Mechanism of invasion by prostate cancer

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Dedicated to my brothers and sisters and to the memory of my parents.

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Summary

The prostate is normally under the control of sex steroids and endocrine therapy is the most effective first line therapy in prostate cancer. Prostate carcinoma is characterised by eventual loss of hormonal sensitivity with increased aggressive behaviour of the tumour. Prostate carcinoma has an initial slow growing non-invasive phase, but, mostly, is invasive by the time of diagnosis. As the tumour progresses, tissue growth factors, such as epidermal growth factor (EGF) and transforming growth factor-ß (TGFß) appear to be more effective than hormones in the control of the cancer cells. Increased tyrosine kinase activity, related to the increased activity of growth factor receptors, has been shown in many prostate cancers.

Plasminogen activators (PA) , particularly urokinase plasminogen activator (uPA), have been implicated in extracellular proteolysis in invasiveness, metastasis and angiogenesis. PA are highly substrate specific enzymes which convert the inactive zymogen plasminogen to plasmin. Plasmin, a proteolytic enzyme of broad specificity, degrades laminin and fibronectin, and activates various tissue metalloproteinases. Degradation of these extracellular proteins facilitates the migration and metastasis of tumour cells to different biological sites. Regulation of uPA activity and pericellular proteolysis, at protein level, is controlled by the combination of PA inhibitors, PAI-1 and PAI-2 which are responsible for the negative regulation and uPA receptor (uPAR) which binds latent uPA. uPA plays a critical role in the invasion and migration of prostate cancer cells and provides a marker of the aggressive phenotype.

This study investigated the regulation by androgen, EGF, and TGFß of the release and function of uPA, PAI-1 and PAI-2 using hormone-insensitive

invasive prostate cancer cells, PC-3 and DU145. These studies include measurement of EGF receptor function, in terms of its sensitivity to a "specific" inhibitor and assay of the various proteases at mRNA, protein and functional levels.

The PC3 and DU145 cells both possess EGF receptor (EGFR). Growth of PC3 cells was stimulated by exogenous EGF but not that of DU145 cells (figs 3.5 and 3.6). However, EGFR-specific tyrosine kinase inhibitors significantly inhibited growth of both cell types (figs 3.7 and 3.8). On the other hand, both cell types were found to be growth insensitive to exogenous TGFß (figs 4.1 and 4.2).

Both cell lines expressed uPA, uPAR and PAI-1 transcripts. uPA, tPA, PAI-1 and PAI-2 proteins were all detectable in the PC3 cells whereas only uPA and PAI-1 proteins (PAI-1 protein was hardly detectable in the control group) were found in DU145 cells.

EGF increased the production of uPA, at both transcriptional and protein level (figs 3.11, 3.15 and 3.16) in addition to increasing uPAR at transcriptional level (figs 3.12 and 3.14) in both cell lines. uPA activity was enhanced by EGF in both cell lines (figs 3.18 and 3.19). The matrigel invasion of the PC3 cells was increased by exogenous EGF in an *in vitro* invasion assay (fig 3.20). The tyrosine kinase inhibitors inhibited all stimulations caused by EGF. uPA production and activity were suppressed well below that in the control group. The tyrosine kinase inhibitor (ZM260603) also reversed the EGF stimulated matrigel invasion by the PC3 cells.

TGFß also stimulated uPA and PAI-1 production in both cell lines (figs 4.8 and 4.9). The TGFß-induced increase in PAI-1 production in both cell lines (figs

4.10 and 4.11) was remarkable (2087 % in PC3, 1433 % in DU145). TGFß also stimulated PAI-2 protein in the PC3 cell line. uPA activity was found to be inhibited by TGFß in the PC3 cell cytosol whereas it was increased in the DU145 cells (figs 4.12 and 4.13). In the matrigel invasion assay TGFß treated PC3 and DU145 cells showed a similar invasion in comparison to the control group.

Overall, EGF is a potent stimulative agent for both growth and invasion in prostate cancer cells. The contribution of TGFß to prostate cancer cell growth and invasiveness still seems to be controversial. The effect of TGFß on the immune system, on angiogenesis and on colony formation by cancer cells all suggest that TGFß plays a positive role in favour of cancer invasiveness and provides a new target for therapy. As can be seen from the results, targetting the EGFR function inhibits not only tumour growth but also its invasiveness. On the basis of these results, inhibition of EGFR-related tyrosine kinase activity may open a new era in the treatment of drug-resistant prostate cancer.

Abbreviations

aa	aminoacid
A_{260}	Absorbance at 260 nm
Ab	Antibody
Ag	Antigen
ALP	Alkaline phospahatase
Approx.	Approximately
ATF	Amino terminal fragment
ATP	Adenosine triphosphate
BMP	Bone morphogenic proteins
BSA	Bovine serum albumin
cyclic AMP	Adenosine-3',5'-cyclic monophosphate
cDNA	Complementary deoxyribonucleic acid
Ci	Curie
C-terminal	Carboxy-terminal of a protein
cpm	Counts per minute
DAG	Diacylglycerol
DEPC	Diethyl pyrocarbonate (1 % w/v) in water
DHIDCCFCS	Dialysed, heat-inactivated, dextran coated charcoal treated foetal calf serum
dH_2O	Distilled water
DHT	Dihydrotestosterone
DMSO	Dimethyl sulphoxide
DNA	Deoxyribonucleic acid
DTT	Dithiothreitol
EDTA	Ethylenediaminetetraacetic acid
EGF	Epidermal growth factor
EGFR	Epidermal growth factor receptor
EGTA	Ethylene glycol-bis (b-aminoethyl ether) N,N,N',N'-

	tetraacetic acid
EHS	extracellular matrix derived from sheep mammary carcinoma
ERK	Extracellular signal regulated kinase
FCS	Foetal calf serum
FGF	Fibroblast growth factor
g	Gram
g_{av}	Average gravitational force
Genistein	4',5,7-Trihydroxyisoflavone
G protein	GTP binding protein
GFD	Growth factor domain
GTP	Guanine triphosphate
Hepes	N-2-Hydroxyethylpiperazine-N'-2-ethane-sulphonic acid
HMW	High molecular weight
HRP	Horse radish peroxidase
IC_{50}	Concentration at which 50% inhibition ensues
IgG	Immunoglobulin G antibody
IGF-1	Insulin-like growth factor-1
IP_3	Inositol-1,4,5-triphosphate
kDa	Kilodaltons
kg	Kilogram
l	Litre
LMW	Low molecular weight
M	Molar
mA	Milliamps
MAPK	Mitogen activated protein kinase
MEK	Mitogen activated protein kinase kinase
ml	Millilitre
mg	Milligram

MTT	3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyl tetrazolium bromide
NGS	normal goat serum
N-terminus	Amino terminus of protein
NK cells	Natural killer cells
^{o}C	Degrees celcius
OPD	0-phenylendiamine
PA	Plasminogen activator
PAI-1	Plasmin activator inhibitor-1
PAI-2	Plasmin activator inhibitor-2
PBS	Phosphate buffered saline
PDGF	Platelet-derived growth factor
pH	$-\log_{10}[H^{+}]$
PI-3-K	Phosphatidylinositol-3-kinase
PMA	phorbol 12-myristate 13-acetate
PMSF	Phenylmethylsulphonylfluoride
PVP	polyvinylpyrolidone-free polycarbonate
RNA	Ribonucleic acid
rpm	revolutions per minute
S-2251	H-D-Val-Leu-Lys-pNA.2HCL
SCID	Severe combined immunodeficiency
SD	Standard deviation
SH2	Src homology 2
SH3	Src homology 3
TEMED	N,N,N',N'-Tetramethylethylene diamine
TGFα	Transforming growth factor alfa
TGFß	Transforming growth factor beta
TK	Tyrosine kinase
TKI	Tyrosine kinase inhibitor

TNF	Tumour necrosis factor
tPA	Tissue-type plasminogen activator
Tris	Tris(hydroxymethyl)methylamine
uPA	Urokinase-type plasminogen activator
uPAR	Urokinase-type plasminogen activator receptor
V	Volts

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INTRODUCTION

1.1 Prostate carcinoma

The incidence of prostatic cancer has dramatically increased in past decades and is becoming the most commonly diagnosed cancer in men in the Western world (Carter & Coffey, 1990). In 1992, 132000 cases were diagnosed, in 1993, 165,000 cases, and 200,000 cases for 1994 (Scher, 1994); more than 317,000 new cases and 45,000 deaths are predicted for 1996 (Perry *et al.*, 1997). Prostate cancer accounts for approximately 13% of all male cancers with 85,000 newly diagnosed cases each year in Europe (Moller-Jensen *et al.*, 1990). In 1992 prostate cancer killed 693 men in Scotland alone with an incidence second only to lung cancer (Scottish Health Statistics 1993).

The incidence and mortality of hormone-dependent cancers, which include tumours of prostate, breast, ovary and endometrium, are high in Western countries relative to the rates in Asia. The highest mortality rates from prostate cancer occur in the black male population in USA, nearly 120 times more than men in China and 30 times more than men in Japan. However, mortality rate is only two times higher than that of Japanese immigrants living in USA (see review Morton *et al.*, 1996) suggesting that the differences are due more too environmental than genetic factors. On the other hand, microscopic (latent) prostatic cancer is commonly found, with similar frequency, in these countries (Yatani *et al.*, 1982). Prostate cancer comprises 2.5% of all cancer deaths in Japan with the corresponding figure for Canada being 10.6% and for Sweden 17.7 % in 1988 (Boyle & Zaridze, 1993).

Several studies have shown there is an increased risk associated with the consumption of meats, milk and fat and a decreased risk associated with cereals, pulses and rice (Carter *et al.*, 1990). Higher plasma isoflavinoid levels were found in Japanese men than in Finnish men (Adlercreutz *et al.*, 1993). The

authors in this study suggested that high concentration of isoflavinoids in plasma may have a preventive role for the progression of prostate cancer. Studies on the relationship between diet and risk for prostate cancer appear of special interest, since nutrition may well influence the hormone balance, especially of steroids (androgens) which have been implicated in the growth of prostate cancer.

A potential role for androgens in the carcinogenesis of the human prostate has been suggested, primarily on the basis that development and growth dependence of the normal prostate require the presence of the hormone. The presence of steroid hormone receptors in prostate cancer, the initial successful treatment with endocrine therapy, a significant association between prolonged administration of testosterone (T) or dihydrotestosterone (DHT) and tumour formation in the rats, and the relations between the tissue DHT content and response to endocrine therapy have all provided further support to the hormonal hypothesis in the aetiology of prostate cancer (extensively reviewed by Carruba, 1994). However, consistent findings of higher DHT levels in benign prostatic hyperplasia (BPH) compared with prostate cancer, combined with the fact that there is no difference in the serum level of hormones between low and high risk groups indicates that there is no simple relationship between androgen levels of prostate cancer incidence in the general population.

Prostate cancer incidence increases rapidly with age. The incidence of latent cancer rises with each decade from 10% in those aged 50-60 years to 50% in those 80 years of age or older (Smith & Parmar, 1993). From 1985-2000, it is predicted that there will be a 37% increase in prostate cancer deaths and a dramatic 90% increase in prostate cancer cases diagnosed (Carter & Coffey, 1990). The incidence of prostate cancer is increased in men with a family

history of the disease. Occupational exposure to heavy metals has also been implicated in the development of prostate cancer (Coffey, 1993).

In some men, prostate cancer metastasises rapidly, killing the patient within a year of diagnosis. In contrast, other patients survive for many years with localised disease without clinically detectable metastases (Johannson *et al.*, 1989). It has been calculated that only 7% of potentially detectable histological prostatic cancers are life threatening (Scardino *et al.*, 1992). Because of prostate cancer incidence increases with age, many patient die of other age-related factors even though they have prostate cancer.

1.2 Growth regulation of the prostate

Over the last 50 years, the endocrinology of the human prostate has been dominated by the belief that androgens are essential for development, growth, differentiation and maintenance of morphology and functional activity of the prostate. The benefits of androgen withdrawal in patients with advanced prostate carcinoma were initially established by Huggins and his colleagues (Huggins & Hodges, 1941). Since then, several other endocrine treatments, including treatment with oestrogens, progestins and anti-androgens have been, in turn, proposed to achieve a decline of androgen stimuli. Nevertheless, after an initial objective response rate of 80-90% to endocrine therapy, patients subsequently fail to respond further and relapse within one or two years, with a median survival of six months after first recurrence (Lepor, 1982; Whitmore, 1973).

The reasons' tumours become androgen independents are still not clear. Almost all advanced prostate cancers ultimately fail to respond to androgen deprivation and there is no effective chemotherapy to cure the disease. Disease progression results from the autonomous growth of clones of hormone-unresponsive cells

(Griffiths *et al.*, 1993). So far the cytotoxic chemotherapy result of prostate cancer has been disappointing, after failure of hormonal manipulation (Yagoda & Petrylak, 1993). The failure of androgen deprivation therapy to limit the progression of prostate cancer indicates that other pathways may be involved in the growth regulation of prostate cancer.

It has been shown that polypeptide growth factors play an important role on the growth management of normal prostate (Peehl & Wong, 1989), BPH (Maddy *et al.*, 1986) and prostate cancer (Carruba, 1994; MacDonald *et al.*, 1990; Schuurmans *et al.*, 1988a). In several studies, it is revealed that androgens have an important role in the production of epidermal growth factor (EGF), transforming growth factor-α (TGFα) and the epidermal growth factor receptor (EGFR) (Schuurmans *et al.*, 1988a; Schuurmans *et al.*, 1988b; Traish & Wotiz, 1987). These studies suggest the possibility that the growth stimulatory effects of androgens are, in part, indirectly mediated by the growth factors. Peptide growth factors appear to be the paracrine mediators of normal prostate and both paracrine and autocrine mediators of prostate cancer (Davies & Eaton, 1991; Steiner, 1993). In contrast to normal prostate, prostate cancer growth regulation is mainly dominated by the autocrine loop (Davies & Eaton, 1991; Steiner, 1993).

EGFR, like platelet-derived growth factor receptor (PDGFR), fibroblast growth factor receptor (FGFR) and insulin like growth factor receptors (IGFR), possesses intrinsic tyrosine kinase activity (Levitzki & Gazit, 1995; Steiner, 1993; Geer *et al.*, 1994). Overexpression of tyrosine kinase (TK) activity has been detected in many carcinomas such as osteosarcoma (Onda *et al.*, 1996), breast carcinoma (Digiovanna *et al.*, 1996; Nagy *et al.*, 1996; Nicholson *et al.*, 1993; Salven *et al.*, 1996), ovary carcinoma (Leake *et al.*, 1995) leukaemia (Chapman *et al.*, 1995), bladder carcinoma (Dinney *et al.*, 1997) and prostate

carcinoma (Fox *et al.*, 1994). Increased productions of EGF, TGFα and overexpression of the EGFR have been associated with neoplastic transformation (Carruba *et al.*, 1994a; Hynes, 1997; Leake *et al.*, 1995). Overexpression of the EGFR has been associated with gene amplification (Saranath *et al.*, 1992; Yamamoto *et al.*, 1986). It is speculated that the carcinoma cells may become more sensitive to the ligands by producing more of the receptors (Marx, 1993). Enhanced expression of both ligands and receptors apparently results in increased TK activity. It has been speculated that increased TK activity can start mutations or influence persistent growth stimulation (Bishop, 1987) in carcinoma. Increased TK activity is thought to lead to persistent growth: "the gas pedal is on all the time, and there is no brake" was the description of Dr. Suzanne Fuque (Marx, 1993) for tamoxifen resistant breast carcinoma, and this also seems appropriate for prostate carcinoma. Increased cellular proliferation rate has been shown in primary prostate cancer (five-fold) and in metastatic prostate cancer (twenty-four fold) when compared with normal prostate (Tu *et al.*, 1996). It has been shown that heterodimerisation of EGFR/*erbB2* complex has greater growth response than homodimerisation of EGFR (Hynes, 1997; Yarden, 1997).

1.3. Protein tyrosine kinases

There are two general classes of protein tyrosine kinases:

The receptor associated tyrosine kinases;

These kinases are mostly members of *src* and *janus* family. There are at least eight members of *src* family in mammals: *src, yes, fgr, fyn, lck, lyn, hck, blk.* They are located on the cytoplasmic side of the plasma membrane and covalently attached to transmembrane proteins by lipid chains (Mustelin & Burn, 1993).

The *janus* family consists of JAK1, JAK2 and Tyk2 (Argetsinger, 1993; Mustelin & Burn, 1993).

The receptor tyrosine kinases;

EGF and TGFα show their effect by binding and down regulating the EGFR in mammalian cells. Ligand binding leads to the receptor dimerization. This dimerization presumably occurs by a conformational change in the external domain of receptor (Geer *et al.*, 1994). The ligand-induced dimerization leads to transphosphorylation between the two receptor molecules in a dimer. Transphosphorylation which results in activation of the kinase activity occurs on at least five tyrosines located in the carboxy terminal region (Ullrich & Schlessinger, 1990). Phosphorylated tyrosine residues attract the SH2 (*src* homology 2) and SH3 (*src* homology 3) domains of signalling molecules such as growth factor binding domain 2 (grb2) phospholipase C (PLC), phophatidylinositol-3' kinase (PI3 kinase) and p21GTPase-activating protein (GAP) (Pawson & Gish, 1992; Waksman *et al.*, 1993). Phosphotyrosine bound Grb2 interacts with a molecule named sos (son of sevenless) which leads to activation of ras signalling pathway. Ras activation appears to play a central role in signal transduction by a large number of protein tyrosine kinases (Geer *et al.*, 1994). Activated ras expression results in the activation of raf-1 and MAP (mitogen activated protein) kinase kinase (MEK). MEK activates MAP kinase (a protein-serine and tyrosine kinase). Activated MAP kinases can migrate from the cytosol into the nucleus (Rosales *et al.*, 1995) and activate early proteins such as myc, jun and fos which results in proliferation-associated gene induction (Langdon & Smyth, 1995). A schematic outline of the receptor tyrosine kinase pathway is shown in fig 1.1.

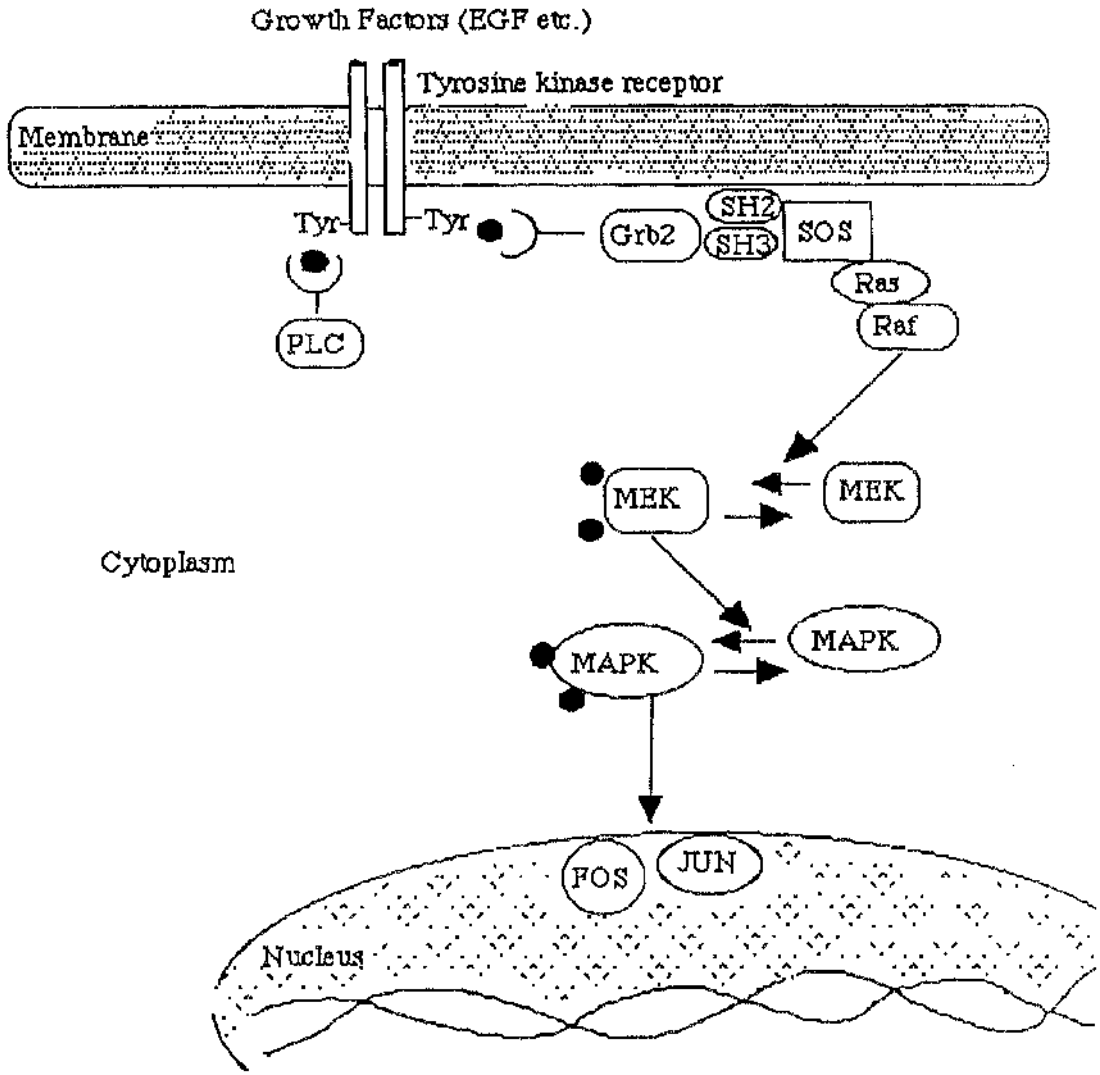

Fig 1.1 Schematic outline of tyrosine kinase pathway. Abbreviations: PLC; phospholipase C, Tyr; tyrosine residues, Grb2; growth factor binding 2, SH2-3; src-homology region 2-3, SOS; son of sevenless, MAPK; mitogen-activated protein kinase, MEK; MAP kinase kinase, EGF; epidermal growth factor, ●; indicates phosphorylated form of kinase

1.3.1. Epidermal growth factor receptor

The EGFR is a 170 kDa transmembrane glycoprotein with a ligand-dependent tyrosine kinase activity (Walker & Burgess, 1994). It is composed of three main domains: an extra cellular ligand-binding domain, a single transmembrane segment and an intracellular tyrosine kinase domain. Three other members of the EGFR family with structures related to EGFR have been identified: *erbB2 (HER2), erbB3(HER3), erbB4(HER4).* Human EGF receptor is also called *HER* or *erbB1* (Walker & Burgess, 1994). Mutation, deletion or overexpression of both EGFR and *erbB2* have been detected in many tumour types (Walker & Burgess, 1994).

Regulation of the *erbB* family receptor function is complex, involving a large family of ligands. EGF, TGFα, amphiregulin and heparin-binding EGF can only activate the EGFR, whereas ß-cellulin can activate either EGFR or *erbB4* (Massague & Pandiella, 1993; Riese *et al.*, 1997). Neurogulins 1 and 2 can bind to either *erbB3* or *erbB4* (Riese *et al.*, 1997). Ligand induced activation of the receptors not only results in homodimerisation of the receptor, but also results in heterodimerization between members of the EGFR family. Transphosphorylation of tyrosine residues on the *erbB2* has been shown in the presence of EGF-related peptides, yet none of them was a specific ligand for *erbB2*. It has been shown that activation of *erbB2* protein potentiates of TK activity (Hynes, 1997;Yarden 1997). Thus heterodimerisation of *erbB2* with EGFR may lead to greater growth response induction than homodimerisation of EGFRs alone.

EGFR expression in carcinoma

The EGFR is overexpressed in various human solid tumours and tumour-derived cell lines, suggesting that it might contribute to tumour progression (Derynck *et al.*, 1987). Enhanced expression of the EGFR or its ligands, EGF and TGFα, can increase signalling via receptor-mediated pathways which may lead to excessive proliferation and cellular transformation (Ching *et al.*, 1993). Several studies have revealed there is a positive correlation between the tumour invasiveness, poor prognosis and EGFR overexpression in pituitary tumours (Petrangeli *et al.*, 1997), in squamous cell carcinoma (Baker-Schreyer *et al.*, 1997) and in breast carcinoma (Klijn *et al.*, 1993; Nicholson *et al.*, 1993).

A significant association between increased EGFR expression and the loss of hormonal control of the tumour has been shown in patients with breast carcinoma (Nicholson *et al.*, 1993). This observation was also paralleled with the finding of increased proliferation rate of the tumour cells which EGFR positive. This finding was supported by Klijn *et al.* (1993;1997) who showed that increased EGFR expression and HER2/neu were associated with both poor prognosis and resistance to endocrine therapy in breast carcinoma.

An *in vitro* study using a hormone-dependent human breast carcinoma cell line, ZR-75-1, has shown that long term tamoxifen treatment resulted in tamoxifen resistance in this cell line which was associated with expression of higher EGFR levels compared to the parent cell line (Long *et al.*, 1992). In an immunohistochemical study, 1 of 18 samples of tamoxifen responsive breast tumours was found to be EGFR positive and none of them was *erbB2* positive. On the other hand 7 of 18 non responsive patient samples displayed the EGFR and 4 of 18 were positive for *erbB2* (Newby *et al.*, 1997).

In the prostate, there is some dispute about the expression of EGFR protein and/or mRNA in correlation with the disease progression. Several studies illustrated that the EGFR protein expression was higher in normal and benign lesions of the prostate than in carcinoma of prostate (Cohen *et al.*, 1994; Ibrahim *et al.*, 1993; Turkeri *et al.*, 1994). However, no difference was observed in the expression of EGFR mRNA between malignant and benign lesions (Turkeri *et al.*, 1994). Fox *et al* showed that tumour *erbB2* expression was associated with a significantly worse prognosis whereas there was no significant association between EGFR expression and survival (Fox *et al.*, 1994; Moul *et al.*, 1996). In another study no correlation was found between the EGFR and other prognostic factors such as tumour size, Gleason score, DNA content and S-Phase fraction (Amatetti *et al.*, 1993). Patients with locally advanced tumours displayed a significant correlation between EGFR expression and the presence of metastases at diagnosis in the study. The authors nevertheless found that both disease-free and overall survival were similar between patients with EGFR positive and receptor-negative tumours.

However, studies have shown greater EGFR mRNA expression in prostate cancer than BPH. Poorly differentiated tumours had greater levels of EGFR mRNA than the well-differentiated tumours (Morris & Dodd, 1990). Ching and associates have also reported higher EGFR mRNA expression in prostate cancer tissues than in BPH tissues (Ching *et al.*, 1993). However, even a small changes in growth characteristic may result in tumour growth after long term. Scher *et al.* (1995) suggested that autocrine stimulation may exist in the majority of metastatic androgen-independent tumours although the paracrine relationship appears to predominate in primary tumours.

EGFR expression in prostate carcinoma cell lines

Degeorges *et al.* (1995) immortalised a prostatic epithelial cell with SV-40 large T antigen. The immortalised cells showed EGFR gene amplification accompanied by an increased number of EGF binding sites and sensitivity to EGF . It is possible to say that increased EGFR level is, therefore, one of the main feature of oncogenic transformation.

Elevated levels of EGFR and TGFα mRNA and protein were observed in carcinoma cells compared with benign, secretory epithelium using *in situ* hybridisation and immunocytochemistry (GlynneJones *et al.*, 1996). GlynneJones *et al* implied that a trend towards increased expression of EGFR and TGFα protein occurs with dedifferentiation, and a similar trend in the growth fraction suggests a role in tumour progression. Although there was a correlation between EGFR and proliferation index, no relationship was found between this latter parameter and TGFα immunoreactivity. They suggested that this growth factor may be linked with other aspects of malignant activity rather than directly stimulating proliferation (GlynneJones *et al.*, 1996).

Blockade of the receptor-ligand interaction by anti-EGFR antibody (ab) resulted in reduced cell proliferation of the prostate cancer cell lines PC3 and DU145 both *in vitro* and *in vivo* (Peng *et al.*, 1996; Prewett *et al.*, 1996).

It has been shown that the number of EGFR sites on a prostate cancer cell, LNCaP, is increased in the presence of androgen (Connolly & Rose, 1990; Schuurmans *et al.*, 1988b), progesterone and estrogen (Schuurmans *et al.*, 1988b). Recently Brass *et al.* (1995) showed that the androgen receptor transfected PC3 cell line expressed an increased amount of EGFR in response to DHT. DHT treatment also increased the ligand binding affinity to the receptor.

However no difference was observed in the amount of production of EGF and TGFα.

Androgen modulation of the EGFR has been demonstrated in normal rat prostate (Traish & Wotiz, 1987) and in non-neoplastic prostate cell lines (Eaton *et al.*, 1991). Traish *et al* showed that the castration of rats resulted in a 3- to 6-fold increase in EGF binding to the receptor. Further treatment of the castrated rats with DHT decreased the numbers of binding sites. In an other study, androgen deprivation resulted in a significant increase of both EGF and IGF-1 receptor levels in BPH tissues (Fiorelli *et al.*, 1991). Amatetti *et al.* (1993) found that EGFR expression was positive in 26.3% of tumours

Inflated EGFR-mRNA levels were found in prostate carcinoma cell lines when compared with BPH (Ching *et al.*, 1993). In parallel, Green *et al* showed that highly metastatic PC3M cells displayed increased EGFR-mRNA in comparison to PC3 cells in nude mice (Greene *et al.*, 1997).

Administration of antisense oligonucleotides directed against mRNAs encoding TGFα and EGFR, resulted in the regression of PC3 tumours in athymic mice (Rubenstein *et al.*, 1996). The authors further showed that the regression was paralleled with the increased expression of bcl-2 (Rubenstein *et al.*, 1997).

In a recent study, a significant correlation between the elevation of erbB2 and poor prognosis has been demonstrated in prostate cancer (Arai *et al.*, 1997). Although erbB2 levels were not always correlated with PSA levels, the increase of erbB2 indicated poor response to endocrine treatment. The patients with high levels of erbB2 had multiple bone metastases.

1.3.2. Epidermal growth factor (EGF)

EGF is low molecular weight (M_r =6045) polypeptide which consists of 53 amino acids (Savage *et al.*, 1972) and is a product of a gene located on chromosome 4. EGF was first identified from the mouse submaxillary gland that causes the opening of the eyelid of a new born animal (Cohen, 1962). Several reports have detected either immunohistochemically or biochemically significant amounts of EGF in a range of human tissues (Kasselberg *et al.*, 1985; Owens & Leake, 1992). Apart from the submandibular salivary gland, the different sites of synthesis include the gastrointestinal tract, anterior pitutiary, bone marrow, prostate, mammary gland, uterus, ovary and placenta. Administration of EGF to animals results in the hyperproliferation of various epithelial tissues and, in cell culture systems, EGF stimulates mitogenesis in a large number of cell types (Soler & Carpenter, 1990). Both *in vivo* and *in vitro* studies demonstrated that, apart from growth regulation, EGF may have profound effects on mechanisms involved in differentiation, motility (Rajan *et al.*, 1996) and function of target cells.

There are five other distinct mammalian gene products that can be identified as EGF-like molecules: Transforming growth factor-α, amphiregulin, betacellulin, crypto and heparin-binding EGF (Massague & Pandiella, 1993; Shing *et al.*, 1993; Shoyab *et al.*, 1989). These ligands are capable of binding to the EGF receptor.

1.3.3 Transforming growth factor-α

TGFα is a 50 amino acid peptide with a molecular weight of 6 kDa and is a product of a single gene located on chromosome 2. TGFα has significant sequence and structural homology with EGF, and shows similar biological actions and activities to EGF and interacts with the EGF receptor (Lyons & Moses, 1990). TGFα has the ability to bring transformation and anchorage

independence to normal cells (Ganesh *et al.*, 1994a). TGFα production has been demonstrated in a variety of human tumours and immortal cell lines, such as MDA-MB436 breast carcinoma, 7860 renal carcinoma, HeLa cervical epithelia carcinoma, A431 epidermoid carcinoma (Derynck *et al.*, 1987; Ganesh *et al.*, 1994a).

Although TGFα binding parameters to the EGFR and its biological effects are very similar to EGF, there are some differences. TGFα was found to be a more potent stimulator of keratinocyte migration and angiogenesis. Chicken EGFR also displayed much higher affinity for TGFα than EGF (see review Massague & Pandiella, 1993).

1.3.4 EGF and TGFα in prostate

Several studies demonstrated the expression of EGF in foetal prostate (Dahiya *et al.*, 1996) and in both normal and benign lesions of prostate and in prostate carcinoma (Amatetti *et al.*, 1993; GlynneJones *et al.*, 1996; Scher *et al.*, 1995). Some studies revealed no difference in the concentration of EGF and TGFα between BPH and prostate carcinoma tissue (Lloyd *et al.*, 1992; Yang *et al.*, 1993). Nevertheless, higher TGFα immunoreactivity was found in less differentiated prostate carcinoma than BPH (Harper *et al.*, 1993) and elevated levels of mRNA for one or both of the ligands were found in prostate cancer samples and in prostate cancer cell lines when compared to BPH (Ching *et al.*, 1993). In an immunohistochemical study, elevated expression of EGF has been found in a large proportion (68%) of prostatic tumours (Fowler *et al.*, 1988). No consensus exist about the involvement of EGF in prostate carcinoma.

The growth effect of EGF on normal prostate, BPH, and prostate cancer cell lines has been widely studied. EGF-induced cell proliferation has been demonstrated in normal prostate cells and BPH epithelial cells (Chopra *et al.*,

1996; Jones *et al.*, 1997b; Sutkowski *et al.*, 1992). In the absence of the growth factors, both normal and BPH cell growth was strongly reduced. It has been shown that EGF stimulates the growth of the PC3 (Carruba *et al.*, 1994a) and the DU145 cells (Jones *et al.*, 1997a). Not all authors report stimulation by EGF. Some prostate cancer cell lines were insensitive to the growth stimulatory effect of exogenous EGF (Chopra *et al.*, 1996; Jarrard *et al.*, 1994). It has been also demonstrated that the blocking of EGF-induced activation and induced internalization of the receptor by anti-EGFR ab significantly inhibited tumour progression of the well-established DU145 and PC-3 xenografts in nude mice (Prewett *et al.*, 1996).

It has been shown that the DU145 cell line secretes TGFα and EGF related polypeptides into conditioned medium (CM) (Connolly & Rose, 1990; MacDonald *et al.*, 1990). CM was shown to have a significant stimulatory effect on DNA synthesis. However, exogenous EGF and TGFα showed a little difference on DNA synthesis or cell proliferation in DU145 cells (Carruba *et al.*, 1994a; Connolly & Rose, 1990; MacDonald *et al.*, 1990). Therefore it has been suggested that the DU145 cell line produces its own growth factors and results in the cells being already stimulated maximally by endogenous growth factors (Connolly & Rose, 1990; MacDonald *et al.*, 1990) that CM either contains additional factors or presents EGF and TGFα in a more active form.

High levels of the EGFR, TGFα and EGF were found in the DU145 cell line (Carruba *et al.*, 1994a). Positive EGF immunoreactivity was also shown in the CM of the LNCaP cell line although secretion by DU145 was 100 fold higher (Connolly & Rose, 1990). The LNCaP cell line has also been demonstrated to secrete TGFα into CM and this secretion was shown to be increased by DHT treatment (Wilding *et al.*, 1989a).

Low levels of the EGFR, TGFα and EGF were found in the PC3 cell line in comparison to the DU145 cell line (Carruba *et al.*, 1994a). Exogenous EGF and anti-EGFR ab did not significantly affect the PC3 cell growth response (Jarrard *et al.*, 1994).

1.3.5 Tyrosine kinase activity inhibitors

Considerable progress has been made in understanding the role of tyrosine kinases in the uncontrolled growth of neoplastic cells in culture. Therefore tyrosine kinases and their signalling pathways have been identified as a potential target for drug design. Several kind of inhibitors have been determined to inhibit the cellular tyrosine kinase activity, including the bioflavinoids, such as quercetin, lavendustin A, herbimycin A and genistein. These compounds have been shown to be competitive inhibitors of ATP binding to TKs and are growth inhibitory to tumour cell lines (Levitzki & Gazit, 1995).

Genistein (5,7,4'-trihydroxyisoflavone), an isoflavinoid found in soy beans, has been shown to inhibit the proliferative growth of human breast carcinoma, prostate carcinoma, epidermoid carcinoma and leukemia cell lines *in vitro* (Barnes *et al.*, 1995). Akiyama and associates demonstrated that genistein is a highly specific inhibitor of tyrosine kinases but rarely inhibits the activity of serine-theronine kinases and other ATP analogue-related enzymes (Akiyama *et al.*, 1987). Inhibition of angiogenesis, induction of apoptosis and of cell differentiation, and inhibition of DNA topoisomerases by genistein have also been demonstrated (Barnes *et al.*, 1995).

Several type of synthetic tyrosine kinase inhibitors were developed, such as tyrophostins (Levitzki & Gazit, 1995; Oshevor *et al.*, 1993), 2-thioindoles (Fry *et al.* 1994a; 1994b) and quinazolines (Jones *et al.*, 1997a; Wakeling *et al.*, 1996). In one study, EGF stimulated the growth of the MCF-7 breast

carcinoma cell line which, in contrast, was inhibited by 4-anilinoquinazolines. The inhibitors showed no effect on either basal growth or insulin-like growth factor 1 (IGF1) stimulated growth (Wakeling *et al.*, 1996). In a recent study, Jones *et al.* (1997a) demonstrated that inhibition of EGFR specific tyrosine kinase activity by 4-anilinoquinazoline inhibits the growth of both the PC3 and the DU145 cell line.

Reddy *et al.* (1992) demonstrated that inhibition of tyrosine kinase activity resulted in inhibition of breast carcinoma cell lines (MCF7, MDA-231, ZR-75). In this study, the tyrosine kinase inhibitor RG-13022, inhibited not only EGF stimulated growth but also estradiol, IGF1 and insulin stimulated growth. The same inhibitor also inhibited the growth of PC3 and LNCaP cell lines (Kondapaka & Reddy, 1996). However, RG-13022 inhibited not only EGF and TGFα stimulated growth, but also FGF and serum stimulated growth. Androgen stimulated growth of LNCaP cell line was also inhibited by RG13022.

EGFR-related TK activity inhibition by 4,5-dianilinophthalimides (CGP 54211 and CGP 53353), (both are ATP competitive protein TK inhibitors), has caused an inhibition of the growth of human bladder cancer cells implanted in nude mice (Dinney *et al.*, 1997). In this study, inhibition of EGFR-related TK activity produced cytostasis *in vitro* of a metastatic human transitional cell carcinoma. Inhibition of EGFR-related tyrosine kinase activity has also inhibited the growth of pancreatic carcinoma cell lines (Gillespie *et al.*, 1993).

1.4.1 Transforming growth factor-ß (TGFß)

TGFß was originally discovered as a secreted factor that induced malignant transformation of non-neoplastic cells (Gitelman & Derynck, 1994). The TGFß family consists of five isoforms, TGFß1-5. Several other proteins are structurally closely related e.g. activins, inhibins, mullerian inhibiting substance and bone morphogenic protein (BMP) (Gitelman & Derynck, 1994). TGFß1 structure is prototypical for all members of the family. Structural analysis reveals TGFß1 to be processed from a precursor of 390 amino acid by proteolysis. The active form of TGFß is a 25 kDa disulphide-linked homodimer. Latent TGFß can be activated by treatment with heat and acid. Nevertheless, the physiological mechanisms have shown the involvement of proteases like plasmin and cathepsins in the activation of TGFß. TGFß is found in a broad variety of normal and tumour tissues and has been purified from platelets, kidney, bone and placenta (Waterfield, 1995).

1.4.2 Biological functions of TGFß

TGFß has complex multifunctional biological characteristics. TGFß generally inhibits the growth of epithelial cells and stimulates mesenchymal cells in normal tissues (Moses *et al.*, 1990). TGFß displays reversible inhibitor activity by arresting cells in the G1 phase of the cycle, in normal as well as transformed epithelial and endothelial cells (reviewed by Massague, 1990).

TGFß has been shown as an immunosupressive agent. It inhibits the proliferation and function of cytotoxic T cells, B cells and natural killer (NK) cells. Furthermore it inhibits cytokine production in lymphocytes and de-activates macrophage function (Massague, 1990). Inactivation of the TGFß gene results in multifocal inflammatory disease and massive lymphocytic infiltration in newborn mice (Shull *et al.*, 1992).

TGFß enhances synthesis and secretion of extracellular matrix proteins, decreases the synthesis of proteases such as metalloproteinases and increases production of protease inhibitor such as tissue inhibitor of metalloprotease (TIMP) and plasminogen activator inhibitor-1 (PAI-1) (Gitelman & Derynck, 1994; Laiho *et al.*, 1986). TGFß also has an important role in wound healing and tissue repair (Adzick & Lorenz, 1994). Increased level of fibronectin, collagen type 1, 2, 3, 4 and 10, thrombospondin and tenascin have been observed in response to TGFß (reviewed by Massague, 1990).

TGFß can induce formation of new blood vessels *in vivo* (Massague, 1990,Sankar *et al.*, 1996). When injected locally, TGFß induces the formation of granulation tissue and angiogenesis (Sankar *et al.*, 1996). However, the mechanism of the angiogenic effects of TGFß *in vivo* are controversial since TGFß is a potent inhibitor of endothelial cells. The widespread distribution of TGFß during development suggests that it also plays important role in embryogenesis (Gitelman & Derynck, 1994).

1.4.3 Transforming growth factor ß receptor

There are three TGFß receptors on the cell surface. These are type 1, 2 and 3 with apparent molecular weights of 55, 80 and 280 kDA, respectively. Type 1 and 2 bind TGFß1 and TGFß3 with higher affinity (Kd 25-50 pM) than TGFß2 (~ 500 pM) (Massague, 1990). Both receptor types 1 and 2 have a biologically active serine/threonine kinase domain. The type 3 receptor has a lower affinity for any TGFß isoforms than types 1 and 2. The type 3 receptor is a transmembrane proteoglycan and has a short cytoplasmic tail with no obvious signalling motif (Wang *et al.*, 1991).

Type 1 and type 2 receptors form a heterodimeric receptor complex. Both are essential for the anti-proliferation effect of TGFß. In the absence of type 2 and

presence of only type 1 receptor, TGFß1 fails to show any inhibitor capacity but still induces gene regulation (Chen *et al.*, 1993). Thus there is evidence that two separate TGFß-triggered signalling pathways are exist which are mediated by different signalling receptor complexes. In contrast, several studies have shown that homodimeric complexes of either type 1 or type 2 receptors alone are incapable of generating a cellular response including gene regulation (Feng *et al.*, 1995; Vivien *et al.*, 1995).

1.4.4 TGFß signal transduction

TGFß action has not been directly linked with the known signalling pathways, such as tyrosine phosphorylation, phosphatidylinositol turnover and modulation of intracellular cAMP levels (Knaus & Lodish, 1994; Massague, 1990).

A heteromeric complex of type 1 and 2 is formed when TGFß binds to the receptor. Type 1 receptor becomes phosphorylated on serine and threonine residues by the receptor 2 kinase. Once phosphorylated, receptor 1 is activated to signal downstream targets (Wrana *et al.*, 1994). Several groups have identified receptor interacting proteins such as TGFß receptor interacting protein (TRIP-1), farnesyl transferase-α, apolipoprotein J/clustrin and TGFß-activated kinase (TAK1) (Attisano & Wrana, 1996). Activation of TAK1 leads to induction of a typical TGFß-mediated transcriptional response.

Mothers against decapentaplegic (MAD) gene from drosophila has been implicated in signalling by the BMP subfamily (Liu *et al.*, 1996). MAD-related (MADR) proteins have been suggested as crucial intracellular mediators in the signalling by serine/threonine kinase receptors (Attisano & Wrana, 1996). The activation of serine/theronine kinase receptors leads to the rapid phosphorylation of MADR protein and its redistribution into the nucleus. Phosphorylated MADR proteins then may transmit signals by interacting with nuclear targets.

Human analogues of MAD genes (hMAD-3 and hMAD-4) activity was regulated by TGFß receptors (Zhang *et al.*, 1996). In this study, hMAD 1-4 genes were transfected in TGFß-unresponsive SW480.7 cells. hMAD-3 and hMAD-4 transfected cells showed an increase in PAI-1 secretion in response to TGFß while hMAD-1 and hMAD-2 did not.

Recently, it was demonstrated that the receptor for glial-cell-line-derived neurotrophic factor (GDNF), one of the most distantly related TGFß family members, have been shown to form a functional receptor complex with an orphan receptor tyrosine kinase, ret (Durbec *et al.*, 1996). This finding is the first example which identifies a relationship between two downstream pathways.

1.4.5 TGFß expression in prostate

TGFß has been found in human and rat prostate and seminal fluid. TGFß has been suggested to act as an autocrine modulator of the growth and differentiation of both human and rat prostate (Atfi & Samperez, 1994; Kyprianou & Isaacs, 1988; Shain & Lin, 1990).

TGFß appears to be a potent inhibitor of epithelial cells in normal prostate (Steiner, 1993). It has been shown that expression of TGFß and its receptor are regulated by androgens. Androgen deprivation of castrated rats resulted in an increase of TGFß receptor numbers in the prostate. Furthermore, androgen administration to the castrated animals decreased the number of TGFß receptors. Thus, TGFß receptor numbers seem to be under negative androgenic control (Kyprianou & Isaacs, 1988). Later studies demonstrated that there is an increased amount of the mRNA expression of TGFß after castration. *In vivo* administration of TGFß to the rat prostate resulted in the death of about 25% of prostatic glandular cells. A dose dependent relationship between the glandular

cell death and TGFß1 concentration was demonstrated *in vitro* in prostate organ cultures (Martikainen *et al.*, 1990). This observation implied that TGFß exhibits an important role in programmed cell death.

On the other hand, increased TGFß1 immunostaining was found in BPH compare to the normal prostate. The staining was mainly placed in the stroma rather than epithelium (Steiner, 1993) as would be expected if TGFß stimulates stromal cell growth.

TGFß expression in prostate carcinoma

Eklov *et al* (1993) demonstrated that there is no significant difference in the staining of TGFß in epithelial cells of normal prostate, BPH and prostate carcinoma. However, the latent-TGFß binding protein staining was positive in normal prostate and BPH tissues, prostate carcinomas were mostly negative. Immunohistochemical localisation of TGFß1 in prostate carcinoma was found to be in the tumour epithelium whereas in BPH TGFß1 was expressed by the stroma (Truong *et al.*, 1993).

TGFß1 mRNA and protein levels were shown to be much higher in rat prostate cancer compared to those in normal prostate (Steiner *et al.*, 1994). They have also demonstrated that the prostate cancer cells are capable of activating endogenously produced latent TGFß. A metastatic mouse model prostate carcinoma cell line secreted relatively high amounts of TGFß in comparison with primary prostate carcinoma cells. Although the metastatic cells were resistant to the inhibitory effect of TGFß, they showed a positive response to TGFß by inducing the secretion of type-4 collagenase matrix metalloproteinase-9 and PAI-1 (Sehgal *et al.*, 1996).

Both PC3 and DU145 cells possess high affinity binding sites for TGFß. Furthermore, both PC3 and DU145 cell lines secreted TGFß into conditioned medium and expressed mRNA for TGFß. However, LNCaP cells did not possess high affinity binding sites for TGFß and there was no detectable TGFß protein nor mRNA in the LNCaP cell line (Wilding *et al.*, 1989b). In a recent study, no difference was observed in the serum level of TGFß in a comparison of prostate carcinoma and BPH patients (Wolff *et al.*, 1997). However, the measured plasma TGFß may derive from damaged platelets. A 3.5 fold higher TGFß1 level found in prostate carcinoma patient urine compared with control group (Perry *et al.*, 1997).

1.4.6. Growth effect of TGFß in prostate carcinoma

The effects of TGFß in the growth regulation of prostate carcinoma, *in vivo* and *in vitro*, have been widely studied. Several studies have demonstrated bifunctional growth modulation of a rat prostate carcinoma cell line by TGFß. Most importantly, TGFß stimulated the cell proliferation at nanogram levels but inhibited at picogram levels (Shain & Lin, 1990). In a recent study, a cell density-dependent sensitivity to TGFß was shown in MATLyLu cells (Morton & Barrack, 1995). When the cells were plated at low density and in the absence of serum, cell growth was inhibited in response to TGFß. In the presence of serum (0.5%) or high density of the plated cells, cell growth was insensitivite to TGFß. Further addition of other growth factors and extracellular matrix components prevented TGFß inhibition (Morton & Barrack, 1995).

Subcutaneous inoculation of TGFß1-overproducing MATLyLu cells into rats produced 50% larger and more extensive metastatic disease than with control MATLyLu cells. However when the cells were cultured *in vitro* with TGFß1, they became growth inhibited and this inhibition was reversed by anti-TGFß1 ab. Nevertheless, this inhibition was transient and there was no net inhibition

(Steiner & Barrack, 1992). It was speculated that poorly differentiated rat prostate carcinoma cells appeared to be resistant to the growth inhibitory effect of TGFß (Steiner *et al.*, 1994). Many carcinoma cells loose their sensitivity to the inhibitory effects of TGFß (Moses *et al.*, 1990; Sehgal *et al.*, 1996). Wilding *et al* demonstrated that although exogenous TGFß administration revealed an initial inhibition in the growth of the PC3 and DU145 cell line, the cells eventually returned to the control levels despite the presence of TGFß (Wilding *et al.*, 1989b). This finding paralleled the findings of Steiner & Barrack (1992).

Although the growth resistance of prostate carcinoma cell lines to TGFß have been demonstrated, there is a large body of literature supporting the growth inhibitory effect of TGFß. Desruisseau *et al* (1996) also demonstrated a 40 % inhibition of the PC3 and DU145 cell lines by TGFß. The growth inhibitory effect of TGFß on PC3 and DU145 cell lines was accelerated by interferon (Goldstein *et al.*, 1991). Carruba and his colleagues illustrated an inhibition of PC3 cells in response to exogenous TGFß (Carruba *et al.*, 1994a). Their later studies showed that the inhibition of PC3 cells by estradiol was accompanied by the increase of TGFß secretion. Addition of anti-TGFß antibody to the medium resulted in a 3 fold increase in cell growth but addition of estradiol restored growth inhibition (Carruba *et al.*, 1994b).

TGFß inhibited the EGF stimulated growth of both a rat prostate carcinoma cell line (Steiner *et al.*, 1994) and human (LNCaP) prostate cancer cell lines (Schuurmans *et al.*, 1988a). Nonetheless, neither basal nor androgen stimulated growth of the LNCaP cell line was affected by exogenous TGFß (Schuurmans *et al.*, 1988a; Wilding *et al.*, 1989b).

Dexamethasone induced growth inhibition of PC3 cells which was accompanied by an increase of TGFß1 mRNA expression. Addition of anti-TGFß ab to the medium resulted in increased cell growth (Reyes Moreno *et al.*, 1995). Bang *et al* (1992) demonstrated that an increased cAMP levels, which inhibited cell growth, increased TGFß2 production and secretion in the PC3 cell line. A human prostate carcinoma cell line (ALVA-101) showed no response to the exogenous TGFß treatment (Meikle *et al.*, 1997). However, addition of anti-TGFß receptor 1 and 2 antibodies exerted a growth stimulatory effect suggesting some residual effect of endogenous TGFß.

The hormone-responsive breast carcinoma cell line, MCF7, secretes TGFß into the medium and this secretion is increased by antioestrogens (Knabbe *et al.*, 1987). Knabbe *et al* (1993) further demonstrated that androgen withdrawal leads to a significant increase in TGFß2 mRNA expression in the LNCaP cell line. Androgen deprivation resulted in an increase of TGFß1 gene expression in the PC82 hormone-responsive prostate carcinoma cell line (Kyprianou *et al.*, 1990). Kim *et al.* (1997a) have demonstrated that immunotherapy using TGFß1 antisense transfected tumour vaccine can successfully treat rat prostate cancer. Transfection of MATLyLu cells with the antisense TGFß1 oligonucleotide has been shown to suppresses TGFß1 protein expression by 60 %. In their study, after inoculation of intact cells into mice, animals were injected with TGFß1 antisense vaccine for 7 weeks. After the treatment, 75% of treated mice were alive without no palpable tumour, however none of the control mice were alive.

1.4.7. TGFß receptors in prostate

The presence of high-affinity binding sites for TGFß were reported in both DU145 and PC3 cells, but not in the LNCaP cell line (Carruba, 1994). Kim *et al.* (1996a) showed that prostate carcinoma frequently lost type 1 and type 2

receptors in contrast to the benign prostate tissues. This study demonstrated a correlation between the loss of receptor type-1 and type-2 expression and increasing Gleason score in prostate carcinoma tissues. Recently a negative correlation between the tumour stages and TGFß type 2 receptor has been demonstrated (Williams *et al.*, 1996). Another study also demonstrated that loss of the type-1 receptor significantly correlates with tumour stage, 5-year survival rate and recurrence of the disease (Kim *et al.*, 1997b). They also illustrated that the type 1 and type 2 receptor are present in PC3 and DU145 cells and these two cell lines are sensitive to the inhibitory effect of TGFß. In this study, LNCaP cells expressed only the type 2 receptor and were insensitive to the inhibitory effect of TGFß. After transfection of the type 1 receptor, the LNCaP cell line became TGFß sensitive (Kim *et al.*, 1996b).

Several studies have shown the presence of other TGFß-related proteins in prostate carcinoma. It has been shown that the DU145 cell line expresses activins and their receptors (Furst *et al.*, 1995). A recent study illustrated that PC3 expresses activin mRNA, protein and receptor (Ying *et al.*, 1995). Both studies displayed the absence of inhibins in the DU145 and PC3 cell line. It has been also shown that PC3, DU145 and LNCaP cell lines displayed mRNA for bone morphogenic protein (Harris *et al.*, 1994).

1.5 Proteolysis

Cancer cell invasion usually involves the localised proteolysis of the surrounding extracellular matrix. This proteolysis is associated with the secretion of lytic enzymes, such as the matrix metalloproteases, collagenases, plasminogen and cathepsins (Vile, 1995).

1.5.1 Plasminogen activator system

Plasminogen

Plasminogen is an inactive proenzyme consisting of a single polypeptide chain with a molecular weight of 92 kDa. Plasminogen can bind to specific receptors through lysine binding sites located in the kringle domain (Masucci & Blasi, 1990). The native form of plasminogen has glutamic acid at the N-terminus and is called Glu-plasminogen. It is converted to a two chain active enzyme, Glu-plasmin, by plasminogen activator (PA). PA catalyses the cleavage of a single polypeptide bond (Arg_{560}-Val_{561}) (Robbins *et al.*, 1967). PA can also catalyse the conversion of Lys-plasminogen which is another inactive form of Glu-plasminogen converted by plasmin, into active Lys-plasmin (Dano *et al.*, 1985).

Plasmin

Plasmin is a two polypeptide chain serine protease derived by plasminogen activation. The two chains are linked by disulfide bonds. The light chain contains the active site and its amino acid sequence has homologue to other serine proteases such as trypsin and chymotrypsin. Plasmin hydrolyses proteins and peptides at their lysyl and arginyl bonds (Dano *et al.*, 1985). Plasmin has a very broad substrate specificity which can itself degrade matrix proteins like fibronectin, laminin, basement membrane and proteoglycans. Plasmin is also able to convert two other zymogens namely pro-uPA and pro-

collagenase to their active forms (Masucci & Blasi, 1990). Plasmin is inactivated by α_2-antiplasmin and α_2-antimacroglobulin (Travis & Salvesen, 1983).

1.6. The component of plasminogen activation system

1.6.1 The plasminogen activators

There are two types of plasminogen activators, urokinase-type plasminogen activator (uPA) and tissue-type plasminogen activator (tPA). These activators have different molecular weights and immunological reactivity (Dano *et al.*, 1980) and are the products of different genes (Edlund *et al.*, 1983).

Tissue-type plasminogen activator

Tissue type plasminogen activator is found in most normal tissues and in blood. The molecular weight for tPA is 70 kDa and plasminogen is the only substrate for tPA. Unlike uPA, tPA has a strong affinity for fibrin (Hoylaerts *et al.*, 1982). Fibrin was found to strongly induce PA activation by tPA. It was demonstrated that anti-tPA ab inhibited the fibrinolytic activity detected in the endothelium (Rijken *et al.*, 1980). The increase of tPA in blood after venous occlusion (Prowse *et al.*, 1984), the presence of tPA but not uPA in endothelial cells (Rijken *et al.*, 1980) and induction of tPA activity but not uPA by fibrin (Tran-Thang *et al.*, 1984) have all pointed to tPA rather than uPA being a key enzyme in physiological thrombolysis.

Urokinase-type plasminogen activator (uPA).

uPA was originally identified in urine. uPA was also identified in blood plasma and it has been reported the purification of uPA from human seminal plasma (Propping *et al* (1978) and from malignant prostate tissue (Kirchheimer *et al.*, 1984). uPA is produced by kidney tubule cells, phagocytic cells, pneumocytes,

fibroblasts and tumour cells. uPA is a 52 kDa glycoprotein and is released as a single-chain proenzyme, pro-uPA. The serine proteases, plasmin, plasma kallikrein and trypsin, and the cathepsins B and L cleave pro-uPA at the peptide bond Lys $_{158}$-Ile$_{159}$ (Schmitt *et al.*, 1992). This cleavage converts pro-uPA into the enzymatically active high-molecular-weight two chain form, HMW-uPA. The two polypeptide chains are linked by disulfide bonds. The A-chain contains 158 aa (1-158) with a M_r 20 kDa and the B-chain contains 252 aa (158-411) with a M_r 32 kDa (Schmitt *et al* 1992; Schmitt *et al.*, 1989). Additional cleavage of the A-chain of HMW uPA results in low molecular weight uPA, LMW-uPA and an amino terminal fragment. This cleavage occurs at peptide bond Lys$_{135}$-Lys$_{136}$ in the presence of plasmin.

Aminoterminal fragment (ATF) of uPA: ATF (135 aa with M_r 16 kDA) consists of a "kringle" structure in its carboxy-terminal, and the growth-factor-like domain (GFD) in its N-terminal. The kringle domain contains three disulfide bonds and characteristic amino acid sequences found within prothrombin and plasminogen (Dano *et al.*, 1985). GFD contains a cysteine rich sequence and binds to the uPA-receptor, uPAR (Schmitt & Janicke, 1992).

Low-Molecular-Weight uPA (LMW-uPA): LMW-uPA contains 276 aa with a M_r 34 kDA. LMW-uPA does not bind the receptor. The N-terminal portion of LMW-uPA has a similar aa sequence to other serine proteases (Dano *et al.*, 1985; Schaller *et al.*, 1982). Pro-uPA, HMW-uPA and ATF bind to the receptor via the GFD (Appella *et al.*, 1987). Pro-uPA, HMW-uPA, ATF domain and LMW-uPA are shown in figure 1.2.

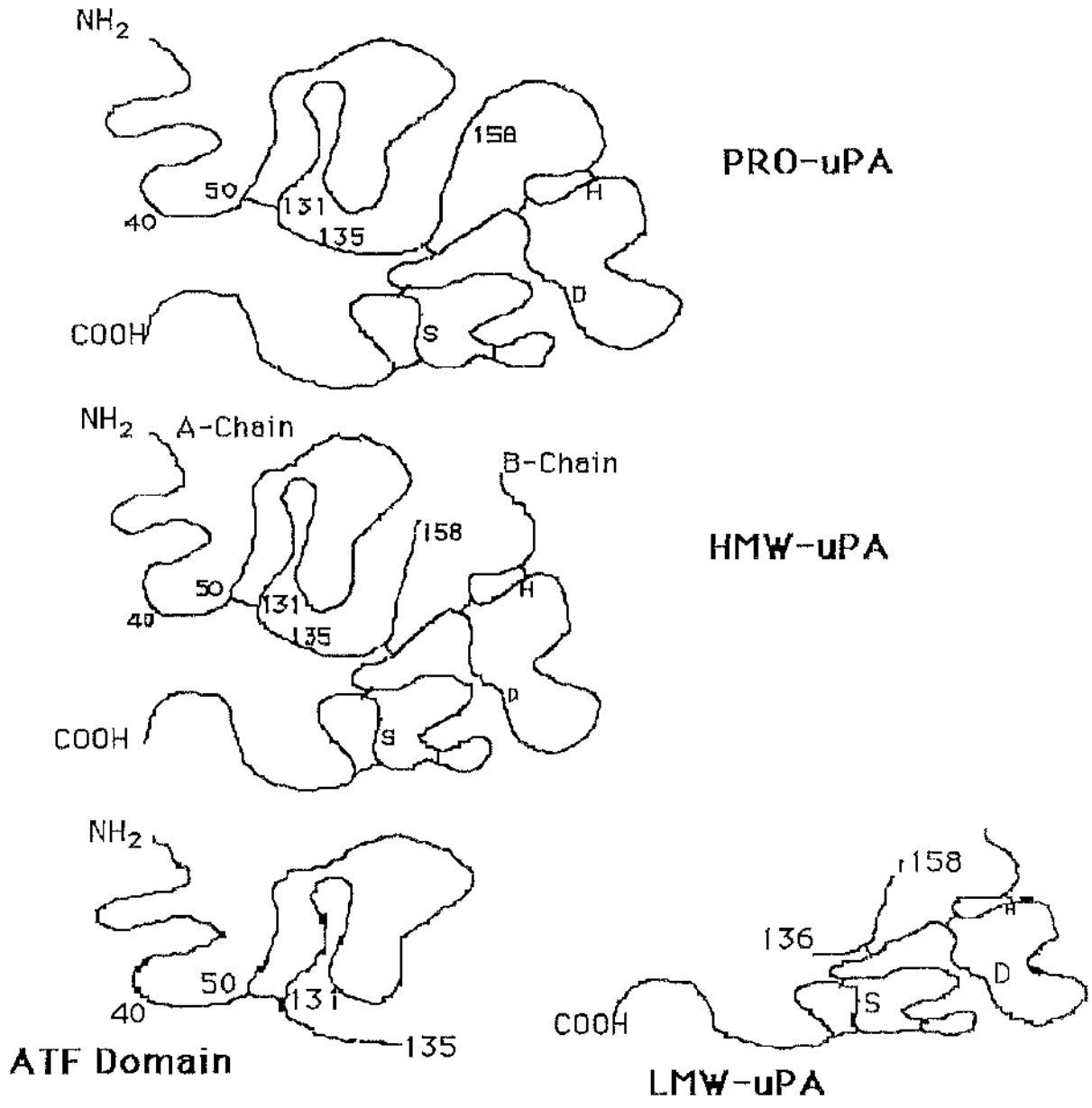

Fig 1.2 The forms of uPA: The pro-uPA is synthesised by cells and is cleaved at the bond Lys^{158}-Ile^{159} by the serine proteases and cathepsins. Active uPA (HMW-uPA) consists of two-chains (A-chain and B-chain) and the A-chain is further cleaved by plasmin at the bond Lys^{135}-Lys^{136}. This cleavage yields the LMW-uPA two chain form of uPA, LMW-uPA and the amino terminal fragment, ATF. ATF consists of the kringle domain and the growth-factor like domain. The figure is adapted from (Schmitt *et al*, 1992).

1.6.2 Urokinase-type plasminogen activator receptor (uPAR)

The presence of the specific binding site for uPA was shown by Vassalli *et al.* (1985). uPAR was first purified from a leukemia cell line by Nielsen *et al.* (1988). uPAR is a 282 aa cysteine-rich glycoprotein of M_r 45-55 kDa. uPAR is attached to the plasma membrane glycolipids via a covalent linkage (Ploug *et al.*, 1991). uPAR is an extracellular molecule, containing a transmembrane domain and a very short intracellular domain (Masucci & Blasi, 1990).

uPAR is present on fibroblasts, monocytes, leukemia cells and on tumour cells derived from solid cancer tissues (Schmitt *et al* 1992; Stoppelli *et al.*, 1986). uPAR binds HMW-uPA, ATF, pro-uPA (but not LMW-uPA) with Kd $\sim 10^{-10}$ M. The receptor is specific for uPA and does not bind EGF, tPA, factors IX and X, plasminogen or thrombin (Blasi *et al.*, 1986). Using the chorioallantoic membrane system, Ossowski found that uPAR was necessary in addition to uPA for tumour cell invasion (Ossowski, 1988).

1.6.3 Plasminogen activator inhibitors

The other components of the PA system are fast-acting plasminogen activator inhibitors, PAI-1 (Cajot *et al.*, 1990), PAI-2 and protease nexin-1 (Low *et al.*, 1981).

PAI-1, the primary PA inhibitor of human plasma, is found in platelets, and is synthesised and secreted by a variety of cells in culture (Erickson *et al.*, 1985). PAI-1 is a 379 aa glycoprotein and is secreted from the cells as a Mr 52kDa glycoprotein (Stephens & Vaheri, 1994a). PAI-1 forms a 1:1 covalent complex with either uPA or tPA (Kobayashi *et al.*, 1994). PAI-1 is able to inhibit uPA mediated proteolysis by tumour cells in vitro (Cajot *et al.*, 1990). Andreasen *et al* demonstrated that PAI-1 inhibited human u-PA and tPA, but not plasmin (Andreasen *et al.*, 1986). PAI-1 quickly loses its activity after secretion.

Vitronectin binds PAI-1 in plasma and in cell culture and this interaction stabilises PAI-1 (Stephens & Vaheri, 1994a).

PAI-2 is present in two forms, a 47 kDa intracellular form and a 60 kDa (glycosylated) form present in the extracellular matrix. PAI-2 remains active in solution and its reaction is slower with tPA, unlike PAI-1 (Stephens & Vaheri, 1994b). In contrast to PAI-1, receptor bound PAI-2 does not internalise (Ragno *et al.*, 1995).

1.7 The mechanism of plasminogen activation

The activation of plasminogen by uPA is simply illustrated in fig. 1.3 adapted from Vile (1995). uPAR is synthesised in the cell and is transported to the cell surface. The extracellular serine proteases convert the uPAR bound pro-uPA into its active form. Active, cell-surface bound uPAR-uPA catalyses the activation of plasminogen to plasmin. Cell surface bound uPAR-uPA complex also catalyses the conversion of other zymogens (such as matrix metalloproteinases). The complex also has the ability to degrade stroma itself (Vile, 1995). Active uPA and plasmin have the potential to generate a powerful proteolytic cascade which degrades the extracellular matrix (Billstrom *et al.*, 1995a). Binding of the specific inhibitor of uPA, PAI-1, to the complex results in inactivation of the uPAR-uPA complex and formation of a uPAR-uPA-PAI complex on the cell surface. This complex is further internalised into the cell. Inside the cell, the uPA-PAI complex is released for degradation and uPAR is recycled to the surface for further proteolysis.

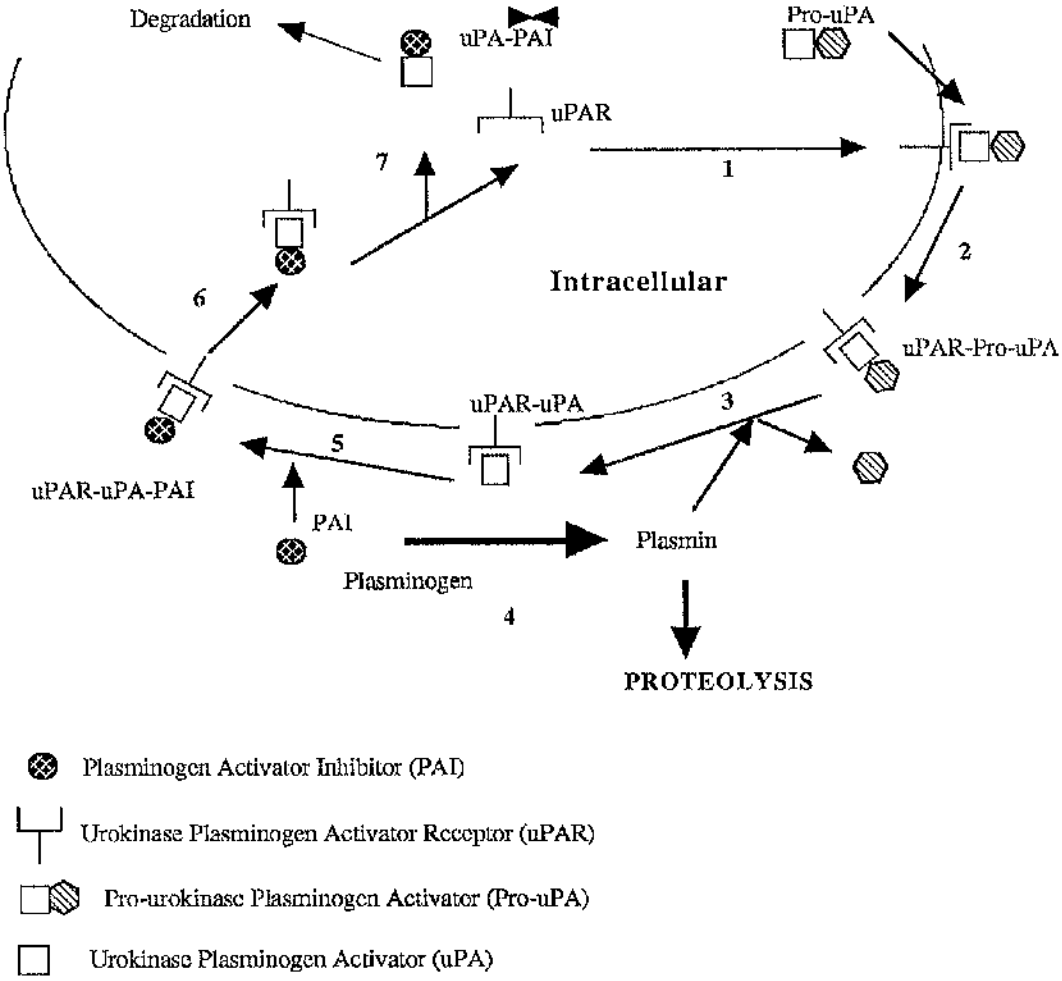

Fig. 1.3 The plasminogen activation mechanism by uPA, leading to focal proteolysis.uPAR is synthesised and/or recycled and transported to the cell surface (1), pro-uPA binds to uPAR and form a pro-uPA-uPAR complex (2), cleavage of pro-uPA by extracellular proteases and release of uPA-uPAR complex (3), plasminogen activation by receptor-bound uPA (4), the inhibition of active uPA-uPAR complex by binding PAI's (5), the receptor-inhibitor complex is internalised into the cell (6) where the uPA-PAI is released for degradation (7). The figure is adapted from the book Cancer Metastasis: From Mechanisms to Therapies edited by Richard G. Vile (1995).

1.8 Plasminogen activator components in carcinoma

It is widely accepted that uPA is a strong and independent prognostic marker in breast cancer and a possible prognostic marker for axillary node-negative patients (Duffy *et al.*, 1994; Duggan *et al.*, 1995; Ferno *et al.*, 1996). uPA has been suggested as a stronger prognostic marker than tumour size or estrogen receptor status in breast carcinoma (Duffy *et al.*, 1992; Duffy *et al.*, 1990). High uPA antigen levels were also prognostic for poor overall survival of patients with colorectal cancer (Ganesh *et al.*, 1994a; Schmitt *et al* 1992; Sim *et al.*, 1988). Elevated expression of uPA protein has been found in malignant oral squamous cell carcinoma tissues (20 times higher than benign tissues) (Zeilhofer *et al.*, 1997).

uPAR also appears to be a prognostic marker in certain cancers. Several studies illustrated that high levels of uPAR correlate with poor prognosis in breast carcinoma (Duggan *et al.*, 1995), in colorectal cancer (Ganesh *et al.*, 1994b) and squamous-cell lung cancer (Pedersen *et al.*, 1994). Expression of uPAR by disseminated carcinoma cells in bone marrow of patients with gastric cancers, was illustrated as a significant predictor of early disease relapse (Heiss *et al.*, 1995). Ganesh *et al.* (1994a) have also demonstrated a positive correlation between PAI-2 expression and poor prognosis in colorectal carcinoma. In contrast, the positive correlation of increased PAI-2 level and good prognosis was observed in breast carcinoma (Foekens *et al.*, 1995). It has been shown that low levels of PAI-2 in breast tumours correlate with shorter disease-free survival (Bouchet *et al.*, 1994).

Not only high uPA expression but also high PAI-1 antigen content have been suggested as poor prognostic factors. Elevated expression of PAI-1 protein has been found in malignant oral squamous cell carcinoma tissues (6 times higher than benign tissues) (Zeilhofer *et al.*, 1997). Janicke and associates showed

that breast carcinoma patients with high PAI-1 antigen levels in their primary tumour have a significantly higher relapse rates than patients with a low PAI-1 content (Janicke *et al.*, 1991). This contradictory situation has implicated the importance of the inhibitors in the reimplantation of circulating tumour cells at metastatic sites (Schmitt *et al* 1992). This reimplantation of metastatic cells requires the blockade of uPA-mediated degradation of the extracellular matrix. Schmitt suggested that the formation of new tumour stroma gives extra support to the generation and growth of metastatic cells. This finding was further supported by showing a significant correlation between S-phase values and PAI-1 levels in breast carcinoma (Schmitt *et al* 1992). A strong correlation between increased levels of PAI-1 and poor prognosis was also illustrated by Bouchet *et al.* (1994) in breast tumours. In a recent study, PAI-1 was found to be a much stronger prognostic indicator than uPA and uPAR in breast carcinoma (Grondahl-Hansen *et al.*, 1997). A significant correlation was demonstrated between uPA, PAI-1 and tumour-associated angiogenesis (Hildenbrand *et al.*, 1995). The correlation of PAI-1 expression and metastatic behaviour was reported in a nude mouse model (Quax *et al.*, 1991). In another study, no difference was observed in the metastasis of melanoma cells in mice over-expressing PAI-1 compared with control mice. Nevertheless, increased expression of PAI-1, by the stable transfection of PAI-1 cDNA to the PC3 cell line, impaired its ability to metastasize in nude mice. Enhanced expression of PAI-1 reduced the density of tumour-associated microvasculature and resulted in an inhibition of lung and liver metastasis (Soff *et al.*, 1995).

Plasminogen activators in prostate

PA has been implicated in tissue remodelling processes such as ovulation (Strickland & Beers, 1976) and in the involution of hormone target tissues such as the rat prostate (Andreasen *et al.*, 1990b; Freeman *et al.*, 1990; Rennie *et al.*, 1984), mammary gland (Ossowski *et al.*, 1979) and rat uterus (Shimada *et al.*,

1985). Deprivation of androgens, by castrating the animals, resulted in an increased expression of plasminogen activity (Andreasen *et al.*, 1990b; Rennie *et al.*, 1984). The increased activity was mainly uPA activity and the inhibition of this activity blocked the involution process of rat prostate (Andreasen *et al.*, 1990b).

Higher uPA activity has been shown in primary prostate carcinoma tissue cultures compared with BPH tissue culture (Festuccia *et al.*, 1995). The authors have also demonstrated higher activity in metastatic prostate carcinoma lesions than in non-metastatic. In another study, 300-fold higher uPA levels were found in the invasive prostate xenograft DU145 when compared with the tumour homogenates of 1013L, a primary human prostate cancer cell line (Billstrom *et al.*, 1995b). Furthermore, although there was no detectable uPA antigen in the plasma of the primary tumour xenograft, the DU145 cell xenografts expressed detectable levels of uPA antigen in plasma. Based on a chromogenic substrate assay, DU145 cells secreted uPA activity at five times more than normal human prostatic epithelium (Waghray & Webber, 1995).

There are several studies on the plasminogen activator contents in prostate carcinoma cell lines. uPA activity and protein levels were correlated with the aggressiveness of the metastatic prostate carcinoma cell lines (Gaylis *et al.*, 1989; Gaylis *et al.*, 1987; Hoosein *et al.*, 1991; Lyon *et al.*, 1995). uPA protein and mRNA expression were found to be undetectable in the LNCaP cell line (Lyon *et al* , 1995). In this study, although PAI-1, uPAR, tPA mRNA transcripts were weakly present in the LNCaP cells, no protein of these components were present. PAI-2 protein and transcripts were also absent in the LNCaP cell line. This finding correlated with the *in vitro* invasion of matrigel by the LNCaP cell line. Less than 0.25% LNCaP cells invaded the matrigel

(Hooscin *et al.*, 1991). The authors illustrated that there were less than 2000 uPAR sites/per cell present in the LNCaP by ligand-binding assay.

It has been shown that uPA plays an important role in the invasion of prostate carcinoma cells and it provides a marker of the aggressive phenotype (Gaylis *et al.*, 1989). Gaylis *et al* have shown that a metastatic component of PC3 tumour (PC3-CALN) has shown higher PA activity than the primary PC3 tumour. PC3-CALN has also greater PA secretion than its parental PC3 cell line. A statistically significant inhibition of Boyden chamber invasion by anti-uPA monoclonal antibodies was demonstrated in cell lines TSU-PR1 and PC3 (Jarrard *et al.*, 1995). The authors then suggested a direct role for uPA in the invasion by prostate cancer. The treatment of prostate cancer cells with N-(4-hydroxyphenyl) retinamide has been illustrated to inhibit *in vitro* cellular adhesion and motility of TSU-PR1 (29%) and PC3 (28%) cell lines (Kim *et al.*, 1995). This inhibition was paralleled with the decrease of uPA's proteolytic activity. The inhibition of in vitro invasiveness by the prostate carcinoma cell line by uteroglobin has also been demonstrated (Leyton *et al.*, 1994). In this study, the DU145 cell line was the most invasive cell line followed by PC3 and LNCaP, with very low invasive potential.

Some studies have demonstrated that inhibition of uPA activity results in both decreased of invasion *in vitro*, and inhibition of tumour growth in prostate cancer xenografts. A competitive inhibitor of uPA, p-aminobenzamidine, caused a dose-dependent inhibition of uPA activity and a dose-dependent decrease in uPA production in the DU145 cell line. P-aminobenzamidine treated mice, inoculated with DU145 cells, showed a 59% decrease in uPA production. Decrease in the uPA production was also correlated with a clear decrease in tumour-growth rate compared to the non-treated mice (Billstrom *et al.*, 1995a). These results were also supported by Jankun and associates, showing inhibition

of prostate cancer xenografts by amiloride and p-aminobenzamidine treatment. (Jankun *et al.*, 1997). Small molecule inhibitors such as Amiloride, p-aminobenzamidine show their inhibitory effects by blocking the active site of uPA. This active catalytic site of uPA is also the binding site for PAI-1 (Jankun *et al.*, 1997)

1.9 The regulation of plasminogen activators

In endothelial cells, tumour necrosis factor (TNF) upregulates uPA at both mRNA and protein levels (Niedbala & Stein-Picarella, 1992). No difference in tPA and PAI-1 antigen levels were noted in endothelial cells after treatment with TNF. Both expression and activity of uPA were also upregulated in response to basic fibroblast growth factor (Gualandris & Presta, 1994).

Increased uPA secretion in response to TGFα was shown in cultured human keratinocytes (Jensen & Rodeck, 1993) and in granulosa cells (Lafrance *et al.*, 1993). EGF also caused a dose-dependent increase in uPA activity in the A431 tumour cell line whereas there was no difference in tPA production (Niedbala & Sartorelli, 1989). In a lung carcinoma cell line, A549, EGF and the tumour promoter phorbol 12-myristate 13-acetate (PMA) caused an increase in uPAR transcription (Lund *et al.*, 1995). EGF increased the secretion and activity of uPA in the MDA-MB 231 breast cancer cell line, and this finding was correlated with increased expression of uPA mRNA (Long & Rose, 1996). EGF also increased uPA gene expression in the PC3 cell line and this increase correlated with the elevated invasive capacity of the cell line (Liu & Rabbani, 1995). The same study also revealed a decrease in uPA secretion and invasiveness of the cell line in response to the dexamethasone. The secretion of PAI-1 is stimulated by EGF, TGF-ß, glucocorticoids and thrombin (Andreasen *et al.*, 1990a). Chemomigration of TSU-PR1 cells across Boyden chambers was stimulated by

EGF and adding anti-EGF ab resulted in the inhibition of cell migration (Zolfaghari & Djakiew, 1996).

TGFß was shown to be an inducer of PAI-1 protein (Albo *et al.*, 1994). Increased uPA and PAI-1 levels were shown in rat osteoblasts in response to TGFß. However a decrease in uPA activity was observed (Allan *et al.*, 1991). Exposure of macrophages to TGFß1 led to a rapid and sustained increase in the levels of uPA mRNA and this increase was reversed by a protein kinase C inhibitor (Falcone *et al.*, 1993). Elevated levels of both uPA and PAI-1 were also demonstrated in SV-40 immortalised bronchial epithelial cells in response to TGFß1. The net effect of TGFß1 on plasminogen activator activity in the medium was inhibited by 50 % (Gerwin *et al.*, 1990). Treatment of endothelial cells with TGFß inhibited uPA production, upregulated PAI-1 and had no effect on uPA receptor levels (Mignatti *et al.*, 1991). In another study with endothelial cells, TGFß stimulation of PAI-1 protein was seen together with increased uPA activity (Sankar *et al.*, 1996). However TGFß reduced PA activity in granulosa cells (Lafrance *et al.*, 1993). Both primary and metastatic mouse prostate cancer cells showed a response to TGFß by inducing PAI-1 (Sehgal *et al.*, 1996). Increased intracellular cAMP level, using cAMP analogs or forskolin, resulted in a decreased level of uPA protein and mRNA expression in PC3 cell line (Wu *et al.*, 1997).

Tyrosine kinase activity and plasminogen activators

A positive correlation has been found between TK activity and uPA in breast carcinoma (Romain *et al.*, 1994). Hepatocyte growth factor/scatter factor (HGF/SF) also stimulated the synthesis of uPA and uPAR in the human leiomyosarcoma cell line SK-LMS-1 via the Met tyrosine kinase signalling pathway. HGF/SF-Met signalling also enhanced its *in vivo* tumorigenicity (Jeffers *et al.*, 1996). In a recent *in vivo* study, the inhibition of EGFR-related

tyrosine kinase activity inhibited the invasion of normal brain tissue by glioblastoma cells in rat brain (Penar *et al.*, 1997). In this study genistein, tyrophostin A25 and an anti-EGFR antibody were used for inhibition of the tyrosine kinase activity.

1.10 Aims of the thesis

The present thesis was designed to examine

(i) the involvement of exogenous EGF and TGFß on prostate carcinoma cell growth.

(ii) the involvement of EGF and TGFß on prostate carcinoma cell invasion

To achieve these aims, I have used the PC3 and DU145 cell lines, which serve as a model for human metastatic and androgen-independent prostate carcinoma. I firstly checked the presence of functional EGF binding sites. After that I checked the growth sensitivity of the cells to EGF, TGFß, EGFR-related TK inhibitor and steroids. Then the research concentrated on the regulation of PA components at the transcriptional and translational levels by the local growth factors. In order to further elucidate these interactions, the changes in uPA and inhibitors, uPA enzyme activity and matrigel invasion of the cells were investigated under exposure to growth factors.

MATERIAL and METHODS

2.1 Materials

2.1.1. Radiochemicals:

ICN International	[γ-^{33}P]ATP
Surrey, UK	
Vienna Lab	^{125}I EGF
Vienna, Austria	
EG&G Berthold	Optiflow (scintillation fluid)
UK	

2.1.2. Tissue culture plastic ware:

Costar Co	Filters (0.22 μm)
U.S.A	Falcon tissue culture flasks (25, 75, 150cm^2)
	24 well plates
	96 well Tissue culture plates
	96 well ELISA plates
	Transwell chambers
	Tissue culture pipettes
Gibco/BRL	Chamber Slides
Paisley, UK	
Medicell International	Dialysis Membrane
London, UK	
Nunc	Cryotubes
InterMed, U.K.	80 cm^2 tissue culture flasks
Whatman Ltd.	Whatman 3 MM Paper
Maidstone, U.K.	Whatman 0.2 mM nitrocellulose paper
Nalgene	0.2 μm Bottle top filters

2.1.3. Tissue culture medium:

Gibco/BRL	RPMI-1640 medium with 25mM
Paisley, UK	HEPES
	Foetal bovine serum
	Glutamine (200 mM)
	Penicillin (10 000 IU/ml)/Streptomycin
	(10 ng/ml)
	Sodium bicarbonate (7.5 %)
	Trypsin (0.25 % w/v)
Sigma	Phenol-red free RPMI-1640
Poole, Dorset, U.K.	

2.1.4. General reagents:

Chemical compounds:

BDH, MERCK Ltd	β-Mercaptoethanol
Glasgow, U.K.	Charcoal (Norit Gsx)
	HEPES
	Methanol
	SDS (Sodium dodecyl sulphate)
Fisher Scientific	Tween 20
Loughborough, U.K	DMSO
	Glycerol
	Sodium acetate
	Sodium carbonate
	Sodium chloride

	Sodium hydrogen carbonate
	Triton X-100
	EDTA
	Calcium chloride
	Potassium chloride
	Magnesium chloride
	Glycine
	Sulphuric acid
	Hydrochloric acid
	Acetic acid
Boehringer	Tris HCl
Lewes, E. Sussex, U.K.	

Biochemical reagents:

American Diagnostica	Mouse anti-uPA ab (Clone No # 394)
Alpha Laboratories	Mouse anti-PAI-1 ab (Clone No # 3785)
Amersham International	Biotrak EGFR-Tyrosine Kinase
Amersham, Buckinghamshire, U.K.	activity detection kit
Bio-rad Laboratories Ltd	Bradford dye reagent
Hertfordshire, U.K.	
BRL	Pre-stained molecular weight markers
Paisley, Scotland	
Calbiochem	Pansorbin
Cambridge, U.K.	uPA
Dako	OPD tablets
Denmark	Monoclonal mouse anti-cytokeratin ab
ICN International	Plasminogen (human)

UK	
Qudratech	S-2251, H-D-Val-Leu-Lys-pNA.2HCL
Surrey, UK	(Chromogenic substrate for plasmin)
R&D System	EGF (Recombinant human)
	TGFß1 (Purified from human platelets)
Sigma	l-dihydrotestosterone
Poole, Dorset, U.K.	Testosterone
	MTT
	Plasminogen
	BSA
	Haematoxylin
	Dithiothreitol
	Dextran
	TEMED
	Trypan Blue (0.4 % w/v)
	HRP conjugated anti-rabbit IgG
	DNA (calf thymus) type XV
	Hoechst 33258
	Ribonuclease A
Gift from Dr. Anneke Geurts	Sheep anti-chicken IgG
Nijmegen, Netherland	Chicken anti-uPA IgG
	Chicken anti-PAI-1 IgG
	Chicken anti-tPA IgG
	Rabbit anti-uPA IgG
	Rabbit anti-PAI-1 IgG
	Rabbit anti-tPA IgG
	uPA standard (Grunenthal)
	PAI-1 standard (Peter Andreasen)

	tPA standard (Actilyse)
Gift from Dr. Susan Jamieson	ALP conjugated goat anti mouse
Western Infirmary, Glasgow	IgG (Dako)
	New Fuchsin Substrate kit
	Normal Goat Serum (Sigma)
	Normal Human Serum (Sigma)
	Mouse anti-cytokeratin ab (Dako)
Gift from Dr. Collin Wilde	EHS
Hannah Research Institute,	
Ayr Scotland	

2.1.5. Molecular Biology reagents:

Pharmacia Biotech	First strand cDNA synthesis kit
Herts, U.K.	
Sigma	Agarose (high melting temperature)
Poole, Dorset, U.K.	DEPC
	Ethidium bromide
	Tri-reagent
Promega	Deoxyribonucleoside triphosphates
Southampton, U.K.	$MgCl_2$
	Phix174 DNA/Hae III Markers
	Taq buffer
	Taq polymerase

2.1.6 Equipment:

Progene	PCR amplification machines
Cambridge, U.K.	
Labsysytems Multiscan	MCC/340 ELISA plate reader
UK	
Wallac	Rack Beta, ß counter
UK	
Packard	Cobra auto-gamma, γ counter
UK	
LKB Biocrom ltd	Ultraspectrophotometer 4050
UK	
Hitachi-Perkin Elmer	MPF-2A fluorescent spectrophotometer
UK	

2.1.7 Solutions

Phosphate buffer saline

170 mM NaCl, 1 mM Na_2HPO_4, 2 mM KH_2PO_4 pH 7.2)

Versene

125 mM NaCl, 2.7 mM KCl, 6.3 mM Na_2HPO_4, 3.2 mM KH_2PO_4, 0.5 mM EDTA and 0.0015 % phenol red

ETN buffer

10 mM EDTA, 10 mM Tris-HCL, 100 mM NaCl, pH 7.0

Hanks modified buffer

1.3 mM $CaCl_2$, 5.4 mM KCL, 0.5 mM $MgCl_2$, 0.5 mM $MgSO_4$, 137 mM NaCl, 4 mM $NaHCO_3$, 0.4 mM NaH_2PO_4, pH 7.2

Lysing buffer for tyrosine kinase activity

50 mM Tris, 1 mM $MgCl_2$, 2 mM EDTA, 20 µg/ml soybean trypsin inhibitor, and 50 µg/ml PMSF

Membrane solubilization buffer

50 mM HEPES, 20% glycerol, 1% TritonX-100, 0.1% BSA, 0.05% sodium azide

Magnesium ATP buffer

1.2 mM ATP in a buffer containing 30 mM HEPES, 72 mM $MgCl_2$ pH 7.4

Substrate buffer

1.5 mM peptide in a buffer containing 135 mM HEPES, 300 µM sodium orthovanadate, 3 mM dithiothreitol, 0.15% Triton X-100, 6% glycerol, 0.05% sodium azide pH 7.4

ELISA sample buffer

0.5M Tris, 0.6M NaCl, 1% Triton X-100 pH 8.5

Coating buffer

15 mmol/l Na_2CO_3, 35 mmol/l $NaHCO_3$, pH 9.6

Dilution buffer

1% w/v BSA, 0.1 % v/v Tween-20 in PBS

Washing buffer

0.1 % v/v Tween-20 in PBS

Substrate buffer

76 mmol/l $NaH_2PO_4.H_2O$ and 35 mmol/l citric acid, pH 5

TBE buffer

45 mM Tris/HCl, 45 mM boric acid, 1 mM EDTA, pH 8.0

Scott's tap water substitute

42 mM NaHCO3, 81 µM MgSO4 pH 7.4

made up in dH_2O.

2.1.8. Cell Lines:

PC3 Cell Line

The PC3 cell line was derived from a vertebral metastasis of a poorly differentiated human prostatic carcinoma (Kaighn *et al.*, 1979). Prostate-specific acid phosphatase levels in these cells are similar to those obtained from normal prostatic epithelial cultures (Kaighn *et al.*, 1979). This cell line is hormone insensitive (figure 4.3). Scatchard plot analysis of ligand binding has shown no or only minimal levels of androgen receptor present in PC3 cells (Carruba, 1994)

DU145 Cell Line

The DU145 cell line was established from a brain lesion (Stone *et al.*, 1978) of metastatic prostate carcinoma. The brain metastasis was a moderately differentiated adenocarcinoma. Low levels of the prostate-specific acid phosphatase have been detected in DU145 cells. DU145 cells are not growth stimulated by androgens (fig 4.4), although Scatchard plot analysis of ligand binding has indicated that significant levels of androgen receptors are present within these cells and it has been suggested that these cells show a metabolic response to androgens (Carruba, 1994).

2.2. Methods

2.2.1. Routine growth and subculture

The cells were grown in RPMI-1640 medium, containing 5 μg/ml phenol red, 25 mM HEPES and supplemented with 10 % (v/v) foetal calf serum (FCS), 100 UI/ml Penicillin, 100 μg/ml Streptomycin. The cells were routinely cultured in 75 cm^2 flasks at 37 °C in atmospheric air enriched with 5% CO_2.

Routine medium was replaced every 48 hours. All manipulations were carried out in a laminar flow cabinet using aseptic techniques.

Cells were passaged when cultures occupied 80-90% of the surface area of the culture flask, and subcultured at ratio of 1:4 or 1:5. The medium was decanted aseptically and the cellular monolayer washed twice with sterile phosphate buffer saline (PBS). Subsequently, 0.05% trypsin, prepared in versene solution, was added to each flask (1 ml for a 25 cm^2 flask; 4 ml for a 75 cm^2 flask, and 6 ml for a 150 cm^2 flask). The flasks were incubated at 37 °C for 2-3 min, then checked under the microscope to make sure that the cells were rounded. The trypsin was then neutralised by adding fresh culture medium (usually 2-3 volumes of trypsin) and the cells evenly dispersed by rapid pipetting. This suspension was then dispensed into new culture flasks containing appropriate volumes of fresh growth medium.

To plate cells for experiments the above procedure was modified slightly as follows. The cells were trypsinised and resuspended as described previously then counted using a haemocytometer. The appropriate dilutions were made to obtain the desired cell density. This cell suspension was then plated on different experimental plates. 30,000 cells/ml were plated in 2 ml per well of routine growth medium for a 6 well plates, 1 ml per well for a 24 well plate and 200 µl per well for a 96 well plate.

Freezing and thawing the cells

To use similar passage number of the cells, the cells were frozen. When freezing cells, cells were washed with PBS, trypsinised, resuspended and transferred to sterile universal and pelleted by centrifugation at 1500 rpm for 5 min before being resuspended in a freezing medium. A 175 cm^2 flask resuspended in 5 ml freezing medium. Freezing medium consisted of routine

growth medium and 10 % (v/v) DMSO. After aliquoting into cryotubes in a 1.5 ml freezing medium, the cells were frozen slowly overnight in a -80 °C freezer and then stored in liquid nitrogen until required. All cells were checked for bacterial contamination in broad agar before freezing down.

On removing cells from liquid nitrogen, cells were thawed rapidly and transferred to a sterile universal containing 10 ml routine medium then centrifuged at 1500 rpm for 5 min and resuspended in routine growth medium in order to remove DMSO. Cells were subcultured at least 3 passages before use in an experiment.

Determination of cell number and viability

An aliquot of the cell suspension was mixed with an equal volume of Trypan Blue solution (0.4 %) and the number and viability of the cells was determined. This was achieved by viewing the cells under a light microscope in a haemocytometer. The viability of cells was established as the percentage of cells that excluded the Trypan Blue dye, thus the proportion of cells whose membranes remained intact and appeared white under the microscope (>95 %). Where determined to be viable the cell number was calculated by using the grid on the slide which ensured that the same area was counted for each determination.

Preparation of Dialysed, Heat-Inactivated, Dextran Coated Charcoal Treated FCS (DHIDCCFCS) and experimental medium

In order to avoid the oestrogenic effect of phenol red, phenol red-free RPMI was used as experimental medium (phenol red-free RPMI-1640, 25 mM HEPES, 2 g/lt $NaHCO_3$ pH 7.2). Growth factors and steroids were removed from the FCS used in experiments by dialysing, heat-inactivating and dextran-charcoal treatment. Dialysis tubing was prepared by boiling in 0.2% w/v

$NaHCO_3$ for 15 min. The strips were then rinsed with distilled water before use. 100 ml FCS was dialysed against 1 litre Hank's modified buffer at 4°C for 48 h, changing the buffer 4 times. Heat inactivation was carried out by incubating dialysed FCS for 45 min at 56°C. The serum was then cooled to 4°C and added to a pellet of dextran coated charcoal (0.25% (w/v) Charcoal, 0.0025% (w/v) Dextran) previously suspended in PBS. The mixture was stirred for 30 min at 4 °C then centrifuged at 10,000g for 30 min at 4°C. The supernatant was finally filtered through 0.22 µm filters to ensure sterility and stored at -20 °C.

2.2.2. Cellular Proliferation Assays

MTT Assay

In order to check the growth effects of EGF, TGFß, steroids and tyrosine kinase inhibitors on the cell lines, a colorimetric assay, MTT was employed. MTT is a yellow water soluble tetrazolium salt which is reduced by the hydrogenase enzymes in the living cells into an insoluble purple formazan product. The method was originally described by Tim Mosmann (1983) and modified by Carmichael *et al.* (1987).

200 µl of 30000 cells/ml were plated onto 96 well tissue culture plates and incubated overnight at 37°C. After washing the cells 3 times with PBS, growth factors or steroids were added in experimental medium containing 2.5% DHIDCCFCS. To examine the effect of TKI on cell growth, medium containing TKI was added to the cells first then serum or EGF was added 30 min later. Cells were incubated at 37°C for 2-3 days then cellular proliferation and viability was determined by MTT assay. 50 µl of MTT (5mg/ml in sterile PBS) was added into each well (final concentration of MTT was 1mg/ml). Plates were wrapped with aluminium foil and kept in the incubator for 4 hours.

After incubation the medium was removed and MTT crystals were dissolved in 200 µl DMSO. 25 µl of glycine buffer (0.1 M glycine, 0.1 M NaCl pH 10) was added to adjust the pH and plates were read at 540 nm using an ELISA plate reader. The mean of triplicate wells was calculated using InStat 2.01 statistical package program. The mean value and standard deviation of each experiments were calculated and graphed using CA-Cricket Graph III. For the TK inhibitor study, a DMSO control was included in the assay which contained the highest DMSO concentration used. In each case, the DMSO concentration (0.1% v/v) was shown to have no effect on the cell proliferation.

The data were analysed by curve-fitting to the appropriate equation using the Levenberg-Marquardt algorithm with the KaleidaGraph package for the Apple Macintosh in order to derive IC_{50} values with associated errors. A correlation between the cell number and absorbance reading at 540 nm (A540), using the MTT assay, is shown in figure 2.1.

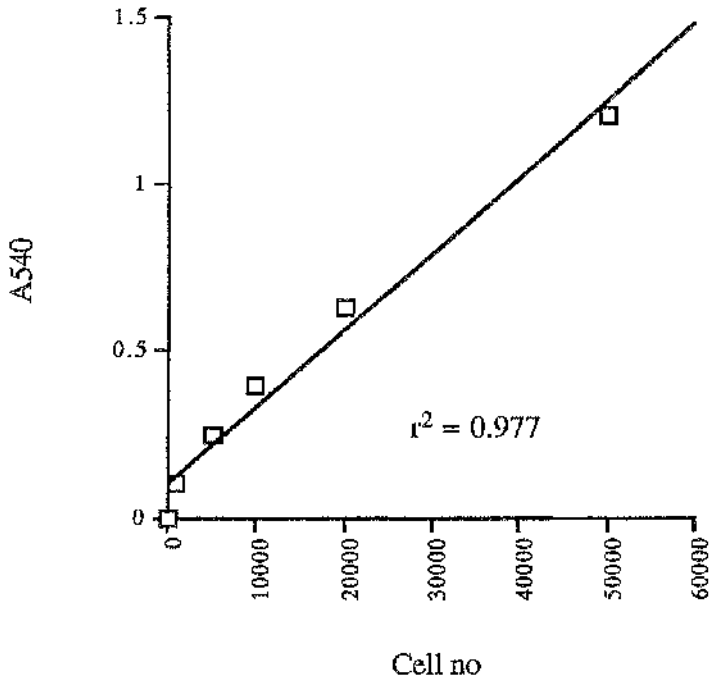

Fig. 2.1 Cell number versus absorbance at 540 nm reading in MTT assay. A range of numbers of cells were plated onto 24 well plates and allowed to attach overnight in routine medium. Then the medium was replaced with serum free experimental medium containing MTT the concentration of 1 mg/ml. MTT crystals were then dissolved by adding 200 µl DMSO after 4 h incubation. Absorbancies were read at 540 nm, subsequently adding the glycine buffer, in the ELISA plate reader. These data are the mean values of three separate experiments.

Hoechst DNA assay

The assay methodology was modified from that of Leake & Habib (1987) and involves the intercalation of Hoechst 33258 with solubilised DNA resulting a complex which fluoresces with a maximum emission at 450 nm.

Cells were plated into 24-well plates and incubated overnight at 37°C. Following the washing with PBS, medium was replaced with experimental medium containing steroids or growth factors. Control wells for steroids received experimental medium containing ethanol 0.1% (v/v). After appropriate incubation time cell monolayers were harvested and the DNA was solubilised using 0.2% SDS in ETN buffer for 30 min at 37 °C. 100 µl aliquots of samples were added into plastic test tubes containing 3 ml ETN buffer, Hoechst 33258 (100 ng/ml) and RNase (5 µg/ml). The mixture was vortexed and incubated in the dark for 30 min at room temperature. The fluorescence enhancement at 450 nm was measured using the fluorescent spectrophotometer with an excitation wavelength of 360 nm.

DNA standards were prepared from a 100 µg/ml solution of calf thymus DNA dissolved in ETN buffer. The standards were used in the range of 0-60 µg/ml DNA. The concentration of DNA present in the samples was extrapolated from a calibration curve which is obtained from the standards. A correlation between the total cellular DNA count and absorbance reading at 540 nm (A540), using MTT assay, is shown in figure 2.2.

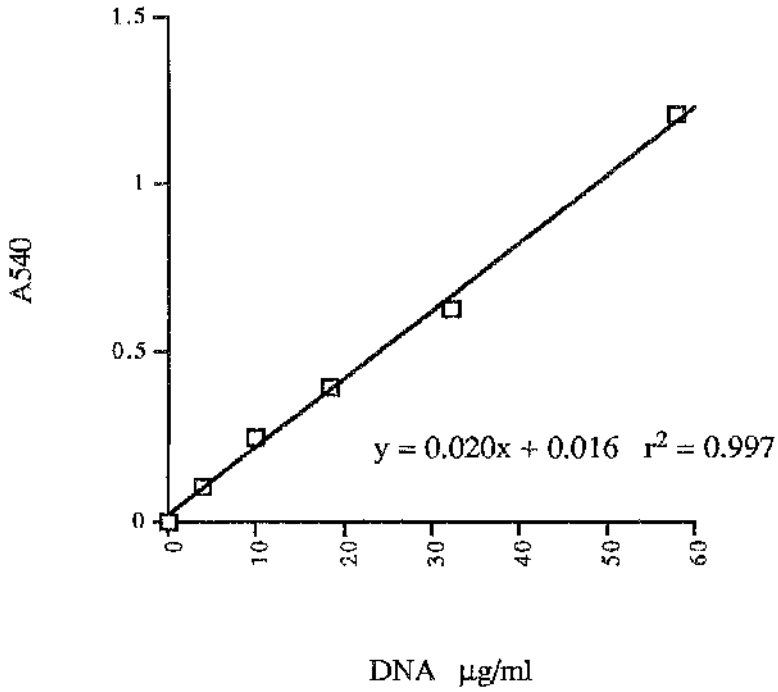

Fig. 2.2 Total cellular DNA counting versus absorbance at 540 nm reading in MTT assay. MTT readings of the increasing number of cells were obtained as described in Fig 2.1. The same number of cells were plated into 24-well plates and left overnight for attachment. Cell culture monolayers then were harvested and DNA was solubilised using 0.2% SDS in ETN buffer. 100 µl aliquots of samples were added into RT-30 tubes containing 3 ml ETN buffer, Hoechst 33258 (100 ng/ml), RNase (5 µg/ml). The mixture was vortexed and incubated in the dark for 30 min. at room temperature. The fluorescence enhancement at 450 nm was measured with an excitation wavelength of 360 nm. These data are the mean values of three separate experiments.

2.2.3. Whole Cell EGF Binding Assay

6×10^4 cells/well were plated into a 24 well tissue culture plate in routine growth medium and left for overnight incubation in a standard incubator. Then cells were washed three times with PBS and left to grow to near confluence in experimental medium containing 1 % (v/v) DHIDCCFCS. Subsequently, cells were washed 3 times with ice-cold PBS-BSA (0.1% w/v) and incubated for 2 h at room temperature in 200 µl binding buffer (the medium with BSA 0.1%). A range of concentrations of ^{125}I-EGF (from 0.15 nM to 3.5 nM) was added to the wells (Koenders *et al.*, 1992). The non-specific binding was determined in the presence of 350 nM unlabelled EGF at the three highest concentrations of labelled EGF. Following 2 h incubation at room temperature, cells were washed 3 times with ice-cold PBS-BSA 0.1% and lysed for 30 min at 37 °C by adding 200 µl SDS (0.1%w/v). Aliquots of cell lysates were finally counted in a gamma-counter. Other wells were used to determine cell number and DNA content.

Results of duplicate experiments were processed using BBC computer program for Scatchard analysis (Leake *et al.*, 1987). The program is a modification of a regression analysis best fit routine yielding both dissociation constant (Kd) and concentration values (fmol/ml homogenate). Data was analysed according to a model for one or two binding sites (Carruba, 1994).

2.2.4. EGF-Receptor Tyrosine Kinase Assay

Preparation of cell homogenates

Cells were grown to 70-80% of confluence in 75 cm^2 cell culture flasks in routine medium. Routine medium was removed, the cells were washed twice with pre-warmed PBS, and a range of concentrations of ZM260603 (0-10 µM) in experimental medium containing 2.5% DHIDCCFCS was added. Control

cells were incubated with experimental medium containing 0.1% DMSO and all cells were incubated for 24 h at 37 °C. Cells were washed 3 times with ice-cold PBS and lysed by incubating with 1.5 ml ice-cold lysis buffer for 1 hour at 4°C. The protease inhibitors (20 µg/ml soybean trypsin inhibitor, 50 µg/ml PMSF) were added to the buffer on the day of the preparation. The cell lysates were centrifuged for 10 min at 1000 rpm to pellet the cell debris and nucleotides. The supernatant was further centrifuged at 50,000 rpm for 30 min at 4°C. The pellet was resuspended by gentle glass-glass homogeniser in 100 µl of ice-cold membrane solubilization buffer. The samples were stored at -70 °C until use.

Determination of EGFR-related TK Activity

γ-[^{33}P]ATP was added to the magnesium ATP buffer to a concentration of 40 µCi/ml. A 5 µl aliquot of this solution was used to determine the total radioactive concentration of the solution. Two controls were used, one with no sample (zero enzyme blank), and one with sample and no EGF. The zero enzyme blank corrects for any non-specific binding of [^{33}P]ATP or its radiolytic decomposition products binding to papers. The zero EGF blank corrects for the basal level of tyrosine kinases present in the sample. The zero EGF control was performed for each sample.

10 µl of each sample or sample buffer for zero enzyme blank was placed in each tube , followed by 5 µl of EGF(6 µM) (5 µl dH_2O for the zero EGF controls). Each tube then received 10 µl of substrate buffer (1.5 mM peptide in a buffer containing 135 mM HEPES, 300 µM sodium orthovanadate, 3 mM dithiothreitol, 0.15% Triton X-100, 6% glycerol, 0.05% sodium azide pH 7.4), and the reaction was started by the addition of 5 µl [^{33}P]ATP. The tubes were mixed then centrifuged for 15 seconds to wash the contents to the base. The tubes were then placed in a 30°C water bath for 30 min. After the incubation, the reaction was terminated by the addition of 10 µl of stop reagent

(300 mM orthophosphoric acid containing carmosine red) to each tube. The terminated reaction mixture was then mixed, and again centrifuged for 30 min. 30 µl of each mixture was then spotted onto the centre of a phosphocellulose binding paper. The papers were washed twice, for 2 min with 250 ml 1% (v/v) acetic acid, followed by two 2 min washes with 250 ml dH_2O. The final wash liquid was decanted and replaced with 200 ml dH_2O, the papers removed and placed in 20 ml scintillation vials to which 10 ml of scintillation fluid was added. The vials were then counted for 1 min.

Calculation of specific activities

The corrected cpm per sample was obtained by subtracting the blank value in each case. Determining the protein content (section 2.2.10.) of each sample allowed the results to be expressed in terms of specific activity. The results were calculated as pmoles phosphate (P) incorporated per minute per mg protein, derived from the calculation shown below;

The [^{33}P] incorporated into the peptide was quantitatively measured by the binding papers. In the presence of enzyme sample and EGF the [^{33}P] counted on the papers represents the sum of non-specific [^{33}P]ATP binding, specific binding of phosphorylated peptide and phosphorylated proteins(A).

In the presence of enzyme and absence of EGF the counts on the papers are the sum of non-specific [^{33}P]ATP binding, and non EGF dependent tyrosine kinase phosphorylation of proteins (B).

Calculation of specific activity (**R**) of 1.2 mM magnesium [^{33}P]ATP

5µl of 1.2 mM Mg[^{33}P]ATP contains $6x10^{-9}$ M ATP

$$\mathbf{R} = \frac{5\ \mu l\ of\ Mg[^{33}P]ATP}{6} \quad cpm/nmole$$

Calculation of total phosphate transferred to peptide and endogenous proteins (T)

30 µl spotted onto binding paper, total terminated volume 40 µl

$T = [A-B] \times 1.33$

Calculation of pmoles P transferred per minute

$$P = \frac{T \times 1000}{I \times R} \text{ pmoles/minute}$$

Where I = Incubation time (minute)

2.2.5 Determination of PA system proteins by ELISA

Sample Preparation

50×10^4 cells/well were plated into 25 cm^2 cell culture flasks in routine medium and incubated at 37 °C. Routine medium was removed, the cells washed twice with prewarmed PBS, and the medium replaced with different doses of EGF, TKI, TGFß in experimental medium containing 2.5% DHIDCCFCS. The medium was used alone or with 0.1% v/v DMSO (for TKI control group) in the control group and cells were incubated for 48 h at 37 °C. After the appropriate incubation time, the medium was removed and centrifuged at 2000 rpm for 10 minutes at 4 °C to remove cell debris. The cells were washed 3 times with ice-cold PBS and lysed for 1h at 4 °C in 1 ml lysis buffer. The lysates were then centrifuged for 10 min at 1000 rpm to pellet the cell debris and nucleotides. For uPA enzyme activity assay, the supernatant and medium were used on the day of preparation. For ELISA experiments, the samples were either used on the day of preparation or stored at -70 °C until use. The total protein content of each sample was determined by using Bradford protein assay (section 2.2.10).

Immunodetection of the PA system proteins

The detection of PA system proteins were performed by the four-span antibody detection system described by Benraad *et al.* (1996) and Grebenschikov *et al.* (1997). The assay detects latent, active and complexed forms of PA system components, such as uPA-uPAR, uPA-PAI-1, uPA-PAI-2, tPA-PAI-1 and tPA-PAI-2. The same procedure was used for all antigents and described below.

ELISA plates were coated with a 1/310 dilution of sheep-anti-chicken antibody in coating buffer and incubated overnight at 4°C. The plates were then blocked with 1% w/v BSA in PBS for 2 h at 37°C. The coated plates were incubated with 100 µl the appropriate catching ab (chicken-anti-uPA or tPA or PAI-1 or PAI-2) for 2 hours at 37°C. These antibodies were used at dilutions of 1/425, 1/475, 1/320 respectively in dilution buffer. The plates were incubated with 100 µl of sample or uPA/PAI-1/tPA standards for overnight at 4°C. This was followed by incubation with tagging ab (rabbit anti-uPA/tPA/PAI-1/PAI-2 IgG), for 2 h at room temperature. The tagging antibodies were diluted 1/150 for uPA, 1/150 for PAI-1 and 1/125 for tPA in dilution buffer. Antibody binding was detected by incubating the plates with horse radish peroxidase (HRP) conjugated goat anti-rabbit IgG (1/40,000 dilution) for 2 hours at room temperature. Colour was developed by adding OPD substrate to the plates. OPD tablets were prepared in substrate buffer and 10 µl H_2O_2 was added freshly per 11 ml of the substrate buffer. The plates were wrapped in aluminium foil and incubated in the dark for 30 min at room temperature. Absorbance was read at 492 nm using the ELISA plate reader. Plates were washed 4 times with washing buffer between all incubations.

The standards for uPA which were pro-uPA standards were used within the range of 0-1.8 ng/ml. The standards for PAI-1 and tPA were used in the range

of 0-2 ng/ml. A linear correlation was obtained within this range (Fig 2.3). In order to get the results linear within the part of the curve, samples were used with and/or without any dilution. For uPA detection both medium and cell cytosol samples were diluted in the dilution buffer (1/50 for PC3, 1/30 for DU145). No further dilution was needed for PAI-1 protein in the EGF, TKI and control group, however 1/20 dilution was used for TGFß stimulated PC3 cells samples and 1/2 dilution was for the DU145 cell samples. For tPA detection, samples were used without dilution.

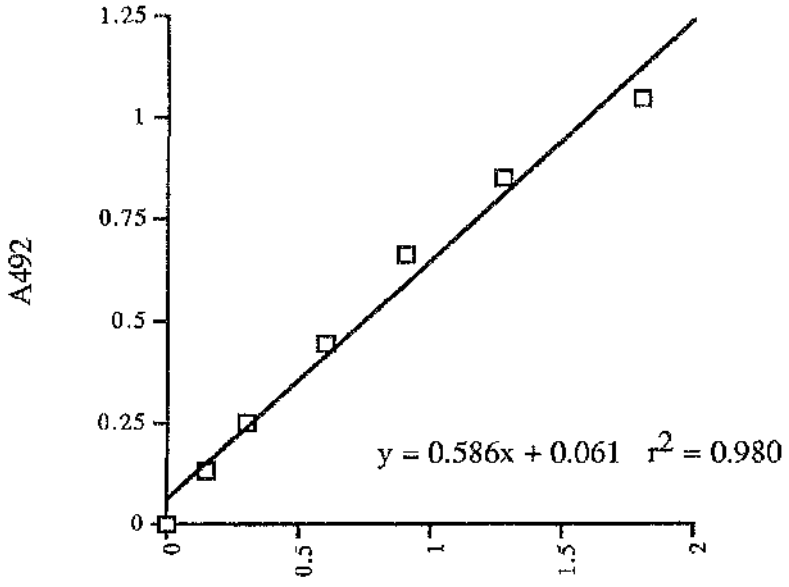

Figure 2.3. Standard curve for an ELISA assay

The samples for the standard curve were prepared as described in section 2.2.5. Pro-uPA concentrations of 0-1.8 ng/ml were used as indicated and the absorbance was read at 492 nm (A492). The blank well (without uPA) was used to zero the plate reader. The assays were carried out in duplicate. This is a representative experiment which has been carried out at least twenty times.

2.2.6 RT-PCR

RNA extraction by Tri-Reagent

The cells were incubated with growth factors and TKI for 48 h and total RNA was extracted from the cultured cells using a modification of the protocol originally described by Chomczynski & Sacchi (1987). Cells were lysed by the addition with 1 ml of Tri-Reagent to either pre-washed 10 x 10^6 cells or to a confluent 75 cm^2 flask. The cell lysate was then transferred to an eppendorf tube and homogenised by 5-10 passages through a Gilson pipette tip. The homogenate was incubated at room temperature for 5 min. Cell membranes/high molecular weight DNA/polysaccharides were pelletted by centrifugation (11000 rpm for 10 min at 4 °C) and the supernatant transferred to a fresh Eppendorf tube. RNA was then separated from DNA and protein by the following phase separation:

0.2 ml RNase-free chloroform per ml of Tri Reagent originally used was added to each tube and vortexed for 15 seconds and then incubated at room temperature for 3 min. Following centrifugation at 11000 rpm for 15 min at 4 °C, the upper aqueous phase was transferred to new tube. The RNA was then precipitated from this phase by adding 0.5 ml of propan-2-ol per 1 ml of Tri-Reagent used initially. Samples were incubated at room temperature for 5-10 min and then centrifuged at 11000 rpm for 10 min at 4 °C to pellet the RNA. The RNA pellet was washed with 1 ml 75 % v/v ethanol. Ethanol was removed by centrifuging at 9000 rpm for 5 min at 4 °C and the RNA pellet either air-dried or dried under vacuum for 5-10 min. The RNA was resolubilised in (1 %) DEPC treated water by pipetting a few times and incubated for 10-15 min at 55-60 °C if necessary to help dissolve it.

The concentration of the RNA was determined by measuring the absorbance, or optical density (OD), of a 10 µl of sample at wavelengths of 260 nm and 280 nm in quartz cuvettes using a Shimadzu UV-1201 spectrophotometer. A 1/100 dilution of the re suspended RNA was used to read the absorbance against a dH_2O blank. The OD_{260}/A_{280} ratio was determined, ideally, a result of 1.8 to 2.0 should be obtained. The concentration of RNA in µg/ml was calculated by the multiplication of the OD_{260} by the dilution factor (100) and by a constant (40) where 40 = 1 OD gives 40 µg RNA at 260 nm. Extracted RNA was either used fresh or stored at -80 °C under 75 % ethanol until use.

First-strand cDNA synthesis

Preparation of first-strand cDNA was achieved using the 'First-strand cDNA synthesis kit' with a total reaction volume of 33 µl. 5 µg of total RNA was diluted with DEPC treated water to a total volume of 20 µl. This was then denatured by heating to 65 °C for 10 min. Denatured RNA was then incubated with murine reverse transcriptase, 6 mM dithiothreitol, 0.6 mM of each dNTPs and 0.2 µg of the supplied poly(dT) primer [*Not*I-d$(T)_{18}$] for 1 h at 37 °C. The resultant first strand cDNA was synthesised using the RNA as a template was then stored at -20 °C.

The polymerase chain reaction (PCR)

The polymerase chain reaction is a technique for the *in vitro* amplification of specific DNA sequences by the simultaneous primer extension of complementary strands of DNA (Eeles *et al.*, 1992). Standard PCR amplification reactions were performed in 1 x PCR buffer (50 mM KCl, 20 mM Tris/HCl) with a total reaction volume of 50 µl. The reaction mix contained final concentrations of 0.2 mM of each dNTP (adenine, guanine, thymidine and cytosine bases), 1.5 mM $MgCl_2$ together with 25 pmoles of 5'-end and 3'-end primers, 5 units of *Taq* DNA polymerase and 3 µl of template DNA. The PCR

machine was programmed for denaturation/annealing and extension of the selected region by the primers. These were performed using various times, temperatures and repeated cycles as follows.

Primers were denatured at 95 °C for 1 min. The uPA and uPAR primers were annealed at 49 °C for 1 min. Annealing at 45 °C for 70 seconds was employed for PAI-1 and ß-Actin primers. The extension step was 1 min 40 seconds at 72 °C for all primers. The primers were run in 35 cycles. The primers sequence were used as described by Noguchi-Takino *et al.* (1996).

The fragments amplified represent nucleotides 644-1122 in uPA with an expected size of 479 bp,

5' (644) AGA ATT CAC CAC CAT CGA GA

ATC AGC TTC ACA ACA CTG AT (1122) **3'**

385-840 in uPAR, recognises 455 bp,

5' (385) TTA CCT CGA ATG CAT TTC CT

TTG CAC AGC CTC TTA CCA TA (840) **3'**

328-780 in PAI-1, expected size 452 bp,

5' (328) ATG GGA TTC AAG ATT GAT GA

TCA GTA TAG TTG AAC TTG TT (780) **3'**

290-881 in ß-Actin expression expected size 591 base pairs

5' (290) GAA ATT CTG GCA CCA CAC CTT

TTG AAG GTA GTT TCG TGG AT (881) **3'**

Agarose gel electrophoresis

High melting temperature agarose (1.5 to 2 %) was dissolved in 0.5 x TBE buffer and melted in a microwave for 1 min. After cooling, the agarose was poured into a horizontal electrophoresis tank and allowed to set. 5 to 10 µl of RT-PCR reaction mixture was loaded. In order to determine the size of the RNA/DNA fragments, Phix174 DNA/Hae III Markers were loaded as well.

The gel was run in the ethidium bromide at 75 mA for about 90 min. After electrophoresis, the gel was visualised by under UV light and photographed.

2.2.7. uPA enzyme activity assay

uPA enzyme activity assay was performed as described by Gaylis and associates (Gaylis *et al.*, 1989) with some modifications. S-2251 was used to determine uPA activity as a chromogenic substrate of plasmin. Samples were prepared as described in 2.2.5, and 10 µl of sample was used for the assay. 1/30 dilution was used for PC3 cell cytosol and medium. The DU145 cell cytosol was diluted 1/5 and medium was used without dilution.

150 µl of buffer (10 mM TrisHCL pH 7.4) and 0.10 µl of plasminogen (final concentration is 0.2 µg/ml) were incubated with 10 µl of samples or uPA standards. Incubations were carried out in 96 well plates for 2 h at 37 °C. 4 µl of the 1 mg/ml S-2251 (final concentration is 20 µg/ml) was added to the mixture and incubated for 10 min at 37 °C. The incubation was then terminated by adding 20 µl of stop mix (1 M acetic acid 10 mM sodium acetate). The absorbance at 403 nm was measured with the plate reader. Control incubations included those of S-2251 with buffer and plasminogen. Active-uPA standards were used in the range of 0-20 u/ml. The standards gave a linear curve between this range (Fig 2.4)

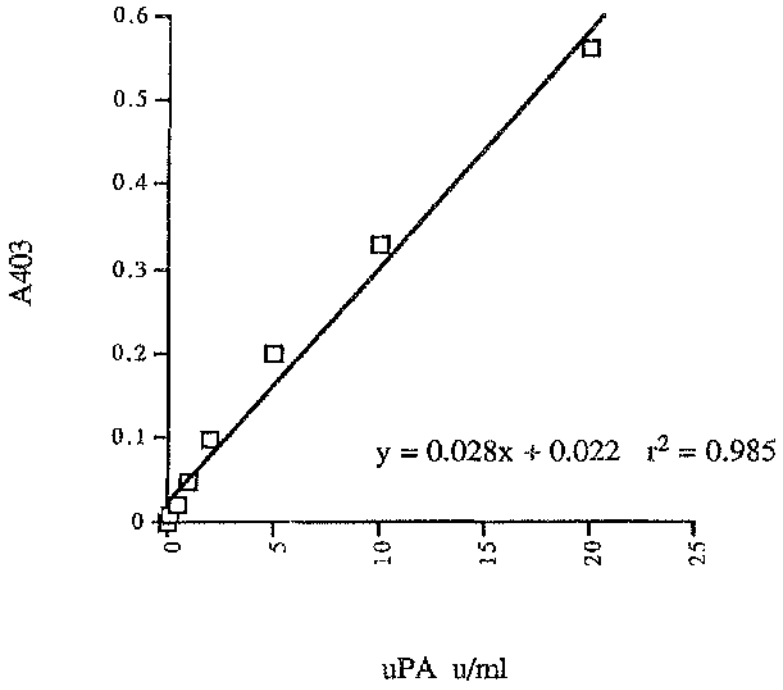

Figure 2.4. Standard curve for an uPA enzyme activity assay

The samples for the standard curve were prepared as described in section 2.2.5. Active-uPA concentrations of 0-20 u/ml were used as indicated and the absorbance was read at 403 nm. The blank well (without uPA) was used to zero the plate reader. The assays were carried out in duplicate. This is a representative experiment which has been carried out at least ten times.

The assay was also performed by omitting plasminogen to ensure plasmin specificity of the substrate. In order to check uPA specific activity in the assay, monoclonal mouse anti-uPA ab (#394, American Diagnostica) was added to the mixture. 1/50 dilution of the ab was incubated with the mixture for 2 h at 37 °C. Control wells were used to zero the plate reader. Results are presented as Plough Units determined using a standard urokinase preparation.

2.2.8. *In vitro* invasion assay

Transwell chambers with 6.5 mm diameter polyvinylpyrolidone (PVP)-free polycarbonate filters of 8 μm pore size were used for the invasion assays (simply illustrated in fig 2.5). This method originally described by Albini *et al.*, (1987) and modified by Repesh (1989) and Schlechte *et al.* (1990). PVP filters were placed in a 24 well tissue culture plates. Matrigel was diluted in ice-cold serum free medium to achieve final protein concentration 1 mg/ml. Plasminogen was mixed with the soluble extracellular matrix to achieve final concentration 50 μg/ml. Each filter was coated with 50 μg of matrigel in 100 μl of serum free medium. The filters were incubated for 1 h at room temperature for gelation of the matrigel. Following incubation the matrigel was rehydrated by adding 50 μl of experimental medium contains 5% DHICDCCFCS for 30 min.

The cell lines were harvested, at approximately 70-80% of plate confluence for all experiments, with tyrpsin/versene and washed two times with serum free medium. Afterwards 5 x 10^4 cells were introduced into the chamber in 200 μl of experimental medium. The experimental medium contained growth factors and inhibitor or was used alone. 0.2% matrigel in serum free medium was used as a chemoattractant and placed in the lower compartment of the wells in 1 ml volume. The cells were incubated at 37 °C for three days. After this time, 5

mg/ml of the vital stain MTT in PBS (filter sterilised) was added into both upper (100 µl of volume) and lower compartments (200µl) and incubated for 3 hours at 37 °C. After incubation, the medium was removed gently. Crystals on the top of filter were removed by cotton swabs and dissolved in 1 ml volume of DMSO. The filters were subsequently immersed with 500 µl DMSO. The glycine buffer was used to increase the pH and absorbance measured at 540 nm in the spectrometer.

The absorbance reading corresponding to the solubilized crystals on the lower aspect of the polycarbonate filter was divided by the sum of this value and the activity in the upper compartment and multiplied by hundred. This is a measure of the number of cells that have managed to invade through the matrigel and reach the filter.

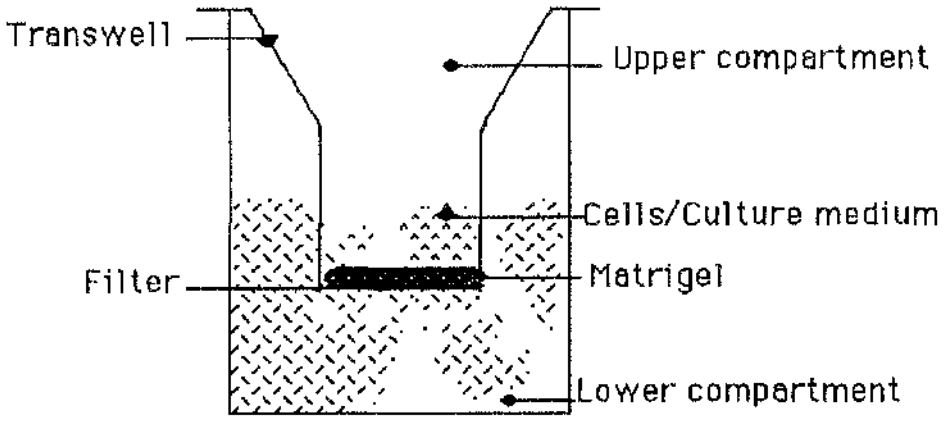

Fig. 2.5 Schematic description of *in vitro* invasion assay. Transwell chambers with 6.5 mm diameter PVP filters of 8 μm pore size were placed in a 24 well tissue culture plates. The matrigel was applied to the upper surface of the filter in the transwell. Each filter was coated with 50 μg matrigel (protein concentration 1 mg/ml) in 100 μl of serum free medium containing 50 μg/ml final concentration of plasminogen. Cells were placed on the top of the filter and left for 72 h. Following the incubation period, the cells were further incubated with MTT for 4 h. Then the medium was removed and top of the filters was swabbed with a cotton swab. The cotton swab and filter were immersed in DMSO to dissolve MTT crystals. After adding glycine buffer, absorbances were read at 540 nm. The absorbance reading corresponding to the solubilized crystals on the lower aspect of the polycarbonate filter was divided by the sum of this value and the activity in the upper compartment and multiplied by hundred.

2.2.9 Immunocytochemistry

Sample Preparation

PC3 cells were seeded into 8-chamber slides in 400 µl routine growth medium per chamber. Then the cells were left in the standard incubator until 70-80% confluent. The medium was removed and cells were washed three times with 0.9% NaCl.

Immunolabelling of sections

Plastic coverage was removed and slides were fixed in acetone for 10 min at room temperature. Then the slides were washed in running tap water for 5 min then in TBS buffer for 10 min. Slides were blocked by incubating with normal goat serum (NGS) (1/5 dilution in TBS buffer) for 15 min. After that, NGS was removed and 100 µl of 1/200 dilution mouse monoclonal anti-uPA (#394) ab was applied on to the slides and incubated in a humidified chamber for 2 hours at room temperatures. After incubation with the monoclonal ab, sections were washed for 10 min in TBS, and alkaline phosphatase (ALP) conjugated goat anti-mouse IgG was added for 30 min. Goat anti-mouse IgG was used 1/20 dilution and contained 10% normal human serum. Bound antibodies were visualised by using new fuchsin detecting substrate kit. The detection system solution consisting of chromogen, activator, substrate and levamisol in tris buffer pH 7.8. After 30 min incubation with the substrate, the sections were washed under running tap water for 10 min. Slides were counter stained with hematoxylin for 10 seconds and washed again under running tap water for 10 min. Then slides were transferred into Scott's tap water substitute. The slides were then dehydrated by successive 5 min incubations in methanol, ethanol, ethanol and xylene substitute incubations then mounted with xylene substitute mountant. An incubation where the first ab was omitted served as a negative

control. The primary ab is shown to be specific for the B chain of human uPA and recognises all known forms of human uPA including receptor bound uPA (Delvecchio *et al.*, 1993). In order to check the performance of all second reagents, anti-cytokeratin ab was replaced with the first ab. The anti-cytokeratin ab was used in 1/100 dilution. All the dilutions were made in Tris-buffer saline and all the incubations took place in a humidified chamber at room temperature.

2.2.10. Protein determination

Protein was routinely measured by the method of (Bradford, 1976) with BSA as a standard. Standard curve was constructed using 0-20 mg range of bovine serum albumin. The standard and unknown protein solutions were made up to a volume of 800 µl with distilled water before the addition of 200 µl of Bradford reagent and the tubes were vortexed. Absorbance was determined at 595 nm using a LKB Biochrom ultra spectrophotometer. Unknown concentrations were determined by plotting the standard curve (x axis = mg/ml of protein concentration, y axis = A_{595}) (Figure 2.6).

2.2.11. Analysis of data and statistics

The data given in figures and tables represent the mean ± SD of n independent determinations. The significance of differences was calculated with the Student's t-test using Instat Mac version 2.0. Instat reports exact P values and states the "significance" of the results using the following arbitrary scheme.

P> 0.10 "not significant"

P< 0.10 "not quite significant"

P< 0.05 "significant"

P< 0.01 "very significant"

P< 0.001 "extremely significant"

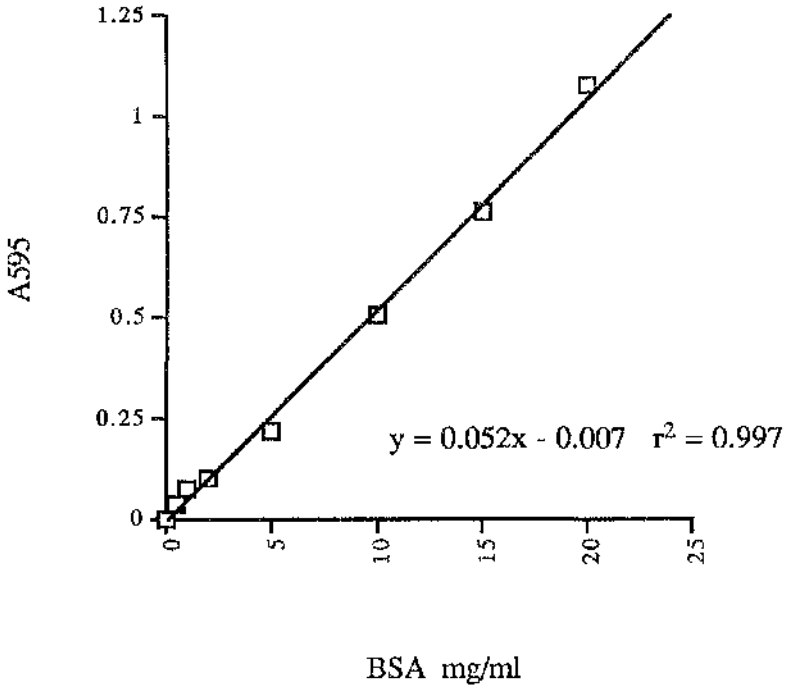

Figure 2.6. Standard curve for a Bradford protein assay

The samples for the standard curve were prepared as described in section 2.2.10. Bovine serum albumin concentrations of 0-20 μg/ml were used as indicated and the absorbance was read at 595 nm. The blank without protein was used to zero the spectrophotometer. The assays were carried out in duplicate. This is a representative experiment which has been carried out at least twenty times.

STUDIES WITH EGF

3.1. Aim of the study

The purpose of this study was to investigate the effect of epidermal growth factor on the proliferation and the invasive potential of two prostate carcinoma cell lines. Firstly the presence of an EGF binding site was determined on the cell lines. To examine the presence of active receptor, EGFR-related tyrosine kinase activity was evaluated in the presence of ligand and the specific inhibitor. Growth responses to EGF and to the inhibitor were evaluated in monolayer cultures. The role of EGF in the regulation of invasion was investigated by examining the effect of EGF on the expression of plasminogen activator system components at both messenger-RNA and protein levels. To both confirm their roles in invasion and to investigate differences among PA family members, activities were assessed in relation to an *in vitro* invasion of the extracellular matrix.

Results

3.2.1 EGFR content of the cell lines

EGF radioreceptor assays of the cell lines were carried out as described in section 2.2.3. Results of Scatchard analysis of EGFR content of cell lines are reported in table 3.1.

Table 3.1 Epidermal Growth Factor receptor levels in PC3 and DU145 cell lines. Values represent the mean ± sd of three different experiments and each experiment was carried out in duplicate.

Cell Line	PC3	DU145
Dissociation Constant K_d (nM)	0.271 ± 0.056	0.546 ± 0.09
Total Specific Receptor (fmol/mg protein)	126.3 ± 23.3	476.4 ± 66.9
Sites per cell	84,260 ± 7510	317,300 ± 25,850

Both cell lines exhibited a single class of high affinity of specific binding site for EGF. EGFR concentrations appeared four times higher in DU145 cells than PC3 cells. These results are consistent with previous findings (Carruba, 1994; MacDonald & Habib, 1992; Wilding *et al.*, 1989b). The Kd value of DU145 cells was also two times higher than PC3 cell line. Scatchard plots of EGFR in these cell lines are illustrated in Figure 3.1 and 3.2.

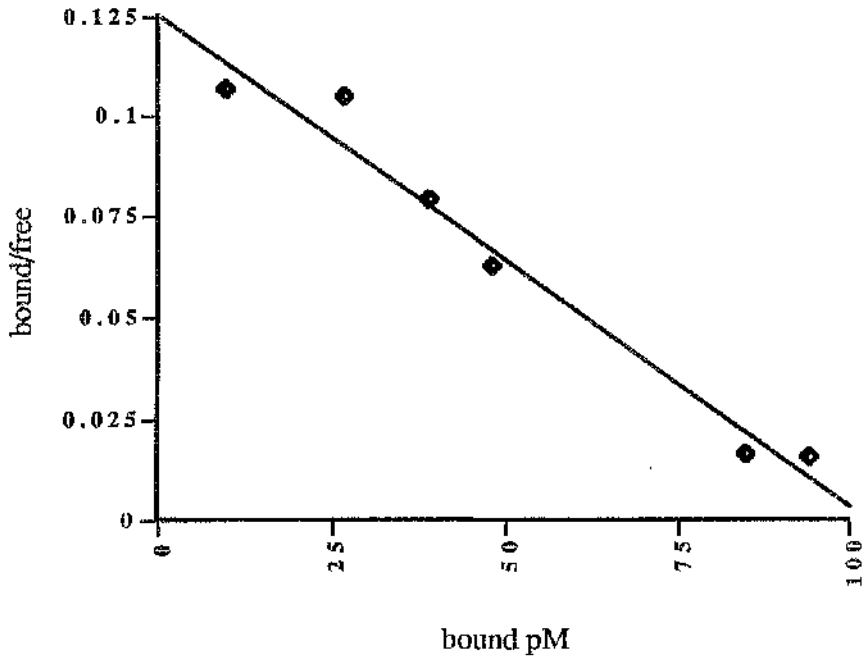

Fig 3.1 Radioreceptor assay of epidermal growth factor receptor in the PC3 cell line. Scatchard plot is shown of specific high affinity EGF binding in PC3 cells. Both dissociation constant and concentration values are shown in table 3.1. The average of three independent determinations is presented.

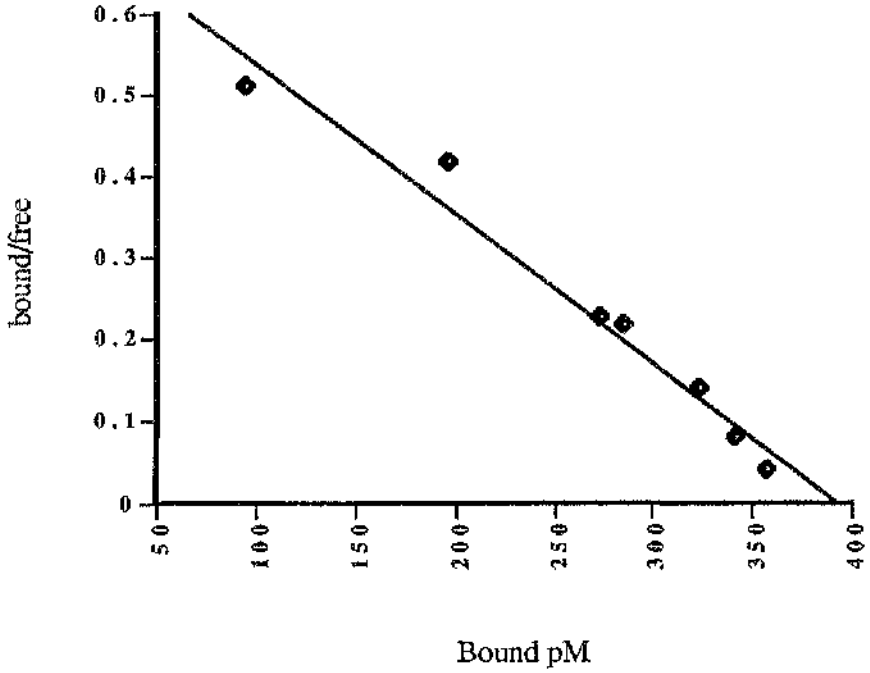

Fig 3.2 Radioreceptor assay of epidermal growth factor receptor in the DU145 cell line Scatchard plot is shown of specific high affinity EGF binding in DU145 cells. Both dissociation constant and concentration values are shown in table 3.1. The average of three independent determinations is presented.

3.2.2 EGFR-related tyrosine kinase activity

EGFR-related tyrosine kinase activity was detected using Biotrak EGFR-Tyrosine Kinase activity detection kit as described in section 2.2.4. To determine if EGFR-related tyrosine kinase activity was present in the PC3 and DU145 cells, the TKI, ZM260603, was employed as a selective inhibitor. 70-80% confluent cell culture flasks were treated with different concentrations of ZM260603 (0-10μM) for 24h. Both cell lines revealed a dose dependent inhibition of the enzyme activity in response to the inhibitor of EGF receptor-related tyrosine kinase activity.

Preliminary experiments showed that EGFR-related TK activity inhibition starts at 0.01 μM concentration of the inhibitor. In the PC3 cell line, the enzyme activity showed a decrease by 39 % at 0.01 μM, 52 % at 0.05 μM, 64 % at 0.1 μM, 76 % at 0.5 μM, 86 % at 1 μM and 94 % at 10 μM (Fig 3.3). IC_{50} value for PC3 cell line was found as 0.021 ± 0.012μM (Table 3.2).

In DU145 cell line, the enzyme activity showed a decrease by 12% at 0.01μM, 47.5 % at 0.05 μM, 51 % at 0.1 μM, 72 % at 0.5 μM, 80 % at 1 μM and 95 % at 10 μM (Fig 3.4). IC_{50} value for DU145 cell line was found as 0.0315 ± 0.021μM (Table 3.2).

Even in the absence of added EGF, the 10 μM dose of TKI reduced enzyme activity by 15 %, suggesting either that there is some endogenous activity of the EGFR or that the TKI does have some inhibitory effect on TK's other than EGFR.

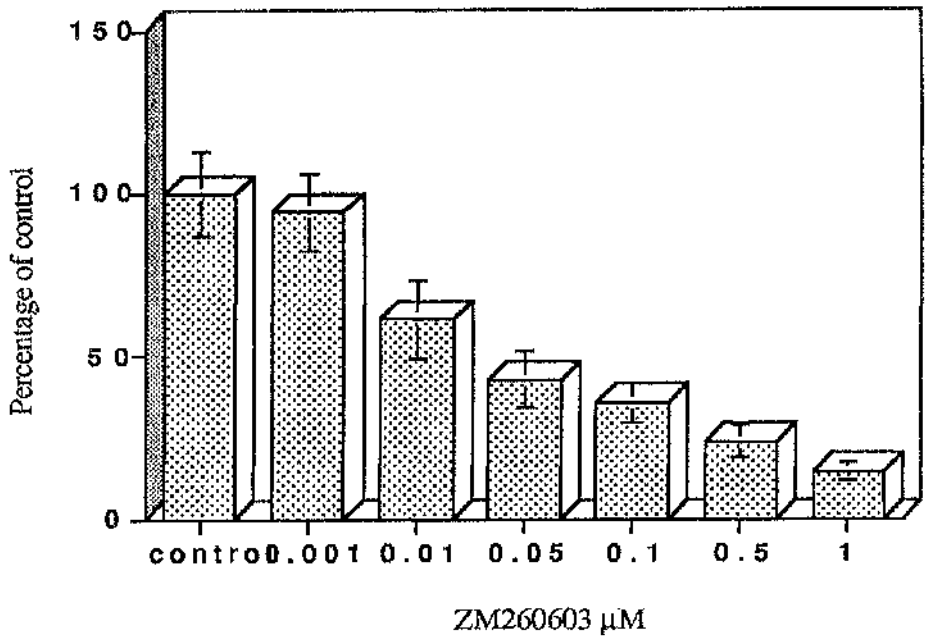

Fig 3.3 The effect of ZM260603 on TK activity in PC3 cells

EGFR related TK activity was detected as described in section 2.2.4. The concentration of EGF used in this experment was 6 μM. In all cases, each sample was assayed in duplicate. The average of three separate determinations ± standard deviation is presented as percentage of control.

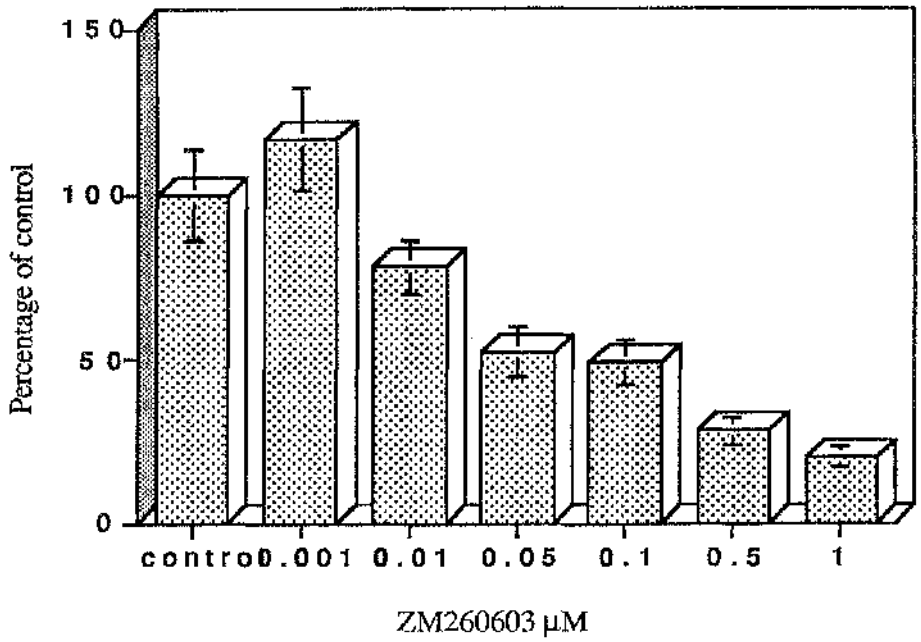

Fig 3.4 The effect of ZM260603 on TK activity in DU145 cells EGFR related TK activity was detected as described in section 2.2.4. The concentration of EGF used in this experment was 6 μM. In all cases, each sample was assayed in duplicate. The average of three separate determinations ± standard deviation is presented as percentage of control.

3.3 Growth Studies

3.3.1 Growth response to EGF

The effects of EGF on growth of the PC3 and DU145 cells was determined by MTT assay. The cells were incubated with different concentrations of EGF for 48 and 72 hours. At 48 h neither PC3 nor DU145 cells exhibited growth stimulation in response to the increasing concentrations of exogenous EGF (from 0.1 to 10 ng/ml).

At 72 hour, treatment with higher doses of EGF (7.5 and 10 ng/ml) resulted in a gradual increase in proliferation in the PC3 cell line in comparison to the control. The PC3 cell line has revealed 29 % and 31 % increase at 7.5 and 10 ng/ml concentration of EGF (Fig 3.5) in 72 hours (P=0.0419, considered as significant). Treatment of the DU145 cell line with EGF did not show any difference in growth at 72 hours (Fig 3.6). Although DU145 cells possess large number of EGF receptors, exogenous EGF had no effect on the cell proliferation. The growth sensitivity of the PC3 cell line and the insensitivity of the DU145 cell line had also been shown by Carruba (1994), and MacDonald & Habib (1992).

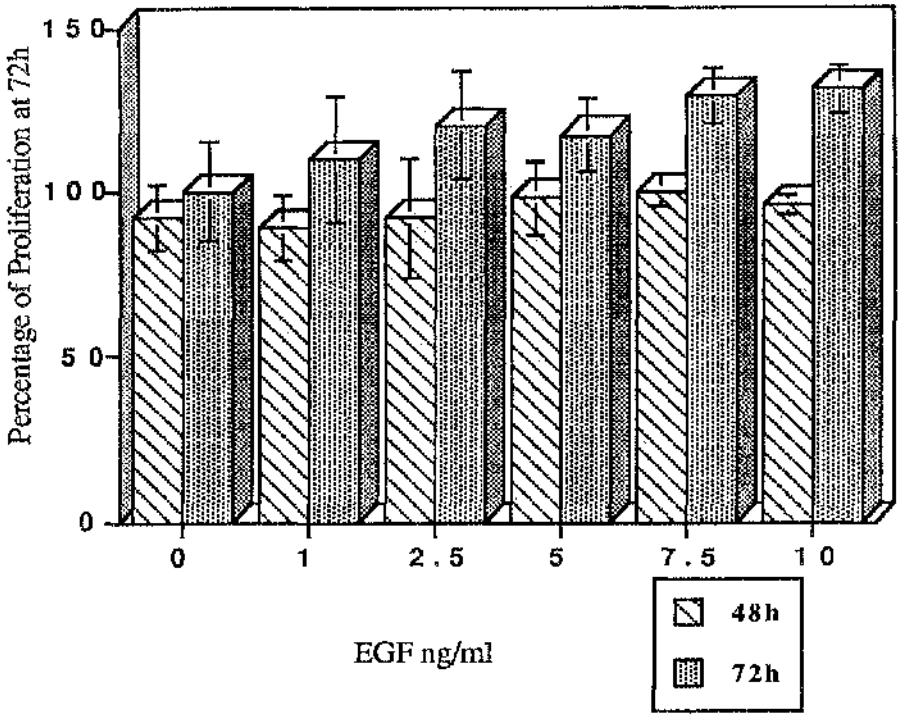

Figure 3.5 The effect of EGF on the growth of PC3 cells

6000 cells per well were plated into the 96 well plates. After overnight incubation, the medium was replaced with experimental medium containing EGF. After 2-3 days incubation, cell growth was assessed by the MTT assay (section 2.2.2). Each of the doses were assayed in quadruplicate, and the average of three independent determinations ± standard deviation is presented.

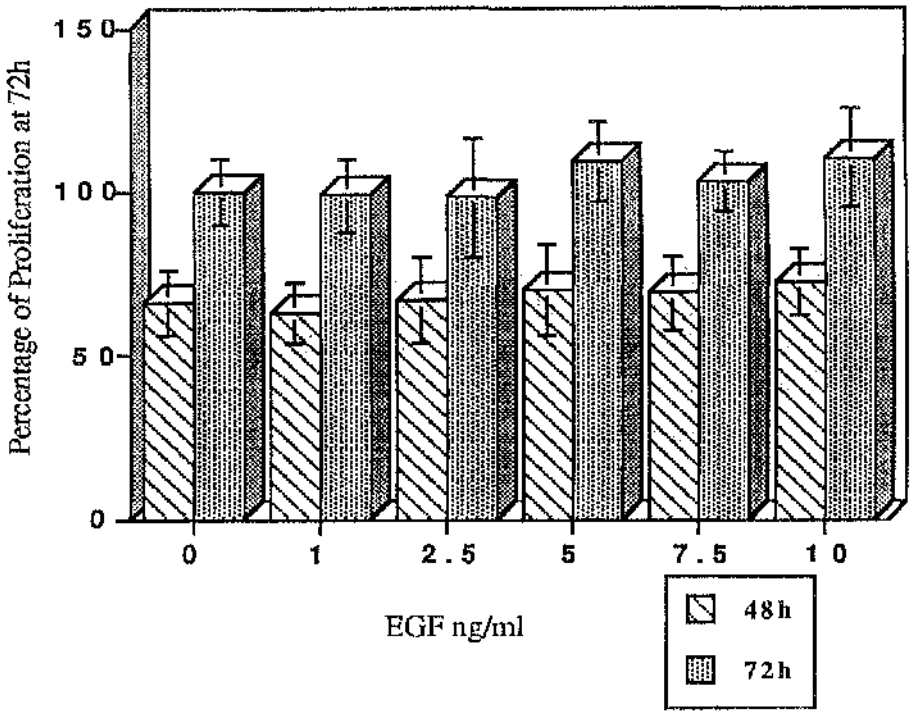

Figure 3.6 The effect of EGF on the growth of DU145 cells

6000 cells per well were plated into the 96 well plates. After overnight incubation, the medium was replaced with experimental medium containing EGF. After 2-3 days incubation, cell growth was assessed by the MTT assay (section 2.2.2). Each of the doses were assayed in quadruplicate, and the average of three independent determinations ± standard deviation is presented.

3.3.2 The growth effect of EGFR-specific TKI

Mitogenic stimulation of EGF involves binding to EGFR and activating the intracellular TK activity (Walker & Burgess, 1994). In order to appreciate if changes in TK activity contributed to the mechanism responsible for the growth of PC3 and DU145 cells, the effect of 4-quinazolines (ZM260603, ZM252868) (Jones *et al.*, 1997a; Wakeling *et al.*, 1996) was investigated. Cell proliferation rates were detected under both serum and EGF stimulation in the presence of the inhibitors for 72 hour. Preliminary experiments showed that cell growth can be inhibited with low μM concentrations and can be detected after both 48 and 72 hours.

ZM252868 led to a significant decrease of the proliferation of PC3 cells at 72 h. Serum stimulated PC3 cell growth was inhibited 43 % at 5 μM and 73 % at 10 μM TKI (Fig 3.7). The ability of the EGF receptor-related tyrosine kinase inhibitor to block prostate cancer cell growth was demonstrated by showing that PC3 cell numbers were reduced by 8.1 % by 1 μM, 33.1 % by 5 μM and 75 % by 10 μM TKI in the presence of EGF (10 ng/ml) over 72 h incubation (Fig 3.8).

The TKI also inhibited the growth of DU145 cell line in a dose dependent manner. DU145 exhibited 4, 32 and 51 % inhibition by 1, 5 and 10 μM TKI (Fig 3.9), in the presence of EGF stimulation and 22, 26 and 33 % inhibition in serum alone (Fig 3.10) at 72 h.

Growth response of the cells was also checked with another EGFR-specific TKI, ZM260603. ZM260603, like ZM252868, caused a dose dependent inhibition of both cell lines. In the presence of EGF, the drug caused 60 % inhibition at 5 μM and 78 % inhibition at 10 μM of PC3 cells. In the presence

of serum, the drug caused 45 % inhibition at 5 μM and 63 % inhibition at 10 μM. In DU145, the drug decreased 50 % at 5 μM and 72 % at 10 μM of the EGF stimulated growth. In the presence of serum, the drug caused 30% at 5μM and 75 % at 10 μM decrease in the growth of the cell in 72 h. The IC50 values of the inhibitor (ZM260603) for both enzyme and cell growth of the drug are shown in table 3.2 (see also fig 3.3 and 3.4).

Table 3.2 IC50 values of ZM260603 in PC3 and DU145 cell lines

Cell Line	PC3	DU145
EGF stimulated growth	3.36 ± 0.64 μM	4.34 ± 0.13 μM
Serum stimulated growth	5.01 ± 0.544 μM	5.52 ± 0.14 μM
Enzyme activity	0.021 ± 0.012 μM	0.0315 ± 0.021 μM

The data in this table clearly suggest that simple inhibition of the tyrosine kinase activity of the EGFR occurs at a much lower (100-fold) concentration of TKI than is required to inhibit growth. This may possibly reflect the problems of uptake across the intact cell membrane in growth experiments or could be due to one of many factors related to the structure of the intact receptor.

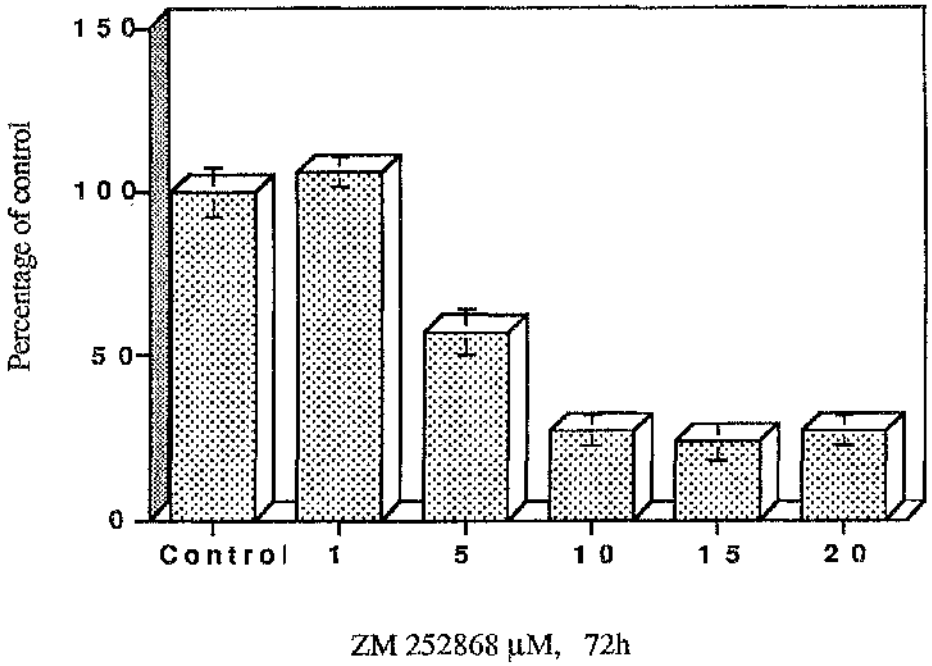

Figure 3.7 The effect of EGFR-related tyrosine kinase activity inhibition on the serum-stimulated growth of PC3 cells

6000 cells per well were plated into the 96 well plates. After overnight incubation, the medium was replaced with experimental medium containing the inhibitor and 30 min. later cells were stimulated by adding 10% FCS in RPMI. After 3 days incubation, cell growth was assessed by the MTT assay (section 2.2.2). Each of the doses were assayed in quadruplicate, and the average of three independent determinations ± standard deviation is presented.

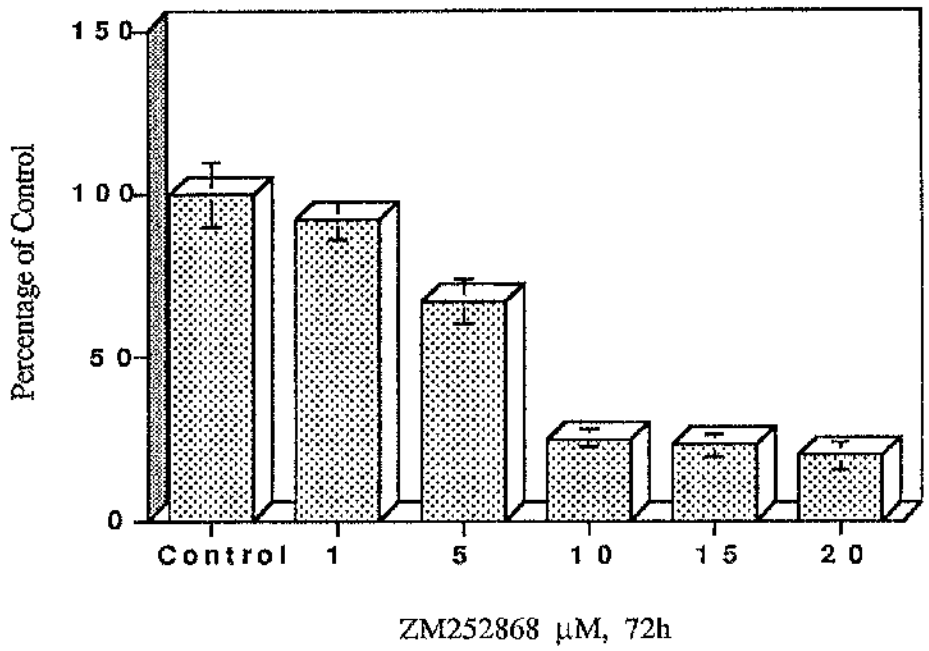

Figure 3.8 The effect of EGFR-related tyrosine kinase activity inhibition on the EGF-stimulated growth of PC3 cells

6000 cells per well were plated into the 96 well plates. After overnight incubation, the medium was replaced with experimental medium containing the inhibitor and 30 min later cells were stimulated by adding 20ng/ml EGF (final concentration of EGF was 10ng/ml) in RPMI. After 3 days incubation, cell growth was assessed by the MTT assay (section 2.2.2). Each of the doses were assayed in quadruplicate, and the average of three determinations ± standard deviation is presented.

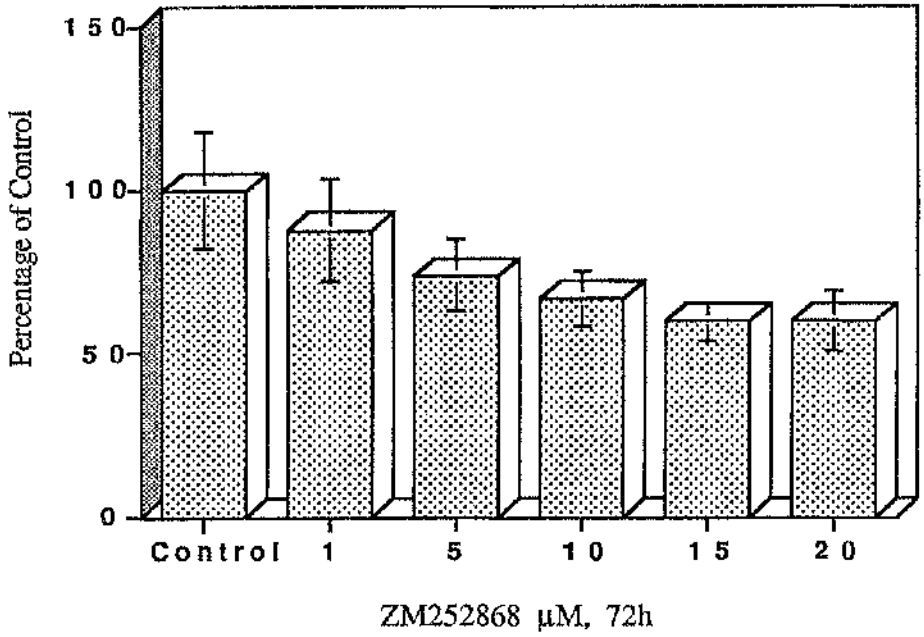

Figure 3.9 The effect of EGFR-related tyrosine kinase activity inhibition on the serum-stimulated growth of DU145 cells

6000 cells per well were plated into the 96 well plates. After overnight incubation, the medium was replaced with experimental medium containing the inhibitor and 30 min. later cells were stimulated by adding 10% FCS in RPMI. After 3 days incubation, cell growth was assessed by the MTT assay (section 2.2.2). Each of the doses were assayed in quadruplicate, and the average of three independent determinations ± standard deviation is presented.

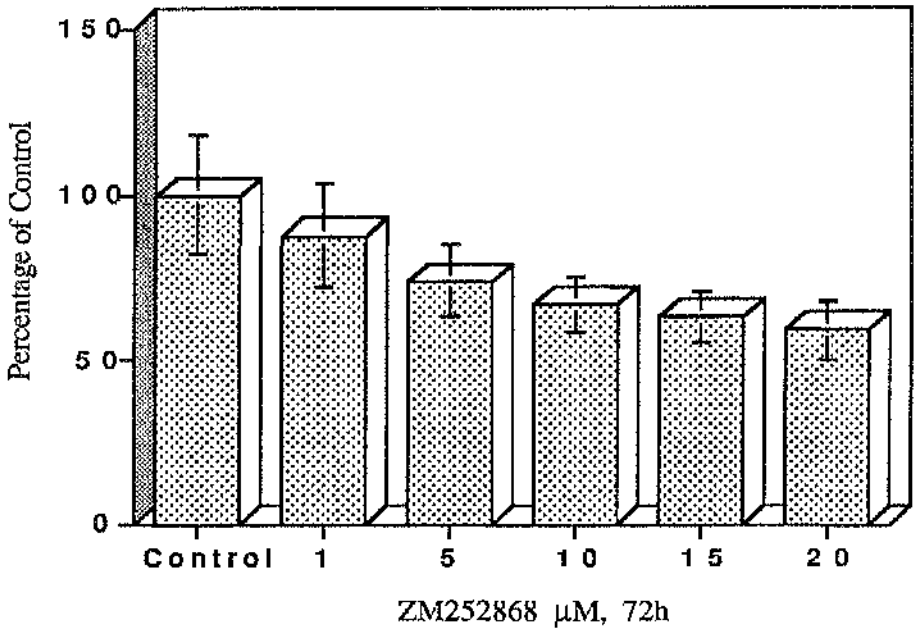

Figure 3.10 The effect of EGFR-related tyrosine kinase activity inhibition on the EGF-stimulated growth of DU145 cells

6000 cells per well were plated into the 96 well plates. After overnight incubation, the medium was replaced with experimental medium containing the inhibitor and 30min later cells were stimulated by adding 20ng/ml EGF (final concentration of EGF was 10ng/ml) in RPMI. After 3 days incubation, cell growth was assessed by the MTT assay (section 2.2.2). Each of the doses were assayed in quadruplicate, and the average of three determinations ± standard deviation is presented.

3.4 The regulation of PA content at mRNA level

The regulation of PA content at the transcription level was detected using RT-PCR. The ratio of uPA mRNA to ß-Actin mRNA was used to evaluate the value of the band density.

Analysis of total cellular RNA revealed an enhanced expression of uPA transcription after exposure to EGF in PC3 cell line. Treatment with EGF (10 ng/ml) resulted in a 96.8 % increase in the uPA mRNA (Fig 3.11) and a 225 % increase in uPAR mRNA expression (Fig 3.12). A 30% decrease was observed in PAI-1 mRNA expression when compared to the control group (Fig 3.13). Inhibition of EGF related TK activity resulted in an 88 % decrease in uPA mRNA (Fig 3.11), a 78 % decrease in uPAR mRNA expression (table 3.3). There was also a slight increase (9 %) in PAI-1 mRNA level when compared to the control group (table 3.3), however, this increase was not statistically significant. The quantification of the density of RT-PCR results for PC3 is summarised in table 3.3

Table 3.3 Quantification of PA components determined as RT-PCR analysed bands by densitometry in PC3 cells. Numbers are expressed as the ratio of RNA expression for uPA/ß-Actin. Values represent the mean of three experiments.

Group	uPA	uPAR	PAI-1
Control	1.57 ± 0.23	0.74 ± 0.01	1.05 ± 0.2
EGF	3.09 ± 0.41	2.41 ± 0.29	0.73 ± 0.09
TKI	0.19 ± 0.07	0.16 ± 0.03	1.14 ± 0.17

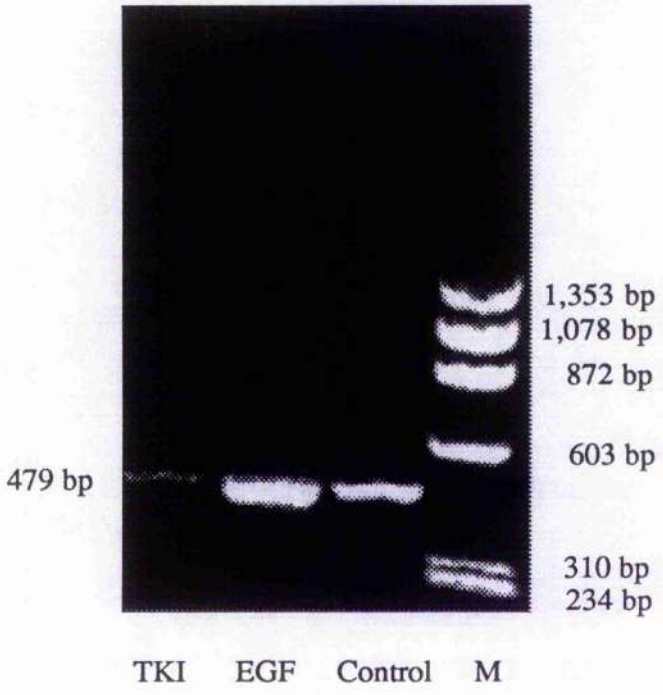

Figure 3.11: The effects of EGF and ZM252868 on uPA transcript in PC3 cells

PC3 cells were untreated or exposed to 10 ng/ml EGF in the presence and absence of 10 μM TKI for 48 h. After the treatment period, RNA was extracted and reverse transcribed into cDNA (sections 2.2.6) then PCR performed using the degenerate primers designed to detect transcripts selectively for uPA isoforms. uPA primers were: **5'** (644), AGAATTCACCACCATCGAGA // ATCAGCTTCACAACACTGAT (1122) **3'**, defining a 479 bp fragment. PCR reaction employed 1 min at 95 °C, 1 min at 49 °C, 40 seconds at 72 °C for 35 cycles. The products were electrophoresed on 2 % agarose gel and visualised with ethidium bromide under UV light. The figure is a representative of three experiments using RNA from different extractions. (M: marker, bp: base pair)

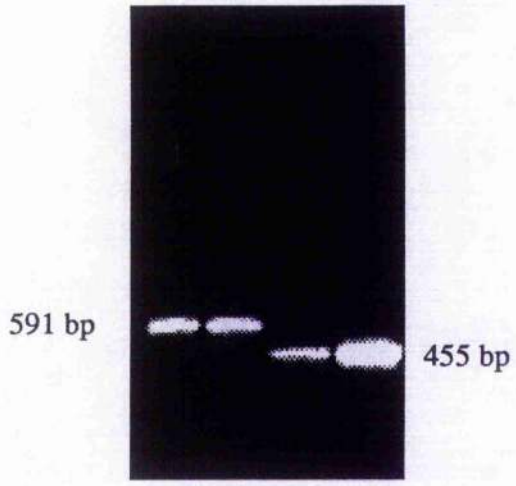

Figure 3.12: The effect of EGF on uPAR transcript in PC3 cells

PC3 cells were untreated or exposed to 10 ng/ml EGF for 48 h. After the treatment period, RNA was extracted and reverse transcribed into cDNA then PCR performed (section 2.2.6) using the degenerate primers designed to detect transcripts selectively for uPAR isoforms. The uPAR oligos were: **5'** (385) TTACCTCGAATGCATTTCCT // TTGCACAGCCTCTTACCATA (840) **3'**, defining a 455 bp fragment. The ß-Actin oligos were **5'** (290) GAAATTCTGGCACCACACCTT // TTGAAGGTAGTTTCGTGGAT (881) **3,** defining a 591 bp fragment'. PCR reaction employed 1 min at 95 °C, 1 min at 49 °C, 40 seconds at 72 °C for 35 cycles for uPAR. PCR reaction employed 1 min at 95 °C, 70 seconds at 45 °C, 40 seconds at 72 °C for 35 cycles for ß-Actin. The products were electrophoresed on 2 % agarose gel and visualised with ethidium bromide under UV light. The figure is a representative of three experiments using RNA from different extractions.

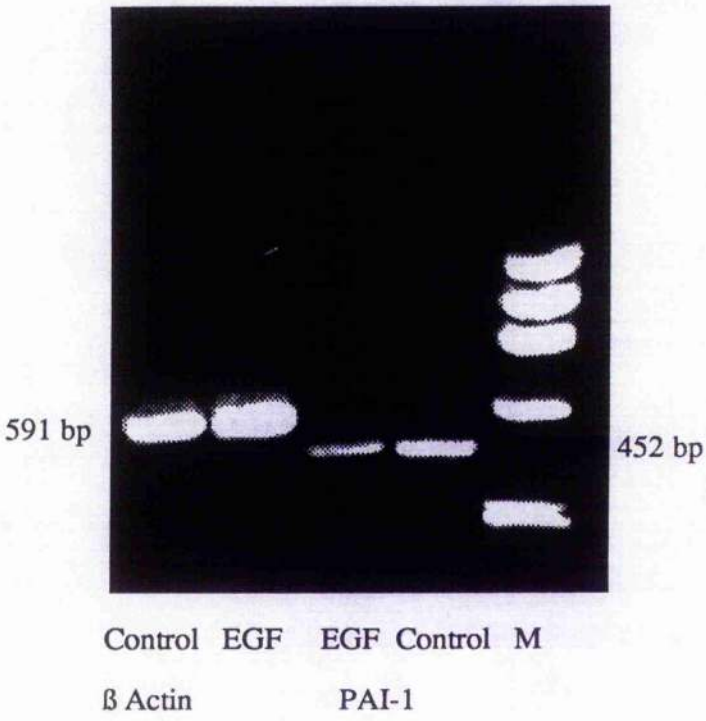

Figure 3.13: The effect of EGF on PAI-1 transcript in PC3 cells

PC3 cells were treated to 10 ng/ml EGF for 48h. After the treatment period, RNA was extracted and reverse transcribed into cDNA (sections 2.2.6) then PCR was performed using the degenerate primers designed to detect transcripts for PAI-1 isoforms. PAI-1 primers were: 5' (328) ATGGGATTCAAGATTGATGA // TCAGTATAGTTGAACTTGTT(780) 3', defining a 452bp fragment. PCR reaction employed 1 min at 95 °C, 70 seconds at 45 °C, 40 seconds at 72 °C for 35 cycles. The products were electrophoresed on 2 % agarose gel and visualised with ethidium bromide under UV light. The figure is a representative of three experiments using RNA from different extractions. (M: marker)

Analysis of total cellular RNA showed that uPA transcription was decreased by 22 % after exposure to EGF in DU145 cell line. However this inhibition was not statistically significant. uPAR mRNA expression was stimulated by 523 % (Fig 3.14) when compared to the control. A 61 % decrease was observed in PAI-1 mRNA expression when compared to the control group (table 3.4).

Inhibition of EGF related TK activity resulted in no difference in uPA mRNA, while totally abolishing the uPAR mRNA expression (table 3.4). The failure of the TKI to reverse the PAI-1 inhibition by EGF in DU145 cells is anomalous. A 68 % decrease was observed in PAI-1 mRNA level when compared to the control group (table 3.4). The quantification of the density of RT-PCR results for DU145 is summarised in table 3.4.

Table 3.4 Quantification of PA components determined as RT-PCR analysed bands by densitometry in DU145 cells. Numbers are expressed as the ratio of RNA expression for uPA/ß-Actin. Values represent the mean of three experiments.

Group	uPA	uPAR	PAI-1
Control	0.94 ± 0.13	0.077 ± 0.01	0.74 ± 0.1
EGF	0.74 ± 0.1	0.48 ± 0.09	0.29 ± 0.06
TKI	0.96 ± 0.11	0	0.24 ± 0.07

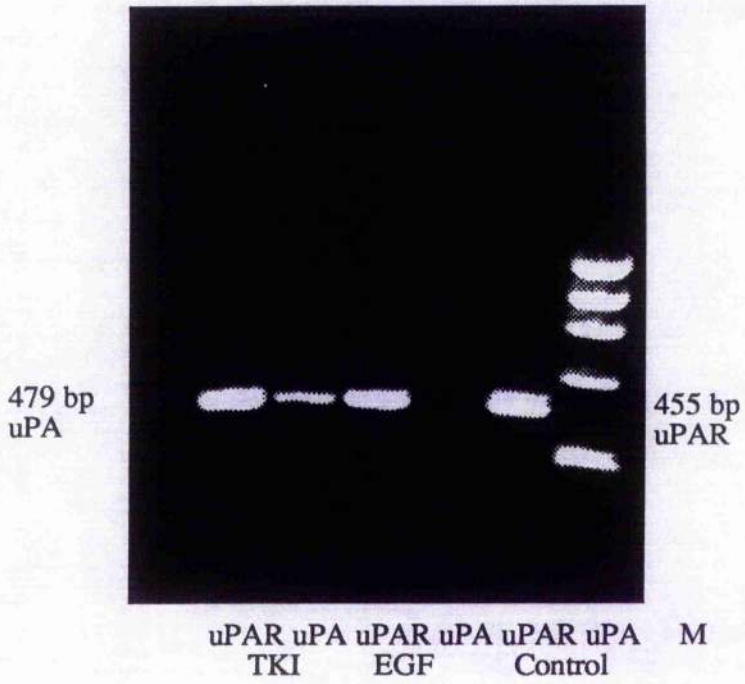

Figure 3.14. The effects of EGF and ZM252868 on uPA and uPAR transcripts in DU145 cells

DU145 cells were untreated or exposed to 10 ng/ml EGF in the presence and absence of 10 μM TKI for 48 h. Then RNA was extracted, reverse transcribed into cDNA and PCR was performed using the primers designed to detect transcripts selectively for uPA and uPAR isoforms. The products were electrophoresed on 2 % agarose gel and visualised with ethidium bromide under UV light. The figure is a representative of three experiments using RNA from different extractions. (M: marker)

3.5. The regulation of uPA, PAI-1 and PAI-2 proteins

3.5.1. The regulation of uPA protein

To determine the amount of both secreted and cell-associated uPA and PAI-1 protein, the four-span antibody detection system was employed, described in section 2.2.5. Both conditioned medium (secreted uPA) and cell homogenate extracts (cell-associated uPA) were analysed. The amount of both secreted and cell-associated uPA by PC3 cells was nearly double than those found in the DU145 cells (27.03 / 15.3 ng/mg ; 18.5 / 9.7 ng/mg respectively) in the control groups in 48h.

Treatment with EGF increased the level of both secreted and cell-associated uPA protein in the PC3 cells. The PC3 cell cytosol fraction showed a 120 % increase in uPA in response to EGF at 10 ng/ml dose in 48h (Fig 3.15). A 77 % increase was also observed in the secreted medium. EGF related TK enzyme activity inhibition reversed the EGF stimulation of uPA production. In the presence of inhibitor, a 40 % decrease in the cytosol and a 63 % decrease in the conditioned medium were observed in the PC3 cells. The levels of uPA protein are shown in table 3.5.

EGF also stimulated the production of cell-associated uPA protein by 100 % in DU145 (Fig 3.16), however secreted uPA levels were similar with the control group. In the presence of inhibitor, cell-associated uPA level was reversed near to the control group. Furthermore, a 200 % decrease was observed in the medium of DU145 cell line in the presence of TKI when compared with the control group, where similar uPA levels were observed in the cytosol. The levels of uPA protein in the DU145 cells are shown in table 3.6.

Table 3.5 The levels of uPA protein in the PC3 cell line. Values represent the mean ± sd of three independent experiments and each experiment was carried out in duplicate.

Group	Cytosol uPA ng/mg ± sd	Secreted uPA ng/mg ± sd
Control	27.03 ± 5.6	18.5 ± 1.5
EGF 5ng/ml	37.63 ± 5.8	31 ± 4.6
EGF 10ng/ml	59.33 ± 4.5	32 ± 4.1
TKI 5μM	18.73 ± 3.4	11.7 ± 3.4
TKI 10μM	16 ± 2.7	6.8 ± 1.7

Table 3.6 The levels of uPA protein in the DU145 cell line. Values represent the mean ± sd of three independent experiments and each experiment was carried out in duplicate.

Group	Cytosol uPA ng/mg ± sd	Secreted uPA ng/mg ± sd
Control	15.3 ± 1.04	9.7 ± 1.2
EGF 5ng/ml	20.5 ± 3.2	8.3 ± 2.3
EGF 10ng/ml	30.1 ± 1.37	9.8 ± 1.7
TKI 5μM	12.37 ± 1.86	5.98 ± 1.3
TKI 10μM	17.57 ± 3.6	2.8 ± 1.3

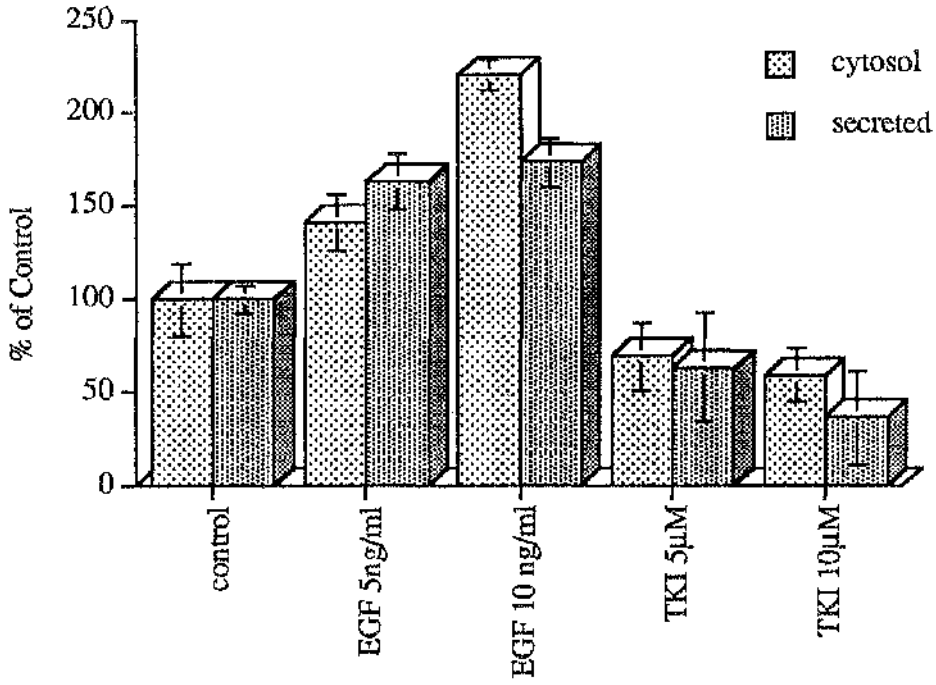

Figure 3.15 Effect of exogenous EGF and ZM252868 on uPA protein in the PC3 cell line

The cell homogenate and the conditioned medium of the cells were assayed by ELISA after 48h. Two different doses were used for each factor (EGF 5ng/ml, 10ng/ml and TKI 5, 10 μM). For both concentration of TKI, cells were stimulated with 10 ng/ml of EGF. In all cases, each sample was assayed in duplicate. The mean of three independent determinations ± standard deviation is presented.

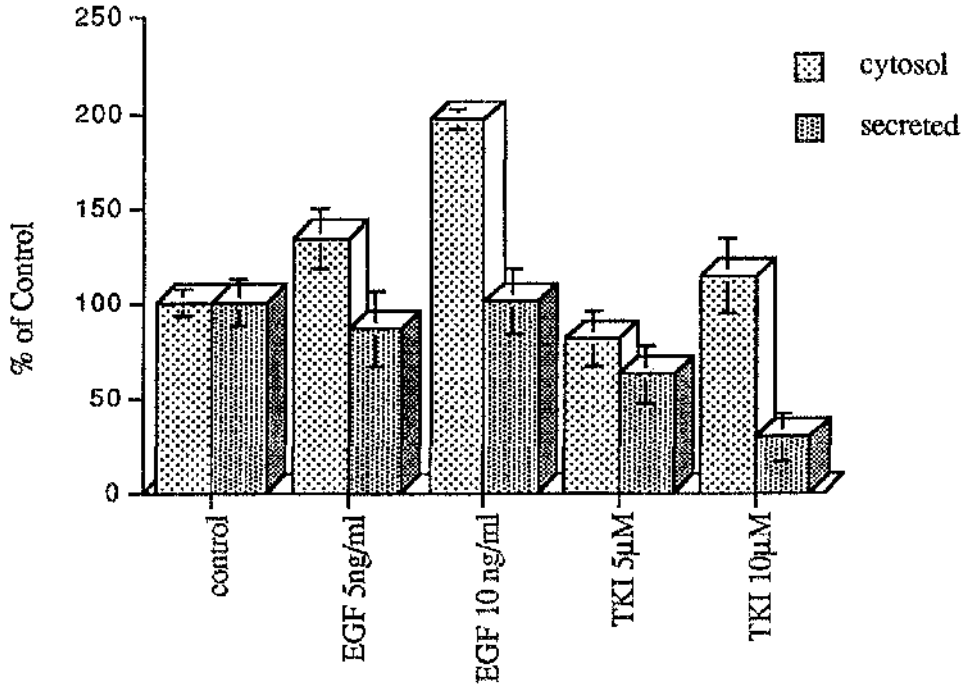

Figure 3.16 Effect of exogenous EGF and ZM252868 on uPA protein in DU145 cells

The cell homogenate and the conditioned medium of the cells were assayed by ELISA after 48h. Two different doses were used for each factor (EGF 5ng/ml, 10ng/ml and TKI 5, 10 μM). For both concentration of TKI, cells were stimulated with 10 ng/ml of EGF. In all cases, each sample was assayed in duplicate. The mean of three independent determinations ± standard deviation is presented.

3.5.2 The regulation of PAI-1 protein

The level of PAI-1 protein was measured by the four-span antibody detection system, described in 2.2.5. PC3 cells were revealed to have a very low amount of PAI-1 protein compared to the uPA protein. Control PC3 cells contained 0.41 ± 0.051 ng/mg PAI-1 protein in the cytosol and 0.32 ± 0.04 ng/mg PAI-1 protein in medium. Treatment with exogenous EGF did not have a significant change in either the cytosol (0.37 ± 0.05 ng/mg) or in medium (0.378 ± 0.04 ng/mg). EGF-related TK inhibition also showed no difference in PAI-1 secretion and production.

PAI-1 protein was hardly detectable in the control group of DU145 cells and there was no detectable PAI-1 protein in either the EGF stimulated group or in the presence of EGF plus TKI.

3.5.3 The regulation of PAI-2 protein

The level of PAI-2 protein was measured by the four-span antibody detection system, described in 2.2.5. PAI-2 protein was only detectable in the PC3 cell line. PC3 cell cytosol was diluted 1 in 20 while conditioned medium was used without dilution. PC3 cell cytosol contained 3 ± 0.6 ng/mg and medium contained 0.18 ± 0.03 ng/mg PAI-2 protein. Exogenous EGF treatment did not change the level of PAI-2 protein either in cytosol (3.18 ± 0.28 ng/mg) or in the medium (0.14 ± 0.03 ng/mg) when compared with the control group (fig 3.17). However in the presence of TKI, a decrease in the level of PAI-2 protein was observed in both cell cytosol (1.61 ± 0.36 ng/mg) and medium (0.09 ± 0.015 ng/mg). These inhibitions were found as significant (p value is 0.0288 for cytosol, p value is 0.0086 for the CM).

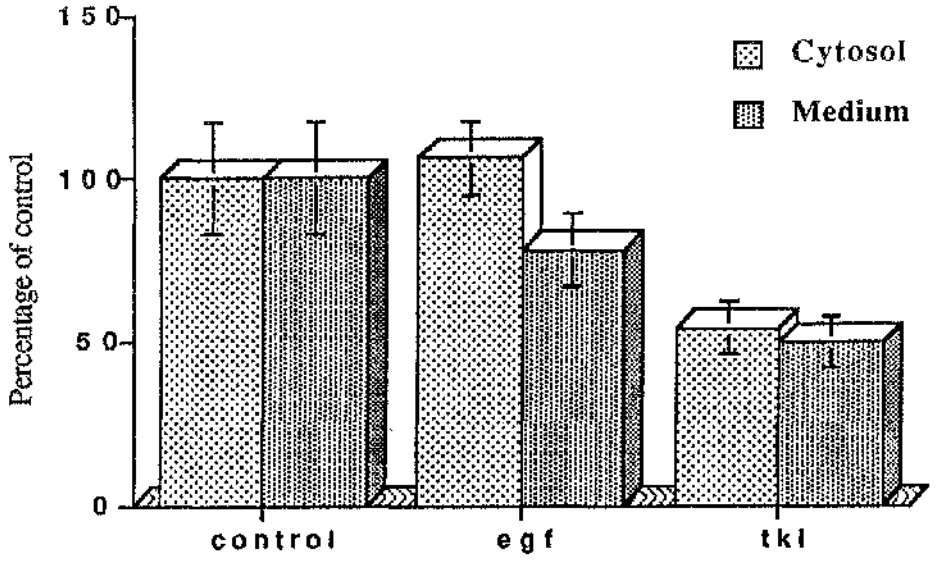

Figure 3.17. Effect of exogenous EGF and ZM260603 on PAI-2 protein in PC3 cells

The cells were incubated with EGF (10ng/ml) with or without the inhibitor (5 μM) for 48 hours. In all cases, each sample was assayed in duplicate. The mean of three independent determinations ± standard deviation is presented.

tPA protein was only detectable in PC3 cell line in a very low level (0.01 ng/mg cell lysate protein, 0.24 ng/mg in medium). Treatment with EGF (alone or in the presence of TKI) showed no effects on the regulation of tPA protein by PC3 cell line.

3.6 Detection of uPA enzyme activity

uPA enzyme activity was detected by using a chromogenic plasmin substrate, S-2251. Inclusion of a monoclonal ab (#394, American Diagnostica, mouse anti-uPA), concentration of 1/50, in the assay mixture abrogated the enzyme activity by 100 %. This result indicates that the plasminogen activation observed was primarily due to uPA. Furthermore there was no activity observed in the absence of plasminogen, indicating that the substrate is specific for plasmin.

Levels of uPA enzyme activity detected in the conditional medium of PC3 cells were 25 times higher than levels in DU145 cells. PC3 cell cytosol also revealed 2.75 times higher uPA activity than DU145 cell cytosol. PC3 cell line has shown a 49 % increase in medium and a 28 % increase (table 3.7) in cytosol in response to EGF (10 ng/ml). In the presence of TK inhibitor (10μM), uPA activity was inhibited by 60 % in cytosol (Fig 3.26) and 72 % in medium (Fig 3.27).

Table 3.7 uPA enzyme activity in the PC3 cell line. Values represent the mean ± sd of three independent experiments and each experiment was carried out in duplicate.

Group	Cytosol uPA Activity u/mg protein	Secreted uPA Activity u/mg protein
Control	98.67 ± 6.17	11.39 ± 1.5
EGF 10ng/ml	126.53 ± 5.71	16.62 ± 0.14
EGF 7.5ng/ml	110.1 ± 6.43	16.25 ± 0.8
EGF 5ng/ml	108.3 ± 8.28	14.64 ± 0.4
EGF 2.5ng/ml	104.3 ± 5.15	13.9 ± 0.8
TKI 10μM	39.1 ± 4.55	3.28 ± 0.69
TKI 7.5μM	50.73 ± 7.51	7 ± 1.72
TKI 5μM	62.2 ± 7.02	9.6 ± 1.04
TKI 2.5μM	81.33 ± 11.29	12.4 ± 1

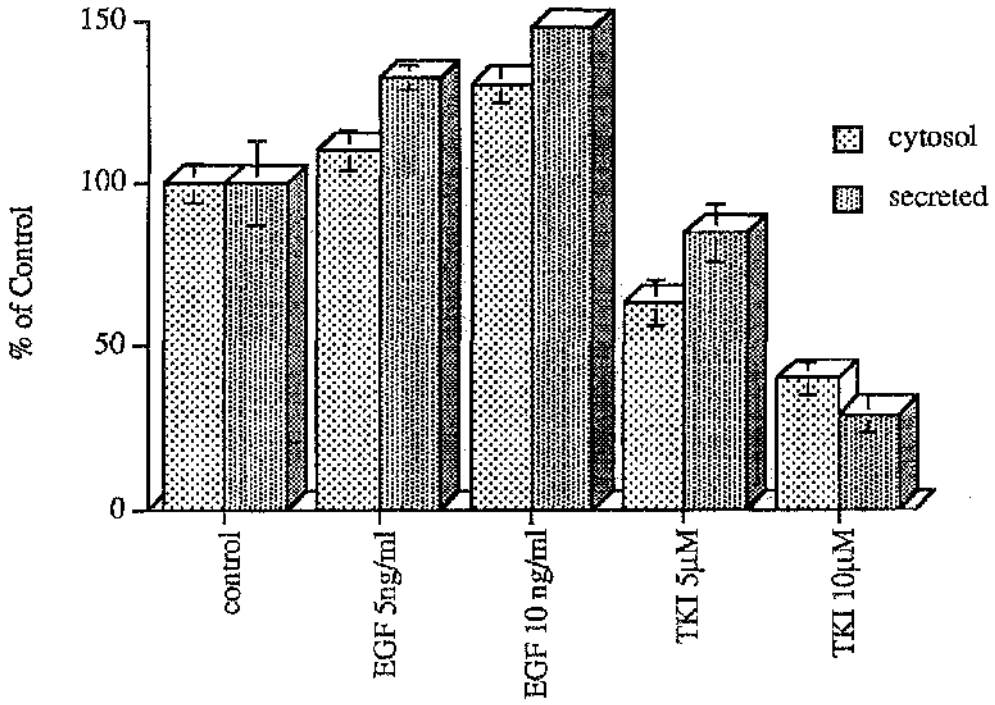

Figure 3.18 Effect of exogenous EGF and ZM252868 on uPA activity in PC3 cells

Effect of exogenous EGF and EGFR-related TK inhibitor (ZM252868) on the uPA activity is shown as a percentage of control. Two different doses were used for each factor (EGF 5 ng/ml, 10 ng/ml and TKI 5, 10 μM). For both concentration of TKI, cells were stimulated with 10 ng/ml of EGF. In all cases, each sample was assayed in duplicate. The mean of three independent determinations ± standard deviation is presented.

EGF treatment also enhanced uPA activity in the DU145 cells. Cell-associated uPA activity was increased by 90 % (Fig 3.19) and secreted uPA activity was increased by 34 % in the DU145 cells by EGF (10 ng/ml concentration). 73 % and 187 % increases were observed at 7.5 ng/ml concentration. Inhibition of the tyrosine kinase activity resulted in a 43 % inhibition in the cytosol fraction of activity whereas no activity was observed in the medium (table 3.8).

Table 3.8 uPA enzyme activity u/mg in DU145 cells. Values represent the mean ± sd of three different experiments and each experiment was carried out in triplicate. For the concentrations of TKI, cells were stimulated with 10 ng/ml of EGF. ND: Not Done

Group	Cytosolic uPA Activity u/mg protein	Secreted uPA Activity u/mg protein
Control	35.85 ± 3.5	0.44 ± 0.3
EGF 10ng/ml	68 ± 8.5	0.59 ± 0.29
EGF 7.5ng/ml	62 ± 4.3	1.27 ± 0.27
EGF 5ng/ml	46 ± 5.1	0.95 ± 0.29
EGF 2.5ng/ml	ND	1.1 ± 0.13
TKI 10μM	20.4 ± 3.4	0
TKI 7.5μM	ND	0.232 ± 0.05
TKI 5μM	25.1 ± 3.7	0.343 ± 0.08
TKI 2.5μM	ND	0.383 ± 0.07

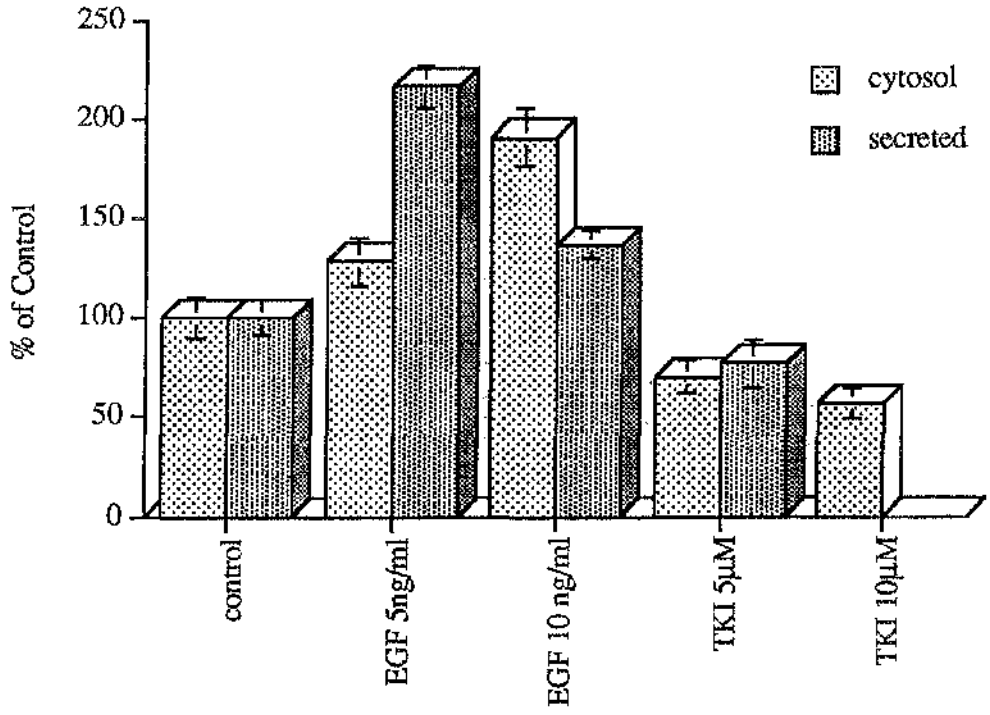

Figure 3.19 Effects of exogenous EGF and ZM252868 on uPA activity in DU145 cells

Effect of exogenous EGF and EGFR-related TK inhibitor (ZM252868) on the uPA activity is shown as a percentage of control. Two different doses were used for each factor (EGF 5 ng/ml, 10 ng/ml and TKI 5, 10 μM). For both concentration of TKI, cells were stimulated with 10 ng/ml of EGF. In all cases, each sample was assayed in duplicate. The mean of three independent determinations ± standard deviation is presented.

3.7 *In vitro* invasion of matrigel

In order to check the effects of EGF and the TK inhibitor on cell invasion, a matrigel invasion assay was performed. The cells were left on matrigel for 72h. In order to optimise the conditions of the method, different amounts of matrigel/well were checked. 50 µg matrigel/well was found to be the optimal amount for PC3 cells invasion at 72h. In order to show uPA dependency of the cell invasion of matrigel, either anti-uPA ab (#394) was added into the culture medium or plasminogen was omitted. Absence of plasminogen in the matrigel revealed a 30 % inhibition of the PC3 cells invasion whereas no difference was observed in the DU145 cells. Addition of the anti-uPA antibody to the medium resulted in a 35 and 36 % inhibition of the invasion in 24 and 48h experiments, respectively, showing that invasion cannot be attributed exclusively to the uPA.

Matrigel invasion of PC3 cells was increased 43 % by EGF at 72h (p value is 0.022, considered significant) compared to the control. There was no difference between TKI, and the control group (table 3.9). EGF increased invasion was reversed by TKI to the control group level (fig 3.20). PC3 cells showed nearly 3 times higher invasive capacity compared to DU145 cells. The DU145 cells exhibited 3-3.5 % invasion in the control group. There were no clear differences between the treated and control group for DU145 cells.

Table 3.9 Percentage of invasion of matrigel by PC3 cell line. Values represent the mean ± sd of at least three independent experiments and each experiment was carried out in duplicate.

Group	% of invasion ± sd
Control	9.8 ± 2.5
EGF	13.98 ± 1.55
TKI	9.28 ± 1.78

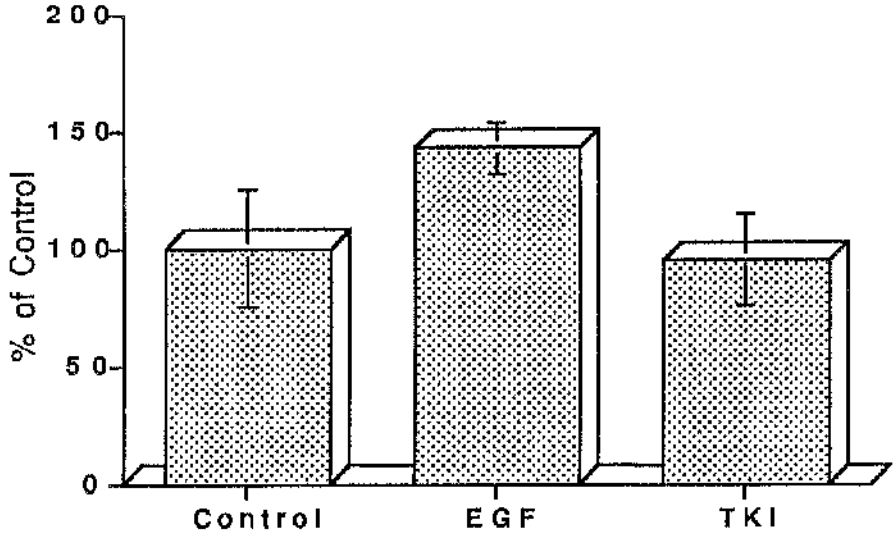

Fig 3.20 Effects of EGF and ZM260603 on matrigel invasion of PC3 cells

5×10^4 cells were plated on to the filter which was coated with 50 μg matrigel containing 50 μg/ml plasminogen. After 3 days incubation, the living cell number was determined by MTT assay. EGF concentration was 10 ng/ml in both EGF alone and in the presence of inhibitor (10 μM). In all cases, each sample was assayed in duplicate. The mean of three independent determinations ± standard deviation is presented.

STUDIES WITH TGFß and STEROIDS

4.1. Aim of the study

The purpose of this study was to investigate the effect of TGFß1 on the proliferation and the invasive potential of two prostate carcinoma cell lines. Growth responses to TGFß1 were evaluated in monolayer culture. The role of TGFß1 in regulation to invasion, using an *in vitro* assay, was investigated by inspecting the difference of the expression of plasminogen activator system components. The regulation of PA components was checked at both messenger-RNA and protein levels. To both confirm their roles in invasion and to investigate differences among family members, activities were assessed in relation to an *in vitro* invasion of extracellular matrix.

4.2 Growth studies

4.2.1 Growth response to TGFß

Both 48 and 72h exposure to TGFß confirmed that neither PC3 (Fig 4.1) nor DU145 (Fig 4.2) cell lines were sensitive to the inhibitory effect of exogenous TGFß. Dose range of TGFß used was 0 to 1ng/ml.

4.2.2 Growth response to sex steroids

To assess the effect on growth of DHT, testosterone, estrogen, progesterone on the cell lines, a range of concentrations from 10^{-6} M to 10^{-11} M of the steroids were tested. The effects of these exogenously added compounds were monitored by counting cells after 48 and 72 hours. It should be noted that in order to eliminate the effects of various growth factors and mitogens, heat inactivated, charcoal stripped serum was used for these studies. Neither PC3 (Fig 4.3) nor DU145 (Fig 4.4) growth were effected by steroids after 48 and 72h incubation. Only 72h data are represented in figures 4.3 and 4.4.

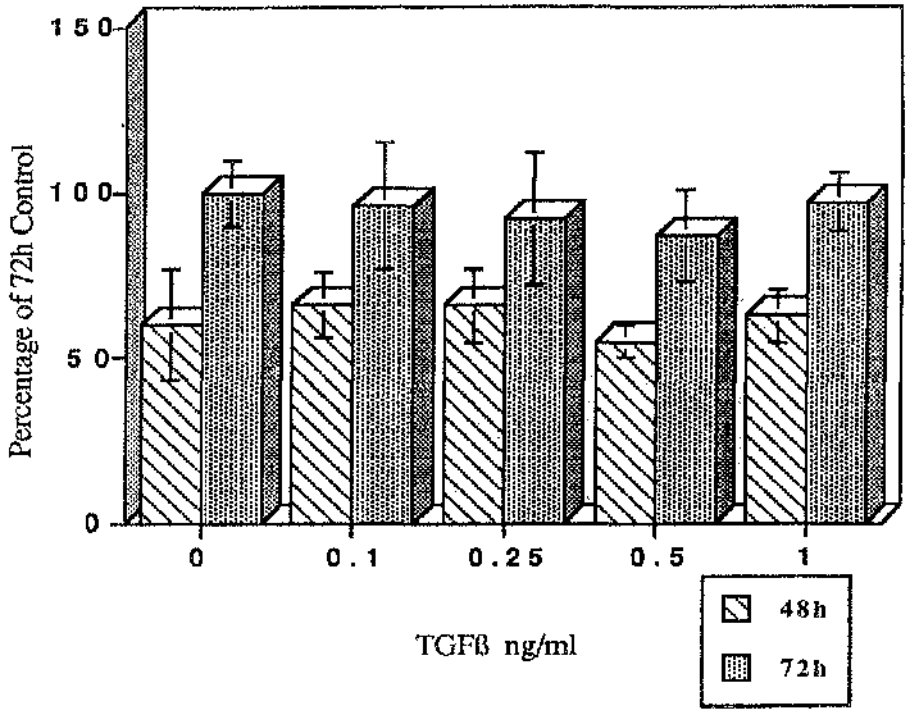

Figure 4.1 The effect of Transforming Growth Factor Beta on growth of PC3 cells

6000 cells/well plated into the 96 well plates. After overnight incubation, the medium of the cells was replaced with experimental medium containing TGFß. Three days incubation was ended by adding MTT. MTT crystals were then dissolved by adding DMSO and absorbances at 540nm were read subsequently after addition of glycine buffer. Each of the doses was assayed in quadruplicate, and the average of three independent determinations ± standard deviation is presented.

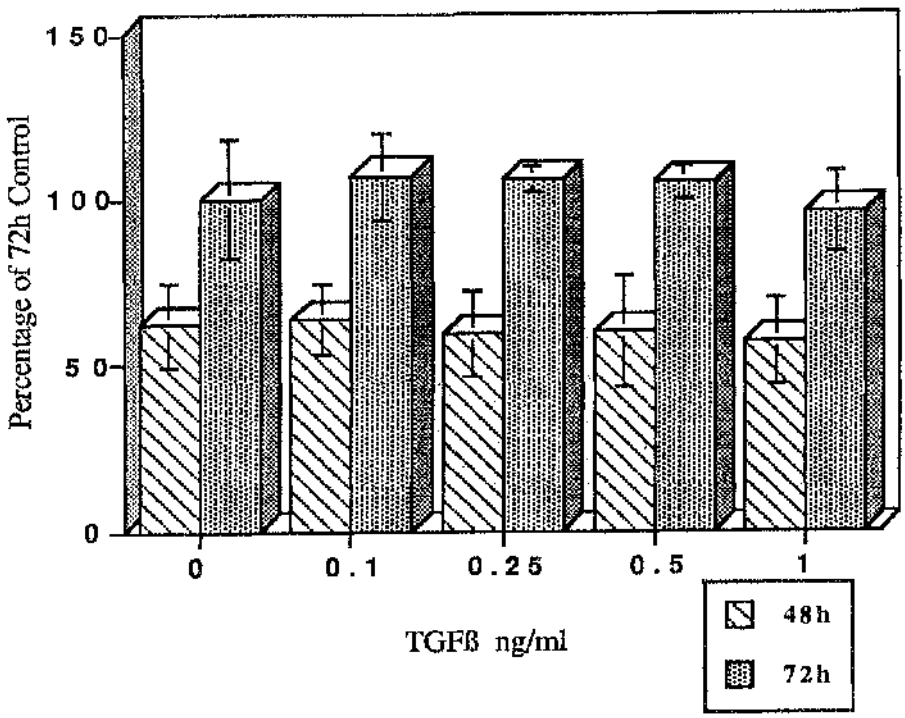

Figure 4.2 The effect of Transforming Growth Factor Beta on growth of DU145 cells

6000 cells/well plated into the 96 well plates. After overnight incubation, the medium of the cells was replaced with experimental medium containing TGFß. Three days incubation was ended by adding MTT. MTT crystals were then dissolved by adding DMSO and absorbances at 540nm were read subsequently after addition of glycine buffer. Each of the doses was assayed in quadruplicate, and the average of three independent determinations ± standard deviation is presented.

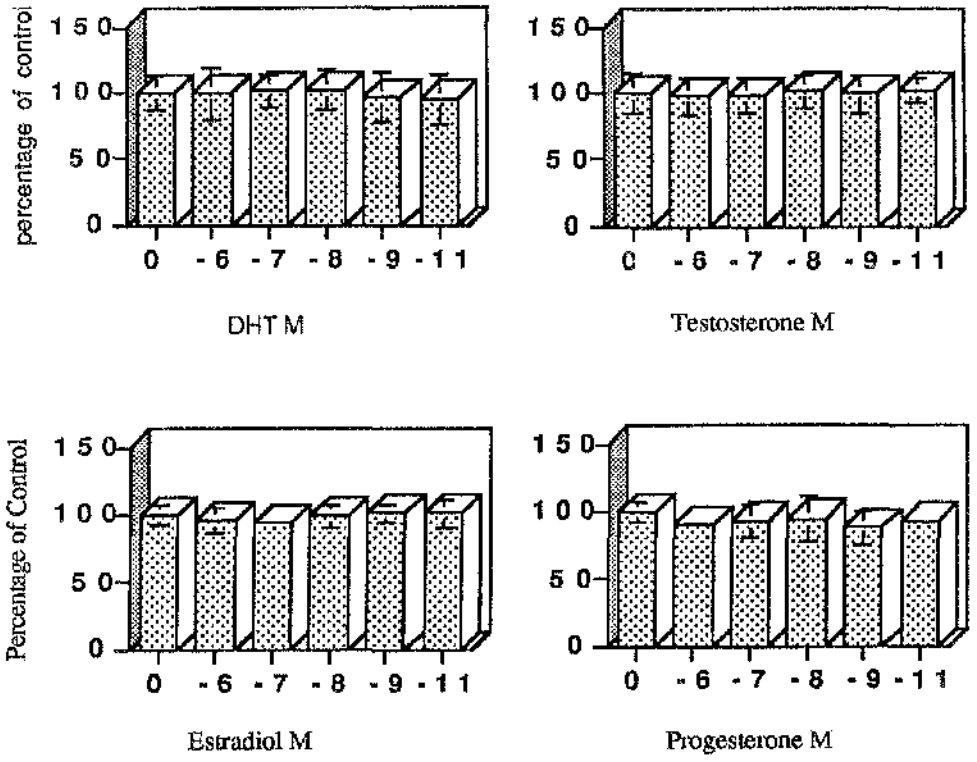

Figure 4.3 The effect of the steroids on growth of PC3 cells 6000 cells/well plated into the 96 well plates. After overnight incubation, the medium of the cells was replaced with experimental medium containing the steroids. Three days incubation was ended by adding MTT. MTT crystals were then dissolved by adding DMSO and absorbances at 540nm were read subsequently after addition of glycine buffer. Each dose was assayed in quadruplicate, and the average of three independent determinations ± standard deviation is presented.

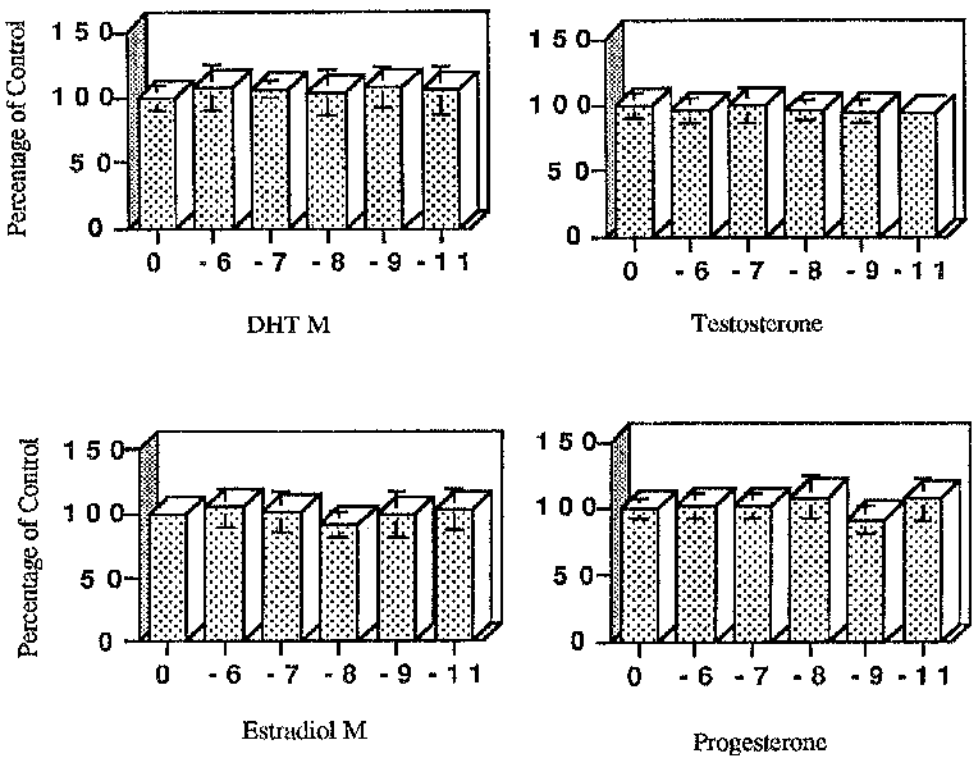

Figure 4.4 The effect of the steroids on growth of DU145 cell line

6000 cells/well plated into the 96 well plates. After overnight incubation, the medium of the cells was replaced with experimental medium containing the steroids. Three days incubation was ended by adding MTT. MTT crystals were then dissolved by adding DMSO and absorbances at 540nm were read subsequently after addition of glycine buffer. Each dose was assayed in quadruplicate, and the average of three independent determinations ± standard deviation is presented.

4.3 The regulation of PA content mRNA

The regulation of PA contents at the transcription level was studied using RT-PCR. The ratio of uPA mRNA to the ß-Actin mRNA was used to evaluate the value of the band density.

Analysis of total cellular RNA revealed enhanced expression of uPA transcription after exposure to TGFß (1ng/ml) in PC3 cell line. In the PC3 cell line, treatment with TGFß (Fig 4.5) increased uPA mRNA levels by 35% (P value is 0.031 and considered significant). The PAI-1 mRNA expression was inhibited by 12 % when compared to the PC3 control group (Fig 4.6). However this inhibition was not statistically significant.

The effect of TGFß was also checked on PAI-1-mRNA in DU145 cell line. Treatment with TGFß did not change the PAI-1-mRNA level (Fig 4.7).

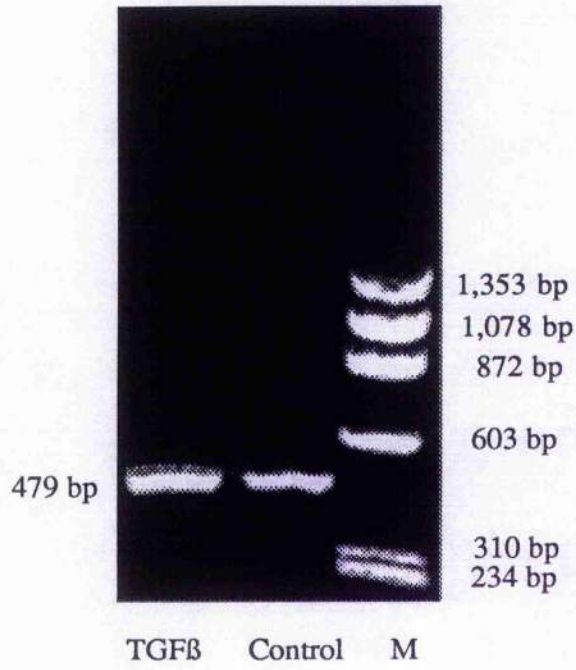

Figure 4.5: The effect of TGFß on uPA transcript in PC3 cells

PC3 cells were untreated or exposed to TGFß at a concentration of 1 ng/ml. After the treatment period, RNA was extracted and reverse transcribed into cDNA (sections 2.2.6) then PCR was performed using the degenerate primers designed to detect transcripts selectively for uPA isoforms. uPA primers were: **5'** (644), AGAATTCACCACCATCGAGA // ATCAGCTTCACAACACTGAT (1122) **3'**, defining a 479 bp fragment. The PCR reaction employed 1 min at 95 °C, 1 min at 49 °C, 40 seconds at 72 °C for 35 cycles. The products were electrophoresed on a 2 % agarose gel and visualised with ethidium bromide under UV light. The results are representative of three experiments using RNA from different extractions. (M: marker)

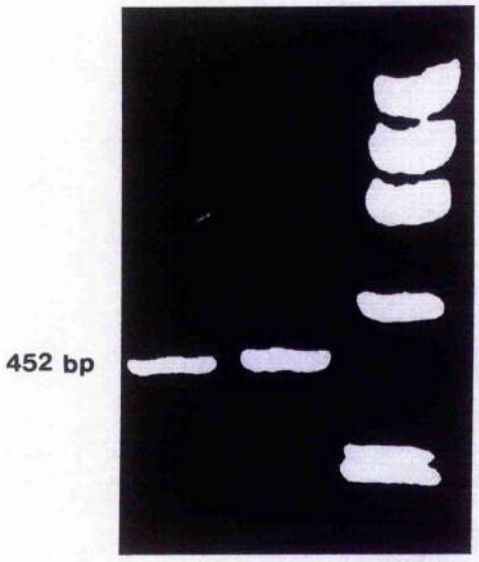

Figure 4.6 The effect of TGFß on PAI-1 transcript in PC3 cells PC3 cells were treated with 1 ng/ml TGFß for 48h. After the treatment period, RNA was extracted and reverse transcribed into cDNA (sections 2.2.6) then PCR was performed using the degenerate primers designed to detect transcripts for PAI-1 isoforms. PAI-1 primers were: **5'** (328) ATGGGATTCAAGATTGATGA // TCAGTATAGTTGAACTTGTT(780) **3'**, defining a 452bp fragment. The PCR reaction employed 1 min at 95 °C, 70 seconds at 45 °C, 40 seconds at 72 °C for 35 cycles. The products were electrophoresed on a 2 % agarose gel and visualised with ethidium bromide under UV light. The results are representative of three experiments using RNA from different extractions.

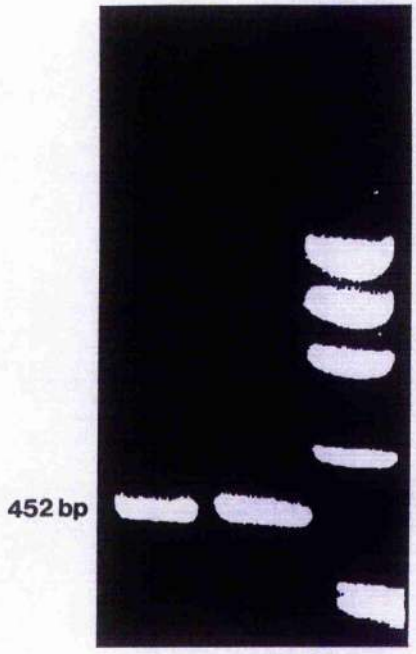

Figure 4.7 The effect of TGFß on PAI-1 transcript in DU145 cells

The cells were exposed to TGFß (1 ng/ml). After the treatment period, RNA was extracted and reverse transcribed into cDNA then PCR was performed using the degenerate primers designed to detect transcripts for PAI-1. PAI-1 primers were: **5'** (328) ATGGGATTCAAGATTGATGA // TCAGTATAGTTGAACTTGTT(780) **3'**, defining a 452bp fragment. The PCR reaction employed 1 min at 95 °C, 70 seconds at 45 °C, 40 seconds at 72 °C for 35 cycles. The products were electrophoresed on a 2 % agarose gel and visualised with ethidium bromide under UV light. The results are representative of three experiments using RNA from different extractions.

4.4. The regulation of uPA, PAI-1 and PAI-2 proteins

4.4.1. The regulation of uPA protein

To determine the amount of both secreted and cytosolic uPA and PAI-1 protein the four-span antibody detection system was used on both conditional medium and cell pellet.

TGFß (1 ng/ml) increased the production of uPA both in cytosol and in the conditioned medium of PC3 cell line (fig 4.8). Cell-associated uPA level was increased by 140 % in TGFß1 treated group (65 ± 8.4 ng/mg) compared with the control group (27.03 ± 5.6 ng/mg). The secreted form of uPA was increased by 58 % in TGFß1 group compared (29.3 ± 3.4 ng/mg) with the control group (18.5 ± 1.5 ng/mg) in PC3 cells in 48h.

uPA protein levels were similar in both cytosol (28.6 ± 6.7 ng/mg) and conditioned medium (16.2 ± 3 ng/mg) compared with control group (fig 4.8) in DHT treated PC3 cells.

In the DU145 cell line, TGFß1 (1 ng/ml) also increased (Fig 4.9) the production of uPA by 57 % in the cytosol (24 ± 4.5 , 15.3 ± 1.04 ng/mg respectively; P value is 0.0082, considered very significant). However, there was no difference in the secreted medium (9.9 ± 1.3, 9.7 ± 1.2 ng/mg respectively).

uPA protein levels were similar in DHT treated and in control group in the DU145 cell cytosol (17.2 ± 2.5 ng/mg) and medium (10 ± 1.9 ng/mg) (fig 4.9).

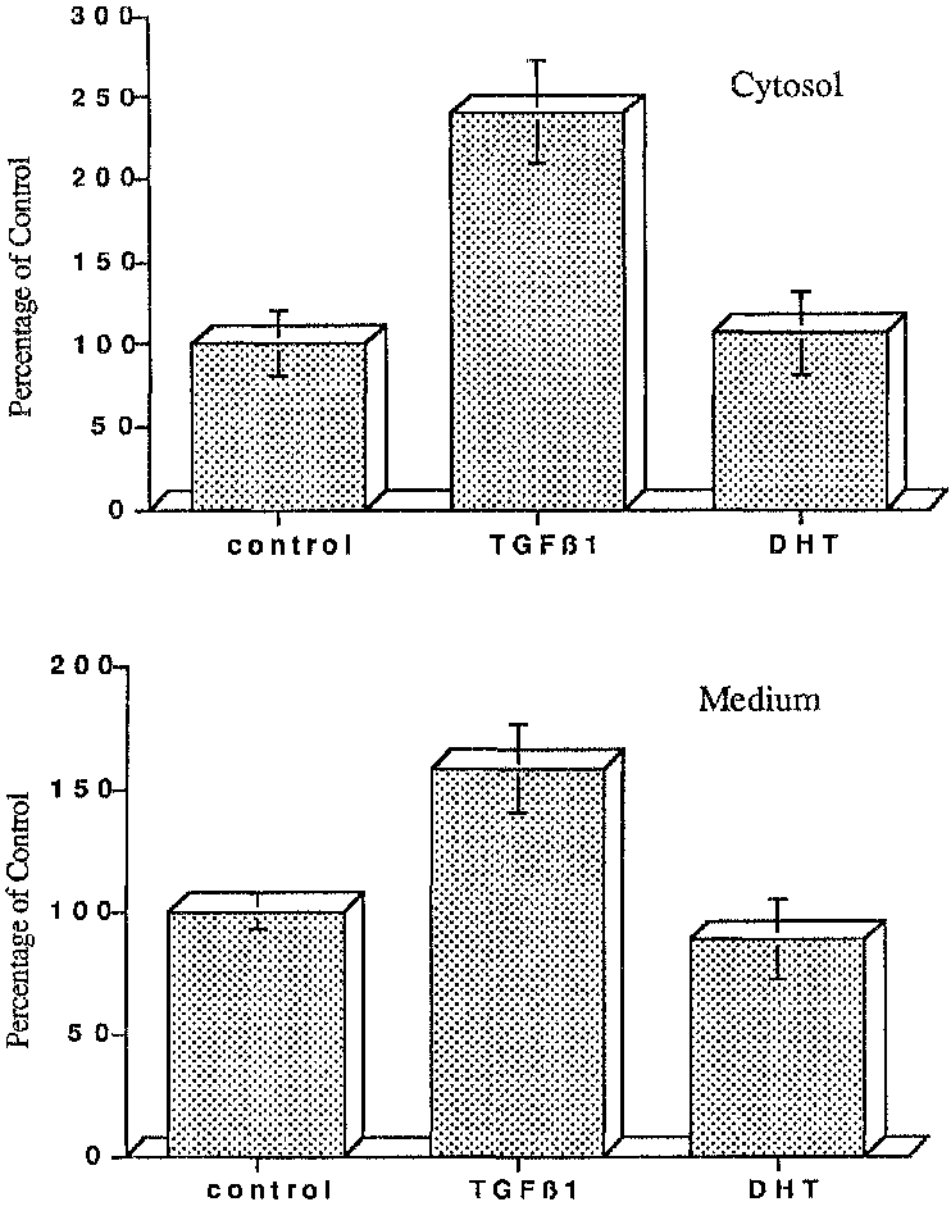

Figure 4.8 The effects of TGFß and DHT on uPA protein in PC3 cells

TGFß 1ng/ml and DHT 1 nM were used. In all cases, each sample was assayed in duplicate using the 4-span ELISA (see section 2.2.5). The average of three independent determinations ± standard deviation is presented.

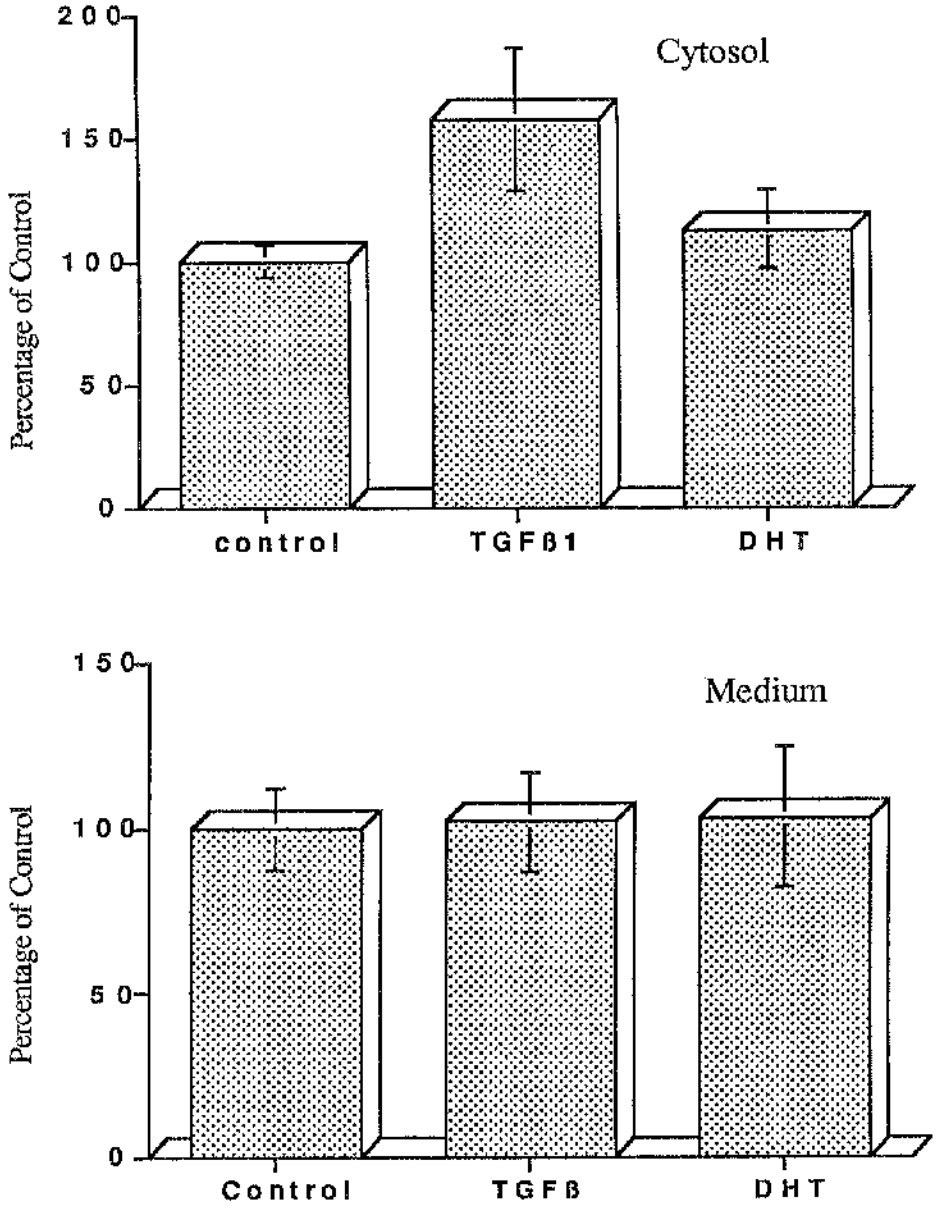

Figure 4.9 The effects of TGFß and DHT on uPA protein in DU145 cells

TGFß 1ng/ml and DHT 1 nM were used. In all cases, each sample was assayed in duplicate using the 4-span ELISA (see section 2.2.5). The average of three independent determinations ± standard deviation is presented.

4.4.2. The regulation of PAI-1 protein

Treatment with TGFß revealed a significant increase in the PAI-1 protein secretion in PC3 (Fig 4.10) Secreted PAI-1 protein levels were nearly 20 times higher in the TGFß1 (1 ng/ml) treated group (6.68 ± 1.4 ng/mg) compared with the control group (0.32 ± 0.04 ng/mg). This stimulation was dose dependent. The induction of PAI-1 protein by TGFß was also demonstrated in the mouse model of prostate carcinoma (Sehgal *et al.*, 1996). In this study both the metastatic and primary mouse prostate carcinoma cell line showed an increase in PAI-1 protein deposition in response to TGFß.

The effect of DHT on PAI-1 protein levels (both secreted and cell-associated levels) was determined and little difference was detected between experimental and control conditions. Detected values are summarised in table 4.1.

Table 4.1 The effects of TGFß1 and DHT on PAI-1 protein in PC3 cell Values represent the mean ± sd of three different experiments and each experiment was carried out in duplicate. ND: Not Done

Group	Cytosol ng/mg	Secreted ng/mg
Control	0.41 ± 0.051	0.32 ± 0.04
TGFß 0.1ng/ml	ND	0.84 ± 0.2
TGFß 0.25ng/ml	ND	1.54 ± 0.3
TGFß 0.5ng/ml	ND	2.89 ± 0.4
TGFß 1ng/ml	ND	6.68 ± 1.4
DHT	0.3 ± 0.04	0.46 ± 0.048

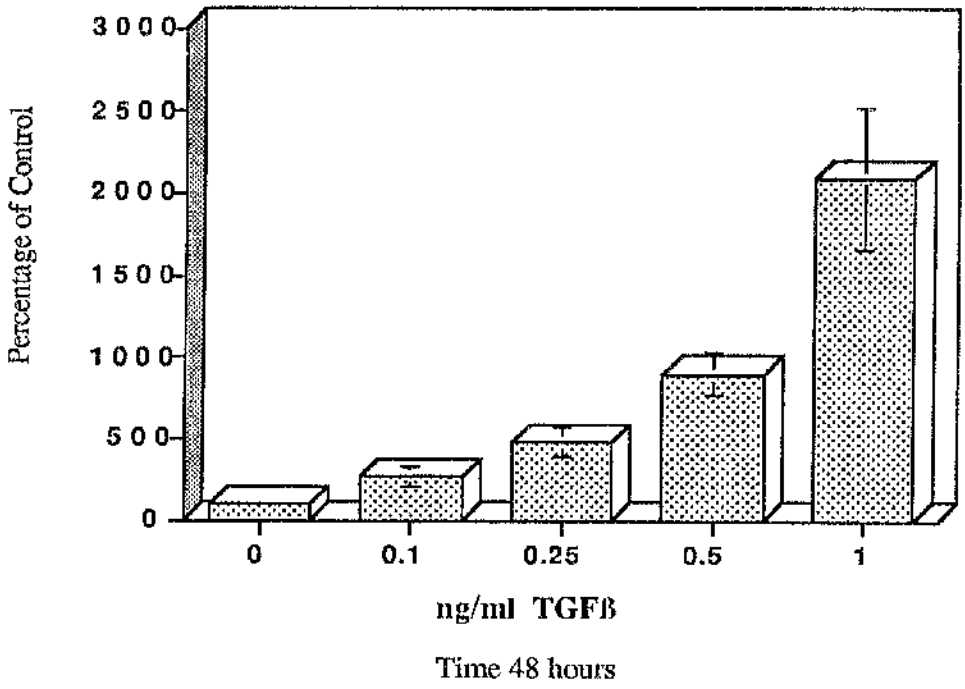

Figure 4.10 Dose response curve of TGFß on the secretion of PAI-1 protein in PC3 cells

The cells were treated with increasing dose of TGFß for 48h. In all cases, each sample was assayed by ELISA in duplicate. The average of three independent determinations ± standard deviation is presented.

Although PAI-1 protein levels were hardly detectable in the medium and cytosol of DU145 cells (0.06 ± 0.02 ng/mg in medium), TGFß (1 ng/ml) treatment revealed a significant increase (0.86 ± 0.17 ng/mg)(fig 4.11). This stimulation was also dose dependent. There was no detectable PAI-1 protein in DHT treated group.

Unlike uPA, PAI-1 transcripts and protein levels did not correlate in response to TGFß in the cell lines. PAI-1 protein values are summarised in table 4.2.

Table 4.2 The effects of TGFß1 and DHT on PAI-1 protein in DU145 cells Values represent the mean ± sd of three different experiments and each experiment was carried out in duplicate. ND: Not Done

Group	Cytosol PAI-1 ng/mg protein	Secreted PAI-1 ng/mg protein
Control	0.01 ± 0.003	0.06 ± 0.02
TGFß 0.1ng/ml	ND	0.035 ± 0.002
TGFß 0.25ng/ml	ND	0.15 ± 0.003
TGFß 0.5ng/ml	ND	0.5 ± 0.03
TGFß 1ng/ml	5.7 ± 0.94	0.86 ± 0.17
DHT	0	0

4.4.3 The regulation of PAI-2 protein

Exposure of TGFß increased PAI-2 production in the PC3 cells. A 59 % increase was observed in the cytosol of TGFß treated group (4.77 ± 0.45 ng/mg) compared with the control group (3 ± 0.6 ng/mg). However, PAI-2 levels in the conditioned medium were similar (0.19 ± 0.015, 0.18 ± 0.03 ng/mg respectively).

Neither cell-associated PAI-2 nor secreted PAI-2 level changed in DHT treated group (3.43 ± 0.4 ng/mg in cytosol, 0.17 ± 0.04 ng/ml in conditioned medium). PAI-2 protein was not detectable either in control or in TGFß treated group of DU145 cells.

4.4.4 The regulation of tPA protein

The PC3 cells displayed tPA protein in both cytosol (0.01 ng/mg) and medium (0.04 ng/mg). TGFß did not effect the production of cytosolic (0.012 ng/mg) and medium (0.01 ng/mg) tPA protein. The tPA protein was not detectable in DU145 cells.

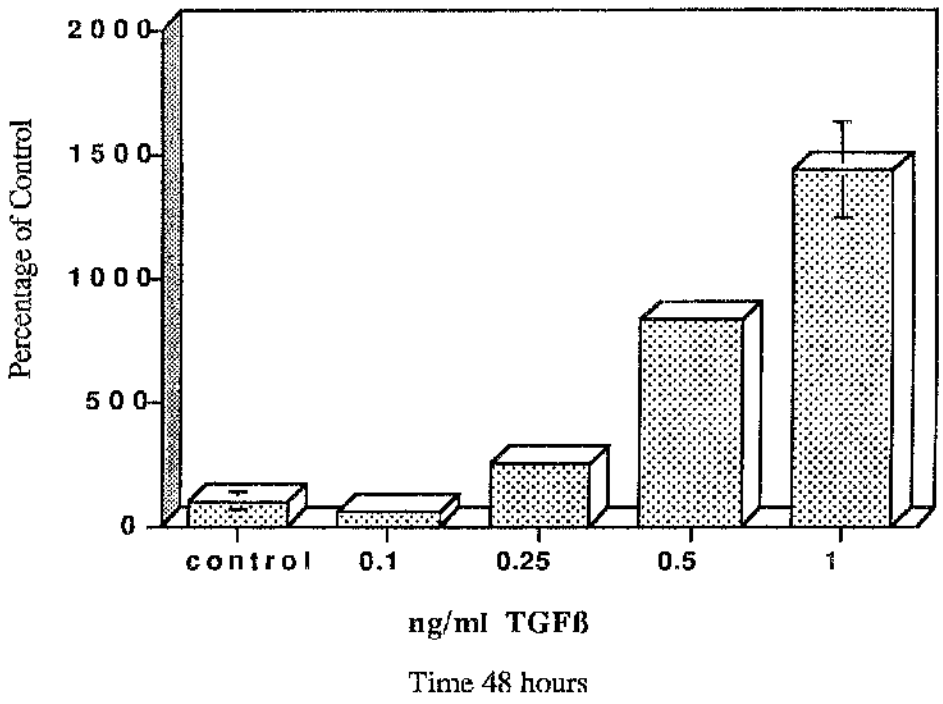

Figure 4.11 Dose response curve of TGFß on the secretion of PAI-1 protein in DU145 cells

The cells were treated with increasing dose of TGFß for 48h. In all cases, each sample was assayed by ELISA in duplicate. The average of three determinations ± standard deviation is presented.

4.5 Detection of uPA enzyme activity

uPA directed invasion and metastasis depend on the presence of active uPA in the system although uPA is initially secreted in an inactive form. To determine how the differences in the uPA activity in response to the differences in protein levels of PA components, uPA activity was analysed by using a chromogenic substrate specific for plasmin as described in the section 2.2.7.

When the PC3 cells were treated with lower doses of TGFß1 (0.5 ng/ml and 0.25 ng/ml), secreted uPA activity was increased compared with control group, nevertheless these increases were not statistically significant. Higher doses of TGFß1 (1 ng/ml and 0.75 ng/ml concentration) treatment did not reveal a significant stimulation.

In cell extracts, the levels of active uPA were decreased in the TGFß1 treated group. There was 28 % of inhibition in the cytosol of 1 ng/ml TGFß1 treated PC3 cells (fig 4.12). This inhibition was statistically significant (p value is 0.016). 0.75 ng/ml and 0.5 ng/ml doses of TGFß1 treatment were also inhibited uPA activity (p values are 0.026 and 0.015 respectively). However 0.25 ng/ml concentration of TGFß1 did not significantly decreased the cell-associated uPA activity.

Similar uPA activity was observed between the DHT and control group in the PC3 cell line. The values of uPA activities are illustrated in table 4.3.

Table 4.3 uPA enzyme activity in PC3 cells. Values represent the mean ± sd of three independent experiments and each dose was carried out in duplicate.

Group	Cytosol uPA Activity u/mg protein	Secreted uPA Activity u/mg protein
Control	98.67 ± 6.17	11.39 ± 1.5
TGFß 1ng/ml	70.77 ± 10.24	12.28 ± 0.53
TGFß 0.75ng/ml	76.73 ± 9.09	14 ± 1.32
TGFß 0.5ng/ml	79.5 ± 5.12	14.9 ± 0.61
TGFß 0.25ng/ml	90.6 ± 7.84	15.66 ± 0.7
DHT 1 nM	92.76 ± 10.5	10.56 ± 1.7

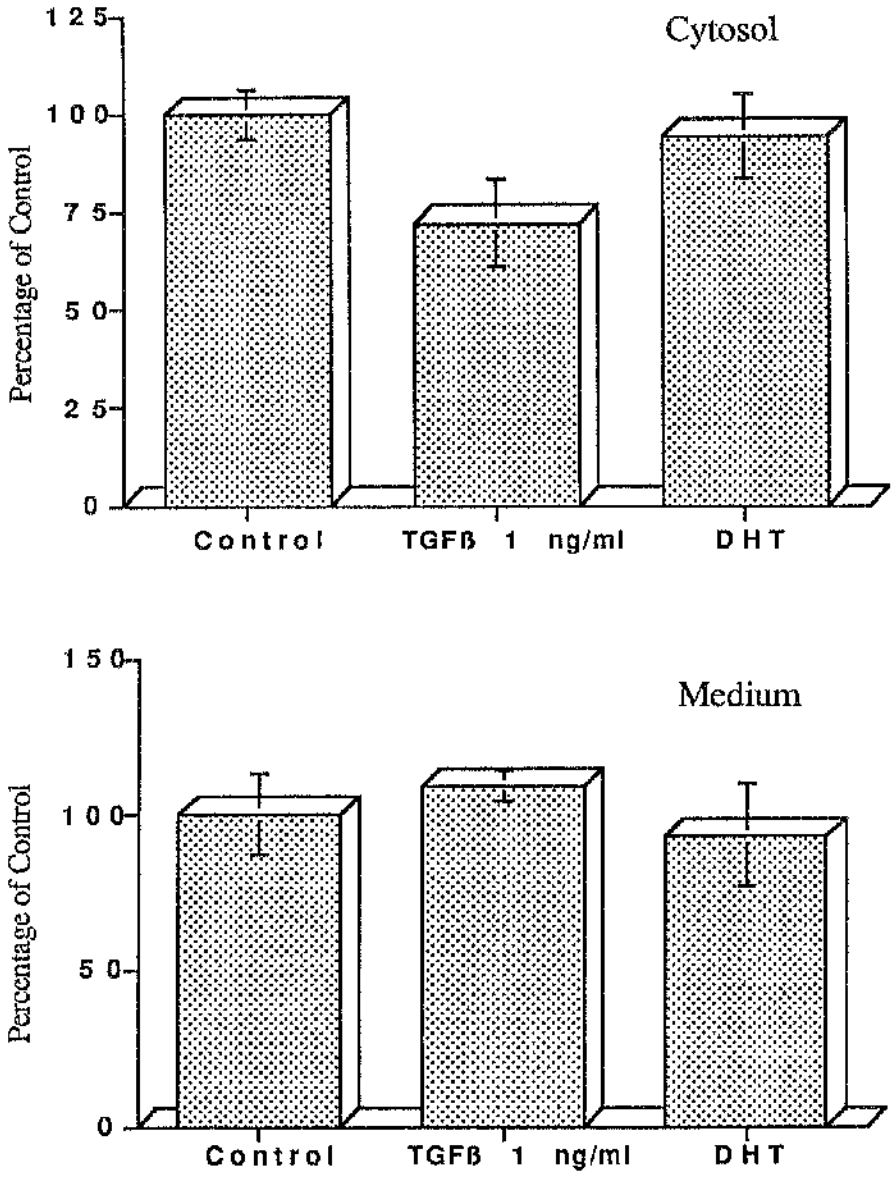

Figure 4.12 The effects of TGFß and DHT on uPA activity in PC3 cells

Effect of exogenous TGFß and DHT on secreted uPA activity is shown as a percentage of the control. The concentrations of TGFß was 1ng/ml, of DHT 1 nM. In all cases, each sample was assayed by an activity assay using S-2251 in duplicate. The average of three independent determinations ± standard deviation is presented.

TGFß increased both cytosolic and secreted forms of uPA activity in the DU145 cell line (fig 4.13). A 131 % of increase in the cytosol fraction and a 225 % increase (table 4.4) were observed at 1 ng/ml concentration of TGFß. Unlike PC3 cells, DU145 cells showed increased uPA activity in both fractions and in all doses of TGFß1 treated group. DHT treated DU145 cells displayed a similar uPA activity with the control group.

Table 4.4 uPA enzyme activity in DU145 cells. Values represent the mean ± sd of three independent experiments and each dose was carried out in duplicate.

Group	Cytosolic uPA Activity u/mg protein	Secreted uPA Activity u/mg protein
Control	35.85 ± 3.5	0.44 ± 0.03
TGFß 1ng/ml	82.9 ± 4	1.44 ± 0.012
TGFß 0.75ng/ml	67.6 ± 9.2	1.75 ± 0.07
TGFß 0.5ng/ml	66.4 ± 6.8	1.5 ± 0.05
TGFß 0.25ng/ml	69 ± 7.3	1.79 ± 0.1
DHT 1 nM	33.2 ± 6.3	0.5 ± 0.059

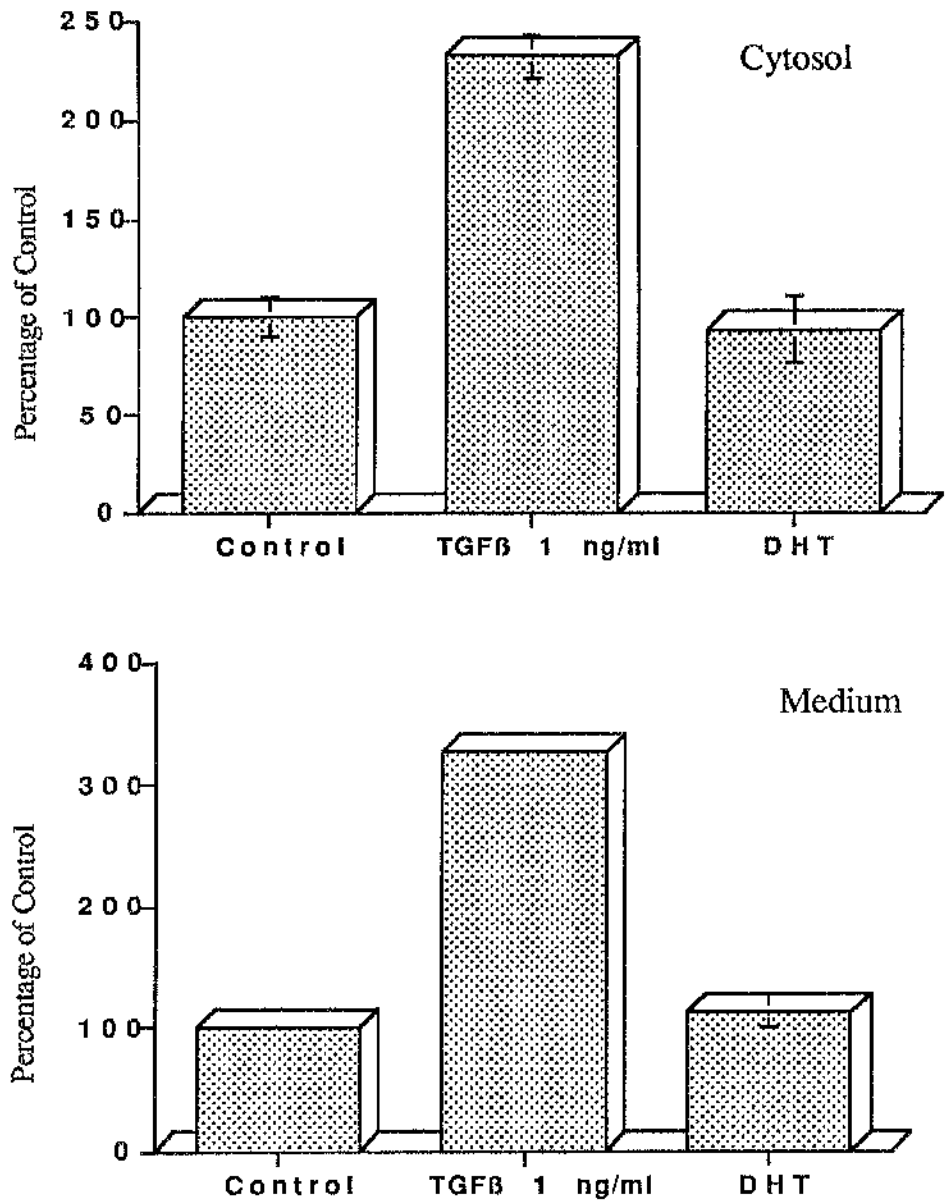

Figure 4.13 The effects of TGFß and DHT on uPA activity in DU145 cells

Effect of exogenous TGFß and DHT on secreted uPA activity is shown as a percentage of the control. The concentrations of TGFß was 1ng/ml, of DHT 1 nM. In all cases, each sample was assayed by an activity assay using S-2251 in duplicate. The average of three independent determinations ± standard deviation is presented.

4.6 *In vitro* invasion of matrigel

In order to check the effects of the TGFß1 on cell invasion, a matrigel invasion assay was performed. The cells were left on matrigel for 72h in the presence of TGFß1 at 1 ng/ml concentration. There were no significant difference between the TGFß1 treated group (% 11.9 ± 5.81) and control group (% 9.8 ± 2.5) in the matrigel invasion of PC3 cell line. The DU145 cell line showed minimal invasion of matrigel, whether or not TGFß was added.

4.7 Immunocytochemical study

The PC3 cells were used for the detection of uPA antigen in immunocytochemistry study. The cells were stained by anti-uPA ab (#394) at the dilution of 1/200. Anti-uPA ab was replaced with normal goat serum in the negative control group (fig 4.14). Some of PC3 cells showed a diffuse cytosol staining for uPA while other showed either cytosolic or membrane dominant staining (fig 4.15).

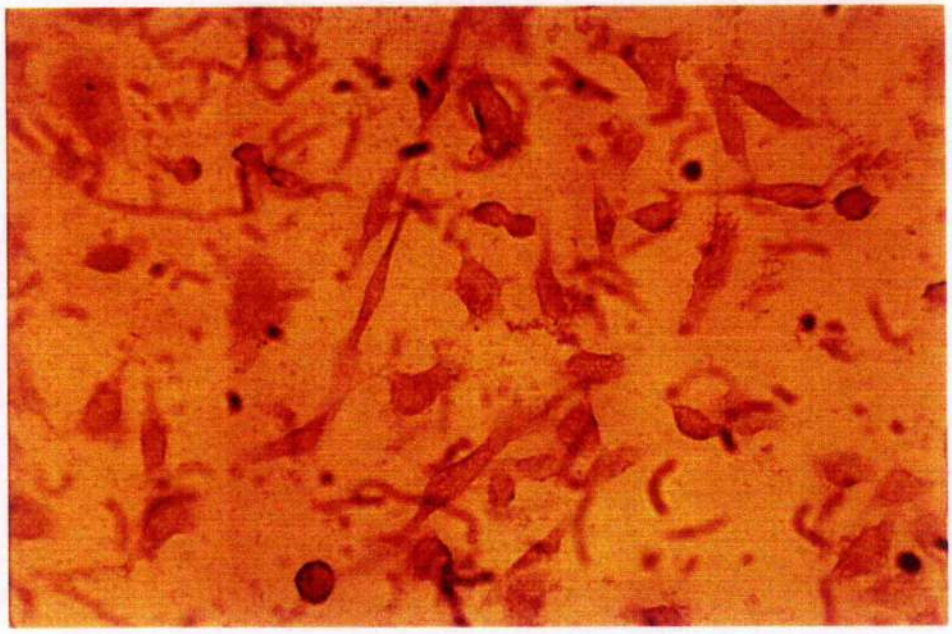

Fig 4.14 Negative control staining of the PC3 cell line.

The cells were incubated with 1/20 dilution of normal goat serum instead of anti-uPA ab. The cells were incubated for 2 hours in room temperature.

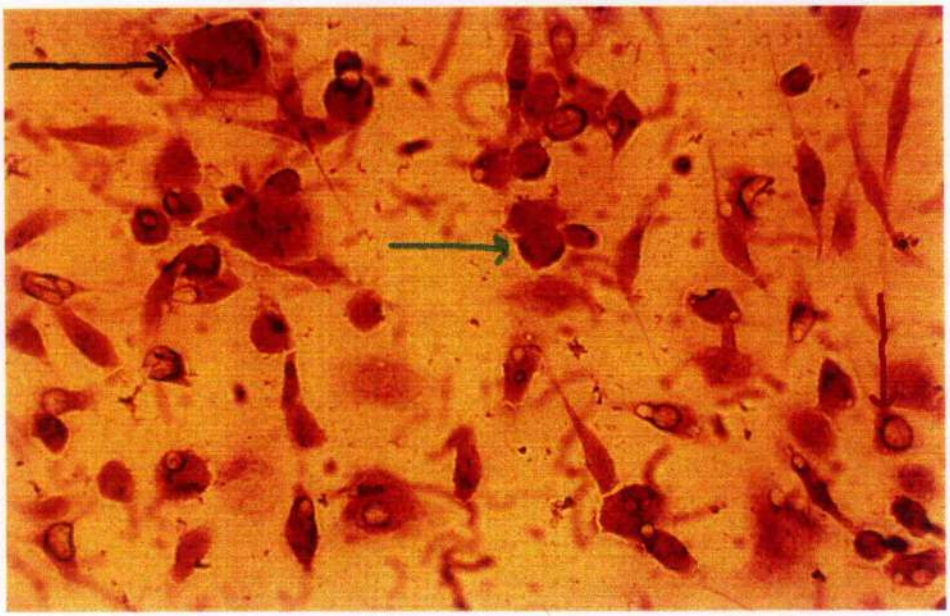

Fig 4.15 uPA staining in the PC3 cell line.

The cells were incubated with 1/200 dilution of uPA ab (#394) for 2 hours. Some of the cells showed diffuse cytosol staining (black arrow). Green arrow indicates the cells which showed both membrane and cytosolic staining. Red arrow indicates only membrane staining.

DISCUSSION

Overview

The incidence of human prostate cancer has been continuously increasing during last decade, so that prostatic carcinoma has become the most prevalent neoplasm and the second cause of cancer death in men (Carter & Coffey, 1990). Prostate carcinoma is recognised as a slow growing malignancy when compared to cancers from other organs. Prostate carcinoma has a relatively slow doubling time, estimated to be between 6 months and 5 years, depending in part on tumour grade (Bostwick *et al.*, 1996). Because prostate carcinoma has a high number of latent cases which do not develop into clinically manifest cancers, the steps in the progression to malignancy are of particular interest. Androgen independent growth and invasion/metastasis are two characteristic features of clinically manifested prostate carcinoma. In an *in vivo* study, androgen-sensitive LNCaP cells were promoted to progress from their androgen-dependent to androgen-independent status after castration of host animals. The androgen-independent LNCaP subline was found to acquire metastatic potential from primary to lymph node and bone (reviewed by Scher & Chung, 1994). In a series of clinical trials, it has been found that 85 % of androgen independent prostate carcinoma had bone metastases (Logothetis *et al.*, 1994). To achieve the process of developing a metastatic focus, prostate cancer cells must be able to proliferate in the secondary site. Specific growth factors have been identified that affect prostate carcinoma cell growth and which seem to be involved in tumour progression (Carruba *et al.*, 1994a). This study was designed to increase understanding of the mechanisms by which local growth factors might regulate growth and invasion

Growth studies

Tumour growth represents a critical balance between cell proliferation and cell death. This balance is usually altered during malignant transformation, resulting in increased growth due to increased cell proliferation, decreased cell death or a combination of these factors.

Although several studies demonstrated higher levels of EGFR in benign lesions of prostate than in prostate carcinoma, considerable data have been published that higher EGFR mRNA levels are found in the malignant lesions of the prostate. The lower numbers of detectable EGFR mRNA in malignant lesions than BPH might be attributed to a rapid turnover of the receptor.

Concerning the proliferation of the cells, the results presented here (see figs 3.5 and 3.6) indicate that there is only a slight difference in the growth characteristics in response to exogenous EGF. Exogenous EGF stimulated the growth of the PC3 cells but not the DU145 cells, at least in the short term. This is in agreement with previous reports indicating that the DU145 cell line is insensitive to the growth stimulatory effect of exogenous EGF (Carruba *et al.*, 1994a; Connolly & Rose, 1990).

It has been shown that DU145 cells secrete higher levels of EGF and TGFα into conditioned medium (CM) than the PC3 and the LNCaP cell lines (Carruba *et al.*, 1994a; Connolly & Rose, 1990). An inverse correlation between the receptor number and growth responsiveness to EGF has been demonstrated in breast carcinoma (Davidson *et al.*, 1987; Godden *et al.*, 1992). Breast carcinoma cell lines which have less than 70,000 EGF binding sites per cell, were stimulated by exogenous EGF, whereas the growth of the cells which have more than 70,000 receptor numbers per cell, were not stimulated. The DU145 cells used in this study exhibit EGF binding sites (317,300 per cell)

nearly four times higher than those found in the PC3 cells (84,260 per cell) (table 3.1). This finding confirms previous reports indicating DU145 cells contain a significantly higher number of EGFR than that expressed in PC3 cells (Carruba *et al.*, 1994a). The growth insensitivity of DU145 cells in response to exogenous EGF might be attributed to their high secretion of EGF and high expression of the receptor. Such a suggestion is supported by our observation that inhibition of endogenous EGFR-related tyrosine kinase activity resulted in a significant inhibition of DU145 cell proliferation (fig 3.10), also confirmed by Jones *et al.* (1997a). It has also been demonstrated that addition of anti-EGFR antibody resulted in growth inhibition of the DU145 cell line (Connoly & Rose, 1991). Given this, it can be concluded that the DU145 cells produce enough EGF for maximal autocrine stimulation and addition of exogenous EGF has no effect on the cell growth, though inhibition of EGFR-related TK activity inhibits the growth of cell line.

On the other hand, a proliferative effect of exogenous EGF was observed in the PC3 cells (fig 3.5). Several other studies have also shown that PC3 cell proliferation was initiated by EGF after 72 hours (Carruba *et al.*, 1994a; Jones *et al.*, 1997a). Inhibition of the TK activity significantly inhibited the EGF-stimulated cell growth (fig 3.8). On the other hand, Jarrard *et al* (1994) showed that addition of neither exogenous EGF nor anti-EGFR ab has effect on the PC3 cell growth. These results are in contrast with our results. In our study specific inhibition of EGFR-related TK activity remarkably inhibited the PC3 cell growth. This differences may be due to either the ab used in this study was not enough to inhibit EGFR function or TK inhibitors inhibit other tyrosine kinases that are important for the growth of PC3 cells.

The IC50 values (3.36 μM for PC3, 4.34 μM for DU145) of the TK inhibitor were found to be very similar for both cell lines. When the cells were

stimulated with the routine serum containing growth medium, the IC50 values were increased by 49 % in the PC3 cells, and 28 % in the DU145 cells in comparison with the EGF stimulated group (table 3.2). It is reasonable to assume that the presence of some factors in serum, which are lost during dialysis, is required for TK-mediated growth stimulation. It has been shown, for example, that IGF-1 significantly stimulates DNA synthesis in both DU145 and PC3 cell line (Iwamura *et al.*, 1993) and other growth factors which work through TK's that are inhibited by this TKI may also be present in serum.

In conclusion, it may be said that EGF is an important autocrine growth modulator in prostate carcinoma and targeting its tyrosine kinase activity would be an effective treatment for drug resistant prostate carcinoma.

In general, TGFß inhibits the growth of normal epithelial cells and stimulates the mesenchymal cells. TGFß also inhibits EGF-induced cell proliferation in benign human prostatic epithelial cells (Sutkowski *et al.*, 1992). Nevertheless, prostate cancer cells develop reduced sensitivity to the antiproliferative effects of TGFß1 with tumour progression (Moses *et al.*, 1990; Sehgal *et al.*, 1996; Steiner *et al.*, 1994). On the other hand, an increase in the expression of TGFß in tumour cells correlates with tumour progression (Sehgal *et al.*, 1996; Steiner *et al.*, 1994). TGFß enhanced MATLyLu tumour growth and metastasis when overexpressed in these prostatic cells *in vivo* (Steiner & Barrack, 1992). This may reflect either immunosuppressive action by TGFß or promotion of metastasis through angiogenesis stimulation.

Neither PC3 nor DU145 cell growth was inhibited by TGFß, at least in short term. These data (figs 4.1 and 4.2) support previous findings that exogenous TGFß1 loses its antiproliferative effect in progressive prostate cancer. There is disagreement on the proliferative effects of TGFß1 on prostate carcinoma cell

lines in the literature. This might be a result of different experimental conditions such as cell density or presence of other factors. It has been demonstrated that prostate carcinoma frequently loose their TGFß receptors when compared with BPH (Kim *et al.*, 1996a). The possible mutations on the receptors, (or somewhere in the TGFß downstream pathway) may change the TGFß sensitivity of the cells. Although some studies (Moses *et al.*, 1990; Sehgal *et al.*, 1996; Steiner *et al.*, 1994) illustrated that prostate carcinoma cells were resistant to the growth inhibitory effect of TGFß, TGFß inhibited the EGF stimulation of these cells (Schuurmans *et al.*, 1988a). The inhibition of LNCaP cell growth by androgen withdrawal (Knabbe *et al.*, 1993) or by dexamethasone (ReyesMoreno *et al.*, 1995) and estrogen (Carruba *et al.*, 1994b) induced growth inhibition of the PC3 cells were all accompanied by the increased TGFß production. In general, the growth inhibition processes of prostate carcinoma cells may involve TGFß action in someway.

The growth of neither the PC3 nor the DU145 cell lines was affected by testosterone, DHT, estradiol or progesterone.

Invasion studies

The first step of the metastatic cascade involves the localised invasion of the surrounding tissues. The spread of malignant tumours involves:
the invasion and infiltration into adjacent normal tissue,
the penetration of lymphatic channels or blood vessels,
survival in the circulation and arrest in the capillary beds of distant organs,
penetration through the vessels and extravasation, then finally
growth in the distant organ.

These processes are largely mediated by proteolytic enzymes that catalyse irreversible disruption of peptide bonds of the proteins in the basement

membrane and extracellular matrix. The regulation of the proteolytic balance of malignant cells is thus an important part of invasion and metastasis processes.

Plasminogen activator components in the cell lines

The PC3 cells showed nearly two times higher uPA level than the DU145 in both cell cytosol and CM (tables 3.5, 3.6). In the uPA activity assay, the PC3 cells showed nearly three times higher activity in cell cytosol than the DU145 cells. These figures confirm that the ELISA assay of uPA protein correlates fairly closely with enzyme activity levels in these two cell lines. PC3 cells again, showed nearly three times higher invasive ability into the matrigel when compared with the DU145 cell line (section 3.7). Cell invasiveness and uPA production in the two cell lines paralleled that of prior reports (Gaylis *et al.*, 1987; Hoosein *et al.*, 1991). Both PAI-1 transcript and protein were present in the two lines. While PAI-2 and tPA proteins were present in the PC3 cell line, neither were detectable in the DU145 cells. It has been shown that DU145 cells express the tPA transcript (Lyon *et al.*, 1995), while the tPA protein was not detectable in the cells. The authors illustrated that higher tPA protein levels (16.16 ng/mg in cell lysate) were present relative to the uPA protein (1.65 ng/mg in cell lysate) in PC3 cells. Both DU145 and PC3 cells showed similar plasminogen activator activity in a plasminogen-dependent fibrin lysis assay. In our experimental system, much higher uPA protein levels (27 ng/mg cell lysate) were observed in comparison with tPA protein (0.01 ng/mg in cell lysate) in the PC3 cell line. Furthermore, using anti-uPA ab (#394) completely abolished the PA activity assay. Therefore it can be concluded that PA activity was due to uPA activity. This difference may simply be due to difference in the sub-clones of DU145 used or to differences in assay techniques.

Both cell line showed higher cell-associated uPA activity than medium-associated activity (tables 3.7, 3.8, 4.3, 4.4). Higher cell-associated uPA

activity than medium-associated activity might be primarily due to uPAR associated active uPA on the cell membrane. Unlike uPA, tPA protein was observed at higher concentration in the CM compared with the cell lysates. So far, no specific binding sites for tPA has been found on the cell membrane. In the case of the PC3 cells, tPA had very little effect on plasminogen activation while uPA was totally responsible for the activation.

Although anti-uPA ab totally inhibited the uPA activity, when the ab was used in an *in vitro* invasion assay, the result showed only 35 % inhibition of the invasion through matrigel (section 3.7) suggesting that invasion is not totally dependent on the presence of active uPA (some of other zymogens may be partially activated). In the absence of plasminogen, PC3 cell invasion was reduced by 30 %. It has been shown that anti uPA antibodies can inhibit the prostate cancer cells invasion (Jarrard *et al.*, 1995). The authors demonstrated the inhibition of the DU145 cell line by 21-30 % and the PC3 cell line by 85 % in the presence of anti-uPA ab. In the present study, 35-36 % inhibition on PC3 cell invasion was observed, while there was no difference in the DU145 cell invasion was noted. Furthermore, omitting plasminogen from matrigel resulted in a similar inhibition level. However the differences between the experimental conditions may cause some alteration on the results.

Although a clear parallel was observed between the uPA activity level and cell invasiveness in this study, uPA activity level does not always correlate with higher invasive capacity. It has been demonstrated that TSU-PR1 cells have a 20-50% higher invasive capacity than DU145 and PC3 cells (Jarrard *et al.*, 1995). However, TSU-PR1 showed two-three fold less uPA activity when compared with PC3 and DU145 cells and anti-uPA ab inhibited the TSU-PR1 cell invasion by 50-60 %. TSU-PR1 cells might use some other effective proteolytic cascades in addition to the PA system. In our experimental system,

the cell invasiveness not only correlated with uPA activity, but also correlated with the expression of other PA components. The PC3 cells expressed PAI-2, tPA and a higher levels of PAI-1 in comparison to the DU145 cells. It seems to be when tumour cells become more aggressive, they express other components of the PA system (and possibly various matrix metalloproteinases).

Effect of EGF on cell invasiveness

From the results (chapter 3), it can be seen that EGF seems to be a potent stimulator of invasion of the prostate carcinoma cells. Increased expression of uPA mRNA, uPAR mRNA and uPA protein levels correlate with the enhanced uPA activity and invasion of matrigel as observed in PC3 cells in response to EGF. These results support previous findings (Jarrard *et al.*, 1994) which demonstrate increased production of uPA and invasiveness of PC3 cells in response to EGF. In an other study, EGF also stimulated the invasion of TSU-PR1 cells (Zolfaghari & Djakiew, 1996). In a recent study, EGF was also found to be an effective chemoattractant for the TSU-PR1 cells (Rajan *et al.*, 1996).

I was unable to demonstrate any significant effect of EGF on matrigel invasion of the DU145 cell line (very low invasion, 3% for control group). This very low level made it impossible to evaluate the effect of ligand and/or inhibitor. EGF stimulated DU145 cells displayed an increase in uPAR transcript, an increase in cell-associated uPA protein levels and uPA activity. Therefore, it can be concluded that DU145 cells do have their invasive potential increased by EGF, at least in terms of increased uPA activity.

EGFR-TK activity inhibition reversed the stimulation of the uPA, uPAR, the uPA activity and cell invasiveness. Furthermore, uPA production and activity

were significantly lower than in the control group in the presence of ZM252868. In a recent *in vivo* study, the inhibition of EGFR-related tyrosine kinase activity inhibited the invasion of normal brain tissue by glioblastoma cells in rat brain (Penar *et al.*, 1997). Although glioblastoma multiforme is a nonepithelial human tumour, it has been consistently linked to overexpression of EGFR. In this study genistein, tyrophostin A25 and an anti-EGFR antibody were used for inhibition of the tyrosine kinase activity. Although nothing mentioned about PA components, the inhibition of the invasion of glioblastoma muliforme, partly, could be, therefore, attributed to the down-regulation of PA activity by TKI.

As can be seen from tables 3.3 and 3.4, there was a decrease in the PAI-1 transcript in response to EGF in each cell line though, oddly, this was not reversed by TKI in the case of DU145 cells. The failure of the TKI to reverse the PAI-1 inhibition by EGF in DU145 cells is unexpected. PAI-1 protein expression was similar between the EGF stimulated and control group. Furthermore, inhibition of TK activity showed no difference in PAI-1 production. Neither PAI-2 nor tPA protein levels were effected in response to EGF. However, PAI-2 protein levels were decreased in the presence of TKI. This inhibition might be non-specific inhibition of the cellular phosphorylation.

It has been previously demonstrated that EGF stimulates an increase in the secretion of uPA in several cell lines including HeLa cells (Lee & Weinstein, 1978), keratinocytes (Jensen & Rodeck, 1993), PC3 (Jarrard *et al.*, 1994), TSU-PR-1 (Rajan *et al.*, 1996). Thus, not surprisingly, EGF was found to be an inhibitor of uPA production in colon carcinoma (Boyd, 1989). In the same study, EGF also increased the uPA binding sites but plasminogen dependent laminin degradation was decreased so that it is not possible to equal rising uPA/uPAR with an increase in all aspects of invasion.

In addition to the plasminogen activator system, the metalloprotease system is also critical in the degradation of extracellular matrix proteins (Reiter *et al.*, 1997). It has been shown that BPH cells, primary prostate carcinoma and PC3 cells express matrix metalloproteinase-9 (MMP-9) (Festuccia *et al.*, 1996). The authors also demonstrated that EGF caused an increase in the secretion of MMP-9, as well as MMP-2, in BPH and primary prostate carcinoma cells. Although direct evidence was not presented in the study, it is possible to assume that an increase on MMP-9 levels might occur in the PC3 cells in response to EGF. In a study with squamous cell carcinoma, MMP-9 levels were also increased by EGF (Shima *et al.*, 1993).

In our study, EGF increased the invasion of matrigel and its inhibitor reversed this invasion. However, it is difficult to conclude that the increase in the invasion of PC3 is solely increase in the plasminogen activator activity. MMP-9 might be involved in the PC3 invasion and EGF might have increased the MMP-9 levels. The interaction between proteases and their inhibitors and their roles in invasion process of prostate carcinoma still remain to be solved.

The effect of TGFß1 on the cell invasiveness

As can be seen from the results (chapter 4), TGFß1 increased uPA transcript and uPA protein levels in both PC3 and DU145 cells. However, the greatest effect of TGFß1 was observed on PAI-1 protein levels. In both cell lines, PAI-1 secretion was increased by TGFß1 in a dose dependent manner (figures 4.10, 4.11). It has been also demonstrated that TGFß1 stimulates PAI-1 expression in lung fibroblasts (Laiho *et al.*, 1986) and in a lung carcinoma cell line (Keski-

Oja *et al.*, 1988). In this study, TGFß also increased PAI-2 levels in the PC3 cell line.

In the activity assay, TGFß1 showed an inhibition of the cytosolic uPA activity in the PC3 cells. However no clear differences were observed in the CM of the PC3 cells (table 4.3). Although a significant increase in PAI-1 protein was found in both cytosol and CM, uPA activity inhibition only occurred in the cytosolic fraction. Later studies with PAI-2 protein correlated with the uPA activity assay. TGFß treated PC3 cells displayed an increase in PAI-2 protein levels only in the cytosol whereas CM levels were similar to the control group (section 4.4.3). Studies with the DU145 cell line, which lacks PAI-2 protein and expresses a very low PAI-1 protein, supported previous results. A dramatic increase in PAI-1 secretion in response to TGFß1 was also observed in both cytosol and CM of the DU145 cells. Both uPA protein expression and activity were also increased by TGFß1 in the DU145 cells. As can be seen from the results (fig 4.13), increased expression of PAI-1 had no effect on the uPA activity in the DU145 cell line. However, enhances in PAI-2 expression correlated with significantly decreased the uPA activity in the PC3 cells.

It has been demonstrated that TGFß significantly reduced matrix degradation by increasing PAI-1 protein levels in HT-1080 human fibrosarcoma cells (Cajot *et al.*, 1990). However, this cell line also expresses PAI-2 transcripts and protein (Noguchi-Takino *et al.*, 1996) and TGFß1 might have increased the PAI-2 production. In another study, TGFß enhanced both PAI-1 and uPA levels in the A549, a lung carcinoma cell line, and TGFß increased PA activity (Keski-Oja *et al.*, 1988). Although nothing is mentioned about PAI-2 protein expression, an increase between PAI-1-uPA complex has been observed. Some other mediators such as tumour necrosis factor α and basic FGF induced both uPA and PAI-1 (Georg *et al.*, 1989; Saksela *et al.*, 1987).

In the DU145 cell line, TGFß1 increased both uPA protein and PAI-1 protein levels. uPA activity was also increased in both fractions of the DU145 cell line. Nevertheless, PAI-1 levels in the PC3 cells were nearly eight times higher than found in the DU145 cells. It is not possible to say whether the increased secretion of PAI-1 was enough to balance for the uPA levels and cause the uPA activity inhibition in the PC3 cells. In addition to the increases in PAI-1 levels, PAI-2 protein level was also enhanced in response to TGFß1. Increases in both inhibitor levels might be enough to balance and further decrease the uPA activity. In the DU145 cell line, unlike PC3, increases in the PAI-1 protein were probably not enough to neutralise uPA activity. These results may raise a question about the effectiveness of the inhibitors on uPA activity. An *in vitro* invasion assay, able to show the invasive capacity of DU145 cells , may help to identify the role of uPA inhibitors in the invasion of the cancer cell lines as DU145 lacks expression of the PAI-2 protein and shows an increase in PAI-1 and uPA protein in response to TGFß1. In addition to TGFß1 effects, the DU145 cells also show an increase in uPA production and activity without affecting the PAI-1 protein levels in the presence of exogenous EGF. However the effect of TGFß on the matrix metalloproteinases needs to be checked to evaluate uPA-PAI-1 relationship to the cell invasion.

Although some *in vitro* and *in vivo* (Soff *et al.*, 1995) experiments showed that PAI-1 expression or increased PAI-1 level inhibited the plasminogen activity and the cell invasiveness, considerable amounts of data support a significant correlation between PAI-1 level and poor prognosis in breast carcinoma, lung adenocarcinoma, gastric carcinoma and ovarian carcinoma (extensively reviewed by Andreasen *et al.*, 1997). However, increased level of PAI-2 correlated with good prognosis in breast carcinoma (Bouchet *et al.*, 1994; Foekens *et al.*, 1995) and in non small-cell lung carcinoma (Nagayama *et al.*,

1994). Thus the full role of PAI-1 in regulating invasion is still to be elucidated.

In the *in vitro* invasion assay, TGFß1 stimulation did not effect the PC3 cell invasion. It has been shown that TGFß increases the colony formation of metastatic cells. Increased TGFß secretion by tumour cells, as well as by platelets at sites of metastatic deposit may help to form a colony. Escaping from the growth inhibitory effect of TGFß while still retaining some responses to TGFß, such as enhancing the level of uPA, PAI-1 and PAI-2, may contribute the metastatic activity of tumour cells. Increased levels of TGFß in the colony site may stimulate tumour development by stimulation of extracellular matrix formation, therefore, increased adhesiveness of cells to the matrix. Furthermore, the ability of TGFß, by stimulating angiogenesis and inhibiting the immune system (Massague, 1990) could additionally support the colony formation. Given this, it can be concluded that the contribution of TGFß to prostate carcinoma progression is positive, based on at least loss of growth inhibition and the stimulation of components of the invasive system.

Conclusions and future aspects:

This study has confirmed that both EGF and TGFß have important roles in the invasion mechanism of androgen-independent prostate cancer. The results reported here provide strong evidences that TK activity is important to the regulation of uPA activity. EGF stimulates the growth and invasive behaviour, which are two main characteristics of clinically manifest prostate cancer. Inhibition of the EGFR-related TK activity not only inhibits the tumour growth but also, potentially, prevents developing metastases. Thus targetting the TK activities may provide a new route for therapy of hormone-independent prostate cancer (as well as breast carcinoma and ovarian carcinoma). However, further *in vivo* studies and clinical trials need to be done. Growth stimulation in both primary and metastatic sites, by EGF, with help from TGFß to promote metastatic colony formation suggests further targets.

The full roles of PAI-1 in invasion process remain to be determined. Further *in vivo* and *in vitro* studies need to be done to elucidate whether PAI-1 has a protective role in invasion processes.

Further studies are need to determine the role of TK activity and its inhibitors in control of tumour growth and metastases in an *in vivo* experimental study. Prostate carcinoma samples from both endocrine therapy-responsive and resistant patients can be used to detect TK activity. To find out the effectiveness of androgens on the regulation of PA system, a possible model could be developed by transfecting androgen-receptor to the PC3 cell line.

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