein/ml were incubated with labelled mEGF (100 µl, 8 nM, 200,000 cpm) at 32°C for 40 min both in the presence and absence of unlabelled mEGF (200 µl, 400 nM). At the end of the incubation each mixture was spun down at 1,780 g for 10 min and the supernatant aspirated off. Disuccinimidyl sulphate (DSS; Sigma; 20 mM, 100 µl) in 5% DMSO (v/v) in distilled water was added to each pellet. The subsequent mixtures were then vortexed and allowed to stand for 20 min at room temperature before the sample buffer (100 µl) was added.The suspensions were then boiled for 5 min and spun down at 500 g for 5 min at 25°C . This enabled dissolution of the receptor and facilitated the removal of undissolved complexes. The resultant supernatants were then taken up with a Hamilton syringe and loaded onto a 7.5% SDS polyacrylamide gel (0.75 mm thickness, 16 x 16 cm area) as well as a solution of standard molecular weights (SDS-6 mix; Amersham) which prior to loading onto the gel had been heated at 32°C for 3 hours. In total three different samples were typically employed for each electrophoretic run, each sample having been incubated in the presence and in the absence of unlabelled mEGF as described above. The gel was then allowed to run at 30 mA for 8 hours in a Bio Rad Protean II cell electrophoresis chamber while being simultaneously cooled. The gel was subsequently stained for 20 min with a "staining solution" containing Brilliant Blue and then washed with a "destaining solution" over a period of 15 hours.

The gel was finally rinsed in distilled water and dried on a Bio-Rad model 443 slab dryer connected to a IEC Lyoprep-3000 Freeze Drier. Autoradiography was performed at -70°C for 3-4 weeks by placing the dried gel in a cassette with 2 intensifying screens and a hyperfilm TM (Amersham). The hyperfilm was developed in Kodak Developer LX-24 (1:5.6 dilution in distilled water) and fixed in Kodak X-ray liquid fixer FX40 (1:5 dilution in distilled water).

2.6 Immunohistochemical Localisation of the EGF Receptor on Human Testicular Tissue

Immunohistochemistry was performed on both frozen and paraffin embedded sections, employing monoclonal antibodies to both the external (EGF-R1: frozen; EGF-R:paraffin) and the internal domains (F4:frozen/paraffin) of the EGF receptor, to ascertain on which cells within the testes the EGF receptor was being expressed and whether such receptors expressed both the internal and external domains.

i) Frozen Sections

Frozen sections were cut on a cryostat to a thickness of 5 microns and placed on a lysine coated slide (0.1% poly-L-lysine hydrobromide, m.w.150-300 kDa, Sigma). The sections were allowed to air dry for 10 min before being fixed in acetone for 20 min. After fixation the sections were used immediately or stored at -20°C. If they were to be used after storage at -20°C then the sections were allowed to reach room temperature prior to use. This avoided moisture penetrating beneath the section which in turn would later cause loss of the section during the washing procedure. Sections were first circled with a wax pen to reduce the amount of antibody required. They were then treated with an avidin blocking reagent for 20 min, followed by a biotin blocking reagent for 20 min, after which the slides were washed twice in Tris Buffered Saline (TBS) for 5 min. The appropriate monoclonal

antibody was then applied at varying concentrations to assess the optimum staining. In the case of the EGF-R1 monoclonal antibody, this was used at dilutions between 1:100 and 1:10 (v/v) in TBS. The F4 antibody was employed at dilutions between 1:8 and 1:3 (v/v) in TBS. It was noted from the titration experiments that the optimum EGF-R1 antibody dilution was 1 in 30 and that the optimum F4 antibody dilution was 1 in 5. The slides were incubated overnight in the monoclonal antibody solution at 4^{o}C. The following day sections were washed twice for 5 min in TBS and then treated with the biotinylated sheep anti-mouse immunoglobulins (1 in 200 v/v in TBS) for 30 min. The sections were washed once more and a streptavidin alkaline phosphatase substrate applied for 20 min. Figure 13 illustrates the step by step method previously sited. Finally the sections were washed in distilled water (1 min) and counterstained in haematoxylin and lithium carbonate and mounted in glycerine jelly. Photographs were taken of the slides within 3 months as the staining was found to fade over time.

ii) Paraffin Embedded Sections

A portion of the same tissue used in the frozen section study was also stored in formalin. By means of a multiple step procedure it was converted to a waxed paraffin block as detailed below.

The tissue was first trimmed to approximately 1.0 x 0.5 x 0.3 cm and placed in a Tissue TEK cassette. It was then processed overnight according to the following procedure in a Miles Scientific Tissue TEK VIP :-

a) 210 min in 40% formalin at 40^{o}C.

b) 50 min in 70% industrial methylated spirits at 40^{o}C.

c) 50 min in 80% industrial methylated spirits at 40^{o}C.

d) 50 min in 90% industrial methylated spirits at 40^{o}C.

e) 50 min in 95% industrial methylated spirits at 40^{o}C.

f) 120 min in absolute alcohol at 40^{o}C.

g) 120 min in xylene at 40°C.

h) 180 min in paraffin wax at 60°C.

Finally the processed specimen was placed in a steel embedding plate and covered in wax using a Tissue TEK 111 embedding wax machine before being placed on a cold plate to solidify.

Sections were then cut from the paraffin waxed block at a thickness of 3 microns using a Leitz 1512 Rotary Microtone and allowed to dry at 37°C for at least 3 hours. The sections were then dewaxed by placing them for two, 2 min periods in xylene followed by two washes in absolute alcohol and one in methanol. The sections were then washed in distilled water and trypsinised in a 50 ml solution of 0.01 M Tris HCl containing 1.8×10^6 ATEE units of trypsin and 9 mM calcium chloride at pH 7.6 for 10 min at 37°C. The staining procedure described above and in Figure 13 was repeated with the exception that the monoclonal antibody for the external domain of the receptor EGF-R was used at the recommended dilution of 1:10 of the stock solution.

For each staining experiment a "negative" control section was included in which the primary antibody was omitted. A "positive" control employing human prostate tissue was also examined. As well as looking at EGF receptor expression on "normal" testicular tissue received from patients who had undergone orchiectomy as first line of treatment for prostatic cancer, tissue from men who had undergone hormonal treatment as first line therapy followed by orchiectomy as second line therapy, were also examined. Sections of cancerous testicular tissue were also looked at to see if they expressed the EGF receptor. Foetal tissue from between 15 and 19 weeks gestation was also examined as well as biopsy samples from men who were undergoing an operation for the reversal of vasectomy. In the latter case the ages of the two patients from which the tissues were obtained, were 30 and 34 years respectively.

At least one section from each of the aforementioned samples was taken for

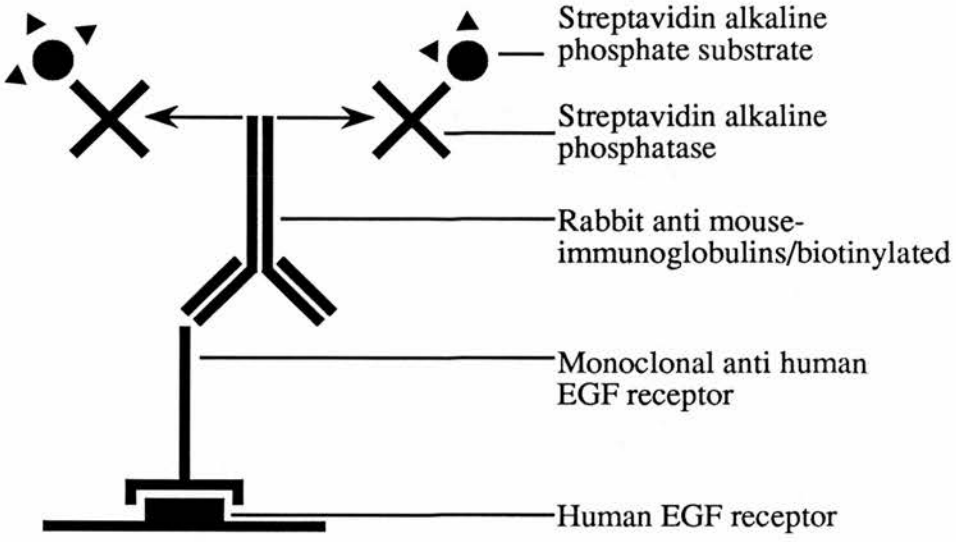


Figure 13
Diagrammatic representation of the final immunohistochemical EGF receptor streptavidin-biotin complex. The streptavidin alkaline phosphatase substrate binds to the fast red chromagen (▲) to produce the final red staining and subsequently identification of the EGF receptor.

histological assessment. The section was stained with Muller's haematoxylin and Putt's eosin for pathological evaluation.

2.7 Determination of Intratesticular EGF Concentrations.

2.7.1 Titration of the Antibody for Human EGF (hEGF)

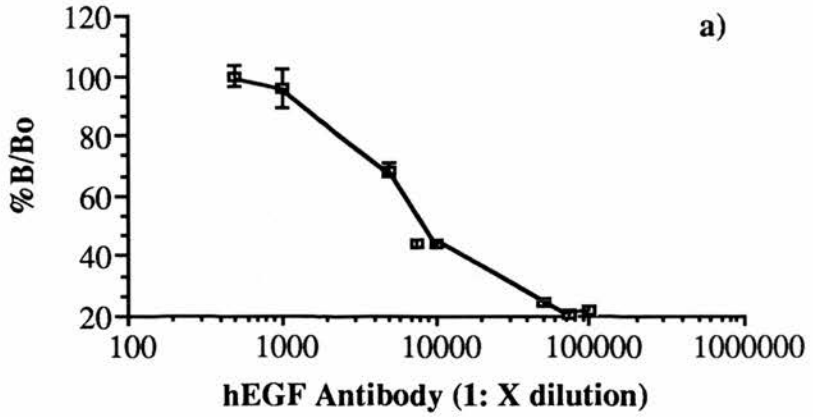
Titration of the rabbit anti urogastrone antibody to be used in the EGF radioimmunoassay (RIA) was performed. Dilutions of 1:500 to 1:100,000 were first employed using a standard concentration of hEGF of 7 ng/ml (Figure 14a). Following this a second, more accurate titration experiment over the concentration range 1:1,000 to 1:40,000 of the antibody was performed employing four different concentrations of hEGF in the range 0.04-20 ng/ml. Titration of the rabbit anti human EGF antibody demonstrated an optimum dilution of 1/10,000 (v/v) in RIA buffer of the stock solution (Figures 14b,c and d).

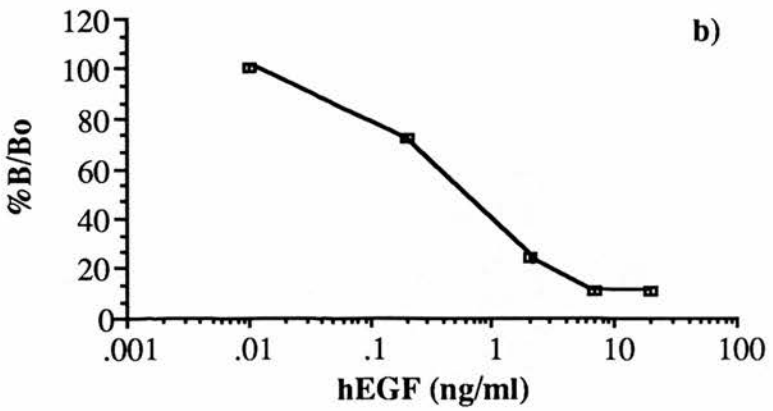
2.7.2 Construction of the Human EGF RIA Curve

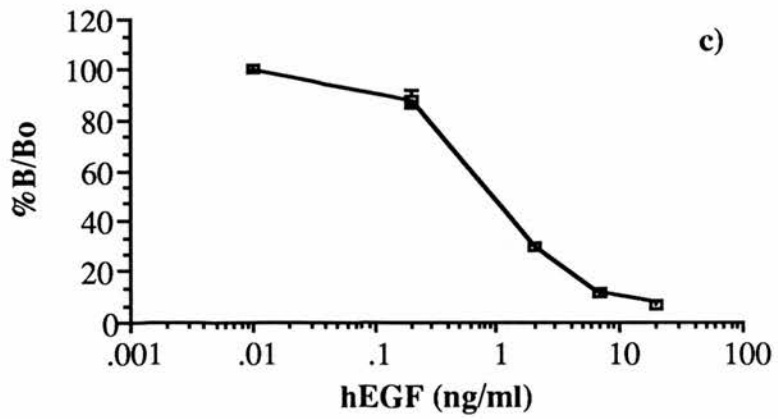
The method of Bynny *et al* (1972) was modified in developing the EGF RIA. All test tubes used in this procedure were presoaked in 0.1% BSA (w/v) in distilled water and dried to prevent non-specific binding. To the tubes was added human EGF (Urogastrone, 100 µl) at concentrations ranging from 0.04 to 20.0 ng/ml . Human EGF antibody (100 µl at a 1:10,000 dilution) and radiolabelled ^{125}I human EGF (100 µl, 50,000 cpm, S.A. 49 TBq/mmol, Amersham) were also added and the tubes vortexed and left for 2 hours at 37°C in a Teram (S13.4) shaking bath at 40 rotations per minute. A second antibody was then added [donkey anti-rabbit antiserum, 250 µl at a 1:20 (v/v) dilution in a 1:200 (v/v) dilution of normal rabbit serum in RIA buffer] to each tube and the tubes vortexed before being stored overnight at 4°C . Finally, distilled water (500 µl) was added to each tube and the contents vortexed and then spun at 1,800 g for 30 min.The supernatant in each tube was then removed by aspiration and the residual pellet counted in a Gamma Counter. Each concentration of urogastrone or unknown sample was run in duplicate or triplicate in order

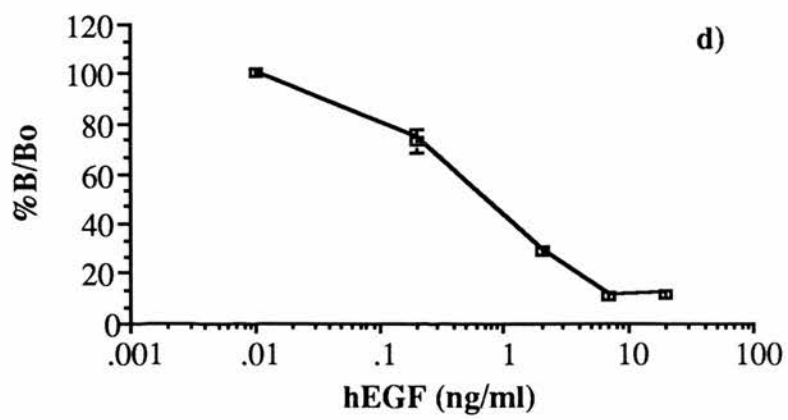
Figure 14

Titration of the antibody for hEGF was performed over the dilution range 1:500 to 1:100,000 employing a standard concentration of 7 ng/ml human EGF (**a**). The percentage of radioactivity bound in the presence of hEGF (B) in comparison to the radioactivity bound in the absence of hEGF (Bo) is plotted according to the dilution of antibody employed. Titration of the antibody for human EGF was further clarified by measuring %B/Bo at concentrations of hEGF from between 0.1 and 20 ng/ml employing dilutions of antibody from between 1:1,000 to 1:40,000 [(**b**)1: 1,000; (**c**) 1:10,000; (**d**) 1:40,000). Values represent mean %B/Bo (n=3)± SEM.


a)
%B/Bo
120
100
80
60
40
20
100
1000
10000
100000
1000000
hEGF Antibody (1: X dilution)


b)
%B/Bo
120
100
80
60
40
20
0
.001
.01
.1
1
10
100
hEGF (ng/ml)


c)
%B/Bo
120
100
80
60
40
20
0
.001
.01
.1
1
10
100
hEGF (ng/ml)


d)
%B/Bo
120
100
80
60
40
20
0
.001
.01
.1
1
10
100
hEGF (ng/ml)

to calculate a mean value. Two control tubes were also set up: Control 1 contained radioactivity (100 µl), 1^{o} Ab (100 µl), 2^{o} Ab (250 µl) and RIA buffer (100 µl) and was used to measure the radioactivity bound in the absence of hEGF (Bo). Control 2 contained radioactivity (100 µl) and RIA buffer (450 µl) and was used to measure the non-specific binding of the radioactivity to the tube (NSB).

The hEGF RIA curve was set up to detect concentrations of hEGF over a range of approximately 0.1 to 10 ng/ml. The inter assay coefficient was calculated to be 4.8% and the intra assay coefficient calculated to be 14.1%. The precision (% error) of the assay was consistently less than 10% for the linear portion of the RIA curve . The RIA was specific for hEGF as no cross reaction was apparent with either rTGF-α or mEGF. The sensitivity of the assay (90% B/Bo) was approximately 0.12 ng/ml and the IC50 value (achievement of half maximal stimulation) approximately 0.9 ng/ml. The standard EGF RIA curve was constructed by plotting %B/Bo against the concentration of hEGF employed to achieve the radioactivity measured (B) (Figure 15). Test samples were then assayed in exactly the same way by substituting 100 µl of the test sample with 100 µl of the standard concentration of hEGF. The concentration of hEGF required to achieve the level of radioactivity measured in the tube was then read from the standard curve.

2.7.3 Sample Preparation

The preparation of the sample was performed at $4^{o}C$. Tissue (approximately 6 g) was washed in sodium chloride (0.15 M, 5 ml) and blotted dry. It was then chopped to a pulp and 5 g weighed out into a large test tube. TEP buffer (3 vols) was added to the tube along with 1,000 cpm of labelled mEGF (100 µl in TEP buffer) to assess recoveries and the mixture vortexed for five 10 second periods. Recoveries were calculated following extraction by measuring the remaining radioactivity in the final extract and reporting it as a percentage of the initial amount added. The mixture was then homogenised by performing

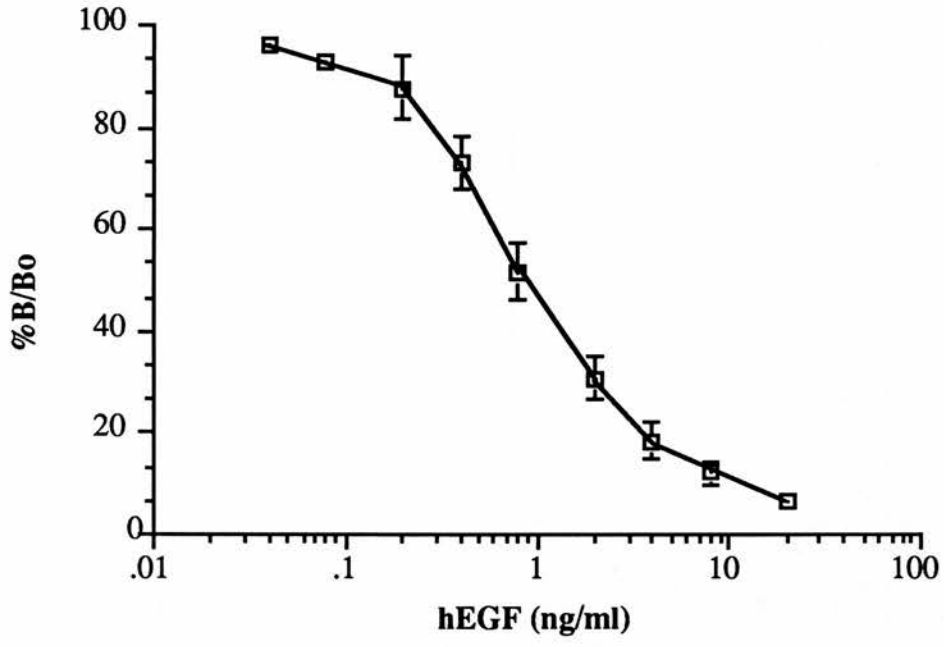


Figure 15
Standard curve for hEGF radioimmunoassay set up to detect concentrations of hEGF over the range 0.1 to 10.0 ng/ml. Points represent mean %B/Bo (± SEM, n=3).

four 15 sec bursts at speed 7 with a Ystral Homogeniser. Cooling periods of 2 min were allowed between each burst. The suspension was then placed in 2 polycarbonate centrifuge tubes and subjected to ultracentrifugation at 77,000 g for 30 min at 4°C. The resulting supernatant was decanted and kept at 4°C , while the pellet was resuspended once more in TEP buffer (1 vol) using a dounce homogeniser with a tight pestle (15 strokes). The suspension thus obtained was then respun for 30 min at 77,000 g. Finally the supernatant was decanted off and combined with the previous one and the pooled supernatants counted in the gamma counter to calculate procedural losses. The solution was then frozen and lyophilised and finally reconstituted in RIA buffer (600 µl), for use in the RIA.

2.7.4 The Effect of Digestion and Sonication on the Release of EGF from Human Testicular Tissue .

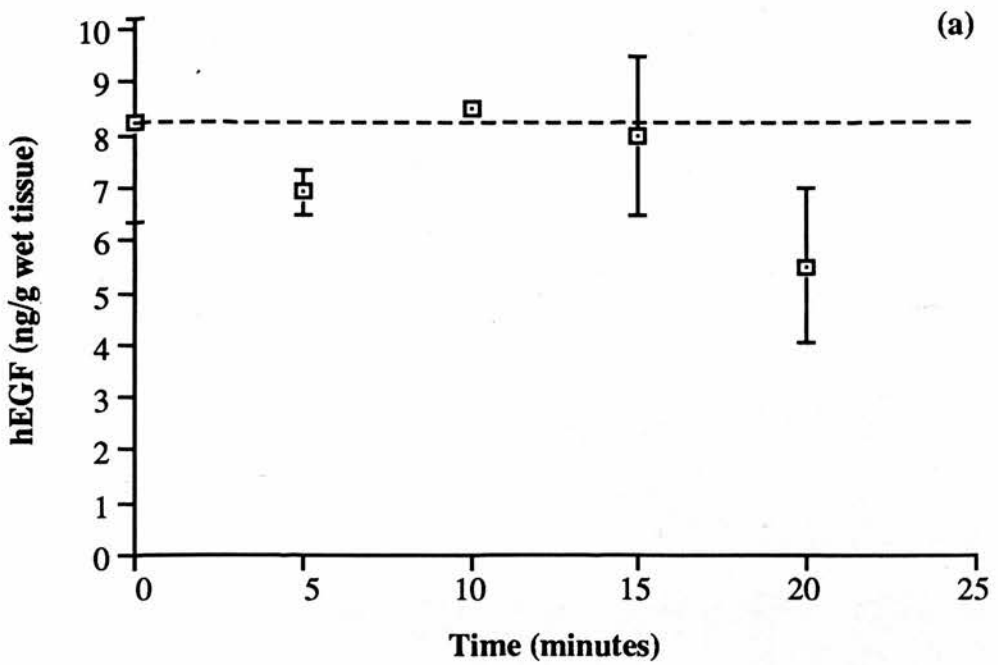
A digestion study was performed using three tissue samples. Following the homogenisation procedure described in section 2.7.3 the crude homogenates were incubated overnight with an equal volume of "digestion buffer" (section 2.2.5) and the following day spun down as described in section 2.7.3. For the sonication study, each of the three crude tissue homogenates were separated into five 1 ml samples and sonicated for either 0, 5, 10, 15 or 20 min using an A180G Sonicator (Ultrasonics Ltd, Manchester, U.K.) at a tuning level of 5 with the power also set at 5. The samples were then assayed for hEGF employing the RIA described in section 2.7.2.

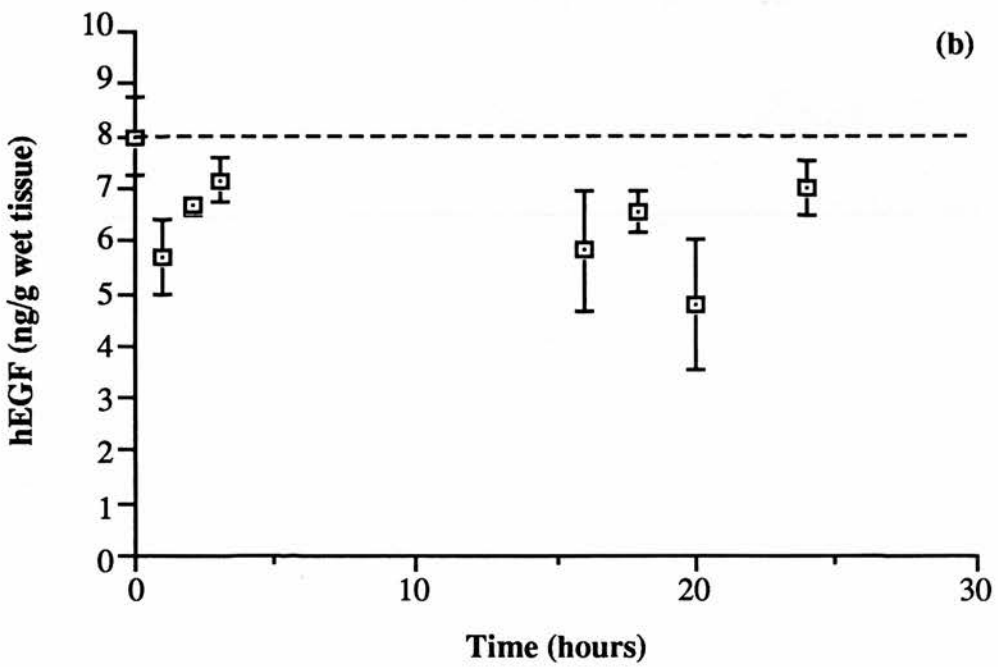
Digestion or sonication of the tissue was shown to be unnecessary. Figures 16a and 16b illustrate that no further liberation of hEGF from the three tissues examined was apparent after a sonication period of between 5 and 20 min or after a digestion period of up to 24 hours. Hence neither sonication or digestion procedures were included in the preparation of the RIA sample.

Figure 16

a) The mean concentration of EGF liberated from human testicular tissue (n=3, ± SEM) plotted against the time (minutes) which the tissue was sonicated for during preparation of the sample.
The dashed line indicates the mean concentration of EGF (n=3) detected in human testicular tissue without the samples having undergone sonication (*ie.* as indicated at time zero).

b) The mean concentration of EGF liberated from human testicular tissue (n=3, ± SEM) plotted against the time (hours) which the tissue was digested for during preparation of the sample.
The dashed line indicates the mean concentration of EGF (n=3) detected in human testicular tissue without the samples having undergone digestion (*ie.* as indicated at time zero).


(a)
hEGF (ng/g wet tissue)
10
9
8
7
6
5
4
3
2
1
0
0
5
10
15
20
25
Time (minutes)


(b)
hEGF (ng/g wet tissue)
10
9
8
7
6
5
4
3
2
1
0
0
10
20
30
Time (hours)

2.8 Determination of Intratesticular TGF-α Concentrations.

A radioimmunoassay kit for rTGF-α (Peninsula Laboratories) was used to detect and measure the presence of hTGF-α in human testicular tissues. The testicular samples were prepared as for the EGF RIA and as described in section 2.7.3. Because the antibody for rTGF-α cross reacts 32% with the hTGF-α (pers. commun. Moores, Peninsula Laboratories) this homology was used to calculate the amount of hTGF-α present in the tissue by first measuring TGF-α immunoreactivity present in the tissue and then multiplying the value by 3.3 (see section 2.2.6).

The antiserum to the rat TGF-α in the RIA kit was raised in rabbits and recognises both rat (100%) and human TGF-α (32%). Ascending rat TGF-α standards (100 µl, ranging from 10 and 1280 pg/100 µl) were placed in BSA coated tubes along with rat TGF-α primary antibody (100 µl). The contents of the tubes were vortexed, incubated overnight at 4°C, then 100µl aliquots of rat ^{125}I TGF-α (20,000 cpm, S.A. 35.8 MBq/mmol) were added and the vortexing/incubating procedure repeated once more. Two secondary antibodies [goat anti-rabbit IgG serum (100 µl) and normal rabbit serum (100 µl)] were then added to each tube, the tubes vortexed and the reaction allowed to proceed at room temperature for a further 2 hours before the addition of RIA buffer (0.5 ml). Finally the tubes were spun down at 1,780 g for 20 min. The supernatants were then aspirated off and the pellets counted in a Gamma Counter. Two controls were run; Control 1 which contained radioactivity (100 µl), 1° antibody (100 µl), 2° antibody (100 µl) and RIA buffer (600 µl) (Bo); control 2 which contained radioactivity (100 µl) and RIA buffer (800 µl). The %B/Bo was then plotted against the concentration of rTGF-α employed to achieve the measured radioactivity(B) to construct the standard RIA curve (Figure 17). The sensitivity of the assay (90% B/Bo) was calculated to be 20 pg/100 µl and the IC50 value 70 pg/100 µl.

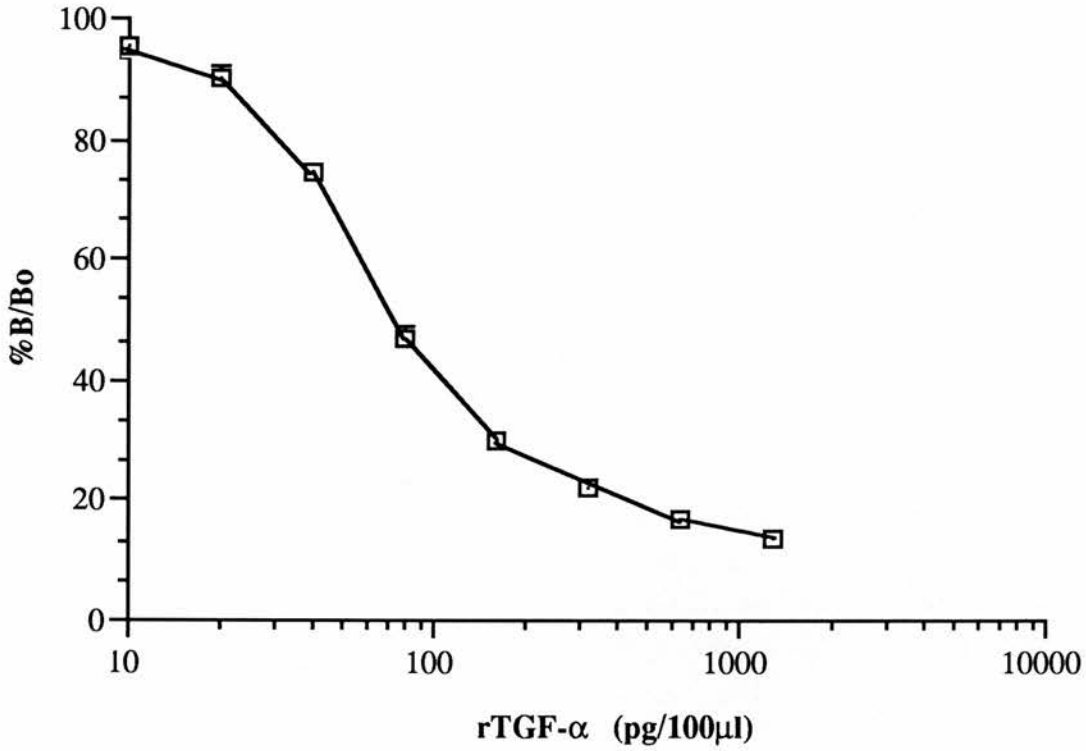


Figure 17

Standard curve for rTGF-α radioimmunoassay set up to detect concentrations of rTGF-α between approximately 20 and 300 pg/100μl. Points represent mean %B/Bo (± SEM, n=3).

2.9 Determination of Intratesticular Steroid Concentrations

2.9.1 Determination of the Quench Curve

Tritiated androgens were employed in the androgen RIAs. In order to convert the tritiated cpm to dpm a quench curve for the Packard Tri-Carb Liquid Scintillation Spectrometer (Model 3255) was evaluated. All tritium labelled radioactivity was measured in a Packard Tri-Carb Liquid Scintillation Counter by placing the radioactive solution (<500 µl) in a vial with 6 ml of scintillation fluid within the counter which in turn was set to the "adjustable" channel. ("Adjustable" indicating that both tritium and carbon radioactive isotopes could be measured on the same channel). The external standard ratio at each measurement was calculated at the same time as the radioactivity in cpms and plotted accordingly (Figure 18). The appropriate level of efficiency was then read from Figure 18 according to the external standard ratio which was measured and the dpms calculated accordingly. The efficiency of the machine was on average approximately 25% as the external standard ratio usually ranged between 0.5 and 0.6. For example a typical reading of 4,000 cpm would perhaps have an external standard ratio value of 0.6. According to Figure 18 such a value indicates that the tritium counter is working at 25% efficiency. Consequently the 4,000 cpm measured is equivalent to 16,000 dpm.

2.9.2 Titration of the Polyclonal Antibodies

Titration of the androstenedione and DHT antibodies for the appropriate steroid RIAs had previously been determined by Mr C. Goodman in these laboratories. However the appropriate titre of the testosterone antibody had not been determined. Briefly RIAs employing antibody dilutions of 1:5,000, 1:10,000 and 1:15,000 were performed employing concentrations of testosterone over the concentration range 7.8-500 pg/100 µl (Figure 19). A dilution of 1:10,000 was found to provide the optimum RIA curve and accordingly this was used in all future testosterone RIAs.

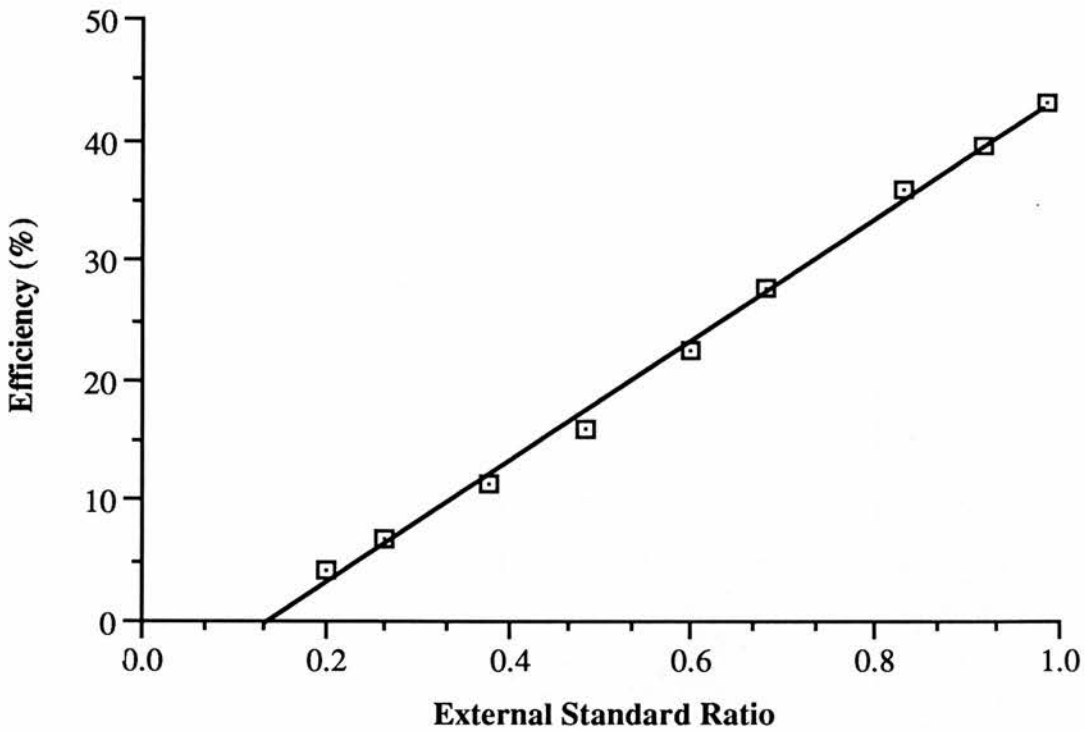


Figure 18
The relationship between the efficiency of the Hewlett Packard Tri-Carb counter (percentage) and the external standard ratio was plotted. This relationship was then used to calculate dpm from cpm for tritium labelled compounds.

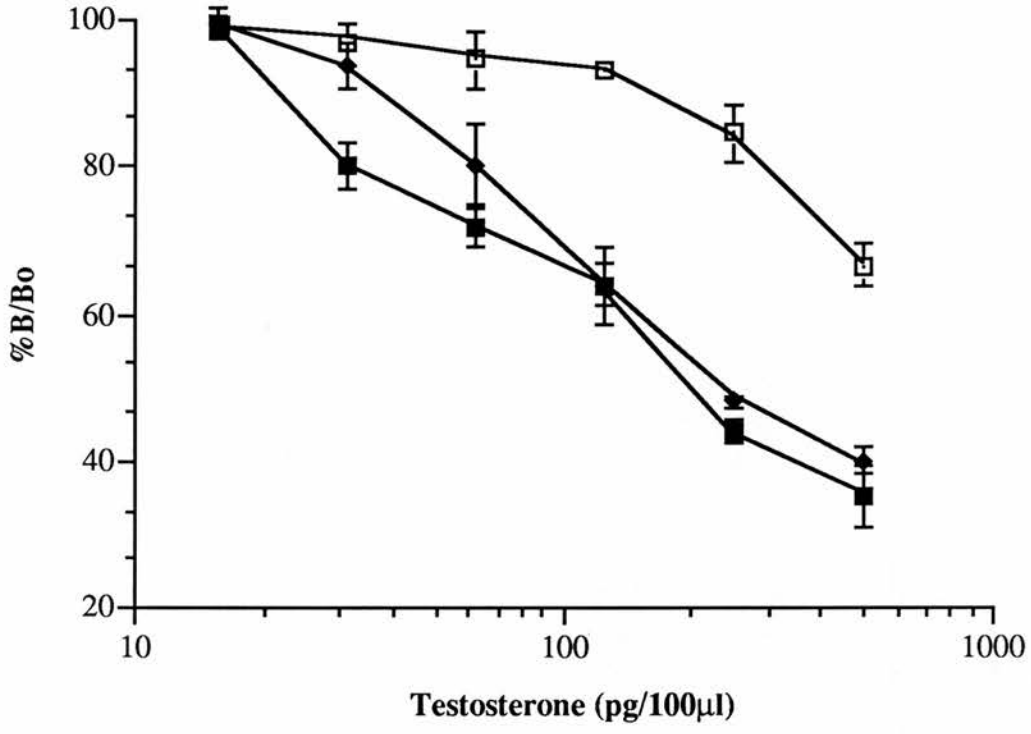


Figure 19
Titration of the antibody for testosterone was performed at three different dilutions of 1:5,000 (⊡), 1:10,000 (◆) and 1:15,000 (■) using testosterone concentrations over the range 7 to 500 pg/100µl. Values represent mean %B/Bo (± SEM, n=3) plotted against the concentration of testosterone employed.

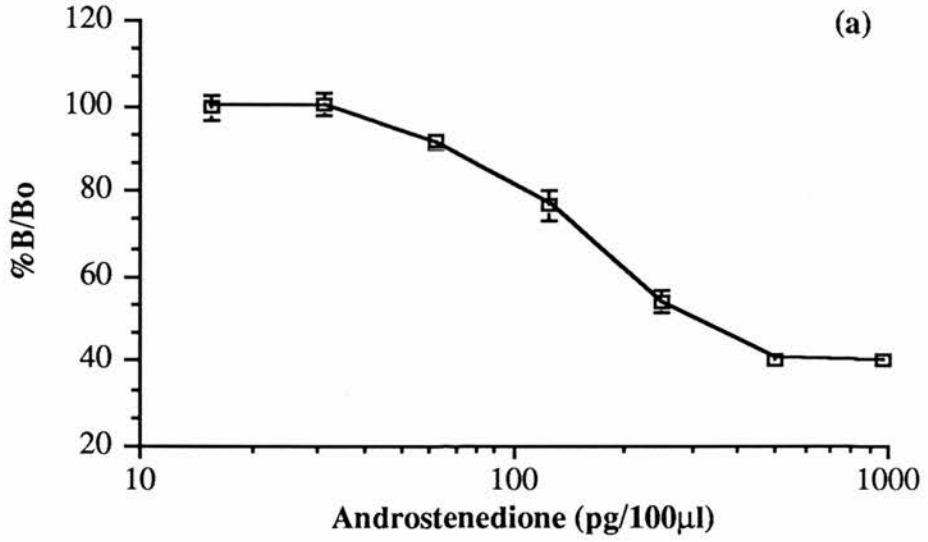
2.9.3 Steroid Radioimmunoassays.

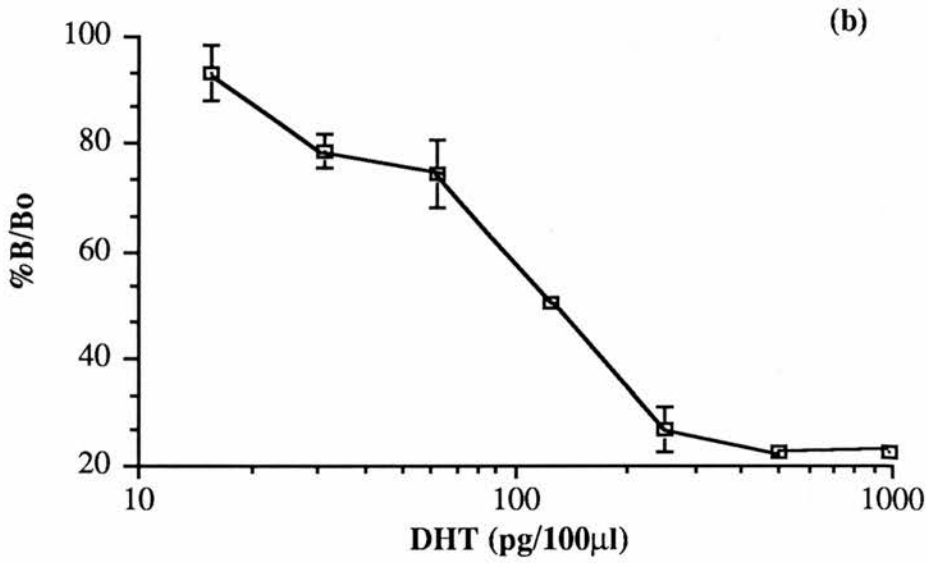
The RIA for assessment of steroids in human testicular was adopted from Abraham (1974) and Habib *et al* (1976). All the test tubes used in the RIA were coated in 0.1% BSA (w/v) in PBS-Gel to prevent binding of radioactivity to the tubes. A series of standards, varying in concentration from 7.8 to 500 pg/100 µl were then made by serially diluting a standard solution of non-radioactive steroid (100 µl, 17.48 nM) with RIA buffer (100 µl) at 4°C . After the addition of each freshly made standard to the required number of tubes (each run in at least duplicate), the antiserum was added (100 µl). Androstenedione antiserum was used at 1:10,000 dilution, DHT at 1:15,000 and testosterone at 1:10,000. The appropriate radioactive steroid (100 µl, 10,000 cpm) was then added to each of the tubes, the tubes vortexed and subsequently placed in a Teram S13.4 shaking water bath set at 40 revolutions per min and at a temperature of 37°C for 1 hour. Two control tubes were also used: Control tube 1 which contained radioactivity (100 µl), antibody (100 µl) and PBS-Gel buffer (100 µl) to measure the radioactivity bound in the absence of steroid (Bo); Control tube 2 which contained radioactivity (100 µl) and PBS-Gel buffer (200 µl) to measure non-specific binding of the radioactivity to the tube. The tubes were then removed and placed on ice for 10 min. Dextran coated charcoal (500 µl) was then added to each tube, the tubes vortexed and once more left on ice for 10 min. Finally the tubes were centrifuged at 1,800 g for 15 min and 500 µl of each supernatant was carefully pipetted into a plastic vial for counting. The count was then multiplied by 8/5 as only 500 µl of the 800 µl present in the tube had been removed for counting. It was then further modified by converting the cpms to dpms according to the tritium quench curve (Figure 18) and %B/Bo plotted against the concentration of the appropriate steroid employed.

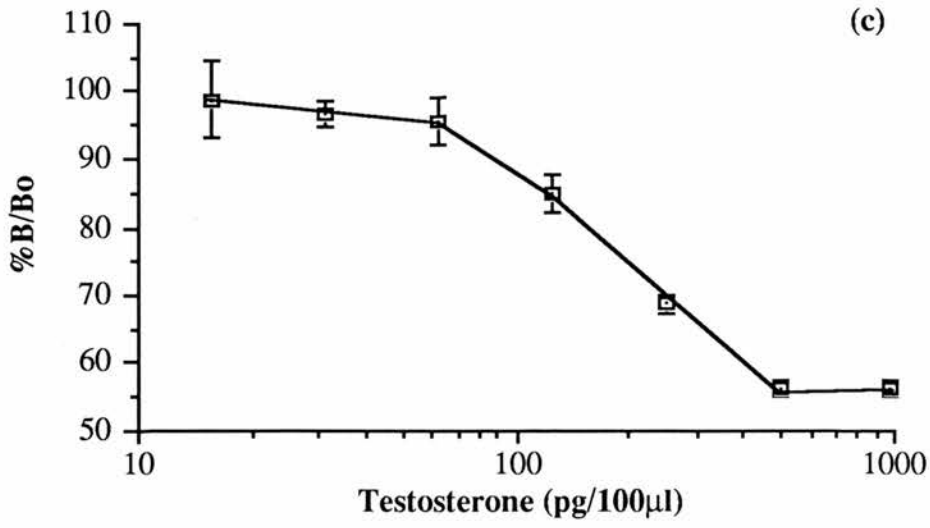
The standard RIA curves for androstenedione, DHT and testosterone are shown in Figures 20 a,b and c . The RIA curves were constructed to detect steroid concentrations in the range 30 to 500 pg/100 µl. All values represent the radioactivity bound (%B/Bo) for each standard concentration of steroid assayed. All measurements

Figure 20

Standard curves were set up for RIA of; (**a**) androstenedione, (**b**) DHT and (**c**) testosterone. The curves were constructed to detect concentrations of the appropriate steroid over the approximate concentration range 30 to 500 pg/100µl. The antibody for androstenedione was employed at a dilution of 1:10,000, DHT at 1:15,000 and testosterone at 1:10,000. All values are represented as the %B/Bo (mean ± SEM n=3) plotted against the concentration of steroid employed.


(a)
120
100
80
60
40
20
%B/Bo
10
100
1000
Androstenedione (pg/100µl)


(b)
100
80
60
40
20
%B/Bo
10
100
1000
DHT (pg/100µl)


(c)
110
100
90
80
70
60
50
%B/Bo
10
100
1000
Testosterone (pg/100µl)

were performed in triplicate or on occasions duplicate. Table 1 illustrates the inter assay and intra assay coefficients for each assay. As illustrated the IC50 for testosterone was approximately 170 pg/100 µl, for DHT 110 pg/100 µl and for androstenedione 175 pg/100 µl.

2.9.4 Sample Preparation.

Wet tissue (0.5 g) was chopped to a pulp then lyopholised in a IEC Lyoprep-3000 Freeze Drier overnight and reweighed. Of this dry tissue approximately 0.05 g was placed in a quickfit tube with Tris buffer (1.5 ml, 0.5 M) and the tissue redispersed by vortexing for 3 x 5 min periods. In order to assess procedural losses 1000 cpm of each radioactive steroid in Tris buffer (100 µl) was added to the quick fit tube which was then vortexed at half hourly intervals during the 1 hour equilibration period to aid equilibration with binding proteins. At the final stage of the preparation the radioactivity present in the extract would then be assessed and expressed as a percentage of the amount originally added. 1000 cpm of radioactive steroid contained negligible amounts of androstenedione, DHT and testosterone (approximately 10^{-17} grammes of each androgen) and therefore this was not thought to hinder the measurement of the actual steroid concentration.

Diethylether (3 ml) was subsequently added to the quick fit tube, the tube vortexed, allowed to stand for 10 min and then centrifuged for 10 min at 1,800 g. The ether phase was then carefully pipetted off and placed in a glass tube. This was repeated once more and the ether combined to give a final volume of 6 ml. The ether was then evaporated off in a vacuum oven at 37^{o}C at a vacuum of 36 in.Hg (Towson and Mercer Ltd, Leeds, U.K.) for approximately 1 hour. The remaining steroids in the glass tube were then reconstituted in ethanol (50 µl). This was then spotted onto thin layered chromatography paper in order to perform Instant Thin Layer Chromatography (ITLC).

	Inter assay coefficient (%)	Intra assay coefficient (%)
Androstenedione	7.48 ± 1.10	4.80 ± 1.02
Dihydrotestosterone	6.04 ± 0.79	4.72 ± 2.43
Testosterone	5.14 ± 1.19	5.15 ± 0.92

Table 1
Inter and intra assay coefficients for androstenedione, DHT and testosterone radioimmunoassays.

2.9.5 Instant Thin Layer Chromatography.

Using this chromatographic technique the different steroids were separated according to their relative mobilities (Rf).

$$Rf = \frac{\text{distance moved by substance}}{\text{distance moved by solvent}}$$

Rf values of 0.52, 0.69 and 0.76 were found for testosterone, DHT and androstenedione respectively when running with 9:1 dichloromethane:diethylether (v/v). 70 ml of the solution was used in a 8 x 25 x 25 cm glass chamber. The positions of the sample steroids were noted by running a standard solution containing 1,000 cpm of tritiated androstenedione, DHT and testosterone in a final volume of 100 µl on the left and right hand sides of the instant thin layer chromatographic polysitic acid gel impregnated glass fibre plate (ITLC plate) (20 x 20 cm) [Gelman Science, Michigan, U.S] on which the test samples were being run. In this way small sections of the migration path taken by the standards solution could be cut and counted in order to assess the position of the steroids. All samples were applied to the plate using micropipettes (Drummond Science, US). Once the relevant positions on the ITLC plate were located the areas corresponding to the unknown samples were cut and placed in 100% pure ethanol (2 ml) for extraction of the steroids. The extraction was repeated and the pooled ethanol placed in glass tubes and dried down. The extracted and separated steroids were reconstituted in PBS-Gel buffer (400µl) and 200µl aliquots were removed from each tube to assess recovery . The remainder was used for the measurement of the steroid concentrations by radioimmunoassay.

2.10 Testicular Cell Culture

The testicular teratoma cell line, Tera- 2 (clone 13, passage 13) was cultured after having been kindly donated by Professor Graham, Department Zoology, University of Oxford.

The cell line was originally established in 1971 by Dr Jorgen Fogh (Fogh and Trempe, 1975) and was set up from a lung metastasis of a primary testicular embryonal carcinoma. The undifferentiated cells are small round carcinomoid cells which appear larger and flatter (resembling epithelial cells) at low density and have the potential to differentiate into large flat cells and neurons (Plate 1).

The epithelial cell line A431 was also cultured as its cells have also been shown to express the EGF receptor (Gregoriou and Rees, 1984). The resultant cells were then subsequently used as positive controls for the various Tera-2 cell experiments which were performed.

The cell types present in teratoma tumours vary greatly. Frequently the tumours contain embryonic carcinoma, seminoma and cells resembling those found in extra-embryonic foetal tissues, such as placental trophoblast and yolk sack. Occurring with lower frequency are groups of cells resembling embryonic and adult tissues, such as muscle, cartilage and nerve (Mostofi and Price, 1973; Pugh, 1976). Cells supplied for the purpose of this study arrived having been cultured in 10% foetal calf serum (FCS) Alpha medium. The cell medium was subsequently changed on arrival to 10% FCS RPMI medium as this was the standard medium used in these laboratories. They were then weaned gradually through 5% and 0.5% FCS serum-free medium (SFM) to grow finally in SFM (15 ml) in a 75 ml flask (Gibco) at 37^{o}C in an atmosphere of 5% CO_2/ 95% air in a KEBO Assab AB incubator (Sweden). The defined serum-free medium was adopted from Mac Donald *et al* (1990) who had previously developed it from Barnes and Sato (1980). Each confluent flask contained approximately 5 x 10^6 cells which were divided into four subsequent to passaging. The doubling time for Tera-2 cells was found to be approximately 48 hours in 10% FCS RPMI media and approximately 96 hours in SFM. On repetition of the process of changing from FCS conditions to SFM conditions it was discovered that the 5% FCS RPMI media stage could be omitted.

All medium was tested for bacterial contamination. This was performed by

(a)

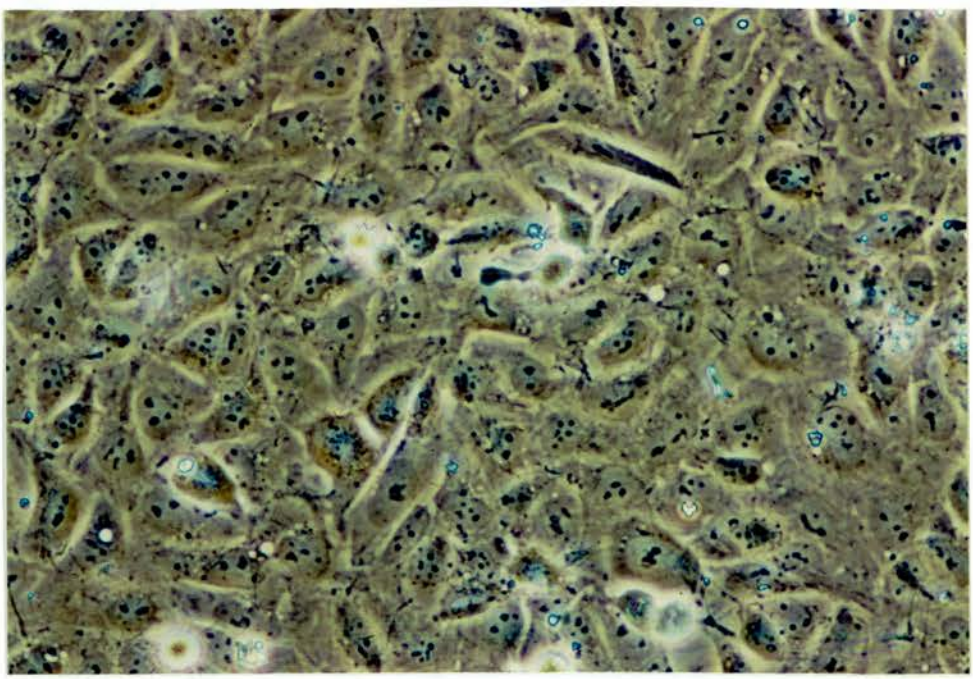


Plate 1 The Tera- 2 cells were grown in 15ml of 0.5% SFM in 75ml flasks until confluent before being passaged.

incubating 2 ml of sterile nutrient broth with 2 ml of the test medium for up to 4 weeks in a humidified atmosphere of 5% CO_2/ 95% air.

2.10.1 Harvesting of Cells.

Detachment of the cells from the 75 ml flasks was achieved by trypsinisation. The medium was decanted and the cells washed with sterile Dulbeccos solution (5 ml) for 10 seconds, the solution decanted once more and trypsin/EDTA (5 ml) added for 10 seconds. This was subsequently discarded and the residual cells incubated at 37°C for 5 min under an atmosphere of 5% CO_2/ 95% air or until they could be seen to have become detached. The cells were then taken up in the appropriate volume of medium and used as required. When the cells were approximately 95% confluent they were split and passaged to facilitate cell growth. If necessary the cells were washed in Dulbeccos solution (5 ml) to remove dead cells once they had become reattached after passaging.

2.10.2 Freezing of Cells

Following detachment, the cells were taken up in 10% FCS RPMI media (10 ml) and the resulting suspension placed in a sterile universal, centrifuged at 800 g for 5 min and the supernatant decanted off. The cells were then reconstituted in "freezing medium" (0.5 ml) and transferred to a 1 ml eppendorf tube which was gradually frozen by placing it in a -70°C freezer overnight before being transferred to a liquid nitrogen storage vessel the following day.

2.10.3 Setting-Up Cell Culture from Frozen Cells

The cells were thawed from liquid nitrogen by placing the eppendorf tube in a water bath at 37°C for 5 min. They were then reconstituted in 10% FCS RPMI media (10 ml) and placed in a universal for centrifugation at 800 g for 5 min. The supernatant was decanted off and the cells reconstituted once more in 10% FCS RPMI media (15 ml) and transferred to a

75 ml flask. The flask was then placed in an incubator at 37°C in an atmosphere of 5% CO_2/95% air.

2.10.4 Counting of Viable Cells.

The cells were stained with trypan blue and counted using a haemocytometer. In brief the cells were harvested as described in section 2.10.1 and reconstituted in an appropriate volume of SFM. 100 µl of the cell suspension was then diluted with an equal volume of 0.5% (w/v) trypan blue in 0.9% (w/v) saline in distilled water. 100 µl of the resultant suspension was then pipetted onto a haemocytometer chamber where it was then taken up by capillary action into the haemocytometer chamber. The cells were then viewed through a microscope and the number of cells within each of the four large squares labelled A counted (Figure 21). The number of cells present per ml of medium was then determined according to the following calculation shown. The cells which appeared clear under the microscope while in trypan blue solution were interpreted as viable cells whereas those that appeared blue were assumed dead.

2.10.5 Attachment of Cells

The cells were plated in 24 well plates for all ligand exchange assays and thymidine incorporation experiments. Initially 2×10^5 cells per well were plated down which gave approximately 85% attachment. Although this was found to be adequate for the ligand binding assay the cells reached confluency after 24 hours. This was detrimental to the thymidine incorporation experiments as cell growth was found to be inhibited. Consequently cells were plated at 0.5×10^5 and 1×10^5 cells per well to assess percentage attachment. 0.5×10^5 cells were found to provide approximately 30% attachment of the original number of cells added and thus provided sufficient room for further growth over a 24 hour period.

Figure 21
The number of viable Tera-2 cells was calculated using the equation and the diagram below which represents the lay out of the haemocytometer chamber employed.

Calculation of Cell Count

$$\text{Total } (10^4/\text{ml}) = \frac{\text{actual count}}{4} \times \text{reciprocal of dilution} \times \text{correction factor}$$

$$= \frac{\text{actual count}}{4} \times 2 \times \text{correction factor}$$

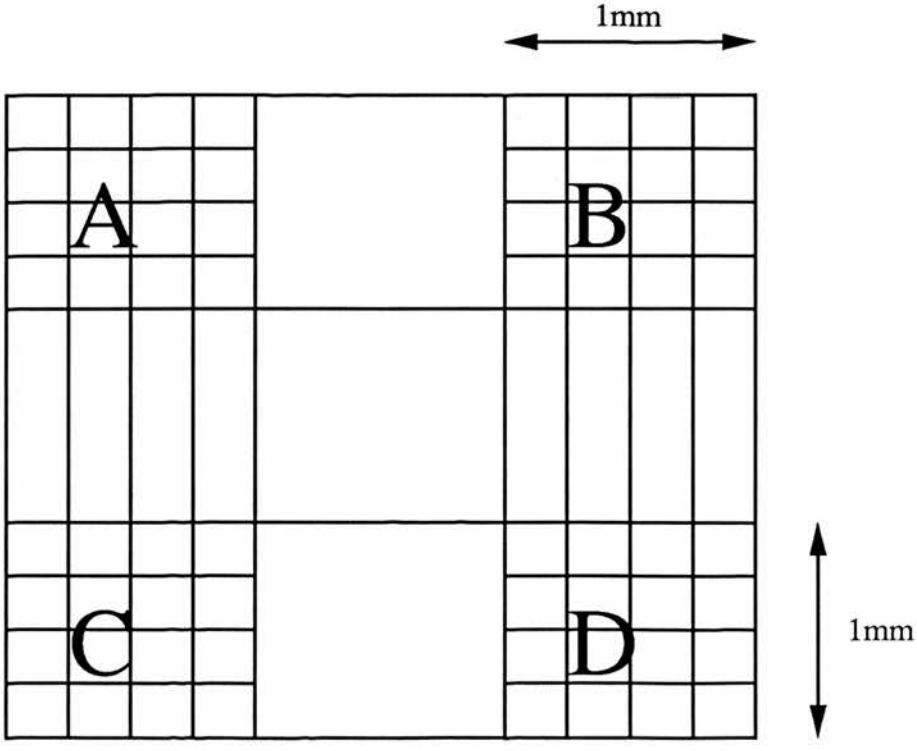
(The actual count is the number of cells counted in **A**, **B**, **C** and **D**.

Using the chamber outlined below the correction factor was calculated to be 1×10^4

This is derived from:

Area of each square = $1\ mm^2$
Area x depth of chamber = $1mm^2 \times 0.1\ mm = 0.1\ mm^3$
$1000mm^3 = 1\ ml$
therefore $0.1 = 1 \times 10^{-4}$

Correction factor = reciprocal value = 1×10^4



2.11 Characterisation of the EGF Receptor on Tera-2 Cells

2.11.1 Mouse EGF Ligand Exchange Assay for Cultured Cells

The EGF ligand exchange assay was performed according to the method developed by Engstrom *et al* (1985) performed at room temperature with some modifications. In brief 2×10^5 cells in 0.5% FCS SFM (1 ml) were plated down in each well of 24 well plates. After being left overnight (approximately 16 hours) the cells were washed twice with Dulbeccos solution (1 ml). Labelled mEGF (1.0 nM, 200,000 cpm) in 300 µl RPMI, was then added to each well and incubated in the presence and absence of unlabelled mEGF (100 nM) in 200 µl RPMI. RPMI (200 µl) was added in the absence of mEGF to bring the final volume to 0.5 ml in each well. The cells were then incubated for 90 min at room temperature with intermittent shaking and the reaction terminated by aspirating off the medium and then washing twice with Dulbeccos solution. The cells were subsequently trypsinised by the addition of Trypsin/EDTA (1 ml) over 5 min and the resultant suspension transferred to a tube and counted in a gamma counter. Non specific binding to the cell culture tray was also measured in each assay by incubating 200,000 cpm in 500 µl RPMI in the absence of any cells.

2.11.2 Displacement Studies with Unlabelled mEGF in Cultured Cells.

In order to assess the concentration of unlabelled mEGF required to give the maximum specific binding, Tera-2 cells were plated down in 24 well plates at a density of 2×10^5 cells/ml as described in section 2.10.5. The cells were subsequently incubated with labelled mEGF (1.0 nM, 200,000 cpm) in 300 µl RPMI, in the presence and absence of increasing concentrations of unlabelled mEGF (ranging from 0.1 nM to 300 nM) in 200 µl RPMI.

2.11.3 Saturation Analysis and Scatchard Plot.

Cells were plated down in 24 well plates at a concentration of 2 x 10^5 cells per well in 0.5% FCS SFM for 16 hours. The cells were then washed in Dulbeccos solution and incubated with increasing concentrations of labelled mEGF (ranging from 1.0-10.0 nM, with between 2 x 10^5 and 2 x 10^6 cpm) in 300 µl RPMI, in the presence and in the absence of 100-fold excess unlabelled mEGF (200 µl) as described in section 2.11.1. Four wells were also run in parallel at the lowest concentration for determination of cell number at the end of the experiment. The non-specific binding, the total count and the amount of radioactivity added were measured at each concentration and the specific binding data then analysed by a computer programme (Munson and Rodbard, 1980) to yield the dissociation constant (Kd) and the number of specific binding sites.

2.12 Western Blot Analysis

Tera-2 and A431 cells were plated in separate 15 cm diameter petri dishes at a density of 1 x 10^7 cells per dish and cultured in SFM for 48 hours. Two petri dishes of Tera-2 cells were cultured and treated as follows, in order to increase the possibility of EGF receptor identification. The cells were first washed in sterile Dulbeccos solution (5 ml) and then scraped off into a small test tube with "lysis buffer" (2 ml for the A431 cells and 1 ml for each dish of the Tera-2 cells). The lysis buffer produced disruption of the cell structure and eventual solubilisation of the receptors. The resultant cell suspensions were spun down at 1,800 g for 10 min and 100 µl of the supernatants boiled for 5 min with sample buffer (100 µl). 200 µl of the solubilised receptors were then loaded onto a Laemmli gel, along with 100 µl of a molecular weight marker solution (range 200-14.3 kDa) and run for 8 hours at 30 mA (see section 2.5). The gel bearing the protein was then removed from the electrophoresis apparatus and equilibrated with transfer buffer (500 ml) for 30 min. The proteins from the gel were then transferred to a 0.45 micron thick nitro-cellulose membrane (Bio-Rad) using a Semi Dry Transfer Cell (Bio-Rad) powered by a Bio-Rad power supply

model 200/2.0 set at 20 V for 40 min. This membrane with the newly transferred proteins was then placed in 10% low fat skimmed milk for 10 min at room temperature to block non-specific binding sites, then washed with TBS (2 x 15 ml) and the lanes (3 x Tera -2 and 1 x A431 receptor proteins) sectioned off accordingly. One lane containing the Tera-2 solubilised receptors was incubated in the monoclonal antibody EGF-R1 (15 ml, 1:30 v/v in TBS), a second in the monoclonal antibody F4 (15 ml, 1:5 v/v in TBS) and a third in TBS buffer (15 ml) overnight at 4°C. The lane containing the A431 solubulised receptors was incubated in the EGF-R1 antibody (15 ml, 1:30 v/v in TBS). The staining procedure as described in section 2.6 was used and the resulting bands photographed.

2.13 Immunocytochemistry.

Both A431 and Tera-2 cells were cultured for this experiment, the former being used as the positive control. Cells at 50%, 75% and 100% confluency were detached from 75 ml flasks and reconstituted in RPMI solution (5 ml) to determine whether the state of cell growth would effect EGF receptor expression and subsequent identification. Aliquots (100µl) of the resulting suspension were then pipetted onto slides and allowed to air dry for approximately 30 min before fixing in 1:1 acetone/methanol solution for 10 min. This enabled the slides to be stored in foil at -20°C until required. When using the slides after storage the cells had to be fixed once again for approximately 5 min and then completely dried to prevent the cells from becoming detached during the washing procedure. Incubation in the monoclonal antibodies and subsequent staining was performed according to the method described in section 2.6. The cells were then mounted in glycerine jelly and the results photographed.

2.14 Preparation of Conditioned Media.

Conditioned medium (1 litre)was collected from six 75 ml flasks of Tera-2 cells which were growing in SFM (15 ml) for up to 48 hours at near confluency (approximately 5×10^6 cells). The medium was collected over a period of 4 weeks and each collection (15 ml) centrifuged at 1,800 g for 15 min and filtered through an acrodisc filter (0.2 μm, Gelman Sciences Inc. Northampton, U.K.) to remove any debris. The aliquots of conditioned media were then stored at -20°C with the addition of PMSF (0.3 mM), until the desired volume had been collected (approximately 1 litre). The conditioned medium was then dialysed overnight at 4°C against ammonium acetate (1.5 litres, 50 mM), using Spectrapor-3 dialysis tubing with a cut off molecular weight of 3,500 Da (Pierce-Warriner, Chester, U.K.) changing the dialysing medium three times during the course of the dialysis. The dialysed product was then frozen, lyophilised and reconstituted in Tris buffer (0.5 M, 3 ml, pH 7.4) before storing it at -70°C . As a control SFM (500 ml) was dialysed as above and reconstituted in Tris buffer (1.5 ml, 0.5 M) pH 7.4 .

2.15 Competition Studies.

Cells were plated down overnight in duplicate in 24 well plates at a density of 2×10^5 in 0.5% FCS SFM. The following morning they were washed twice in Dulbeccos solution before being used in a ligand exchange assay as described in section 2.11.1. The binding site for EGF was competed for by labelled mEGF (1.0 nM, 200,000 cpm, 100 μl) with each of the following peptides ; bFGF, vNGF, hIGF-I, rTGF-α (200 μl in RPMI, 100 nM) and concentrated conditioned media (200 μl).

2.16 Rat TGF-α and Conditioned Medium as Competitors for Radiolabelled mEGF.

In order to assess the effect of rTGF-α and concentrated conditioned media on the binding of labelled mEGF to the receptor site, various concentrations of rTGF-α and concentrated conditioned media were used in the ligand exchange assay described in section 2.11.1 in place of the unlabelled mEGF. Rat TGF-α was employed at concentrations ranging between 0.1 and 100 nM and the conditioned media at serial dilutions of the concentrated solution.

2.17 Radioimmunoassays for rTGF-α and hEGF.

A RIA for hEGF was set up according to section 2.7 to measure the amount of immunoreactive hEGF present in the concentrated conditioned media (100 µl) as well as in the concentrated SFM (100 µl). In addition the concentration of rat TGF-α in the concentrated conditioned media and concentrated SFM was also investigated employing the commercial RIA kit described in section 2.2.6.

2.18 Effect of M bolerone on the binding of radiolabelled mEGF to Tera-2 Cells.

Cells were plated down overnight at a density of approximately 200,000 cells per ml in 0.5% FCS SFM in quadruplicate for each concentration of mibolerone to be tested. The following day they were then washed in Dulbeccos solution (2 x 5 ml) before being incubated in mibolerone (1 ml) at concentrations ranging from 0.1 to 100 nM in SFM for 24 hours. The number of cells present at the end of this period were then measured at each concentration. The remaining cells were washed in Dulbeccos solution (1 ml) and a ligand exchange assay performed as described in section 2.11.1.

2.19 Thymidine Incorporation by Tera-2 cells.

Cells were seeded in quadruplicate for each individual point in 24 well plates. Parallel plates were set up to measure total cell count at each point as well as to act as controls. Cells were plated down at a density of 50,000 per ml in 0.5% FCS SFM and after incubating overnight the cells were washed twice in Dulbeccos solution(1ml) before commencement of the experiment. Methyl-^{3}H thymidine (500 µl, 0.1 GBq/well in SFM) and an equal volume of the appropriate concentration of test substance in SFM was added to each well. The cells were then incubated for the appropriate period of time and at the end of the incubation 100 µl of the cell medium was pipetted off for tritium counting. The remaining medium was then aspirated off and the cells washed in Dulbeccos solution (2 x 1 ml), before being exposed to TCA [10% (w/v) in distilled water, 1 ml] for 30 min at 4^{o}C to remove free radioisotope and then washed once more in Dulbeccos solution (1 ml). The cells were solubilised in sodium hydroxide (0.5 ml, 0.5 M) and the solution transferred to scintillation vials for counting in the tritium counter.

2.19.1 Time Course Studies For Thymidine Incorporation by Tera-2 cells.

Cells were incubated with tritiated thymidine (0.5 ml, 0.1 GBq/well in SFM) as described in section 2.19 in the presence of 10 nM mEGF (0.5 ml), 10 nM rTGF-α (0.5 ml) and 10 nM mibolerone (0.5 ml) in SFM both for a period of up to 24 hours and a period of up to 3 days to assess the length of time required for each substance to affect thymidine incorporation. Parallel plates were set up as controls to assess the effect of thymidine incorporation on cells incubated in SFM (0.5 ml) and to determine cell number at each time interval under the various conditions. All measurements were performed in quadruplicate.

2.19.2 Effect of mEGF, rTGF-α and Mibolerone on Thymidine Incorporation

The effect of mEGF (0.5 ml over the range 0.3 to 100 nM in SFM), rTGF-α (0.5 ml over the range 1.0 to 300 nM in SFM) and mibolerone (0.5 ml over the range 0.3 to 100 nM in SFM) over a 24 hour period on the incorporation of thymidine by the Tera-2 cells in comparison to the incorporation of Tera-2 cells incubated in SFM (0.5 ml) was investigated. The experiment was performed according to section 2.19. Parallel plates were also set up to determine the effect of each concentration of mEGF, rTGF-α and mybolerone on cell number.

2.20.0 Data Analysis.

2.20.1 Mean ± Standard Error of the Mean (SEM).

All experiments were performed in at least triplicate so that the mean values ± SEM could be calculated according to the following formulae:-

$$\text{Mean} = \bar{x} = \frac{1}{n} \sum x \qquad x = \text{the variables}, \quad n = \text{the no. variables}$$

$$\text{SEM} = \frac{\text{S.D}}{\sqrt{n}} \qquad \text{S.D.} = \frac{\sum (\bar{x} - x)}{n}$$

2.20.2 Saturation Analysis

Saturation Analysis was performed using the weighted, nonlinear least-squares curve fitting program 'Ligand' (Munson and Rodbard, 1980), run on an IBM-PC. The curves were analysed according to a model of one or two binding sites. 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 and

Yamamura, 1985). The programme makes some assumptions: (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 a total and true separation of bound ligand from free ligand.

2.20.2.1 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 data significantly better ($P<0.05$ partial F test) than a model for a single binding site.

2.20.2.2 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 smaller variance (more precise) usually receive more weight (Rodbard, 1974a; 1974b; Rodbard *et al,* 1976).

2.20.2.3 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.

2.20.3 Inter Assay and Intra Assay Coefficients.

The coefficient of variance for RIAs can be calculated as follows:-

$$CV = \frac{100\ S.D.}{mean}$$

In the case of a RIA the values for the inter assay coefficient formula are taken from the group of data representing the unknown samples which were run in the various RIAs. In contrast the data for the intra assay coefficient is taken from running extra samples of the standard concentrations which were used to construct the RIA curve. Sensitivity was taken as 90% B/Bo.

2.20.4 t-Test

The probability of two measurements being statistically significantly different was calculated using the following t test formulae:-

$$t = \frac{\bar{x}_1 - \bar{x}_2}{\sqrt{(SEM_1^2 + SEM_2^2)}} \qquad \text{Formula 1}$$

$$t = \frac{\bar{x}_1 - \bar{x}_2}{s\sqrt{\left(\frac{1}{n_1} + \frac{1}{n_2}\right)}} \qquad \text{Formula 2}$$

where $s^2 = \dfrac{\Sigma_1 (x - \bar{x}_1)^2 + \Sigma_2 (x - \bar{x}_2)^2}{n_1 + n_2 - 2}$

s is an estimate of the standard deviation based on both samples jointly.

Then according to the number of degrees of freedom involved (n-1) and the final value of

t calculated, the probability of the two measurements being statistically significantly different from each other can be calculated by looking at the appropriate t table. Formula 1 was the formula that was used when the two groups were of equal size whereas formula 2 was used when they were not of equal size.

2.20.5 Calculation for Correlation.

Attempts were made to correlate the concentration of EGF or TGF-α in particular tissues with the concentration of androgens present. Correlation (r) measures a co-relation, a joint property of two variables. This was performed employing the following equations:

$$r = \frac{\Sigma (x - \bar{x})(y - \bar{y})}{\sqrt{[\Sigma (x - \bar{x})^2 \Sigma (y - \bar{y})^2]}}$$

Results Chapter 3

3.1 Characterisation of the EGF Receptor.

The binding site for EGF was characterised by means of a radioreceptor ligand exchange assay. The radioreceptor assay was optimised according to a) the subcellular fraction employed and its' protein concentration, b) the duration, temperature and pH of the incubation employed in the radioligand exchange assay and c) the concentration of labelled and unlabelled mEGF used to detect the hEGF binding site. The affinity (dissociation constant) of the receptor was subsequently determined by Scatchard analysis and the affinity of mEGF for the receptor site compared with that of other growth factors. The effect of heat and trypsin as well as storage at -70^{o}C on the EGF receptor present in the tissues was also investigated.

3.1.1 Subcellular Fractionation

Subcellular fractions were prepared from three different tissues. Following subcellular fractionation the specific EGF binding in each fraction was assessed using the radioligand exchange assay. Approximately 72% of the specific binding for hEGF found in the non-fractionated tissue was associated with the 800 g "crude" fraction while 12% was associated with the mitochondrial and 18% with the microsomal fraction (Figure 22). No specific binding was detected in the cytosolic fraction. In all subsequent experiments the specific binding of EGF was determined by preparing a crude homogenate and centrifuging at 105,000 g for two periods of 40 min and 20 min to yield a particulate fraction. This fraction (the particulate fraction) represented all the previous fractions apart from the cytosolic fraction and was subsequently shown to contain 101% ± 6.7 (SEM) (n=3) of the binding sites detected in the tissue prior to fractionation.

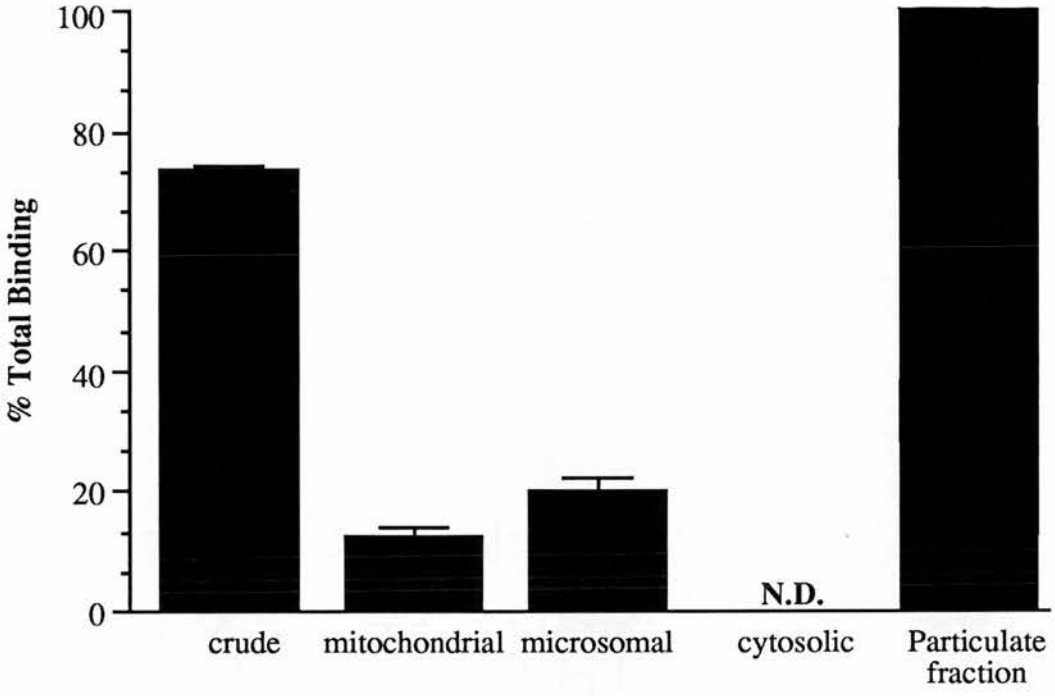


Figure 22
Subcellular distribution of the EGF receptor in human testicular tissue was investigated. Subcellular fractions of human testicular tissue (1 mg/ml protein concentration) were incubated with labelled mEGF (8 nM, 200,000 cpm) at 32 C for 40 min in the presence and the absence of unlabelled mEGF (50-fold excess). Each value plotted represents the mean specific binding of labelled mEGF (± SEM) of three different particulate fractions, each of which was analysed in duplicate shown as a percentage of the specific binding detected in the three non-fractionated tissue preparations.

3.1.2 Time and Temperature Studies.

Figure 23 illustrates the specific binding patterns of labelled mEGF to the 105,000 g particulate fraction at 4°C, 25°C, 32°C and 37°C over a period of three hours. Maximum binding of labelled mEGF to its receptor was achieved after incubating at a temperature of 32°C and 37°C for a period of 40-50 min and at 25°C after an incubation period of 90 min. The maximum binding achieved at 4°C was approximately 80% of the maximum binding seen at 32°C and 37°C, after an incubation period of 180 min.

3.1.3 Protein Concentration.

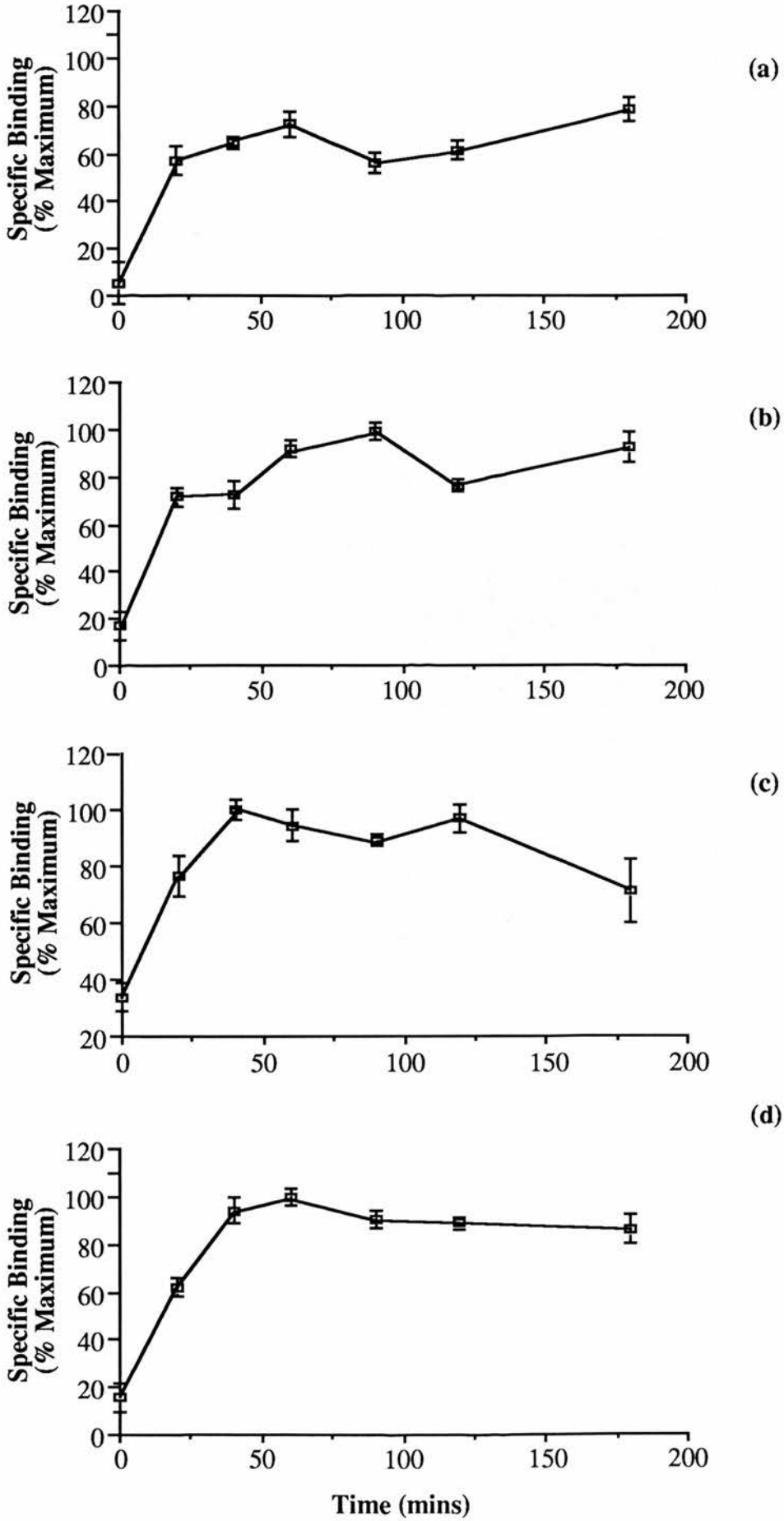
The effect of protein concentration on the number of EGF specific binding sites was investigated. Particulate fractions obtained from three different tissue samples were examined, over a protein concentration range of 0.5-8.0 mg/ml. The number of specific EGF binding sites observed rose linearly with increasing protein concentration of the particulate fractions employed (Figure 24). All measurements were converted to a percentage of the maximum specific binding achieved with each particulate fraction and mean data presented ± SEM (n=3).

3.1.4 Displacement of Radiolabelled mEGF with Unlabelled mEGF.

In order to assess the displacement of radiolabelled mEGF from the EGF binding site, increasing concentrations of unlabelled mEGF were used to compete with 8.0 nM labelled mEGF for the receptor. Figure 25 illustrates the percentage bound (% Bound) of three particulate fractions (mean ± SEM) which were incubated with labelled mEGF (8 nM, 200,000 cpm) in the presence and absence of unlabelled mEGF (10-400 nM). The optimum specific binding for all three samples was obtained when >200 nM of unlabelled mEGF was used to compete for the EGF binding site.

Figure 23

Determination of optimal duration and temperature of the ligand exchange assay employed in measuring the specific binding of mEGF to the particulate fraction. Incubation was carried out at 4°C (**a**), 25°C (**b**), 32°C (**c**) and 37°C (**d**). Values represent the mean percentage (± SEM, n=3) of the maximum specific binding obtained during the 32°C incubation .


(a)
(b)
(c)
(d)
Specific Binding (% Maximum)
120
100
80
60
40
20
0
0
50
100
150
200
Time (mins)

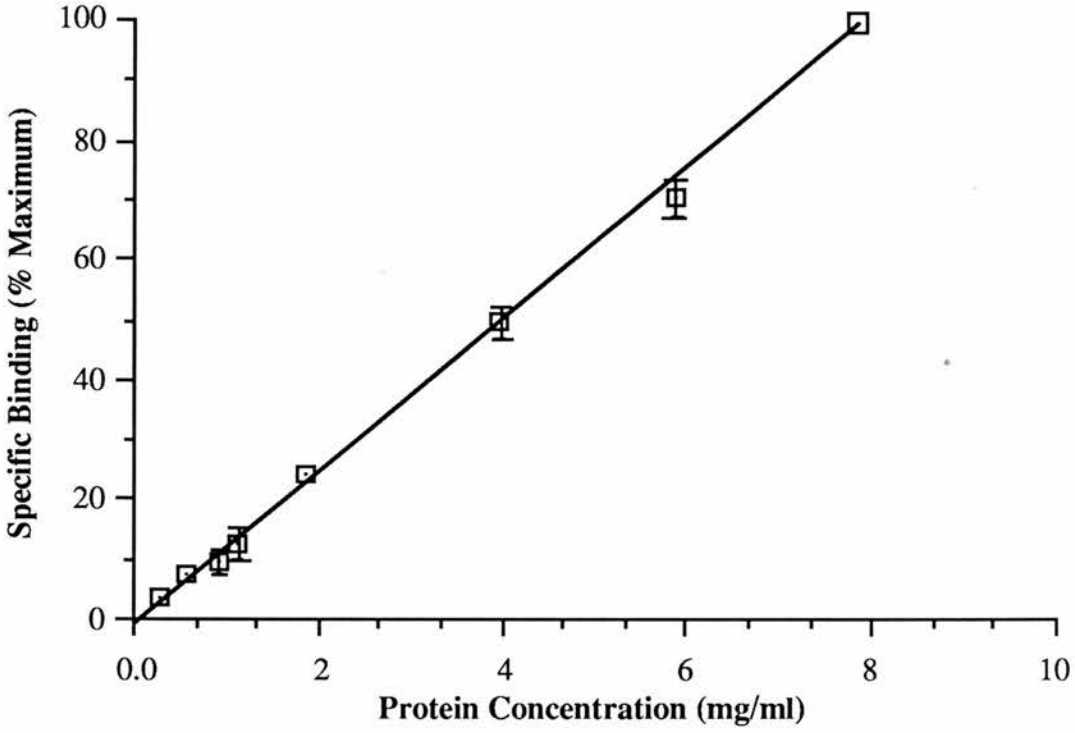


Figure 24
The relationship between specific binding of mEGF and the protein concentration of the particulate fraction employed was investigated. The data is represented as the mean percentage (± SEM, n=3) of the maximum specific binding obtained at each concentration of protein employed.

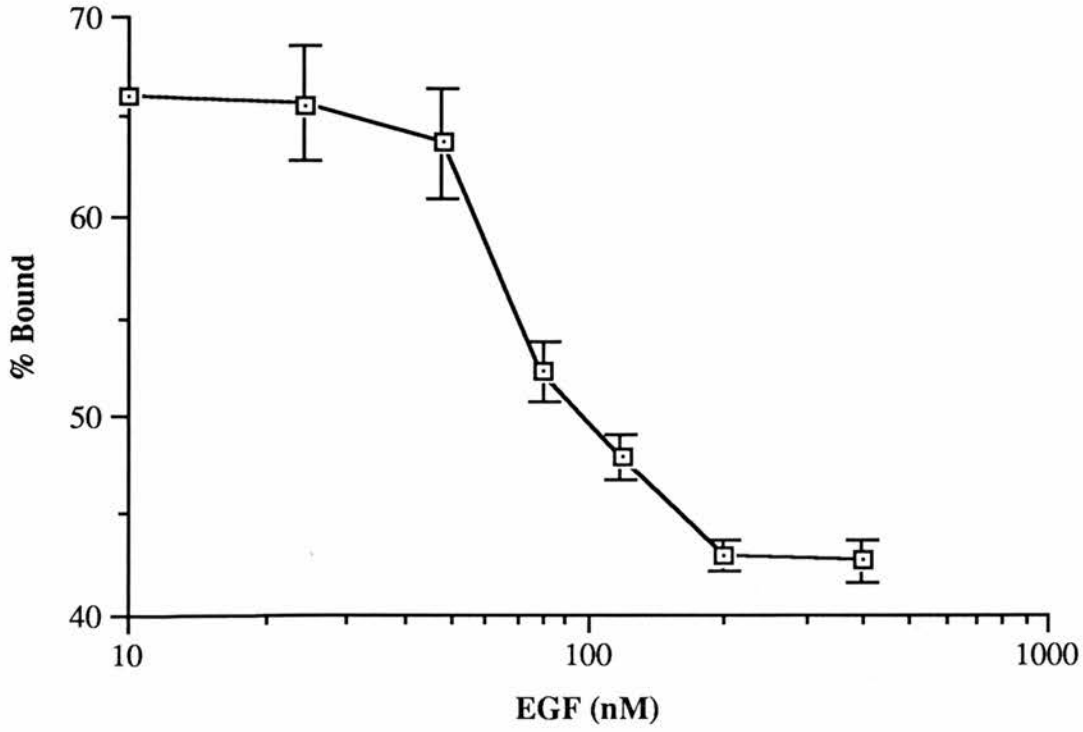


Figure 25
Labelled mEGF (8 nM) was competed with various concentrations of unlabelled mEGF ranging from 10 to 400 nM. The specific binding of labelled mEGF at each concentration was then converted to % Bound meaned (± SEM, n=3) and plotted against the log concentration of the unlabelled mEGF employed.

3.1.5 Saturation Analysis and Scatchard Plot.

In order for a binding site to be described as specific for one ligand the ligand must show a high affinity saturable interaction with the site in question (Adamson and Rees, 1981).

The saturability of the EGF receptor with radiolabelled mEGF was investigated as described in section 2.4.10 and saturation was reached with concentrations ≥8 nM labelled mEGF.

Figure 26a illustrates the binding of labelled mEGF to the particulate fraction in the presence and absence of unlabelled mEGF over the concentration range 1.0-10.0 nM of labelled mEGF. Figure 26b illustrates the specific binding determined at each concentration of labelled mEGF.

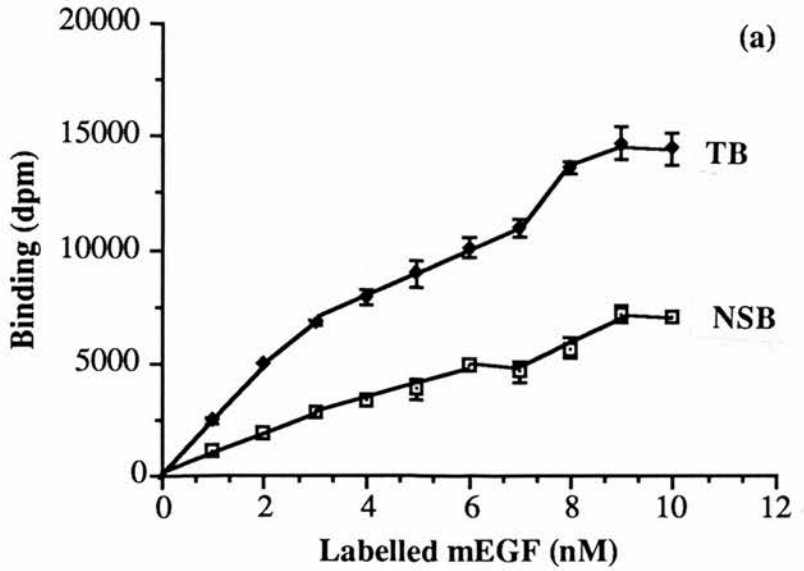
The saturation data obtained from the experiments performed in section 2.4.10 were then analysed by the Scatchard method using a computer programme written by Munson and Rodbard (1980). The maximum confidence limit set was 100%. This limit yielded a single high affinity binding site with a mean Kd of $1.18 \pm 0.32 \times 10^{-9}$ M (n=6) and 528 ± 116 fmoles binding sites per mg of protein.

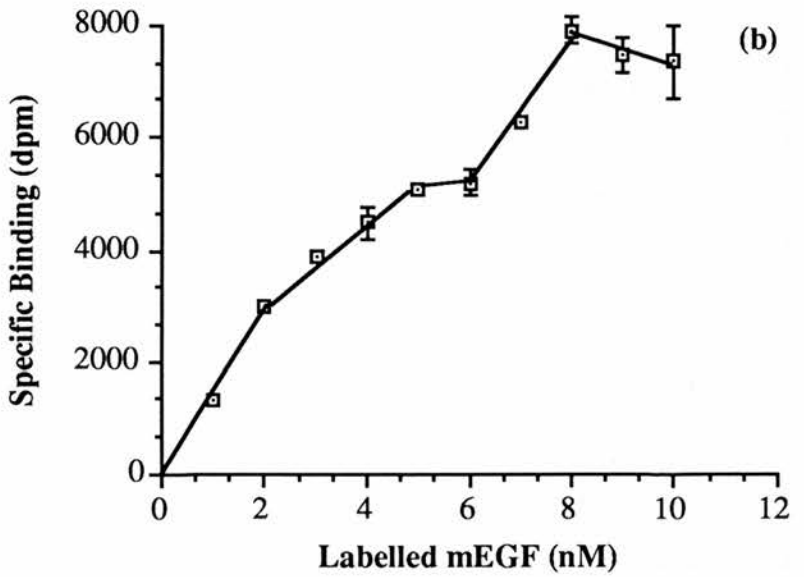
3.1.6 Competition Studies.

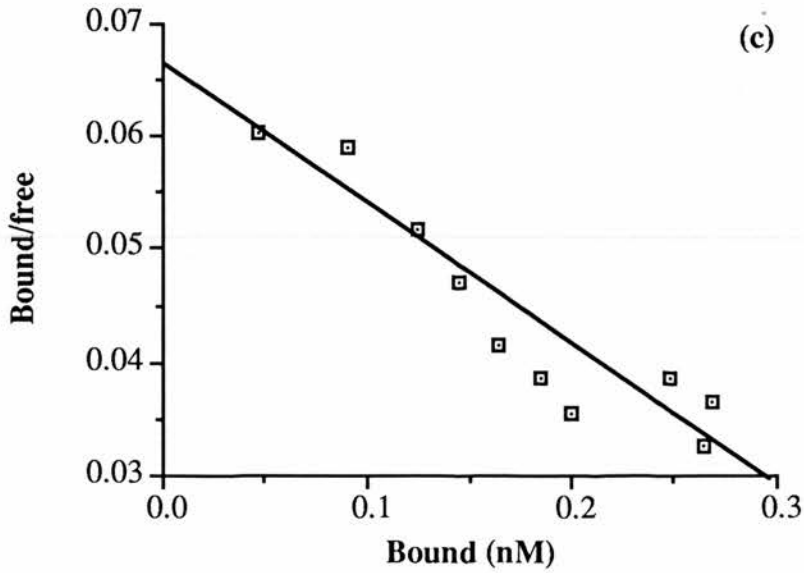
The various factors used to compete for the EGF binding site were employed at concentrations which were 50-fold in excess of the 8 nM labelled mEGF (approximately 400 nM). Figure 27a illustrates that specifically bound EGF was only partly displaced by rTGF-α (24%), hI (11%), hPrl (2%), hLH (5%) and hFSH (16%) whereas IGF-I, hGH, bFGF and vNGF did not compete for the binding site. As hI, hPrl, hLH and hFSH did illustrate some competition for the EGF binding site an experiment was performed in which the aforementioned peptides were used at approximately 100-fold excess of the radiolabelled mEGF employed (approximately 800 nM) (Figure 27b). It was determined from this experiment that no further

Figure 26

Saturation of the EGF binding site on human testicular tissue with radiolabelled mEGF over the concentration range 1.0 to 10.0 nM was performed. The non-specific binding (**NSB**) and total binding (**TB**) (**a**) and specific binding (**b**) were plotted against the concentrations of labelled mEGF employed. The specific binding at each concentration was then converted to Bound and Bound/Free to construct a Scatchard plot (**c**). From this the Kd of the EGF binding site was calculated to be approximately 1.18 nM and the number of binding sites 528 fmoles/mg protein.


(a)
Binding (dpm)
20000
15000
10000
5000
0
TB
NSB
0
2
4
6
8
10
12
Labelled mEGF (nM)


(b)
Specific Binding (dpm)
8000
6000
4000
2000
0
0
2
4
6
8
10
12
Labelled mEGF (nM)


(c)
Bound/free
0.07
0.06
0.05
0.04
0.03
0.0
0.1
0.2
0.3
Bound (nM)

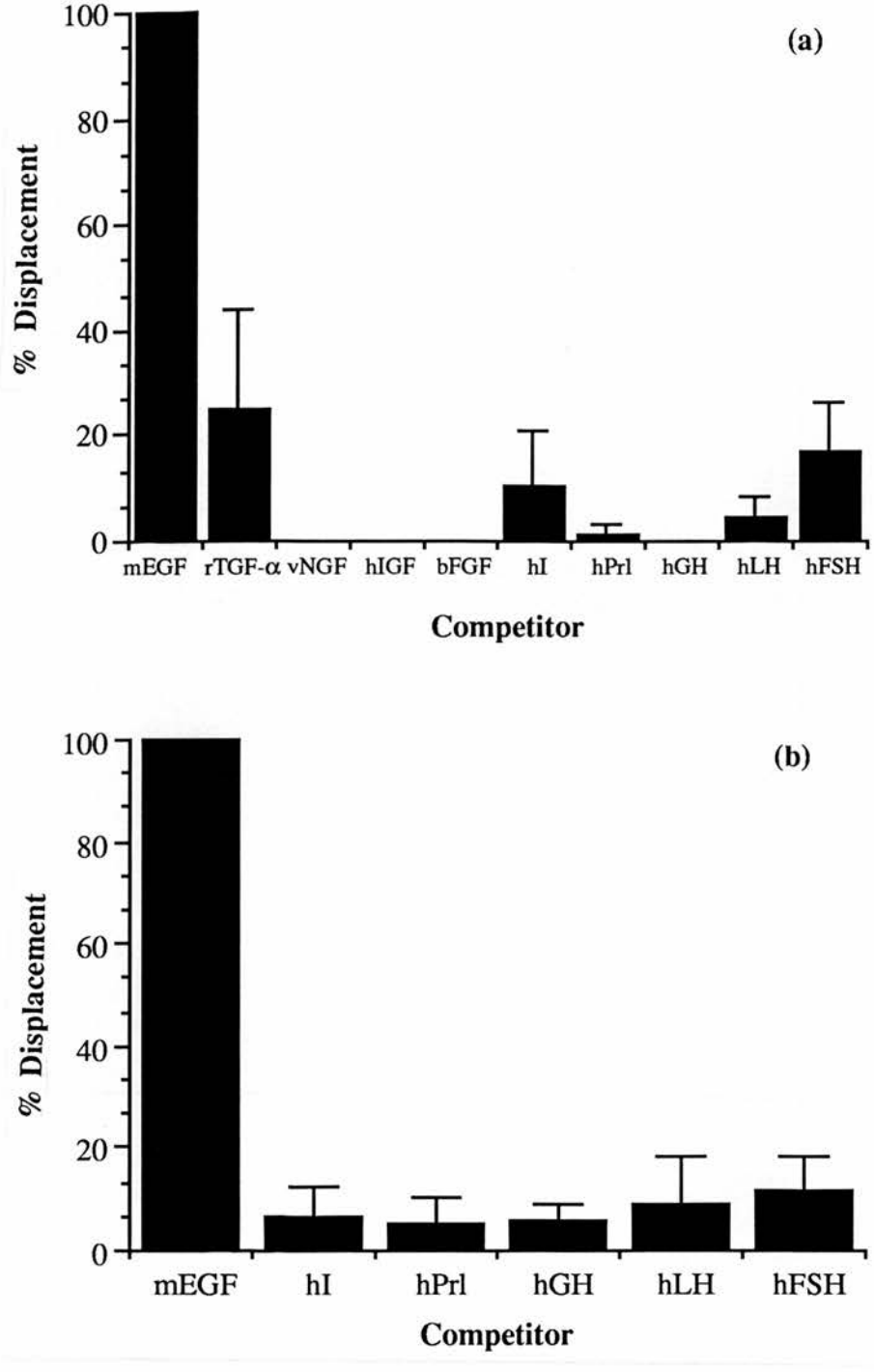


Figure 27
Competition of the receptor site with other peptide growth factors and hormones was performed. The mean specific binding of labelled mEGF (± SEM, n=3) achieved with each competitor was compared with the mean specific binding achieved with unlabelled mEGF and converted to a percentage. In Figure (**a**) the competitor was used at a concentration 50-fold in excess of labelled mEGF and in Figure (**b**) at a concentration 100-fold in excess.

To test the specificity of the receptor multiple concentrations of competitor should be used but unfortunately research finance does not always permit this.

significant increase in the level of competition was achieved with any of the aforementioned competitors and therefore binding was not concentration dependent. All measurements were expressed as a percentage of the specific binding achieved with 400 nM mEGF which on Figures 27a and 27b is represented as the maximum competition, 100%.

3.1.7 Trypsin and Thermal Sensitivity.

A significant decrease in the binding of EGF to its receptor was observed when particulate fractions which had been preincubated with trypsin were employed in a ligand exchange assay as described in section 2.4.6. The mean reduction in binding of the three particulate fractions following incubation in trypsin for 30 min at 37°C in comparison to the control which had not been incubated in trypsin are shown (Table 2).

Preheating of the particulate fractions before assay was investigated. Preheating of the particulate fractions at 45°C and 60°C for 10 min produced mean decreases of 49% and 55% in EGF binding in comparison to identical samples which had been left on ice. On heating the particulate fractions at 80°C the sample became too thick to dispense accurately through a pipette tip and because of this could not be assayed.

3.1.8 Effect of pH on the Specific Binding of Labelled mEGF in Human Testicular Tissue.

The pH of the buffer employed in the ligand exchange assay described in section 2.4.6 was varied between 6.6 and 8.2. As illustrated in Figure 28 this was found to have no effect on the specific binding of radiolabelled mEGF to three testicular tissue samples except at the lower pH of 6.6 which produced a slight decrease in the specific binding. Once again the binding data obtained in each individual tissue sample was expressed as a percentage of the maximum specific binding achieved. The mean ± SEM of the data

(a)

Trypsin	-	+
^{125}I mEGF Binding	100%	13.1% ± 1.3

(b)

Heat	Control	45° C	60° C
^{125}I mEGF Binding	100%	49% ± 8.6	55% ± 14.9

Table 2

The effect of trypsinisation (**a**) and heat pretreatment (**b**) at 45°C and 60°C on specific radiolabelled mEGF binding by the 105,000g membrane particulate fraction was assessed. The results are expressed as percentage change from the untreated control and the values represent mean (n=3, ± SEM).

Binding was found to be statistically significantly decreased after trypsin treatment (***, $P<0.0001$) and after heat treatment at 45°C (**, $P<0.01$) but not after heat treatment at 60°C.

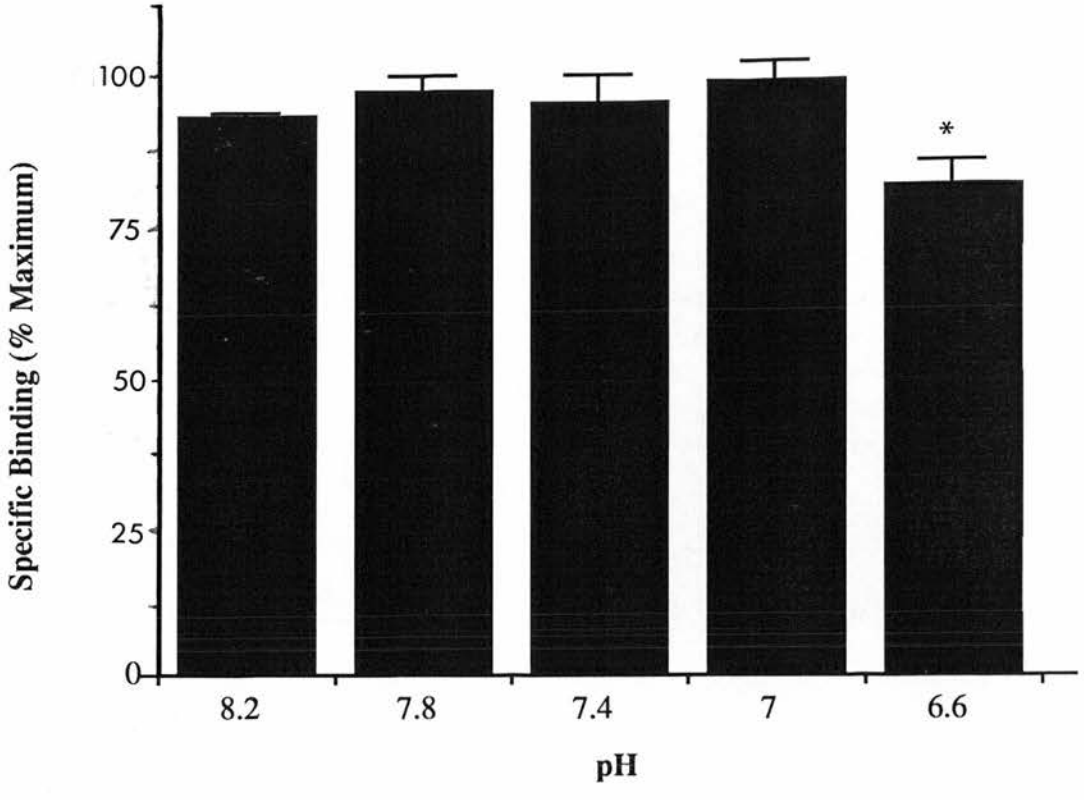


Figure 28

The effect of buffer pH on specific binding of labelled mEGF to three different particulate fractions was investigated. Particulate fractions (100 µl) were incubated with labelled mEGF (200 µl, 8 nM, 200,000 cpm) at 32°C for 40 min in the presence and absence of unlabelled mEGF (200 µl, 50-fold excess) employing buffers with a pH of between 6.6 and 8.2. Each value plotted represents the mean specific binding (± SEM, n=3) of labelled mEGF as a percentage of the maximum specific binding achieved.

Binding was only found to be statistically significantly decreased at pH 6.6 compared to a pH of 7.0 (*, $P<0.05$).

from all three tissues was then calculated and represented in Figure 28.

3.1.9 Effect of Freezing in Liquid Nitrogen and Storage at -70°C on the Specific Binding Sites for hEGF in Testicular Tissue and Particulate Fractions.

Freezing of testicular tissue in liquid nitrogen was found to have no effect on the specific binding of EGF present in the three testicular tissues examined (Figure 29a). The tissue once frozen was then kept for a period of up to six months and during this time no significant loss of the EGF receptors was observed (Figure 29a). However storage of the particulate fractions prepared from the above mentioned tissues for a period of 14 days produced a decrease in specific EGF binding after the tenth day of storage (Figure 29b). Consequently storage of the particulate fractions was limited to ten days only. The binding data was expressed as a percentage of the maximum specific binding achieved within each tissue sample and the mean data from all the samples is shown ± SEM.

3.2 EGF Receptor in Normal, Treated and Cancerous Testicular Tissue.

The specific binding of EGF to its receptor in individual samples was achieved by employing the radioligand exchange assay described in section 2.4.6. The specific binding data is represented in Figure 30 as the molarity of radiolabelled mEGF specifically bound to the EGF receptor in 2 mg/ml of protein.

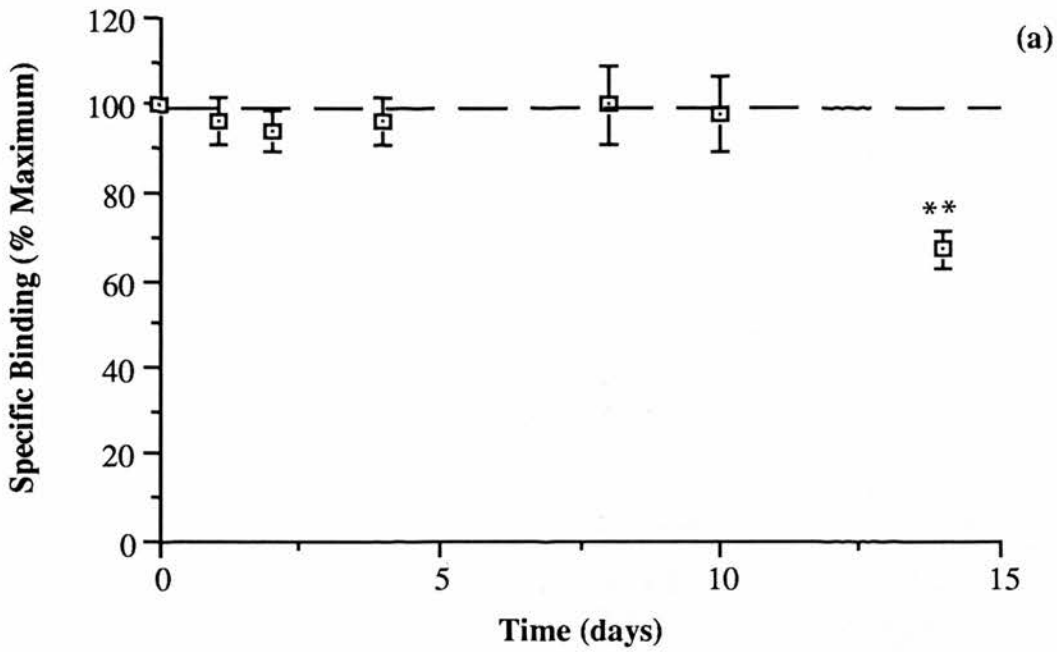
a) Normal testicular tissue.

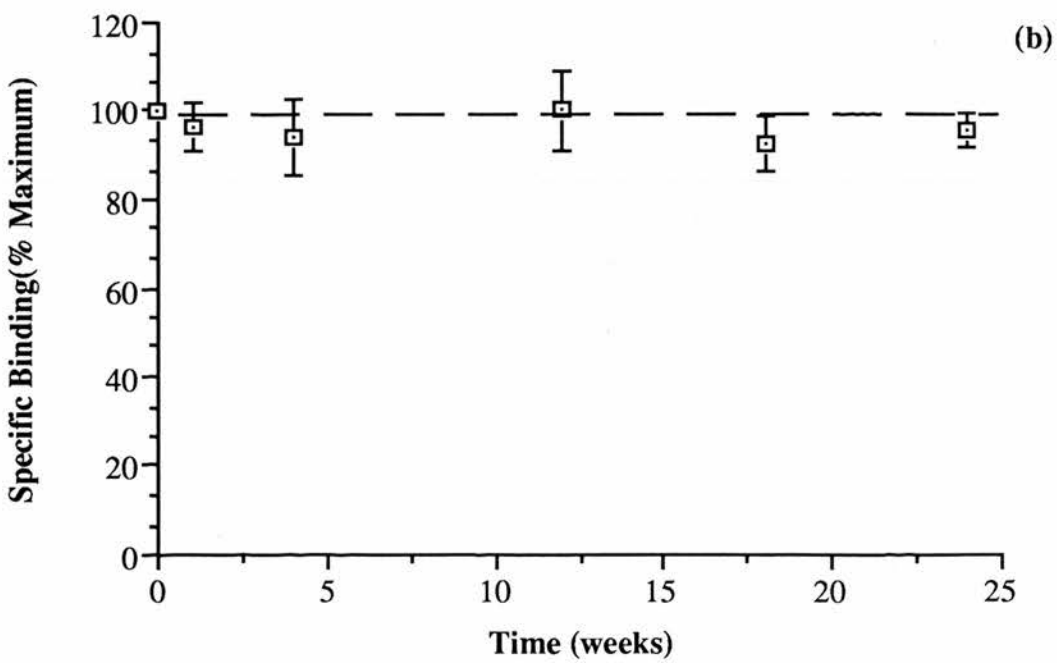
It was found that in all of the 36 normal testicular tissue samples investigated EGF receptors were expressed (Figure 30). The specific binding ranged from

Figure 29

a) The effect of freezing human testicular tissue in liquid nitrogen and its subsequent storage at -70^{o}C on the binding of labelled mEGF to its over 14 days was investigated. The data is represented as the mean percentage(± SEM, n=3) of the maximum specific binding achieved. Binding was found to be statistically significantly decreased after storage of the particulate fraction for more than 10 days (**, P<0.01).

b) The effect of storage at -70^{o}C on EGF binding in the testicular particulate fractions was investigated over a 24 week period. The specific binding data is represented as the mean percentage (± SEM, n=3) of the maximum specific binding achieved.


(a)
Specific Binding (% Maximum)
120
100
80
60
40
20
0
**
0
5
10
15
Time (days)


(b)
Specific Binding(% Maximum)
120
100
80
60
40
20
0
0
5
10
15
20
25
Time (weeks)

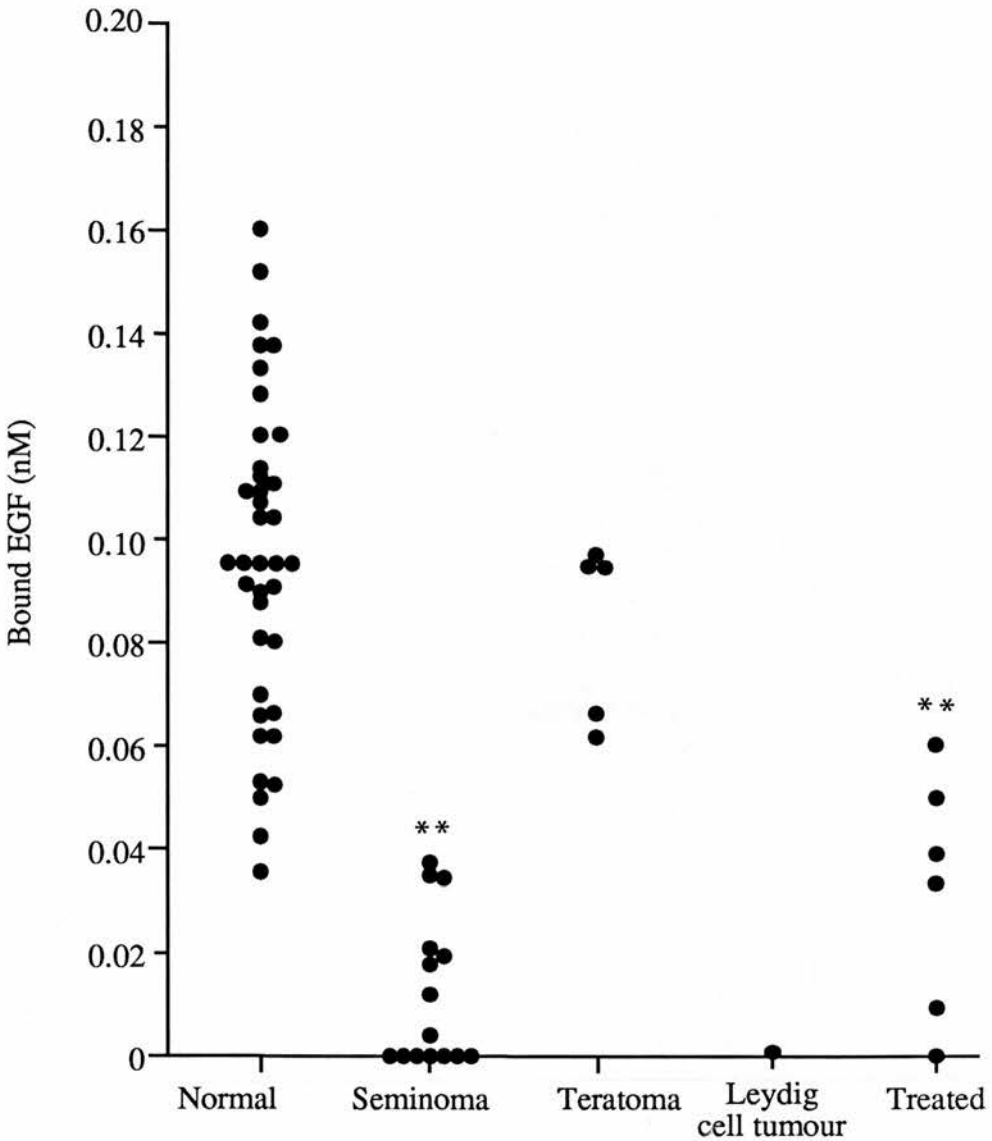


Figure 30
The specific binding of labelled mEGF for each tissue used in the study was plotted according to their designated group. Each value was obtained by incubating the particulate fraction (100 µl) with labelled mEGF (200µl, 8 nM, 200,000 cpm) at 32 C for 40 min in the presence and the absence of unlabelled mEGF (200 µl, 50-fold excess). Each value represents the mean molarity (± SEM, n=3) of EGF bound to each particulate fraction.

Normal testicular tissue was found to express 0.09 ± 0.01 nM EGF receptors in the 36 normal tissue samples which were assessed, for seminoma 0.01 ± 0.01 nM (n=15), for teratoma 0.08 ± 0.01 nM (n=5) and for treated tissue 0.03 ± 0.01 nM (n=6).

The concentration of EGF receptors expressed in seminoma and treated tissues was found to be significantly lower than the concentration expressed in normal tissue (**, $P<0.001$ and **, $P<0.001$ respectively).

0.04 to 0.16 nM (mean = 0.09 ± 0.01) in the 36 particulate fractions which were analysed.

b) Cancerous testicular tissue.

Three types of cancerous testicular tissue were obtained (see section 1.11). In the 15 seminoma tissues which were investigated EGF receptors were found to be either absent or "poorly" expressed. The number of specific binding sites which were identified were found to range from 0 to 0.04 nM (mean = 0.01 ± 0.01). In the case of the 5 teratoma tissues investigated the concentration of binding sites were found to range from 0.06 to 0.1 nM (mean = 0.08 ± 0.01). One Leydig cell tumour was also investigated for EGF receptor expression but no EGF binding was detected (Figure 30).

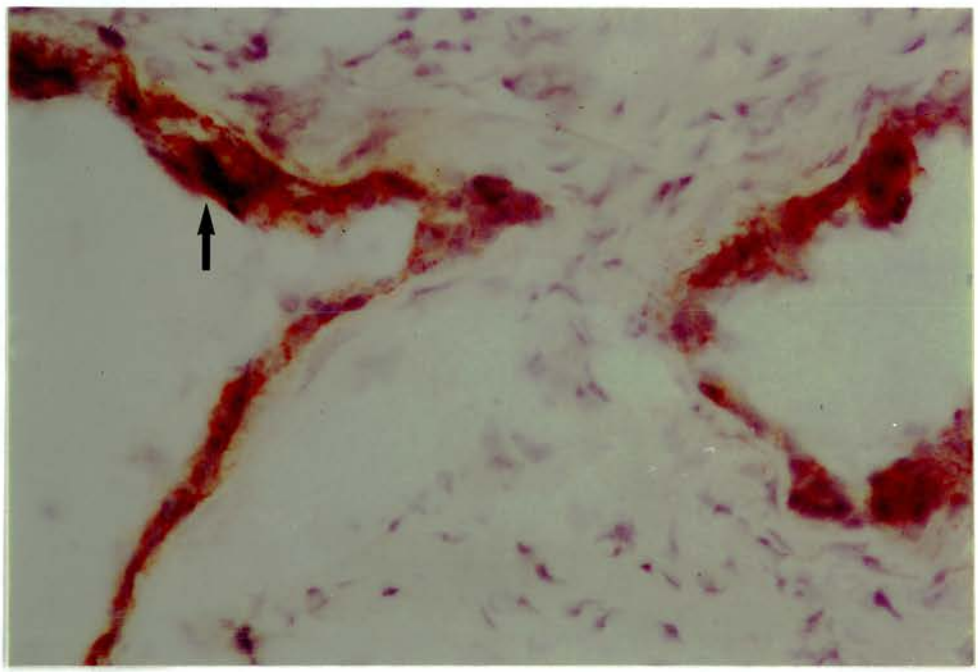
c) Treated testicular tissue.

Treated testicular tissue was obtained from patients who had been treated for prostatic cancer by orchiectomy as a second line of therapy. Initially the patients had received either cyproterone acetate or stilboestrol as a course of medication. In this group of tissues specific binding of EGF ranged from between 0 and 0.06 nM (mean = 0.03 ± 0.01) in the 6 tissue samples which were investigated (Figure 30).

3.3 Immunohistochemical Localisation of the EGF Receptor.

Immunohistochemical staining of 21 frozen sections of human testicular tissue with monoclonal antibodies for the hEGF receptor illustrated that EGF receptors are present in the tissue. In employing the immunohistochemical streptavidin-biotin technique a prostate section was included in all the immunohistochemical studies as a positive control (Plate 2a and 2b). A negative control of each section used in the staining procedure was also performed (Plate 3a) by emitting the primary antibody. In the early development of this procedure false positive staining was apparent due to the presence of biotin in the testes. Consequently an avidin biotin blocking kit was used to eliminate this

(a)



(b)

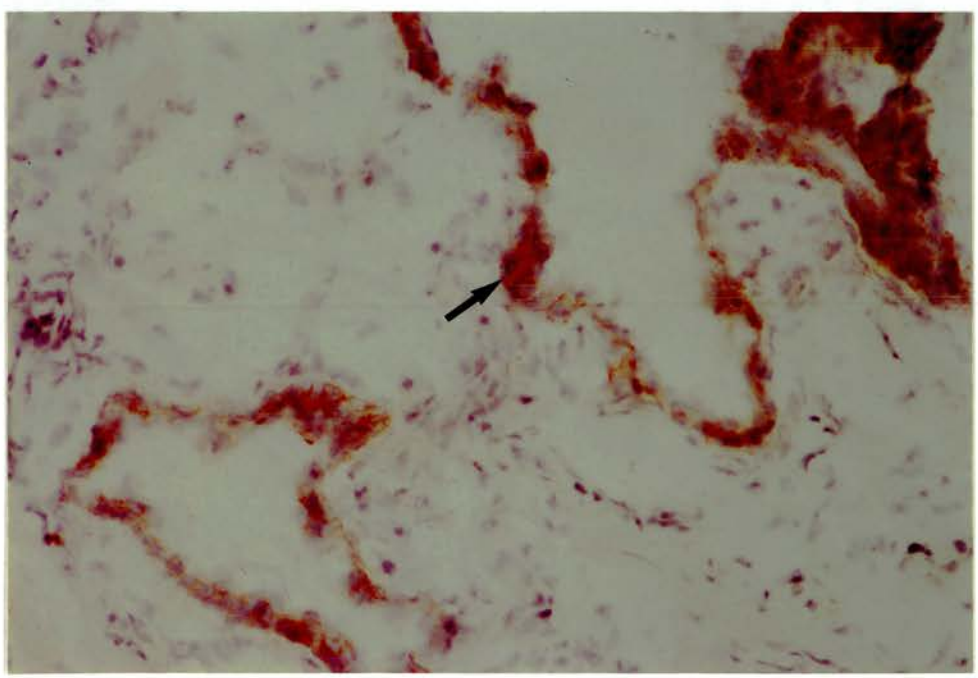


Plate 2 Frozen sections of prostate were used as positive controls for the F4 and the EGF-R1 monoclonal antibodies. Plate (**a**) [x400] illustrates the binding sites detected with the F4 antibody and plate (**b**) [x160] those detected with the EGF-R1 antibody.

problem. Lipofuskin staining, another common source of false positive was easily distinguishable from EGF receptor staining and this eliminated a further source of potential errors (Plate 3b).

In using the streptavidin-biotin technique and the monoclonal antibody EGF-R1, EGF receptors were located on both Leydig cells and peritubular cells. This is clearly seen in plates 4a and 4b where the staining appears as strands of red around both the thin peritubular cells surrounding the seminiferous tubules and also around the large round Leydig cells present in clusters within the interstitium. This was confirmed when using 6 paraffin sections with the monclonal antibody for the external domain of the EGF receptor, EGF-R (Plates 5a and 5b). In neither procedure was staining observed in Sertoli or germ cell areas. It was also noted that when using the antibody for the internal domain of the receptor (F4) no staining was apparent on the paraffin or frozen sections of human testicular tissue which were employed despite titrating the antibody over a concentration range of 1/3 to 1/8 (v/v).

Testicular tissues from men in the age group 30-35 (treated for vasectomy reversal) were also examined for EGF receptor expression using the EGF-R1 monoclonal antibody and the F4 monoclonal antibody. These were also found to express only the external domain of the EGF receptor (Plate 6a and 6b). Foetal testicular tissue obtained from foetus of between 15 and 19 weeks gestation was also found to express only the external domain of the EGF receptor and not the internal domain (Plate 7).

EGF receptors were also shown to be present in the 3 teratoma tissues by immunohistochemical staining with the monoclonal antibody for the external domain of the receptor (EGF-R1) and the monoclonal antibody for the internal domain F4 (Plates 8a and 8b). The staining was never diffuse but always appeared in and around defined structures which were apparent within the teratoma section. This complemented the biochemical findings previously mentioned where a ligand exchange assay was

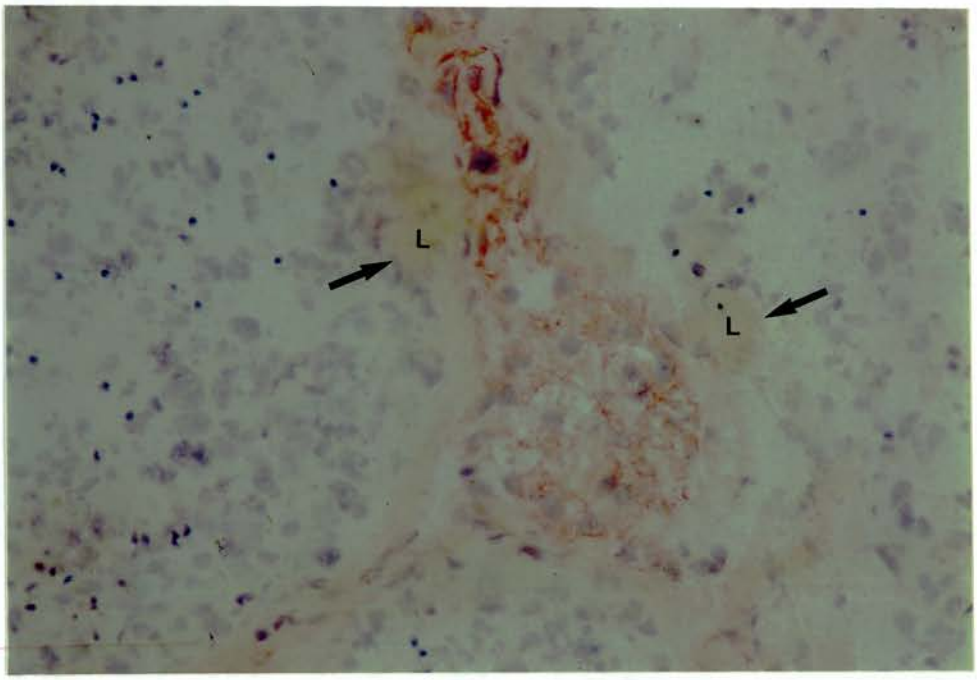


Plate 3b Lypofuskin staining is easily interpreted as non-specific staining (**L**).

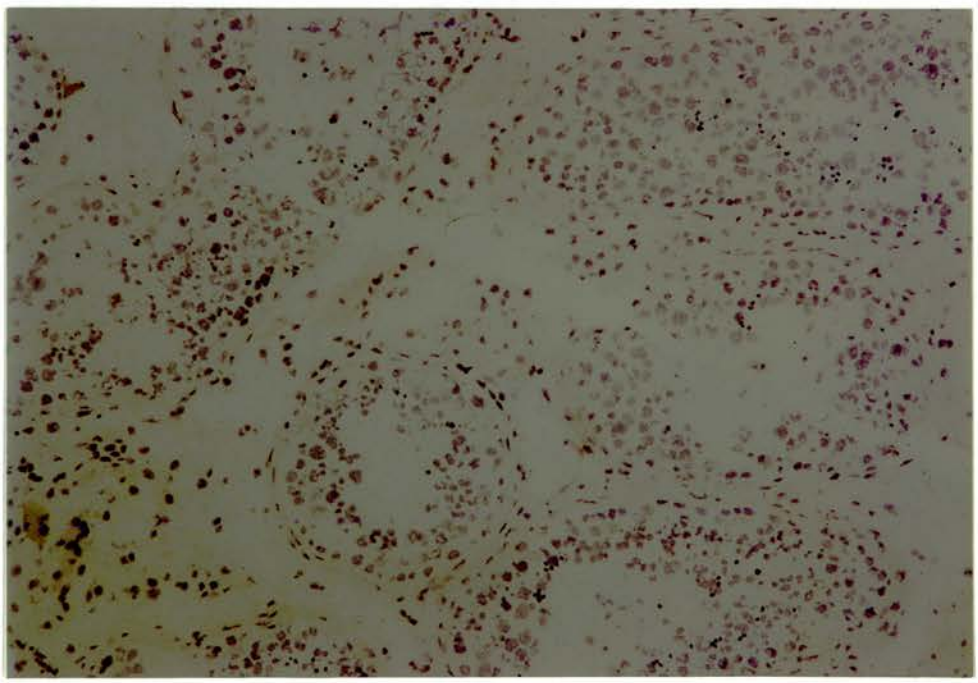
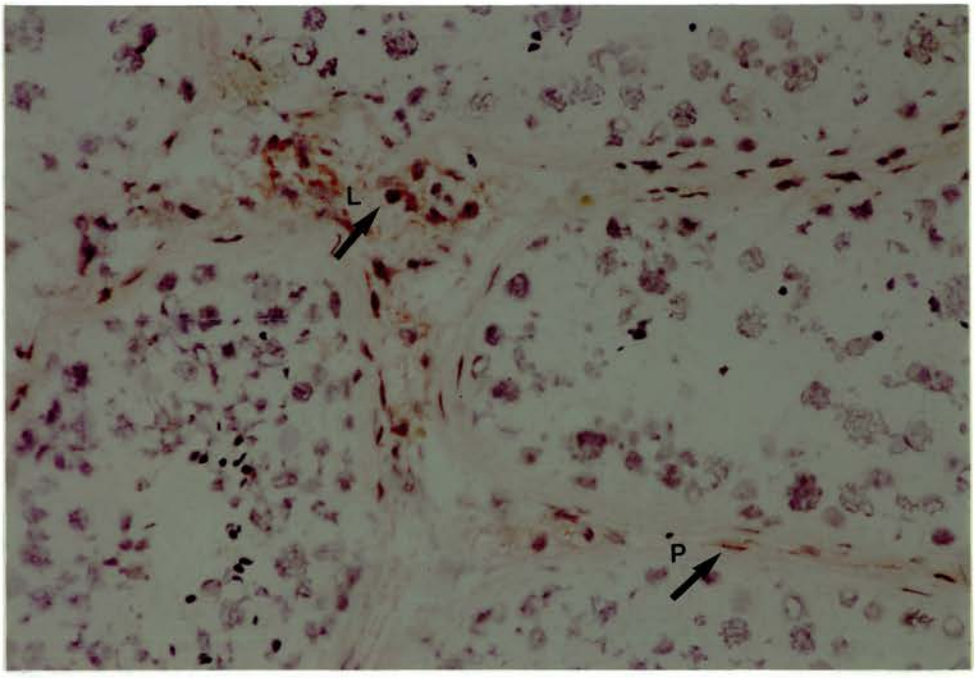


Plate 3a Negative controls were used to show the selectivity of the EGF-R1, EGF-R and F4 monoclonal antibodies for the EGF receptor on human testicular tissue. The section was incubated in Tris buffer with the omission of primary antibody. There is clearly no red staining on the section illustrated (x 160).

(a)



(b)

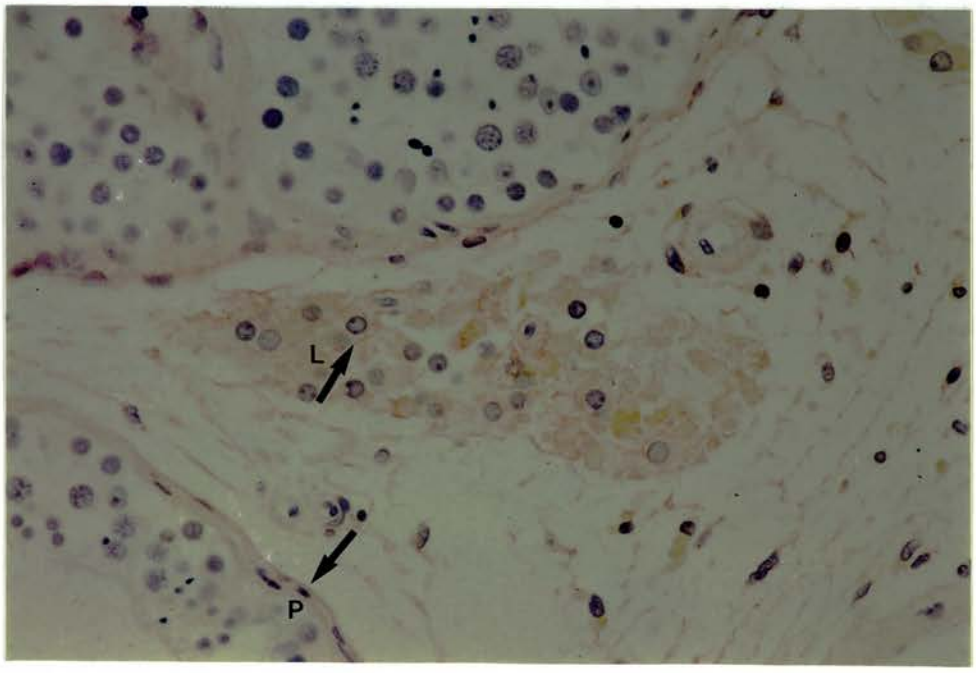
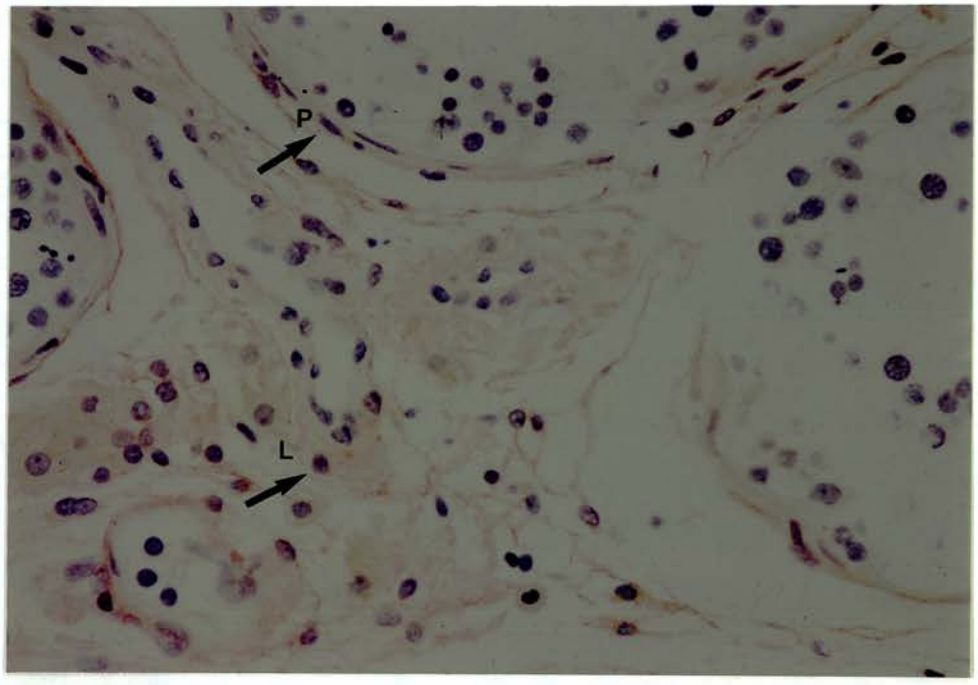


Plate 4 Immunohistochemical staining using the streptavidin-biotin labelled method on frozen sections of human testicular tissue was performed (**a**) and (**b**) [x 400] . The primary monoclonal antibody EGF-R1 was used at a dilution of 1:30 (v/v) and the sections lightly counterstained with haematoxylin. A positive reaction for the EGF receptor appears as dark diffuse staining in the interstitium both around the peritubular (**P**) and Leydig cells (**L**).

(a)



(b)

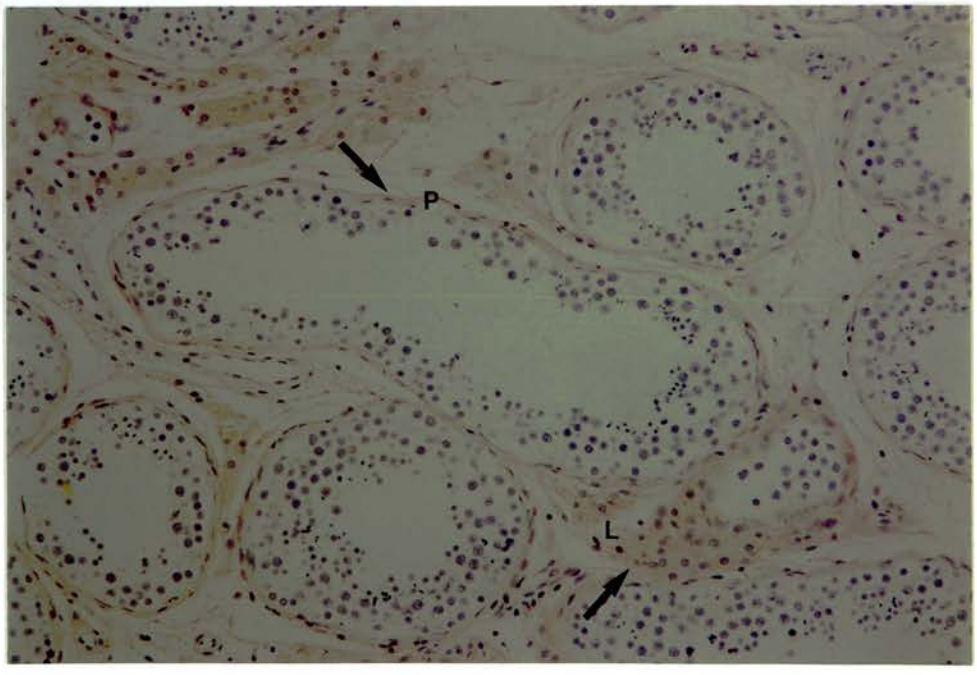
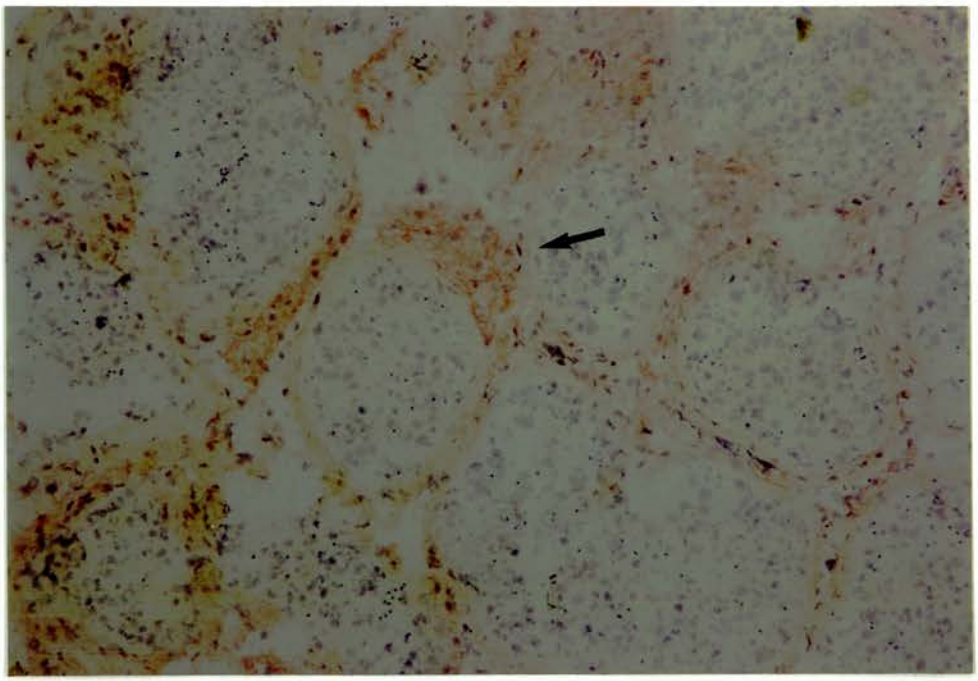


Plate 5 Immunohistochemical staining using the streptavidin-biotin labelled method on paraffin sections of human testicular tissue [x 400 (**a**), x 160 (**b**)]. The monoclonal antibody EGF-R was employed at a 1:10 dilution (v/v). Leydig cells (**L**) became stained along with the peritubular cells surrounding the seminiferous tubules (**P**).

(a)



(b)

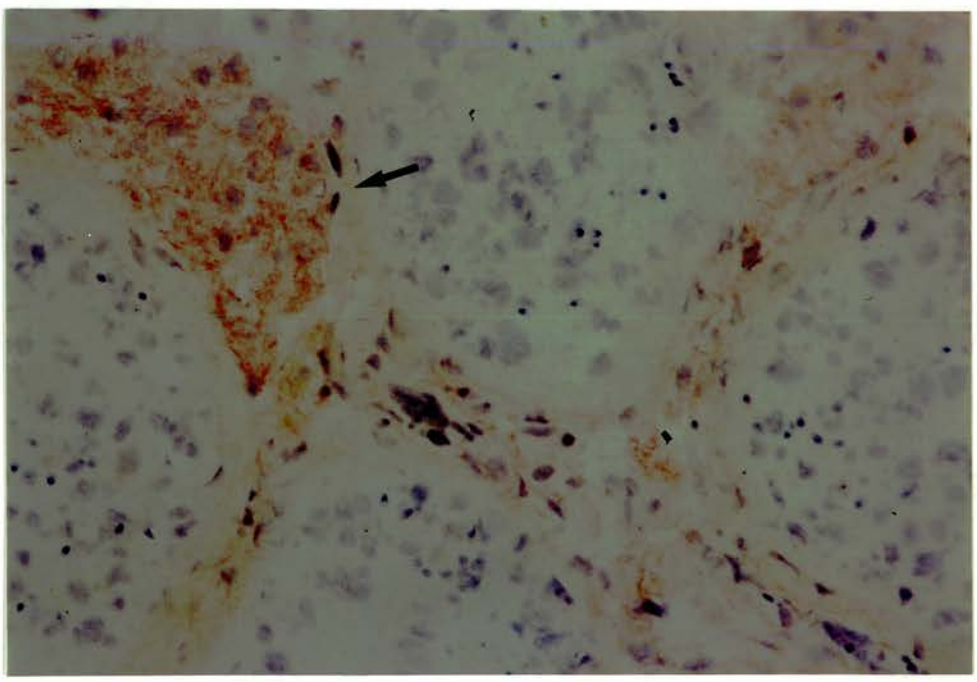


Plate 6 Staining of frozen sections [x160 (**a**), x400 (**b**)] with the monoclonal antibody EGF-R. The sections were cut from a biopsy specimen received from a normal, healthy 34 year old male. The staining appears to be slightly more dense than that observed in the older specimens looked at. However it is located in the interstitial areas as previously seen and around the seminiferous tubules.

(a)

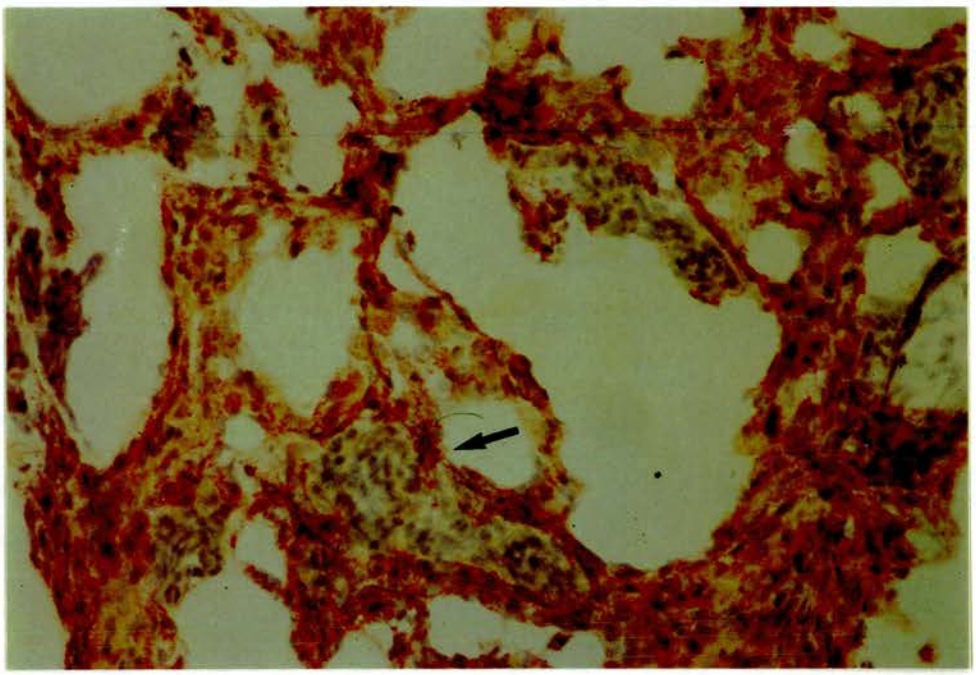
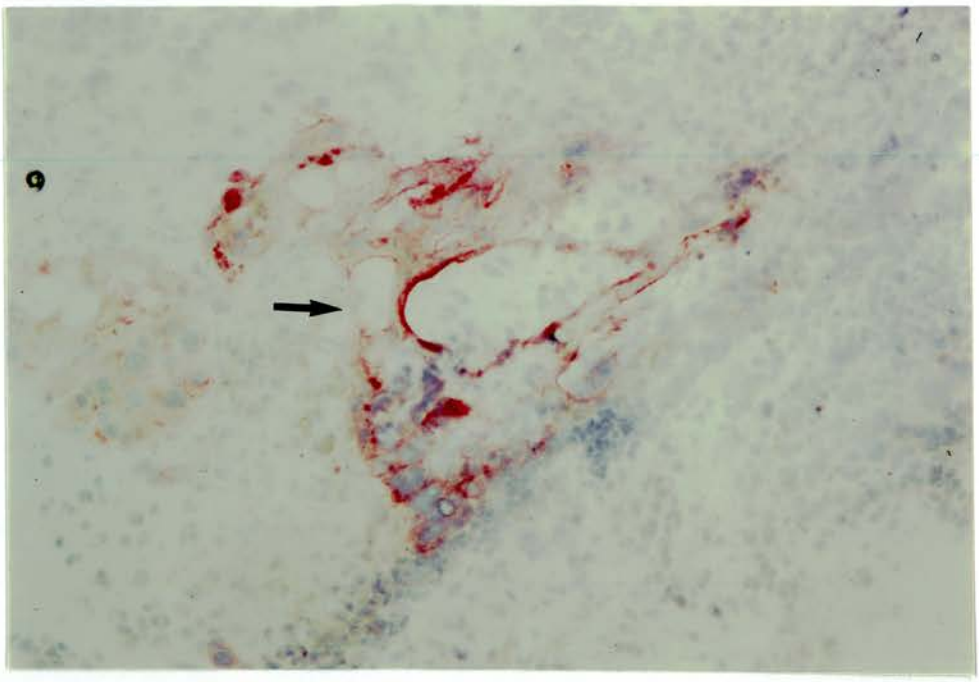


Plate 7 Frozen sections of foetal tissue (between 15 and 19 weeks of gestation) were incubated with the monoclonal antibody EGF-R1 (**a**) and stained accordingly. Although the sections were not of the highest quality EGF receptor staining is apparent in the interstitial areas.

a)



b)

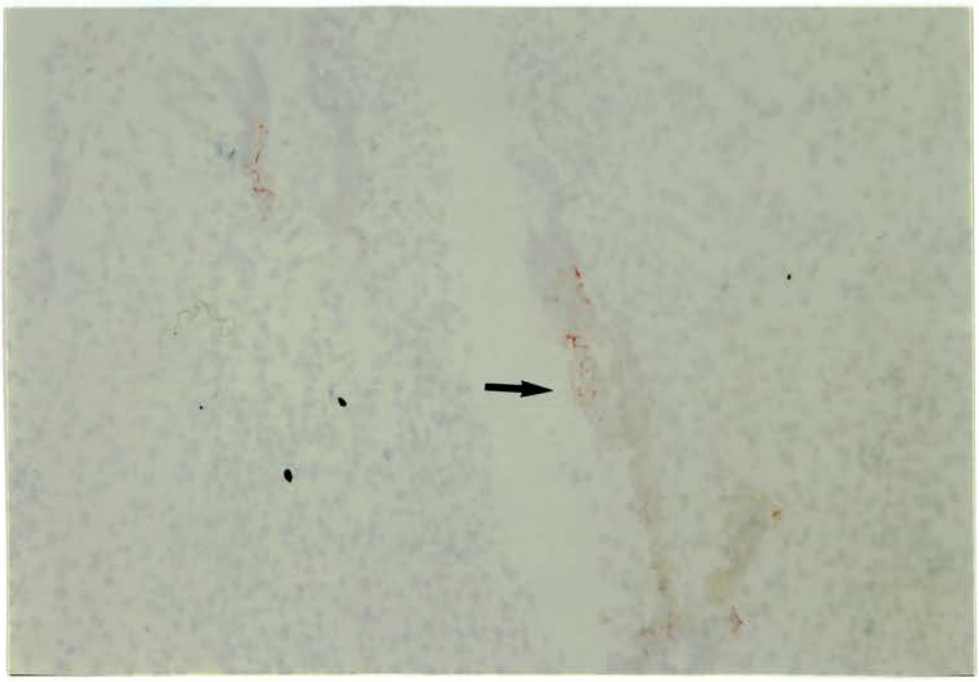


Plate 8 Frozen sections of two teratomas were incubated with the EGF-R1 monoclonal antibody **(a)** [x400] and with the F4 monoclonal antibody (**b**) [x160] . The external domain of the EGF receptor as well as the internal domain of the receptor were both found to be expressed. The staining was always confined to certain areas of the tissue and often appeared to be extremely dense.

used to identify the EGF receptor. The immunohistochemical results for the other tissue groups also confirmed that receptors were either completely absent or distributed in very small diffuse patches on either the seminoma or treated testes.

Experiments in which the F4 monoclonal antibody was employed for detection of the internal domain of the receptor on seminoma or treated tissue illustrated that the internal domain of the EGF receptor on human testicular was not available for binding.

3.4 Molecular Characterisation of the EGF Receptor: Affinity Labelling and Crosslinking of the EGF Receptor Complex Followed by Electrophoresis (SDS-Page) and Autoradiography.

Molecular characterisation of the EGF receptor by an affinity labelling and crosslinking procedure enabled visualisation of the EGF binding site. The bound labelled mEGF/receptor complex was prepared in the absence and in the presence of unlabelled mEGF, solubilised and then crosslinked before being transferred onto a 7.5% SDS polyacrylamide gel for electrophoresis. The protein bands of the solubilised receptor complex which were prepared in the absence of unlabelled mEGF can be seen in lanes A1, A2 and A3. Lanes B1, B2 and B3 represent the protein bands of the solubilised receptor complex which were prepared in the presence of unlabelled mEGF (Plate 9). In lanes A1, A2 and A3 there are dark bands around the 125 kDa molecular weight region which are absent in lanes B1, B2 and B3. These dark bands represent the ^{125}I labelled EGF receptor/mEGF complex protein. It is absent in lanes B1, B2 and B3 because in place of labelled mEGF, unlabelled mEGF which was used as a competitor is bound to the receptor.

The molecular weight of the protein band corresponding to the EGF receptor complex was estimated by use of standard molecular weight markers which

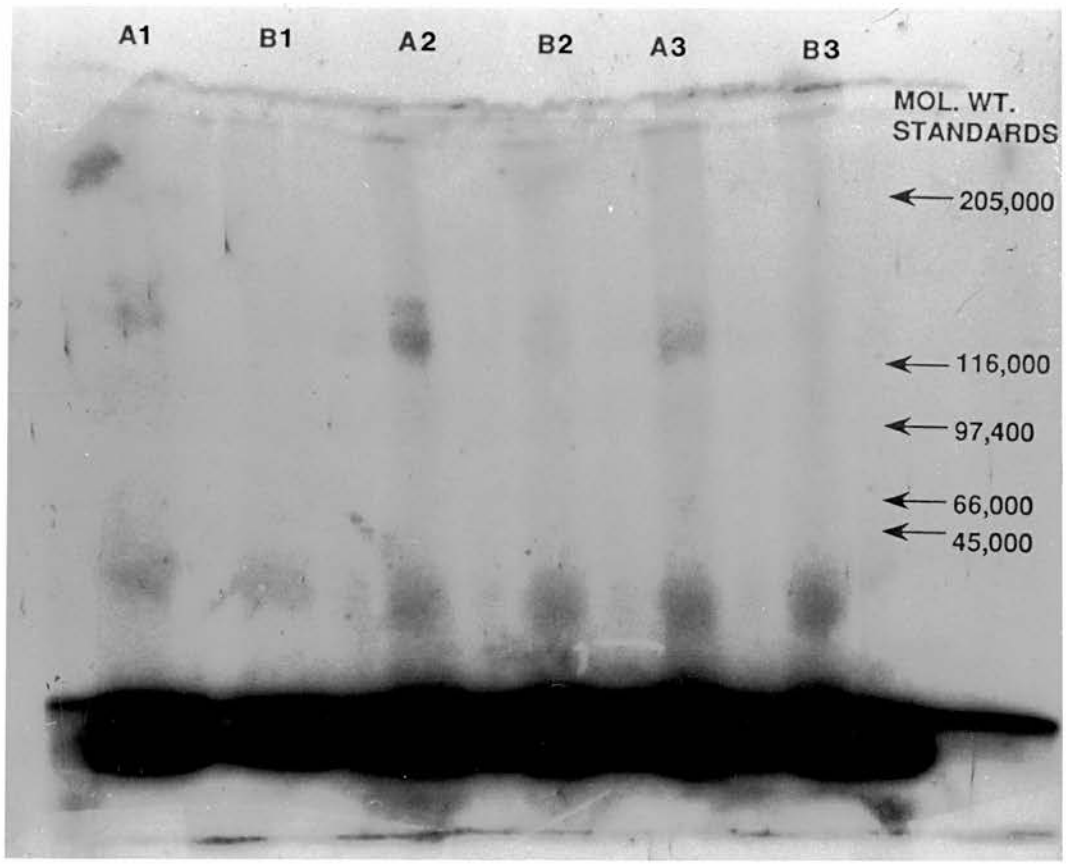


Plate 9 Autoradiography was performed on three testicular tissue samples (1,2 and 3) that had been pre-incubated with 8 nM ^{125}I mEGF at 32^{o}C for 40 min in the presence (lane **B**) and absence (lane **A**) of unlabelled EGF at a 50-fold excess concentration. The receptor complex from each sample was then crosslinked with 1 mM DSS and run on a 7.5% SDS-polyacrylamide gel, along with standards ranging from 29,000 to 205,000 in molecular weight. The gel was subsequently counterstained, dried and autoradiography was carried out at -70^{o}C with an Amersham hyperfilm TM for up to 4 weeks.

were run concurrently on the gel. Labelling of some smaller molecular weight compounds was also observed although these were thought to be non-specific with relation to the EGF receptor as unlabelled EGF did not displace binding to these bands.

3.5 EGF Concentrations in Human Testicular Tissue.

The concentration of EGF in normal human testicular tissue (n=12) was statistically greater than that found in the cancerous seminoma tissues ($p<0.05$, n=6) or the treated testicular tissues ($p<0.05$, n=6) (Figure 31). In the normal group of tissue the concentrations ranged from 1.4 to 11.5 ng/g wet tissue with a mean of 5.2 ± 1.0 ng/g. In the seminoma group the concentrations ranged from 2.0 to 3.6 ng/g wet tissue with a mean of 2.5 ± 0.3 ng/g and in the treated group from 1.5 to 2.5 ng/g wet tissue with a mean of 2.2 ± 0.2 ng/g. The mean recovery of EGF from normal tissue was approximately 48%, from seminoma tissue 51% and from treated tissue 53%.

3.6 TGF-α Concentrations in Human Testicular Tissue.

A rTGF-α RIA was established to detect concentrations of hTGF-αover the concentration range 10 to 1200 pg/100 µl . The sensitivity of the assay was calculated to be approximately 22 pg/tube and the IC50 value approximately 70 pg/tube.

The hTGF-α concentrations found in normal, seminoma and treated testicular tissue are illustrated in Figure 32. For normal tissue (n=12) the concentration of hTGF-α detected was 2.8 ± 0.2 ng/g wet tissue and ranged from 1.8 to 4.0 ; for seminoma tissue (n=6) 2.1 ± 0.1 ng/g wet tissue ranging from 1.6 to 2.5 ng/g and for treated tissue (n=6) 1.9 ± 0.3 ng/g wet tissue ranging from 1.2 to 3.0 ng/g.

The concentration of TGF-α in normal tissue was found to be statistically significantly higher than that found in seminoma ($p < 0.05$) and in treated tissue ($p < 0.001$).

Figure 33 illustrates the concentrations of EGF (n=12) and TGF-α

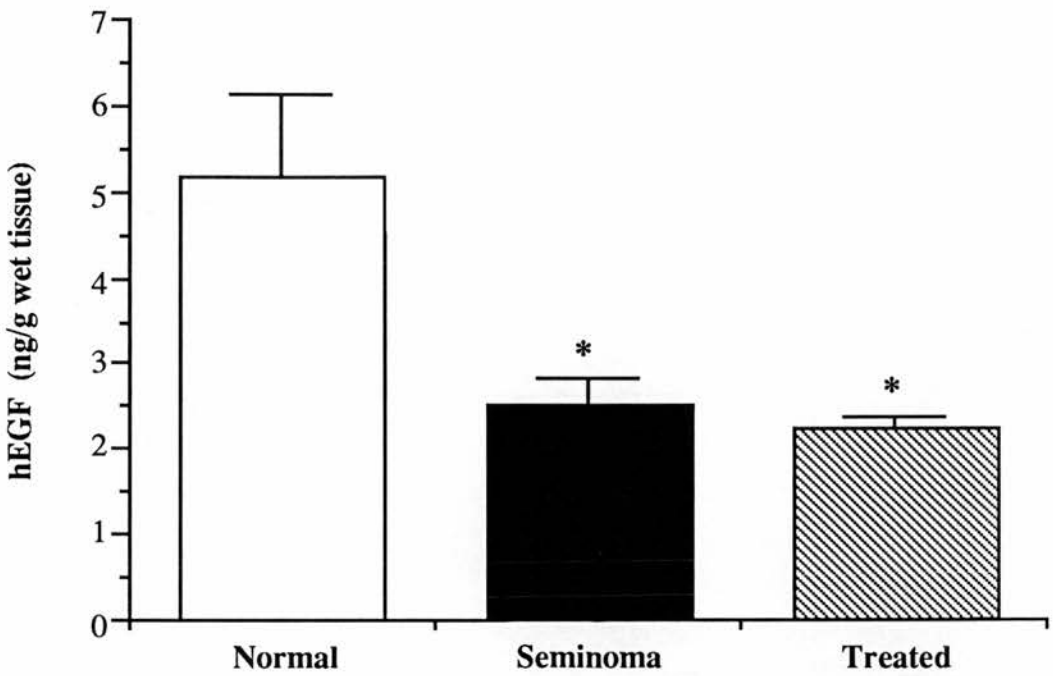


Figure 31
The concentration of hEGF in normal (n=12), seminoma (n=6) and treated (n=6) tissues was assessed. The normal tissues contained a significantly higher concentration of hEGF than did the seminoma (*, $p<0.05$) or treated (*, $p<0.05$) tissues. Values represent mean concentration (± SEM).

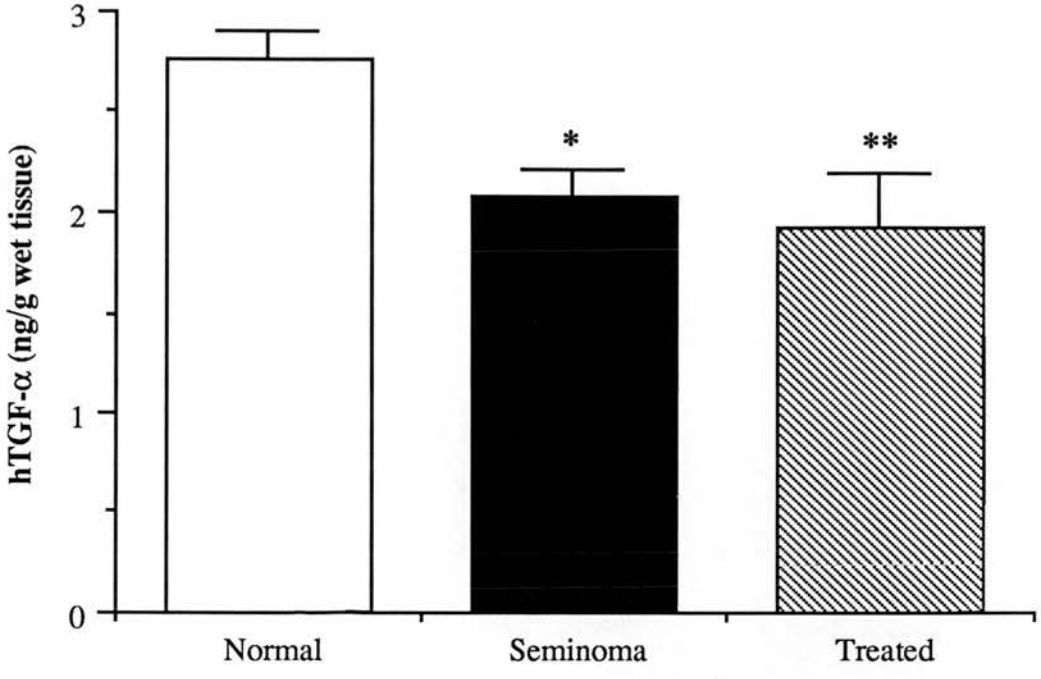


Figure 32

The concentration of hTGF-α found to be present in normal tissue (n=12) was significantly higher than the concentration found in seminoma (n=6) (*, $p<0.05$) and treated (n=6) (**, $p<0.001$) tissues. Values represent mean concentration (± SEM).

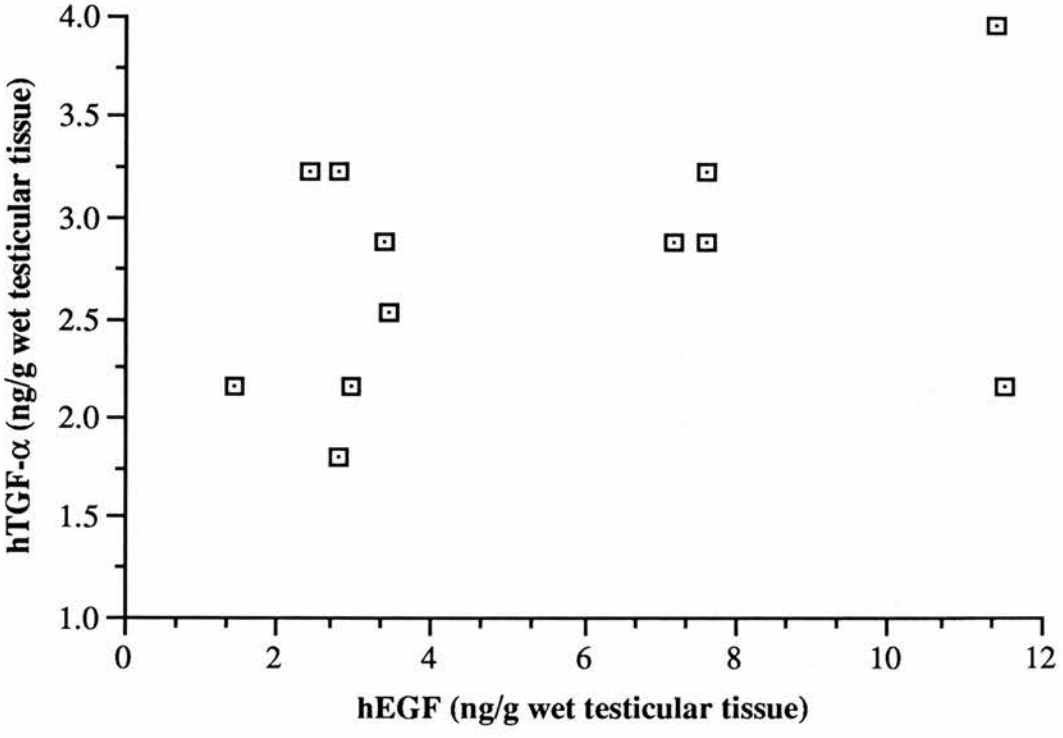


Figure 33

Concentrations of hEGF and hTGF-α in the same testicular tissues were plotted against each other for the 12 tissues which were analysed in an attempt to evaluate any correlation which may exist between the two growth factors *in vivo*. No correlation was found at the $p<0.05$ level of significance.

(n=12) in a number of normal testicular tissue samples, as an attempt to investigate any possible correlation which might exist between the concentrations of EGF and TGF-α . However no statistically significant correlation at the $P<0.05$ level was illustrated between the concentrations of EGF and TGF-α within each individual tissue sample.

3.7 Steroid Concentrations in Human Testicular Tissue.

The concentrations of androstenedione, DHT and testosterone were measured in human testicular tissue. Recoveries of each of the individual steroids were estimated by measuring half of the final separated extract for radioactive counts as described in section 2.9.4. Recovery of the steroids from the normal (n=16), seminoma (n=5) and treated (n=4) tissues are shown in Table 3.

The concentration of steroid in each sample was read from the appropriate standard curve according to the %B/Bo value. (%B/Bo was calculated as previously described in section 2.7.2) This value was then readjusted depending on dilution factors and the amount of tissue from which the sample was prepared. In normal tissue the mean concentrations of steroids were: for androstenedione 0.46 ± 0.13 nmoles/g dry tissue, ranging from 0.11 to 1.61 nmoles/g; for DHT 0.29 ± 0.06 nmoles/g dry tissue, ranging from 0.05 to 0.44 nmoles/g and for testosterone 15.58 ± 2.55 nmoles/g dry tissue, ranging from 0.45 to 31.8 nmoles/g (Figure 34)

In the 5 cancerous tissues the mean concentration of steroids were: for androstenedione 0.025 ± 0.005 nmoles/g dry tissue, ranging from 0.012 to 0.042 nmoles/g; for DHT 0.093 ± 0.017 nmoles/g dry tissue, ranging from 0.043 to 0.173 nmoles/g and for testosterone 0.83 ± 0.25 nmoles/g dry tissue, ranging from 0.40 to 1.75 nmoles/g.

In treated testicular tissue the mean concentration of steroids were: for androstenedione 0.29 ± 0.07 nmoles/g dry tissue, ranging from 0.11 to 0.42 nmoles/g;

Recoveries for	Normal tissue (%)	Seminomas (%)	Treated tissue (%)
Androstenedione	34.7 ± 2.7	45.6 ± 8.2	49.1 ± 2.5
Dihydrotestosterone	31.7 ± 4.0	43.3 ± 4.1	38.2 ± 3.2
Testosterone	47.0 ± 3.9	57.0 ± 1.2	51.1 ± 6.1

Table 3
Estimated mean percentage recoveries (± SEM) for steroids extracted from normal (n=16), seminoma (n=5) and treated (n=4) human testicular tissues.

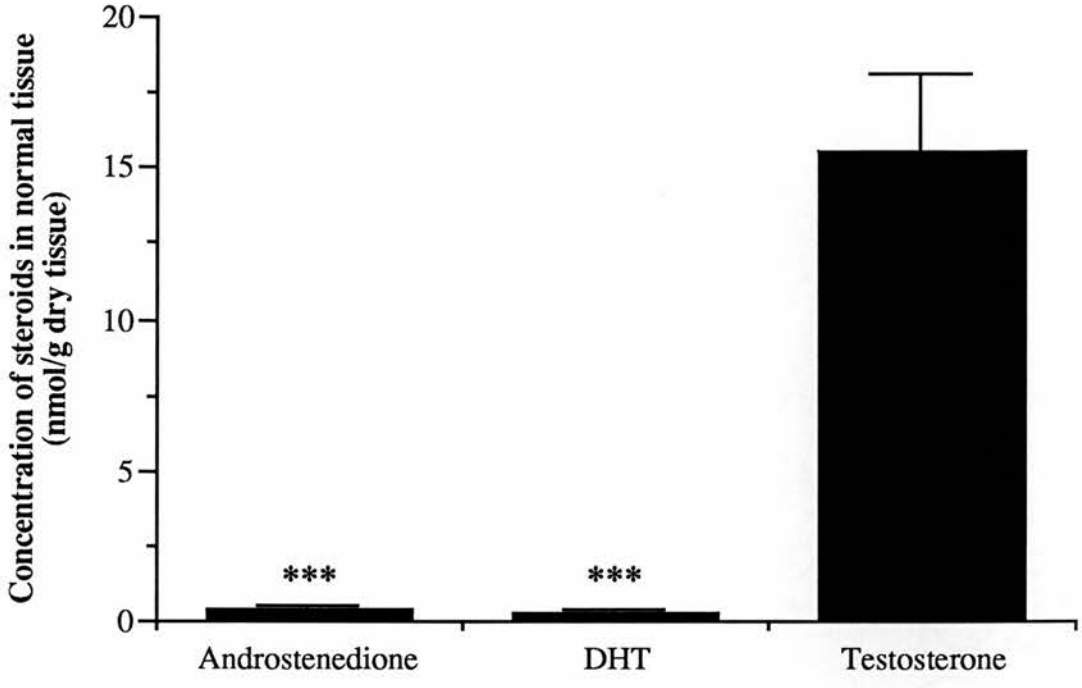


Figure 34
The concentrations of androstenedione, DHT and testosterone in normal testis received from 16 patients of approximately 72 years of age were measured. The concentration of testosterone in the normal tissue was found to be significantly higher than the concentrations of DHT (***, $p<0.0005$) and androstendione (***, $p<0.0005$). Values represent mean concentration (± SEM).

for DHT 0.31 ± 0.05 nmoles/g dry tissue, ranging from 0.19 to 0.44 nmoles/g and for testosterone 14.52 ± 0.40 nmoles/g dry tissue, ranging from 13.86 to 15.56 nmoles/g.

Figures 35a, b and c show the differences between androstenedione, DHT and testosterone in normal, seminoma and treated tissue. As is illustrated in Figure 35a the concentration of androstenedione is significantly higher in normal tissue than in the seminoma tissue ($p< 0.0005$) but not significantly greater than that in the treated tissue. Similarly the concentration of DHT is significantly higher in the normal tissue than in the seminoma tissue ($p<0.01$) but not significantly higher than that in the treated tissue and correspondingly the concentration of testosterone is significantly higher in the normal tissue than in the seminoma tissue ($p< 0.0005$) but not significantly higher than that in the treated tissue.

As steroid secretion has been found to be regulated by growth factors and similarly growth factor receptor expression by steroids it was important to investigate the possible correlation which may exist between steroid concentration and EGF and TGF-α concentrations in each sample analysed. Figures 36a, b and c illustrate any possible correlation which may exist between the concentration of EGF and the concentrations of androstenedione, DHT and testosterone. Similarly Figures 36d, e and f illustrate any possible correlation which may exist between the concentration of TGF-α and the concentrations of androstenedione, DHT and testosterone. However no statistically significant correlation between any of the aforementioned factors/hormones at the $p<0.05$ level was found.

The correlation which may exist between androstenedione, DHT and testosterone concentrations within the testis was also investigated on an individual tissue basis (Figure 37a, b and c). However once again no statistically significant correlation between any of the aforementioned hormones at the $P<0.05$ level was found.

Figure 35

The concentrations of androstenedione (**a**), DHT (**b**) and testosterone (**c**) in normal [n=16], seminoma [n=5] and treated [n=4] tissue were measured. The concentration of steroids in the normal group were not significantly greater than those measured in the treated group. However the concentrations of androstenedione, DHT and testosterone measured in the seminoma subgroup were found to be significantly lower than those measured in the normal group (***, $p< 0.0005$,**, $p< 0.01$ and ***, $p< 0.0005$ respectively).

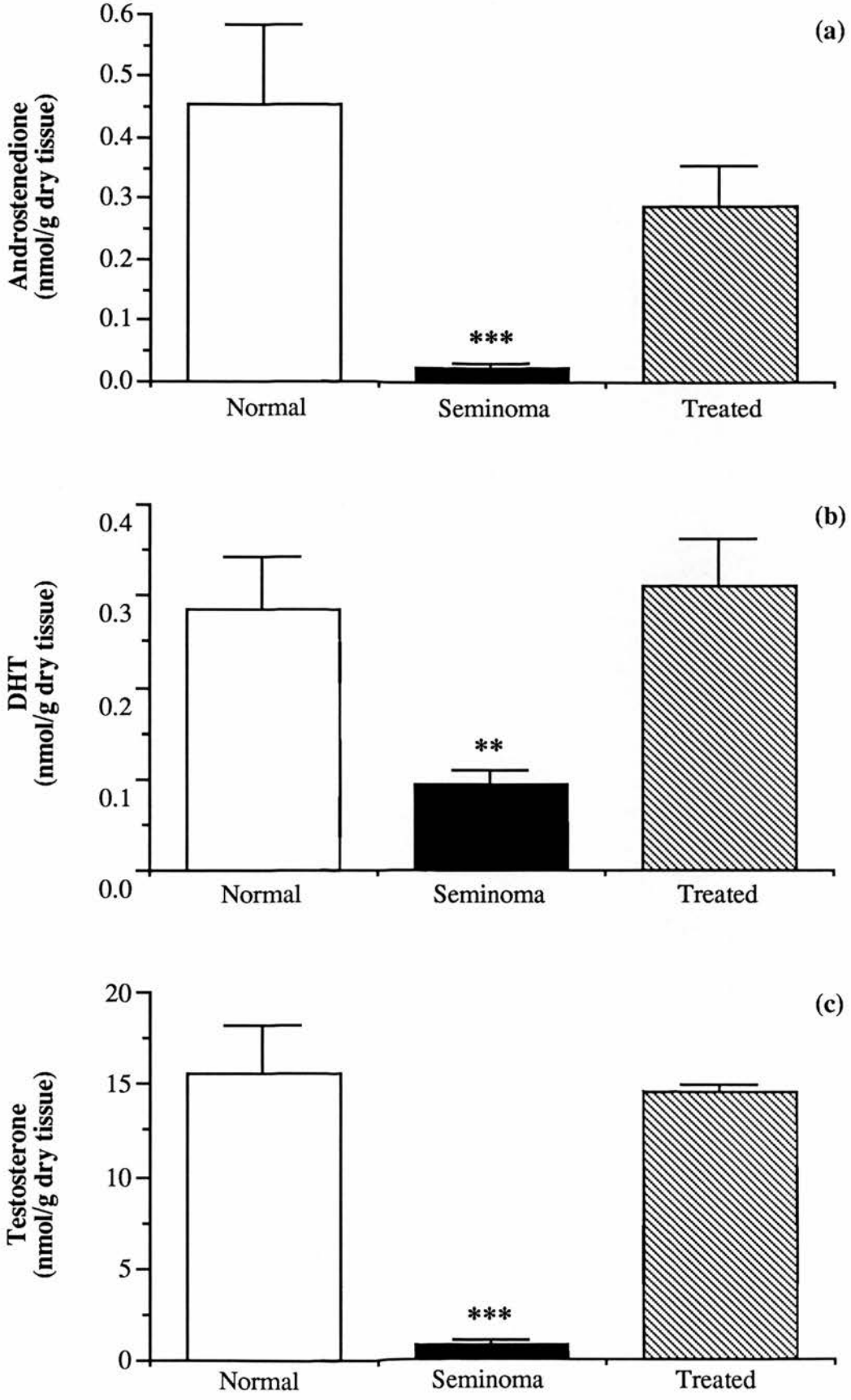

(a)
Androstenedione (nmol/g dry tissue)
0.6
0.5
0.4
0.3
0.2
0.1
0.0
***
Normal
Seminoma
Treated
(b)
DHT (nmol/g dry tissue)
0.4
0.3
0.2
0.1
0.0
**
Normal
Seminoma
Treated
(c)
Testosterone (nmol/g dry tissue)
20
15
10
5
0
***
Normal
Seminoma
Treated

Figure 36

An attempt was made to correlate the concentration of hEGF found in human testicular tissues with the intratesticular concentrations of androstenedione (**a**), DHT (**b**) and testosterone (**c**). A similar correlation was also attempted between hTGF-α and androstenedione (**d**), DHT (**e**) and testosterone (**f**). No correlation between any of the aforementioned factors/hormones was found at a significance level of $p<0.05$. Values represent mean concentration.

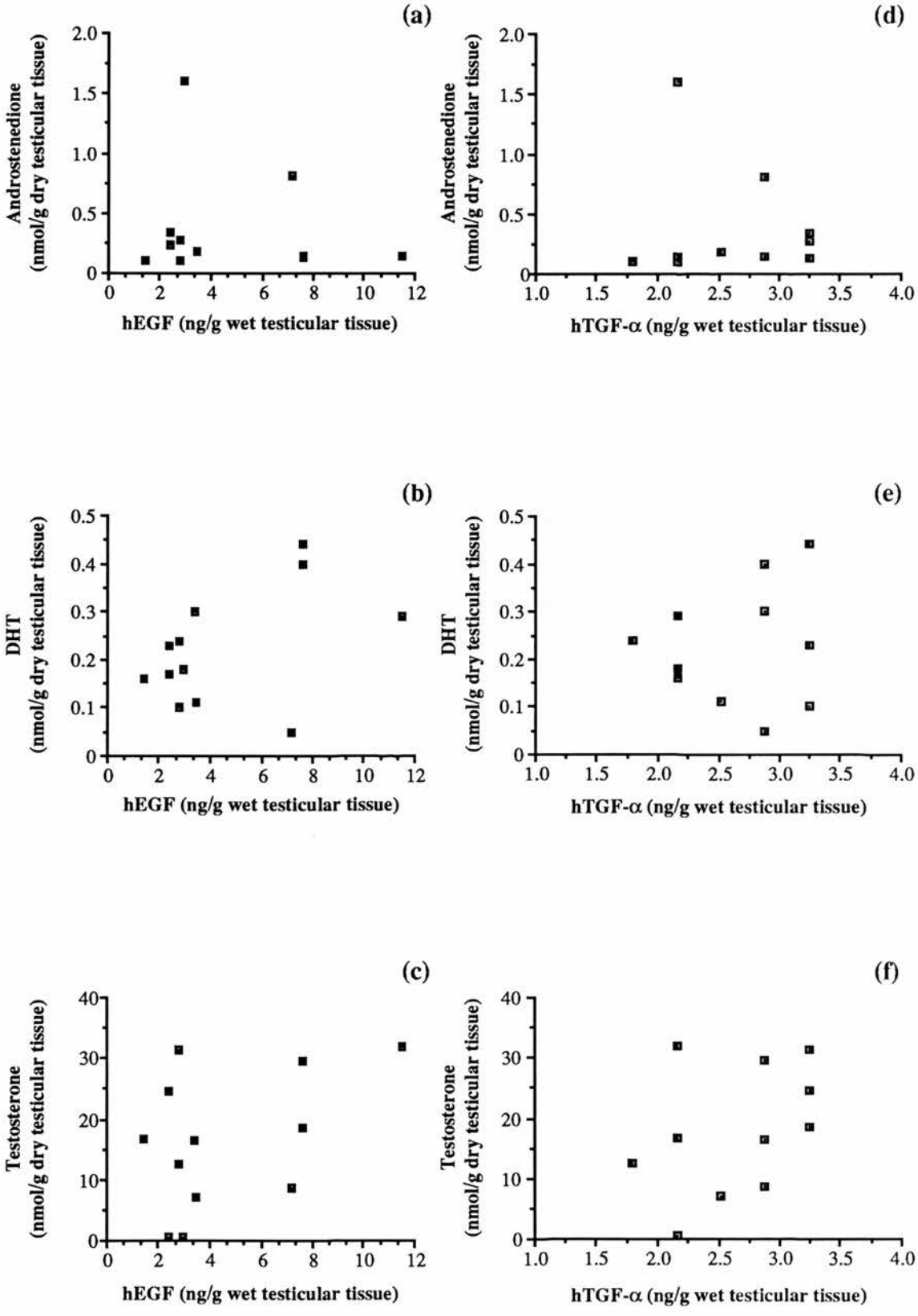
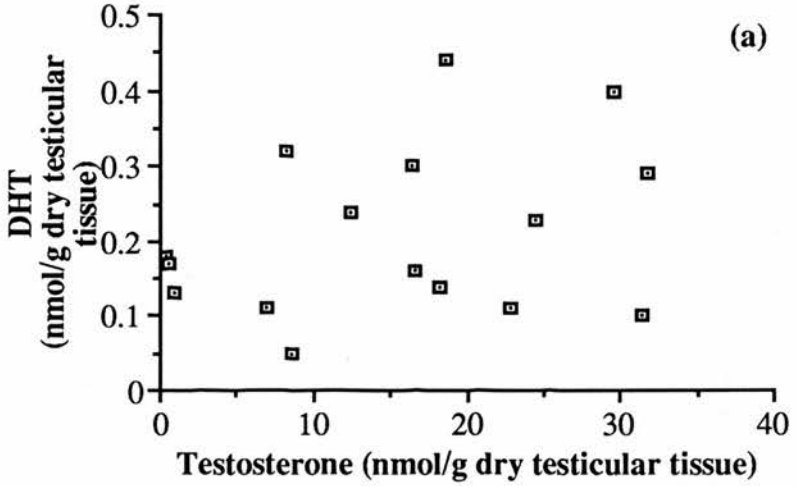
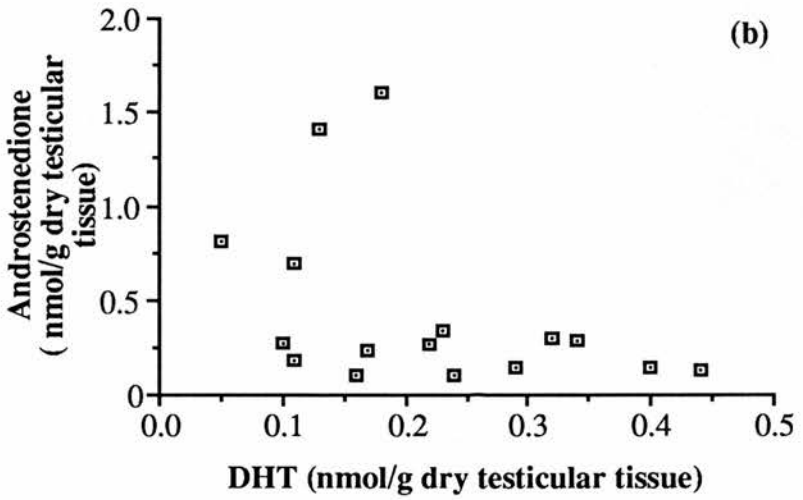
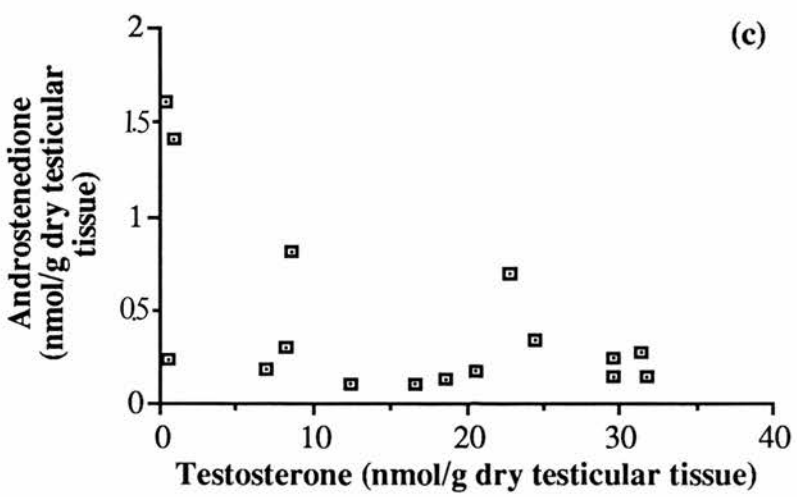

(a)
Androstenedione (nmol/g dry testicular tissue)
hEGF (ng/g wet testicular tissue)
(d)
Androstenedione (nmol/g dry testicular tissue)
hTGF-α (ng/g wet testicular tissue)
(b)
DHT (nmol/g dry testicular tissue)
hEGF (ng/g wet testicular tissue)
(e)
DHT (nmol/g dry testicular tissue)
hTGF-α (ng/g wet testicular tissue)
(c)
Testosterone (nmol/g dry testicular tissue)
hEGF (ng/g wet testicular tissue)
(f)
Testosterone (nmol/g dry testicular tissue)
hTGF-α (ng/g wet testicular tissue)

Figure 37

The correlation between the concentration of androstenedione and DHT (**a**), androstenedione and testosterone (**b**) and DHT and testosterone (**c**) within testicular tissue was investigated. No correlation between any of the aforementioned hormones was found at a significance level of $p<0.05$. Values represent mean concentration.


(a)
DHT (nmol/g dry testicular tissue)
0.5
0.4
0.3
0.2
0.1
0
0
10
20
30
40
Testosterone (nmol/g dry testicular tissue)


(b)
Androstenedione (nmol/g dry testicular tissue)
2.0
1.5
1.0
0.5
0
0.0
0.1
0.2
0.3
0.4
0.5
DHT (nmol/g dry testicular tissue)


(c)
Androstenedione (nmol/g dry testicular tissue)
2
1.5
1
0.5
0
0
10
20
30
40
Testosterone (nmol/g dry testicular tissue)

3.8 Tissue Culture

3.8.1 EGF Receptor Radioligand Exchange Assay

EGF binding on Tera-2 cells was identified using the radioligand exchange assay as described by Engstrom *et al* (1985). Optimum binding was achieved employing labelled mEGF at a concentration of 1.0 nM with unlabelled mEGF in 100 fold excess. In addition displacement studies with unlabelled mEGF followed by saturation analysis and Scatchard plot as well as competition studies were performed.

3.8.2 Displacement Studies with mEGF

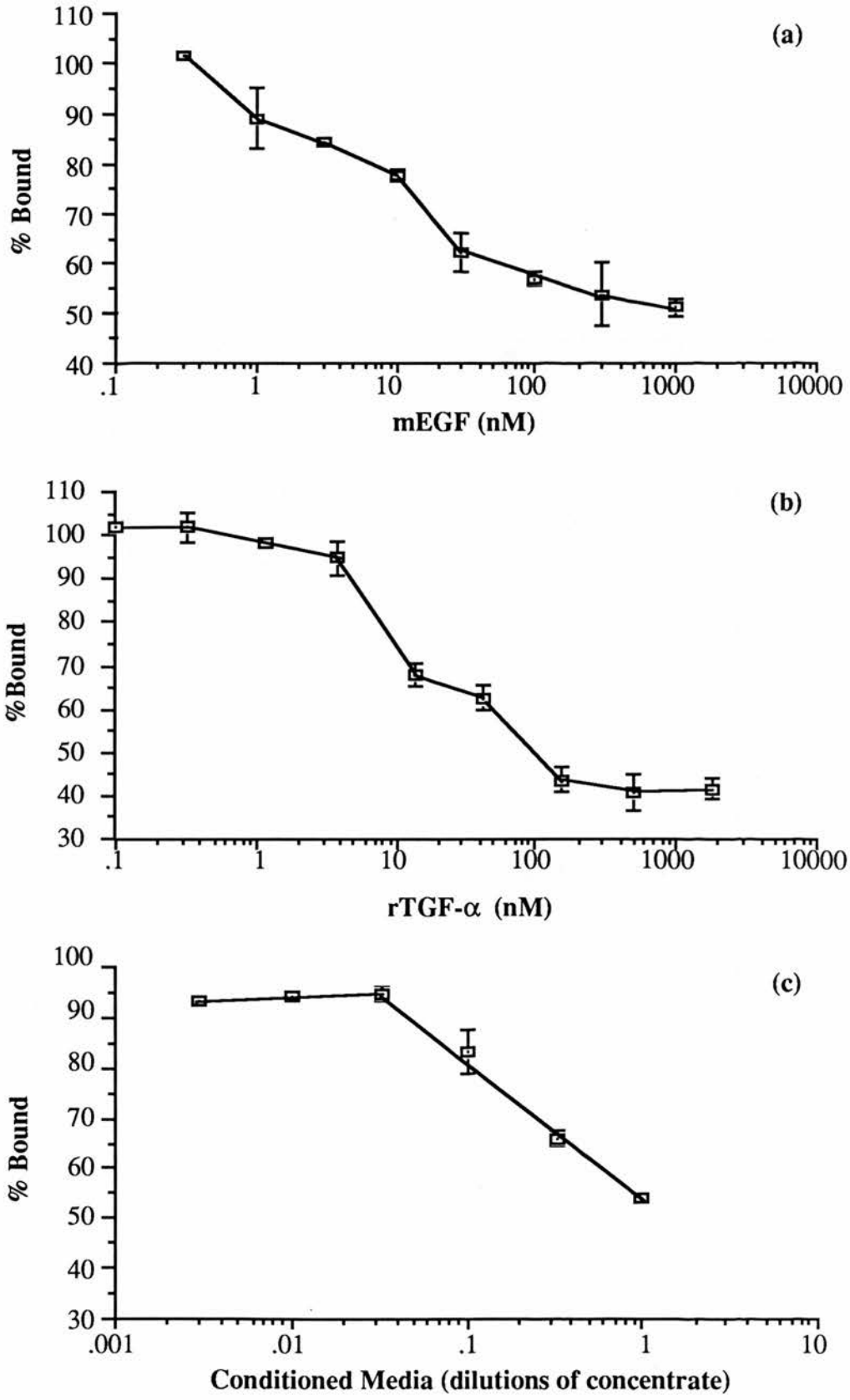
The radiolabelled mEGF specifically bound to the EGF receptor on the Tera-2 cell line was found to be displaced by competing increasing concentrations of unlabelled mEGF (0.3-1000 nM) against a single concentration of radiolabelled mEGF (1 nM, 200,000 cpm). Maximum displacement was achieved when the concentration of unlabelled mEGF was in excess of the labelled mEGF (1 nM) by 100-fold. Radiolabelled mEGF was displaced in a sigmoidal fashion from the binding site by ascending concentrations of unlabelled mEGF (Figure 38a).

3.8.3 Displacement Studies with rTGF-α .

The EGF receptor on the Tera-2 cell line was competed for with various concentrations of rTGF-α (0.1-2000 nM) and labelled mEGF (1 nM, 200,000 cpm). It was found that similar concentrations of rTGF-α were required to compete for the EGF receptor site (Figure 38b) to those already described in the saturation with mEGF study (Figure 38a).

Figure 38

The competition between various concentrations of mEGF, rTGF-α and the concentrated conditioned media with labelled mEGF for the EGF receptor on the Tera-2 cell line was investigated. Concentrations between 0.3 and 1000 nM were chosen for mEGF (**a**), concentrations between 0.1 and 2000 nM for rTGF-α (**b**) and for the concentrated conditioned media (**c**) serial dilutions of the stock solution. Mean percentage bound of labelled mEGF (± SEM, n=3) was then plotted against the concentration of competitor competing for the EGF receptor site.


(a)
% Bound
110
100
90
80
70
60
50
40
.1
1
10
100
1000
10000
mEGF (nM)
(b)
%Bound
110
100
90
80
70
60
50
40
30
.1
1
10
100
1000
10000
rTGF-α (nM)
(c)
% Bound
100
90
80
70
60
50
40
30
.001
.01
.1
1
10
Conditioned Media (dilutions of concentrate)

3.8.4 Displacement Studies with Concentrated Conditioned Medium.

Various dilutions of the concentrated conditioned medium were used to compete for the EGF receptor along with labelled mEGF. The concentrated conditioned media was used in its most concentrated form (1.0) and then at subsequent dilutions (0.3, 0.1 *etc.*). The sigmoidal shaped curve obtained (Figure 38c) is very similar in shape to the curve obtained with rTGF-α (Figure 38b). In fact if the two graphs are superimposed then the concentrated conditioned media (1.0) would represent approximately 50 nM of rTGF-α activity. According to the RIA study the concentrated conditioned media contains only 0.28 pg/ml hTGF-α which represents 0.09 pg/ml rTGF-α which is approximately 0.02 pM of rTGF-α.

3.8.5 Saturation Analysis and Scatchard Plot.

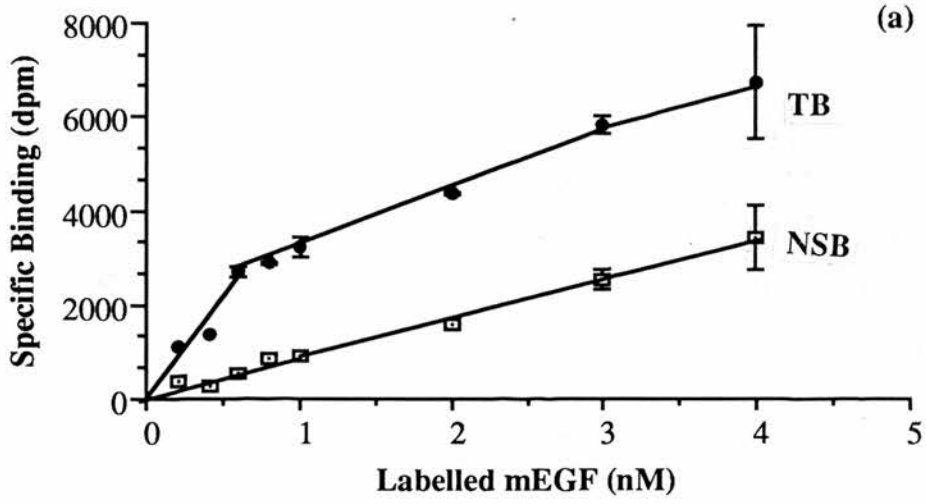
Saturation of the EGF binding site on the Tera-2 cell line with radiolabelled mEGF was performed over the range 0.2-4.0 nM labelled mEGF (1×10^5 to 2×10^6 cpm) with unlabelled mEGF over the range 20-400 nM. Figure 39a illustrates the I^{125}mEGF binding achieved over the concentration range 0.2-4.0 nM while in the presence and absence of unlabelled mEGF. Figure 39b illustrates the specific binding achieved over the same concentration range of radiolabelled mEGF. The saturation data was then analysed by Munson and Rodbards computer programme (1980) to yield a single binding site with a Kd of $0.21 \pm 0.08 \times 10^{-9}$ (n=3) where a maximum confidence limit of 100% was enforced (Figure 39c). The number of binding sites per cell was calculated to be approximately $6.73 \pm 0.81 \times 10^4$.

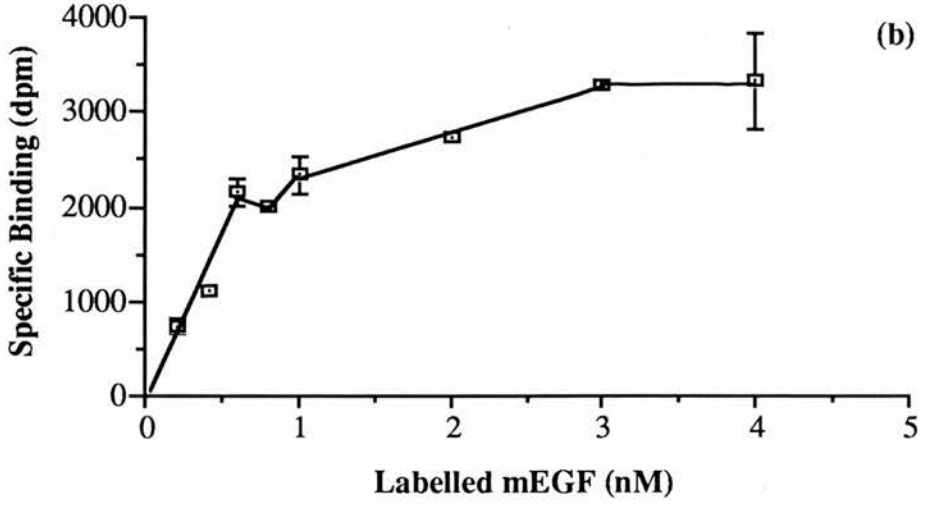
3.8.6 Competition Studies.

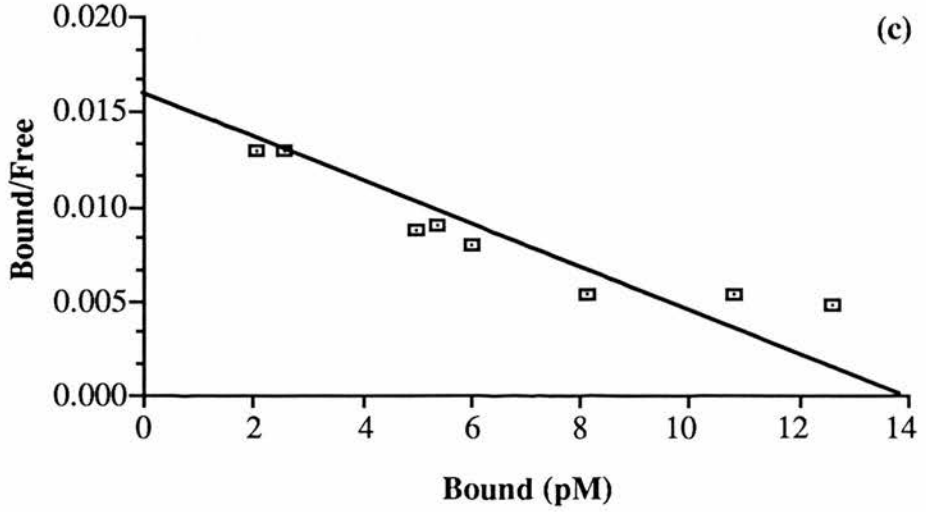
Competitors for the EGF receptor which were tested included rTGF-α , hIGF-I, vNGF, bFGF and concentrated conditioned media (CCM). The results were expressed

Figure 39

Saturation of the EGF binding site on the Tera-2 cell line was performed over the concentration range 0.02 to 4.0 nM of labelled mEGF. Mean nonspecific binding (**NSB**) and mean total binding (**TB**) (**a**) and mean specific binding (± SEM) (**b**) measured at the various concentrations of labelled mEGF were plotted. The specific binding data was than converted to Bound and Bound/Free and a Scatchard plot constructed (**c**). The affinity constant (Kd) of the receptor with respect to EGF was then calculated and found to be approximately 0.25 nM and the number of binding sites 6.7×10^4 per cell .


(a)
Specific Binding (dpm)
8000
6000
4000
2000
0
TB
NSB
0
1
2
3
4
5
Labelled mEGF (nM)


(b)
Specific Binding (dpm)
4000
3000
2000
1000
0
0
1
2
3
4
5
Labelled mEGF (nM)


(c)
Bound/Free
0.020
0.015
0.010
0.005
0.000
0
2
4
6
8
10
12
14
Bound (pM)

as a percentage of the % Bound obtained with unlabelled mEGF. Rat TGF-α showed a near identical competitive reaction to mEGF for the EGF binding site (approximately 99%) (Figure 40). The concentrated conditioned media also showed competition of 84% and vNGF 37% and bFGF 32% competition (Figure 40).

3.8.7 Transmodulation by Mibolerone.

The effect of various concentrations of mibolerone (0.1-100 nM) on EGF receptor expression on the Tera-2 cells was investigated. The cells were incubated in the various concentrations of mibolerone for 24 hours prior to the commencement of the EGF receptor assay. The specific binding at each concentration was found to be similar to that found in the control cells which had been incubated in ordinary serum-free medium prior to the assay (Figure 41). All specific binding data was adjusted according to cell number at each concentration, although the cell number in the control group was not found to be significantly different from the cell number in each of the mibolerone groups.

3.8.8 Western Blot Analysis.

Lane 1 of Plate 10 shows the positions of the molecular weight markers which were run on an SDS-PAGE gel alongside solubilised receptors. The protein bands in lane 2 represent the solubilised EGF receptors from A431 cells which were used as a positive control. The receptors have been immunolabelled with the monoclonal antibody for the external domain of the receptor (EGF-R1). Bands of protein are identifiable in the 170 and 125 kDa molecular weight regions. A band is also noticeable around the 70 kDa area which corresponds to the molecular weight of the internal domain of the receptor. Lane 3 represents solubilised receptors from Tera-2 cells stained in a similar fashion. The intensity of staining in lane 2 is less than lane 3. Proteins from the Tera-2 cells

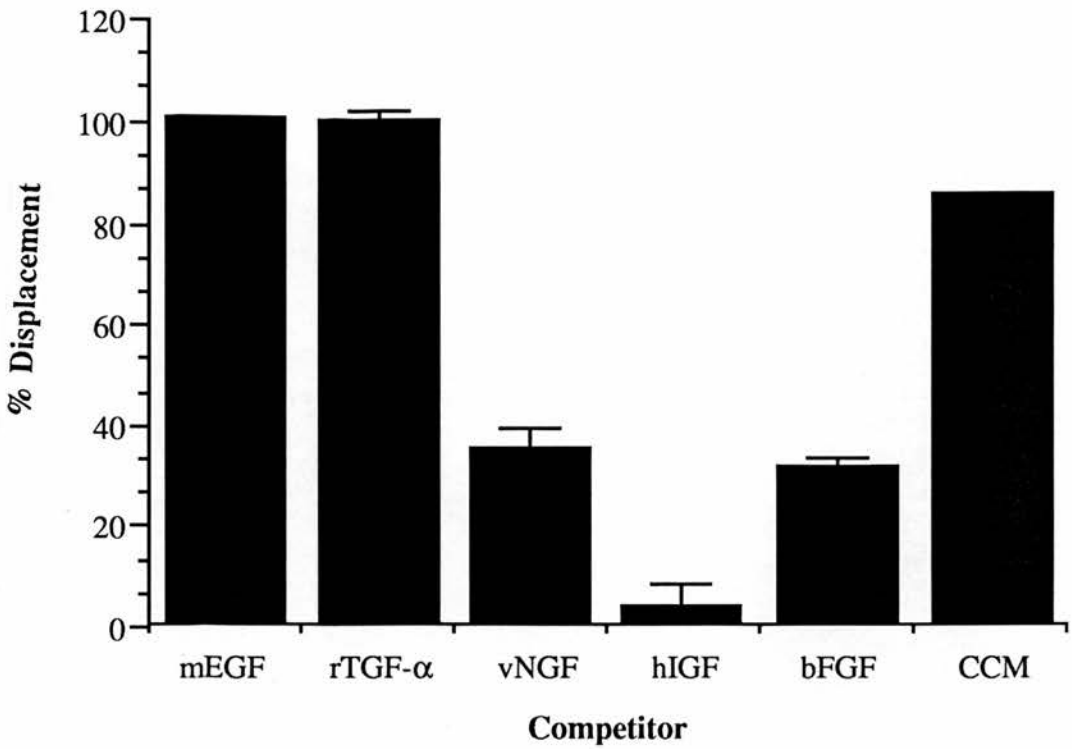


Figure 40
Competition for the EGF receptor was performed by replacing mEGF in the radioligand exchange assay with other peptide growth factors. All growth factors were used 50-fold in excess of the concentration of labelled mEGF employed. The specific binding obtained with each peptide is represented as a mean percentage (± SEM, n=3) of the specific binding achieved with unlabelled mEGF.

To test the specificity of the receptor multiple concentrations of competitor should be used but unfortunately research finance does not always permit this.

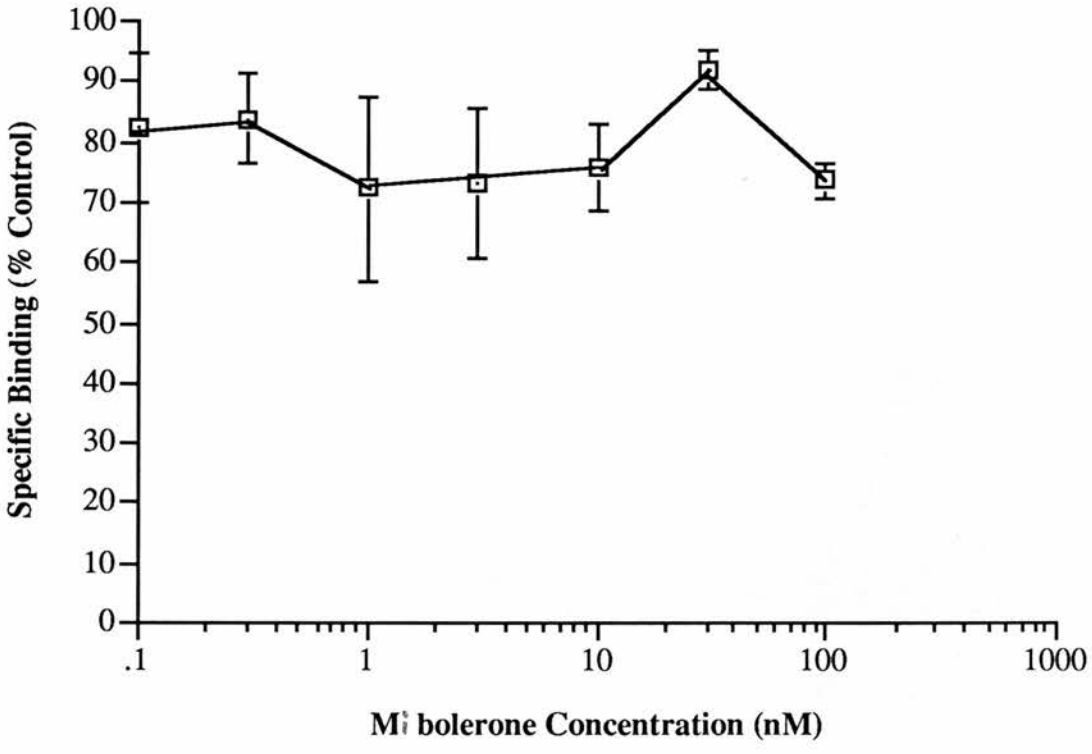


Figure 41
The transmodulatory effect of mibolerone on the expression of EGF receptors in the Tera-2 cell line was investigated. Cells were incubated in concentrations of mibolerone ranging from 0.1 to 100 nM for 24 hours. At the end of the incubation period the cells were employed in a ligand exchange assay to measure the specific binding of labelled mEGF. Data is expressed as the mean percentage (± SEM, n=3) of specific binding obtained when the cells were incubated in serum-free medium (*i.e.* control).

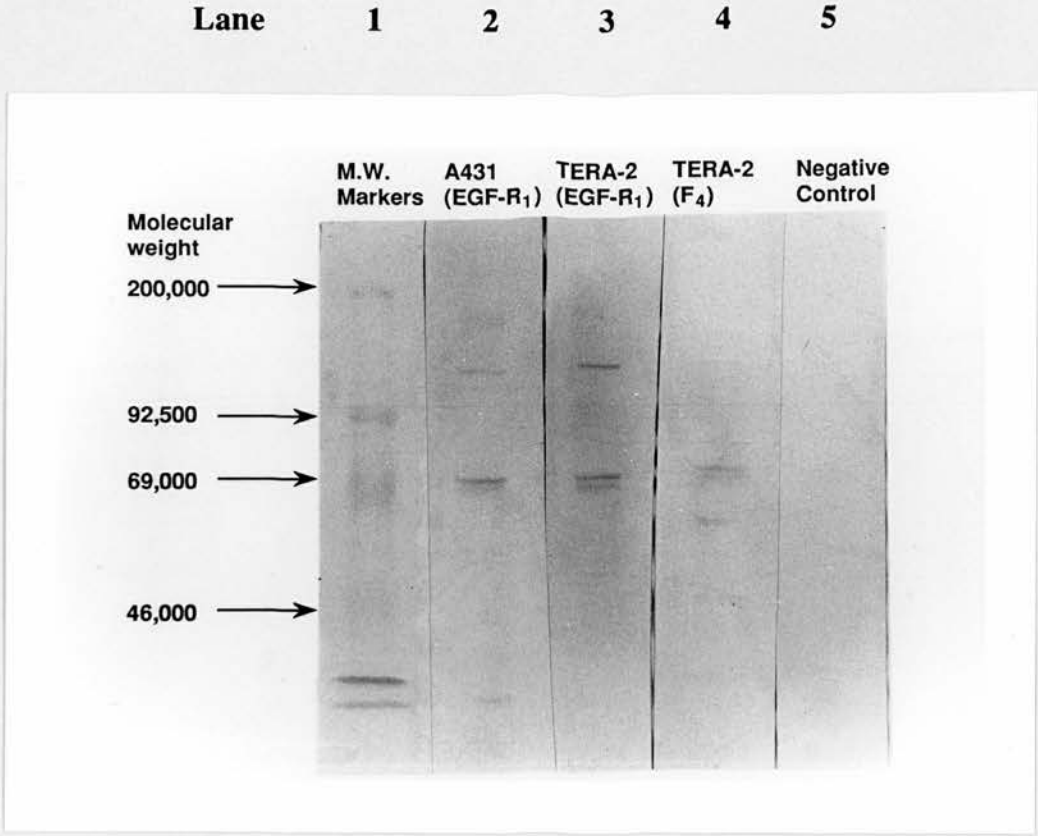


Plate 10

Solubilised receptors from the Tera-2 cell line and the A431 cell line were run electrophoretically on a 7.5% SDS- polyacrylamide gel, along with standard molecular weights ranging from 14.6 to 200 kDa. The gel was then equilibriated in transfer buffer before being transferred to a nitrocellulose membrane. The membrane was then incubated with the appropriate monoclonal antibody and stained employing the streptavidin-biotin technique. Lane **1** illustrate the positions of the molecular weight markers. Lane **2** shows the proteins of the A431 solubilised receptors which were detected by the EGF-R1 monoclonal antibody (the positive control). Similarly lane **3** illustrates the proteins of the Tera-2 solubilised receptors detected with the same antibody where as lane **4** illustrates the proteins detected with the F4 monoclonal antibody. Finally lane **5** was run as a negative control for the Tera- 2 cell line and was incubated in TBS Buffer for 16 hours instead of the monoclonal antibodies.

were employed at twice the concentration as the A431 cells. In lane 4 the solubilised receptors have been immunolabelled with the monoclonal antibody for the internal domain of the receptor (F4). Finally lane 5 shows a negative control for the Tera-2 solubilised receptors (incubated in Tris buffer) which is completely free of labelled protein bands and therefore indicates that the procedure is specific only to immunolabelled proteins.

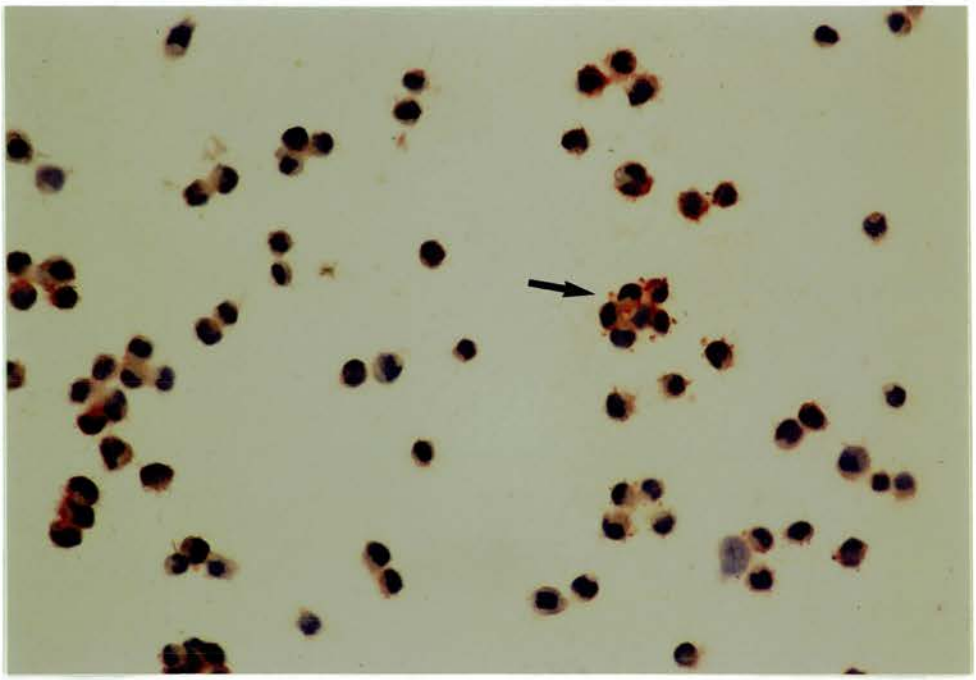
3.8.9 Immunocytochemistry.

Plates 11a and 11b show staining of the Tera-2 cells with both the EGF-R1 antibody and the F4 antibody . Staining for the external domain of the receptor was not observed although staining for the internal domain was observed. A431 cells were used as a positive control for both antibodies both the external and the internal domains of the EGF receptors were expressed on the A431 cells (Plates 12a and 12b). For the Tera-2 cell line the cells were plated down after being detached from a flask where they were at approximately 50% confluency. This was investigated further by performing immunocytochemistry on cells which had been detached from 80 and 100% confluent flasks. It was however found that the percentage of confluency from which the cells had been detached had no effect on the EGF receptor expression with respect to immunolabelling.

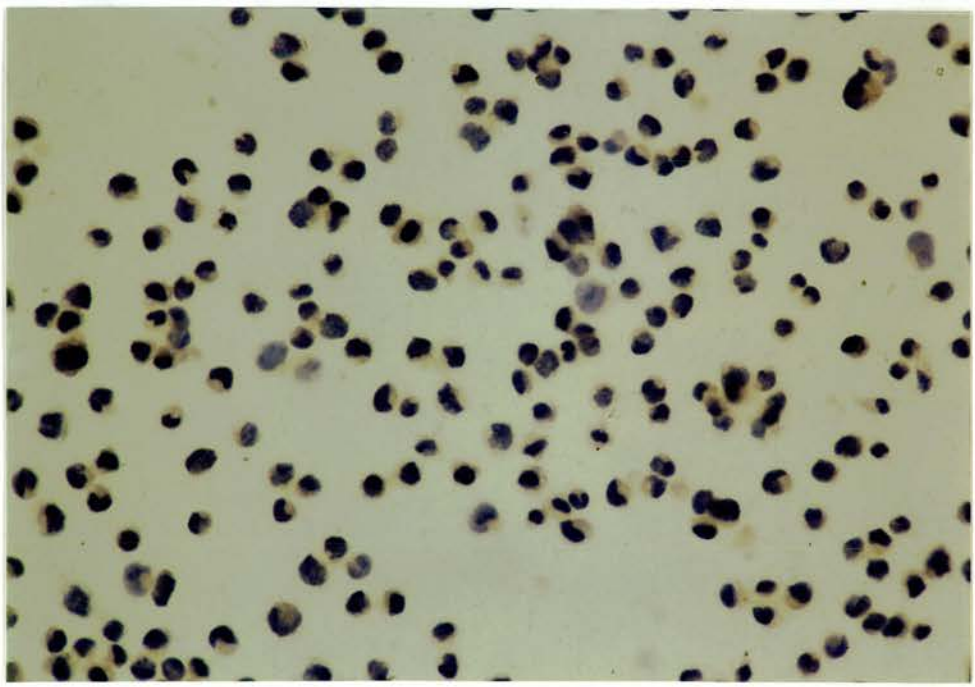
3.8.10 Human EGF and hTGF-α Concentrations in Tera-2 Cell Conditioned Media.

The conditioned media obtained from the Tera-2 cell culture medium werefound to contain 2.40 ± 0.33 pg of hEGF and 2.55 ± 0.78 pg of hTGF-α per ml of conditioned media (Figure 42). To detect such small amounts of EGF and TGF-α in the conditioned media 1000 ml was collected over a 4 week period from 8 flasks of Tera-2 cells being grown in serum-free medium. The medium was then filtered and

(a)

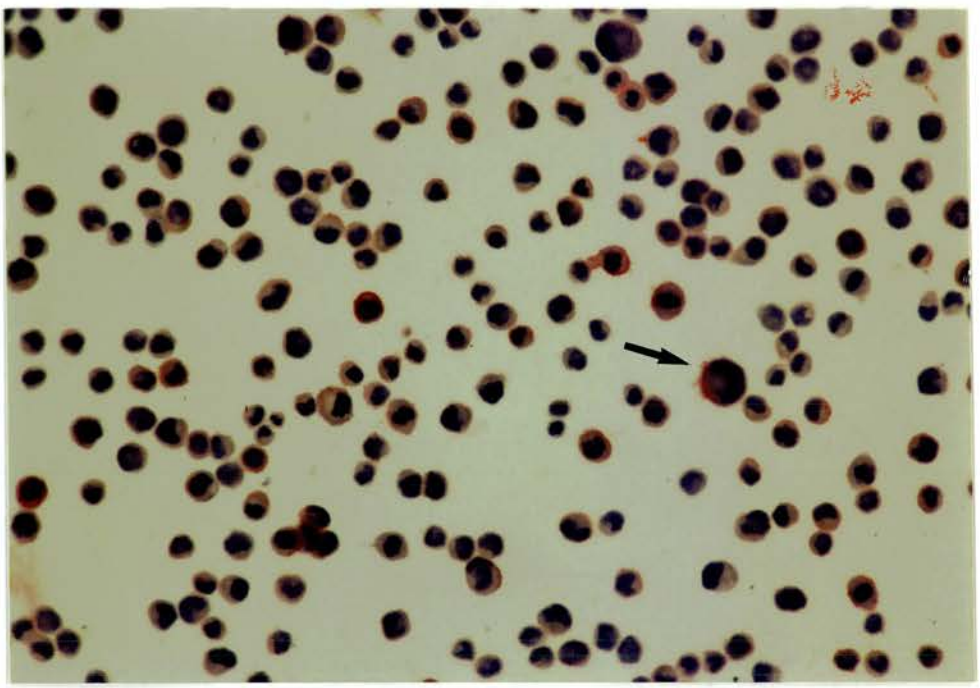


(b)



Plates 11 The internal domain of the EGF receptor was detected on the Tera-2 cells employing the monoclonal antibody F4 (**a**) (*ie*.the red staining). However the external domain was not detected (**b**).

(a)



(b)

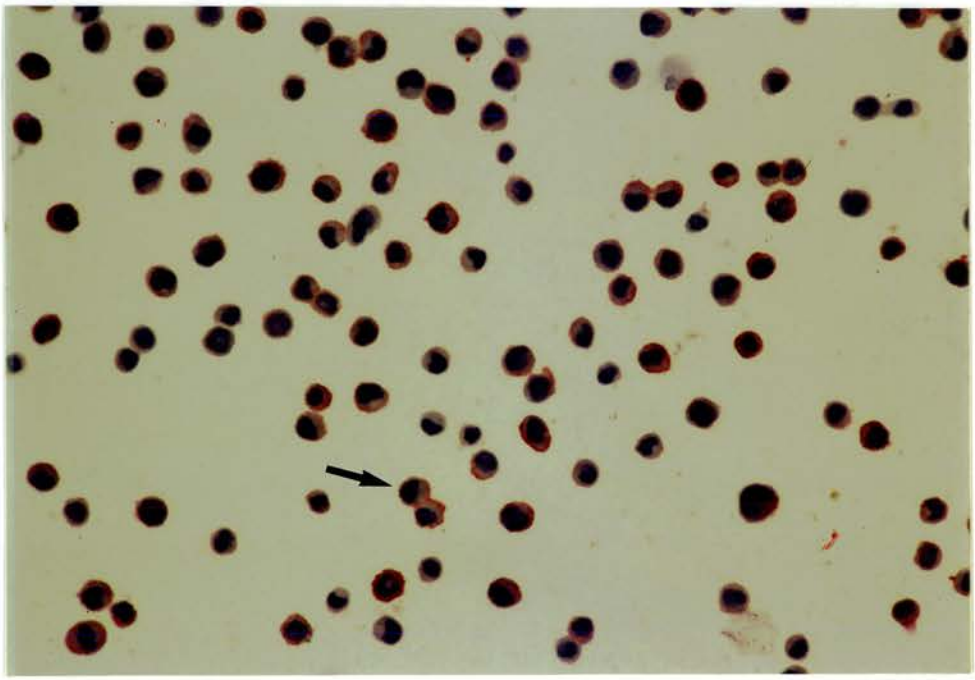


Plate 12 In order to check that both antibodies were working A431 cells were incubated with the F4 monoclonal antibody **(a)** and the EGF-R1 monoclonal antibody **(b)**. As illustrated both domains of the EGF receptor were identified on the A431 cells (*ie*.the red staining).

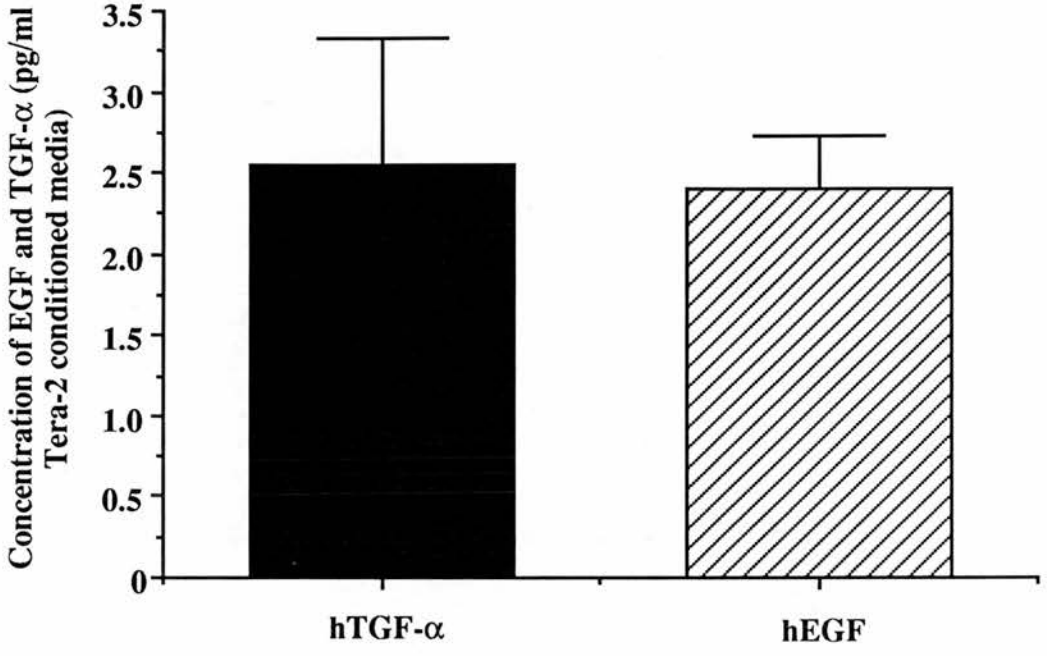


Figure 42

The concentrations of hEGF and hTGF-α per ml of conditioned media taken from the Tera-2 cell line were approximately 2.40 pg/ml and 2.55 pg/ml . The conditioned media was collected from eight 75 ml flasks, all grown at near confluency over a 4 week period until 1 litre of the conditioned media had been obtained. Each flask at near confluency would contain approximately 1 x 10^6cells. The conditioned media was filtered, dialysed, frozen and then lyophilised. Finally it was reconstituted in 3 ml Tris buffer pH 7.4 and used accordingly in the hEGF and rTGF-α radioimmunoassays. Values represent mean concentration (±SEM, n=3).

centrifuged before adding PMSF (0.5 M) and lyophilising the material. The lyophilised material was then reconstituted in 3 ml RIA buffer. As a control serum-free medium was processed in a similar fashion and assayed to see if it contained hEGF or hTGF-α. It was found that the control contained no hEGF or rTGF-α.

3.11 Growth and Thymidine Incorporation Experiments.

Thymidine incorporation experiments were performed in order to assess the growth of the cells in the presence of certain ligands at various concentrations, over variable periods of time. At first cells were plated down at a density of 200,000/ml in 24 well plates (section 2.10.5) However this was found to be too high a number, as after an 8 hour incubation period the cells were found to be dying due to confluency having been achieved (Figure 43). Consequently a cell attachment study was performed (section 2.10.5)and from the results (Table 4) a density of 50,000 cells/ ml was chosen for the thymidine incorporation into DNA experiments.

Cell number in each experiment at each particular point was always measured concurrently as was the amount of free thymidine still available. Cell number was of importance because the cells could have been dying at the very high concentrations of ligand which were used and this could have been observed as a decrease in cell growth. Free thymidine was also measured to ensure that the cells were always saturated.

3.11.1 Effect of mEGF and Mibolerone on Thymidine Incorporation by Tera-2 cells.

No response was noted when the Tera-2 cells were incubated for 24 hours in various concentrations of mEGF from 0.3-100 nM (Figure 44a) or mibolerone from 0.3-100 nM (Figure 44b). The viable cell number remained approximately constant for each concentration in the dose-response study. Mouse EGF was also found to have no

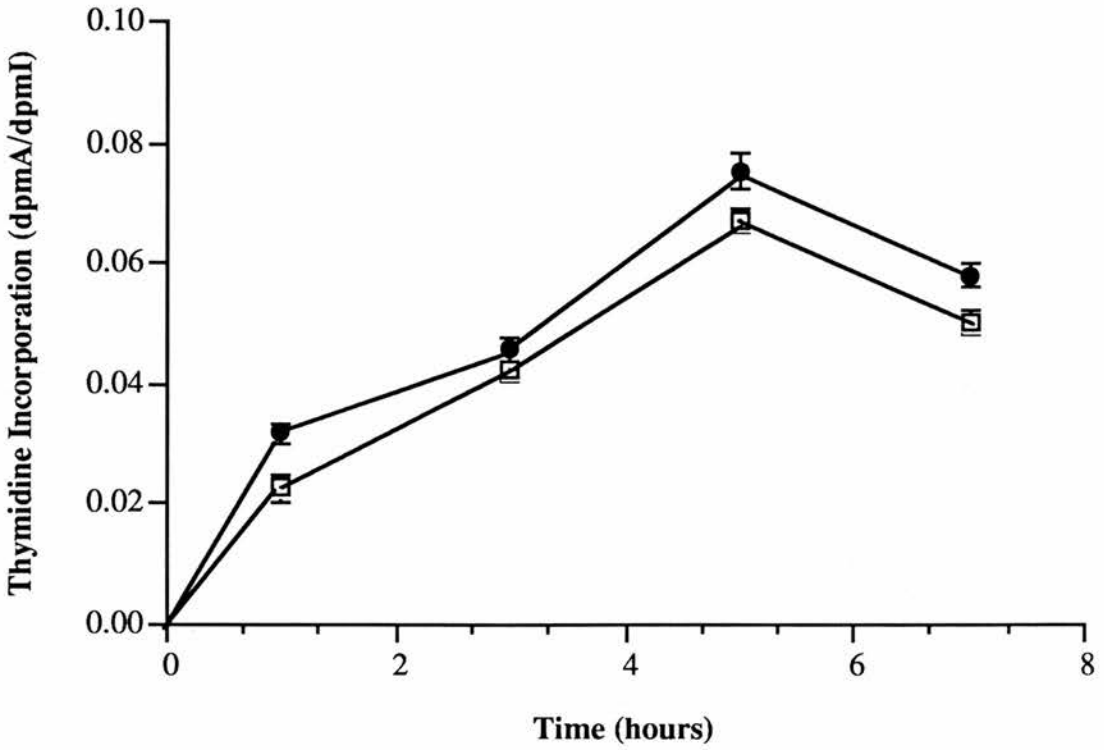


Figure 43
To perform thymidine incorporation experiments, Tera-2 cells were first plated down (in quadruplate) at 2 x 10^5 cells per ml and incubated in the test substance (mEGF, ⊡) or in serum-free medium (●) for upto 24 hours. Thymidine incorporation is represented as the mean ratio (± SEM, n=4) of the actual thymidine incorporated (dpmA) over the amount initially added (dpmI).

Cell Number/ml	2×10^5	1.5×10^5	1×10^5	0.5×10^5
Plating Efficiency	85% ± 3	87% ± 5	78% ± 6	30% ± 10

Table 4
The effect of cell density on cell attachment to 24 well plates was investigated. Cells were plated down at varying densities and after an interval of 24 hours the mean number of cells which had actually become attached was calculated. Values represent mean percentage (± SEM, n=6).

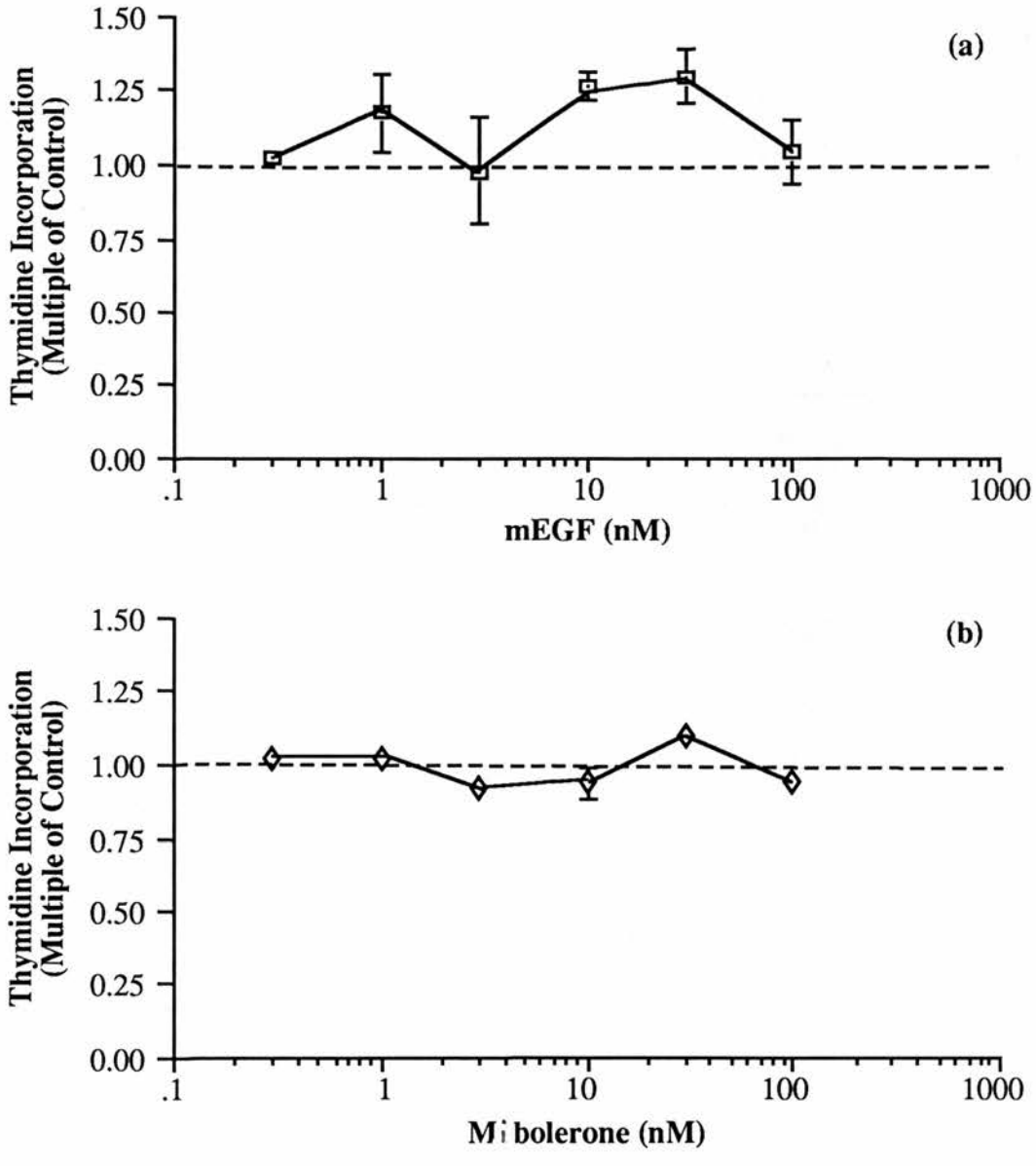


Figure 44

The effect of mEGF and mibolerone on the incorporation of thymidine by Tera-2 cells was assessed. The concentrations of mEGF (**a**) and mibolerone (**b**) employed ranged from 0.3 to 100nM. The cells were incubated in the presence of these substances for 24 hours. The mean thymidine incorporation (± SEM, n=4) at each concentration of test substance is expressed as a multiple of the thymidine incorporation observed with the control (serum-free medium). The mean values were also adjusted according to the number of cells present in the control and test groups.

effect on cell growth over a period of either 24 or 72 hours at a concentration of 10 nM (Figures 45a and 45b). This was also the case for 10 nM mibolerone (Figure 45a and 45b). Cell number at each time point was measured once again and compared to control but the cells were only found to have increased by an equal amount to that observed in the control group.

3.11.2 Effect of rTGF-α on Thymidine Incorporation by Tera-2 Cells.

The effect of various concentrations of rTGF-α on cell growth was also investigated. Concentrations from 1.0 -300 nM were chosen and incubated over a period of 24 hours. Rat TGF-α was found to stimulate thymidine incorporation at concentrations of 10 nM and above (approximately 1.95 compared to control at its maximum effect) (Figure 46). However the cell number was not found to have increased even when compared to the control group. Time course studies were also performed in which the cells were incubated for 24 and 72 hours (Figures 47a and 47b). An increase in thymidine incorporation was apparent after 24 hours, although when incubated for 72 hours no further significant increase was noted. Once again cell number at each time point was measured, although despite an increase in the test group this was not found to differ from the increase in the control group.

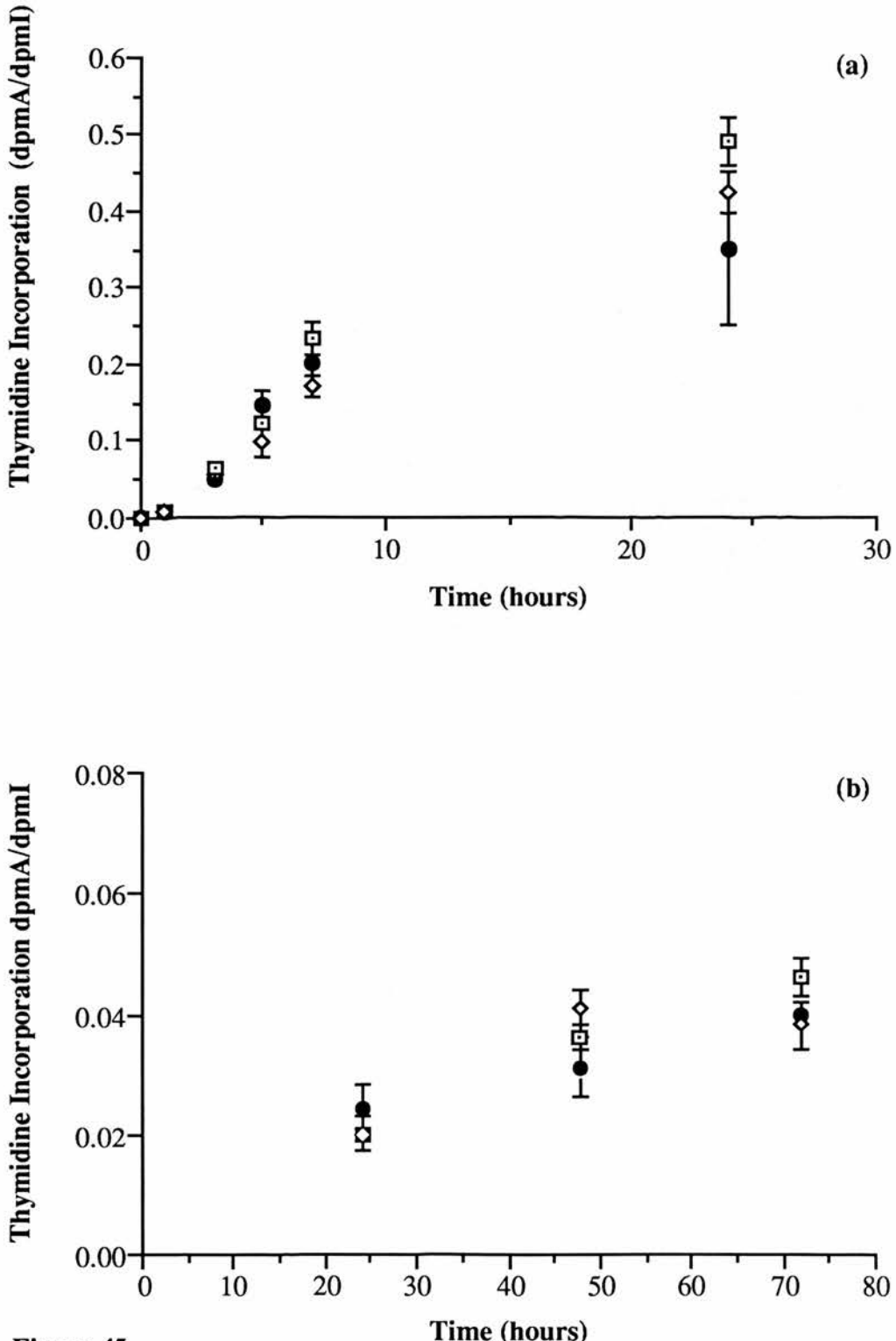


Figure 45
The effect of 10 nM mEGF (⊡), 10 nM mi bolerone (◇) and serum-free medium (●) on thymidine incorporation over a 24 hour period (**a**) and a 72 hour period (**b**) was assessed. The incorporation of thymidine by the cells in each group was adjusted according to the number of viable cells present. The results of each study are expressed as the mean (± SEM, n=4) of the ratio of the actual thymidine incorporated (dpmA) over the amount initially added.

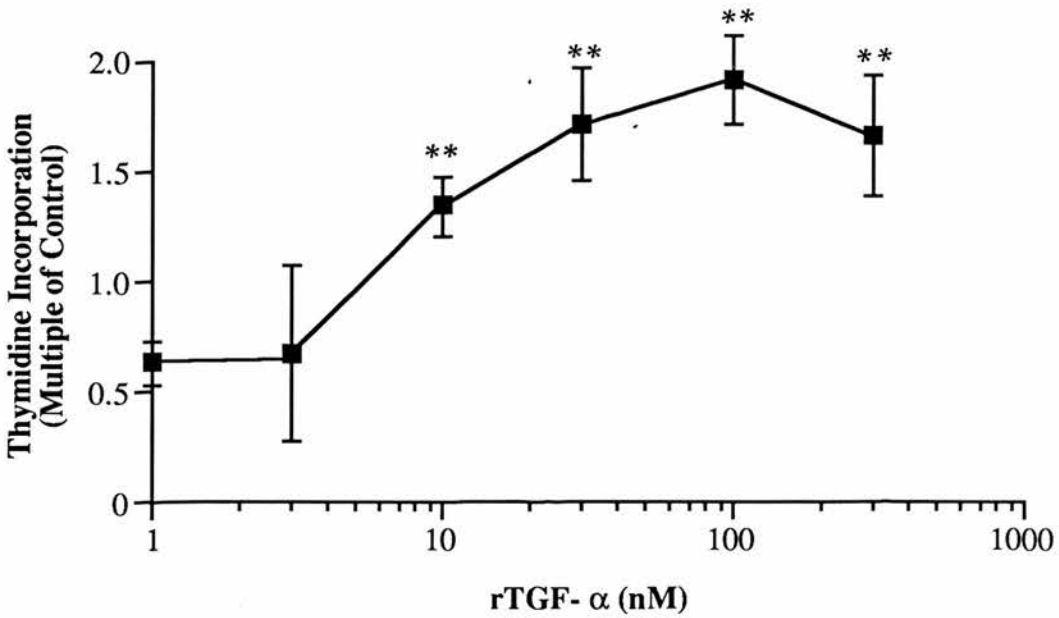


Figure 46

The effect of rTGF-α at various concentrations on the incorporation of thymidine by Tera-2 cells was assessed. The concentrations of rTGF-α employed ranged from 1.0 to 300 nM. The cells were incubated in the presence of rTGF-α for 24 hours. The mean thymidine incorporation (± SEM, n=4) at each concentration of test substance is expressed as a multiple of the thymidine incorporation observed with the control (serum-free medium). The mean values were also adjusted according to the number of cells present in the control and test groups.

Rat TGF-α was found to significantly increase thymidine incorporation at concentrations > 3 nM (**, P>0.01).

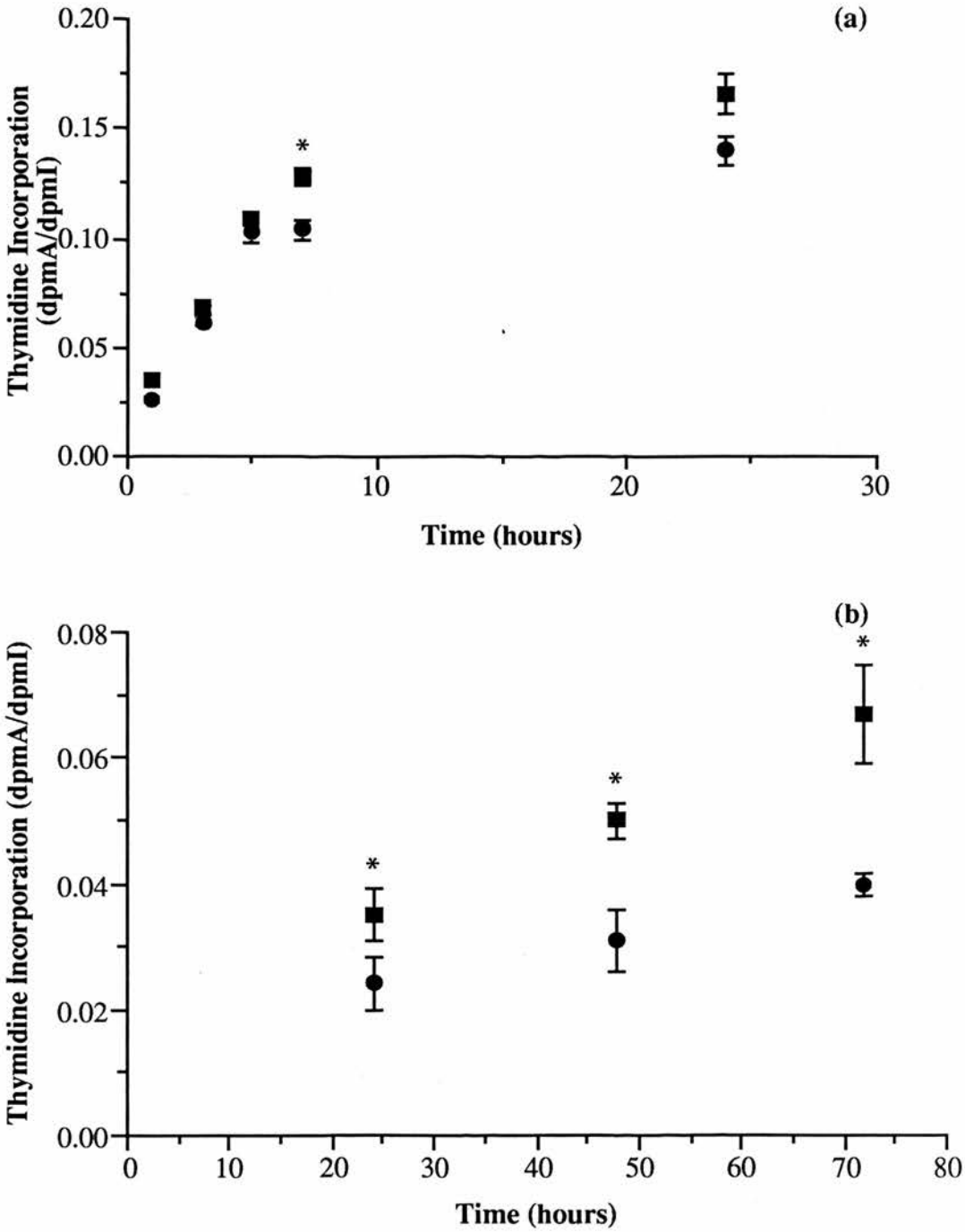


Figure 47

The effect of 10 nM rTGF-α (■) and serum-free medium (●) on thymidine incorporation over a 24 hour period (**a**) and a 72 hour period (**b**) was assessed. The incorporation of thymidine by the cells in each group was adjusted according to the number of viable cells present. The results of each study are expressed as the mean (± SEM, n=**4**) of the ratio of the actual thymidine incorporated (dpmA) over the amount initially added (dpmI).

Rat TGF-α was found to significantly increase thymidine incorporation when incubated with the cells for more than 7 hours (*, $P < 0.05$).

DISCUSSION Chapter 4

4.1 Summary

The results presented above show that EGF receptors are present in human testicular tissue and that they are located on Leydig and peritubular cells. Using biochemical and immunohistochemical techniques EGF receptors were also identified in cancerous testicular tissue. However EGF receptors were only found to be present on tumour tissues of the subtype teratoma and not on the tumour tissues of the subtype seminoma. Both cancerous and normal tissue were assayed for EGF and TGF-α like activity. Both peptides were found to be present in normal tissue and all subgroups of cancerous tissue. The cell line Tera-2 (derived from a lung metastasis of a testicular teratoma) was also assayed for the EGF receptor. EGF receptors were expressed and it was further established that this cell line secreted both EGF and TGF-α like molecules *in vitro*. The concentrations of androstenedione, DHT and testosterone were also determined within each group of tissue although no correlations between the concentration of androgens and the concentration of EGF or TGF-α were found.

4.2 The EGF Receptor

Employing the ligand exchange assay described in section 2.4.6 it was discovered that 8 nM labelled mEGF was sufficient to saturate the binding sites for EGF in human testicular tissue. Saturation of the binding site was evident by interpretation of the saturation curve which was found to plateau at concentrations ≥ 8 nM labelled mEGF. This value is similar to values for EGF receptors identified in human bladder cancer (Neal *et al*, 1989), primary human breast tumours (Sainsbury *et al*, 1985a) and human prostate cancer (Maddy *et al*, 1989). In each instance the Kd values for the high affinity EGF receptor, 1.2 nM, 1.9 nM and 1.6 nM respectively were in close

agreement with the affinity constant for human testicular tissue of 1.8 nM in the present study. In each case a single binding site was detected, represented by a linear Scatchard plot.

Having evaluated a saturating dose of labelled mEGF, studies were performed to ascertain whether the binding process was time and temperature dependent and also to optimise the ligand exchange assay. This was of particular importance as when measuring mEGF binding in some normal and all cancerous tissues Scatchard analyses were not performed because of the small quantities of tissue available. Therefore EGF binding within different tissues was compared, measuring the amount of radioactivity bound to each sample expressed as dpm/mg protein. Each sample was assayed under the same optimum conditions. The assay was performed over 40 min at 32^{o}C. A similar pattern of binding was also reported by Mukku and Stancel (1985b) who identified the presence of EGF receptors in rat uterus. In that study an incubation period of 60 min at 25^{o}C was employed.

Specific components of normal testicular tissue were also examined for the expression of the EGF receptor. As detailed in section 3.1.1 it was found that the crude fraction, the mitochondrial and the microsomal fractions all contained EGF receptors whilst the cytosolic fraction did not. In obtaining the crude fraction, the nuclear fraction was also retained (see section 2.4.3). Ichii *et al* (1988) had previously shown that on separation of the nuclear fraction from the crude fraction of rat livers, the nuclear fraction was found to express the EGF receptor.

The results on EGF receptor cellular distribution in this study are in close agreement with the results published by Maddy *et al* (1987) who fractioned human prostate tissue. Maddy *et al* (1987) found that 68% of the total binding of EGF was present in the crude fraction, 21% in the mitochondrial fraction, 10% in the microsomal fraction and 0% in the cytosolic fraction whilst in this study approximately 72% of the total EGF binding was found in the crude fraction, 12% in

the mitochondrial fraction, 18% in the microsomal fraction and 0% in the cytosolic fraction. For the purpose of this study the binding was maximised by spinning the homogenised tissue (described in section 2.4.2) twice at 105,000 g to produce what was termed the 'particulate' fraction which was composed of all the previous fractions mentioned excluding the cytosolic fraction, *i.e.*the 'crude' fraction which also contains the tissue nuclear fraction, was shown to contain the highest percentage of EGF receptors per mg of protein within the tissue. This may suggest that some EGF receptors within human testicular tissue are actually located intracellularly (*i.e.* in the nucleus) as well as extracellularly]. It has recently been postulated by Logan (1990) that growth factors may exert their effect by intracrine regulation of the nucleus. Until recently the majority of polypeptide growth factors have been thought to act by either paracrine or autocrine modes of action, with some specific examples of growth factors which may also act in an endocrine manner (for *e.g.* IGFs; Holly and Wass, 1989). However it is now postulated that growth factors may act in an intracrine fashion which does not require the growth factor to be secreted by the cell, nor does it require receptors on the cell surface to mediate growth factor activity. It is proposed that growth factors remain within the cell of origin and act on intracellular messengers to regulate cell function (Auron *et al,* 1984, Stockli *et al,* 1989, Ishikawa *et al,* 1989). The growth factors involved in postulated intracrine regulations exist within the cytoplasm. It would therefore have been of interest to assay the above mentioned subcellular fractions for EGF-like activity as EGF was later found to be present in the testis (section 3.5). Ramani *et al* (1986) who studied the distribution of EGF receptors in the human placenta organelles found that EGF receptors were expressed in the lysosomes, rough and smooth endoplasmic reticulum and the Golgi apparatus as well as microvillus plasma membranes. EGF receptors had previously been detected in placenta cells by Lai *et al* (1984; 1986).

The effect of the protein concentration of the particulate fraction on

specific binding of mEGF to its receptor was also investigated. Unlike the results reported by Maddy *et al* (1987) in which human prostate tissue was used, it was found that in human testicular tissue the specific binding of EGF rose linearly with the increasing concentration of protein of the particulate fraction employed. This was useful as the protein concentration of the particulate fraction could be calculated after a ligand binding experiment had been performed. The data was then adjusted if a particulate fraction with a > 2 mg/ml protein concentration had been employed. In using the fraction immediately after it had been prepared, freezing of the tissue was avoided. It was found that storage of the particulate fractions at -70^{o}C produced a decrease in EGF binding over 10 days. However snap freezing and storage of the tissue at -70^{o}C caused no loss of EGF receptors in tissues which had been stored over a 6 month period. Subsequently all tissues were used within a six month period and all particulate fractions either fresh or within 10 days of having been prepared.

The optimum pH of the buffer employed in the radioligand exchange assay was also investigated. It was found that the buffer could be used at a pH of between 7.0 and 8.2 with no significant change in EGF binding and although at a lower pH of 6.6 EGF binding was reduced, this difference was not of statistical significance. The pH of the buffer was subsequently optimised to pH 7.4. Massague (1983) also reported that binding of radiolabelled mEGF to A431 and human placenta membranes was optimal over the pH range 7.0-9.0. However, the binding of radiolabelled rat transforming growth factor alpha (TGF-α) to A431 cells and human placenta membranes was highly sensitive to pH only being optimal within the pH range 8.0 to 8.5 (Massague, 1983). This may indicate that the EGF receptor site, although thought to be common to both EGF and TGF-α, may be in a more advantageous conformation for binding of TGF-α when the assay is performed at a pH between 8.0 and 8.5.

The optimum concentration of unlabelled mEGF required to compete with labelled mEGF for the receptor site was also determined. Bennett and Yamamura

(1985) found the amount of unlabelled mEGF required to compete for the EGF receptor site to be approximately 100-300 -fold in excess of the concentration of radiolabelled mEGF employed to produce maximum specific binding. This would ensure that the pool of unlabelled mEGF available for binding to the EGF receptor would be at a constant concentration with respect to the competition process taking place, despite some unlabelled mEGF having been lost to the receptor sites. In this study a 50-fold excess of unlabelled mEGF was found to produce maximum specific EGF binding and this accords with Maddy *et al* (1987).

After optimising the radioligand exchange assay, saturation of the EGF binding sites on human testicular tissue with radiolabelled mEGF was performed. The saturation data was than used to construct a Scatchard plot from which the affinity constant and the number of EGF receptors present per mg of protein was calculated. A single high affinity binding site was identified with a Kd of 1.18×10^{-9} M and approximately 528 fmoles binding sites per mg protein. The presence of a high affinity binding site for EGF was first suggested by the evidence produced by Schechter *et al* (1978) who observed that occupancy of a small fraction of EGF receptors resulted in maximal stimulation of growth using cells. Schechter *et al* (1978) subsequently proposed that a small population of high affinity receptors may be responsible for the growth promoting effects of EGF.

4.3 Low and High Affinity Binding Sites for EGF

EGF binding sites have since been identified in numerous tissues although the binding site does not always appear to have the same characteristics. King and Cuatrecasas (1982), Hofmann *et al* (1984), Fanger *et al* (1984) and Maddy *et al* (1987) reported two EGF receptor sites. King and Cuatrecasas (1982) described two binding sites: a high affinity site (Kd 0.1-0.3 nM) and a low affinity site (Kd 2.0-15.0 nM). Hofmann

et al (1984) reported finding two binding sites on human uterine leiomyomas: a high affinity site (Kd 0.1 nM) and a low affinity site (Kd 3.7 nM). In comparison Fanger *et al* (1984) described finding two classes of receptors on HeLa S_3 cells: a high affinity site (Kd 0.16 nM) and a low affinity site (Kd 1.5 nM) and Maddy *et al* (1987) found two binding sites on human prostate with a high affinity site (Kd 0.8 nM) and a low affinity site (Kd 7.6 nM) respectively. In summarising the aforementioned data it appears that a high affinity site must have a Kd in the range of 0.1-0.8 nM. Edery *et al* (1985) however detected a low affinity binding site of 0.5 nM and a high affinity binding site of 0.08 nM EGF on mouse mammary glands. Both affinities are much higher than the Kd values previously mentioned.

In evaluating binding data one must consider whether two sites exist or just one. In some studies (*e.g.* Edery *et al,* 1985) the two binding affinities are well defined whereas in the case of Delarue *et al* (1988) on breast cancer the binding affinities were only 2-fold different (3.2 nM and 6.3 nM respectively). In the latter case it is possible that the data has been misinterpreted and that only a single binding site is expressed although in the former case the difference in affinities suggests that there are in fact two binding sites.

Boni-Schnetzler and Pilch (1987) illustrated that the presence of high and low affinity binding sites was the result of the formation of active receptor dimers from inactive receptor monomers. The dimer formation being the high affinity site with a Kd value of 4.9 nM and the low affinity binding site with a Kd of 19.0 nM. Active dimer formation was postulated to be necessary for the generation and maintenance of the mitogenic signal elicited by EGF binding to its receptor. This was also confirmed by the work of Bellot *et al* (1990) who reported that the high affinity site was necessary for transduction of the EGF signal. However this was in contrast to the work published by Biswas *et al* (1985) who reported that the monomer was in fact the high affinity binding site and that EGF bound to the dimer to produce an activated monomer.

In the study performed by Biswas *et al* (1985) freshly isolated receptors were used as a source of monomers and aged receptors as a source of dimers. This in itself could have lead to an ambiguous result as the EGF receptor kinase has a low stability which will be potentiated during the course of time. Schlessinger has also more recently postulated that the EGF receptor is a multifunctional allosteric protein (Schlessinger, 1988). It has already been established that EGF receptors are randomly distributed on the surface of certain types of cell (Schlessinger *et al,* 1978; Haigler *et al,*1978; 1979) and that they undergo rapid lateral (Hillman and Schlessinger, 1982) and rotational (Zidovetzki *et al,* 1981; 1986) diffusion. An allosteric oligomerisation model has subsequently been proposed to explain how ligand binding to the extracellular domain can activate the tyrosine kinase domain which is on the cytoplasmic side of the plasma membrane (Schlessinger *et al,* 1983; Schlessinger, 1986; Yarden and Schlessinger, 1987a; 1987b). According to Schlessinger's model, monomeric EGF receptors are in equilibrium with oligomeric receptors over the surface of the cell. The oligomeric receptors are postulated to have a higher ligand binding affinity than the receptor monomers and that the binding of EGF to the receptor will act to stabilise the oligomeric state. This subsequently leads to the activation of the catalytic properties of the kinase domain by subunit interaction between neighbouring cytoplasmic domains. This model provides an explanation for the detection of two binding sites with different affinities as well as supporting the detection of a single binding site with a high affinity. In cases where a single binding site was detected the equilibrium between the receptor conformations is postulated as being well over to the oligomeric receptor conformation. Bellot *et al* (1990) recently reported that contrary to the notion that receptor affinity is regulated by the off-rate of a ligand attaching to its receptor, high affinity binding is due to an "elevated rate of association", the high affinity EGF receptor being more accessible or perhaps more electrostatically attractive to diffusing ligand. Bellot *et al* (1990) also pointed out that that there is no direct evidence to prove that there are two

different populations of EGF receptor with different affinities for EGF but only that a single receptor may exist in either a monomeric or oligomeric form (Schlessinger, 1988) and subsequently exhibit two different affinities. Weber *et al* (1984) performed quantitative binding experiments with radiolabelled EGF which indicated that the stoichiometry of ligand binding to the EGF receptor is 1:1. This agrees with the findings of Schlessinger (1988) and Bellot *et al* (1990) that indeed two different affinity binding sites can exist but that one site exists which associates more quickly with EGF (*i.e.* the oligomeric receptor which illustrates a higher affinity for the EGF molecule by having to available sites). The fact that the receptor may be in a dimeric form also correlates with the work published by Schreiber *et al* (1983), Boni-Schnetzler and Pilch (1987), Schlessinger (1988) and Defize *et al* (1989) .

Defize *et al* (1989) used a monoclonal antibody to inhibit the lower affinity binding site (mAb E9) and Bellot *et al* (1990) a monoclonal antibody (mAb 108) to inhibit the high affinity binding site. In both cases the results derived suggested that the high affinity binding site plays the major role in transduction of the EGF signal. Gill *et al* (1987) postulated that in comparison to receptor aggregation suggested by Schreiber *et al* (1983) and to the disaggregation of dimers to monomers suggested by Biswas *et al* (1985), that activation of cytoplasmic tyrosine protein kinase is the result of a single molecule act transmitted via the transmembrane alpha helix to alter the ATP binding site. However, this describes an energetically unfavourable state. In the allosteric model suggested by Schlessinger (1988) the energetically unfavorable conformational change which transmits the signal through the transmembrane region is bypassed as monomeric EGF receptors are in equilibrium with oligomeric receptors which exhibit a higher affinity for EGF and where upon binding of EGF to the oligomeric receptor the oligomeric state is stabilised. Ligand binding is the major regulator of the enzyme activity which in turn results in a conformational change that increases the tyrosine kinase catalytic rate as well as phosphorylation of the

various cellular substrates and hence regulates signal transduction at the same time as regulating the affinity of the receptor. Receptor oligomerisation has been confirmed by various methods including morphological (Haigler *et al,*1979), biophysical (Hillman and Schlessinger, 1982, Zidovetzki *et al,* 1981; 1985) and biochemical (Boni-Schnetzler and Pilch, 1987; Cochet *et al,* 1988; Fanger *et al,*1986; Yarden and Schlessinger, 1987a) approaches.

4.4 Single EGF Binding Sites: Low or High Affinity Binding Sites ?

Single EGF binding sites have been reported by Banks-Schlegel and Quintero (1986), Traish and Wotiz (1987) and Schuuramans *et al* (1988), with respective Kd values of 0.2-2.7 nM, 1.16 nM and 0.45 nM. In the case of Banks-Schlegel and Quintero (1986) the single binding site is quoted as having an affinity of between 0.2 and 2.7 nM. To interpret this result as a single binding site is possibly incorrect as there is a 13-fold difference between the highest and lowest Kds determined. It is possible that the data represents two binding sites. In researching the literature available on the affinity of the EGF receptor the majority of the single studies which report Kds are in the range previously quoted for high affinity binding sites (0.1-0.8 nM) (section 4.3) although on ovine skin Wynn *et al* (1989) detected a single binding site with a Kd value of 0.06 nM (a much higher affinity binding site to those previously reported). For instance the single affinity constants determined by Neals *et al* (1989) and Sainsbury *et al* (1985) are closest to the values quoted as being in the higher affinity binding group (*i.e.*between 0.1 and 0.8 nM). However in contrast they are also closer to the values quoted in the low affinity binding group published by King and Cuatrecasas (1982) of 2.0 to 15.0 nM. It has been suggested that differences in experimental techniques employed as well as the variance in the methods chosen for analysis of the data may be

preventing or even biasing the detection of two binding sites. In detecting two different affinity binding sites one must also consider the possibility of the two sites existing on two types of cell within the same tissue homogenate. For example Edery *et al* (1985) illustrated that two EGF receptors were expressed in the membrane preparation prepared from mammary gland membranes, but that once the epithelial cells were separated, only one class of the EGF receptors previously found was identified.

In the studies performed by Mukku and Stancel (1985b) it was suggested that the various methods used for Scatchard analysis may in fact lead to ambiguity in the affinity constants which are detected. For example it was suggested that curvilinear Scatchard plots are sometimes obtained if binding studies are performed by adding increasing concentrations of unlabelled EGF to the reactions, but that linear plots are obtained if binding studies are performed by the isotope dilution method (the latter being the method employed in this study). In this study it was found that the method whereby radiolabelled EGF was diluted actually limited the range in which the affinity constant(s) of EGF could be detected. This was because when very low concentrations of radiolabelled ligand were used the specific binding was difficult to interpret as much higher standard errors were incurred. In this study the saturation analysis binding data was analysed using a curve fitting programme written and developed by Munson and Rodbard (1980) which evenly weights all of the ligand concentrations used. It therefore eliminates difficulties in interpreting the binding data which may occur when using Scatchard analysis (Scatchard, 1949) which has previously been shown to unevenly weight the binding data obtained when very low concentrations of ligand were employed (Bennett and Yamamura, 1985). Another advantage gained from using the computer programme was that the data could be analysed for any number of binding sites. In this study the saturation results were analysed for both single and dual binding sites but only a single binding site was identified.

It is however, likely, that given the data available in the literature both high and low affinity binding sites for EGF exist but that the high affinity site is an allosteric binding site. The results which represent single binding sites may be explained by the dis-association of such oligomeric states during the sample preparation or alternatively by the possibility that the equilibrium between the oligomeric receptor and the monomeric receptor is displaced. In turn it is possible that identification of a single binding site on certain types of tissues could be a characteristic of a non-functional binding site as Schlessinger's model postulates that the receptor must be in an oligomeric state for the EGF signal to be transmitted (Schlessinger, 1988).

4.5 Signal Transduction and Non-functional Receptors

The question of the functionality of the EGF receptor on certain types of tissue has been discussed by Livneh *et al* (1986; 1988), Chen *et al* (1987), Honegger *et al* (1987a,b) and Moolenaar (1988). Each have reported that the tyrosine kinase activity of the EGF receptor appears to be a necessary part of the signal transduction process, because site directed mutagenesis of this activity results in a receptor that binds and internalises EGF but is completely dysfunctional in signal transduction and normal receptor routing (Honegger *et al,* 1987b). The binding of EGF to the receptor stimulates a series of rapid responses, including phosphorylation of tyrosine residues within the EGF receptor itself and within many other cellular proteins to produce hydrolysis of phosphatidyl inositol and release of calcium from intracellular stores (Carpenter and Cohen, 1979). However the mutant receptor kinase is unable to stimulate $[Na^+]/[H^+]$ exchange, Ca^{2+} influx and inositol phosphate formation Moolenaar (1988). Whether EGF receptor affinity (and therefore possibly oligomerisation) is related to functionality (with respect to kinase activity and signal transduction) remains an open question. It would be of interest to define whether the single binding site for EGF discovered on human testicular tissue was functionally active by assessing whether the tyrosine kinase

enzyme which phosphorylates tyrosine^{1173} was stimulated on binding of EGF to the membrane. It is known that the mechanism by which protein kinase C produces autophosphorylation of the EGF receptor at threonine^{654}, is independent of the tyrosine protein kinase activity which phosphorylates tyrosine^{1173} and leads to transduction of the EGF signal (Davis, 1988). However both phosphorylation of threonine^{654} and tyrosine^{1173} lead to agonist induced down regulation of the receptor (Davis, 1988). It is therefore as yet not clearly understood how the two kinase enzymes are capable of regulating the expression of the EGF receptor which is known to be important in the transduction of the signal and yet only tyrosine protein kinase has been found to be involved in transduction of the EGF signal.

4.6 Further Characterisation of the EGF Receptor

Competition of the receptor site with other unlabelled peptides besides EGF was performed to further clarify the specificity of EGF for its receptor. Several other growth factors and hormones of similar composition, and in some instances structure were used. It was found that only TGF-α competed (approximately 24%) for the EGF receptor site. It is well known that TGF-α binds to the EGF receptor and therefore competition for the receptor site on human testicular tissue is not unexpected. However the fact that only 24% competition was observed may suggest that the receptor site may favor binding of EGF in comparison to TGF-α. However as reported previously in section 4.2, pH of the buffer employed in the ligand exchange assay can effect the relative affinity of the EGF receptor for EGF and TGF-α.

The EGF receptor has in fact been found to be activated by three distinct growth factors encoded by separate genes: EGF, TGF-α and vaccinia virus growth factor (VVGF). These growth factors have similar disulphide backbone structures but their overall sequence is only 24% identical (for review see Apella *et al,* 1988). All three growth factors have been found to compete for the EGF receptor and in doing so

express similar affinities (for review see Apella *et al,* 1988). This suggests that they may be binding to a similar region of the EGF receptor. However a report recently by Winkler *et al* (1989) suggests that EGF and TGF-α bind to the EGF receptor but in different ways. Winkler *et al* (1989) reported that a monoclonal antibody to the EGF receptor (13 A9) was capable of producing a stable conformation of the EGF receptor which was not favorable for TGF-α binding. Alternatively, it has been suggested that the monoclonal antibody blocked a part of the surface of the exterior portion of the receptor site which was necessary for TGF-α binding but not EGF binding. The results from this study support the theory put forward by Winkler *et al* (1989) which suggests that the EGF receptor can vary in its affinity for EGF and TGF-α according to its environment.

The EGF binding site on human testicular tissue was further found to be both heat labile and trypsin sensitive, indicating that the receptor was of a proteinaceous nature. However the heating process only destroyed 50% of the specific binding observed with respect to controls . It is possible that the preheating period of 10 min was not sufficient to produce total inactivation of all the EGF receptors. Hock and Hollenberg (1980) reported having found irreversible inactivation of the EGF receptor at 55^{o}C and 65^{o}C respectively in human placenta when the membranes were incubated for a period of 10 minutes. However they did not report whether the 'irreversible inactivation' accounted for 100% of the EGF binding previously noted in the control. It has also been assumed that the consequence of heat shock is always followed by denaturation of proteins. However Munro and Pelham (1984) have shown that stabilisation of proteins after heat shock can occur which in this case could explain the presence of 50% of the EGF receptor sites which remained available for binding after heat treatment.

Molecular characterisation of the receptor using the crosslinking agent Disuccinimidyl suberate illustrated that labelled mEGF was binding to a protein and that

the radiolabelled mEGF/protein complex had a molecular weight of 125 kDa. In the past such a molecular weight complex has been interpreted as EGF having bound to a degraded form of the EGF receptor (Weisman *et al,*1987). It is possible that in this study the 170 kDa form of the receptor reported by Mukku (1984) and Cohen *et al* (1980) may have been degraded by a protease. In the past it has been shown that a calcium dependent protease 'calpain' acts by removing a 20 kDa fragment from the EGF receptor (Cassel and Glaser, 1982). In this study calcium sequestering agents were incorporated in the appropriate buffers to avoid such degradation. There have been various other reports identifying degraded forms of the EGF receptor, for example Decker (1989) identified a 130 kDa, 125 kDa and 105 kDa receptor species using immunoprecipitation on NIH-3T3 cells. This is in support of the findings in this study. The 125 kDa and 130 kDa species were shown to have been derived from the 170 kDa receptor and the 105 kDa species from the 130 kDa species. Similarly, Cohen *et al* (1982), Cassel and Glaser (1982) and Weisman *et al* (1987) reported a 150 kDa receptor which again was thought to be a proteolytic fragment of the EGF receptor. Linsley and Fox (1980a; 1980b; 1980c) also reported 160 kDa, 145 kDa and 115 kDa EGF receptor complexes using the epidermoid carcinoma cell line A431. Weisman *et al* (1987) reported finding a receptor complex with a 125 kDa molecular weight in human meningioma again confirming the results of this study. Chinkers and Brugge (1988) had earlier shown that tryptic cleavage (1 μg/ml) of purified EGF receptors from A431 cells resulted in receptor fragments of molecular weights 165 and 125 kDa. It may therefore be assumed that the 125 kDa EGF receptor species identified in this study is a fragment of the 170 kDa form and that a trypsin-like endogenous protease must be present in human testicular tissue. Weisman *et al* (1987) however also illustrated that the 125 kDa species was able to bind EGF in a specific manner. The 125 kDa species although a degraded product of the 170 kDa may therefore still be able

to function as a functional receptor.

4.7 Location of the EGF Receptor on Human Testicular Tissue

Localising the EGF receptor in normal testicular tissue would provider greater insight into the the particular cells affected by EGF*in vivo*. The F_4 monoclonal antibody for the internal domain of the receptor (Gullick *et al,* 1986) and the EGF-R (Starkey *et al,* 1975) and EGF-R1 (Waterfield *et al,* 1982) monoclonal antibodies for the external domain of the receptor were employed with an indirect immunohistochemical streptavidin-biotin technique employing alkaline phosphatase (section 2.6). This method was chosen because of its sensitivity and specificity in contrast to the direct ABC peroxidase method (Bains and Miller, 1988) which is often employed. In the ABC peroxidase method the peroxidase enzyme was found to label endogenous peroxidase which could not be distinguished from the specific staining.

In the alkaline phosphatase streptavidin-biotin method the endogenous alkaline phosphatase is blocked by levamisole which is introduced in the substrate reagent. In the case of the ABC peroxidase technique the endogenous peroxidase can be quenched by incubating the section in 0.6% hydrogen peroxide but unfortunately this leads to a reduction or even a loss of staining intensity due to the destruction of antigens which became evident when the sections were processed. This is particularly a problem when cryostat sections are used. The streptavidin reagents also have a longer shelf life compared to the ABC peroxidase reagents. The streptavidin solution has a neutral pH and therefore does not have any preference to charged ions which may be present within the section, whereas the avidin in the ABC method has (pers. commun. Mr Lawrence Brett, M.L.S.O., Western General Hospital). Streptavidin does also not attach to carbohydrates in the tissue section although avidin alone does. One other advantage of the streptavidin technique was that it enabled the areas of lipofuskin to be detected, as when using an ABC peroxidase technique with

diaminobenzadine (DAB) the staining of lipofuskin could not be distinguished from the positive staining. In the case of streptavidin alkaline phosphatase the fast red pigment which was used is only partly absorbed by the lipofuscin material to take a pink rather than a red stain in contrast to the EGF receptors. However in employing the peroxidase technique with DAB the lipofuscin becomes stained to the same extent as true positive staining. The streptavidin biotin procedure overall provides a much clearer and more specific staining pattern as well as being more economical.

Employing the indirect streptavidin biotin technique EGF receptors were found to be located in the interstitium of normal testicular tissue obtained from orchiectomy samples (mean age 72 years), on foetal tissues (obtained from abortions carried out after a period of 15 to 19 weeks gestation and on biopsy specimens (mean age 32 years) obtained from men undergoing vasectomy reversal. It is therefore evident that EGF receptors are present during the course of human testicular development and function and therefore may play some role in the intricate control of testicular function.

Localisation of EGF receptors on sections of human testicular tissue has not been reported before, although, EGF receptors have been reported to be expressed on rat Sertoli and Leydig cells (Morris and Mather, 1984). A recent report (Suarez-Quian *et al,* 1989) identified EGF receptors in testes from mature and immature rats and immature monkeys. The receptors were found to be present on the Sertoli cells (mesenchymal) of the immature rats and monkeys and also in the interstitial areas of the mature rats (Suarez-Quian *et al,* 1989). This accords with the results of Skinner (1989) who also found the EGF receptor to be present on the Sertoli cells of immature rat testes. It is therefore possible that EGF receptors are only expressed on Sertoli cells in immature mammals, hence the reason why they were not detected on human testicular samples obtained from men with a mean age of 72 years.

Suarez-Quian *et al* (1989) employed immunofluorescence as the

method for detection of the EGF receptor, which in comparison to immunohistochemical detection is often thought of as more difficult to interpret because the morphology of the tissue section and the staining itself can not be viewed simultaneously. Also when using immunofluorescense the clarity of the staining is often less sharp and a more diffuse pattern is apparent. This in turn can produce difficulties when determining on which cell the staining is appearing if the cells are in close proximity (*i.e.* peritubular and Sertoli cells). As previously reported Suarez-Quain *et al* (1989) identified EGF receptor expression on both Leydig and Sertoli cells. It is possible that the interpretation of Sertoli cell staining may well include peritubular cell staining as identified in the present study. Also Suarez-Quian *et al* (1989) used a polyclonal antibody rather than a monoclonal antibody which can often decrease the specificity of the detection procedure.

To clarify the location of the EGF receptor on human testicular tissue two different antibodies EGF-R and EGF-R1 were employed; the former for paraffin sections and the latter for frozen sections. As a direct comparison frozen sections and paraffin sections obtained from the same patient were processed and labelled with the appropriate antibodies. The morphology of the frozen sections was found to be poor. EGF receptors were clearly present in interstitial tissue although the cell type which appeared stained could not be positively identified. Subsequently tissue sections were examined which were cut from paraffin blocks. The morphology of these sections was superior to that of the frozen sections and while using the antibody EGF-R, the staining was very similar to that seen with EGF-R1 on frozen sections. However as staining was much clearer it could be definitely localised to Leydig and peritubular cells. However the EGF-R1 antibody employed on frozen sections produced variable results in terms of intensity of staining especially when repeated on the same section. Because of this variation no attempt was made to relate intensity of staining to the level of EGF receptor expression although this has been done in the past by Maddy *et al* (1987) on

prostate tissue. When using the F4 antibody on either the paraffin or frozen sections no staining was apparent indicating that the internal domain of the EGF receptor was absent. The absence of the internal domain of the EGF receptor may indicate that the receptor is non-functional. There is no evidence in the literature to suggest why the internal domain of the receptor should be absent. It is possible that the internal domain of the receptor has morphologically changed due to stimulation by endogenous EGF in the testis which in turn inhibits the F4 antibody from binding to the internal domain. Alternatively the receptor may have been degraded during the preparation of the section and thus is not available for binding.

4.8 EGF and TGF-α in Human Testicular Tissue.

As early as 1971 the activity of ornithine decarboxylase in the testes of neonatal mice was found to be markedly enhanced by EGF (Stastny and Cohen, 1971; Statsbury and Cohen, 1972). Since then it has been identified in numerous tissues and body fluids (Hirata and Orth, 1979; Shikata *et al,* 1984; Gregory *et al,* 1986). With respect to the testis EGF has been shown to stimulate lactate production in cultured rat testicular cells (Mallea *et al* ,1986) and in the human Leydig cell tumour line MA 10, mEGF has been shown to potentiate the activation of steroid biosynthesis via hCG and cAMP stimulation (Ascoli *et al,* 1981; 1984; 1987). Epidermal growth factor activity has already been measured in human testicular tissue (Elson *et al,*1984) at a concentration of 3.2 ng of EGF per gram of tissue. In this study EGF was found to be present at a concentration of 5.16 ng/g wet testicular tissue in normal tissue which was statistically significantly higher than the 2.23 ng/g detected in treated testicular tissue. This would suggest that the concentration of EGF in the testis is affected by anti-androgens or synthetic oestrogens administered systemically. This in turn could reflect the low if not negligible expression of EGF receptors previously noted in treated testicular tissue

employing biochemical techniques (section 4.2). The patients from which the treated testicular tissue was obtained, had received cyproterone acetate or diethylstilboestrol as treatment for prostatic cancer. Cyproterone and stilboestrol are known to affect the testis by acting on the anterior pituitary to inhibit LHRH secretion (Bruchovsky *et al,* 1988). In inhibiting LHRH secretion, LH secretion is also inhibited which in turn reduces steroidogenesis. One may postulate therefore that in the absence of LH or possibly reduced steroidogenesis the expression of EGF receptors on the Leydig cell is reduced. However as suggested previously a decrease in LH secretion would manifest itself as a decrease in testosterone production. As shall be discussed later, a decrease in intratesticular concentrations of androstenedione, DHT and testosterone in treated tissue was seen but it was not statistically significantly different from the concentrations measured in normal tissue.

Mori *et al* (1989) also detected endogenous EGF in testicular tissue by using a monoclonal antibody for hEGF. Human EGF was present in both the interstitium and the seminiferous tubules of the testis using a polyclonal antibody to hEGF. Feminised testes were also studied and hEGF was only identified in the interstitial areas of the tissue. Bartlett *et al* (1990) reported that in stage synchronised spermatogenesis in the rat testicular EGF concentrations were higher in testes synchronised between stages IX and II than at other stages of the cycle of the seminiferous epithelium. This elevation in testicular EGF concentrations correlated well with mitotic division of type A spermatagonia at stages IX, XII and XIV of the the cycle of the seminiferous epithelium. Tsutsumi *et al* (1986) also reported that sialoadenectomy in the mature male produced a decrease in the number of mature sperm in the epididymis by as much as 55%. The number of spermatids in the testis were also found to have decreased by 40 to 50% and the number of spermatocytes to have increased by approximately 20% (Tsutsumi *et al,* 1986). Administration of EGF to the sialoadenectomised mice restored both the sperm content of the epididymis and the

number of spermatids in the testis to normal. Thus it was suggested that EGF may play a role in male reproductive function by stimulating the meiotic phase of spermatogenesis. If this is the case then perhaps EGF is also involved in infertility. Human epidermal growth factor or urogastrone has already been detected in human seminal plasma from both infertile and fertile males (Richards *et al,* 1988).

TGF-α was also detected in both the normal and treated groups of testicular tissues which were studied. In normal tissue 2.76 ng/g wet testicular tissue was measured which was again statistically significantly different from 1.92 ng/g measured in treated testicular tissue. No correlation between the concentrations of hTGF-α and hEGF in normal testicular tissue was found.

In measuring human TGF-α a rat TGF-α RIA kit was employed because no human TGF-α RIA kits were available. However it was suggested by the manufacturers that because there is a high degree of sequence homology between rat and human TGF-α (as only four amino acids are different in rTGF-α in comparison to hTGF-α , Connolly and Rose, 1989), the rTGF-α RIA kit could be used to detect hTGF-α (see section 2.2.6). The difference in structure of rat and human TGF-α affects antigen antibody interaction, such that only 32% of hTGF-α is detected. This factor was taken into consideration in all hTGF-α calculations. The fact that hTGF-α was found in the normal and treated groups of testicular tissue, suggests that TGF-α as well as EGF may play an important role in testicular function. P-Mod-S, a factor first identified by Skinner and Fritz (1985) and reported to be secreted by peritubular cells to act on Sertoli cell function in coculture, may modify TGF-α secretion (Skinner, 1989). The factor P-Mod-S which was synthesised by the rat peritubular cells and secreted into the medium to act on the Sertoli cells specifically sustained rates of ABP production by these cells.

4.9 Other Factors in the Testis

In the past few years various reports have been published on other factors in the testis which may assist in its normal function. Sertoli cells have been shown to produce an EGF like substance (Holmes *et al,* 1986; Buch *et al,* 1988). Holmes *et al* (1986) investigated the ability of conditioned medium obtained from rat Sertoli cell culture to displace labelled mEGF binding to its receptor. The conditioned medium was found to contain EGF competing activity as well as producing a mitogenic response when used in culture of Sertoli cells. However, when the conditioned medium was assayed for EGF like activity the medium was found to contain no EGF immunoreactivity (Holmes *et al,* 1986). This result could have been attributed to the fact that this EGF like material was in fact TGF-α as recently characterised by Skinner *et al* (1989) which is produced by the peritubular cells and competes for the EGF receptor yet has no EGF immunoreactivity. Skinner *et al* (1989) also found that this protein has an autocrine stimulatory effect on the peritubular cells as EGF receptors were also found to be expressed by the peritubular cells. This is in support of the findings in this study.

Brown *et al* (1989) have shown that in rams depilatory doses of mouse EGF temporarily impaired gonadotrophin and androgen secretion by inhibiting LHRH release from the hypothalamus. Such treatment appeared to have no effect on the responsiveness of the pituitary to LHRH and it was therefore postulated that an already established mechanism for decreasing LHRH could have been activated by the EGF Brown *et al* (1989). One such regulator is inhibin which is known to be the messenger involved in the negative feedback mechanism to the anterior pituitary for the control of steroid production (Hao Li and Ramasharma, 1987). Gonzales *et al* (1989) recently found that inhibin secretion is increased by EGF when administered to isolated segments of rat seminiferous tubules *in vitro*. Morris *et al* (1988) also found that inhibin accumulation is stimulated by EGF when rat Sertoli cells are cultured in vitro.

It has been established that inhibin is produced by the Sertoli cells which are in contact with the peritubular cells through the neighbouring basement membrane. Rat Sertoli cells have also been shown to express EGF receptors by Skinner (1989) although this was not corroborated for human tissues in this study. It is however possible that TGF-α which is secreted by the peritubular cells acts in an autocrine manner to regulate its own synthesis as well as acting in a paracrine manner on the Sertoli cell to stimulate inhibin production. The fact that EGF receptors were found on Sertoli cells in rat and not in human may suggest a species difference. However as mentioned previously in section 4.7 the absence of EGF receptors on Sertoli cells could be attributed to the fact that the testes employed in this study were mature. In other studies EGF receptors have only been found to be expressed on Sertoli cells when the testis under investigation are immature (Mori *et al,* 1989; Skinner, 1989; Suarez-Quain *et al,* 1989).

Skinner and Moses (1989) have found that TGF-β was produced by both peritubular and Sertoli cells through the identification of gene expression and protein synthesis. TGF-β was found to have no effect on Sertoli cell function but it did inhibit the ability of TGF-α to promote peritubular cell growth. TGF-β has also been found to inhibit human chorionicgonadotropin (hCG) stimulated steroidogenesis in primary rat Leydig cell cultures (Lin *et al,* 1987). In contrast TGF-α had no effect on either the basal or hCG-stimulated testosterone production and did not modify the effect of TGF-β on steroidogenesis. The effect of TGF-β on steroidogenesis has been further supported by the *in vitro* work of Avallet *et al* (1987) and Fauser and Hsueh (1988). The data indicates that inhibin and TGF-β which are released by the Sertoli cells may serve as intragonadal paracrine signals in the modulation of LH-stimulated androgen

biosynthesis.

In reviewing the literature it has been found that authors have often commented on the fact that it is the prepubertal cells that seem to be producing the maximum response being measured. For example Feig et al (1980) found that the mitogenic activity in the seminiferous tubules isolated from newborn mice is five times greater than that in the tubules of adult mice. In this study it was found that the EGF receptors appeared to be much more dense in the foetal tissues (obtained from abortions carried out after a period of 15 to 19 weeks gestation) which were employed in the immunohistochemistry procedure in comparison to the adult testis which were used.

It is possible that EGF-like activity is under the control of androgens and that an increase in mitogenic activity in the seminiferous tubules of foetal tissue would coincide with an increase in intratesticular steroids. In the foetal testes it is well documented that there is a surge of steroid production during the 15-19 weeks gestation period (Rommerts and van der Molen, 1989) although it is possible that numerous other factors may contribute or be responsible for this event.

4.10 The Role of Steroids in the Testis

As previously discussed in section 1.5 one of the primary functions of the testes is the manufacture of androgens. Testosterone, which is the essential androgen manufactured by the testis, exerts its effects both internally and externally to the testes. Within the testes spermatogenesis cannot be maintained in its absence (Sharpe *et al,* 1988). It is understood that testosterone mediates its effect on spermatogenesis via the androgen receptor located within the Sertoli cell (Sanborn *et al,* 1975; Grootegoed *et al,* 1977; Wright and Frankel, 1980). Testosterone has also been found to stimulate the

secretion of ABP and seminiferous tubular fluid in the rat (Sharpe, 1983; 1986). Receptors for testosterone have also been identified in peritubular cells (Verhoeven, 1980). As discussed earlier Skinner and Fritz (1985) reported that androgen stimulation of Sertoli cell function was enhanced by peritubular cells via P-Mod-S.

EGF receptors have also been found to be expressed by the Leydig cell. It is therefore possible that Leydig cells are somehow modulated by TGF-α or EGF via a direct action or via modification of Sertoli cell and peritubular cell functions. The concentrations of androstenedione, DHT and testosterone for sixteen individual tissue samples were measured in this study as well as the levels of endogenous TGF-α and EGF for 12 of these samples. The steroid levels in normal testes were found to be 0.46 nmoles/g androstenedione, 0.29 nmoles/g DHT and 15.58 nmoles/g testosterone whereas in the treated tissue they were 0.29 nmoles/g, 0.31 nmoles/g and 14.52 nmoles/g respectively. The levels of androgens in normal testicular tissue were further supported by the values reported by Purvis *et al* (1978) and De La Torre *et al* (1982) (Table 5). Despite the steroid levels in both papers having been measured from wet tissue, the results in this study could be converted from the concentration measured in 1 gram of dry testicular tissue to 1 gram of wet testicular tissue. This was performed by taking into consideration the mean percentage water evaporated after lyophilisation (85%). Purvis *et al* (1978) and De La Torre *et al* (1982) also used a similar age group to the one used in this study. The concentrations of androstenedione, DHT and testosterone in the treated group of testis were also found to be similar to those found in the normal testis. However despite no change in intra-testicular androgen levels the anti-androgens appeared to have produced a decrease in the expression of the EGF receptor. The effect of the anti-androgens (cyproterone and stilboestrol) on the body is to produce a decrease in androgen production by acting centrally on the pituitary to inhibit the secretion of LHRH (Bruchovsky *et al,* 1988). Cyproterone acetate for example, owing to its progestational activity, overrides the

	Tamm et al (1987) ng/g wet tissue	Present Study ng/g wet tissue
Testosterone	724	670
DHT	6.95	12.0

	de la Torre et al (1982) nmol/ kg wet tissue	Present Study nmol/kg wet tissue
Testosterone	2740	2337
DHT	96.0	42.9

Table 5
Comparison of the intratesticular steroid concentrations reported in this study with those published by Tamm *et al* (1987) and de la Torre *et al* (1982).

negative feedback inhibition of the hypothalamus by testosterone (Bruchovsky *et al,* 1988). Stilboestrol (diethylstilboestrol) also acts by overriding the negative-feedback inhibition of the hypothalamus by testosterone. This again reduces the secretion of both LHRH and LH accompanied by marked lowering of plasma testosterone levels into the castrate range (Bruchovsky *et al,* 1988). However the concentration of androgens measured in the treated tissues was not statistically significantly lower than the concentrations measured in the normal tissues. This might best be attributed to the delay in time of the patients receiving their last dose of drug and them having an orchiectomy. In addition patients with intact anterior pituitaries can "escape" from anti-androgen therapy and this is illustrated by an increase in LH secretion which overrides the effect of the anti-androgen (Varenhorst, 1988). Unfortunately however LH was not measured as a routine parameter in these patients and hence this theory could not be evaluated.

4.11 Endogenous EGF in the Human Testis and Its Possible Regulation by Androgens.

In view of EGF receptors having been localised to Leydig cells the possible relationship between EGF and steroidogenesis was also investigated. This was performed by comparing the concentration of EGF with the concentration of androgens present within each individual tissue sample. The results for each tissue sample were then grouped together and analysed to see if overall there was any correlation between EGF and TGF-α concentrations and androgen concentrations. No correlation was observed between any of the steroid hormones and growth factors which were measured. This can perhaps be attributed to the very small groups of tissues which were involved.

Steroid regulation of EGF production has been illustrated in the submandibular gland (SMG) of the mouse (Bynny *et al,* 1974). Tuomela *et al* (1989) recently reported that mouse EGF concentrations were altered by gonadectomy and treatments with oestradiol and progesterone. EGF concentrations were found to

decrease in either the absence of androgens or in the presence of oestrogens/progesterones. In support of this is the fact that in the SMG of the male mouse the concentration of EGF is at least ten times higher than that found in the SMG of the female mouse. Hiramatsu *et al* (1988) have shown that castration of the male mouse produces a marked reduction in the concentration of EGF in the prostate gland and that this reduction is restored by the treatment of castrated animals with testosterone. These results indicate that the synthesis of immunoreactive EGF in the prostate is regulated by endogenous androgens as is the case for its synthesis in the submandibular gland (Bynny *et al,* 1974). Pascal *et al* (1989) reported that castration of the male mouse produced a decrease in EGF mRNA levels in the submandibular gland, thus providing further evidence for regulation of EGF gene expression in response to steroid hormones. EGF has also been found to regulate the production of certain hormones. EGF stimulated thyroid hormone secretion in the mouse (Ahren, 1987) and decreased progestin production in cultured rat granulosa cells (Jones *et al,* 1982). EGF has been found to stimulate steroidogenesis *in vitro* (Verhoeven and Cailleau, 1986) directly stimulating the output of C19 steroids (testosterone and androstenedione) and C21 steroids (progesterone, 17α-hydroxyprogesterone and 20α-hydroxypregn-4-en-3-one) in freshly prepared Percoll-purified Leydig cells from prepubertal and adult rats and mice, and in interstitial cells from immature rats cultured in the presence or absence of LH.

In several other types of tissue it has already been reported that EGF receptors can be regulated by certain steroids (Mukku and Stancel, 1985; Traish and Wotiz, 1987; Murphy *et al,*1986). For example, in the prostate gland Traish and Wotiz (1987) found that androgens decreased EGF receptor expression. Castration of mature rats resulted in a 3-to 6-fold increase in labelled mEGF binding, while treatment of 7-day castrated rats with 5α-dihydrotestosterone decreased the number of EGF binding sites. Mukku and Stancel (1985) looked at the effects of oestrogen on EGF

receptor expression in vitro within uterine tissue of immature female rats. It was found that following hormone treatment EGF receptor levels increased between 6 and 12 hours and remained elevated for up to 18 hours before declining between 18 and 24 hours. Non-oestrogenic hormones such as progesterone, dexamethasone and DHT all failed to elevate EGF receptor levels. In contrast Murphy *et al* (1986) found that progestin upregulated EGF receptors in mammary carcinoma cells. It is therefore evident that EGF receptors can be regulated by steroids but that response is dependent upon the tissue, the state of the tissue (*i.e.* cancerous or normal) and possibly the species. Also steroidogenesis is principally controlled by LH which is regulated centrally, any possible effects which EGF may have on steroidogenesis are therefore likely tobe minor in relationship to LH.

4.12 EGF Receptors in Cancerous Tissues.

The possible role of the EGF receptor in oncogenesis has become a major topic of investigation over the last ten years due to the detection of the EGF receptor on numerous types of cancerous tissues. For example they have been found to be expressed by normal mammary glands in mice (Edery *et al,* 1985), normal human uterine tissue (Hofmann *et al,* 1984) and by normal human prostate (Maddy *et al,* 1987). Receptors have also been found to be present in all of these tissues but in the cancerous state for example human breast cancer (Sainsbury *et al,*1985a; 1985b; 1985c; 1985d; Skoog *et al,* 1986; Crawford *et al,* 1987; Macias *et al,* 1987; Pekonen *et al,* 1988; Horne *et al,* 1988; Boluffer *et al,* 1990), cancerous human uterine tissue (leiomyomas, Hofmann *et al,* 1984) and human prostate cancer (Maddy *et al,* 1989). Numerous other tissues such as bladder (Berger *et al,* 1987a; Neal *et al,* 1985; 1989), ovary (Bauknecht *et al,* 1984; 1986), human meningioma cells (Weisman *et al,* 1987) gastric and colonic carcinomas (Sugiyama *et al,* 1989; Yasui *et al,* 1988a; 1988b),

lung cancer (Berger *et al,* 1987b; Bepler *et al,* 1988) and human eosophogeal carcinoma (Banks-Schlegel and Quintero, 1986) have also been found to express the EGF receptor. In some of these tissues EGF receptor mRNA has also been identified for example in breast cancer (Travers *et al,* 1988). Derynck (1987) found that the level of EGF receptor mRNA and EGF mRNA was higher in tumours than in the corresponding normal tissue and that TGF-α mRNA was also present in higher concentrations in the tumour tissue.

In human testicular cancer EGF receptors were found to be expressed on teratoma tissues but not on seminoma tissues which were both investigated using biochemical techniques. As for Leydig cell tumours, only one specimen was obtained during the course of the study and this was found to be devoid of EGF receptors using biochemical analysis. However, having found that the teratoma tissues expressed the EGF receptor it was unfortunate that the receptor could not be characterised in terms of its affinity due to the lack of tissue available for biochemical analysis. Therefore in order to investigate the presence of EGF receptors on teratoma tissue further, a cell line Tera-2 was cultured. The receptor was again characterised using saturation analysis. The binding procedure adopted was taken from the methods of Engstrom *et al* (1985) who had previously studied the EGF receptor on the Tera-2 cell line. Engstrom *et al* (1985) also reported finding a single high affinity binding site of 0.2 nM with 26,000 receptors present per cell. This is similar to the results obtained in this study where a high affinity binding site of 0.21 nM was detected with approximately 67,000 receptors per cell employing Munson and Robard's (1980) curve fitting programme to analyse the saturation data.

Breast cancer is one of the more widely investigated types of cancer, the reason for this being that an inverse relationship between oestrogen receptor (ER) expression and EGF receptor expression was discovered which was later found to be related to prognosis of the cancer. That is to say that if ER expression is low then EGF

receptor expression is found to be high which correlates with a poor prognosis for the patient (Sainsbury *et al,* 1983; Sainsbury, 1988). This has also been found to be the case in breast cancer (Sainsbury *et al,* 1985b; 1985c; 1987; Macias *et al,* 1987; Horne *et al,* 1988; Boluffer *et al,* 1990) and ovarian cancer (Harding *et al.* 1989) in which ER expression correlates with good prognosis and bladder cancer in which high EGF receptor expression correlates both with poor prognosis and the stage of the tumour (Berger *et al,* 1987a). However this is not always the case as Bauknecht *et al* (1984) associated a good prognosis with positive EGF receptor status for ovarian carcinomas. It has further been confirmed that EGF receptors are linked to the status of the histological subtype of breast cancer (Sainsbury *et al,* 1987; Perez *et al,* 1984; Reubi and Torhorst, 1989). The oestrogen receptor is also used as prognostic indicator on its own (Crawford *et al,* 1987) as well as combined with progesterone receptors (Clarke and McGuire, 1989; Harding *et al,* 1990). It is already known that patients with teratomas have a poorer prognosis than patients with seminomas (pers. commun. Mr T.B.Hargreave, Consultant Urologist, Western General Hospital). This corroborates the findings in breast cancer where EGF receptor expression is related to poor prognosis, for as demonstrated in this study, it is the teratoma tissue which expresses EGF receptors.

4.13 Localisation of the EGF Receptor on Cancerous Testicular Tissues and Cells.

In employing immunohistochemical techniques to identify the EGF receptor, receptors were not detected on the various seminoma sections, although they were discovered on all three of the teratoma sections processed. On the teratoma sections the staining always appeared in dense localised regions. The presence of EGF receptors on teratoma tissue is perhaps not unexpected as the cells present are differentiating in multiple directions to form keratinising epithelium, cartilage and nerve (Hogan and Tilley,

1981). Thus the growth of these tissues may in fact require EGF receptors to be expressed for normal development. Detection with the F4 antibody of the internal domain of the receptor was only observed on the teratoma tissues and not on any of the other testicular tissues whether normal or cancerous. The apparent absence of detection of the internal domain of the EGF receptor on such tissues may indicate that the binding area to which the F4 antibody becomes attached was either changed or missing as previously mentioned in section 4.7. With the Tera-2 cells the A431 cell line was used as a positive control and although the Tera-2 cells became stained when using the F4 antibody they did not become stained when using the EGF-R1 antibody. This may suggest that the external domain of the receptor on the Tera-2 cells was absent and that the receptors were in fact truncated. The reason for this may be that the cells were grown in 10% FCS RPMI before plating down. 10% FCS was shown to contain EGF which could have down regulated the receptors. If this was the case then down regulation would be accompanied by truncation of the receptor which in turn would mean that the extracellular domain of the EGF receptor would not be available for binding by the monoclonal antibody. The fact that this did not hinder detection of the A431 cell receptors may be explained by the fact that A431 cells express a much higher number of EGF receptors (10^6 in comparison to 6.7×10^4).

4.14 Further Characterisation of the EGF Receptor on Tera-2 Cells

The EGF receptor on Tera-2 cells was further characterised by performing Western blot analysis. The receptor was identified in the 170 kDa form and also in the 125 kDa degraded form. The 125 kDa protein had previously been identified by Decker (1989) on NIH-3T3 cells where he had used an antiserum prepared against a trypE-EGF receptor fusion protein to bind to the EGF receptor site in an immunoprecipitation procedure. The antibody was found to bind to the carboxy terminal of the EGF

receptor. However if an antibody which bound to the amino terminus of the receptor was employed then the 125 kDa receptor species was not identifiable. It was also indicated in Decker's report that proteolysis of the receptor to the 125 kDa form could be part of the receptors normal function, as before the immunoprecipitation procedure, the cells had continuously been exposed to 10 nM EGF (Decker, 1989). He proposed that this treatment lead to proteolysis of the EGF receptor site in a similar fashion to EGF producing internalisation of the receptor (Carpenter *et al,* 1976; Carpenter, 1987) and subsequently degradation of the receptors in lysosomes (Beguinot *et al,* 1984; Dunn, 1984). In this study however the cells had not been treated with EGF for at least 4 days.

Competition of the receptor site on the Tera-2 cells with various other growth factors (vNGF, bFGF, hIGF-I) clarified further that the binding site was specific to EGF. However rTGF-α and concentrated conditioned medium also offered similar competition. Venom NGF was found to offer 37% competition and bFGF 32% competition for the receptor site. This result could possibly be attributed to the cells still being alive during the incubation period of the assay, as the cells were not homogenised. Transmodulation of the EGF receptor by vNGF and bFGF could have occurred affecting the binding of radiolabelled mEGF to the receptor in an indirect way rather than the growth factors directly competing for the EGF receptor. This could be investigated by first lysing the cells before use in the radioligand exchange assay. Rat TGF-α and concentrated conditioned medium offered 99% and 84% competition respectively for the EGF binding site both of which were not significantly different from the percentage competition illustrated with mEGF.

4.15 EGF and TGF-α in Cancerous Tissues

EGF and TGF-α have been found to be present in various cancerous tissues as well as normal tissues (Osborne *et al,* 1982; Kawamota *et al,* 1983; Perroteau *et al,* 1986; Stoscheck and King 1986; Salomon *et al,* 1983; 1987; Connolly and Rose, 1989; Imanishi *et al,* 1989; Wilding *et al,* 1989; Bates *et al,* 1990). In this study both hEGF and hTGF-α were found to be present in seminoma tissues as well as in the conditioned medium of the Tera-2 cell line. Unfortunately the endogenous concentrations of hTGF-α and hEGF were not identified in the Leydig cell tumour or the teratoma tumours due to the lack of tissue available. Mori *et al* (1989) also identified hEGF in both seminoma and embryonal carcinoma tissues, further supporting our finding of hEGF like activity in seminoma tissue and the concentrated conditioned medium from the Tera-2 cell line. Interestingly hTGF-α was found in the concentrated conditioned medium of the Tera-2 cell line at approximately the same concentration as that of the hEGF. However no cross reactivity between the antibody for rTGF-α and hEGF was identified at 95% limit. Human TGF-α as discussed previously (section 4.3) was also found to be present in normal testicular tissue, although the concentration was not statistically significantly different from that found in seminoma tissues. The fact that no EGF receptors were expressed in seminoma tissue possibly suggests that the growth factor is being secreted in an ectopic manner. The role of transforming growth factors in the transformation of normal to cancerous cells is being investigated (Coffey *et al,* 1986; Keski-oja *et al,* 1987; Knabbe *et al,* 1987; Schuurmans *et al,* 1988b; Imanishi *et al* 1989). It has been shown that both EGF and TGF-α induce differential processing of the EGF receptor (Decker, 1990) and that certain cells can lose growth responsiveness to EGF with a concomitant increase in TGF-α production (Salomon, 1987). The EGF receptor has also been shown to be critical in the regulation of the degree of maturation of malignant epidermal cells (King and Sartorelli, 1989). Subsequently monoclonal antibodies have been developed to

block the effects of growth factors in the treatment of cancers which are dependent on growth factors (Rodeck *et al,* 1987; Bronchud and Dexter, 1989). However the majority of human anti-tumour monoclonals to date have been disappointing (Campbell *et al,* 1988; Sunada *et al,*1990). The effect which anti-oestrogens may exert on EGF stimulated growth (Cormier and Jordan, 1989) as well as the actions of anti-neoplastic agents have also been investigated (Hanauske *et al,* 1987). In Tera-2 cells, IGF-I and IGF-II have also been shown to stimulate growth (Biddle *et al,* 1988).

The effect of hEGF, rTGF-α and concentrated conditioned media on the Tera-2 cell line was investigated further by competing several concentrations of hEGF, rTGF-α and concentrated conditioned media with a single concentration of labelled mEGF. It was discovered that hEGF, rTGF-α and the concentrated conditioned media all compete at various concentrations for the EGF receptor site with labelled mEGF to produce dissociation curves. However the curves differ in that the TGF-α curve has a slightly steeper gradient than the EGF curve. This may indicate that the EGF binding site characterised on the Tera-2 cell line has a higher affinity for TGF-α than for EGF. Winkler *et al* (1989) reported that there is a different conformation of the receptor required in binding of TGF-α to the EGF receptor in comparison to EGF. This difference in conformation may be dependent on whether the cell is normal or cancerous. The curve for the competition of concentrated conditioned media is also of a different shape compared to both the TGF-α and EGF curves. This could be attributed to both EGF and TGF-α being present in the concentrated conditioned media and competing for the EGF receptor site synergistically. However the competition achieved with the 0.14 nM of TGF-α and the 0.13 nM of EGF (present in the concentrated conditioned media) does not in any way approach the 84% competition which is observed with the concentrated conditioned media for the EGF binding site illustrated in Figure 40. Ideally this percentage of competition should be achieved with concentrations of TGF-α and EGF in the 100 nM range. It is therefore

possible that other growth factors are present in the concentrated conditioned media which also compete for the EGF binding site or possibly which augment the effect which EGF and TGF-α may be having on the receptor.

The fact that EGF and its receptor and TGF-α are found on the Tera-2 cell line suggests that the cells may be responsive to one or both of these substances. As a result the effect of TGF-α and EGF on the growth of the Tera-2 cell line was investigated. Tritiated thymidine incorporation was employed to assess the possible increase in DNA synthesis on addition of EGF or TGF-α. EGF was found to have no effect on cell growth over a 24 hour period at concentrations ranging from 0.3 to 100 nM. This was also reported by Engstrom *et al* (1985) who thought that the lack in response was possibly due to the experiment being performed in bulk cultures as difficulty was experienced in the plating down of the cells. In this study TGF-α was found to produce an increase in thymidine incorporation compared with controls. At its maximum the thymidine incorporation increased by approximately 2-fold in comparison to the control which was obtained when the cells were incubated in 10 nM TGF-α over 24 hours. Cell number in both the control and TGF-α treated wells was also determined and showed no significant difference in actual cell growth between the two differently treated groups of cells.

Time course studies were also performed to evaluate the effect of TGF-α and EGF on thymidine incorporation over a 72 hour period, however, no further increase in thymidine incorporation was noted when cells were incubated over 72 hours. In MCF-7 cells Osborne *et al* (1980) reported that EGF increased the proportion of cells active in DNA synthesis by nearly 2-fold . It was found that stimulation of incorporation of uridine and leucine by the MCF-7 cells was evident after 3 hours, whereas EGF stimulation of thymidine incorporation was delayed until 12-18 hours (Osborne *et al*,1980). In this study the increase in thymidine incorporation was noted after a 24 hour incubation period in 10 nM TGF-α, although this response did

not alter when the cells were incubated in 10 nM TGF-α over 72 hours. No change in the response to EGF was noted when the cells were incubated over 24 or 72 hours in 10 nM EGF or at concentrations of 0.3 to 100 nM EGF over 24 hours. Cell number was again monitored at each individual time point and no significant difference between the cells incubated in the TGF-α, the EGF and the control wells was noted.

It is well documented that cell lines that produce TGF-α show very little mitogenic response to exogenous EGF, despite EGF receptors having been expressed (Coffey *et al,* 1986; Salomon *et al* ,1987). Coffey *et al* (1986) reported that colon cancer cell lines produced measurable amounts of TGF-α and had detectable levels of EGF receptors, although exogenous EGF did not enhance cell growth of these cells. Similarly Saloman (1987) detected levels of EGF receptors in *ras*-transformed mouse mammary epithelial cells. The cells were unresponsive to exogenous EGF and TGF-α in a growth assay despite EGF-like material being detected in the concentrated conditioned medium. The loss of response to exogenous growth factor may, as is suggested, be due to autocrine secretion of a growth factor resulting in maximal autostimulation of that cell line. However some human breast cell lines do show a proliferative response to EGF and TGF-α (Dickson *et al,* 1986) and also secrete EGF/TGF-α like material (Bates *et al,* 1983). In relation to this study the fact that TGF-α produced an effect on the Tera-2 cells and not EGF suggests that the EGF receptor when activated by TGF-α initiates a different intracellular signal to that initiated by EGF binding to the receptor.

4.16 Steroids in Cancerous Tissues

In measuring the concentration of androgens in seminoma tissues it was found that the levels of androstenedione, DHT and testosterone were lower in comparison to the concentrations measured in normal tissues. The concentration of androstenedione was

found to be 0.025 nmoles/g for DHT 0.093 nmoles/g and for testosterone 0.83 nmoles/g of dry testicular tissue. It can therefore be postulated that testicular tissue loses its steroid producing capabilities in the transformation from normal testicular tissue to seminoma tissue. However it is not clear from this finding alone whether seminomas require androgens for differentiation and proliferation. Because such low concentrations of androgens are present in the seminoma tissue in comparison to normal testicular tissue one may speculate that seminoma tissue does not depend on androgens as a source of stimulus which is in contrast to breast cancer and prostate cancer.

In the progression of prostatic cancer testosterone is thought to be the main promotor of growth although there is an indication of the possible involvement of EGF and TGF-α, as EGF receptors have been found to be present on the epithelial cells of the tissue (Maddy *et al,* 1989). At present the main method of treatment for the prevention of the progression of prostatic cancer is to inhibit the effects of androgens on the gland. One way of achieving this is to remove the main organ supplying the testosterone which is the testes (*i.e.*orchiectomy); the main source from which 'normal testicular tissue' for this study was derived. Another possible approach is to introduce anti-androgens (cyproterone acetate) or oestrogens (diethylstilboestrol) into the systemic circulation in order to decrease the production of testosterone by acting on the anterior pituitary to decrease the release of LH which normally stimulates the Leydig cell to increase androgen output (Bruchovsky *et al,* 1988). LHRH analogues have also been investigated and are presently being used in treatment of prostatic cancer as they produce inhibition of testosterone secretion by inhibiting LH release (Bruchovsky *et al,* 1988). However in treatment with certain types of steroid analogues there is evidence that some stimulate rather than inhibit cell proliferation. This raises certain implications with regard to the selection of patients for hormone therapy aswell as the long term use of certain analogues (Braunsberg *et al,* 1987a; 1987b).

Recently steroid/thyroid type receptor-like proteins have been identified in tissues which show oncogenic potential (Slusyer, 1990). The receptors are thought to be mutated or truncated forms of the original steroid/thyroid receptor family (Slusyer, 1990).

In the case of teratoma tissues experiments were once again performed employing the Tera-2 cell line to evaluate the possibility of the cells being androgen sensitive. Mibolerone was the synthetic androgen chosen because of its low degradability. Its affect on DNA synthesis was investigated by performing thymidine incorporation experiments. Both time course studies and dose response studies were performed employing mibolerone but no response was noted. Cell number was also measured but no significant difference between controls and mibolerone treated cell numbers was apparent. The effect of various concentrations of mibolerone on EGF receptor expression was also investigated. No change in specific EGF binding was observed when cells were incubated in 0.1-100 nM concentrations of mibolerone over 24 hours. These findings indicate that although EGF and testosterone have been shown to be required for spermatogenesis (in which germ cells are constantly dividing), testosterone and EGF are not required in the proliferation of Tera-2 cells.

Steroids have been shown to influence cancerous growth as well as EGF receptor expression and EGF and TGF-α secretion on other tissues (Bynny *et al,* 1974; Dickson *et al,* 1986; Traish and Wotiz, 1987; Schuurman *et al,*1988). It has been shown that androgen independent tissues require different hormones depending on whether they are normal or tumour epithelial cells (McKeehan *et al,* 1984; 1987).

In the human breast cancer cell line MCF-7 oestrogen has been found to stimulate the production of TGF's. When TGF-α concentrations increased an increase in tumour growth was also noted (Dickson *et al,* 1986a). Blockade of the EGF receptor on the MCF-7 cell line was found to inhibit TGF-α secretion but it did not inhibit growth which was thought to have been oestrogen induced (Dickson *et al,*

1986a; 1986b; 1986c). It is therefore likely that as well as hormones and growth factors acting synergistically they may also act separately in certain types of cancer to augment their own effects. In the case of seminomas androgens may indeed be present specifically for this reason. Both androgens and TGF-β have been shown to modulate the growth response of LNCaP cells to EGF (Schuurmans *et al,* 1988a; 1988b).

4.17 The Role of Steroids and Growth Factors in the Progression of Cancerous Tissues.

As previously stated EGF and testosterone are both thought to be required in spermatogenesis (Bartlett *et al,* 1990; Sharpe *et al,* 1988). It was therefore of interest to investigate any possible role which androgens or EGF/TGF-α may have in the maintenance of germ cell tumours, since, despite the enormous advances made in the treatment of these tumours, very little is known about their aetiology. The risk factors that have been identified at present offer little clue as to how to prevent the disease. Given the increase in incidence reported in 1981 by Davies it is clearly important to try and understand what causes this form of cancer rather than depend on surgical and chemotherapeutic treatment. However it is possible that Davies (1981) did not report an increase in testicular cancer but rather an increase in the number of patients who come forward for treatment. Returning to the possible increase in testicular cancer, information is now available from epidemiological studies which indicate a role for genetic, viral and physical factors in the causation of testicular cancer (Schottenfeld and Warshauer, 1982; Henderson *et al,* 1983) although at present the only clearly identifiable risk factor is a prior history of cryptorchidism (Chivers *et al,* 1984; Strader *et al,* 1988). Investigations have also been centered on the possible involvement of exposure to oestrogens during the gestation period (Depue *et al,* 1983 and Depue, 1984) as well as on the possibility of inheritance (Dieckmann *et al,* 1987). Evidence has become available which suggests that exposure to certain hormones, for example in

utero, might have an aetiological role. It is pointed out that the pattern of incidence of testicular tumours in relation to age is significant as the peak incidence occurs around the age of 30-35 years. Depue (1984) suggests that as the development of cancer from initiation to diagnosis usually takes some 20-30 years, it seems reasonable to postulate that the critical and limiting period for germ cell initiation is the period *in utero*, as very few germ cell tumours are diagnosed after 45 years of age. However there is no evidence in the literature to support the suggestion that the development of cancer takes up to 20-30 years. It is equally valid to suggest for instance that puberty initiates the onset of testicular cancer as around that period androgen levels are higher than at any other time of life in the male. The incidence of testicular cancer in childhood, for example, is very rare although the cases that are seen may be attributable to the increasing concentrations of steroids which occur during both foetal development and through the first few years post gestation (Bidlingmaier *et al,* 1983; Rommerts and van der Molen, 1988).

It has been established for a number of years that oestrogens play a critical role in the aeitiology of breast cancer (Leake *et al,* 1979; 1981). Furthermore it has now been suggested that oestrogen control of hormone dependent breast cancer is mediated by autocrine and paracrine growth factors secreted by the breast cancer cells themselves. Lippman *et al* (1986) has shown direct unmediated effects of estrogen on specific cell functions as well as demonstrating that cancer cells secrete a number of growth factors (IGF-I, TGF-α TGF-β, a PDGF-like competency factor and at least one new epithelial colony stimulating factor). Some of these have been shown to be oestrogen regulated in hormone dependent cells as well as more numerous in cells which acquire independence either spontaneously or by *ras* transformation (Lippman *et al,* 1986). It has been shown by Travers *et al* (1988) that growth factors are expressed differently in several types of breast tissue depending on whether the tissue is malignant, benign or normal. This particularly applies to the growth factors TGF-α

and TGF-β. It has therefore been postulated that such growth factors may play an important role in controlling growth of human breast cancers, particularly those that are hormone independent. This was corroborated by the results of Lippman *et al* (1987) who illustrated that growth regulation of human breast cancer occurs through regulated growth factor secretion. The fact that growth factor secretion can also be regulated by hormones in hormone dependent tissues signifies that there is some interaction between steroids and growth factors in cancerous tissues. In the past estrogen receptors have been used as prognostic indicators for breast cancer but now it appears that the more reliable indicators of prognosis are the proteins which are regulated by or are related to the oestrogen receptor (Horne *et al,* 1988).

Epidermal growth factor receptor status has in the past been used as a predictor of early recurrence of and death from breast cancer (Sainsbury *et al,* 1987) as have both ER and EGF receptor status (Sainsbury *et al,* 1985d). It is possible that the hormones themselves cause the transformation of cells from hormone dependent to hormone independent. The hormones stimulate an increase in growth factor secretion which in turn enables the cells to eventually become growth factor dependent and hormone independent. It has been shown by Westley and Rochefort (1980) that human breast cancer cells secrete a 46 kDa glycoprotein when stimulated by oestrogen, and that oestradiol induces EGF related polypeptide production in MCF-7 cells (Dickson *et al,* 1986b; 1986c). However in the transformation of tissues from hormone dependent to hormone independent it has been shown by Arteaga *et al* (1988) that blockade of the EGF receptor on MCF-7 cells inhibits TGF-α induced but not oestrogen induced growth. It is therefore evident that when both growth factors and hormones are present in the cells environment cell growth is under the control of both of them. It is interesting to note that in salivary gland tumours no high affinity steroid hormone receptors were detected indicating that such a cancer is not dependent on endocrine

secretion. It is however well established that the SMG is one of the major sites of EGF production.

Clinically the expression of growth factors in hormone dependent tissues is of significance because it has been demonstrated that tumours arising in hormone sensitive cells (for example breast) frequently progress from the hormone sensitive to the hormone insensitive state (King and Darbre, 1989). Often some of the regulatory controls of their normal progenitors are retained, but even these controls may be lost or altered during the progression. Altered involvement of growth factor activity in the transition from endocrine responsive to unresponsive breast cancer is an attractive idea because of its simplicity and compatibility with two clinical features. Endocrine insensitive human breast tumours grow faster and recur earlier than their endocrine responsive counterparts (Hahnel, 1982; Clark and McGuire, 1989). Also the EGF receptor is over expressed in unresponsive relative to responsive tumours, and over expression correlates with increased rate of recurrence (Harris, 1989; Nicholson *et al,* 1989). However this picture is not as simple as it appears. By investigating the expression of growth factor receptors and growth factor concentrations in both normal and cancerous tissue a greater insight into the control and regulation of cell growth can perhaps be evolved.

5.1 Conclusion

The results from this study indicate the possible involvement of EGF/TGF-α and the EGF receptor in the regulation of testicular function. It is possible that EGF/TGF-α are involved in testicular cell-cell communication although LH and FSH are the prevailing hormones which control testicular function. TGF-α mRNA has been found to be present in both peritubular and Sertoli cells (Skinner *et al*, 1989a). It may therefore be assumed that TGF-α is involved in peritubular-Sertoli cell communication. In this study EGF receptors were not found to be present on human Sertoli cells but they were found to be present on human peritubular and Leydig cells. EGF and TGF-α were also both found to be present in testicular extracts. One may therefore postulate that as well as TGF-α being involved in peritubular-Sertoli cell communication it may also be involved in peritubular-Leydig cell communication(Figure 48).

EGF and TGF-α were also found to be secreted by the Tera-2 cell line and the EGF receptor found to be expressed by Tera-2 cells as well as teratoma tissues. In performing competition studies with EGF and TGF-α on both normal tissue and cancerous cells it was found that EGF offered the greatest competition for the EGF receptor site in normal tissue (100% and 24% respectively) but that TGF-α offered equal competition for the receptor in the cancerous state (100% and 99% respectively).

In cancerous testicular tissue it may be concluded from the results of this study, that as yet there is no evidence to suggest that EGF or TGF-α play a role in the development and progression of seminoma tumours, but that the growth factors may be involved in teratoma tumour development. As mentioned previously EGF and its receptor were both identified in teratoma tissue/cells; the EGF receptor on both teratoma

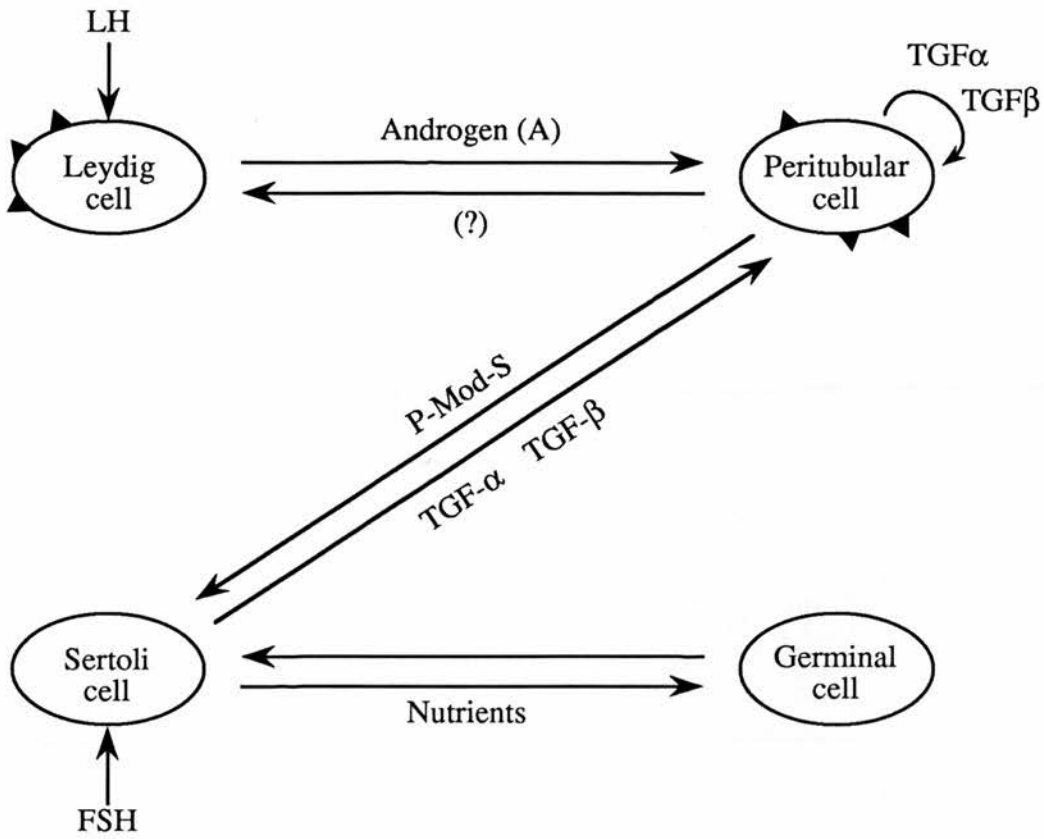


Figure 48
EGF receptors within the testes: As EGF receptors (▲) are expressed on Leydig and peritubular cells one may postulate that TGF-α could be the missing factor (?) in peritubular - Leydig cell communication. TGF-α has recently been characterised by Skinner *et al* (1989a) and is thought to be involved in peritubular - Sertoli cell communication and *vice versa*. Thus it appears that TGF-α may play a significant role in cell-cell communication within the testes via the EGF receptor.

tissue and the Tera-2 cells and EGF in the media of the Tera-2 cells. In addition TGF-α also appeared to be secreted by Tera-2 cells (*in vitro*) and when added to the cells *in vitro* produced stimulation of thymidine incorporation. This effect was not evoked by EGF. As discussed previously the EGF receptor identified on cancerous testicular cells appeared to have a higher affinity for TGF-α than the receptor identified on normal testicular tissue. It could therefore be postulated that the EGF receptor may be modified in the transformation of human testicular tissue from normal to cancerous and that in the cancerous state the EGF receptor has a higher affinity for TGF-α than EGF.

In looking at treated testicular tissue EGF receptors were found to be absent. This may indicate that in the event of a reduction in circulating androgen levels, expression of the EGF receptor is decreased. It could therefore be postulated that EGF receptors within the testes are under the control of androgens and that as demonstrated by Pascall *et al* (1989) in the mouse submandibular gland, a reduction in androgen concentrations can produce a decrease in mRNA encoding the EGF receptor. As reported by Hiramatsu *et al* (1988) and Pascall *et al* (1989) a decrease in circulating androgen levels also produces a decrease in EGF levels. This is again corroborated by our findings in which 5.16 ng per gram of EGF were found in normal testicular tissue in comparison to 2.23 ng per gram in treated testicular tissue. The possibility that EGF and indeed EGF receptors are regulated by circulating androgen concentrations may implicate EGF and its receptor in steroid production, especially as the EGF receptor is located on the Leydig cell, the site of steroidogenesis.

5.1 Future studies

(i) It is now well established that specific peptides (growth factors) bind to cell surface proteins (receptors) and initiate or maintain the biochemical events required for cellular proliferation. However these receptors are not always functional, in that the receptor is not always coupled to the second messenger. Further experimentation should therefore

be carried out to investigate the possibility of the EGF receptor found on human testicular tissue being non-functional. This could be achieved by performing autophosphorylation experiments or a tyrosine kinase assay.
The results of these studies may explain why the internal domain of the EGF receptor was not detected with the F4 monoclonal antibody.

(ii) The expression of EGF receptors on testicular tissues exposed to cyproterone acetate or stilboestrol should be investigated with a view to determining whether gene reduction or receptor down regulation resulted in non-detection of the receptor in such samples.

(iii) Ideally the role of growth factors in the communication of human testicular cells should be investigated using a primary testicular cell culture. Unfortunately the tissue required for such an experiment can not easily be acquired. Juvenile/foetal human testicular tissue is the most favorable tissue as it is in its growing state, however, ethical consent for such a study which would employ foetal testicular tissue may present a problem.
However juvenile/foetal tissue represents the growing state of the testes where as after puberty the majority of testicular cells are no longer proliferating. With this in mind post pubertal tissue would perhaps be more relevant for culture and for the investigation of paracrine control in the adult testes.

References

Abraham, G.E. (1974). Radioimmunoassay of Steroids in Biological Materials. Acta Endocrinologica, Suppl. 183, 75, 1-42.

Ahren, B. (1987). Epidermal growth factor (EGF) inhibits stimulated thyroid hormone secretion in the mouse. Peptides, 8, 743-745.

Akiyama, T., Kadooka, T., Ogawara, H. and Sakakibara, S. (1986). Characterisation of the Epidermal Growth Factor Receptor and the *erb*-B Oncogene Product by Site-Specific Antibodies. Arch. Biochem. Biophys. 245, 531-536.

Andrews, P.W. (1982). Human embryonal carcinoma cells in culture do not synthesise fibronectin until they differentiate. Int. J. Cancer. 30, 567-571.

Angeletti, R.J. and Bradshaw, R.A. (1971). Nerve growth factor from mouse submaxillary gland: amino acid sequence. Proc. Natl. Acad. Sci. USA. 68, 2417-2420.

Anzano, M.A., Roberts, A.B., DeLarco, J.E., Wakefield, L.M., Assoian, R.K., Roche, N.S., Smith, J.M., Lazarus, J.E. and Sporn, M.B. (1985). Increased secretion of type beta transforming growth factor accompanies viral transformation of cells. Mol. Cell. Biol. 5, 242-247.

Apella, E., Weber, I.T. and Blasi, F. (1988). Structure and function of epidermal growth factor-like regions in proteins. FEBS letters. 231, 1-4.

Armelin, H.A. (1973). Pituitary extracts and steroid hormones in the control of 3T3 cell growth. Proc. Natl. Acad. Sci. 70, 2702-2706.

Arteaga, C.L., Coronado, E. and Osborne, C.K. (1988). Blockade of the Epidermal Growth Factor Receptor Inhibits Transfroming Growth Factor α-Induced but Not Estrogen-Induced Growth of Hormone-Dependent Human Breast Cancer Cells. Mol. Endocrinol. 2, 1064-1069.

Ascoli, M. (1981). Regulation of gonadotropin receptors and gonadotropin responses in a clonal strain of Leydig tumour cells by epidermal growth factor. J. Biol. Chem. 256,179-183.

Ascoli, M. Euffa, J. and Segaloff, D.L. (1987) Epidermal Growth Factor Activates Steroid Biosynthesis in Cultured Leydig Tumour cells without Affecting the Levels of cAMP and Potentiates the Activation of Steroid Biosynthesis by Chorionadotropin and cAMP. J. Biol. Chem. 262, 9196-9203.

Assoian, R.K., Komoriya, A., Meyers, C.A., Miller, D.M. and Sporn, M.B. (1983). Transforming growth factor-β in human platelets: Identification of a major storage site, purification and characterisation. J. Biol. Chem. 258, 7155-7160.

Assoian, R.K. and Sporn, M.B. (1986). Type beta transforming growth factor in human platelets: release during platelet degranulation and action on vascular smooth muscle cells. J. Cell Biol. 102, 1217-1223.

Assoian, R.K. (1987). Purification of type-β transforming growth factor from human platelets. Methods. Enzymol. 146, 153-163.

Auron, P.E., Webb, A.C., Rosenwasser, L.J., Mucci, S.V., Rich, A., Wolff, S.M. and Dinarello, C.A. (1984). Nucleotide sequence of human monocyte interleukin 1 precursor cDNA. Proc. Natl. Acad. Sci. USA, 81, 7907-7911.

Avallet, O., Vigier, M., Perrard-Sapori, M.H. and Saez, J.M. (1987). Transforming growth factor-β inhibits Leydig cell functions. Biochem. Biophys. Res. Comm. 146, 575-581.

Bains, R.M. and Miller, K.D. (1988). Peroxidase labelling in immunocytochemistry: a critical comparison of five systems. Med. Lab. Sci. 45, 30-35.

Baker, J.B. and Cunningham, D.D. (1978). Glucocorticoid-mediated alteration in growth factor binding and action: analysis of the binding change. J. Supramol. Struc. 9, 69-77.

Banks-Schlegel, S.P. and Quintero, J. (1986). J. Biol. Chem. Human Esophageal Carcinoma Cells Have Fewer, but Higher Affinity Epidermal Growth Factor Receptors. J. Biol. Chem. 261, 4359-4632.

Bardin, C.W., Musto, N.A., Gunsalus, G.S., Kottie, N., Cheng, S-L., Larrea, F. and Becker, R. (1981). Extracellular androgen binding protein. Ann. Rev. Physiol. 43, 189-198.

Bardin, C.W., Chen, C-L. C., Morris, P.L., Gerendai, I. Boitani, C., Liotta, A.S., Margioris, A. and Krieger, D.T. (1987). Pro-opiomelanocortin-derived peptides in testis, ovary and tissues of reproduction. Recent Prog. Horm. Res. 43, 1-28.

Barnes, D. & Sato, G. (1980). Serum-free Cell Culture: a Unifying Approach. Cell, 22, 649-655.

Bartlett, J.M.S., Spiteri-Grech, J. and Nieschlag, E. (1990). Regulation of Insulin-Like Growth Factor-I and Stage-Specific Levels of Epidermal Growth Factor in Stage Synchronised Rat Testes. Endocrinol. 127, 747-758.

Bates, S.E., Valverius, E.M., Ennis, B.W., Bronzert, D.A., Sheridan, J.P., Stampfer, M.R., Mendelsohn, J., Lippman, M.E. and Dickson, R.B. (1990). Expression of the Transforming Growth Factor-α/Epidermal Growth Factor Receptor Pathway in Normal Human Breast Epithelial Cells. Endocrinol. 126, 596-607.

Bauknecht, T., Rau, B., Meerpohl, H.G. and Pfleiderer, A. (1984). The prognostic value of the presence of EGF receptors in ovarian carcinomas. Tumour-Diagnostik, 5, 62-66.

Bauknecht, T., Kiechle, M., Bauer, G. and Siebers, J.W. (1986). Characterisation of Growth factors in Human Ovarian Carcinomas. Cancer Res. 46, 2614-2618.

Bawa, S.R. (1963). Fine structure of the Sertoli cell of the human testis. J. Ultrastruct. Res. 9, 459-474.

Beguinot, L., Lyall, R.M., Willingham, M.C. and Pastah, I. (1984). Down-regulation of the epidermal growth factor receptor in KB cells is due to receptor internalisation and subsequent degradation in lysosomes. Proc. Natl. Acad. Sci. USA. 81, 2384-2388.

Bellot, F., Moolenar, W., Kris, R., Mirakhur, B., Verlaan, I., Ullrich, A., Schlessinger, J. and Felder, S. (1990). High-Affinity Epidermal Growth Factor Binding is Specifically Reduced by a Monoclonal Antibody and Appears Necessary for Early Responses. J. Cell Biol. 110, 491-502.

Bellve, A.R. (1979). The molecular biology of mammalian spermatogenesis. In: Oxford Reviews in Reproductive Biology. Ed. C.A. Finn. Clarendon, Oxford, U.K. Vol. 1, 159-261.

Bellve, A.R. and Feig, L.A. (1984). Cell Proliferation in the Mammalian Testis: Biology of the Seminiferous Growth Factor (SGF). Rec. Prog. Horm. Res. 40, 531-567.

Bellve, A.R. and Zheng, W. (1989). Growth factors as autocrine and paracrine modulators of male gonadal functions. J. Reprod. Fertil. 85, 771-793.

Benahmed, M., Grenot, C. Tabone, E. and Saez, J.M. (1985). FSH regulates cultured Leydig cell function via Sertoli cell proteins: An in vitro study. Biochem. Biophys. Res. Commun. 132, 720-734.

Benhamed, M., Morera, A.M., Chauvin, M. and de Peretti, E. (1987). Sm C/IGF-I as a possible intratesticular regulator of Leydig cell activity. Mol. Cell. Endocrinol. 50, 69-77.

Benahmed, M., Cochet, C., Keramidas, M., Chauvin, M.A. and Morera, A.M. (1988). Evidence for a FSH dependent secretion of a receptor reactive transforming growth factor-β like material by immature Sertoli cells in primary culture. Biochem. Biophys. Res. Commun. 154, 1222-1231.

Bennett, J.P. and Yamamura, H.I. (1985). Neurotransmitter, Hormone, or Drug Receptor Binding Methods. In: Neurotransmitter Receptor Binding, Second Edition. Ed. H.I. Yamamura, Raven Press. 61-89.

Bepler, G., Rotsch, M., Jaques, G., Haeder, M., Heymanns, J., Hartogh, G., Kiefer, P. and Havemann, K. (1988). Peptides and Growth Factors in small cell lung cancer: production binding sites and growth effects. J. Cancer Res. Clin. Oncol. 114, 235-244.

Bergami, F., Caione, P. and Rivosecchi, M. (1977). Testicular tumors in infancy and childhood. Zeitschrift Fur Kinderchirurgie, 20, 57-70.

Berger, M.S., Greenfield, C., Gullick, W.J., Haley, J., Downward, J., Neal, D.E., Harris, A.L. and Waterfield, M.D. (1987a). Evaluation of epidermal growth factor receptors in bladder tumours. Br. J. Cancer, 56, 533-537.

Berger, M.S., Gullick, W.J., Greenfield, C., Evans, S., Addis, B.J. & Waterfield, M.D. (1987b). Epidermal growth factor in lung tumours. J. Pathol. 152, 297-307.

Bergh, A. (1982). Local differences in Leydig cell morphology in the adult rat testis: evidence for a local control of Leydig cells by adjacent seminiferous tubules. Int. J. Androl., 8, 325-330.

Bergh, A. (1983). Paracrine regulation of Leydig cells by the seminiferous tubules. Int. J. Androl., 6, 57-65.

Bernier, M., Chatelain, P., Mather, J.P. and Saez, J.M. (1986). Regulation of gonadotropin receptors, gonadotropin responsiveness and cell multiplication by somatomedin-C and insulin in cultured pig Leydig cells. J. Cell Physiol. 129, 257-263.

Berns, E.M.J.J., de Boer, W. and Mulder, E. (1986). Androgen-Dependent Growth Regulation of and Release of Specific Protein(s) by the Androgen Receptor Containing Human Prostate Tumor Cell Line LNCaP. The Prostate, 9, 247-259.

Biddle, C., Li, C.H., Schofield, P.N., Tate, V.E., Hopkins, B., Engstrom, W., Iiuskisson, N.S. and Graham, C.F. (1988). Insulin-like growth factors and the multiplication of Tera-2, a human teratoma-derived cell line. J. Cell Sci. 90, 475-484.

Bidlingmaier, F., Dorr, H.G., Eisenmenger, W., Kuhnle, U. and Knorr, D. (1983). Testosterone and Androstenedione Concentrations in Human Testis and Epididymis during the First Two Years of Life. J. Clin. Endocrinol. Metab. 57, 311-315.

Biswas, R., Basu, M., Sen-Majumdar, A. and Das, M. (1985). Intrapeptide autophosphorylation of the EGF receptor: regulation of kinase catalytic function by receptor dimerization. Biochemistry, 24, 3795-3802.

Boluffer, P., Miralles, F., Rodriguez, A., Vazquez, C., Lluch, A., Garcia-Conde, J. and Olmos, T. (1990). Epidermal Growth Factor Receptor in Human Breast Cancer: Correlation with the Cytosolic and Nuclear ER Receptors and with Biological and Histological Tumor Characteristics. Eur. J. Cancer Clin. Oncol. 26, 283-290.

Boni-Schnetzler, M. and Pilch, P.F. (1987). Mechanism of epidermal growth factor receptor autophosphorylation and high-affinity binding. Proc. Natl. Acad. Sci. USA. 84, 7832-7836.

Bowen-Pope, D.F., DiCorletto, P.E. and Ross, R.J. (1983). Interactions between the receptors for platelet-derived growth factor and epidermal growth factor. J. Cell Biol. 96, 679-683.

Bradford, M.M. (1979). A rapid and sensitive method for the quantitation of microgram quantities of protein utilising the principle of protein dye binding. Analytical Biology, 72, 248-254.

Bradshaw, R.A. (1978). Nerve growth factor. Annu. Rev. Biochem. 47, 191-216.

Braunsberg, H., Coldham, N.G., Leake, R.E., Cowan, S.K. and Wong, W. (1987a). Actions of a Progestogen on Human Breast Cancer Cells: Mechanisms of Growth Stimulation and Inhibition. Eur. J. Cancer Clin. Oncol. 23, 563-571.

Braunsberg, H., Coldham, N.G. and Leake, R.E. (1987b). Effects of Serum Type on Steroid Receptor Binding Sites and Response to Progestin of Cultured Human Breast Cancer Cells. Eur. J. Cancer Clin. Oncol. 23, 573-576.

Bringman, T.S., Lindquist, P.B. and Derynck, R. (1987). Different Transforming Growth Factor-α Species Are Derived From A Glycosylated And Palmitoylated Transmembrane Precursor. Cell, 48, 429-440.

Bronchud, M.H. and Dexter, T.M. (1989). Clinical use of growth factors. Br. Med. Bull. 45, 590-599.

Brown, B.W., Mattner, P.E., Panaretto, B.A. and Brown, G.H. (1989). Effect of mouse epidermal growth factor on plasma concentrations of LH, FSH and testosterone in rams. J. Reprod. Fertil. 87, 649-655.

Brown, K.D., Blakeley, D.M., Henville, A. and Setchell, B.P. (1982). Rete testis fluid contains a growth factor for cultured fibroblasts. Biochem. Biophys. Res. Commun. 105, 391-397.

Brown, N.J. (1976). Yolk-sac tumour (orchioblastoma) and other testicular tumours of childhood. In: Pathology of the Testis. Ed. R.C.B. Pugh. Blackwell Scientific Publications, London, U.K., 356-370.

Bruchovsky, N., Rennie, P.S. and Goldenberg, S.L. (1988). Mechanism and effects of androgen withdrawal therapies. In: New Developments in Biosciences. Walter de Grutyer and Co. Berlin. New York, 4, 3-14.

Buch, J.P., Lamb, D.L., Lipschultz, L.I. and Smith, R.G. (1988). Partial characterisation of a unique growth factor secreted by human Sertoli cells. Fertility and Sterility, 49, 658-665.

Burgess, A.W. (1989). Epidermal growth factor and transforming growth factor-α. Br. Med. Bull. 45, 401-424.

Bynny, R.L., Orth, D.N.and Cohen, S. (1972). Radio-immunoassay of epidermal growth factor. Endocrinol. 90, 1261-1266.

Campbell, G., Lidereau, R. and Callahan, R. (1988). Lack of evidence for the prognostic significance of *c-erb*-2 amplification in human breast carcinoma. Oncogene Res. 3, 139-146.

Carpenter, G., Lembach, K.J., Morrison, M.M. and Cohen, S. (1975). Characterisation of the binding of ^{125}I-labelled epidermal growth factor to human fibroblasts. J. Biol. Chem. 259, 4297-4304.

Carpenter, G. and Cohen, S. (1976). ^{125}I-labelled human epidermal growth factor: Binding, internalisation and degradation in human fibroblasts. J. Cell Biol. 71, 159-71.

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

Carpenter, G., King, L. and 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. and Cohen, S. (1979). Epidermal Growth Factor. Ann. Rev. Biochem. 48, 193-216.

Carpenter, G., Stoscheck, C.M., Preston, Y.A. and 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. Methods Enzymol. 109, 101-110.

Carpenter, G. (1987). Receptors for epidermal growth factor and other polypeptide mitogens. Annu. Rev. Biochem. 56, 881-914.

Cassel, D. and Glaser, L. (1982). Proteolytic cleavage of the epidermal growth factor receptor. A Ca^{2+} dependent, sulfphydryl-sensitive proteolytic system in A431 cells. J. Cell. Biochem. 257, 9845-9848.

Catt, K.J. and Dufau, M.L. (1973). Interaction of LH and hCG with testicular gonadotropin receptors. Adv. Exptl. Med. Biol. 36, 379-418.

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

Chen, W.S., Lazaar, C.S., Poenie, M., Tsien , R.Y., Gill G.N.and Rosenfeld, M.G. (1987). Requirement for intrinsic protein kinase in the immediate and late actions of the EGF receptor. Nature, 328, 820-823.

Cheng, C.Y. and Bardin, C.W. (1986). Rat testicular testibumin is a protein responsive to follicle stimulating hormone and testosterone that shares immuno-determinants with albumin. Biochem. 25, 5276-5288.

Cheifetz, S., Weatherbee, J.A., Tsang, M.L-S., Anderson, J.K., Mole, J.E., Lucas, R. and Massague, J. (1987). The transforming growth factor-β system, a complex pattern of cross-reactive ligands and receptors. Cell, 48, 409-415.

Chinkers, M. and Brugge, J.S. (1984). Characterisation of structural domains of the human epidermal growth factor receptor obtained by partial proteolysis. J. Biol. Chem. 259, 11534-11542.

Chivers, C., Pike, M.C. and Forman, D. (1984). Apparent doubling of frequency in undescended testis in England and Wales in 1962-1981. Lancet, i, 329-323.

Christensen, A.K. (1975). Leydig cells. In: Handbook of Physiology. Eds. R.O. Greep and E.B. Ashwood. Am. Physiol. Soc. Washington, USA. 5, 57-94.

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

Clermont, Y. (1963). The cycle of the seminiferous epithelium in man. Am. J. Anat. 112, 35-45.

Clermont, Y. (1970). In: The Human Testis. Eds. E. Rosemberg and C.A. Paulsen. Plenum Press.

Clermont, Y. (1972). Kinetics of spermatogenesis in mammals. Seminiferous epithelium cycle and spermatagonail renewal. Physiol. Rev. 52, 198-236.

Cochet, C., Gill, G.N., Meisenhelder, J., Cooper, J.A. and Hunter, T. (1984). C-kinase phosphorylates the epidermal growth factor-stimulated tyrosine protein kinase activity. J. Biol. Chem. 259, 2553-2558.

Cochet, C., Kashles, O., Chambaz, E., Borrelo, I., King, C.R. and Schlessinger, J. (1988). Demonstration of epidermal growth factor-induced receptor dimerization in living cells using a chemical covalent cross-linking agent. J. Biol. Chem. 263, 3290-3295.

Coffey, R.J., Shipley, G.D. and Moses, H.L. (1986). Production of Transforming Growth Factors by Human Colon Cancer Lines. Cancer Res. 46, 1164-1169.

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

Cohen, S. and Carpenter, G. (1975). Human Epidermal Growth Factor: Isolation and chemical and biological properties. Proc. Natl. Acad. Sci. U.S.A. 72, 1317-1321.

Cohen, S., Carpenter, G. and King, L. (1980). Epidermal Growth Factor-Receptor Protein Kinase Interactions. J. Biol. Chem. 255, 4834-4842.

Cohen, S., Ushiro, H., Stoscheck, C. and Chinkers, M. (1982). A Native 170,000 Da Epidermal Growth Factor Receptor-Kinase Complex from Shed Plasma Membrane Vesicles. J. Biol. Chem. 257, 1523-1531.

Collins, D.H. and Pugh, R.C.B. (1964). Classification and frequency of testicular tumours. Brit. J. Urol. (Suppl.) 36, 1-11.

Connolly, J.M. and 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, T.G. (1986). Maturation of Spermatozoa in the Epididymis. In: The epididymis, sperm maturation and fertilisation. Springer-Verlarg, Heidelberg. Part I, 1-8.

Cormier, E.M. and Jordan, V.C. (1989). Contrasting Ability of Anti-oestrogens to Inhibit MCF-7 Growth Stimulated by Estradiol or Epidermal Growth Factor. Eur. J. Cancer Clin. Oncol. 25, 57-63.

Costrini, N.V. and Bradshaw, R.A, (1979). Binding characteristics and apparent molecular size of detergent-solubilised nerve growth factor receptor of sympathetic ganglia. Proc. Natl. Acad. Sci. USA. 76, 3242-3245.

Coussens, L., Yang-Feng, L.T.L., Liao, Y.C., Chen, E., Gray, A., McGrath, J., Seeburg, P.H., Libermann, T.A., Schlessinger, J., Francke, U., Levinson, A. and Ullrich, A. (1985). Tyrosine kinase receptor with extensive homology to the EGF-receptor shares chromosomal location with *neu* oncogene. Science, 230, 1132-1139.

Crawford, D.J., Cowan, S., Fitch, R., Smith, D.C. and Leake, R.E. (1987). Stability of oestrogen receptor status in sequential biopsies from patients with breast cancer. Br. J. Cancer, 56, 137-140.

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

Daugaard, G., von der Masse, H., Olsen, J., Rorth, M. and Skakkebaek, N.E. (1987). Carcinoma-in-situ testis in patients with assumed extragonadal germ cell tumours. Lancet, ii, 528-530.

Davies, T.W., Williams, D.D.R. and Whitaker, R.H. (1980). Risk factors for undescended testis. Int. J. Epidemiol. 15, 197-201.

Davies, J.M. (1981). Testicular cancer in England and Wales: some epidemiological aspects. Lancet, ii, 928-932.

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

Davis, R.J. (1988). Independent Mechanisms Account for the Regulation by Protein Kinase C of the Epidermal Growth Factor Receptor Affinity and tyrosine-Protein Kinase Activity. J. Biol. Chem. 263, 9462-9469.

Decker, S.J. (1989). Epidermal Growth Factor-Induced Truncation of the Epidermal Growth Factor Receptor. J. Biol. Chem. 25, 17641-17644.

Defize, L.H.K., Ardndt-Jovin, D.J., Jovin, T.M., Boonstra, J., Meisenhelder, J., Hunter, T., de Hey, H.T. and de Laat, S.W. (1988). A431 cell variants lacking the blood group A antigen display increased high affinity EGF receptor number, protein tyrosine kinase activity and receptor turnover. J. Cell Biol. 107, 939-949.

Defize, L.H.K., Boonstra, J., Meisenhelder, J., Kruijer, W., Tertoolen, L.G. J., Tilly, B.C., Hunter, T., van Bergen en Henegouwen, P.M.P., Moolenaar, W.H. and de Laat, S.W. (1989). Signal Transduction by Epidermal Growth Factor Occurs Through the Subclass of High Affinity Receptors. J. Cell Biol. 109, 2495-2507.

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

Delarue, J.C., Friedman, S., Mouriesse, H., May-Levin, F., Sanch-Garnier, H. and Contesso, G. (1988). Epidermal growth factor receptor in human breast cancers: correlation with estrogen and progesterone receptors. Breast Cancer Res. Treat. 11, 173-178.

De la Torre, B., Noren, S., Hedman, M., Ritzen, M. & Diczfalusy, E. (1982). Intratesticular and plasma steroid profiles in fertile and infertile men. Int. J. Androl. 5, 367-378.

De Mellow, J.S., Handelsman, D.J. and Baxter, R.C. (1987). Short-term exposure to insulin-like growth factors stimulates testosterone production by testicular interstitial cells. Acta. Endocrinol. 115, 483-489.

Depue, R.H., Pike M.C. and Henderson, B. (1983). Estrogen exposure during gestation and risk of testicular cancer. J. Natl. Cancer Inst. 71, 1151-1155.

Depue, R.H. (1984). Maternal and gestational factors affecting the risk of cryptorchidism and inguinal hernia. Int. J. Epidemiol. 13, 311-318.

Derynck, R., Jarret, J.A., Chen, E.Y., Eaton, D.H., Bell, J.R., Assoian, R.K., Roberts, A.B., Sporn, M.B. and Goeddel, D.V. (1985). Human TGF-β complementary DNA sequence and expression in normal and transformed cells. Nature, 316, 701-705.

Derynck, R. (1986). Transforming Growth Factor-α : Structure and Biological Activities. J. Cell. Biochem. 32, 293-304.

Derynck, R., Goeddel, D.V., Ullrich, A., Gutterman, J.U., Williams, R.D., Bringman, T.S. and Berger, W.H. (1987). Synthesis of Messenger RNAs for Transforming Growth Factors α and β and the Epidermal Growth Factor Receptor by Human Tumours. Cancer Res. 47, 707-712.

Deslypere, J.P. and Vermeulen, A. (1984). Leydig Cell Function in Normal Men: Effect of Age, Life-Style, Residence, Diet, and Activity. J. Clin. Endocrinol. Metab. 59, 955-962.

Deuel, T.F. (1987). Polypeptide Growth Factors: Roles in Normal and Abnormal Cell Growth. Ann. Rev. Cell Biol. 3, 443-492.

Dickson, R.B., McManaway, M.E. and Lippman, M.E. (1986a). Estrogen induced factors of breast cancer cells partially replace estrogen to promote tumour growth. Science, 232, 1540-1543.

Dickson, R.B. Huff, K.K. Spencer, E.M. and Lippman, M.E. (1986b). Induction of Epidermal Growth Factor-Related Polypeptides by 17β-Estradiol in MCF-7 Human Breast Cancer Cells. Endocrinol. 118, 138-142.

Dickson, R.B., Bates, S.E., McManaway, M.E. and Lippman, M.E. (1986c). Characterisation of estrogen responsive transforming activity in human breast cancer cell lines. Cancer Res. 46, 1707-1713.

Dieckmann, K.P., Becker, T., Jonas, D. and Bauer, H.W. (1987). Inheritance and Testicular Cancer. Oncology, 44, 367-377.

Dorrington, J.H., Roller, N.F. and Fritz, I.B. (1975). Effects of follicle-stimulating hormone on cultures of Sertoli cell preparations. Mol. Cell. Endocrinol. 3, 57-70.

Downward, J., Parker, P. and Waterfield, M.D. (1984a). 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. and Waterfield, M.D. (1984b). Close similarity of epidermal growth factor and v-*erb*-B oncogene protein sequences. Nature, 307, 521-527.

Dulak, N.C. and 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.

Dunn, W.A. (1984). Receptor-mediated endocytosis of epidermal growth factor by hepatocytes in the prefused rat liver: ligand and receptor dynamics. J.Cell Biol. 98, 2148-2159.

Edery, M., Pang, K., Larson, L., Colosi, T. and Nandi, S. (1985). Epidermal Growth Factor Receptor Levels in Mouse Mammary Glands in Various Physiological states. Endocrinol. 117, 405-411.

Elson, S.D., Browne, C.A. and 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.

Engstrom, W., Rees, A.R. and Heath, J.K. (1985). Proliferation of a human embryonal carcinoma-derived cell line in serum-free medium: Inter-relationship between growth factor requirements and membrane receptor expression. J. Cell Sci. 73, 361-373.

Engstrom, 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.

Erhart, J.C., Creuzet, C., Rollet, E. and Loeb, J. (1981). Epidermal growth factor-stimulated phosphorylation of tyrosine residues on a 120,000 dalton protein in mouse liver plasma membrane subfractions. Biochem. Biophys. Res. Commun. 102, 602-609.

Evan, G. (1986). Growth factors, receptors and oncogenes. TIG, 2, 2-3.

Ewing, L. and Brown, B.L. (1977). Testicular steroidogenesis. In: The Testis. Eds. A.D. Johnson and W.R. Gomes. Academic Press, New York, U.S.A. Vol. 4, 239-287.

Ewing, T.M., Murphy, L.J., Ng, M.L., Pang, G.Y.N., Lee, C.S.L., Watts, C.K.W. and Sutherland, R.L. (1989). Regulation of epidermal growth factor receptor by progestins and glucocorticoids in human breast cancer cell lines. Br. J. Cancer, 44, 744-752.

Fablicant, R.N., DeLarco, J.E. and Todaro, G.J. (1977). Nerve growth factor on human melanoma cells in culture. Proc. Natl. Acad. Sci. USA. 74, 565-569.

Fabri, A., Knox, G., Buczko, E. and Dufau, M.L. (1988). β-Endorphin Production by the Fetal Leydig Cell: Regulation and Implications for Paracrine Control of Sertoli Cell Function. Endocrinol. 122, 749-755.

Fanger, B.O., Viceps-Madore, D. and Cidlowski, J.A. (1984). Regulation of High- and Low-Affinity Epidermal Growth Factor Receptors by Glucocorticoids. Arch. Biochem. Biophys. 235, 141-149.

Fauser, B.C.J.M. and Hsueh, A.J.W. (1988). Effect of transforming growth factor-β on human chorionic gonadotropin induced testosterone production by cultured rat testicular cells. Life Sciences. 43, 1363-1370.

Fawcett, D.W. (1975). Ultrastructure and function of the Sertoli cell. In: Handbook of Physiology. Eds. D.W. Hamilton and R.O. Greep. American Physiological Society, Washington, U.S.A. 5, 21-55.

Feig, L.A., Bellve, A.R., Erickson, N.H. and Klagsburn, M. (1980). Sertoli cells contain a mitogenic peptide. Proc. Natl. Acad. Sci. 77, 4774-4778.

Feig, L.A., Klagsburn, M. and Bellve, A.R. (1983). Mitogenic polypeptide of the mammalian seminiferous epithelium: Biochemical characterisation and partial purification. J. Cell Biol. 97, 1435-1443.

Feyrter, F. (1946). Ueber die These von den peripheren endokrinen Druesen. Wien Zeitschrift Inn Med. 27, 9-38.

Fitzpatrick, S.L., LaChance, M.P. and Schultz, G.S. (1984a). Characterisation of epidermal growth factor receptor and action on human breast cancer cells in culture. Cancer Res. 44, 3443-3447.

Fitzpatrick, S.L., Brightwell, J., Wittliff, J.L., Barrows, G.H. and Schultz, G.S. (1984b). Epidermal growth factor binding by breast tumour biopsies and relationship to estrogen receptor and progestin receptor levels. Cancer Res. 44, 3448-3452.

Fogh, J. and Trempe, G. (1975). New human tumour cell lines. In: Human Tumor cell in vitro. Ed. J. Fogh. Plenum Press. New York, pages 115-120.

Folkman, J. (1983). Angiogenesis: initiation and modulation. Symp. Fundam. Cancer Res. 36, 201-208.

Fox, C.F., Vale, R., Peterson, S.W. and Das, M. (1979). The EGF receptor identification and functional modulation. In: Hormones and Cell Culture. Eds. G.H. Sato and R. Ross. Cold Spring Harbor Conference on cell proliferation, Cold Spring Harbor Laboratory, New York, U.S.A. pages 143-165.

Fraker, P.J. & Speck, J.C., Jr. (1978). Protein and cell membrane iodinations with sparingly soluble chloramide,1,3,4,6-tetrachloro-3 ,6 -diphenylglycouril. Biochem. Biophys. Res. Commun. 80, 849-857.

Fraser, H.M. and Sandow, J. (1977). Gonadotrophin release by highly active analogue of luteinizing hormone releasing hormone in rats immunized against luteinizing releasing hormone. J. Endocrinol. 74, 291-296.

Fritz, I.B. and Tung, P.S. (1987). Morphogenetic restructuring and formation of basement membranes by Sertoli cells and testis peritubular cells in co-culture: inhibition of the morphogenetic cascade by cyclic AMP derivatives and by blocking direct cell contact. Dev. Biol. 120, 139-153.

Galdieri, M., Monaco, L. and Stefani, M. (1984). Secretion of ABP by Sertoli cells is influenced by contact with germ cells. J. Androl. 5, 409-415.

Gill, G.N., Santon, J.B. and Bertics, P.J. (1987). Regulatory features of the Epidermal Growth Factor Receptor. J. Cell. Physiol. (Suppl.) 5, 35-41.

Gonzales, G.F., Risbridger, G.P. and de Kretser, D.M. (1989). Epidermal growth factor increases inhibin synthesis by isolated segments of rat seminiferous tubules. J. Endocrinol. 123, 213-219.

Gospodarowicz, D. (1975). Purification of a fibroblast growth factor from bovine pituitary. J. Biol. Chem. 250, 2515-2520.

Gospodarowicz, D., Neufeld, G. and Schweigerer, L. (1986a). Molecular and biological characterisation of fibroblast growth factor, an angiogenic factor which also controls the proliferation and differentiation of mesoderm and neuroectoderm derived cells. Cell Differ, 19, 1-17.

Gospodarowicz, D., Neufeld, G. and Schweigerer, L. (1986b). Fibroblast growth factor. Mol. Cell. Endocrinol. 46, 187-204.

Goustin, A.S., Leof, E.B., Shipley, G.D. and Moses, H.L. (1986). Growth Factors and Cancer. Cancer Res. 46, 1015-1029.

Gray, A., Dull, T.J. and Ullrich, A. (1985). Nucleotide sequence of epidermal growth factor cDNA predicts a 128,000 molecular weight protein precursor. Nature, 313, 228-231.

Greene, L.A. and Tischler, A.S. (1976). Establishment of a noradrenergic clonal line of rat adrenal pheochromocytoma cells which respond to nerve growth factor. Proc. Natl. Acad. Sci. USA. 73, 2424-2428.

Gregoriou, M. and Rees, A.R. (1984). Properties of a monoclonal antibody to epidermal growth factor receptor with implications for the mechanisms of action of EGF. EMBO J. 3, 929-937.

Gregory, H. (1975). Isolation and structure of urogastrone and its relationship to epidermal growth factor. Nature, 257, 325-327.

Gregory, H., Willshire, R., Kavanagh, J.P., Blacklock, W.V., Chowdury, S. and 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.

Grinsted, J. and Byskov, A.G. (1981). Meiosis-inducing and meiosis-preventing substances in human male reproductive organs. Fertil. Steril. 35, 199-204.

Grootegoed, J.A., Peters, M.S., Muller, E., Rommerts, F.F.G. & Van Der Molen, H.J. (1977). Absence of a nuclear androgen receptor in isolated germinal cells of rat testis. Mol. Cell. Endocrinol. 9, 159-167.

Grootegoed, J.A. (1987). Towards a better understanding of Sertoli cell function. Int. J. Androl. 10, 727-729.

Grotjan, H.E. and Heindel, J.J. (1982). Effect of spent media from Sertoli cell cultures on *in vitro* testosterone production by the rat testicular interstitial cells. Ann. N.Y. Acad. Sci. 383, 456-457.

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

Habib, F.K., Lee, I.R., Stitch, S.R. and Smith, P.H. (1976). Androgen levels in the plasma and prostatic tissues of patients with benign hypertrophy and carcinoma of the prostate. J. Endocrinol. 71, 99-107.

Hagenas, L., Ritzen, E.M., Ploen, L., Hansson, V., French, F.S. and Nayfeh, S.N. (1975). Sertoli cell origin of testicular androgen-binding protein (ABP). Mol. Cell. Endocrinol. 2, 339-350.

Hahnel, R., Kelsall, G.R., Martin, J.P., Masters, A.M., McCartney, A.J. and Twaddle, E. (1982). Oestrogen receptors and progesterone receptors in tumours of the human ovary. Gynaecol. Oncol. 13, 145-151.

Haigler, H.T., Ash, J.F., Singer, S.J. and Cohen, S. (1978). Visualisation by fluorescence of the binding and internalisation of epidermal growth factor in human carcinoma cells A-431. Proc. Natl. Acad. Sci. USA. 75, 3317-3321.

Haigler, H.T., McKanna, J.S. and Cohen, S. (1979). Direct visualisation of the binding and internalisation of a ferritin conjugate of epidermal growth factor in human carcinoma cells A-431. J. Cell Biol. 81, 382-395.

Hall, P.F. (1970). Endocrinology of the testis. In: The Testis. Eds. A.D. Johnson, W.R. Gomes and N.L. VanDemark. New York Academic Press, U.S.A. Ch. 2, 2-72.

Hanauske, A.R., Osborne, C.K., Chamness, G.C., Clark, G. M., Forseth, B.J., Buchok, J.B., Arteaga, C.L. and Von Hoff, D.D. (1987). Alteration of EGF-Receptor Binding in Human Breast Cancer Cells by Anti-neoplastic Agents. Eur. J. Cancer Clin. Oncol. 23, 545-551.

Handelsman D.J., Spaliviero, J.A., Scott, C.D. and Baxter, R.C. (1985). Identification of insulin-like growth factor-I and its receptors in the rat testis. Acta Endocrinol. 109, 534-549.

Hansson, V., McLean, W.W., and Smith, A.A. (1974). Androgen receptors in rat testis. Steroids, 23, 823-832.

Hansson, J., Calandra, R., Purvis, K., Ritzen, M. and French, F.S. (1976). Hormonal regulation of spermatogenesis. In: Vitamins and Hormones: Advances in Research and Aplications. Eds. P.L. Munson, E. Diczfalusy, J. Glover and R.E. Olson. Academic Press, New York, U.S.A. 5, 249-255.

Hao Li, C. and Ramasharma, K. (1987). Inhibin. Ann. Rev. Pharmacol. Toxicol. 27, 1-21.

Harding, M., Cowan, S., Hole, D., Cassidy, L., Kitchener, H., Davis, J. and Leake, R. (1990). Estrogen and Progesterone Receptors in Ovarian Cancer. Cancer, 65, 486-491.

Harman, S.M. and Tsitouras, P.D. (1980). Reproductive hormones in aging men. I. Measurement of sex steroids, basal luteinizing hormone and Leydig cell response to human chorionic gonadotropin. J. Clin. Endocrinol. Metab. 51, 35-40.

Harris, A.L., Sainsbury, J.R.C., Smith, K., Neal, D.E., Hall, R.R.and Farndon, J.R. (1987). Epidermal growth factor receptors in primary human breast and bladder cancer: Relation to tumor differentiation, invasion and patient survival. In: Proceedings of Peptides, Growth Factors, and New (Anti)Steroidal Agents. Eds. J.G.M. Klijn, R. Paridaens and J.A. Foekens. EORTC Monograph Series. Raven Press. 18, 415-424.

Harris, A.L. (1989). Epidermal growth factor receptors in human breast cancer. Recent Results Cancer Rev. 113, 70-77.

Hayman, M.J., Ramsay, G.M., Savin, K., Kitchener, G., Graf, T. and Beug, H. (1983). Identification and characterisation of the avian erythroblastosis virus *erb*-B gene product as membrane glycoprotein. Cell, 32, 579-588.

Hayman, M.J. and Beug, H. (1984). Identification of a form of the avian erythroblastosis virus *erb*-B gene product at the cell surface. Nature, 309, 460-462.

Hayman, M.J., Kitchener, G., Knight, J., McMahon, J., Watson, R. and Beug, H. (1986). Analysis of autophosphorylation activity of transformation defective mutanats of avian erythroblastosis virus. Virol. 150, 270-275.

Headon, D.R. and Allan, G. (1989). Steroid hormones and growth factor receptors. Biochem. Soc. Transactions. 628th meeting, Galway, 598-599.

Heller, C.G. and Clermont, Y. (1964). Kinetics of the germinal epithelium in man. Rec. Prog. Horm. Res. 20, 545-575.

Henderson, B.E., Ross, R.K., Pike, M.C. and Depue, R.H. (1983). In: Epidemiology of testis cancer, Urologic Cancer. Eds. M.K. Skinner, D.G. Grune and Stratton, New York, U.S.A., pages 237-250.

Hillman, G.M. and Schlessinger, J. (1982). Lateral diffusion of epidermal growth factor complexed to its surface receptors does not account for the thermal sensitivity of patch formation and endocytosis. Biochem. 21, 1667-1672.

Hiramatsu, M., Kashimata, M., Minami, N., Sato, A., Murayama, M. and Minami, N. (1988). Androgenic regulation of epidermal growth factor in the mouse ventral prostate. Biochem. Int. 17,311-317.

Hirata, Y. and Orth, D.N. (1979). Epidermal Growth Factor (urogastrone) in human tissues. J. Clin. Endocrinol. Metab. 48, 667-672.

Hock, R.A. and Hollenberg, M.D. (1980). Characterisation of the Receptor for Epidermal Growth factor-Urogastrone in Human Placenta Membranes. J. Biol. Chem. 255, 10731-10736.

Hofmann, G.E., Rao, C.V., Barrows, G.H., Schultz, G.S. and Sanfilippo, J.S. (1984). Binding Sites for Epidermal Growth Factor Receptor in Human Uterine Tissues and Leiomyomas. J. Clin. Endocrinol. Metab. 58, 880-884.

Hogan, B., Fellous, M., Avner, P. and Jacob, F. (1977). Isolation of a human teratocarcinoma cell line which expresses the F9 antigen. Nature, 270, 515-517.

Hogan, B.L.M. (1981). From embryo to teratocarcinoma in tissue culture. Nature, 290, 737-738.

Holden, S., Bernard, O., Artzt, K., Whitmore, W. and Bennett, D. (1977). Human and mouse embryonal carcinoma cells in culture share and embryonic antigen (F9). Nature, 270, 518-520.

Hollenberg, M.D. and Cuatrecasas, P. (1973). Epidermal Growth Factor: Receptors in human fibroblasts and modulation of action by cholera toxin. Proc. Natl. Acad. Sci. 70, 2964-2968.

Holly, J.M.P. and Wass, J.A.H. (1989). Insulin-like growth factors; autocrine, paracrine or endocrine ? New perspectives of the somatomedin hypothesis in the light of recent developments. J. Endocrinol, 122, 611-618.

Holmes, S.D., Lipshultz, L.I. and Smith, R.G. (1984). Regulation of transferrin secretion by human Sertoli cells cultured in the presence or absence of human peritubular cells. J. Clin. Endocrinol. Metab. 59, 1058-1062.

Holmes, S.D., Spotts, G. and Smith, R.G. (1986). Rat Sertoli Cells Secrete a Growth Factor That Blocks Epidermal Growth Factor (EGF) Binding to Its Receptor. J. Biol. Chem. 261, 4076-4080.

Holmes, S.D., Spott, G. and Smith, R.G. (1986). Characterisation of a growth factor secreted by rat Sertoli cells in culture. In: Progressive and Developmental Biology. Ed. Alan R. Liss, Inc. Part A, pages 389-392.

Holstein, A.F., Schutte, B., Becker, H. and Hartmann, M. (1987). Morphology of normal and malignant germ cells. Int. J. Androl, 10, 1-18.

Honegger, A.M., Dull, T.J., Felder, S., Van Obberghen, E., Bellot, F., Szapary, D., Schmidt, A., Ullrich, A. and Schlessinger J. (1987a). Point mutation at the ATP bindig site of the EGF receptor abolishes protein tyrosine-kinase activity and alters normal receptor cellular routing. Cell, 51, 199-209.

Honegger, A.M., Szapary, D., Scmidt, A., Lyall, R., Van Obberghen, E., Dull, T.J., Ullrich, A. and Schlessinger, J. (1987b). A mutant epidermal; growth factor receptor with defective protein tyrosine-kinase is unable to stimulate proto-oncogene expression and DNA synthesis. Mol. Cell Biology, 7, 4568-4561.

Hoogerbrugge, J.W. and van der Molen, H.J. (1986). Stimulation of steroid production in isolated rat Leydig cells by unknown factors in interstitial fluid differs from the effects of luteinising hormone on luteinising hormone releasing hormone. J. Endocrinol. 109, 111-117.

Horne, G.M., Angus, B., Wright, C., Needham, G., Nicholson, S., Harris, A.L., Innes, B. and Horne, C.H.W. (1988). Relationships between oestrogen receptor, epidermal growth factor receptor, ER-D5 and P24 oestrogen regulated protein in human breast cancer. J. Pathol. 155, 143-150.

Hsu, A-F. and Troen, P. (1978). An androgen binding protein in the testicular cytosol of human testis. Comparison with human plasma testosterone-estrogen binding globulin. J. Clin. Invest. 61, 1611-1619.

Hsueh, A.J.W., Dahl, K.D., Vaughan, J., Tucker, E., Rivier, J., Bardin, C.W. and Vale, W. (1987). Hetrodimers and homodimers of inhibin subunits have different paracrine action in the modulation of luteinizing hormone-stimulated androgen biosynthesis. Proc. Natl. Acad. Sci. U.S.A. 84, 5082-5086.

Huang, H.F.S. and Nieschlag, E. (1986). Suppression of intratesticular testosterone is associated with quantitative changes in spermatogonial populations in intact adult rats. Endocrinol. 118, 619-627.

Hunter, T. and Cooper, J.A. (1981). Epidermal growth factor induces rapid tyrosine phosphorylation of proteins in A431 human tumor cells. Cell, 24, 741-752.

Hunter, T. and Cooper, J.A. (1985). Protein-tyrosine kinases. Ann. Rev. Biochem. 54, 897-930.

Hunter, T., Ling, N. and 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.

Hutson, J.C. and Stocco, D.M. (1981). Peritubular cell influence on the efficiency of androgen-binding protein secretion by Sertoli cells in culture. Endocrinol. 108, 1362-1368.

Ichii, S., Yoshida, A. & Hoshikawa, Y. (1988). Binding Sites for Epidermal Growth Factor in Nuclear Fraction from Rat Liver. Endocrinol. Japan, 35, 567-575.

Imai, Y., Leung, C.K.H., Frisen, H.G. and Shiu, R.P.C. (1982). Epidermal growth factor receptors and effect of epidermal growth factor on growth of human breast cancer cells in long term tissue culture. Cancer Res. 42, 4394-4398.

Imanishi, K., Yamaguchi, K., Suzuki, M., Yanaihara, N. and Abe, K. (1989). Production of transforming growth factor-α in human tumour cell lines. Br. J. Cancer, 59, 761-765.

Ishiwaka, F., Miyazono, K., Hellman, U., Drexler, H., Wernstedt, C., Hagiwara, K., Usuki, K., Takaku, F., Risau, W. and Heldin, C.H. (1989). Identification of angiogenic activity and the cloning and expression of platelet derived endothelial cell growth factor. Nature, 338, 557-562.

Janecki, A., Jalcubowick, A. and Lukaszyk, A. (1985). Stimulatory effect of Sertoli cell secretory products on testosterone secretion by purified Leydig cells in primary culture. Mol. Cell. Endocrinol. 42, 235-243.

Jegou, B., Laws, A.O. and de Kretser, D.M. (1984). Changes in testicular function induced by short-term exposure of the rat testis to heat: further evidence for interactions of germ cells, Sertoli cells and Leydig cells. Int. J. Androl. 7, 244-257.

Jewitt, M.A.S. (1978). Testis carcinoma: transplantation into nude mice. Nat. Cancer. Inst. Monogr. 49, 65-66.

Johnson, L. Zane, R.S., Petty, C.S. and Neaves, W.B. (1984). Quantification of the human Sertoli cell population: Its distribution, relation to germ cell numbers and age-related decline. Biol. Rperod. 31, 785-795.

Johnson, L., Petty, C.S. and Neaves, W.B. (1986). Age-related variation in seminiferous tubules in men: a stereologic evaluation. J. Andrology, 7, 316-322.

Jones, P.G. and Campbell, P.E. (1976). Tumours of the Testis and Breast. In: Tumors of infancy and childhood. Eds. P.G. Jones. Blackwell Scientific Publications, Oxford, U.K. 25, 869-897.

Jones, P.B.C., Welsh, T.H. and Hsueh, A.J.W. (1982). Regulation of ovarian progestin production by epidermal growth factor in cultured rat granulosa cells. J. Biol. Chem. 257, 11268-11273.

Jutte, N.H.P.M., Jansen, R. Grootegoed, J.A., Rommets, F.F.G., Clausen, O.P.F. and van der Molen, H.J. (1982). Regulation of survival of rat pachytene spermatocytes by lactate supply from Sertoli cells. J. Reprod. Fertil. 65, 431-438.

Kaplan, P.L., Anderson, M. and 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.

Karamehmedovic, O., Woodtli, W. and Pluss, H.J. (1975). Testicular tumors in childhood. J. Pediat. Surg. 10, 109-110.

Kasson, B.G. and Hsueh, A.J.W. (1987). Insulin-like growth factor-I augments gonadotropin-stimulated androgen biosynthesis by cultured rat testicular cells. Mol. Cell. Endocrinol. 52, 27-34.

Kasuga, M., Van Obberghen, E., Nissley, S.P. and Rechler, M.M. (1981). Demonstration of two subtypes of insulin-like growth factor receptors by affinity cross-linking. J. Biol. Chem. 256, 5305-5308.

Kawamoto, T., Sato, J.D., Lee, A., Polikoff, J., Sato, G. and Mendelson, J. (1983). Growth stimulation of A431 cells by epidermal growth factor: identification of high-affinity receptors for epidermal growth factor by an anti-receptor monoclonal antibody. Proc. Natl. Acad. Sci. U.S.A. 80, 1337-1341.

Keski-Oja, J., Leof, E.B., Lyons, R.M., Coffey, R.J. and Moses, H.L. (1987). Transforming Growth Factors and Control of Neoplastic Cell Growth. J. Cell. Biochem. 33, 95-107.

Khanum, A. and Dufau, M.L. (1988). Angiotensin II receptors and inhibitory actions on Leydig cells. J. Biol. Chem. 263, 5070-5074.

King, L.E., Carpenter, G. and Cohen, S. (1980). Characterisation of epidermal growth factor stimulated phosphorylation using A-431 membranes. Biochem. 19, 1524-1528.

King, A.C. and Cuatrecasas, P. (1982). Resolution of high and low affinity epidermal growth factor receptors. Inhibition of high affinity component by low temperature, cycloheximide and phorbol esters. J. Biol. Chem, 257, 3053-3060.

King, I. and Sartorelli, A.C. (1989). Epidermal Growth Factor Receptor Gene Expression, Protein Kinase Activity and Terminal Differentiation of Human Malignant Epidermal Cells. Cancer Res. 49, 5677-5681.

King, R.J.B. and Darbre, P.D. (1989). In: Endocrine Therapy of Breast Cancer. Ed. F. Cavalli. Berlin, Springer-Verlag. Ch. III, 3-15.

Kleinsmith, L.J. and Pierce, B. (1964). Multipotentiality of single embryonal carcinoma cells. Cancer Res. 24, 1544-1552.

Knabbe, C., Lippman, M.E., Wakefield, L.M., Flanders, K.C., Kasid, A., Derynck, R. and 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.

Knecht, M. and Catt, K.J. (1983). Modulation of cAMP-mediated differentiation in ovarian granulosa cells by epidermal growth factor and platelet-derived growth factor. J. Biol. Chem. 258, 2789-2794.

Kothari, L.K. and Gupta, A.S. (1974). Effect of ageing on the volume, structure and total Leydig cell content of the human testis. Int. J. Fertil. 19, 140-146.

Kris, R.M., Lax, I., Gullick, W., Waterfield, M.D., Ullrich, A., Fridkin, M. and 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.

Lai, W.H. and Guyda, H.J. (1984). Characterisation and regulation of epidermal growth factor receptors in human placental cell cultures, J. Clin. Endocrinol. Metab. 58, 344-352.

Lai, W.H., Guyda, H.J and Bergeron, J.J.M. (1986). Binding and internalisation of epidermal growth factor receptors in human placental cells in culture. Endocrinol. 118, 413-423.

Lamb, D.J., Spotts, G., Holmes, S.D. and Smith, R.G. (1987). Unique characteristics of rat Sertoli cell secreted growth factor. In: Cell Biology of the Testis and Epididymis. Eds. M-C. Orgebin-Christ and B.J. Danzo. New York Academy of Science. New York, U.S.A., pages 497-499.

Landreth, G.E. and Shooter, E.M. (1979). Nerve growth factor receptors on PC12 cells: ligand-induced conversion from low- to high-affinity states. Proc. Natl. Acad. Sci. USA. 77, 4751-4755.

Lax, I., Kris, R., Sasson, I., Ullrich, A., Hayman, M.J., Beug, H. and Schlessinger, J. (1985). Activation of c-*erb*-B in avian leukosis virus-induced erythroblastosis leads to the expression of a truncated EGF-receptor kinase. EMBO. J. 4, 3179-3182.

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

Leake, R.E., Laing, L. and Smith, D.C. (1979). A role for nuclear oestrogen receptors in prediction of therapy regime for breast cancer patients. In: Steroid Receptor Assays in Human Breast Tumours: Methodological and Clinical Aspects. Ed. R.J.B. King. Alpha Omega Press. Cardiff, U.K., pages 73-81.

Leake, R.E., Laing, L., Calman, K.C., MacBeth, F.R., Crawford, D.J. and Smith, D.C. (1981). Oestrogen receptor status and endocrine therapy of brest cancer. Response rates and status stability. Br. J. Cancer, 43, 59-64.

Le Blond, C.P. and Clermont, Y. (1952). Definition of the stages of the cycle of the seminiferous epithelium in the rat. Ann. N.Y. Acad. Sci. 55, 548-573.

Lee, L.S. and Weinstein, B. (1978). Epidermal growth factor, like phorbol esters, induces plasminogen activator in Hela cells. Nature, 274, 696-697..

Le Magueresse, B., Le Gac, F. Loir, M. and Jegou, B. (1986). Stimulations of rat Sertoli cell secretory activity *in vitro* by germ cells and residual bodies. J. Reprod. Fertil. 77, 489-498.

Levi-Montalcini, R. and Hamburger, V. (1951). Selective growth stimulating effects of mouse sarcoma on the sensory and sympathetic nervous system of the chick embryo. J. Exp. Zool. 116, 321-351.

Lin, T., Haskell, J., Vinson, N. and Terracio, L. (1986). Direct stimulatory effects of insulin-like growth factor-I on Leydig cell steroidogenesis in primary culture. Biochem. Biophys. Res. Commun. 137, 950-956.

Lin, T., Blaisdell, J. and Haskell, J.F. (1987). TGF-β inhibits Leydig cell steroidogenesis in primary culture. Biochem. Biophys. Res. Commun. 146, 387-394.

Linsley, P.S. and Fox, C.F. (1980a). Controlled proteolysis of EGF receptors: evidence for transmembrane distribution of the EGF binding and phosphate receptor site. J. Supramol. Struct. 14, 461-71.

Linsley, P.S. and Fox, C.F. (1980b). Direct linkage of EGF to its receptor: characterisation and biological release J. Supramol. Struct. 14, 441-459.

Linsley, P.S. and Fox, C.F. (1980c). Properties of receptors for epidermal growth factor in detergent solution: evidence for heterogenous aggregated states. J. Supramol. Struct. 14, 511-525.

Lippman, M.E., Dickson, R.B., Bates, S., Knabbe, C., Huff, K., Swain, S., McManaway, M., Brozert, D., Kasid, A. and 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., Bronzert, D., Huff, K. and Kasid, A. (1987). Growth Regulation of Human Breast Carcinoma Occurs Through Regulated Growth Factor Secretion. J. Cell. Biochem. 35, 1-16.

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

Livneh, E., Prywes, R., Kashles, O., Reiss N., Sasson, I., Mory, Y., Ullrich, A. Moolenar, W.H., Bierman, A.J., Tilly, B.C., Verlaan, I., Defize, L.H.K., Honegger, A.M., Ullrich, A. and Schlessinger, J. (1988). A point mutation at the ATP binding site of the EGF receptor abolishes signal transduction. EMBO J. 7, 707-710.

Lloyd, C.E. and Ascoli, M. (1983). On the mechanisms involved in the regulation of the cell-surface receptors for human choriogonadotropin and mouse epidermal growth factor in cultured Leydig tumour cells. J. Cell Biol. 96, 521-526.

Logan, A. (1990). Intracrine regulation at the nucleus - a further mechanism of growth factor activity ? J. Endocrinol. 125, 339-343.

MacDonald, A., Chisholm, G.D. and Habib, F.K. (1990). Production and response of a human prostatic cancer line to transforming growth factor-like molecules. Br. J. Cancer. 62, 579-584.

Macias, A., Azavedo, E., Hagerstrom, T., Klintenberg, C., Perez, R. and Skoog, L. (1987). Prognostic Significance of the Receptor for Epidermal Growth Factor in Human Mammary Carcinomas. Anticancer Res. 7, 459-464.

Maddocks, S. and Sharpe, R.M. (1989). Assessment of the contribution of Leydig cells to the secretion of inhibin by the rat testis. Mol. Cell. Endocrinol. 67, 113-118.

Maddy, S.Q., Chisholm, G.D., Hawkins, R.A. & Habib F.K. (1987). Localisation of Epidermal Growth Factor receptors in the human prostate by biochemical and immunocytochemical methods. J. Endocrinol. 113, 147-153.

Maddy, S.Q., Chisholm, G.D., Busuttil, A. and Habib, F.K. (1989). Epidermal growth factor receptors in human prostate cancer: correlation with histological differentiation of the tumour. Br. J. Cancer, 60, 41-44.

Mallea, L. E., Machado, A.J., Navaroli, F. and Rommerts, F.F.G. (1986). Epidermal growth factor stimulates lactate production and inhibits aromatization in cultured Sertoli cells from immature rats. Int. J. Androl. 9, 201-208.

March, C.J., Mosley, B., Larsen, A., Ceretti, P.D., Braedt, G., Price, V., Gillis, S., Henney, C.S., Kronheim, S.R., Grabstein, K., Conlon, P.J., Hopp, P.T. and Cosman, D. (1985). Cloning sequence and expression of the distinct human interleukin-1 complementary DNAs. Nature, 315, 641-647.

Margolis, B., Bellot, F., Honegger, A.M., Ullrich, A., Schlessinger, J. and Zilberstein, A. (1990). Tyrosine Kinase Activity Is Essential for the Association of Phospholipase C with the Epidermal Growth Factor Receptor. Mol. Cell. Biol. 10, 435-441.

Massague, J. (1983). Epidermal growth factor-like transforming growth factor II. Interaction with epidermal growth factor receptors in human placenta membrane and A431 cells. J. Biol. Chem. 258, 13614-13620.

Massague, J. and Czech, M.P. (1983). The subunit structures of two distinct receptors for insulin-like growth factor-I and -II, synthesis and regulation in rat fibroblasts. Nature, 82, 5028-5045.

Massague, J. and Like, B. (1985). Cellular receptors for type-β transforming growth factor: ligand binding and affinity labelling in human and rodent cell lines. J. Biol. Chem. 260, 2635-2645.

Massague, J. (1985a). Type-β transforming growth factor receptor in cells chronically exposed to ligand. In: Growth Factors and Transformation, Cancer Cells 3. Eds. J. Feramisco, B. Ozanne and C. Stiles. Cold Spring Harbor Press, New York, U.S.A., pages 73-78

Massague, J. (1985b). Subunit structure of a high-affinity receptor for type β-transforming growth factor: evidence for a disulphide-linked glycosylated receptor complex. J. Biol. Chem. 260, 7059-7066.

Massague, J. (1987). The TGF-β family of growth and differentiation factors.Cell, 49, 437-438.

Mc Ginley, D.M., Posalaly, M. and Russell, L. (1979). Gap junctions between Sertoli and germ cells of rat seminiferous tubule. Tissue and Cell, 4, 741-754.

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

McKeehan, W.L., Adams, P.S. and Fast, D. (1987). Different hormonal requirements for androgen-independent growth of normal and tumor epithelial cells from rat prostate. *In vitro* Cell. Develop. Biol. 23, 147-152.

Means, A.R., Fakunding, J., Huckins, C. (1976). Follicle stimulating hormone, the Sertoli cell, and spermatogenesis. Rec. Prog. Horm. Res. 32, 477-527.

Meidan, R., Lim, P., McAllister, J.M. and Hsueh, A.J.W. (1985). Hormonal regulation of androgen biosynthesis by primary cultures of testis cells from neonatal rats. Endocrinol. 116, 2473-2482.

Moolenaar, W.H., Bierman, A.J., Tilly, B.C., Verloan, I., Defize, L.H., Honegger, A.M., Ullrich, A. and Schlessinger, J. (1988). A point mutation at the ATP-binding site of the EGF-receptor abolishes signal transduction. EMBO. J. 7, 707-710.

Moore, C.R. (1924). Properties of the gonads as controllers of somatic and physiological characteristics. VIII Heat application and testicular degeneration; the function of the scrotum. 34, 337-345.

Mori, H. and Christensen, A.K. (1980). Morphometric analysis of Leydig cells in the normal rat testis. J. Cell Biol. 84, 340-354.

Mori, M., Naito, R., Tsukitani, K., Okada, Y. & Tsujimura, T. (1989). Widespread distribution of human epidermal growth factor (hEGF) in human tissues and organs under normal and tumorous conditions - Immunohistochemical studies with the use of polyclonal and monoclonal antibodies. Acta Histochem. Cytochem. 22, 15-34.

Morris, P.L. and Mather, J.P. (1984). Epidermal growth factor binding sites in testicular cells. Fed. Proc. 43, 522-527.

Morris, P.L., Vale, W.W., Cappel, S. and Bardin, C.W. (1988). Inhibin production by primary Sertoli cell-enriched cultures: Regulation by follicle stimulating hormone, androgens and epidermal growth factor. Endocrinol. 122, 717-725.

Moses, H.L., Tucker, R.F., Leof, E.B., Coffey, R.J.Jr, Halper, J. and Shipley, G.D. (1985). Type-β transforming growth factor is a growth stimulator and a growth inhibitor. In: Cancer Cells. Eds. Feramisco, J. Ozanne, B. Stiles, C. Cold Spring Harbor Press, New York, U.S.A. 3, 65-71.

Mostofi, F.K. (1974). International histological classification of tumors. A report by the Executive Committee of the International Council of Societies of Pathology. Cancer, 33, 1480-1483.

Mostofi, F.K. and Price, E.B. (1973). Tumors of the Male Genital System. AFIP Washington, U.S.A.

Mukku, V.R. (1984). Regulation of Epidermal Growth Factor receptor levels by thyroid hormone. J. Bio. Chem. 259, 6543-6547.

Mukku, V.R. and Stancel, G.M. (1985a). Regulation of Epidermal Growth Factor Receptor by Estrogen. J. Biol. Chem. 260, 9820-9824.

Mukku, V.R. and Stancel, G.M. (1985b). Receptors for Epidermal Growth Factor in the Rat Uterus. Endocrinol. 117, 149-154.

Mulder, E., Peters, M.J., Buerden, W.M.O. and van der Molen, H.J. (1974). A receptor for testosterone in mature rat testes. FEBS Lett. 47, 209-211.

Mulder, E., Peters, M.J., de Vries, J. and van der Molen, H.J. (1975). Characterisation of a nuclear receptor for testosterone in seminiferous tubules of mature rat testes. Mol. Cell. Endocrinol. 2, 171-182.

Muller, J. and Skakkebaek, N.E. (1988). Carcinoma-in-situ of the testis: Aspects of the prepubertal lesion. In: Carl Schirren Symposium: Advances in andrology. Ed. A.F. Holstein. Diesbach-Verlag, Berlin., pages 173-178

Munro, S. and Pelham, H.R.B. (1984). Use of peptide tagging to detect proteins expressed from cloned genes: deletion mapping functional domains of Drosophila hsp 70. EMBO. J. 3, 3087-3093.

Munson, P.J. and Rodbard, D. (1980). Ligand: A versatile computerised approach for characterisation of ligand binding systems. Analytical Biochemistry, 107, 220-239.

Murphy, L.J., Sutherland, R.L., Stead, B., Murphy, L.C. and Lazarus, L. (1986). Progestin Regulation of Epidermal Growth Factor Receptor in Human Mammary Carcinoma Cells. Cancer Res. 46, 728-734.

Naess, O., Attramadal, A. and Hansson, V. (1977). Receptors for testosterone and 5α-dihydrotestosterone in the anterior pituitary and various areas of the brain. In: The Testis in Normal and Infertile Men. Eds. P. Troen and H.R. Nankin. Raven Press, New York, U.S.A. 227-241.

Neal, D.E., Marsh, C., Benett, M.K., Abel, P.D., Hall, R.R., Sainsbury, J.R.C. and Harris, A.L. (1985). Epidermal growth factor receptors in human bladder cancer: comparison of invasive and superficial tumours. Lancet, i, 366-368.

Neal, D.E., Smith, K., Fennelly, J.A., Bennett, M.K., Hall, R.R. and Harris, A.L. (1989). Epidermal Growth Factor Receptor in Human Bladder Cancer: A Comparison of Immunohistochemistry and Ligand Binding. J. Urol. 141, 517-521

Neaves, W.B., Johnson, L., Porter, J.C., Parker, C.R. and Petty, C.S. (1984). Leydig Cell Numbers, Daily Sperm Production, and Serum Gonadotropin Levels in Aging Men. J. Clin. Endocrinol. Metab. 59, 756-763.

Nicholson, S., Sainsbury, J.R.C., Halcrow, P., Chambers, P., Farndon, J.R. and Harris, A.L. (1989). Expression of epidermal growth factor receptors associated with lack of response to endocrine therapy in recurrent breast cancer. Lancet i, 182-185.

Nilsen, T., Maroney, P., Goodwin, R., Rottman, R., Crittenden, L., Raines, M. and Kung, H.J. (1985). C-*erb*-B activation in AVL-induced erythroblastosis: novel RNA processing and promoter insertion result in expression of an amino-truncated EGF receptor. Cell, 41, 719-726.

Nissley, S.P., Short, P.A., Rechler, M.M., Podskalny, J.M. and Coon, H.G. (1977). Proliferation of Buffalo rat liver cells in serum-free medium does not depend upon multiplication-stimulating activity (MSA). Cell, 11, 441-446.

O'Keefe, E., Hollenberg, M.D. and Cuotrecasas, P. (1974). Epidermal Growth Factor characteristics of specific binding in membranes from liver, placenta and other tissues. Arch. Biochem. Biophys. 164, 518-526.

Osborne, K., Hamilton, B., Titus, G. and Livingston, R.B. (1980). Epidermal Growth Factor Stimulation of Human Breast Cancer Cells in Culture. Cancer Res. 40, 2361-2366.

Osborne, C.K., Hamilton, B. and Nover, M. (1982). Receptor binding and processing of epidermal growth factor by human breast cancer cells. J. Clin. Endocrinol. Metab. 55, 86-93.

Ozawa, S., Ueda, M., Ando, N., Shimizu, N. and Abe, O. (1989). Prognostic Significance of Epidermal Growth Factor Receptor in Esophageal Squamous Cell Carcinomas. Cancer, 63, 2169-2173.

Pandiella, A., Beguinot, L., Vicentini, L.M. and Meldolesi, J. (1989). Transmembrane signalling at the epidermal growth factor receptor. TIPS, 10, 411-414.

Papadopoulos, V., Carreau, S. and Drosdowsky, M.A. (1985). Effects of phorbol ester and phospholipase C on LH stimulated steroidogenesis in purified rat Leydig cells. FEBS Lett. 188, 312-316.

Papadopoulos, V., Carreau, S. and Drosdowsky, M.A.(1986). Effects of seminiferous tubule secreted factors on Leydig cell cyclic AMP production in mature rats. FEBS Lettt. 202, 74-78.

Papadopoulos, V., Kamtchouing, P., Drosdowsky, M.A., Hochereau de Reviers, M.T. and Carreau, S. (1987). Adult rat Sertoli cells secrete a factor or factors which modulate Leydig cell function. J. Endocrinol. 114, 459-467.

Parvinen, M. (1982). Regulation of the seminiferous epithelium. Endocr. Rev. 3, 404-417.

Parvinen, M., Vihko, K.K. and Toppari, J. (1986). Cell interactions during the seminiferous epithelial cycle. Int. Rev. Cytol. 104, 115-151.

Pascall, J.C., Saunders, D.M., Blakeley, D.M., Laurie, M.S. & Brown, K.D. (1989). Tissue-specific effects of castration and ovariectomy on murine epidermal growth factor and its mRNA. J. Endocrinol. 121, 501-506.

Pekonen, F., Partanen, S., Makinen, T. and Rutanen, E.M. (1988). Receptors for Epidermal Growth Factor and Insulin-like Growth Factor-I and their relation to Steroid Receptors in Human Breast Cancer. Cancer Res. 48, 1343-1347.

Pera, M.F., Blasco Lafita, M.J. and Mills, J. (1987). Cultured stem-cells from human testicular teratomas: the nature of human embryonal carcinoma, and its comparison with two types of yolk-sac carcinoma. Int. J. Cancer, 40, 334-343.

Pereira, M.E., Segaloff, D.L. and Ascoli, M. (1988). Inhibition of gonadotropin responsive adenylate cyclase in MA-10 Leydig tumour cells by epidermal growth factor. J. Biol. Chem. 263, 9761-9766.

Perez, R., Pascual, M., Macias, A. and Lage, A. (1984). Epidermal growth factor receptors in human breast cancer. Breast Cancer Res. Treat. 4, 189-193.

Perrard-Sapori, M.H., Chatelain, P.C., Rogemond, N. and Saez, J.M. (1987). Modulation of Leydig cell functions by culture with Sertoli cells or with Sertoli cell-conditioned medium: effect of insulin, somatomedin-C and FSH. Mol. Cell. Endocrinol. 50, 193-201.

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

Persson, H., Ayer-Le Lievre, C., Soder, O., Villar, M.J., Metsis, M., Olson, L., Ritzen, M. and Hokfelt, T. (1990). Expression of β-Nerve Growth Factor Receptor mRNA in Sertoli Cells Downregulated by Testosterone. Science, 247, 704-707.

Pierce, J.G. and Parsons, T.F. (1981). Glycoprotein hormones: structure and function. Ann. Rev. Biochem. 50, 465-495.

Pike, L.J., Marquardt, H., Todaro, G.J., Gallis, B., Casnellic, J.E., Bornstein, P.E. and Krebs, G. (1983). Transforming growth factors and epidermal growth factor stimulate phosphorylation of a synthetic tyrosine-containing peptide in a similar manner. J. Biol. Chem. 257, 14628-14631.

Pike, L.J. and Krebs, E.G. (1986). Protein tyrosine kinase activity of hormone and growth factor receptors. In: Receptors. Ed. P.M. Conn. New York, Academic, U.S.A. 3, 93-134.

Pilch, P.F. and Czech, M.P. (1979). Interaction of cross-linking agents with the insulin effector system of isolated fat cells. J. Biol. Chem. 254, 3375-3381.

Pilch,, P.F. and Czech, M.P. (1980). The subunit structures of the high affinity insulin receptor. Evidence of disulfide-linked receptor complex in fat cell and liver plasma membranes. J. Biol. Chem. 255, 1722-1731.

Pryor, J.P., Cameron, K.M., Chilton, C.P., Ford, T.F., Parkinson, M.C., Sinokrat, J. and Westwood, C.A. (1983). Carcinoma-in-situ in testicular biopsies from men presenting with infertility. Br. J. Urol. 55, 780-784.

Pugh, R.C.B. (1976). Pathology of the Testis. Blackwell's Scientific, Oxford, U.K.

Puma, P., Buxser, S.E., Watson, L., Kelleher, D.J. and Johnson, G.L. (1983). Purification of the receptor for NGF from A875 melanoma cells by affinity chromatography. J. Biol. Chem. 258, 3370-3375.

Purvis, K., Calandra, R., Sander, S. and Hansson, V. (1978). Androgen Binding Proteins and Androgen Levels in the Human Testis and Epididymis. Int. J. Androl. 1, 531-548.

Purvis, K., Cusan, L. and Hansson, V. (1981). Regulation of steroidogenesis and steroid action in Leydig cells. J. Steroid. Biochem. 15, 77-86.

Radford, H.M., Avenell, J.A. and Panaretto, B.A.. (1987). Some effects of epidermal growth factor on reproductive function in Merino sheep. J. Reprod. Fertil. 80, 113-118.

Ramani, N., Chegini, N., Rao, C.V., Woost, P.G. and Schultz, G.S. (1986). The presence of epidermal growth factor binding sites in the intracellular organelles of term human placenta. J. Cell Sci. 84, 19-40.

Reubi, J.C. and Torhorst, J. (1989). The relationship between somatostatin, epidermal growth factor and steroid hormone receptors in breast cancer. Cancer, 64, 1254-1260.

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

Richards, R.C., Lewis-Jones, D.I., Walker, J.M. and Desmond, A.D. (1988). Epidermal growth factor (urogastrone) in human seminal plasma from fertile and infertile males. Fertil. Steril. 50, 640-643.

Rinderknecht, E. and Humbel, R.E (1978). The amino acid sequence of human insulin-like growth factor-I and its structural homology with pro-insulin. J. Biol. Chem. 253, 2769-2776.

Ritzen, E.M., Boitani, C., Parvinen, M., French, F.S. and Feldman M. (1982). Stage-dependent secretion of ABP by rat seminiferous tubules. Mol. Cell. Endocrinol. 25, 25-53.

Ritzen, E.M. (1983). Chemical messengers between Sertoli cells and neighbouring cells. J. Steroid Biochem. 19, 499-504.

Ritzen, E.M., Hansson, V. and French, F.S. (1989). The Sertoli Cell. In: The Testis. Second Edition. Eds. H. Burger and D, de Kretser. Raven Press, Ltd. New York, U.S.A., pages 269-302.

Roberts, A.B., Newton, D.L., Lamb, L.C., Sporn, M.B., De Larco, J.E. and Todaro, G.J. (1980). Transforming growth factors: Isolation of polypeptides from virally and chemically transformed cells by acid/ethanol extraction. Proc. Natl. Acad. Sci. U.S.A. 77, 3494-3498.

Roberts, A.B., Anzano, M.A., Lamb, L.C., Smith, J.M. and Sporn, M.B. (1981a). Isolation from murine sarcoma cells of normal transforming growth factors potentiated by EGF. Proc. Natl. Acad. Sci.USA, 78, 5339-5343.

Roberts, A.B., Anzano, M.A., Lamb, L.C., Smith, J.M. and Sporn, M.B. (1981b). New class of transforming growth factors potentiated by epidermal growth factor: isolation from non-neoplastic tissues. Proc. Natl. Acad. Sci. U.S.A. 78, 5339-5343.

Roberts, A.B., Anzano, M.A., Wakefield, L.M., Roche, N.S., Stern, D.F. and Sporn, M.B. (1985). Type-β transforming growth factor: a bifunctional regulator of cellular growth. Proc. Natl. Acad. Sci. U.S.A. 82, 119-123.

Rodbard, D. (1974a). Statistical quality control and data processing for radioimmunoassays. In: Radioassay: clinical concepts. Ed. O.B. Hunter. Stockie I, Searle, pages, 201-207.

Rodbard, D. (1974b). Statistical analysis if radioimmunoassays and immunoradiometric (labelled antibody assays). A generalised weighted, iterative least squares method for logistic curve fitting. In: Radioassay: clinical concepts. Ed. O.B. Hunter. Stockie I, Searle, pages, 209-233.

Rodeck, U., Herlyn, M., Herlyn, D., Molthoff, C., Atkinson, B., Varello, M., Steplewski, Z. and Koprowski, H. (1987). Tumour Growth Modulation by a Monoclonal Antibody to the Epidermal Growth Factor Receptor: Immunologically Mediated and Effector Cell-Independent Effects. Cancer Res. 47, 3692-3696.

Rodriguez-Rigau, L.J., Zukerman, Z., Weiss, D.B., Smith, K.D. and Steinberger, E. (1980). Hormonal Control of Spermatogenesis in Man: Comparison with the Rat. In: Testicular Development, Structure and Function. Eds. A. Steinberger and E. Steinberger, Raven Press, New York, U.S.A.

Rommerts, F.F.G., de Jong, F.H., Brinkman, A.O. and van der Molen, H.J. (1982). Development and cellular localisation of rat testicular aromatase activity. J. Reprod. Fertil. 65, 281-288.

Rommerts, F.F.G. and Themmen, A.P. (1986). LHRH the role of LHRH (agonists) in the regulation of gonadal function. Acta. Endocrinol. (Suppl.) 276, 76-84.

Rommerts, F.F.G. and van der Molen, H.J. (1989). Testicular Steroidogenesis. In: The Testis. Second Edition. Eds. H. Burger and D, de Kretser, Raven Press, Ltd. New York. 303-328.

Root, A., Steinberger, E. and Smith, K.(1972). Isosexual pseudo-precocity in a 6-year old boy with testicular tissue cell adenoma. J. Pediatr. 80, 264-268.

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

Rosen, D.M., Stempien, S.A., Thompson, A.Y. and Seyedin, S.M. (1988). Transforming growth factor-beta modulates the expression of osteoblast and chodroblast phenotypes *in vitro*. J. Cell. Physiol. 134, 337-346.

Ross, M.H. (1976). The Sertoli cell junctional specialisation during spermiogenesis and at spermiation. Anat. Rec. 186, 79-103.

Ross, R. and Vogel, A. (1978). The platelet-derived growth factor. Cell, 14, 203-210.

Rowley, M.J. and Heller, C.G. (1971). Quantitation of the cells of the seminiferous epithelium of the testis. Employing the Sertoli cell as a constant. Z. Zellforsch, 115, 461-472.

Russell, L.D. and Clermont, Y. (1977). Degeneration of germ cells in normal, hypophysectomised and hormone treated hypophysectomised rats. Anatomical Record, 187, 347-366.

Russell, L.D. (1978). The blood-testis barrier and its formation relative to spermatocyte maturation in the adult rat. A lanthanum tracer study. Anat. Rec. 190, 99-112.

Russell, L.D. (1980). Sertoli-germ cell inter-relations. A review. Gamete Res. 3, 178-202.

Russell, L.D. and Peterson, R.N. (1985). Sertoli cell junctions morphological and functional correlates. Int. Rev. Cytology, 99, 177-221.

Sabio, H., Burget, E.O., Farrow, G.M. and Kelalis, P.P. (1974). Embryonal carcinoma of the testis in childhood. Cancer, 43, 2118-2121.

Saez, J.M., Tabone, E., Perrard-Sapori, M.H. and Rivarola, M.D. (1985). Paracrine regulation of testicular function. Med. Biol. 63, 249-329.

Saez, J.M., Perrard-Sapori, M.H., Chatelain, P.G., Tabone, E. and Rivarola, M.A. (1987). Paracrine regulation of testicular function. J. Steroid. Biochem. 27, 317-329.

Sainsbury J.R.C., Farndon, J.R., Harris, A.L. and Sherbet, G.V. (1985a). Epidermal growth factors are present on human breast cancers Br. J. Surg. 72, 186-188.

Sainsbury, J.R.C., Farndon, J.R., Sherbert, G.V. and Harris, A.L. (1985b). Epidermal growth factor receptors and oestrogen receptors in human breast cancer. Lancet, i, 364-366.

Sainsbury, J.R.C., Sherbert, G.V., Farndon, J.R. & Harris, A.L. (1985c). Epidermal Growth Factor receptors and oestrogen receptors in human breast cancer. Lancet i, 660-672.

Sainsbury, J.R.C., Malcolm, A., Appleton, D., Farndon, J.R. and Harris, A.L. (1985d). Presence of epidermal growth factor receptors as an indicator of poor prognosis in patients with breast cancer. J. Clin. Pathol. 38, 1225.

Sainsbury, J.R.C., Needham, G.K., Malcolm, A., Farndon, J.R. and Harris, A.L. (1987). Epidermal growth factor as predictor of early recurrence of and death from breast cancer. Lancet, i, 1398.

Salmon, W.E. and Daughaday, W.H. (1957). A hormonally controlled serum factor which stimulated sulfate incorporation by cartilage *in vitro*. J. Lab.Clin. Med. 49, 825-829.

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

Salomon, D.S., Perroteau, I., Kidwell, W.R., Tam, J. and 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. Physiol. 130, 397-409.

Sanborn, B.M., Steinberger, A., Meistrich, M. and Steinebrger, E. (1975). Androgen binding sites in testis cell fractions as measured by nuclear exchange assay. J. Ster. Biochem. 6, 1459-1456.

Savage, C.R. Jr., Inagami, T. and Cohen, S. (1972). The primary structure of Epidermal Growth Factor. J. Biol. Chem. 247, 7612-7621.

Savage, C.R. Jr., Hash, J.H. and Cohen, S. (1973). Epidermal Growth Factor: Location of disulfide bonds. J. Biol. Chem. 248, 1669-7672.

Scatchard, G. (1949). The attractions of proteins for small molecules and ions. Ann. N. Y. Acad. Sci. 51, 660-672.

Schechter, Y., Hernaez, L. and Cuatrecasas, P. (1978). Epidermal growth factor: biological activity requires persistent occupation of high affinity cell surface receptors. Proc. Natl. Acad. Sci. U.S.A. 75, 5788-5791.

Schechter, A.L., Hung, M.C., Vaidyanathan, L., Weinberg, R.A., Yang-Feng, T.L., Francke, U., Ullrich, A. and Coussens, L. (1985). The *neu* gene: An *erb* B-homologous gene distinct from and unlinked to the gene encoding the EGF receptor. Science, 229, 976-978.

Schlessinger, J., Schechter, Y., Willingham, M.C. and Pastan, I. (1978). Direct visualisation of binding, aggregation and internalisation of insulin and epidermal growth factor on living fibroblastic cells. Proc. Natl. Acad. Sci. U.S.A. 75, 2659-2663.

Schlessinger, J., Schreiber, A.B., Levi, A., Lax, I., Libermann, T. and Yarden, Y. (1983). Regulation of cell proliferation by epidermal growth factor. CRC Crit. Rev. Biochem. 14, 93-111.

Schlessinger, J. (1988). The Epidermal Growth Factor Receptor as a Multifunctional Allosteric Protein. Biochemistry, 27, 3119-3123.

Schlessinger, J., Schreiber, A.B., Levi, A., Lax, I., Liberman, T. and Yarden, Y. (1988). Regulation of cell proliferation by epidermal growth factor. CRC Critical Rev. Biochem. 14, 93-111.

Schottenfeld, D. and Warshauer, M.E. (1982). Testis. In: Cancer Epidemiology and Prevention. Eds. J.S. Schottenfeld and W.B. Fraumi. Sanders, Philadelphia, U.S.A., pages 947-957

Schreiber, A.B., Lax, I., Yarden, Y., Eshhar, Z. and Schlessinger, J. (1981). Monoclonal antibodies against receptor for epidermal growth factor induce early and delayed effects of epidermal growth factor. Proc. Natl. Acad. Sci. USA, 78, 7535-7539.

Schreiber, A.B., Libermann, T.A., Lax, I., Yarden, Y. and Schlessinger, J. Biological role of epidermal growth factor-receptor clustering. Investigations with monoclonal anti-receptor antibodies. J. Biol. Chem. 258, 846-853.

Schulze, W. and Rehder, U. (1984). Organisation and morphogenesis of the human seminiferous epithelium. Cell Tissue Res. 237, 395-407.

Schuurmans, A.L.G., Bolt, J. and Mulder, E. (1988a). Androgens Stimulate Both Growth Rate and Epidermal Growth Factor Receptor Activity of the Human Prostate Tumor Cell LNCaP. The Prostate, 12, 55-63.

Schuurmans, A.L.G., Bolt, J. and 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-Pescador, R., Fong, N., Selby, M., Rutter, W.J. and Bell, G.I. (1983). Structure of a mouse submaxillary messenger RNA encoding epidermal growth factor and and seven related proteins. Science, 221, 236-240.

Setchell, B.P. (1969). Do the Sertoli cells secrete the rete testis fluid? J. reprod. Fertil. 19, 391-392.

Setchell, B.P. (1978). The Mammalian Testis. Ed. B.P. Setchell. Elek, London, U.K.

Setchell, B.P. (1980). The Functional Significance of the Blood-Testis Barrier. J. Androl. 1, 3-10.

Setchell, B.P. and Sharpe, R.M. (1981). Effect of injected human chorionic gonadotrophin on capillary permeability, extracellular fluid volume and the flow of lymph and blood in the testes of rats. J. Endocrinol. 91, 245-254.

Seyedin, S.M., Thompson, A.Y., Bentz, H., Rosen, D.M., McPherson, J.M., Conti, A., Siegel, N.R., Stalluppi, G.R. and Piez, K.A. (1986). Cartilage-inducing factor-A: Apparent identity to transforming growth factor-β. J. Biol. Chem. 261, 5693-5695.

Sharpe, R.M., Fraser, H.M., Cooper, I. and Rommerts, F.F.G. (1981). Sertoli cell Leydig cell communication via an LHRH-like factor. Nature, 290, 785-787.

Sharpe, R.M. (1982). The hormonal regulation of the Leydig cell. In: Oxford Reviews in Reproductive Biology. Ed. C.A.Finn. Oxford University Press, England. 4, 241-317

Sharpe, R.M. (1983). Local control of testicular function. Q.J. Exp. Physiol. 68, 265-287.

Sharpe, R.M. (1984a). Intragonadal hormones. Bibliography of Reproduction, 44, C1-C16.

Sharpe, R.M. (1984b). Intratesticular factors controlling testicular function. Biol. Reprod. 30, 29-49

Sharpe, R.M. and Cooper, I. (1984). Intra-testicular secretion of a factor(s) with major stimulatory effects on Leydig cell testosterone secretion *in vitro*. Mol. Cell. Endocrinol. 37, 159-168.

Sharpe, R.M. (1986). Paracrine control of the testis. Clin. Endocrinol. Metab. 15, 185-207.

Sharpe, R.M., Kerr, J.B., Fraser, H.M. and Bartlett, J.M.S. (1986). Intratesticular factors and testosterone secretion: effect of treatments that alter the level of testosterone within the testis. J. Androl. 7, 180-189.

Sharpe, R.M. and Cooper, I. (1987). Comparison of the effects of purified Leydig cells of four hormones (oxytocin, vasopressin, opiates and LHRH) with suggested paracrine roles in the testis. J. Endocrinol. 113, 89-96.

Sharpe, R.M. (1988). Endocrinology and Paracrinology of the Testis. In: Physiology and Toxicology of Male Reproduction. Eds. J.C. Lamb IV and P.M.D. Foster. Academic Press, London, U.K., pages 71-99.

Sharpe, R.M. Donachie, K. and Cooper, I. (1988a). Re-evaluation of the intratesticular level of testosterone required for quantitative maintenance of spermatogenesis in the rat. J. Endocrinol. 117, 19-26.

Sharpe, R.M., Kerr, J.B. and Maddocks, S. (1988b). Evidence for a role of the Leydig cells in the control of the intratesticular secretion of inhibin. Mol. Cell. Endocrinol. 60, 243-247.

Sharpe, R.M., Bartlett, J.M.S. and Kerr, J.B. (1988c). Interactions Between the Seminiferous Tubules and Leydig Cells in the Testis. Serono Symposium Publications, 49, 119-128.

Shikata, H., Utsumi, N., Hiramatsu, M., Minami, N., Nemoto, N. and Shikata, T. (1984). Immunohistochemical localisation of nerve growth factor and epidermal growth factor in guinea pig prostate gland. Histochemistry, 80, 411-413.

Sikora, K., Evan, G. and Watson, J. (1987). Oncogenes and germ cell tumours. Int. J Androl. 10, 57-67.

Skakkebaek, N.E. (1972). Possible carcinoma-in-situ of the testis. Lancet, ii, 516-517.

Skakkebaek, N.E. (1975). Atypical germ cells in the adjacent "normal" tissue of testicular tumours. Acta. Pathol. Microbiol. Scand. 83, 127-130.

Skakkebaek, N.E., Berthelsen, J.G., Giwercman, A. and Muller, J. (1987) Carcinoma-in-situ of the testis: Possible origin from the gonocytes and precursor of all types germ cell tumours except spermatocytoma. Int. J. Androl. 10, 19-28.

Skinner, M.K. and Griswold, M.D. (1982). Secretion of testicular transferrin by cultured Sertoli cells is regulated by hormones and retinoids. Biol. Reprod. 28, 211-221.

Skinner, M.K. and Fritz, I.B. (1985). Androgen stimulation of Sertoli cell function is enhanced by peritubular cells. Mol. Cell. Endocrinol. 40, 115-122.

Skinner, M.K. and Fritz, I.B. (1986). Identification of a non-mitogenic paracrine factor involved in mesenchymal-epithelial cell interactions between testicular peritubular cells and Sertoli cells. Mol.Cell. Endocrinol. 44, 85-97.

Skinner, M.K., Fetterolf, P.M. and Amnthony, C.T. (1988). Purification of a Paracrine Factor, P-Mod-S Produced by Testicular Peritubular Cells That Modulates Sertoli Cell Function. J. Biol. Chem. 263, 2884-2890.

Skinner, M.K.(1989). Peritubular myoid cell-Sertoli cell interactions which regulate testis function and growth. In: Perspectives in andrology. Ed. M. Serio. Serona Symposia, Raven Press. 53, 175-182.

Skinner, M.K. and Moses, H.L. (1989). Transforming Growth Factor-β Gene expression and Action in the Seminiferous Tubule: Peritubular Cell-Sertoli Cell Interactions. Mol. Endocrinol. 3, 625-634.

Skinner, M.K., Takacs, K. and Coffey, R. (1989a). TGF-α gene expression and action in the seminiferous tubule: peritubular cell-Sertoli cell interactions. Endocrinol. 124, 845-854.

Skinner, M.K., Schlitz, S.M. and Anthony, C.T. (1989b). Regulation of Sertoli cell differentiated function: testicular transferrin and ABP expression. Endocrinol. 124, 3015-3024.

Skoog, L., Macias, A., Azavedo, E., Lombardero, J. and Klintenberg, C. (1986). Receptors for EGF and oestradiol and thymidine kinase activity in different histological subgroups of human mammary carcinomas. Br. J. Cancer, 54, 271-276.

Slamon, D.J. (1987). Proto-oncogenes and human cancers. New Eng. J. Med. 317, 955-957.

Slamon, D.J., Clark, G.M., Wong, S.G., Levin, W.J., Ullrich, A. and McGuire, W.L. (1987). Human breast cancer: Correlation of relapse and survival with amplification of the *Her*-2/*neu* oncogene. Science, 235, 177-182.

Sluyser, M. (1990). Steroid/Thyroid Receptor-like Proteins with Oncogenic Potential: A Review. Cancer Res. 50, 451-458.

Soder, O., Pollanen, P., Syed, V., Granholm, M., Arver, S., Euler, M., Gustafsson, B., Froysa, B., Parvinen, M. & Ritzen, E.M. (1989). Mitogenic factors in testis. In: Perspectives in Andrology. Ed M. Serio. Serona Symposia, Raven Press. 53, 215-225.

Sporn, M.B. and Todaro, G.J. (1980). Autocrine secretion and malignant transformation of cells. New Eng. J. Med. 303, 878-880.

Sporn, M.B., Roberts, A.B., Wakefield, L.M. and Assoian, R.K. (1986) Transforming growth factor-a: biological function and chemical structure. Science, 233, 532-534.

Sporn, M.B. and Roberts, A.B. (1988). Peptide growth factors are multifunctional. Nature, 332, 217-219.

Starkey, R., Cohen, S. and Orth, D.N. (1975). Epidermal Growth Factor : identification of a new hormone in human urine. Science, 189, 800-803.

Statsbury, M. and Cohen, S. (1972). The stimulation of ornithine decarboxylase activity in testis of the neonatal mouse. Biochem. Biophys. Acta, 261, 177-182.

Stastny, M. and Cohen, S. (1970). Epidermal growth factor IV. The induction of ornithine decarboxylase. Biochem. Biophys. Acta. 204, 578-589.

Steinberger, E. (1971). Hormonal Control of Mammalian Spermatogenesis. Physiol. Rev. 51, 1-22.

Steinberger, A. and Steinberger, E. (1972). Testis: basic and clinical aspects. In: Reproductive Biology. Eds. H. Balin and S.R. Glaser. Amsterdam. Excerpta Medica Foundation. 4-267.

Steinberger, A. and Steinberger, E. (1976a). The Sertoli cell. In: The Testis. Eds. A. Johnson and W. Gomes. Academic Press, New York, USA. Part IV, 371-394.

Steinberger, A. and Steinberger, E. (1976b). Secretion of an FSH-inhibiting factor by cultured Sertoli cells. Endocrinol. 99, 918-921.

Stockli, K.A., Lottspeich, F., Sendtner, M., Masiakowski, P., Carroll, P., Gotz, R., Lindholm, D. and Thoenen, H. (1989). Molecular cloning, expression and regional distribution of rat ciliary neurotrophic factor. Nature, 342, 920-923.

Stoscheck, C.M. and Carpenter, G. (1984a). Characterisation of the metabolic turnover of epidermal growth factor receptor protein in A-431 cells. J. Cell. Physiol. 120, 296-302.

Stoscheck, C.M. and Carpenter, G. (1984b). Down regulation of epidermal growth factor receptors: Direct demonstration of receptor degradation in human fibroblasts. J. Cell Biol. 98, 1048-1053.

Stoscheck, C.M. and King, L.E. Jr. (1986). Role of Epidermal Growth Factor in Carcinogenesis. Cancer Res. 46, 1030-1037.

Strader, C.F., Weiss, N.S., Daling, J.R., Karagas, M.R. and McKnight, B. (1988). Cryptorchism, orchiopexy and the risk of testicular cancer. Am. J. Epidemiology, 127, 1013-1018.

Stroobant , P., Rice, A.P., Gullick, W.J., Cheng, D.J., Kerr, I.M and Waterfield, M.D. (1985). Purification and characterisation of vaccinia virus growth factor. Cell, 42, 383-393.

Suarez-Quian, C.A., Dai, M., Onoda, M., Kriss, R.M. and Dym, M. (1989). Epidermal Growth Factor Localisation in the Rat and Monkey Testes. Biology of Reproduction. 41, 921-932.

Sugiyama, K., Yonemura, Y. and Miyazaki, I. (1989). Immunohistochemical Study of Epidermal Growth Factor and Epidermal Growth Factor Receptor in Gastric Carcinoma. Cancer, 63, 1557-1561.

Sunada, H., Yu, P., Peacock, J.S. and Mendelsohn, J. (1990). Modulation of Tyrosine, Serine and Threonine Phosphorylation and Intracellular Processing of the Epidermal Growth Factor Receptor by Anti-receptor Monoclonal Antibody. J. Cell. Physiol. 142, 284-292.

Svoboda, M.E., Van Wyk, J.J., Klapper, D.J., Fellows, R.E., Grissom, F.E. and Schlueter, R.J. (1980). Purification of somatomedin-C from human plasma: chemical and biological properties, partial sequence analysis and relationship to other somatomedins. Biochem. 19, 790-797.

Tahka, K.M. (1986). Current aspects of Leydig cell function and its regulation. J. Reprod. Fert. 78, 367-380.

Taketani, Y. and Oka, T. (1982). Biological action of epidermal growth factor and its functional receptors in normal mammary epithelial cells. Proc. Natl. Acad. Sci. USA, 80, 2647-2650.

Tamm, J., Volkwein, U., Kurniawan, E. and Becker, H. (1987). Concentrations of unconjugated 5α-Androstane-3α,17β-diol and 5α-Androstane-3β,17β-diol and their precursors in human testicular tissue. Comparison with testosterone, 5α-dihydrotestosterone, estradiol-17β, and with steroid concentrations in human epididymis. J. Steroid Biochem. 26, 345-348.

Tauber, J.P. and Tauber, M.T. (1987). Growth factors. Nucl. Med. Biol. 14, 407-419.

Taylor, J.M., Mitchell, W.M. and Cohen, S. (1972). Epidermal Growth Factor: physical and chemical properties. J. Biol. Chem. 247, 5928-5934.

Teerds, K.J., Rommerts, F.F. and Dorrington, J.H. (1990). Immunohistochemical detection of transforming growth factor-alpha in Leydig cells during the development of the rat testis. Mol. Cell. Endocrinol. 69, R1-6.

Temin, H., Pierson, R.W. and Dulak, N.C. (1972). The role of serum in the control of multiplication of avian and mammalian cells in culture. In: Growth, Nutrition and Metabolism of cells in culture. Eds. G.H. Rothblatt and V.J. Cristofalo. New York, Academic Press, U.S.A., pages 50-81.

Teslenko, L.V., Kornilova, E.S., Sorkin, A.D. and Nikolsky, N.N. (1987). Recycling of epidermal growth factor in A431 cells. FEBS. Lett. 221, 105-109.

Thompson, S., Stern, P.L., Webb, M., Walsh, F., Engstrom, W.S., Evans, E.P., Shi, W.K., Hopkins, B.R. and Graham, C.F. (1984). Cloned human teratoma cells differentiate into neuron-like cells and other cell types in retinoic acid. J. Cell Sci. 72, 37-64.

Tindall, D.J., Miller, D.A. and Means, A.R. (1977). Characterisation of androgen receptor in Sertoli cell-enriched testis. Endocrinol. 101, 13-23.

Todaro, G.J., Fryling, C. and DeLarco, J.E. (1980). Transforming growth factors produced by certain human tumour cells: polypeptides that interact with epidermal growth factor receptors. Proc. Natl. Acad. Sci. USA, 77, 5258-5262.

Tonelli, Q.J. and Sorof, S. (1980). Epidermal growth factor requirement for development of cultured mammary gland. Nature, 285, 250-252.

Traish, A.M. and Wotiz, H.H. (1987). Prostatic Epidermal Growth Factor Receptors and Their Regulation by Androgens. Endocrinol. 121, 1461-1467.

Travers, M.T., Barrett-Lee, P.J., Berger, U., Luqmani, Y.A., Gazet, J.C., Powles, T.J. and Coombes, R.C. (1988). Growth factor expression in normal, benign, and malignant breast tissue. Br. Med. J. 296, 1621-1623.

Tres, L.L., Smith, E.P., Van Wyk, J.J. and Kierszenbaum, A.L. (1986). Immunoreactive sites and accumulation of somatomedin-C in rat Sertoli-spermatogenic cell co-cultures. Exp. Cell Res. 162, 33-50.

Tsitouras, P.D. (1987). Effects of Age on Testicular Function. Endocrinol. Metab. Clinincs, 16, 1045-1059.

Tsutsumi, O., Kurachi, H. & Oka, T. (1986). A physiological role of Epidermal Growth Factor in male reproductive function. Science, 233, 975-977.

Tung, P.S. and Fritz, I.B. (1980). Interactions of Sertoli cells with myoid cells *in vitro*. Biol. Reprod. 23, 207-217.

Tuomela, T., Viinikka, L. and Perheentupa, J. (1989). Mouse epidermal growth factor concentrations are altered by gonadectomy and treatments with estradiol and progesterone. Life Sci. 44, 1815-1821.

Twardzik, D.R. (1985). Differential expression of transforming growth factor-α during prenatal development of the mouse. Cancer Res. 45, 5413-5416.

Ullrich, A., Coussens, L., Hayflick, J.S., Dull, T.J., Gray, A., Tam, A.W., Lee, J., Yarden, Y., Libermann, T.A., Schlessinger, J., Downward, J. Mayes, E.L.V., Whittle, N., Waterfield, M.D. and Seeburg, P.H. (1984). Human epidermal growth factor receptor cDNA sequence and aberrant expression of the amplified gene in A431 epidermoid carcinoma cells. Nature, 309, 418-425.

Ullrich, A., Bell, J.R., Chen, E.Y., Herrera, R., Petruzzelli, L.M., Dull, T.J., Gray, A., Coussens, L., Liao, Y.C., Tsubokawa, M., Mason, A., Seeburg, P.H., Grunfeld, C., Rosen, O.M. and Ramachandran, J. (1985). Human insulin receptor and its relationship to the tyrosine kinase family of oncogenes. Nature, 313, 756-761.

Van Obberghen, E., Rossi, B., Kowalski, A., Gazzano, H. and Ponzio, G. (1983). Receptor-mediated phosphorylation of the hepatic insulin receptor: evidence that the Mr 95,000 receptor unit is its own kinase. Proc. Natl. Acad. Sci. USA. 80, 945-949.

Van Wyk, J.J., Underwood, L.E., Baseman, J.B., Hintz, R.L., Clemons, D.R. and Marshall, R.N. (1975). Explorations of the insulin-like and growth promoting properties of somatomedin by membrane assays. Adv. Metab. Disord. 8, 127-150.

Van Wyk, J.J., Underwood, L.E., D'Ercole, A.J. Clemmons, D.R., Pledger, W.J., Wharton, W.R. and Leof, E.B. (1981). Role of somatomedin in cellular proliferation. In: The Biology of Normal Human Growth. Ed. E.R. Ritzen. New York, Raven Press, U.S.A., pages 223-233.

Varenhorst, E. (1988). Prostate cancer treatment: tolerance of different endocrine regimens. In: New Developments in Biosciences. Walter de Grutyer and Co. Berlin. New York, U.S.A. 4, 105-137.

Verhoeven, G. (1980). Androgen receptors in cultured interstitial cells derived from immature rats. J. Ster. Biochem. 13, 469-474.

Verhoeven, G. and Cailleau, J. (1985). A factor in spent media from Sertoli cell-enriched cultures that stimulates steroidogenesis in Leydig cells. Mol. Cell. Endocrinol. 40, 57-68.

Verhoeven, G. and Cailleau, J. (1986). Stimulatory effect of Epidermal Growth Factor on steroidogenesis in Leydig cells. Mol. Cell. Endocrinol. 47, 99-106.

Verhoeven, G. and Cailleau, J. (1988a). Testicular peritubular cells secrete a protein under androgen control that inhibits induction of aromatase activity in Sertoli cells. Endocrinol. 123, 2100-2110.

Verhoeven, G. and Cailleau, J. (1988b). The androgen responsiveness of Sertoli cells depends on a complex interplay between androgens, FSH and peritubular cell factors. Miniposter Book. 5th European Workshop on Molecular and Cellular Endocrinology of the Testis. Abs: G.1.

Vihko, K.K. and Huhtaniemi, I. (1989). A rat seminiferous epithelial factor that inhibits Leydig cell cAMP and testosterone production: mechanism of action, stage-specific secretion, and partial characterisation. Mol. Cell. Endocrinol. 65, 119-127.

von der Masse, H., Giwercman, A. and Skakkebaek, N.E. (1986). Radiation treatment of carcinoma in situ of the testis. Lancet, i, 624-625.

von der Masse, H., Giwercman, A., Muller, J.and Skakkebaek, N.E. (1987). Management of carcinoma in situ of the testis. Int. J. Androl. 10, 209-220.

Waites, G.M.H. and Gladwell, R.T. (1982). Physiological significance of fluid secretion in the testis and blood-testis barrier. Physiol. Rev. 62, 624-671.

Walker, D.H. and Pike, L.J. (1987). Phosphatidylinositol kinase is activated in membranes derived from cells treated with epidermal growth factor. Proc. Natl. Acad. Sci. U.S.A. 84, 7513-7517.

Waterfield, M.D., Mayes, E.L.V., Stroobant, P., Bennett, P.L.P., Young, S., Goodfellow, P.N., Banting, G.S. and Ozanne, B. (1982). A monoclonal antibody to the human epidermal growth factor receptor. Journal of Cellular Biochemistry, 20, 149-161.

Waterfield, M.D. (1989). Epidermal growth factor and related molecules. Lancet, i, 1243-1246.

Wathes, D.C. (1984). Possible actions of gonadal oxytocin and vasopressin. J. Reprod. Fertil. 71, 315-345.

Waxman, M. (1976). Malignant germ cell tumour *in situ* in a cryptorchid testis. Cancer, 38, 1452-1456.

Weber, W., Gill, G.N. and Spiess, J. (1984). Production of an epidermal growth factor-related protein. Science, 224, 294-297.

Wegienka, L.C. and Kolb, F.O. (1967). Hormonal studies of a benign cell tumor of the testis producing androstenedione and testosterone. Acta Endocrinol. 56, 481-489.

Weisman, A.S., Raguet, S.S. and Kelly, P.A. (1987). Characterisation of Epidermal Growth Factor Receptor in Human Meningnoma. Cancer Research, 47, 2172-2176.

Welsh, T.H. and Hsueh, A.J.W. (1982). Mechanism of the Inhibitory Action of Epidermal Growth Factor on Testicular Androgen Biosynthesis *in vitro*. Endocrinol. 110, 1498-1506.

Westley, B. and Rochefort, H. (1980). A Secreted Glycoprotein Induced by Estrogen in Human Breast Cancer Cell Lines. Cell, 20, 353-362.

Whitley, B. and Glaser, L. (1986). Epidermal Growth Factor (EGF) Promotes Phosphorylation at Threonine-654 of the EGF Receptor: Possible Role of Protein Kinase C in Homologous Regulation of the EGF Receptor. J. Cell Biol. 103,1355-1362.

Wilding, G., Zugmeier, G., Knabbe, C., Valverius, E., Flanders, K. and 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, 241-246.

Wilding, G., Valverius, E., Knabbe, C. and 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. and Fendly, B. (1989). Epidermal Growth Factor and Transforming Growth Factor-α Bind Differently to the Epidermal Growth Factor Receptor. Biochemistry, 28, 6373-6378.

Wright, W.W. and Frankel, A.I. (1980). An androgen receptor in the nuclei of late spermatids in testes of male rats. Endocrinol. 107, 314-318.

Wynn, P.C., Maddocks, I.G., Moore, G.P.M., Panaretto, B.A., Djura, P., Ward, W.G., Fleck, E. and Chapman, R.E. (1989). Characterisation and localisation of receptors for epidermal growth factor in ovine skin. J. Endocrinol. 121, 81-90.

Yamamoto, T., Nishida, T., Miyajima, N., Kawai, S., Oooi, T. and Toyoshima, K. (1983). The *erb*-B gene to avian erythroblastosis virus is a member of the *src* gene family. Cell, 35, 71-78.

Yarden, Y. and Schlessinger, J. (1987a). Self-phosphorylation of epidermal growth factor receptors: evidence for a model of intermolecular allosteric activation. Biochem. 26, 1434-1442.

Yarden, Y. and Schlessinger, J. (1987b). Epidermal growth factor induces rapid reversible aggregation of the purified epidermal growth factor receptor. Biochem. 26, 1443-1451.

Yasui, W., Hata, J., Yokozaki, H., Nakatini, H., Ochiai, A., Ito, H. and Tahara, E. (1988a). Interaction between epidermal growth factor and its receptor in the progression of human gastric carcinoma. Int. J. Cancer, 41, 211-217.

Yasui, W., Sumiyoshi, H., Hata, J., Kameda, T., Ochiai, A., Ito, H. and Tahara, E. (1988b). Expression of Epidermal Growth Factor Receptor in Human Gastric and Colonic Carcinomas. Cancer Res. 48, 137-141.

Ying, S.Y. (1989). Inhibins, Activins and Follistatins. J. Ster. Biochem. 33, 705-713.

Zachary, I. and Rizengurt, E. (1983). High affinity receptors for peptides of the bombesin family in Swiss 3T3 cells. Proc. Natl. Acad. Sci. U.S.A. 82, 7616-7620.

Zeillinger, R., Kury, F., Czerwenka, K., Kubista, E., Sliutz, G., Knogler, W., Huber, J., Zielinski, C., Reiner, G., Jakesz, R., Staffen, A., Reiner, A., Weba, F. and Spona, J. (1989). *Her*-2 amplification, steroid receptors and epidermal growth factor receptors in primary breast cancer. Oncogene, 4, 109-114.

Zetterberg, A., Engstrom, W. and Dafgard, E. (1984). The relative effects of different types of growth factors on DNA replication, mitosis and cellular enlargement. Cytometry, 5, 368-375.

Zeuthen, J., Norgaard, J.O.R., Avner, P., Fellous, M., Wartiovaara, J., Vaheri, A., Rosen, A. and Giovanella, B.C. (1980). Characterisation of a human ovarian teratocarcinoma derived cell line. Int. J. Cancer, 25, 19-25.

Zidovetzki, R., Yarden, Y., Schlessinger, J. and Jovin, T.M. (1981). Rotational diffusion of epidermal growth factor complexed to cell surface receptors reflects rapid microaggregation and endocytosis of occupied receptors. Proc. Natl. Acad. Sci. U.S.A. 78, 6981-6985.

Zidovetzki, R., Yarden, Y., Schlessinger, J. and Jovin, T.M. (1986). Microaggregation of hormone-occupied epidermal growth factor receptors on plasma membrane preperations. EMBO J. 5, 247-250.

Localization and characterization of epidermal growth factor receptors on human testicular tissue by biochemical and immunohistochemical techniques

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ABSTRACT

In the present study attempts were made to characterize the epidermal growth factor (EGF) receptor on human testicular tissue. A radioligand exchange assay with ^{125}I-labelled EGF was used to detect a high affinity, low capacity, single binding site in the 105 000 *g* particulate fraction of human testicular tissue. Binding was optimal at 32 °C following a 40-min incubation with a mean (± S.D.) dissociation constant of 327 ± 59 pmol/l (d.f. 9). The number of binding sites ranged from 0·07 to 0·21 pmol/mg protein. Competition studies with other peptide hormones including LH, FSH, prolactin, insulin-like growth factor-I, fibroblast growth factor and nerve growth factor have confirmed the specificity of EGF for its receptor. The receptor was also found to be heat-labile and sensitive to trypsinization. Cross-linking experiments using disuccinimidyl suberate revealed major binding species at the 125 kDa region and this is thought to represent a proteolysed form of the receptor. Immunohistochemical localization of the receptors demonstrated their presence in the interstitial tissue and not within the seminiferous tubules. The presence of specific EGF binding in the interstitial tissue suggests that EGF may play some role in testicular steroidogenesis.

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INTRODUCTION

The epidermal growth factor (EGF) receptor is a transmembrane glycoprotein with intrinsic protein-tyrosine kinase phosphotransferase activity (Cohen & Carpenter, 1975). So far the receptor has been located on numerous tissues, including the uterus (Hofmann, Rao, Barrows *et al.* 1984), placenta (Hock & Hollenberg, 1980) and prostate (Maddy, Chisholm, Hawkins & Habib, 1987). On binding to the receptors the peptide EGF has been found to initiate a variety of responses according to the cell on which the receptor is expressed (Carpenter & Cohen, 1979; McKeehan, Adams & Rosser, 1984). EGF has also been found in various body fluids, including prostate fluid (Gregory, Willshire, Kavanagh *et al.* 1986) and human seminal plasma (Elson, Browne & Thorburn, 1984) but no one has so far investigated the presence of EGF receptors in human testicular tissue.

In an earlier study, Bellve & Zheng (1985) reported the presence of growth factors in the gonads during the onset and maintenance of spermatogenesis in pubertal and adult animals. Although EGF was not singled out it is possible that the peptide might be one of the growth factors present. However, later reports by Holmes, Spott & Smith (1986) and Buch, Lamb, Lipschultz & Smith (1988) on the characterization of a unique growth factor secreted by human Sertoli cells suggest that EGF and this secreted growth factor might not be the same. Nonetheless, evidence is accumulating on a role for EGF in the testis. Tsutsumi, Kurachi & Oka (1986) have observed a 50% reduction in spermatid production in the absence of EGF. Furthermore, EGF has been found to stimulate steroidogenesis in primary cultured Leydig cells of the rat testes (Verhoeven & Cailleau, 1986).

In an attempt to define the mode of action of EGF in this target tissue, we have undertaken the present study to evaluate the presence of EGF receptors in the human testis.

MATERIALS AND METHODS

Hormones and growth factors

Mouse EGF, transforming growth factor-α (TGF-α), bovine fibroblast growth factor (FbGF) and venom

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nerve growth factor (vNGF) were obtained from Sigma, Poole, Dorset, U.K. Insulin-like growth factor-I (IGF-I) was obtained from Sera Laboratories, Crawley Down, Sussex, U.K. Human prolactin, human follicle-stimulating hormone (hFSH), human luteinizing hormone (hLH), human growth hormone (hGH) and human insulin were generously donated by NIADDK, Bethesda, MD, U.S.A.

Other reagents

EGF was iodinated using the method of Fraker & Speck (1978). Briefly, EGF (10 μg) reacted with $Na^{125}I$ (specific activity 350/600 mCi/ml; Amersham International plc, Bucks) in the presence of 1,3,4,6-tetrachloro-3α-6α-diphenyl-glycouril (Iodogen; Sigma). The iodinated peptide was purified by chromatography on a Sephadex G-50 column (1 × 46 cm). The percentage bound was calculated after precipitation with trichloroacetic acid at 20% and 10% concentrations. The final specific activity varied between 20 and 70 μCi/μg.

Monoclonal antibodies

The anti-human EGF receptor monoclonal antibody for the external domain of the receptor for use on frozen sections was kindly donated by Dr M. D. Waterfield, Imperial Cancer Research Fund Laboratory, London (Waterfield, Mayes, Stroobant *et al.* 1982). The anti-mouse EGF receptor antibody for the external domain of the receptor for use on paraffin sections was obtained from Oncor, Gaithersburg, MD, U.S.A. (Starkey, Cohen & Orth, 1975). The third monoclonal antibody recognizing the internal domain of the receptor was a gift from Dr W. J. Gullick of the ICRF Oncology Group, Hammersmith Hospital, London (Gullick, Marsden, Whittle *et al.* 1986).

Other reagents

Normal rabbit serum (NRS) was obtained from the Scottish Antibody Production Unit, Carluke, Lanarkshire, U.K., streptavidin alkaline phosphatase from ICN Biomedicals, High Wycombe, Bucks, U.K. and biotinylated secondary antibody sheep anti-mouse immunoglobulins from Amersham International plc. Disuccinimidyl suberate (DSS) along with a molecular weight standard kit for proteins in the molecular weight range of 50–250 kDa and bovine serum albumin (BSA; fraction V) were purchased from Sigma.

Buffers

The following buffers were prepared: buffer A containing Tris (10 mmol/l), EDTA (1 mmol/l), EGTA (1 mmol/l), sucrose (0·25 mmol/l) and phenylmethylsulphonyl fluoride (0·05 mmol/l), pH 7·4; buffer B containing Tris (10 mmol/l), sodium chloride (0·9%, w/v) and BSA (0·1%, w/v), pH 7·4; 10% and 20% polyethylene glycol 8000 mol. wt (PEG) made up with buffer B; 1 mmol/l DSS dissolved in dimethylsulphoxide (DMSO) to give a final concentration of 5% of DMSO on the gel for cross-linking. All other cross-linking reagents are identical to those described by Laemmli (1970).

In addition the following buffers were also prepared for immunohistochemistry: Tris-buffered saline buffer (TBS) containing Tris (10 mmol/l), sodium chloride (0·9%, w/v) pH 7·6; Michaelis veronal buffer (pH 9·2) containing sodium acetate trihydrate (0·14 mol/l), sodium barbitone (0·14 mmol/l), formaldehyde (0·04%, w/v); steptavidin alkaline phosphatase substrate/chromogen in Michaelis veronal buffer containing Fast Red ITR (1·3 mmol/l), levamisole (1·0 mmol/l) and Napthol 6-bromo-2 hydroxy-3 napthoic acid-2 methoxyanilidine phosphate (0·75 mmol/l) in 100% dimethylformamide.

Tissue preparation

Human testicular tissue was obtained from patients with prostatic cancer who had undergone orchiectomy as first line of treatment. The tissue was used either fresh or snap-frozen in liquid nitrogen and stored at −70 °C until required. Specimens were examined by a pathologist and only those with a normal and well-defined morphology were included in the present study. The following steps were carried out at 4 °C: 1 g tissue was cut into small pieces and homogenized with 3 ml buffer A for two periods of 15 and 20 s with an Ystral homogenizer (Scottish Scientific Instruments Centre Ltd, Edinburgh, U.K.). The homogenate was then ultracentrifuged for 40 and then 20 min at 105 000 ***g***. At the in-between stage the pellet was resuspended in 2 ml buffer A and dispersed using a glass Dounce homogenizer. Following the last centrifugation the pellet was finally resuspended in 3 ml buffer B and was again redispersed in the glass homogenizer using a tighter fitting pestle. The protein concentration was then measured by the Bradford (1976) method and adjusted to 2 mg/ml. Unless specified otherwise this particulate membrane fraction was used in all subsequent incubations.

Subcellular fractionation of the tissue was performed according to the method of Leake, Chisholm & Habib (1983).

Radioligand exchange assay

For each study the assay when used was performed on at least three different tissues, each in triplicate. Particulate fraction (100 μl) containing 2 mg protein/ml concentration was incubated with 100 μl ^{125}I-labelled EGF (8·0 nmol/l; 200 000 c.p.m.) in the presence and

absence of a 50-fold excess concentration of the unlabelled EGF (200 µl); all dilutions of materials were made in buffer B. The reaction vessels coated previously with 0·1% BSA solution were incubated at 32 °C for 40 min and the reaction was stopped by the addition of 1 ml ice-cold 20% PEG. The mixture was then vortexed and centrifuged at 4 °C at 3500 r.p.m. for 20 min. The tubes were then aspirated and the pellets resuspended in 1 ml 10% PEG. They were then vortexed and centrifuged again as before. Finally the supernatant was aspirated once again and the tubes counted in a Packard Crystal 2 Multidetector Radioimmunoassay System. The specific binding was calculated by subtracting the non-specific binding from the total binding. This standard procedure was then used in all subsequent binding studies.

For time and temperature studies, binding experiments were performed at 4, 25, 32 and 37 °C. All studies were undertaken for varying times up to 18 h. The concentration of the labelled EGF used was as above.

The effect of pH on specific binding was also examined. A pH range between 6·6 and 8·2 was chosen. Binding studies were performed as previously described, having adjusted the solutions to the required pH. In addition the relationship between increase in protein concentration and the number of specific binding sites present was investigated. Protein concentrations varied between 0·5 and 8·0 mg/ml.

Saturation analysis

This was performed over a range of 0·5–8·0 nmol ^{125}I-labelled EGF/l. Non-specific binding at each concentration of radiolabel was determined in the presence of the appropriate unlabelled competitor at a 50-fold excess concentration. Binding was carried out at 32 °C for 40 min and the specific binding data was analysed by a computer program (Munson & Rodbard, 1980) to yield the dissociation constant (K_d) and the number of specific binding sites.

Competition studies

The specificity of the receptor for EGF was examined in the presence of 50- and 100-fold excess of the following competitors: TGF-α; IGF-I, FbGF, vNGF, insulin, hLH, hFSH and prolactin. The radioligand exchange assay was used but with the substitution of the various above-named peptides for unlabelled EGF.

Effect of heat and trypsinization

Homogenate preparations were heated before use in the assay at 45, 60 and 80 °C or preincubated with 0·5% (w/v) trypsin for 15 min at 32 °C. After one wash with buffer B (1 ml), the pellets were spun at 3500 r.p.m. and the supernatant was aspirated. The assay was then performed as previously described.

Cross-linking and autoradiography

Following the preparation of the membrane-bound ^{125}I-labelled EGF the supernatant was aspirated off and 100 µl of the 1 mmol DSS/l solution was added to the pellet. The mixture was vortexed and left for 20 min at room temperature. The sample buffer (25 µl) containing mercaptoethanol (Laemmli, 1970) was added and the pellet solution allowed to boil for 5 min following which the solution was once again spun down at 25 °C for 5 min. The resultant supernatant was then taken up with a Hamilton syringe and loaded onto the gel which was set up using a Bio-Rad Protean 2 Slab Cell along with standards (SDS-6 mix; Amersham International plc). Each standard had been heated at 32 °C for 3 h. The gel was then allowed to run at 30 mA overnight. Gels were subsequently stained with Brilliant Blue mixture for 20 min and washed with a mixture of 25% methanol/10% acetic acid solution over a period of 15 h. The gels ultimately rinsed in distilled water and dried on a Bio-Rad model 443 slab dryer connected to an IEC lyoprep-3000 freeze drier. Finally autoradiography was performed at −70 °C for 3-4 weeks by placing the dried gels in a cassette along with two intensifying screens and a Hyperfilm B_{max} (Amersham International plc).

Immunohistochemistry

Immunohistochemistry was performed on both frozen and paraffin sections, employing monoclonal antibodies to both the external (EGF-R1, frozen; EGF-R, paraffin) and internal domains (F4, frozen/paraffin) of the EGF receptor. The methods employed were as follows.

Frozen sections

The frozen sections were first fixed in acetone for 20 min. They were then treated with streptavidin-biotin blocking reagents (Vector, Peterborough, Cambs, U.K.) and then covered with NRS (1:5 dilution) to block non-specific binding. After 20 min the NRS was removed and the EGF-R1 antibody in a 1:30 dilution or the F4 antibody in a 1:5 dilution were applied and left overnight at 4 °C. The following day the sections were washed for 5 min in TBS and then treated with biotinylated sheep anti-mouse immunoglobulins (1:200) for 30 min. The sections were then washed once more and an alkaline phosphatase-labelled streptavidin as supplied by ICN was applied for 30 min. Finally freshly prepared streptavidin–alkaline phosphatase substrate was applied for 20 min. The

sections were then counterstained in haematoxylin and lithium carbonate, and mounted in glycerine jelly.

Paraffin-fixed sections

Paraffin sections of the same tissue as used for the frozen sections were dewaxed and trypsinized in 1·8 × 10·6 ATEE units trypsin and CaCl (9 mmol/l) solution (pH 7·6) for 15 min at 37 °C. (1 ATEE unit = ΔA_{237} of 0·001/min in 3 ml at pH 7·0 at 25 °C.) The staining procedure as described above was subsequently followed except that the monoclonal antibody for the external domain of the receptor EGF-R was used at a 1:10 dilution of the stock solution.

Haematoxylin and eosin sections were also prepared for each tissue sample to enable the pathologist to identify the areas of specific staining. Furthermore, for each staining experiment a 'negative' control section was included in which the primary antibody was omitted. Occasionally a 'positive' control employing human prostate tissue was also examined.

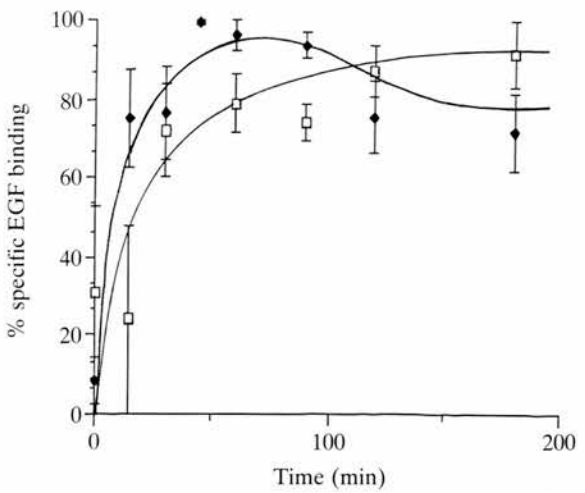
RESULTS

Binding studies

Text-figure 1 illustrates the specific binding patterns of ^{125}I-labelled EGF to the 105 000 ***g*** particulate fraction at 4 and 32 °C. Binding was clearly time- and temperature-dependent with the maximal binding attained at 32 °C after 40 min. Although maximum binding was also achieved at 4 °C this was over a longer period of time. Binding was also examined at 25 and 37 °C (results not shown) but, in common with the experiments at 4 °C, the reaction at those temperatures was slower to yield the maximum binding. The optimum pH experiments which demonstrated that the highest levels of specific uptake was achieved at pH 7·4 are also not shown. There was, however, not a great deal of variation over the physiological pH range chosen.

In addition, subcellular fractions were prepared from specimens obtained from three patients and specific ^{125}I-labelled EGF binding was assessed in each component. It was found that over 68% of the specific binding for EGF was associated with the 800 ***g*** crude fraction and that the rest occurred in the mitochondrial (15%) and microsomal (17%) fractions. No specific binding of radiolabelled EGF was detected in the cytosol. In order to maximize the binding values, it was decided to undertake all subsequent incubations on the 105 000 ***g*** membrane particulate fraction which would also contain the 800 ***g*** crude pellet as well as the mitochondrial and microsomal fractions.

In a separate experiment we also found that the number of EGF specific binding sites rose linearly with protein concentration over the concentration used.



TEXT-FIGURE 1. Time- and temperature-dependent specific binding studies for epidermal growth factor (EGF) on human testicular tissue were carried out at 4 °C (◆) and 32 °C (□) over a period of 3 h. Aliquots (100 µl) of the particulate membrane fraction containing 2 mg protein/ml were incubated with ^{125}I-labelled EGF (8 nmol/l) in the presence and absence of 50-fold excess unlabelled EGF. Each value represents the % mean ± S.E.M. of the maximum binding at 32 °C and 4 °C for three different specimens each analysed in triplicate.

Saturation analysis

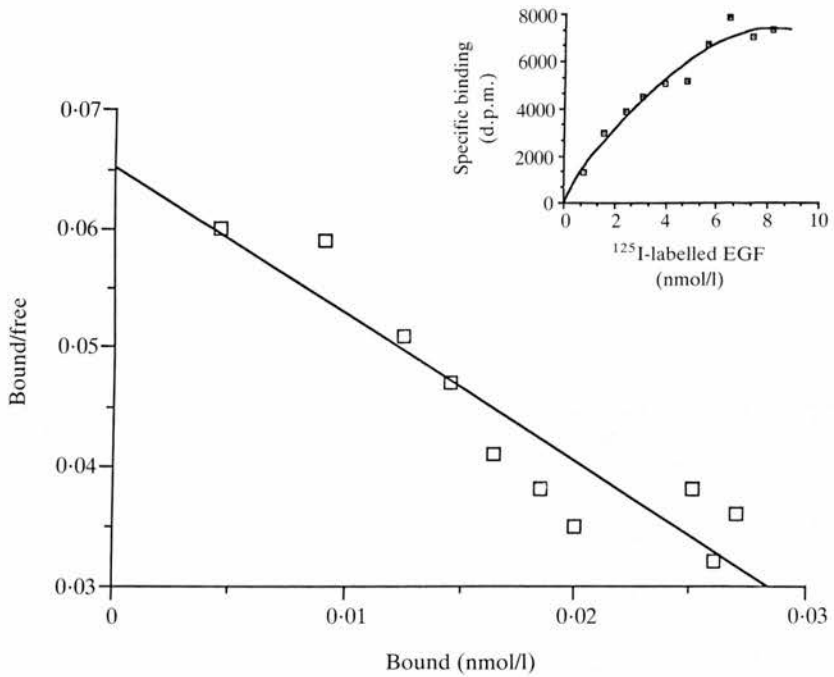
Incubation of the membrane fractions with increasing concentrations of ^{125}I-labelled EGF (0·5–8 nmol/l) produced a saturation of the binding protein at 8 nmol/l (Text-fig. 2). Scatchard (1949) analysis of the data employing a computer program written by Munson & Rodbard (1980) yielded one binding site with a mean ± S.D. K_d of 327 ± 59 pmol/l (d.f. 9). The binding capacity for the same ten patients ranged from 0·07 to 0·21 pmol/mg protein. A typical Scatchard plot is also shown in Text-fig. 2.

Competition studies

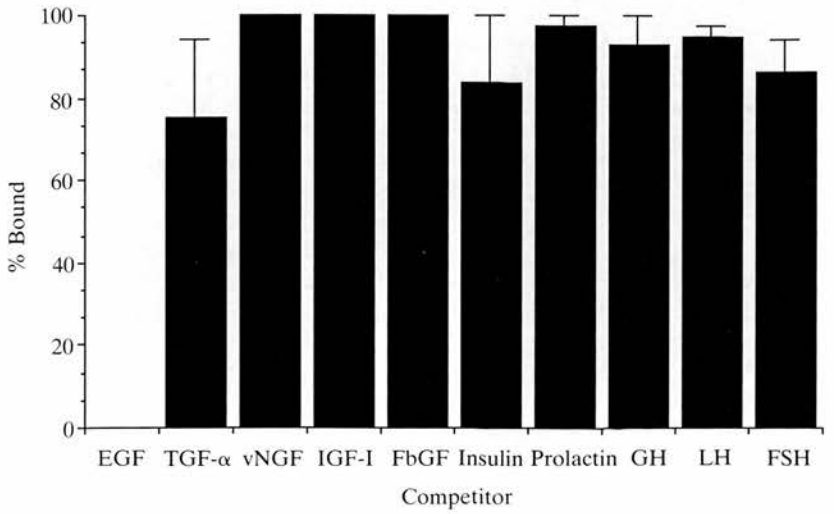
Competition studies showed that the specially bound EGF was not displaced from its receptor by any of the other peptides used at either a 50- (Text-fig. 3) or a 100-fold excess (results not shown) of the concentration of labelled EGF used.

Effect of heat and trypsinization

Binding of EGF to its receptor was abolished after preincubation with trypsin but was only reduced by 50% after the homogenate had been preheated at 45 and 60 °C (Table 1). Unfortunately agglutinization of the homogenate occurred at 80 °C and therefore could not be used to perform a binding assay.



TEXT-FIGURE 2. Saturation analysis of ^{125}I-labelled epidermal growth factor (EGF) binding in human testicular tissue. Aliquots (100 µl) of the particulate fraction suspension were incubated with concentrations of ^{125}I-labelled EGF ranging from 0·05 to 8 nmol/l in the presence and absence of a 50-fold excess unlabelled EGF at 32 °C for 40 min. The data (insert) were analysed by the Scatchard (1949) method to yield the K_d and the number of specific binding sites.



TEXT-FIGURE 3. Specificity of the epidermal growth factor (EGF) binding sites. Aliquots (100 µl) of the particulate fraction suspension were incubated with ^{125}I-labelled EGF (8 nmol/l) for 40 min at 32 °C in the presence and absence of a 50-fold excess of the unlabelled competitor. % Bound was taken as the amount of ^{125}I-labelled EGF displaced by a 50-fold excess of unlabelled competitor. Each value represents the mean ± S.E.M. of three different specimens each analysed in triplicate. vNGF, nerve growth factor; FbGF, bovine fibroblast growth factor; TGA-α, transforming growth factor-α.

TABLE 1. Effect of heat pretreatment at 45 and 60 °C and trypsinization on specific ^{125}I-labelled epidermal growth factor binding by the 105 000 ***g*** membrane particulate fraction. Results are expressed as % change from untreated control and the values represent means ± S.E.M. of three different specimens each analysed in triplicate

Pretreatment	Specific binding (% of control)
Heat (45 °C)	53·0 ± 7·7
Heat (60 °C)	52·0 ± 12·0
Trypsin	7·73 ± 0·73

Cross-linking and autoradiographic studies

Plate 1 shows that in the presence of labelled EGF (lane A) there is a much more intense protein band appearing around the 125 kDa region in comparison with the band seen in experiments carried out in the presence of excess unlabelled EGF (lane B).

Immunohistochemical studies

Immunohistochemical staining of a frozen section of human testicular tissue with the monoclonal antibody (EGF-R1) is shown in Pl. 2, fig. 1. The staining appears to be around the Leydig cells and the thin fibroblastic cells of the interstitial tissue. This is also shown on the paraffin sections on which the external domain monoclonal antibody EGF-R was used (Pl. 2, fig. 2). In neither procedure was any staining observed in the Sertoli cell or germ cell area. It was also found that when using the monoclonal antibody for the internal domain of the receptor (F4) no staining became apparent on either of the two types of sections used.

DISCUSSION

The results from this study show for the first time that EGF receptors are present in human testicular tissue. The receptor has been shown to have a single high affinity binding site with an affinity constant of 327 ± 59 pmol/l (d.f. 9). The number of specific binding sites ranged between 0·07–0·21 pmol/mg protein. These figures are similar to those already published by others in human placenta (Hock & Hollenberg, 1980), breast cancer (Sainsbury, Sherbert, Farndon & Harris, 1985) and human fibroblasts (Carpenter & Cohen, 1979). However, unlike the reports published by O'Keefe, Hollenberg & Cuatrecasas (1974), Hofmann *et al.* (1984) and Maddy *et al.* (1987), our data suggest that there is only one binding site. The experiments performed also indicate that 68% of the EGF binding sites exist in the 800 ***g*** crude membrane pellet and that the receptor sites are specific to EGF and are not displaced by TGF-α, IGF-I, FbGF, vNGF, prolactin, insulin, LH, FSH or GH. Specificity is indicated by the fact that the maximum percentage competition of another peptide for the EGF binding site was 24% by TGF-α, which is known to bind to the EGF-R (Derynck, 1986).

Autoradiographic/cross-linking studies also confirm the presence of a specific receptor for EGF in human testes. However, in direct contrast to previous reports (Mukku, 1984), the autoradiographic results suggest that the protein is in the 125 kDa region. Since earlier reports identify a protein of 170 kDa, we suspect that the 125 kDa product may be a degraded form of the receptor, possibly a subunit due to proteolysis taking place during the experimental procedure by the calcium-dependent protease calpain (Cassel & Glaser, 1972). The protease is known to remove a 20 kDa fragment from the receptor, which could have still occurred despite attention being given to calcium-sequestering agents in the buffers used in the present study. Similar results have also been reported by Weisman, Raguet & Kelly (1987) on the characterization of the EGF receptors in human meningioma.

Immunohistochemical studies confirmed the biochemical results for the presence of EGF receptors in human testicular tissue. The receptors were exclusively located in the interstitial tissue of the testes with no staining in the Sertoli cells; a finding which has not been reported before in any of the species so far examined. The immunostaining was confirmed on both the frozen and paraffin sections and was of a grainy appearance. In some instances the staining also appeared as small strands interwoven between the seminiferous tubules; this may be due to staining of the fibroblastic type cells which surround the seminiferous tubules. All of the ten patients included for immunohistochemistry showed some degree of staining in the interstitial tissue when the antibodies for the external domain of the receptor were used (EGF-R1, frozen; EGF-R, paraffin). However, there was no staining with the antibody to the internal domain of the receptor. Although no one has so far undertaken any immunohistochemistry on human testicular tissue employing antibodies to the EGF receptor, staining with antibodies to EGF has been reported by Mori, Naito, Tsukitani *et al.* (1989) who found an immunoreaction in both the Leydig cells of the testes and the seminiferous tubules, and particularly in spermatozoa and cells undergoing spermatogenesis.

So far very little work has been published on EGF in relation to the functioning of the human testes, although there are a few studies on the rat testes. Holmes *et al.* (1986) characterized a growth factor secreted by rat Sertoli cells in culture. Since then

Skinner (1989) has identified this EGF-like material as TGF which has also been found to be produced by the peritubular cells. In view of the present findings of EGF receptors probably in human Leydig cells, the potential action of TGF or EGF on the Leydig cell is of extreme interest not least because of earlier reports on the effect of EGF on testicular cell lines. Ascoli (1981) found that there was a 97% reduction in the expression of human chorionic gonadotrophin receptors in MA-10 Leydig tumour cell lines grown in the presence of EGF. Furthermore, it was also found that in the same cell line EGF produced an increase in steroid biosynthesis (Ascoli, Euffa & Segaloff, 1987). Similarly, Verhoeven & Cailleau (1986) reported a stimulatory effect of EGF on steroidogenesis in normal rat Leydig cells.

As a result of this study we can now confirm that epidermal growth factor receptors are present on human testicular tissue and that because of their probable location on the Leydig cell these may have some control over steroidogenesis—a possibility which we are at present investigating.

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REFERENCES

Ascoli, M. (1981). Regulation of gonadotropin receptors and gonadotropin responses in a clonal strain of Leydig tumour cells by epidermal growth factor. *Journal of Biological Chemistry* **256,** 179–183.

Ascoli, M., Euffa, J. & Segaloff, D. L. (1987). Epidermal growth factor activates steroid biosynthesis in cultured Leydig tumour cells without affecting the levels of cAMP and potentiates the activation of steroid biosynthesis by choriogonadotropin and cAMP. *Journal of Biological Chemistry* **262,** 9196–9203.

Bellve, A. R. & Zheng, W. (1985). Growth factors as autocrine and paracrine modulators of male gonadal functions. *Journal of Reproduction and Fertility* **85,** 771–793.

Bradford, M. M. (1976). A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein dye binding. *Analytical Biology* **72,** 248–254.

Buch, J. P., Lamb, D. J., Lipschultz, L. I. & Smith, R. G. (1988). Partial characterization of a unique growth factor secreted by human Sertoli cells. *Fertility and Sterility* **49,** 658–665.

Carpenter, G. & Cohen, S. (1979). Epidermal growth factor. *Annual Review of Biochemistry* **48,** 193–216.

Cassel, D. & Glaser, L. (1982). Proteolytic cleavage of the epidermal growth factor receptor ACa + dependent, sulfphydryl-sensitive proteolytic system in A431 cells. *Journal of Cellular Biochemistry* **257,** 9845–9848.

Cohen, S. & Carpenter, G. (1975). Human epidermal growth factor: Isolation and chemical and biological properties. *Proceedings of the National Academy of Sciences of the U.S.A.* **72,** 1317–1321.

Derynck, R. (1986). Transforming growth factor alpha: structure and biological activities. *Journal of Cellular Biochemistry* **32,** 293–304.

Elson, S. D., Browne, C. A. & Thorburn, G. D. (1984). Identification of epidermal growth factor-like activity in human male reproductive tissues and fluids. *Journal of Clinical Endocrinology and Metabolism* **58,** 589–594.

Fraker, P. J. & Speck, J. C., Jr. (1978). Protein and cell membrane iodinations with sparingly soluble chloramide 1,3,4,6-tetrachloro-3,6-diphenylglycouril. *Biochemical and Biophysical Research Communications* **80,** 849–857.

Gregory, H., Willshire, R., Kavanagh, J. P., Blacklock, W. V., 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.

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 Research* **46,** 285–292.

Hock, R. A. & Hollenberg, M. D. (1980). Characterization of receptor for epidermal growth factor-urogastrone in human placenta membranes. *Journal of Biological Chemistry* **255,** 10731–10736.

Hofmann, G. E., Rao, Ch. V., Barrows, G. H., Schultz, G. S. & Sanfilippo, J. S. (1984). Binding sites for epidermal growth factor in human uterine tissues of leiomyomas. *Journal of Clinical Endocrinology and Metabolism* **58,** 880–884.

Holmes, S. D., Spott, G. & Smith, R. G. (1986). Characterization of a growth factor secreted by rat Sertoli cells in culture. *Progressive Clinical Biological Research* **217A,** 389–392.

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

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

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

Maddy, S. Q., Chisholm, G. D., Hawkins, R. A. & Habib, F. K. (1987). Localization of epidermal growth factor receptors in the human prostate by biochemical and immunocytochemical methods. *Journal of Endocrinology* **113,** 147–153.

Mori, M., Naito, R., Tsukitani, K., Okada, Y. & Tsujimura, T. (1989). Widespread distribution of human epidermal growth factor (hEGF) in human tissues and organs under normal and tumorous conditions – immunohistochemical studies with the use of polyclonal and monoclonal antibodies. *Acta Histochemistry and Cytochemistry* **22,** 15–34.

Mukku, V. R. (1984). Regulation of epidermal growth factor receptor levels by thyroid hormone. *Journal of Biological Chemistry* **259,** 6543–6547.

Munson, P. J. & Rodbard, D. (1980). Ligand: A versatile computerized approach for characterization of ligand binding systems. *Analytical Biochemistry* **107,** 220–239.

O'Keefe, E., Hollenberg, M. D. & Cuatrecasas, P. (1974). Epidermal growth factor characteristics of specific binding in membranes from liver, placenta and other tissues. *Archives of Biochemistry and Biophysics* **164,** 518–526.

Sainsbury, J. R. C., Sherbert, G. V., Farndon, J. R. & Harris, A. L. (1985). Epidermal growth factor receptors and oestrogen receptors in human breast cancer. *Lancet* i, 660–672.

Scatchard, G. (1949). The attractions of proteins for small molecules and ions. *Annals of the New York Academy of Sciences* **51,** 660–672.

Skinner, M. K. (1989). Peritubular myoid cell–Sertoli cell interactions which regulate testis function and growth. *Perspectives in*

andrology, Serono Symposia, vol. 53, pp. 175–182. Ed. M. Serio. New York: Raven Press.

Starkey, R., Cohen, S. & Orth, D. N. (1975). Epidermal growth factor: Identification of a new hormone in human urine. *Science* **189,** 800–803.

Tsutsumi, O., Kurachi, H. & Oka, T. (1986). A physiological role of epidermal growth factor in male reproductive function. *Science* **233,** 975–977.

Verhoeven, G. & Cailleau, J. (1986). Stimulatory effect of epidermal growth factor on steroidogenesis in Leydig cells. *Molecular and Cellular Endocrinology* **47,** 99–106.

Waterfield, M. D., Mayes, E. L. V., Stroobant, P., Bennett, P. L. P., Young, S., Goodfellow, P. N., Banting, G. S. & Ozanne, B. (1982). A monoclonal antibody to the human epidermal growth factor receptor. *Journal of Cellular Biochemistry* **20,** 149–161.

Weisman, A. S., Raguet, S. S. & Kelly, P. A. (1987). Characterization of epidermal growth factor receptor in human meningioma. *Cancer Research* **47,** 2172–2176.

DESCRIPTION OF PLATES

Plate 1

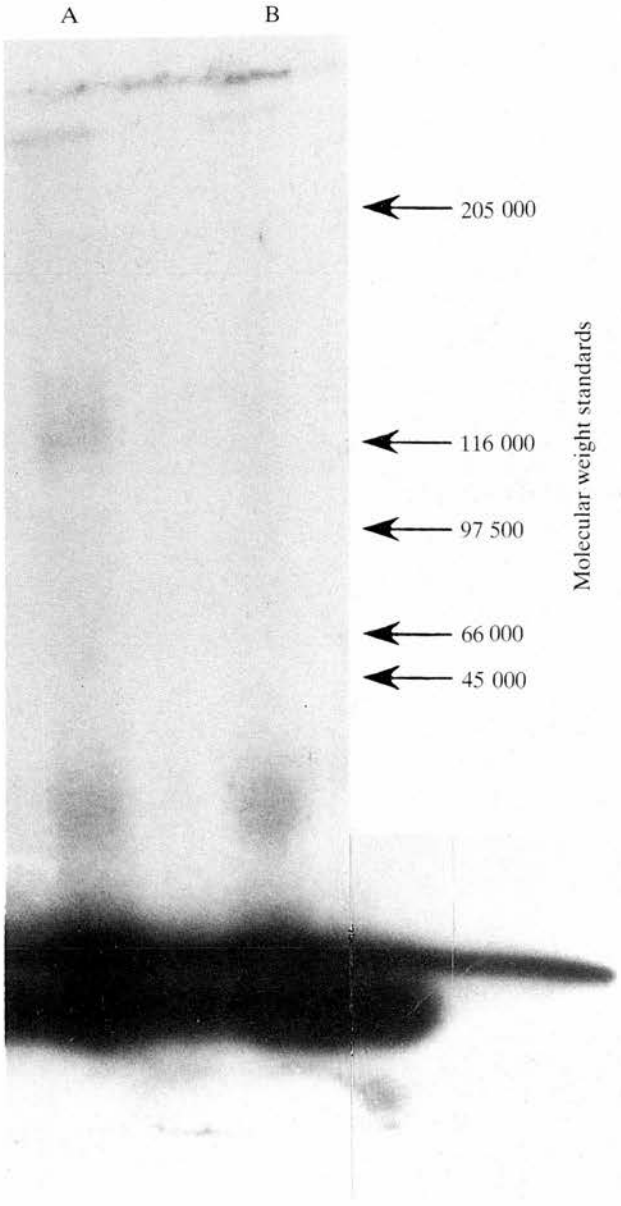
Autoradiography was performed on a testicular tissue sample that had been preincubated with ^{125}I-labelled epidermal growth factor (EGF; 8 nmol/l) at 32 °C for 40 min in the presence (lane B) and absence (lane A) of unlabelled EGF at a 50-fold excess concentration. The receptor complex was then cross-linked with disuccinimidyl suberate (1 mmol/l) and run on 7·5% SDS–polyacrylamide gels, along with standards ranging from 45 000 to 205 000 in molecular weight. The gel was subsequently counterstained, dried and autoradiography was carried out at −70 °C with an Amersham Hyperfilm B_{max} for up to 4 weeks.

Plate 2

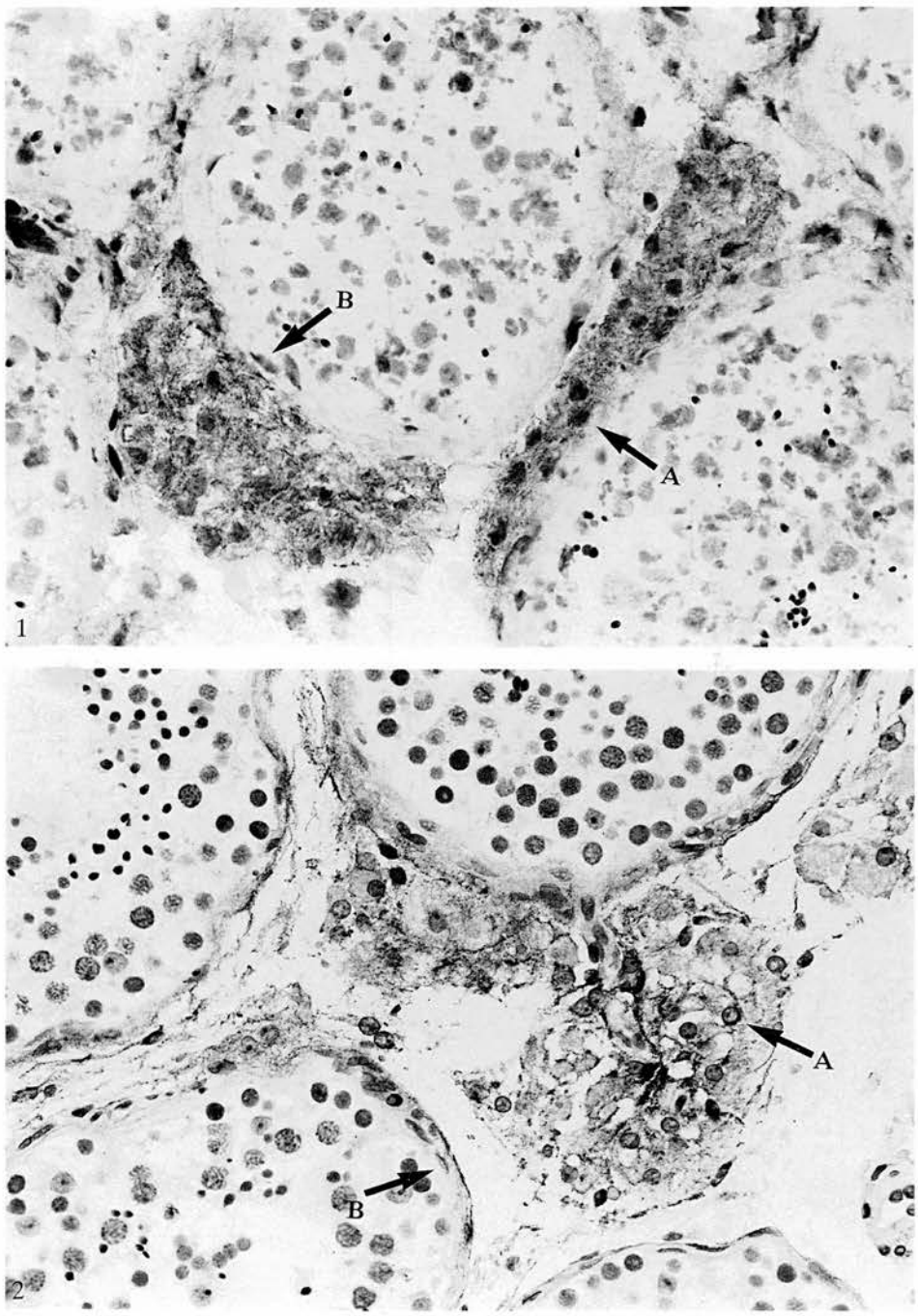
Immunohistochemical staining using the streptavidin–biotin labelled method on frozen sections of human testicular tissue.

FIGURE 1. Primary monoclonal EGF receptor (EGF-R1) antibody was used at a dilution of 1:30. The sections were lightly counterstained with haematoxylin. A positive reaction for the EGF receptor appears as dark diffuse staining in the interstitial tissue area (B) although it is more intense where there is a large population of Leydig cells (A) (× 280).

FIGURE 2. A paraffin section of human testicular tissue on which immunohistochemical staining has been performed using the monoclonal antibody EGF-R at a 1:10 dilution is shown. Again Leydig cells (A) have become stained along with fibroblastic cells surrounding the seminiferous tubules (B) (× 280).


A
B
205 000
116 000
97 500
66 000
45 000
Molecular weight standards

(Facing p. 492)


B
A
1
A
B
2