The Role of the Urokinase Family in Invasion by Breast Cancer

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by

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Dedicated to my most beloved and wonderful parents

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Abbreviations Used

A_{260}	Absorbance at 260 nm
Ab	Antibody
alpha-2 MR	Alpha-macroglobulin receptor
APS	Ammonium persulphate
ATF	Amino terminal fragment
BSA	Bovine serum albumin
^{o}C	Degrees centigrade
cDNA	Complementary DNA
Ci	Curie
C-terminal	Carboxy-terminal of a protein
CM	Conditioned medium
CMF	Cyclophosphamide methotrexate and 5-fluorouracil
Cont	Control
cpm	Counts per minute
DCC	Dextran coated charcoal
DEPC	Diethyl pyrocarbonate (1 % w/v) in water
DES	Diethylstilboestrol
DHIDCCFCS	Dialysed, heat-inactivated, dextran coated

	charcoal stripped fetal calf serum
DMSO	Dimethyl sulphoxide
DNA	Deoxyribonucleic acid
DTT	Dithiothreitol
EDTA	Ethylene diamine tetra-acetic acid
EGF	Epidermal growth factor
EGFR	Epidermal growth factor receptor
EHS	Engelbreth-Holm-Swarm (extracellular matrix derived from sheep mammary carcinoma)
E2	17β estradiol
ELISA	Enzyme-linked-immunosorbent assay
ER	Estrogen receptor
ETN	EDTA-Tris-NaCl buffer
FCS	Fetal calf serum
FGF	Fibroblast growth factor
FITC	Fluorescein isothiocyanate
GFD	Growth factor domain
HED	HEPES-EDTA-dithiothreitol
HEPES	4-(2-hydroxyethyl-1-piperazine)-ethanesulphonic acid
HIDCCFCS	Heat-inactivated dextran coated charcoal stripped FCS

HMW	High molecular weight
HRT	Hormone replacement therapy
IgG	Immunoglobulin G antibody
LMW	Low molecular weight
LRP	Low density lipoprotein receptor-related protein
mAb	Monoclonal antibody
MCF	Michigan Cancer Foundation
MDA	MDA-MB-231 cell line
MTT	3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyl tetrazolium bromide
MW	Molecular mass
NGS	Normal goat serum
N-terminus	Amino terminus of protein
OPD	O-phenylendiamine
PA	Plasminogen activator
PAGE	Polyacrylamide gel electrophoresis
PAI-1	Plasminogen activator inhibitor type-1
PAI-2	Plasminogen activator inhibitor-2
PBS	Phosphate buffered saline
PBS-A	Phosphate buffered saline with bovine serum albumin

PCR	Polymerase chain reaction
PDGF	Platelet-derived growth factor
Pg	Progesterone
PgR	Progesterone receptor
PKC	Protein kinase-C
PMA	phorbol 12-myristate 13-acetate
PMSF	Phenylmethylsulphonylfluoride
PVP	polyvinylpyrolidone
RNA	Ribonucleic acid
RPMI-1640	Roswell Park Memorial Institute
RT	Reverse transcriptase
S-2251	H-D-Val-Leu-Lys-pNA.2HCL
SDS	Sodium dodecyl sulphate
SN	Cell cytosol/lysate/supernatant
TAM	Tamoxifen
TAE	Tris-acetate EDTA
TBS	Tris buffered saline
TBST	Tris buffered saline Tween
TEMED	N, N, N', N'-tetramethylene-diamine
Tα	Transforming Growth Factor α

Tβ	Transforming Growth Factor-β
TGFα	Transforming growth factor alpha
TGF-β	Transforming growth factor beta
TAM	Tamoxifen
TPA	Tetradecanoyl-phorbol-acetate
tPA	Tissue-type plasminogen activator
TRIS	N-Tris (hydroxymethyl) methyl-2-amino -ethanesulphonic acid
uPA	Urokinase plasminogen activator
uPAR	Urokinase plasminogen activator receptor

ABSTRACT

Breast cancer is the leading cause of cancer mortality in women in the Western world. Its growth and development is directly governed by endogenous steroids and growth factors. Endocrine therapy is the most effective form of treatment for a proportion of breast cancer patients. Work from several laboratories has shown that breast cancer cells metastasise via a pathway that involves the plasminogen activator system and metalloproteinases. Thus, the plasminogen activator system, which consists of urokinase (uPA), its inhibitor (PAI-1) and receptor (uPAR), is central to the process of invasion and metastasis. Given their important role, it is hardly surprising that these three proteins have been used as biological markers of patient prognosis. uPA and PAI-1 has been significantly implicated as markers of aggressive breast cancer.

The first step of invasion involves the conversion of plasminogen into plasmin by uPA. uPA is localised on the cell surface (bound to its receptor uPAR) and is principally regulated by two inhibitors, plasminogen activators type-1 and type-2 (PAI-1 and PAI-2). Possessing a wide substrate specificity, plasmin in turn activates a variety of enzymes which degrade different components of the extracellular matrix such as laminin, fibronectin, collagenase(s), proteoglycans etc.

The present study was conducted to further understand the effect(s) of different growth factors and steroid hormones on the proliferation and invasive potential of two well characterised human breast cancer cell lines, MCF-7 (hormone sensitive) and MDA-MB-231 (hormone insensitive). Furthermore, extracellular matrix (EHS) was used to determine the effect, if any, that support substrate might have on the levels of plasminogen activators and growth rate. Plasminogen activators were quantified both at the mRNA and

protein levels, using RT-PCR and ELISA, respectively. uPA activity was determined by chromogenic assay and the invasive properties of the two cell lines were determined using the Boyden invasion chamber.

In the initial phase, proliferation of each cell line on both substrata, plastic and EHS, was studied after treatment with growth factors (TGFα, TGFβ and EGF), steroid hormones (E2 and Pg) and anti-oestrogen, tamoxifen. Different amounts of each growth factor and hormone were used and cell numbers were obtained after 24, 48 and 72 hours. These studies showed that growth of MCF-7 cells was stimulated significantly by exogenous TGFα and EGF, on both EHS and plastic with cells being more sensitive to growth factors on EHS. Proliferation of MDA cells was only slightly increased by the same growth factors on either substrate. By contrast, TGFβ inhibited the growth of MDA cells, on both surfaces. However, the concentrations required for inhibition on the two substrata were enormously different. E2, Pg, and TAM (an anti-oestrogen) also stimulated the growth of MCF-7 cells on both plastic and EHS.

For quantification of uPA, uPAR and PAI-1, a combination of Western blotting and ELISA, using specific monoclonal antibodies, was employed to monitor the relative amounts of these proteins after treating the cell lines with growth factors and hormones. These studies showed that the MDA cell line expressed uPA and PAI-1 proteins whereas uPA was hardly detectable in the MCF-7 cell line. Moreover, qualitative RT-PCR analysis clearly showed that uPAR and PAI-1 transcripts were present in both MDA and MCF-7 cells. However, uPA mRNA could not be detected in MCF-7.

EGF and TGFα induced an increase in uPA, PAI-1 and uPAR mRNA levels in MDA cells. In MCF-7 cells, both factors induced an increase in uPAR and PAI-1 transcripts. Corresponding changes in invasive potential were measured after growth factor treatment.

TGFβ increased plasminogen activator components both at the transcriptional and the translational levels in MDA cells. It caused an increase in both cell secreted and cell associated uPA and PAI-1 proteins, the amounts of which varied depending on the substratum. The percentage increase in cell secreted and cell associated proteins was higher on EHS than on plastic. Amounts of secreted uPA and PAI-1 were higher than cell associated levels. TGFβ stimulated the secreted form of PAI-1 by about 420% (Fig 33A) as compared to 349% (Fig 29A) for uPA on EHS. On the same substratum, PAI-1 and uPA levels in cell extracts were 170% (Fig 35A) and 151% (Fig 31A), respectively. Interestingly, cell associated PAI-1 and uPA levels on plastic were only 30% and 23%, respectively. With the passage of time, however, the antigen levels fluctuated in response to certain growth factors, and their response to different doses also varied with time and substratum. TGFβ increased the amounts of uPA enzyme activity in MDA cells. *In vitro* invasion of MDA and MCF-7 cell lines were also enhanced by TGFβ.

The TGFβ induced increase in invasion by MDA cells was reduced to the value of control in the presence of a neutralising anti-uPA antibody, whereas MCF-7 cell invasion, in the presence of same antibody, remained above the level of control. Fetal calf serum on the other hand, inhibited the *In vitro* invasion of both cell lines in a dose dependent manner. Addition of plasminogen to the FCS medium also could not revert the inhibitory affect of FCS on invasion of matrigel. Increased uPA and PAI-1 and uPAR mRNA and protein expression correlated with increased invasion.

E2 significantly increased cell secreted and cell associated PAI-1 protein levels in MCF-7 cells whereas tamoxifen induced an increase in cell associated antigen only. E2 also increased the invasion of the same cell line by about 50% while TAM caused a decrease in invasion. In the MDA cell line TAM did not change the level of invasion. Thus tamoxifen is seen to be

positively anti-oestrogen in relation to invasion, rather than a weak agonist as it is in relation to proliferation.

In conclusion, these results clearly show that various growth factors and/or hormones not only have an effect on the proliferative properties of cell lines but they also change the relative amounts of uPA, PAI-1 and uPAR and that these changes can be reflected in invasive activity. The ECM contributes to the overall response of these cells.

CHAPTER 1

INTRODUCTION

1.1 Overview

In Westernised societies, breast cancer is the leading cause of cancer-related death among women aged 30-70 years (Blackwood & Weber, 1996) It is estimated that approximately 8-10% of all women in the United States and Western Europe suffer from this disease at some point in their lifetime (Clarke *et al.*, 1992 ; Li *et al.*, 1994; Schmitt *et al.*, 1997a). In the UK alone, 25,000 new cases are diagnosed every year and 15,000 die of the disease every year. Furthermore, its incidence is increasing world-wide, for example in the UK, the incidence is rising at about 1-1.5% per year but this is coupled with falling mortality (Lochter & Bissell, 1995; Sainsbury, 1997). 5 year survival for breast cancer is 68% (Campaign, 1996). Breast cancer can not only occur sporadically but can also be inherited. The incidence of sporadic breast cancer, however, is much higher than genetically inherited disease(Cavenee & White, 1995; Wooster & Straton, 1995). It is estimated that about 3-10% of all the patients suffering from breast cancer are genetically susceptible to the disease (Stratton & Wooster, 1996; Wooster & Straton, 1995). 60% of patients die because of metastasis.

In the US, breast cancer incidence is four to seven times higher than it is in China or Japan. Moreover, studies have shown that the incidence of the disease increases in Chinese, Japanese or Phillipino women upon migration to the United States and eventually approaches that among US white women. Similarly, Asian women born in the West have a breast cancer risk 60% higher than those born in the East.

Among white and black Americans, there are dramatic differences in not only the incidence of breast cancer but also in survival and mortality rates. Incidence rates in white women are 20% higher than in black women but the mortality rate, as estimated in 1990, was lower in white women (27.4) and

higher (31.7) in black women per 100,000, respectively (Marchant, 1994). Similarly, black Americans have a higher mortality rate from prostate cancer. In the USA, the black population have an incidence rate for prostate cancer that is 30 times more than that for Japanese men. However, the mortality rate is only two times higher than that of Japanese immigrants living in USA (see review by (Verde *et al.*, 1984) The increasing incidence of breast cancer in the United States and immigrant populations have led to the speculation and/or suggestion that the differences are due more to environmental risk factors rather than genetic factors. National Cancer Institute (NCI), reported to the White House that an estimated 2 million women will succumb to breast cancer between 1990 and the year 2,000, 460,000 of whom will die of the disease (Marchant, 1994). Recent data from the NCI, for the period 1989-1992, show that in the USA there was an overall 4.7% reduction in mortality. This reduction was mainly confined to the white population (5.5% reduction) as mortality amongst the black population was still on the increase (2.6%) (Sainsbury, 1997). The incidence of breast cancer is similar among all the Western countries such as the United States and Western Europe. It is very likely therefore that certain common factors, such as diet or life style may influence the rate of breast cancer.

Over the years, a tremendous amount of effort has gone into trying to understand breast cancer. Unfortunately, due to complexity of the disease, there is little evidence that significant progress has been made, since the introduction of anti-oestrogens, in improving overall survival. Although there are various endocrine and cytotoxic therapies which have been used to treat the disease, it is disturbing to note that the incidence of breast cancer has been on the rise (Cufer, 1995; Hankey, 1992; Parkin & Nectoux, 1991). Many factors have been associated with breast cancer but there has not been any explanation for its continuous increase in western societies as compared to the Asian societies (Stephens, 1997). Most oncologists, in spite of some

spectacular success in the tumour suppressor genes, feel that there has been almost no progress in the therapy of solid tumours including breast cancer (Bissell & Werb, 1995) in the last 30 years. If the present trend continues it is predicted that there will be more than one million sufferers of the disease by the turn of the century. In order to combat this disease it is imperative that a clearer understanding of the biology of tumours at both the cellular and molecular level is achieved. Breast cancer is a problem of differentiation, and in order to understand the problems associated with breast, it is necessary to understand the normal differentiated breast. Clearly, this knowledge in turn can be used to invent innovative therapies to prevent breast cancer growth and progression.

1.2 Aetiology of Breast Cancer

Several factors appears to play important roles in triggering the disease (Boyle and Leake, 1988) and in the aetiology of breast cancer (Kuller, 1995), including family history, early menarche, late menopause, age at first conception, use of oestrogen containing oral contraceptives, nulliparity, late pregnancy and the lack of breast feeding and endogenous and exogenous reproductive hormones (Tavassoli, 1997; Vessey, 1997). External factors such as ionizing radiation, exposure to chemicals, and excessive consumption of alcohol have also been thought to play a role in causing breast cancer. Alcohol consumption may have a carcinogenic effect by altering hepatic metabolism and interference with clearance, or by products of ethanol metabolism in the circulation (Stampfer *et al.*, 1993). Other controversial and putative risk factors include cigarette smoking, x-rays and high fat diets. The effects of cigarette smoking on breast and or other cancers have been extensively studied but the conclusions are still vague e.g. the inverse relationship between smoking and endometrial cancer could not be corroborated nor there has been

a significant relationship between smoking and colorectal or breast cancer (Nordlund *et al.*, 1997). Others have suggested an association of smoking with breast cancer depending on the receptor status of the tumour. Morabia and associates have shown that smoking is related to both oestrogen receptor positive (ER^{+}) and oestrogen receptor negative (ER^{-}) tumours but the strength of the association is greater for ER- tumours among post menopausal women (Morabia *et al.*, 1998). A study by a Russian group has also suggested some environmental factors (smoking) play a great role in the modification of breast cancer risk. They have suggested that these factors change the endogenous hormonal and metabolic parameters that modify the autocrine-paracrine relationship in the tumour tissue and of the hormone sensitivity of the latter (Khanson *et al.*, 1998). However, studies have shown no practical importance between cigarette smoking and breast cancer risk (Braga *et al.*, 1996). Recently tobacco use has been shown to be the most preventable cause of cancer (Yu *et al.*, 1997). Due to the huge discrepancy in the field, cigarette smoking cannot totally be eliminated as a risk factor in breast cancer. A high fat diet has been associated with a decreased excretion of oestrogen in the faeces. On the other hand, high fibre diet increased the secretion of oestrogens in faeces and therefore, lower blood oestrogen levels and decreased breast cancer risk (Rose, 1990). A vegetarian diet high in lignin's and isoflavonoids appears to reduce the risk of breast cancer (Stephens, 1997). The mechanism of action of these factors is not known but could involve prolonged hormonal stimulation of cellular proliferation, DNA damage, disregulation of growth factors, and cell-cycle associated genes in the breast (Service, 1998).

1.3 Prognostic Factors in Breast Cancer

Reliable prognostic markers are required for predicting the risk of developing breast cancer. It is also used to divide the patient groups into low versus high risk of recurrence and identification of appropriate treatments (Leinster, 1998).

Prognostic markers may reflect growth potential e.g. Ki-67, p53, and bcl-2, or invasive potential, e.g. uPA, tPA, PAI-1 and uPAR (Barendsz-Janson *et al.*, 1998). Tumour cell invasion and metastasis depends on the content of fibrinolytic parameters i.e. the proteolytic enzymes in the tumour. Extensive research has suggested the association of elevated levels of uPA and PAI-1 and metastasis in breast cancer (Grondahl-Hansen *et al.*, 1997b; Janicke *et al.*, 1993; Pedersen *et al.*, 1997; Schmitt *et al.*, 1990; Schmitt *et al.*, 1997b; Sumiyoshi *et al.*, 1991). A similar correlation has been reported in cervical cancer (Kobayashi *et al.*, 1994), pulmonary adenocarcinoma (Pedersen *et al.*, 1994b), squamous and large cell lung carcinoma (Pedersen *et al.*, 1994a) and in colon carcinoma (Gandolfo *et al.*, 1996). Cathepsin D (a lysosomal aspartic protease) has also been associated with a poor prognosis in breast cancer. Relapse rate for patients with high cathepsin D and PAI-1 is high (Kute *et al.*, 1998). tPA on the other hand, is an index of longer survival and therefore a good prognostic factor in breast tumours (Grebenshikov *et al.*, 1997). The authors have also described uPA and PAI-1 as markers of poor prognosis in node negative breast cancer patients.

1.4 Role of Steroid Hormones in Breast Carcinogenesis

The natural oestrogen 17-β-estradiol (E2) has been known to influence proliferation and neoplastic transformation of mammary epithelium. An early intermediate event in carcinogenesis is the aberrant proliferation which is

associated with an altered estradiol metabolism (Telang *et al.*, 1997b; Zhu & Conney, 1998). Telang and co-workers have implicated an altered cellular E2 metabolism as an endocrine biomarker in the modulation of human mammary carcinogenesis (Telang *et al.*, 1997a). Female sex hormones, oestrogens and progesterones, have long been known as potential stimulators of cell proliferation and thus as promotors of breast cancer. Since cells which harbour cancer causing mutations proliferate rapidly after growth stimulation by steroid hormones they promote carcinogenesis (Service, 1998). A positive correlation of breast cancer risk and high concentrations of endogenous estradiol has been strongly suggested, in post menopausal women (Thomas *et al.*, 1997).

Early surgical observations by Beatson (1896) established the basis for our understanding that ovarian endocrine influences are of primary importance in growth control of some breast cancer (Beatson, 1896). Epidemiological evidence in women indicates that the length of exposure of the mammary glands to oestrogen and progestogenic stimuli is directly proportional to breast cancer risk (Kuller, 1995). Women who have their first pregnancy after age 35 have a higher risk of breast cancer than those who remained nulliparous. Prolonged exposure to endogenous oestrogens as a result of early menarche, late menopause and late age at first full term pregnancy increase the time window for the initiation of breast cancer (Kelsey & Whittemore, 1994). The increased levels of oestrogens and progesterone and other hormones, at late age (35+) pregnancy, will result in increased breast cancer growth, if their are transformed cells present because of their increased mitotic activity. Obesity also appears to increase the risk of developing breast cancer, perhaps as a result of increasing peripheral aromatisation of circulating androgens to oestrogen (Boyle & Leake, 1988; Kuller, 1995). Oral contraceptives (containing both oestrogen and progesterone) do not appear to be a major risk

factor but this is currently controversial (Malone *et al.*, 1993). Hankinson has suggested an increased risk of breast cancer with longer duration of the use of contraceptives (Hankinson & Stampfer, 1997). Oestrogen stimulation is more clearly implicated as an aetiologic agent in vaginal adenocarcinoma, endometrial carcinoma and ovarian cancer. Recent evidence of involvement of oestrogen metabolites (which can bind to DNA and remove two bases, adenine and guanine, and thereby triggering damage) have been implicated in breast cancer by different groups, and this has further signified the link of oestrogen to breast cancer (Service, 1998).

The mechanism of interaction of oestrogens and progestins requires E2, acting through its receptor (ER) to induce the expression of PR. Both of these hormones work in harmony to regulate other genes, such as those for growth factors and cell cycle associated genes.

1.4.1 Oestrogen and Progesterone Receptors

Oestrogen and progesterone receptors are members of the steroid/ thyroid receptor super family. ER modulates gene expression in an oestrogen-dependent manner and thus functions as a transcription factor (Yamamoto, 1985). These specific, high affinity, low capacity binding sites are essential for the cells to respond to hormonal stimulation. Oestrogen receptor undergoes transformation after binding to its ligand, (oestrogen), ER then dimerizes and complexes with additional proteins to form the complete ER. Recent work has shown mutated forms of ER in breast cancer. The presence of ER in normal mature human breast tissues has been reported. 6-7% epithelial cells in normal breast are ER positive while 29% are PR positive. The level of ER varies according to different phases e.g. in menstrual cycle and during cell cycles. Progesterone receptors levels are at their highest and oestrogen receptor at

their lowest, in the luteal phase of the menstrual cycle, when the epithelial mitoses are at their peak suggesting the pivotal role of progesterone in breast epithelial cells in mitosis (Clarke *et al.*, 1992).

It is clear that oestrogens can act both directly and indirectly on normal mammary tissue to induce events that are closely associated with carcinogenic potential. The ability of E2 to stimulate the proliferation of some ER-positive breast tumour cells both *In vivo* and *In vitro* clearly implicates oestrogens as regulators of tumour growth, subsequent to carcinogenesis (Clarke *et al.*, 1992). Thus it is possible that oestrogens function as carcinogens, tumour promoters and as regulators of tumour growth (Thijssen & Blankenstein, 1994).

The presence of ER in a proportion of breast tumours may indicate that these tumours arise from ER positive epithelial cells. Since breast cancer is often characterised by hormonal control of its growth, measurements of ER levels in the tumour are used to predict patients' prognosis (Leake, 1997). About 60% of human breast cancers are ER positive (have an ER content above a minimum clinical cut-off value) and from these only 2/3 respond to endocrine therapy. Approximately 1/3 of breast cancers are hormonally responsive at the time they become metastatic. 5-10% of ER negative breast cancers by the biochemical assay respond to endocrine therapy (Dickson *et al.*, 1989). The mechanism for loss of ER expression in malignant progression of breast cancer is unknown and may only be a minor phenomenon since about 80% of ER^+ breast cancers, at first presentation, remain ER positive for the majority of their subsequent history (Crawford *et al.*, 1987; Robertson, 1996). However, it has been shown that in ER negative cell lines there is no transcription of the gene due to extensive methylation of 5'-promoter of the gene. The knowledge that oestrogen action promotes the synthesis of PR improves the predictive value of steroid receptor status. Clinical studies have

shown that 70% of ER^+/PR^+ breast cancers respond to hormone therapy. Not all the ER positive breast cancers respond to endocrine treatment and it may be because of non-functional ER in ER^+ cells. Some measure of ER functionality much improves the clinical value of ER determination (Leake *et al.*, 1981). However, discrepancies among the predicted values and levels of receptors may also be due to different laboratory errors, differential metabolism of tamoxifen, mutated ER that can modulate regulate gene expression in the absence of ligand or ability of defective or phosphorylated ER to bind ligand but not regulate gene expression. ER- and PR- negative breast cancers differ from their receptor-positive counterparts. ER- negative tumours are often highly proliferative and invasive *In vivo* and *In vitro*, and have altered expression of certain growth factors such as EGF and TGFα etc. Growth factor production in ER-negative cells can be constitutive, uncontrolled and non-oestrogen regulated (Dickson *et al.*, 1989).

It has been shown that protein kinase-C (PKC) remarkably increased the invasiveness of ER positive (MCF-7 and ZR-75) cell lines, after modulation of PKC activity with tetradecanoyl-phorbol-acetate (TPA), whereas it decreased the invasiveness of ER negative (MDA-MB-231 and MDA-MB-435) cell lines (Platet *et al.*, 1998). Oestrogen also down regulates ER mRNA by inhibition of ER gene transcription (Dickson *et al.*, 1989). It has also been shown that ER-negative and drug resistant breast cancers express elevated levels of PKC as compared to ER- positive breast cancer. Future investigation into modulation of ER function could have an impact both on diagnosis and therapy of breast cancer.

1.5 Growth Factors : Overview

Growth factors are specialised polypeptides that co-ordinate and regulate cellular activity. These growth factors are normally secreted at only basal levels to maintain homeostasis, but the levels vary according to the hormonal environment and age of the individual. Growth factor levels increase in response to injury, carcinogens and in the presence of neoplastic cells. They are responsible for rapid cell division in embryogenesis, cancer and early wound healing. Growth factors are also mediators by which oestrogens and progesterone stimulate both normal and neoplastic mammary tissue.

1.5.1 Epidermal Growth Factor (EGF)

It is now known that growth factors are the mediators of several of the actions of steroid hormones. Oestrogens stimulate the production of endogenous growth factors in mammary gland, which can function in autocrine, paracrine and juxtacrine manner (Duffy, 1992). The EGF family is a very important group in the development of the mammary gland and in the pathogenesis of breast cancer.

The EGF family is comprised of EGF, TGFα, heparin binding EGF-like growth factor (Whiltelock *et al.*, 1991), amphiregulin (Albini *et al.*, 1991) and betacellulin (Khokha *et al.*, 1989). These peptides are potent mitogens that interact with the EGF receptor, a structural homologue of the c-erbB oncogene family.

Native EGF is a 53 amino acid long polypeptide (6kDa) which is derived from a much longer precursor containing nine other copies of EGF- like units. EGF was first identified from mouse sub maxillary gland that causes eyelid opening of a new born animal (Murphy *et al.*, 1989), and later from human urine and

thus designated as urogastrone (Uria *et al.*, 1994). EGF is synthesised as a much larger transmembrane glycosylated, biologically active precursor of 128kDa. Precursor molecules have an N-terminal extracellular domain, a single transmembrane domain of 25 amino acids and a C-terminal domain of 150 amino acids, as predicted from the cDNA sequence. Mature EGF spans the cell membrane. The function of the other 8 units of EGF remains to be elucidated. Immunological studies have suggested that amino acids represent the receptor binding domain and are also responsible for signal transduction, while amino acids 35-53 stabilise the ligand receptor interactions.

Epidermal Growth Factor Receptor (EGFR)

The EGFR is a 170kDa transmembrane glycoprotein and is expressed in most normal epithelial tissues. It is expressed or overexpressed in many human epithelial malignancies, including lung, breast, head and neck, and bladder cancers. EGFR has intrinsic tyrosine kinase activity. It is composed of three domains: an extracellular domain that binds receptor specific ligands, a lipophilic transmembrane segment and intracellular tyrosine kinase domain that initiates a signal cascade from the cytoplasm to the nucleus. Signal transduction through EGFR occurs upon ligand binding followed by dimerization of the receptor, activation of tyrosine kinase, autophosphorylation of the receptor and phosphorylation of other substrates. EGFR structure, functions, mechanism of action and its role in carcinogenesis have been extensively reviewed by (Liotta *et al.*, 1991; Lochter & Bissell, 1995). Autophosphorylation processes are thought to result in internalisation and down regulation of the receptor after ligand binding (Sappino *et al.*, 1989). Whether the receptor is recycled or degraded is yet unknown though there is probably an element of both. EGFR is expressed in 30-40% of human

breast cancer. Expression of EGFR is an indicator of poor prognosis (Nicholson *et al.*, 1990). EGF dependent transformation of rodent fibroblasts due to overexpression of EGFR again implicate its role in the process of cellular transformation. Patients with EGFR positive tumours have shorter disease free intervals and lower overall survival than EGFR negative patients. The presence of EGFR can be correlated with the histological grade of tumours, the involvement of lymph nodes, the number of metastases, and poor prognosis for the patient. EGFR is detected in invasive ductal carcinomas, but not in lobular or colloid carcinoma (Klijn *et al.*, 1992). An uncontrolled autocrine loop of TGFα/EGFR results in a rapidly growing tumour as in squamous carcinoma of the head and neck, in which EGFR is frequently overexpressed. Although overexpression of EGFR is often present in epithelial carcinomas and is an indicator of poor prognosis, its role in tumour initiation, growth and progression needs to be better defined.

The proto-oncogene c-erbB2 is a related EGFR system. It is a 185kDa tyrosine kinase receptor and is homologous to EGFR. 20-30% of invasive breast carcinomas express c-erbB2. There is an inverse correlation between the expression of c-erbB2 and survival particularly in lymph node negative cases (Paget, 1889). erbB2 positive tumours also have minimal response to endocrine therapy and a poor response to chemotherapy regimens such as CMF.

EGF and Breast Cancer

Breast and other endocrine glands like ovaries, endometrium and prostate are under the hormonal modulation for normal growth and also for tumour progression. In this respect, sex steroids play an important role in the

regulation of locally acting growth factors such as the EGF family (Martinez-Lacaci & Dickson, 1996)

EGF has been detected in a variety of breast cancer cell lines (Johnson *et al.*, 1993) and human breast tumours (Albini *et al.*, 1986). EGF is mitogenic for several breast cancer cells. Low levels of EGFR are associated with growth response to EGF whereas cell lines with higher EGFR are growth inhibited by EGF. This observation is clinically very important (Kirby, 1965; Zetter *et al.*, 1990) because ER-/EGFR positive tumours are associated with metastasis and shorter survival time whereas ER+/EGFR negative tumour types are better differentiated and is good prognosis. EGF promotes anchorage dependent growth of human mammary epithelial cells (Sato & Rifkin, 1989). Interestingly TGFα mRNA levels are also higher in hormone responsive endometrial cells (Murphy, 1994) and prostate cancer thus suggesting hormonal stimulation of an autocrine loop in hormone responsive cell lines. EGF mRNA levels can further be increased by progestins in T47-D breast cancer cells (Del-Rosso *et al.*, 1990). This shows a possible hormonal regulation of EGF. EGF is probably the principal milk derived growth factor and appears to be an important regulator of growth and differentiation of the mouse mammary gland (Yagel *et al.*, 1989) especially during pregnancy, lactation, and spontaneous formation of mammary tumours.

1.5.2 Transforming Growth Factor α (TGFα)

TGFα and TGFβ, have the ability to bring transformation and anchorage independence to normal cells. TGFα was first isolated from human solid tumours and from the culture media of virus-transformed cells. It has been demonstrated in a variety of retrovirally transformed cells, human tumours and normal embryonic cells (Guidice & Saleh, 1995)

TGFα is a 47-50 amino acid peptide with a molecular weight of 6kDa. It has 3 disulphide bonds between six cysteine residues (Graham & Lala, 1991). Human TGFα is derived from a large membrane bound precursor protein of 160 amino acids that is biologically active. The N-terminal sequence consists of a 23 amino acid signal peptide, 74-75 amino acids constitute the mature growth factor domain followed by 39 amino acids at the C-terminus. This latter sequence is a cysteine rich part of the growth factor and remains in the cytoplasm. Pro-TGFα is juxtacrine in function (Lala & Graham, 1990). Human TGFα shares 42% homology with human EGF while it is thought to have 27% amino acid sequence homology with murine EGF. Three dimensional studies have revealed that its tertiary structure resembles that of EGF and is able to bind to EGF receptor with the same affinity.

TGFα and Breast Cancer

TGFα has the same biological effects as EGF in cultured human and mouse mammary cell lines and mouse mammary explants (Yagel *et al.*, 1989). A number of studies have clearly shown that TGFα is present in both primary and metastatic human breast cancers. TGFα is expressed in mammary epithelial cells and breast cancer cells and is regulated by oestrogens. Physiological concentration of E2 is sufficient to induce TGFα mRNA transcription and protein translation (Flaumenhaft *et al.*, 1992). It has been demonstrated that this induction is mediated through ER by using anti-oestrogens. The mitogenic effect of E2 is mediated by TGFα in ER positive human breast cancer cells (Flaumenhaft *et al.*, 1992; Testa & Quigley, 1990) Availability of purified antibodies has made it easy to find the expression of any factor. Experiments using in situ hybridisation analysis have detected TGFα mRNA during the proliferative stage and during human pregnancy.

Similar studies have indicated that breast cancer cells synthesised the growth factor. (Irving & Lala, 1995) have demonstrated that 31% of primary tumours and 50% of metastastic tumours analysed were positive for TGFα. Further, during mouse mammary gland studies, immunohistochemistry has revealed TGFα expression in the basal epithelia, proliferative and bud cap cells.

1.5.3 Transforming Growth Factor β (TGFβ)

TGFβ was first studied in the 1970s in association with mammalian and avian retroviruses that could induce sarcomas in animals. It was found that retrovirus could transform normal fibroblasts by certain substances later called Sarcoma Growth Factor (SGF). It had been observed that SGF from transformed cells was composed of TGFα and TGFβ, operating in tandem. (Duffy *et al.*, 1988)

The chromosomal locations of the human TGFβ genes are 19q13 for TGFβ-1, 1q41 and 14q24 for TGFβ-2 and 3, respectively. The corresponding mRNAs range from 2.5kb (TGFβ-1) to 6.5kb (one of the TGFβ-2 transcripts). It is indicative of a differential regulation of these three isoforms that they have distinguishing promoter sequences.

Mature TGFβ is a 25kDa polypeptide that belongs to a much larger superfamily of sequence related molecules in the inhibin family. This family of multifunctional regulatory peptides is involved in a range of processes including regulation of growth, development, and differentiation. The 3 mammalian isoforms TGFβ1, β2, and β3 are all homodimers. As the most predominant member of its family, TGFβ 1is found essentially in all mammalian tissues, with the highest concentrations being found in platelets and bone. Each of the members consist of two peptide chains of 112 amino acids and nine cysteine residues with a molecular weight of 25kDa. The

25kDa form represents the C-terminal end of a precursor molecule. TGFβ-1 is encoded as a 390 amino acid precursor while TGFβ-2 as a 412 amino acid precursor. All three precursors have N-terminal signal peptides of 20-23 hydrophobic amino acids (for reviews see Sainsbury, 1997; Knabbe & Zugmaier 1994; Benson & Colletta, 1995).

Latency in TGFβ is conferred by dimerization of N-terminal part of associated proteins which folds itself around TGFβ1 (called latency associated peptide β1-LAP). An additional component called latent TGF (LTBP) is also found in the latent complex released from blood platelets. The function associated to LTBP is to target TGF to extracellular matrices (Heidtmann *et al.*, 1989).

TGFβ isoforms have been identified in normal tissues of both developing and adult organisms, as well as neoplastic tissue (Patthy, 1985; Wolf *et al.*, 1993a) Most cells, normal or transformed, release TGFβ as a latent, biologically inactive molecule.

TGFβ can be activated *in vitro* by acidic pH (Kobayashi *et al.*, 1990; Schmitt *et al.*, 1989). However *in vivo* mechanisms of activation require proteases such as plasmin and cathepsin D (Goretzki *et al.*, 1992). Latent TGFβ has a longer plasma half life thus providing stability to the molecule to act at a distant site from its origin (Gunzler *et al.*, 1982a). It has been shown that the half life of recombinant TGFβ is about 90 minutes *in vivo*. The active form of TGFβ has a very short half life and is removed from the circulation by the liver (in 2-3 minutes) (Gunzler *et al.*, 1982a).

Biological Functions of TGFβ

TGFβ is a multifunctional growth factor. It plays a central role in most processes of intercellular communication, including differential regulation of

the extracellular matrix, angiogenesis and immunosuppression. In general it is inhibitory to a range of epithelial and endothelial cells, human keratinocytes, hepatocytes and human carcinoma cell lines but stimulatory to mesenchymal cells (Schwann cells, Osteoblasts, Chondrocytes, W138 human fetal lung fibroblast). TGFβ regulates the expression of a wide variety of genes and proteins, including several nuclear proteins involved in cell growth control. TGFβ regulates c-jun and c-fos. There is inhibition of keratinocyte DNA synthesis by down regulation of c-myc by TGFβ. However, mouse NIH3T3 cells are stimulated by TGFβ by the induction of c-myc, thus having a more direct mitogenic effect. Picomolar quantities of TGFβ can regulate proliferation. TGFβ regulates cyclin D function and Retinoblastoma (Rb) protein phosphorylation. TGFβ stimulates the anchorage independent growth of human and rat fibroblasts. For example, NRK-49 fibroblasts are stimulated by TGFβ but inhibited when transformed by Kirsten sarcoma virus thus showing the bifunctional nature of TGFβ. TGFβ1 has a role in wound healing processes indicated by its high content in blood platelets (Lyons *et al.*, 1990). It is believed that many cancers may arise due to dysfunction of TGFβ regulation. Solid tumours require vascularization to gain a clinically detectable size. TGFβ stimulate the production of stromal components like fibronectin, proteoglycan and tenascin, their overexpression favouring stroma formation and thus angiogenesis. Increased expression of TGFβ allows the tumour to escape from immunological response. The overexpression of TGFβ2 is likely to be responsible for the immune deficiency in patients with Glioblastoma. Thrombospondin, a stromal element, promotes angiogenesis and tumour invasion. These effects may be due to its ability to activate latent TGFβ1.

TGFβ and Breast Cancer

Growth factors exert their functions by autocrine or paracrine loops. Over activity of any of these loops results in excessive proliferation, a characteristic of the preneoplastic state or tumour progression.

In the field of cancer, dysfunction of TGFβ has been implicated. It has a growth inhibitory effect on the progression of cancers derived from epithelial origin like breast cancer. Exogenous TGFβ inhibits the growth of breast cancer cell lines indicating its principal inhibitory autocrine loop. Breast cancer cells resistant to anti-oestrogens are still growth inhibited by TGFβ *in vitro* (Gunzler *et al.*, 1982b). The anti-oestrogen, tamoxifen, can also inhibit the growth of the hormone insensitive (anti-oestrogen insensitive) cell line MDA-MB-231 when co-cultured with ER positive (MCF-7) cells in a system permitting interchange of conditioned media. This is due to enhancement of TGFβ secretion by the MCF-7 cells. This could explain how endocrine insensitive cells respond to endocrine therapy, since tumours are often heterogenous in ER content. *In vivo* studies have also confirmed the induction of TGFβ by tamoxifen in ER positive cells by antagonising the stimulatory effect of oestrogen and so tamoxifen may inhibit ER negative cells. *In vivo* studies, using immunohistochemistry on tissue from a group of breast cancer patients following tamoxifen therapy, further confirmed the elevation of TGFβ in both ER positive and ER negative cells (Schmitt *et al.*, 1997a).

Other growth inhibitors such as retinoic acid are of interest. These are naturally occurring derivatives of vitamin A (retinol) and exert a key role in maintenance of epithelial homeostasis through regulation of cellular proliferation and differentiation. Retinoic acid induces the secretion of TGFβ-

1 and TGFβ-2 depending upon the cell types (reviewed in Benson & Colletta, 1995; Knabbe & Zugmaier, 1994)).

Induction of TGFβ is not only confined to breast tissue/cells. Up regulation of TGFβ has been observed in the peritumoral fibroblasts *in vivo*, suggesting involvement of paracrine loop between stromal and epithelial cells (Schmitt *et al.*, 1997a). Experimental evidence has suggested that overexpression is associated with enhanced metastatic potential of rat prostate cancer cell lines. Withdrawal of androgen leads to enhanced expression of TGFβ-2 in the androgen responsive human prostate cancer cell line LNCaP (Blasi, 1988). TGFβ-2 has been suggested as a marker of the hormonally regulated growth state of tumour tissue. A more aggressive stage of tumour is said to be accompanied by the loss of the inhibitory function of TGFβ *in vivo*.

Mechanism of Action of TGFβ

The mechanism of action of TGFβ is not fully understood. A complex pattern of cross-reactive ligands and cell surface receptors has been described (Blasi *et al.*, 1987). Three major transmembrane receptors are known to bind TGFβs (Heldtmann *et al.*, 1989). These are type I (53kDa), type II (70-80kDa) and type III (250kDa) receptors. These were found by cross linking studies of iodinated TGFβ with cell surface proteins of human breast cancer cells. TGFβ interacts with two types of membrane receptors, which functionally cooperate to generate an intracellular signal (Janicke *et al.*, 1990). These two types i.e. type 1 and type 2 contain a serine-threonine kinase domain and are thought to mediate the growth inhibitory action of TGFβ. (Derynck, 1994; Duffy *et al.*, 1990; Pennica *et al.*, 1983). The three types of TGFβ receptors have been cloned. Growth inhibitory functions of TGFβ remain to be elucidated but it has been shown that growth inhibition by TGFβ is linked to suppression of the

retinoblastoma protein (pRB) phosphorylation (Grondahl-Hansen *et al.*, 1991). However, inactivation of retinoblastoma gene alone does not lead to the loss of TGFβ receptors or of response to TGFβ in breast cancer cell lines (Skriver *et al.*, 1984)

1.5.4 Insulin like Growth Factor (IGF)

Insulin like growth factors were discovered as the somatomedins in 1957 but were purified and sequenced in the mid 1970s. Insulin like growth factor (IGF) is named due to its close homology to pro-insulin. The IGF family consists of two ligands IGFI and IGFII with their respective receptors IGFRI and IGFRII and some six different binding proteins IGFBP are described. IGFI and IGFII are single chain molecules of 70 and 67 amino acids respectively with high affinities for their respective membrane bound receptors. The single chain 7.5kDa IGFI and IGFII are produced in many body tissues, specially in liver, and are under the control of hormonal regulation. IGFI is known to have a role in bone elongation in an endocrine manner under GH control, while IGFII is thought to be involved in fetal development. Recently, their role in development, growth and transformation has been described.

IGFRI is a heterotetrameric protein consisting of two alpha and two beta chains. Like insulin receptor, it has intrinsic tyrosine kinase activity. It has been observed that ligand binding causes autophosphorylation of the receptor and this transduces the signal of the ligand. The receptor binds, both ligands IGFI and IGFII, with equal affinity. On the other hand, IGFRII is a single chain molecule with affinity for only IGFII. Unlike IGFRI, IGRFII has no tyrosine kinase activity and therefore the signalling mechanism is obscure.

IGFs and Breast Cancer

IGF increases the rate of proliferation of some breast cancer cells (Ossowski & Reich, 1983) (Ossowski, 1988). Kullen et al have described that IGFII mRNA is expressed in T47-D cells and that both T47-D and MCF-7 cells are growth stimulated by both IGFI and IGFII *In vitro* (Wei *et al.*, 1996) IGF may stimulate mitogenesis by reducing the levels of active TGFβ. 50% of breast carcinomas appear to have IGFII mRNA while it is present in all normal breast tissues.

The levels of IGFI and IGFII appear to be low in epithelial cells but their confirmed presence in the stromal components of a number of breast tumours have suggested a potential paracrine role for IGFs, whose level can be modulated by the anti-oestrogen, tamoxifen (Vassalli *et al.*, 1985; Xing & Rabbani, 1996). It is believed that stromal secretion of IGFI can be induced by platelet derived growth factor present in breast epithelial cells with stromal cells possessing PDGF receptors. IGFI mRNA is absent in primary breast tumours but is present in normal breast. Stromal cells are rich sources of IGFs which may serve as positive paracrine growth factors. The functions of IGFs in breast cancer are not only controlled by levels of ligands and receptors but also by the presence of IGF binding proteins. These extracellular binding proteins may either stimulate or inhibit IGF action and they may determine both activity and bioavailability of these growth factors (Yee *et al.*, 1991). Breast cancer cell lines secrete significant levels of IGF binding proteins and their addition to the culture media has been shown to inhibit cancer cell growth.

Breast cancer patients have higher plasma IGFI levels than their normal counterparts (Felsenfeld *et al.*, 1996) and 25% reduction in IGFI has been observed in patients treated with tamoxifen. The levels of IGF binding

proteins are also subject to modulation by oestrogens and possibly by anti-oestrogens (Lonning *et al.*, 1992).

1.6 Tumour Development and Metastasis

Breast carcinomas are mixtures of genetically and phenotypically distinct cells, despite having arisen from a single cell. Genetic disposition, either by external (UV-irradiation or chemical carcinogens) or internal stimuli (hormones or growth factors) leads to transformation in a cell. One of the earliest detectable changes in transformed cells is a several fold increase of genomic instability compared with normal cells (Tisty, 1997). Both these properties, genetic instability and tumour heterogeneity determine the ability of the cells to metastasise (Welch & Wei, 1998). Transformation is the initial stage of a tumour development. Proliferation of the transformed cell is followed by formation of a primary tumour. The cells in the primary tumour are confined within its place and therefore cancer is localised. Primary tumours can be treated surgically, so patients rarely die of carcinoma *in situ*. It is the undetected spread of tumour cells throughout the body, which is life threatening. This establishment of tumour at a site distant from the primary site, is metastasis. The term "metastasis" was first described by a French physician, Joseph Claude Recamier, in his 1829 treatise Recherches du Cancer. He was the first to show that cancer cells can form distant tumours in the body through the circulatory system. He observed the growth of a tumour in the brain of a patient with breast cancer. He also noted that the veins in the same site were invaded by tumour cells. A clearer understanding of the mechanisms underlying invasion and metastasis is required.

Steps Involved in Metastasis

This process of invasion and metastasis requires a series of sequential steps (Liotta & Stetler-Stevenson, 1991) begining with dissociation of the cells from primary mass, to move through a basement membrane surrounding the tumour, making its way through the interstitial connective tissue (extracellular matrix) and then through another basement membrane into the bloodstream. After escaping the immunosurveillance in circulatory system, the cells have to cross all the barriers of basement membranes and extracellular matrix (ECM) on reaching the suitable destination, but in a reverse order. The tumour cell in the new environment establishes the micro metastasis and only grow in size in the presence of ample supply of blood vascular system. Tumours 1-2mm in diameter can receive all the nutrients by diffusion but for further growth it needs to develop its own blood supply (Folkman, 1990).

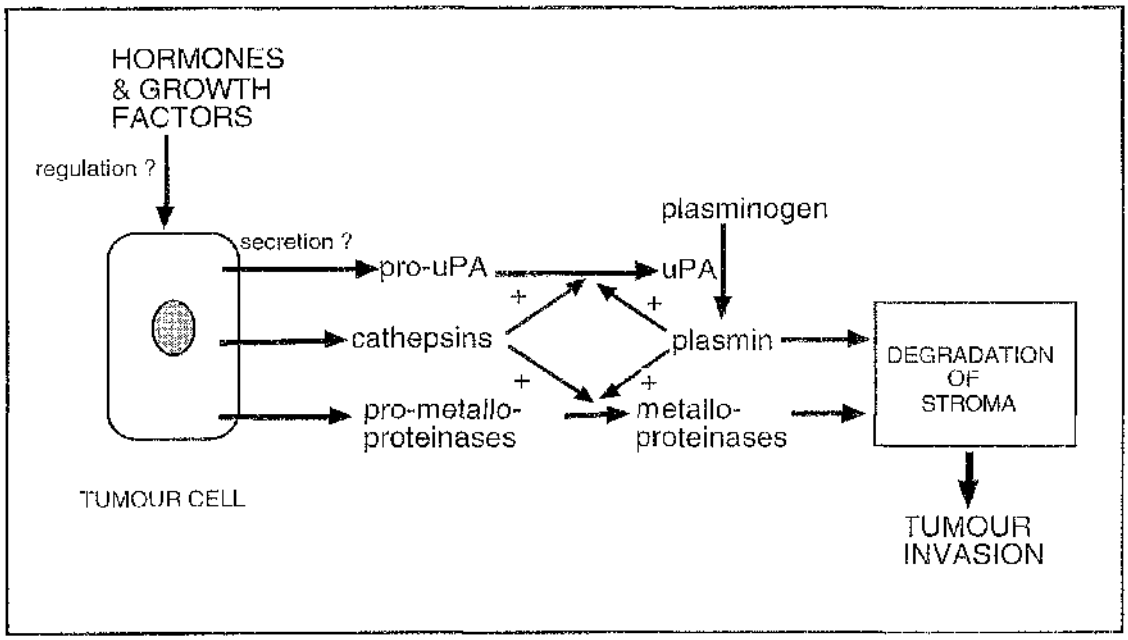


Fig. 1 Tumour associated proteases are involved in the degradation of stroma and basement membrane, which leads to tumour invasion. The metalloproteinases include collagenase, stromelysins, cathepsins and gelatinases.

This figure is modified fromSchmitt *et al* 1990

1.7 Metastatic Spread: Where, Why?

Normally, tumour cells are carried away through the blood and/or lymphatic circulation until a favourable environment is found. One might anticipate that malignant cells would metastasise to an organ of the same somatic origin. This is not the case. However, metastasis is not random but displays a selective pattern e.g. cancers of the breast and large bowel exhibit different metastatic foci, despite both originating in epithelial cells (Picford & Birnie, 1992). One may speculate, on the basis of the lymphatic system and the blood flow, that 60% of the metastasis from the primary tumour may deposit in the lung, liver and bone marrow. The frequency with which metastasis occurs in lung and liver may be because the metastasising cell passes through the heart before it finds the resting place. Because the heart pumps all the blood through the lung capillaries before sending it to other parts of the body, lungs therefore, are the most common metastatic site, for many cancers. Liver is the favoured site of metastasis from colon cancer because of the direct drainage of the venous blood from the large intestine, into the liver (Liotta, 1992). Sometimes, the metastatic foci are different from that mentioned and the only explanation applicable may be the hypothesis proposed by (Paget, 1889). The consensus of "seed and soil" theory was proposed by him after his studies on breast cancer. He referred to the circulating tumour cells as "seed" and the favourable environment that is provided by the organs for the cells to metastasise, as the "soil". It appears that hormones and growth factors provide greater affinity for the circulating tumour cells and therefore induce them to move out of the bloodstream and into cells/tissues which have the appropriate balance of the desired endocrine and paracrine functions (Liotta, 1992).

It has been observed that not all cells, that have survived mechanical turbulence and escaped the host immune system, can grow in an organ. Only as few as one in 10000 tumour cells leaving the primary mass can initiate a

new tumour at a distant site. Once in the new environment tumour cells proliferate. Cell proliferation is controlled by growth factors (Zetter *et al.*, 1990). Another important factor for a tumour cell to grow and establish metastasis beyond about 3mm in diameter, must establish its own blood supply i.e. angiogenesis is essential (Pickford & Birnie, 1992).

1.8 Extracellular Matrix and Epithelial Cells Interactions

Cells are dependent on their interaction with the substratum for proper growth and differentiation. For instance, it has been shown that whereas cells grown on plastic respond poorly to lactogenic hormones, those grown on collagen gels, or EHS, induce the gene expression of milk producing genes in response to lactogenic hormones. Protein secretion is also dependent on substratum and has been shown to be enhanced generally in cultures on floating gels. Epithelial differentiation can be induced and maintained on and within a variety of matrices derived from ECM components. Both normal and neoplastic mouse mammary epithelium displayed some morphogenesis and also high growth rate when grown on and within collagen gels. These as well as other results clearly underscore the role that a substratum plays on the proper biological functioning of a normal or a malignant cell.

Although surgeons and oncologists can treat cancers that are in their early stages, very rarely are they able to save patients whose tumours have metastasised to other parts of their body. Tumour cell invasion across the basement membrane (BM) and tumour associated angiogenesis are considered to be the key steps of metastasis (Figure 1). Although it is a complicated process that requires the co-ordinated action of numerous proteins to degrade the BM, understanding how metastasis occurs is of obvious importance. Thus, in order to study the *in vitro* invasive potential of a certain cancer cell line and

how different hormones and growth factors affect it, it was important to use a substratum that contained different membrane proteins and was therefore more physiological than plastic. Matrigel, which is an extract of Engelbreth-Holm-Swarm (EHS) tumour basement membrane (BM), is a substratum which has been widely used since 1986 (Albini *et al.*, 1986) for such invasion studies. Matrigel is comprised largely of laminin, type IV collagen, heparan sulphate proteoglycan, fibronectin and entactin. Recently, however, trace amounts of plasminogen have also been found in Matrigel (Farina *et al.*, 1996). In its reconstituted form it has been used extensively as a BM barrier to tumour cell invasion and to study the effect that the BM has on vasculargenesis *In vitro*.

Several laboratories have noted the importance of the major proteins in Matrigel on cellular growth and differentiation. Specifically, it has been found that all five proteins in Matrigel are required for proper growth and maintenance of different cell lines. In one study it was found that proper alveolar morphogenesis and mammary-specific functional differentiation required the presence of Matrigel. Growth of these cells on individual components of the ECM, however, was not associated with proper alveolar formation and differentiation. Another study showed that the growth and differentiation of the mammary epithelium cells is dependent on the substratum. In this case protein synthesis and secretion by mouse epithelial cells on floating collagen gels and EHS-matrix were compared and it was found that protein synthesis was consistently higher in cells that were attached to EHS matrix. Similarly, cells associated with the EHS matrix also had higher rates of growth and protein secretion into the culture medium than those which were grown on plastic. The much greater rates of growth suggest that the matrigel may facilitate both two and three dimensional interaction of epithelial cells

In a comprehensive study (Li *et al.*, 1994) showed the influence of a reconstituted basement membrane and its various components on casein gene expression and subsequent secretion in mouse mammary epithelial cells. Consistent with previous reports the results show that when these cells were plated on plastic they immediately stopped synthesising and secreting most milk proteins even in the presence of lactogenic hormones. More importantly, cells cultured on type-I collagen gels were found to have increased amounts of β-casein mRNA and protein in addition to other milk proteins. On Matrigel, most of the cells produced high amounts of the β-casein. Interestingly, they found that while individual components of the basal lamina such as type IV collagen, and fibronectin had no effect on morphology and β-casein mRNA levels, both laminin and heparan sulfate proteoglycan caused an increase in β-casein mRNA levels. A dramatic difference in the morphology of mammary epithelial cells was seen when they were plated on plastic and matrigel. Whereas no changes were evident on plastic, on EHS the same cells formed ducts, ductules, and lumina which resembled secretory alveoli. Taken together these results clearly show the important role of substratum on cellular metabolism.

1.9 Proteases In Invasion and Metastasis

Cells in the body are scaffolded in a basement membrane which is responsible for the regulation of many cellular functions. The components of basement membrane consists of type IV collagen, laminin, heparan sulphate and proteoglycan and the destruction of these components correlates with invasion and metastasis (Liotta & Stetler-Stevenson, 1991; Tryggvason, 1989). This step requires the coordinated expression of destructive enzymes and their inhibitors. Several lines of evidence show that particular proteases are responsible for the breakdown of basement membranes (involved in

compartmentalisation and many cellular functions) and subsequently the extracellular matrix. All the evidence points to a common set of enzymes and their inhibitors that are involved in many forms of cancer. These enzymes consists of 4 main classes namely; serine proteases, matrix metalloproteases, cysteine proteases and aspartyl proteases which allow tumour cells to escape from its primary site and to intra and extravasate the basement membrane.

The key enzymes appears to be plasminogen activators (Mignatti & Rifkin, 1993). While the uPA/plasminogen system is undoubtedly very important in the spread of metastasis, the other proteases i.e. metalloproteases (Bassat *et al.*, 1990; Matrisian, 1990; Woessner, 1991) such as the stromelysins (Bassat *et al.*, 1990) and cathepsins (Duffy *et al.*, 1991; Foucre *et al.*, 1991; Moscatelli & Rifkin, 1988; Rochefort *et al.*, 1990; Rochefort *et al.*, 1996) have also been implicated to play roles in the dissemination of tumour cells.

Matrix Metalloproteases

Matrix metalloproteases are an important group of zinc enzymes responsible for degradation of extracellular components, including interstitial collagens, basement membrane collagen (type IV), fibronectin, laminin and various proteoglycans (Moscatelli & Rifkin, 1988) in normal embryogenesis and tissue remodelling, as well as in disease processes such as arthritis, cancer, and osteoporosis.

The chief characteristics of matrix metalloproteases (MMPs) are no different from other proteases involved in normal and cancer situations in that these proteases are secreted in zymogen form. The inactivity is because of a highly conserved sequence of nine amino acids. This sequence contains a reactive cysteine residue. The zymogen can be activated by other enzymes, plasmin and chymotrypsin (Duffy, 1992), or by organomercurials (Woessner, 1991).

The question arises as to how these metalloproteases are activated *In vivo*? Since organomercurials or trypsin are not present in the tissues it is expected that plasminogen enters from the blood and that the local tissue can produce plasminogen activator (PA) forms plasmin by PA and in turn MMPs are activated either directly or indirectly by plasmin. This again emphasises the role of PAs in invasion. The activity of these enzymes is inhibited by tissue inhibitors of matrix metalloprotcases (TIMPs) (Matrisian, 1990).

Several members of the MMPs family have been linked to cancer cell biology. Liotta *et al.* (1992) have found that in general, augmented levels of metalloproteases in tumours correlated with the development of invasion and metastases in cancer of the human breast, colon, stomach, thyroid, lung and liver. These include Type IV collagenase or gelatinase (known as MMP-2) which is found in a wide variety of tumours including mammary adenocarcinoma cells where the degree of its expression is correlated to metastatic potential (Nakajima *et al.*, 1987). Type IV collagenase is expressed in human skin and colon cancers (Tryggvason *et al.*, 1993) at the invading front and in bladder carcinoma. Type I collagenase (MMP-1), also showed elevated expression in mammary carcinoma cell lines (Whiltelock *et al.*, 1991).

Tissue Inhibitors of Matrix Metalloproteases (TIMPs)

Control of extracellular proteolysis is critical to the normal, healthy organism. Just as there is a family of metalloproteases, there is also a family of TIMPs. Different forms of these inhibitors are described e.g. TIMP-1, -2 and -3. Several groups have shown that TIMP-1 and TIMP-2 can inhibit the process of tumour cell invasion (Parish, 1994). TIMP-1 is a glycoprotein with a molecular mass of 28kDa and it inhibits invasion and metastasis in animal

models. This has been observed by transfecting antisense TIMP-1 RNA in 3T3 cells (Khokha *et al.*, 1989). TIMP-2 is non-glycosylated and inhibits invasion of ECM (Albini *et al.*, 1991). It has been shown that TIMP-3 is expressed in human breast tumours (Uria *et al.*, 1994). TIMP forms a 1:1 stoichiometric complex with MMP-1 and MMP-3. The enzyme binds reversibly and can be dissociated by acid treatment or EDTA (Murphy *et al.*, 1989).

Stromelysins

Stromelysins are also MMP family members involved in tumour biology. They are broad spectrum proteases which can cleave ECM components. There are different forms of stromelysins i.e. stromelysin-1,-2 and -3. In addition to others, the metalloprotease stromelysin 3 is often found in breast cancer and is rarely present in benign tumours of the breast. The prognostic value of stromelysin 3 in invasive breast cancer however has not been established. However the correlation with other known prognostic factors, point out that it may play an important role. Stromelysins-1 and -2 are present in elevated levels in most invasive breast carcinomas (Wolf *et al.*, 1993b).

Cathepsins

Cathepsin D is the most studied protease of the group cathepsins. It is secreted as a 52kDa precursor form. It is a lysosomal aspartic protease whose expression is regulated by oestrogens and certain growth factors in breast cancer cells. Normal breast tissue contains little cathepsin D while its content is higher in cancer tissues (Duffy *et al.*, 1991). A number of studies have shown that high levels of cathepsin D offer a bad prognosis, reduced overall survival and early metastatic spread (Foucre *et al.*, 1991). Its involvement in

metastasis is controversial. On one hand no correlation was found between cathepsin D and invasiveness (Johnson *et al.*, 1993), while others have shown a link between elevated levels of tumour cathepsin D and poor prognosis in either node-negative or node positive patients. The influence of other cathepsins, such as cathepsins B, H, and L, on the prognosis of breast cancer patients is under study.

Plasminogen Activators

There are two independent structural genes that code for plasminogen activators. These are molecularly, functionally and immunologically distinct (Pennica *et al.*, 1983) but have about 40% homology on the basis of their amino acid sequences (Pennica *et al.*, 1983; Verde *et al.*, 1984). They are called urokinase type (uPA) and tissue type (tPA) plasminogen activators. They are termed after their original identification in urine and tissue extracts, respectively (Dano *et al.*, 1985). The molecular weights of uPA and tPA are 50kDa and 70kDa, respectively (Laiho & Oja, 1989). tPA has a role in fibrinolysis, whereas uPA is involved in tissue remodelling, development and also in cancer invasion and metastasis (Mignatti & Rifkin, 1993) .

1.10 Plasminogen Activator System

Plasminogen activation system has been shown to play a pivotal role in tumour cell invasion (Dano *et al.*, 1985) Plaminogen activators mediate the conversion of inactive plasminogen, by limited proteolysis, to active plasmin, which has a broad substrate specificity (Robins *et al.* 1967).

1.10.1 Plasminogen

Plasminogen is an inactive proenzyme consisting of a single polypeptide chain with a molecular weight of 92,000. It exists in two forms; the Glu-plasminogen and the Lys-plasminogen. Both these variants differ slightly due to the differences in the glycosylation. The Glu-plasminogen has glutamic acid and Lys-plasminogen has lysine at the N-terminus. The molecular weight of Lys-plasminogen is approximately 8kDa lower than Glu-plasminogen (Dano *et al.*, 1985).

Glu-plasminogen is converted into its active Glu-plasmin form by cleavage of a single polypeptide bond (Arg 560-Val 561) that is catalysed by the plasminogen activator (PA). The active two chain Glu-plasmin is held together by disulphide bonds. The formation of Lys-plasminogen from Glu-palsminogen is catalysed by plasmin by the cleavage of another peptide bond (Lys76-Lys77). Plasminogen is widely available in the body, having a circulating concentration of approximately 2 mM and a half-life of 2.2 days (Saksela, 1985). Numerous studies have shown that plasminogen predominantly appears to be located extravascularly (Grondahl-Hansen *et al.*, 1991).

Plasminogen regulation

Plasminogen activation is regulated at several stages. Firstly, the activation of inactive plasminogen to active plasmin. Secondly, plasmin can be inhibited by its inhibitors e.g. α_2-antiplasmin. Thirdly, binding of plasminogen and/or plasmin to its receptor is required, close to the uPA receptor on the cell surface. Fourthly, there is regulation of the activators of plasminogen, i.e. uPA and other molecules like plasmin that activate plasminogen.

1.10.2 Plasmin

Plasmin, is generated by a single proteolytic cleavage at ^{560}Arg by plasminogen activators. Plasmin has an important role in physiological fibrinolysis and is also ascribed other important roles in several physiological and pathological circumstances characterised by tissue remodelling and cell motility. Amongst the two types of plasminogen activators, uPA has a specific substrate, plasminogen. Plasmin can, either directly or through its ability to activate some prometalloproteases (e.g. procollagenase type IV), degrade components of the tumour stroma such as fibrin, fibronectin, proteoglycans and laminin (Blasi *et al.*, 1987; Duffy, 1992; Ellis *et al.*, 1991; Janicke *et al.*, 1990; Schmitt *et al.*, 1992). In addition, plasmin affects various growth factor systems, e.g. activation of latent TGFβ (Lyons *et al.*, 1990) and dissociation of IGF from its binding protein (Campbell *et al.*, 1992).

Plasmin is a trypsin-like protease with a molecular weight of approximately 25kDa. It is a two chain polypeptide held together by disulphide bonds. Like other serine proteases such as trypsin, chymotrypsin and pancreatic elastase, it has a relatively broad substrate specificity and hydrolyses proteins and polypeptides at lysine and arginine residues.

Regulation of Plasmin Activity

Plasmin is inhibited by several protease inhibitors occurring in plasma but the most important are α2-antiplasmin and α2-macroglobulin. The latter seems to be involved only when there is a depletion of the fast reacting α2-antiplasmin. Plasmin activity can be controlled at several levels but most important steps include 1) Availability of plasminogen activator 2) Levels of plasminogen activator inhibitor.

1.11 Components of Plasminogen Activation System

Two well documented activators of plasminogen are urokinase type of Plasminogen Activator (uPA) and tissue type Plasminogen Activator (tPA).

1.11.1 Urokinase Type of Plasminogen Activator (uPA)

uPA is a very well characterised 50kDa protein which is glycosylated at Asn302. uPA is secreted as an inactive single-chain protein (pro-uPA) with no intrinsic enzymatic activity. Pro-uPA is activated by a variety of proteases including plasmin, cathepsins B/L, Kallikrein, trypsin-like enzymes, and nerve growth factor-γ, leading to the enzymatically active high molecular weight two chain form, HMW-uPA. Pro-uPA is cleaved at a peptide bond lysine (158)-isoleucine (159), by these proteases (Blasi *et al.*, 1987). uPA is a multidomain protein (Gunzler *et al.*, 1982a) whose N-terminal A-chain (20kDa) and the C-terminal B-chain (34kDa) are linked by a single disulphide bond (Verde *et al.*, 1984; Dano *et al.*, 1985).

Over years many forms of uPA have been identified. The precursor protein called high-molecular weight (HMW)-uPA is 411 amino acids in length (≈52 kDa) and is secreted from normal fibroblasts, trophoblast cells of the placenta and kidney tubules etc, as well as from tumour cells and is then subsequently activated extracellularly (Saksela, 1985; Schmitt *et al.*, 1992). The HMW-uPA is further converted into an enzymatically active LMW-uPA which is 276 amino acids (≈34 kDa) and an inactive form called amino-terminal-fragment (ATF) which is 135 amino acids in length (≈16 kDa). Subsequently, ATF is converted into a 49 amino acid polypeptide termed growth factor like domain, GFD (≈6 kDa) and 86 amino acid Kringle domain (Gunzler *et al.*, 1982a; Gunzler *et al.*, 1982b). The GFD domain has a sequence homology to EGF

(Patthy, 1985) and is involved in the binding of uPA to its receptor, uPAR (Appella *et al.*, 1987) (see Fig 2). The Kringle domain binds to heparin-related cell surface entities (Brunner *et al.*, 1994; Schmitt *et al.*, 1995). Many hormones and cytokines control the expression of uPA in a cell specific way (Laiho & Oja, 1989).

Conversion of the inactive form of uPA to active uPA requires its interaction with its cognate receptor (uPAR). Pro-uPA, active uPA and the amino terminal 135 residue region of uPA (ATF) bind to cell surface receptor uPAR with high affinity with a K_d ranging from 0.05-3 nM, while low molecular weight uPA does not bind to uPAR (Blasi, 1988). The mechanism of pro-uPA activation is not fully elucidated. Once active, uPA activates plasminogen into its active form plasmin which in turn activates other proteases to breakdown and/or remodel the extracellular matrix (ECM).

The conversion of plasminogen into plasmin by uPA is controlled by two inhibitors, termed PAI-1 and PAI-2, which can rapidly inactivate enzymatically active uPA by binding to uPA when present alone or when bound to its receptor, uPAR (Fig 2) (Schmitt *et al.*, 1992; Schmitt *et al.*, 1995). These two inhibitors belong to the serpin family but are the product of different genes.

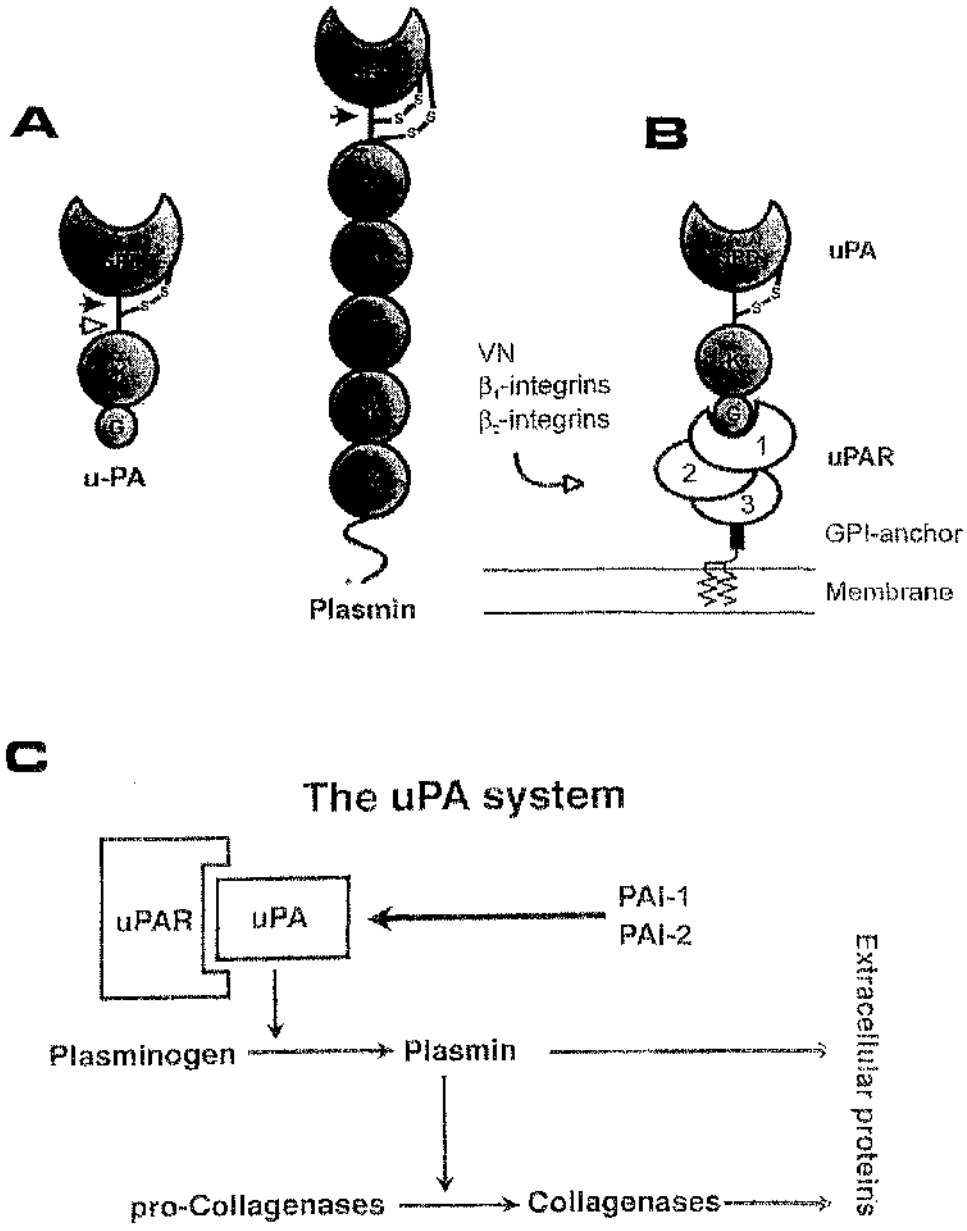


Figure 2. Structure of plasminogen activator components.

(A) The domain structure of pro-u-PA and plasminogen. SPD, serine proteinase domain; K, kringle: G, growth factor domain. Closed arrows indicate the position of the cleavage sites at the conversion of the inactive, 1-chain zymogen forms pro-u-PA and plasminogen to the active, 2-chain proteinase u-PA and plasmin. Open arrow shows the position of the cleavage site at conversion of u-PA to low-m.w. u-PA. Inter-domain disulfide bridges also are indicated.
(B) Domain structure of u-PAR. Numbering of domains is given. A bound u-PA molecule is indicated, as well as the reported interactions of u-PAR with VN and $ß_1$- and $ß_2$-integrins.
(C) Simple schematic diagram of plasminogen activator system.

The figures, A and B, are adapted from Andreasen *et al.*, 1997.

uPA and Carcinoma

The urokinase type plasminogen activator (uPA), is widely distributed in body fluids and tissues but is more abundant in urine (Laiho & Oja, 1989). Several cell lines have now also been found to be rich sources of uPA. For instance, mouse sarcoma virus infected 3T3 cells are an exceptionally rich source of uPA. A strong correlation has been found between uPA secretion and tumour formation and metastasis in nude mice using carcinogens or chemical agents (Sisskin *et al.*, 1980). High levels of uPA were found in malignant epithelial cells of human breast and colon cancer as well as in lewis lung carcinoma (Duffy *et al.*, 1990; Grondahl-Hansen *et al.*, 1991; Janicke *et al.*, 1990; Skriver *et al.*, 1984). uPA and other components of the plasminogen activation system have been demonstrated in adeno-, squamous cell-, and large cell lung carcinoma cell lines (Dosne *et al.*, 1991; Heidtmann *et al.*, 1989; Pappot & Brunner, 1995). It has been demonstrated that tissues of primary cancer and/or metastasis of the breast, ovary, prostate, cervix uteri, bladder, lung, and of the gastrointestinal tract contain high amounts of uPA compared to control tissues (Dano *et al.*, 1985; Schmitt *et al.*, 1990).

Although uPA is predominantly responsible for the conversion of enzymatically inactive plasminogen (Kobayashi *et al.*, 1990), it can also break down a variety of lysine and arginine esters and amides as well as other low molecular weight substrates. Specifically, uPA hydrolyses the arginine-valine bond in plasminogen and this reaction has been shown to be inhibited by various compounds including heavy metals such as Zn.

1.11.2 Tissue Type Plasminogen Activator (tPA)

tPA structurally resembles uPA. It has been found in blood and normal tissues. tPA has been mostly associated with fibrinolytic function, while uPA has been associated with the regulation of extracellular proteolysis. tPA is secreted as an inactive polypeptide and is converted into its active two chain form by digestion with plasmin. Native tPA is a single polypeptide chain with a 70kDa molecular weight. The two chains, A and B, are linked together by disulphide bonds. Similar to uPA, tPA A-chain contains a region of sequence homology with EGF and is responsible for the fibrin binding property (Pennica *et al.*, 1983).

1.11.3 Urokinase-Type Plasminogen Activator Receptor (uPAR)

uPAR, also known as CD87, was first described by (Vassalli *et al.*, 1985) on human monocytes and on the promyelocytic leukemia cell line U937. uPAR is expressed in peripheral blood monocytes, granulocytes and T-lymphocytes (Blasi *et al.*, 1986; Stoppelli *et al.*, 1985).

Human uPAR, encoded by a gene on chromosome 19, is a 313 amino acid, highly glycosylated, long single polypeptide. The molecular weight is reduced to 35kDa after deglycosylation (Behrendt *et al.*, 1991). uPAR essentially contains three homologous domains of 90 amino acids (Behrendt *et al.*, 1991; Roldan *et al.*, 1990). The polypeptide, once synthesised is processed at the C-terminus. uPAR is attached to the plasma membrane covalently via an anchor known as GPI (glycosyl-phosphatidyl inositol) (Ploug *et al.*, 1991). It possesses five potential attachment sites for N-linked carbohydrate and has an unusually high content of cysteine. The glycosylated forms of the protein range in size from 45-60 kDa (Ploug *et al.*, 1991; Behrendt *et al.*, 1991) and this apparent variation in the molecular weights may be due to the extent of N-linked glycosylation.

The two important parameters of uPAR for binding to uPA are (1) the conformation of uPAR (Chucholowski *et al.*, 1992) and (2) glycosylation (Ploug *et al.*, 1991). Both of them are important from a regulatory point of view, since the unfolded, chemically reduced form of uPAR does not block the binding of pro/HMW uPA to cell surface anchored uPAR (i.e., it does not compete). Secondly, a mutant uPAR has decreased affinity for uPA in comparison to wild type uPAR (Schmitt *et al.*, 1995). Any alteration in the five sites of glycosylation of uPAR results in an inactive uPAR. The authors have shown that recombinant uPAR missing the GPI-anchor, blocks uPA binding to native uPAR.

High affinity uPA-uPAR binding sites have been described on the surface of several cultured tumour cells and in membranes of human breast carcinoma (Dano *et al.*, 1994). The complex, consisting of cell bound uPAR, uPA, and PAI-1 is readily internalised by normal and tumour cells. Recently it has been shown that a multiligand α–2 macroglobulin receptor/low density lipoprotein receptor (CD 91) is involved in the binding and endocytosis of the uPAR/uPA/PAI1 complex .

1.12 Regulation of Plasminogen Activators

Regulation of extracellular proteolysis appears to be the result of the concerted action involving various types of malignant cells and host cells. It is assumed that tumour cells and host cells do interact in a co-operative manner e.g. uPA may be produced by adjacent fibroblast like cells or tumour cells and may bind to uPAR, expressed at the invasive foci of the cancer cells or host cells (Stoppelli *et al.*, 1986). PAI-1, present in endothelial cells may bind the uPA/uPAR complex. Several experiments have shown that the inhibition of uPA leads to the inhibition of invasion thus showing the important role of uPA

in cancer invasion. These studies have been performed with *in vitro* system measuring the penetration and degradation of a human amniotic membrane (Mignatti *et al.*, 1986) and extracellular membrane (Cajot *et al.*, 1990; Hollas *et al.*, 1991; Kobayashi *et al.*, 1992). The results in this thesis also show the inhibition of invasion in breast cancer cells, in a Boyden chamber, by using antibodies against uPA. The same results have been demonstrated in experiments with prostate cancer cells by other members in our laboratory. Extensive studies on inhibition of invasion have been performed by Ossowski and co workers. They have found that inhibition of uPA by anti catalytic antibodies inhibits invasion and metastasis of human cancer cells transplanted on the chorioallantoic membrane of chicken embryos (Ossowski & Reich,. 1983; Ossowski, 1988). uPA is selectively expressed at invasive foci in some experimental and human cancers.

1.12.1 Regulation of Plasminogen Activator Synthesis

The activation of plasminogen, under physiological conditions requires uPA at the cell surface and the presence of uPAR. uPA has high affinity for its receptor, uPAR and the dissociation rate is low (Vassalli *et al.*, 1985). The receptor bound uPA provides focused proteolysis and therefore, can be taken as a regulatory target. The receptor bound uPA is not internalised in the absence of PAI-1 (Cubellis *et al.*, 1990), which binds to uPA/uPAR complex (Conese & Blasi, 1995a) and initiates internalisation and then subsequent degradation of uPA by lysosomal enzymes.

The synthesis of each of these components is regulated by a variety of hormones, growth factors and cytokines (Andreasen *et al.*, 1990; Dano *et al.*, 1985). The pattern of regulation varies among different cell types. If, for example, a certain growth factor induces uPA synthesis in one cell type, it

may inhibit it in another and leave it unchanged in most cell types. However, it has been shown that the interaction of growth factor and plasminogen activator is bi-directional, in that growth factors regulate plasminogen activation and are regulated by plasminogen activators. For example, EGF stimulated uPA production in brain astrocytes while IGF stimulated only tPA and PDGF had no effect on PA activities in astrocytes (Tranque *et al.*, 1994). Changes induced by TGFβ in the PA system differ depending on the cell types. TGFβ upregulates uPA in MCF-7 cells (Albo *et al.*, 1997) and induces both uPA and PAI-1 in MDA-MB-231 cells (Arnoletti *et al.*, 1995; Farina *et al.*, 1998) and in the human adenocarcinoma cell line A549 (Keski-Oja *et al.*, 1988). It has been shown by Graham (1997) that TGFβ, as low as 0.1ng/ml increases PAI-1 by 63%, in human trophoblast cells. While Lund *et al.*, (1987) demonstrated that uPA and tPA are decreased by TGFβ in embryonic fibroblasts. On the other hand, growth factors including IGF (Campbell *et al.*, 1992) and TGFβ (Lyons *et al.*, 1990) are regulated by the plasminogen system. For example, PAI-1 is known to induce TGFβ in certain cells. Cell surface bound plasminogen activates latent growth factors such as TGFβ, insulin and interleukins (Schmitt *et al.*, 1995).

Hormones like oestrogens, progesterone and prolactin are also involved in the regulation of the plasminogen activation system (M-y-Lopez & Ossowski, 1987). E_2 has been shown to induce tPA in MCF-7 cells (Ryan *et al.*, 1984). However, it down regulates uPA in S30 cells (a transfectant stably ER-expressing cell line) (Long & Rose, 1996). Ossowski *et al.*, (1979) has suggested a very distinct difference in the response of plasminogen activator synthesis to hormonal modulation between normal and neoplastic cells. The bi-directional regulation and also the differential regulation of plasminogen activation varies among different cell lines under different circumstances. This, therefore, provides a versatile system for regulation of cell surface plasminogen activation that allows regulatory factors to turn on plasminogen

activation on one cell type but not on others. Such a system may form the basis for a strict regulation of plasminogen activation during non malignant physiological processes such as trophoblast invasion, wound healing and prostate involution, while defects in this regulatory system may lead to the apparently uncontrolled plasminogen activation and result in invasive cancer.

Plasminogen activation can be controlled at several levels. Pro-uPA is converted more efficiently when bound to its receptor than when present in soluble form. The activation is 20 times higher (Ellis *et al.*, 1989). In addition to other enzymes, plasmin appears to be the main enzyme involved in the activation of inactive uPA. Plasmin also has cell surface receptors and therefore presents an efficient mechanism of uPA activation. The mechanism of the initiation itself, however, has not been clarified. The overall efficiency of plasminogen activation due to receptor bound plasmin is because receptor bound plasmin,(which has low affinity and high dissociation rate towards its receptor) has some degree of protection from its inhibitors, alpha-2 antiplasmin (Ellis *et al.*, 1990) and other serum inhibitors.

PAI-1 induced internalisation and degradation of receptor bound uPA, signifies another level of regulation (Conese & Blasi, 1995b). It has been shown in a number of studies that the localisation of the components may itself regulate the proteolytic processes. e.g. in HT1080 fibrosarcoma cells PAI-1 and uPA do not co-localise; uPA is found at the cell-cell or cell-substratum sites whereas PAI-1 has been found in the extracellular matrix. Cell migration brings the receptor bound uPA and PAI-1 in contact and therefore cell migration may be thought to regulate surface proteolysis.

If internalisation and degradation of receptor bound uPA by PAI-1 go through an endocytotic step, then migrating cells have the ability to carry out a complete plasminogen activation sequence, starting with the synthesis and secretion of inactive pro-uPA, followed by binding to uPAR, activation,

production of surface plasmin, inhibition by PAI-1 and internalisation and degradation of the receptor bound uPA/PAI-1 complex.

Another type of regulation however, may also be possible. Enzymatic activity is not necessary for binding to uPAR (Schmitt *et al.*, 1992). The amino terminal fragment, with no enzymatic activity can bind to uPAR. The occupation of the receptor with an inactive uPA (ATF) results in the decrease of its actual as well as surface bound activity.

uPAR binds both active and inactive pro-uPA with high affinity (K_d 10^{-9}-10^{-11} M) (Ellis *et al.*, 1991). uPAR is bound to cell surface by a GPI anchor (Ploug *et al.*, 1991) and can be released by its specific enzyme phospholipase C (Blasi *et al.*, 1990). This suggests still another type of regulatory mechanism. Concomitant binding of pro-uPA and plasminogen to cell surface suggests the enhancement of plasmin formation.

1.12.2 Regulation of Plasminogen Activators by Growth Factors and Steroid Hormones

Growth factors including TGFα, TGFβ and EGF (Arnoletti *et al.*, 1995; Tranque *et al.*, 1994) and hormones (Mira-y-Lopez *et al.*, 1983) are not only able to promote cell proliferation but can also modulate urokinase expression in various tumour cells.

Furthermore, a mitogenic effect of urokinase itself was found in certain human tumour cell lines e.g. prostate cell lines derived from hyperplasia or carcinoma (Kichheimer *et al.*, 1987) and the human ovarian cancer cell line OV-MZ-6 (Wilhem *et al.*, 1994). A positive correlation was shown by Vecchio *et al.* (1993) between uPA concentration and rate of proliferation. They have assessed proliferation by immunostaining with monoclonal Ab Ki67. uPA can

also activate latent growth factors directly or through a plasmin mediated mechanism and thus contribute to the proliferative activity.

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 (M-y-Lopez & Ossowski, 1987). EGF increases the plasminogen activator activity in both intracellular and extracellular levels in HeLa cells and also in other cell lines such as cultured human foreskin fibroblasts and the rat kidney cell line NRK-536-3-1. Since TGFα induces its affects via the same receptor as EGF it has been observed that TGFα also stimulates the activity of plasminogen activators.

With the exception of glucocorticoids which suppress uPA activity in a variety of cultured cells and tissues (like primary cultures of involuting murine mammary glands and explants of rat mammary tumours), steroid hormones generally increase plasminogen activator activity. Oestrogens, for instance, increase plasminogen activator activity in both primary culture and in the MCF-7 cell line (Pourreau-Schneider *et al.*, 1989). They also increase the PA activity in mammary tumours induced by chemicals and promote the prolactin stimulation of uPA activity. Progesterone blocks prolactin stimulation of uPA activity. Similarly, anabolic steroids and androgens increase the plasminogen activator activity in different cell lines.

uPA activity is increased by LH, LHRH and HCG in granulosa cells of preovulatory follicles of rat ovaries. It should also be noted that polypeptide hormones and cAMP have been reported to cause parallel changes in intracellular and extracellular levels of uPA by affecting de novo synthesis of uPA by a mechanism dependent on mRNA synthesis.

1.12.3 Regulation, interaction and functions of uPAR

Critical balance of the protease uPA, the inhibitor PAI-1 and cell surface receptor uPAR, are the prerequisites of the efficient tumour cell invasiveness, focal proteolysis and metastasis (Liu *et al.*, 1995). For instance, overexpression of not only uPA and PAI-1 (Schmitt *et al.*, 1997b) but also uPAR in breast cancer cells resulted in increased invasion and metastasis (Xing & Rabbani, 1996). In addition to PAI-1, uPAR is another major binding protein to the vitronectin rich extracellular matrix (Kanse *et al.*, 1996). uPAR even may influence cell adhesion and direct migration of adherent cells by regulating integrin functions of the cells (Felsenfeld *et al.*, 1996; Wei *et al.*, 1996).

It had been shown earlier that uPAR does not internalise uPA (Vassalli *et al.*, 1985). However, uPAR is responsible for mediating internalisation of uPA/PAI complexes (Cubellis *et al.*, 1990; Olson *et al.*, 1992). Both uPA and pro-uPA binds with the same affinity to uPAR with a K_d of 0.1-2 nM (Appella *et al.*, 1987). In uPA, the receptor binding site is located in the amino terminal fragment (ATF) of the growth factor domain (GFD). LMW uPA that lacks GFD does not bind to uPAR (Schmitt *et al.*, 1995). The ligand (uPA) binding domain in uPAR is located within the first 90 amino acids, of the N-terminus (Behrendt *et al.*, 1991). uPAR is specific for uPA and does not bind tPA, plasminogen or EGF (Blasi *et al.*, 1986). Ossowski showed that mere production of high levels of uPA in the absence of uPAR will not provide a tumour cell with maximal invasive potential (Ossowski, 1988). Vecchio and colleagues strongly support the idea that uPAR plays a central role in invasive phenotypes in breast cancer patients and therefore suggests its potential use as a prognostic marker in such patients (Vecchio *et al.*, 1993).

1.13 Plasminogen Activator Inhibitors (PAI)

There are several classes of protease inhibitors in the plasma but the predominant class of inhibitors is referred as SERPINS (serine protease inhibitors), including PAI-1, PAI-2 and PN-1. The serpin family is diverse. This class includes inhibitors of the serine proteases involved in physiological and pathophysiological processes.

The main function of these inhibitors is to negatively regulate proteolytic events associated with numerous biochemical pathways (Potempa *et al.*, 1994). The distinct protease inhibitors specific for plasminogen activators are endothelial type PAI (PAI-1) (Kruithof *et al.*, 1987), the placental type PAI (PAI-2) and protease nexin (PN-1). These are different in immunological and physiological characteristics (Springer & Kluft, 1987) but all have arginine in their reactive centre (Hill & Hastie, 1987).

1.13.1 Plasminogen Activator Inhibitor Type-1 (PAI-1)

PAI-1, mainly produced by endothelial cells (Springer & Kluft, 1987), is present in high concentration in plasma. PAI-1 is also produced by a number of other cell types including platelets (Erickson *et al.*, 1984), normal rat kidney cells (NRK) (Ryan *et al.*, 1996) and different tumour cells (Andreasen *et al.*, 1990). The expression of PAI-1 is differentially regulated in different human cancer cell lines by inhibitors of protein synthesis (Lund, 1996).

PAI-1 is a secreted protein. Amino acid sequencing and cDNA nucleotide sequencing have revealed the structure of human PAI-1 as a 381 amino acid long, single chain glycoprotein, with a signal peptide of 21-23 amino acids. PAI-1 has a 50kDa molecular weight (Andreasen *et al.*, 1986). The authors have shown that PAI-1 has arginine in its reactive centre towards the carboxy-

terminus of the protein. Human PAI-1 efficiently inhibits tPA and uPA but not pro-uPA (Andreasen *et al.*, 1990) and is present across all components of the tumour. PAI-1 reacts with thrombin, albeit slowly, until some cofactor like heparin is added to the system.

PAI-1 is secreted as an active protease inhibitor but is spontaneously converted into its inactive form. The half life of active PAI-1 is about 2 hours in culture medium (Levin & Santell, 1987). The inactivation may be because of conformational changes that hide the reactive centre of the protein (Springer & Kluft, 1987). The inactive stable form is termed latent. The latent form is stable because the inhibitory activity can be restored by denaturation or renaturation (Hekman & Loskutoff, 1985). Recently, Mottonen and associates have shown the crystal structure of the latent form. The authors have shown that vitronectin (Vitronectin is serum spreading protein or S protein and is a 75kDa adhesive glycoprotein found in plasma and in connective tissues-it binds strongly to glass and plastic surfaces and promotes the spreading of a variety of cell types on cultures dishes) maintains the active conformation of PAI-1, once it is bound to this ECM protein, and can inhibit uPA mediated proteolysis (Mottonen *et al.*, 1992). PAI-1 is heterogenously distributed in the tumour. This was shown by immunocytochemical studies. Fukao and associates, using an immunohistochemical technique, have shown that PAI-1 is mostly present in the carcinoma itself and not in the normal mucosa in stomach and colorectal tissues (Fukao *et al.*, 1991). Whereas Cajot and others have shown even distribution of PAI-1 in ECM and also its localisation not only in microgranular and fibrillar material but also as lining of the cell membrane, plasma membrane protrusions and surface pits (Cajot *et al.*, 1990).

Molecules that affect the synthesis and secretion of PAs, also affect PA-inhibitors. For example, the secretion of PAI-1 is stimulated by TGFβ1 treated

cells, whereas uPA and tPA are decreased (Lund *et al.*, 1987). PAI-1 is decreased by gonadotrophins in granulosa cells (Ny *et al.*, 1985).

1.13.2 Plasminogen Activator Inhibitor Type-2 (PAI-2)

PAI-2 is produced mainly by placenta and macrophages (Kruithof *et al.*, 1987). PAI-2 is a 60kDa glycosylated protein with 393 amino acids. PAI-2, also belongs to the SERPIN family. It binds uPA specifically but less efficiently than PAI-1 (Conese & Blasi, 1995a). PAI-1 and PAI-2 are immunologically different, their genes are located on different chromosomes and are expressed differentially in individual cell types.

PAI-2 was first purified from placenta extracts but is also released by tumour cells and phagocytic cells. Higher concentrations of PAI-2 have been observed in the plasma of pregnant women during the 3rd trimester of pregnancy suggesting a role of PAI-2 in the control of plasminogen activation by uPA during parturition (Springer & Kluft, 1987). The clearance of PA and inhibitory activity takes place primarily in the liver (Emeis, 1985).

1.13.3 Protease Nexin-1 (PN-1)

A third PA inhibitor is protease-nexin-1 (PN-1). It is a 43kDa protein and is present in human fibroblasts and placental cells (Baker *et al.*, 1980) and in murine kidney (Moll *et al.*, 1996). In contrast to the PA-specific inhibitors, PN-1 also inhibits plasmin , thrombin , and other trypsin like serine proteases (Scott *et al.*, 1985).

It has been shown recently by Conese and his colleagues that PN-1 binds uPA and this complex follows the same pattern of internalisation and degradation as uPA-PAI-1 complexes. (Conese *et al.*, 1994). Both complexes involves α_2-MR, in addition to uPAR (Hertz *et al.*, 1992). Therefore its role in the control of uPA mediated plasminogen activation cannot be ruled out. PN-1 has an important function in reproduction as indicated by its presence in the mouse seminal vesicle (Vassalli *et al.*, 1993) and human placenta (White *et al.*, 1993).

1.14 Regulation of PAI-1 and PAI-2

Both PAI-1 and PAI-2 react rapidly and specifically with both tPA and uPA but not with pro-uPA (Kruithof *et al.*, 1987). PA-inhibitors form stable covalent complexes with uPA and tPA in equimolar ratios (1:1) (Kobayashi *et al.*, 1994). The complex formation between uPA and its inhibitor is formed between the reactive sites of the respective proteins. It has been shown by many authors that receptor bound uPA is available for efficient inhibition by PAIs (Cubellis *et al.*, 1989; Ellis *et al.*, 1990; Pyke *et al.*, 1991).

1.14.1 Regulation of PAI-1 and PAI-2 in Cell Cultures

The inhibitors of plasminogen activators are modulated in a similar fashion to the plasminogen activators themselves. The effects of TGFβ on cell proliferation and urokinase plasminogen activator system have been studied extensively on both normal and cancer cells and tissues. TGFβ is known to regulate the PA system both at the transcription and translational levels. Bourhis and colleagues have shown recently that TGFβ1 and sodium butyrate differentially modulate components of the PA system. e.g. TGFβ inhibited

proliferation and uPA activity but increased PAI-1 levels in normal human breast epithelial cells (HBECs) whereas, an increased uPA activity and PAI-1 antigen levels were observed in response to TGFβ in MDA-MB-231 cells (Bourhis *et al.*, 1998). Sodium butyrate showed no effect on PAI-1 levels. A close correlation of PAI-1 and TGFβ mRNA levels has been associated with perturbance in PA/PAI-1 balance thereby resulting in glomerulosclerosis (Moll *et al.*, 1995). PAI-1 gene expression is regulated by both serum and the nature of the substratum. This has been observed in normal rat kidney cells (NRK) by Ryan *et al.* They have shown an increase in PAI-1 expression by serum both, in agarose suspension, and in monolayer cultures (Ryan *et al.*, 1996). TGFβ induces increased PAI-1 activity and antigen in medium conditioned by the different cell lines e.g. human lung fibroblast cell lines WI-38 and Hel 229, in HT 1080 and in the human epidermoid cell line A431 etc.

Autocrine activity of growth factors may be involved in the regulation of the PA system e.g. basic fibroblast growth factor (bFGF) is known to induce PAI-1 mRNA in bovine endothelial cells (Pepper *et al.*, 1992). EGF does not only increase PAI-1 protein level but also increased the mRNA levels in prostate cancer cell lines. This has been shown recently by a work in our laboratory. Insulin also induced PAI-1 levels.

A time dependent increase in the components of plasminogen activators was observed by Lund and colleagues, after treating mouse skin with phorbol esters, such as phorbol 12-myristate 13-acetate (PMA), which are tumour promoters and activators of protein kinase C. PAI-1 mRNA could be detected after 24 hours of PMA treatment (Lund *et al.*, 1996). PMA also increased PAI-2 protein in U937 cells due to an increase in PAI-2 mRNA and gene transcription rate (Kruithof *et al.*, 1987).

1.15 Relation of Tumour Invasion and Metastasis to Normal Processes

Tumour invasion and metastasis are the major causes of treatment failure for cancer patients. Metastases do not occur randomly and/or accidentally but it is an active process of tumour invasion, which involves a complex phenomenon of responses by both cancerous and normal cells.

The complex series of host-tumour interactions involves invasion of the healthy tissues, by penetrating the ECM at multiple steps in the metastatic cascade by cyclic repetition of 3 biochemical steps: 1) Tumour cell attached to the basement membrane or ECM. 2) Breakdown of the basement membrane or ECM by the proteolytic enzymes. 3) Increased motility of the tumour cells to the distant sites with the help of the tumour associated proteases.

uPA is the key enzyme required for the invasiveness of cells, both in cancer and inflammation and for the normal physiological processes including mammary gland involution (Duffy, 1987), embryogenesis, tissue remodelling, wound healing (Del-Rosso *et al.*, 1990), angiogenesis (Sato & Rifkin, 1989), trophoblast implantation (Saksela, 1985) and gametogenesis (Sappino *et al.*, 1989).

The biochemical and molecular biological steps in physiologically invasive processes and cancer cell invasion and metastasis are qualitatively identical, e.g. during both mammary gland involution and cancer invasion, basement membranes are destroyed. Both require uPA dependent proteolysis for plasmin generation and thereby activation of a cascade of events leading to the degradation of extracellular matrix. Thus, if proteases i.e. plasminogen activator/plasmin system play a role in normal destructive processes they may also be involved in pathological destructive processes such as cancer invasion (Duffy, 1987).

Proteolysis in normal tissue occurs for only a limited duration and is strictly regulated at many levels. e.g. trophoblast cells, like malignant tumour cells, are highly invasive which is necessary for implantation and penetration of the maternal endometrial stroma and blood vessels. However, the invasion of uterus is under strict control, in contrast to tumour cells.

In malignant neoplasms the imbalance of proteolysis favours unchecked invasion and metastasis. The difference between normal and cancer invasion is that proteolysis in tumour cells couples with motility of cells to the places and at times which is inappropriate for normal cells (Liotta *et al.*, 1991). More importantly, there is a very large difference in the amounts of uPA synthesis, its inhibitor and receptor that are secreted. A large number of studies have demonstrated the increased levels of uPA, PAI-1 and uPAR in cancer invasion and metastasis. High levels of uPA in cancer made it a significant prognostic indicator in many of the cancers e.g. breast, colon, ovary, prostate, oesophagus, and lung etc. uPA is a marker of poor prognosis. Extensive studies on trophoblast implantation have been performed to compare the invasive trophoblast and tumour cell. The intrinsic invasiveness was determined (Kirby, 1965) by using mouse blastocyst and showed that *In vivo* invasion was greater in non-pregnant uteri than pregnant uteri. Mignatti *et al..*, (1986) also demonstrated the *in vitro* invasiveness of the trophoblast using the human trophoblast cultures in *in vitro* invasion assays. These and other studies showed that trophoblast invasion is under strict control which limits its invasion beyond certain required point. Some early studies by (Kirby, 1965; Mignatti *et al.*, 1986; Yagel *et al.*, 1989) have shown that invasion during pregnancy is controlled by decidual tissues of the uterus, to limit implantation. It was later demonstrated by Graham, (1991 and 1992) that decidua derived factors play a role in the *in vivo* control of trophoblast invasion.

One of the molecules thought to regulate the trophoblast invasion is TGFβ (Lala & Graham, 1990). It has been shown that TGFβ limits implantation by inducing differentiation of the human cytotrophoblast cells into non invasive syncytiotrophoblasts (Graham & Lala, 1992). The exact mechanism of TGFβ action in not clear but it is known that TGFβ induces the expression of TIMPs (Laiho & Oja, 1989) and also PAI-1 (Lund *et al.*, 1987). Furthermore, Graham, (1991 and 1992) confirmed that the trophoblast invasiveness is increased by using antibodies against TGFβ. It may be because the presence of this antibody decreases the level of TIMP, at both protein and message levels. TGFβ also up regulates integrin expression and reduces the migratory ability of the invasive trophoblast. This was confirmed by using antibodies against integrins where stimulated migration was observed (Irving & Lala, 1995).

The interesting feature of the regulatory pathway is the activation of latent TGFβ by plasmin (Flaumenhaft *et al.*, 1992). uPA activates plasminogen into plasmin, which then activates latent TGFβ in the extracellular matrix. TGFβ increases the inhibitor levels and therefore, in the presence of increased inhibitor expression, the invasion process is terminated (Strickland & Richards, 1992). Thus proteases that are normally involved in tumour invasion are also responsible for trophoblast implantation (Testa & Quigley, 1990). Proteases are found at highest levels in the invasive front, where degradation of the normal tissue occurs, both during normal and invasive processes. If TGFβ controls the trophoblast invasion, it has the potential to be used as an inhibitor of cancer invasion and metastasis, since metastasis is the cause of mortality.

1.16 Plasminogen Activator Components as Prognostic Markers in Cancers?

The importance of plasminogen activator components, uPA and PAI-1, and recently uPAR, has been strongly suggested in the process of invasion and metastasis. Their involvement in cancer metastasis has been proved by results from experimental model systems with animal tumour metastasis. Furthermore, results from the studies on many human cancers have shown that high levels of uPA, PAI-1 and uPAR predict poor patient prognosis.

Considerable evidence indicates that uPA is a strong and independent prognostic marker in breast cancer (Duffy *et al.*, 1996; Duffy *et al.*, 1990; Ferno *et al.*, 1996; Hildenbrand *et al.*, 1998; Holst-Hansen *et al.*, 1996; Janicke *et al.*, 1991; Janicke *et al.*, 1993; Schmitt *et al.*, 1997a; Schmitt *et al.*, 1990). Clinical studies have suggested that expression of the uPA system directly influences malignant behaviour. For example, Foekens and associates have shown that the level of uPA in tumour cytosols have been correlated with metastatic disease and shortened survival among breast cancer patients (Foekens *et al.*, 1992). Further studies on the major subgroups of patients with breast cancer (ER^+, Positive axillary nodes, women younger or older than 50 years of age) have shown that uPA was an indicator of prognosis in all sub-groups. In all sub-grouping in node negative breast cancer patients, uPA has been suggested to be a more significant prognostic marker than tumour size or oestrogen receptor status (Duffy *et al.*, 1998; Duffy *et al.*, 1994). High uPA antigen levels were shown to be a stronger prognostic marker than uPA activity in breast cancer (Reilly *et al.*, 1991). It has been demonstrated by Janicke and colleagues that uPA is 12 times higher in cancer tissues extracts than in the extracts of normal breast tissue. The same group has further shown that patients whose tumours have levels of uPA as high as 3.49 ng/mg protein had a statistically significant high relapse rate and shorter life expectancy

(Janicke *et al.*, 1991). Many groups have shown that established markers of poor progression in breast cancer (lymph node involvement and negative hormone receptor status etc) were associated with higher uPA content. The impact of uPA on prognosis is even stronger than hormone receptor and lymph node status (relative risk 21.1> 5.8> 3.0), respectively (Schmitt *et al.*, 1990). There is a significant correlation between uPA and PAI-1 and tumour associated angiogenesis (Hildenbrand *et al.*, 1995a; Hildenbrand *et al.*, 1995b).

uPA has been suggested as a marker of metastasis in colorectal cancer (Mulcahy *et al.*, 1994). High uPA antigen levels were correlated with poor overall survival in patients with colorectal cancer (Schmitt *et al.*, 1992). Elevated uPA levels were found to be associated with poor prognosis in gastric cancer and therefore has been suggested as a new prognostic factor in gastric cancer predicting shorter survival in clinically important subgroups of patients. They have further shown that uPA levels in tumour tissue extracts significantly correlated with vascular invasion (Cho *et al.*, 1997; Nekarda *et al.*, 1994). A 4-fold elevated uPA content, in addition to increase in other fibrinolytic components, was observed in metastatic ovarian cancer. These elevated levels at the site of metastases may contribute to the aggressive potential of cancer cells. Therefore uPA can be used as a prognostic marker in selecting more aggressive cancer (Schmalfeldt *et al.*, 1995). Furthermore, high uPA levels, as measured by immunohistochemistry have been reported to be associated with poor prognosis in pulmonary adenocarcinoma (Oka *et al.*, 1991). The adenocarcinomas constitute about one fourth of all lung cancers. The prognostic value of uPA has also been determined in other types of lung carcinomas (Pedersen *et al.*, 1994a).

Not only uPA but elevated levels of PAI-1 has been associated with poor prognosis of patients in many cancer types. A plethora of data has shown that

PAI-1 is an independent and significant prognostic marker in breast and other cancer types. PAI-1 was found to be an independent prognostic variable predicting short overall survival with a relative risk of 2.27 in node positive cancer patients (Grondahl-Hansen *et al.*, 1997b). In another study Grondahl-Hansen and associates have shown that PAI-1 is a much stronger prognostic factor than uPA and uPAR in breast carcinoma (Grondahl-Hansen breast *et al.*, 1997a). Janicke and associates have found an increased risk of relapse and death in patients with PAI-1 levels greater than 2.18 ng/mg protein and uPA levels >2.97 ng/mg protein (Janicke *et al.*, 1993). The same group have found that in node negative patients, PAI-1 follows the impact of uPA. In a time dependent risk profile of prognostic markers Schmitt and associates have shown that the prognostic significance of uPA and oestrogen receptor status has been decreased whereas PAI-1 prognostic impact is increased with time and become similar in prognostic strength to the lymph node status (Schmitt *et al.*, 1997b). Elevated amounts of PAI-1 in patients with primary tumour has a significant correlation with high relapse rate (Schmitt *et al.*, 1992). This high risk of relapse in breast cancer patients with high PAI-1 antigen is confounding and contradictory to what one would expect given the biochemical role of PAI-1, which has been known to serve as a local attenuator of uPA mediated proteolysis (Bacharach *et al.*, 1992). Cajot and associates in their early studies on the role of PAI-1 in the degradation of the extracellular matrix by fibrosarcoma and colon carcinoma cells have shown that PAI-1 inhibited the breakdown of the extracellular matrix whereas, monoclonal antibodies to PAI-1 increased the degradation of the matrix (Cajot *et al.*, 1990). This clearly implies that PAI-1 acts as an inhibitor of uPA mediated proteolysis but the enormous amount of data regarding elevated levels of PAI-1 and poor prognosis indicate that PAI-1 protects the tumour from autoproteolysis and thereby helps in reimplantation of tumour cells at a site distant from its primary site (Schmitt *et al.*, 1992). It has recently been

shown that PAI-1 may promote tumour progression, by (a) promoting the intra- or perivascular tumour cell development, or (b) by either disrupting uPAR-integrin complex or by interfering with integrins, or both, and thereby causing tumour cell migration (Chapman, 1997). Kanse and colleagues have also observed that PAI-1 can modulate the uPAR binding to vitronectin, again enabling tumour cells to migrate (Kanse *et al.*, 1996). PAI-1 may also conserve tumour tissue integrity and its involvement in promoting cancer progression and metastasis formation.

A significantly higher level of PAI-1 (5.08 ng/mg) was found in the higher stages (G2/G3) as compared to PAI-1 level (2.19ng/mg) in lower (G1) stage of the endometrial carcinoma (Kohler *et al.*, 1997) but longer observation time has been proposed by the authors to use uPA and PAI-1 as prognostic markers. It has been shown recently in clinical studies of various lung cancer cells that PAI-1 expression in tumour tissues is an adverse prognostic feature. In assays for invasiveness, a cell line with no PAI-1 antigen and expressing only uPA and uPAR displayed no invasive capability despite levels of secreted uPA more than 20-fold higher than other cell lines. So, they concluded that both uPA and PAI-1 are necessary for optimum invasiveness of lung cancer cells (Liu *et al.*, 1995). In another study on gastric cancer, it has been demonstrated that PAI-1 showed a significant correlation with advanced lymph node involvement which was associated with decreased survival. The relative risks of failure were 2.9 and 5.0-fold for PAI-1 and lymph node involvement, respectively.

It has been shown that, unlike PAI-1, low levels of PAI-2 had a worse prognosis in breast cancer patients than those with elevated levels and therefore high levels of PAI-2 has been suggested as a marker of good prognosis in breast cancer (Duggan *et al.*, 1997; Foekens *et al.*, 1995). PAI-2 mRNA was found in cancer cells. Low levels of PAI-2 in breast cancer is

correlated with shorter disease-free survival in post menopausal women and women without lymph node involvement (Bouchet *et al.*, 1994).

uPAR has recently been implicated as a possible prognostic marker in breast cancer as well as other types of cancer. The potential prognostic value of uPAR in breast cancer tissues was determined by ELISA and significant correlations were found between the levels of uPAR relative to the uPA and PAI-1 levels (Ronne *et al.*, 1995). In another study, a significant correlation between elevated levels of uPAR and poor prognosis has been demonstrated in breast cancer (Grondahl-Hansen *et al.*, 1995). This group has shown that when the clinically relevant subgroups were analysed separately, the prognostic value of uPAR was particularly strong in node-positive post menopausal women with considerable shorter overall survival. The multivariate analysis including other established factors such as uPA, PAI-1 and ER status, however showed that PAI-1 was the only independent variable for both relapse free and overall survival.

AIMS AND OBJECTIVES

The plasminogen-plasmin system represents a complex cascade of proteolytic enzymes which, together with other enzyme systems such as collagenases and other metalloproteases, is involved in the degradation of extracellular matrix (Blasi *et al.*, 1987; Dano *et al.*, 1985; Liotta & Stetler-Stevenson, 1991; Mignatti & Rifkin, 1993). Urokinase type of plasminogen activator (uPA) is the key enzyme in this system in that it activates plasminogen into a broad trypsin like substrate, plasmin. Proteolytic activity of uPA is potentiated and localised by binding to its receptor on cell surface (Chucholowski *et al.*, 1992; Ellis & Dano, 1991; Schmitt *et al.*, 1997b). The net uPA activity is determined not only by the amount of enzyme but also by the specific inhibitor, such as plasminogen activator inhibitor type 1 (PAI-1).

Breast cancer cell proliferation, invasion and metastasis is under the control of multiple factors including steroid hormones and growth factors (Ossowski *et al.*, 1979; Springer & Kluft, 1987). However less is known about the exact role of hormones and growth factors in modulating the invasiveness and progression of metastasis of breast cancer. Many studies showed earlier the morphological changes of cells grown on different substrata, e.g. plastic, collagen, fibrinogen etc., but it was only recently that EHS was used to study the morphological changes of the cells. There were no data available, at least for breast cancer cell lines, to determine the effect of EHS on the regulation of cellular function.

In the light of the foregoing, the aim of the study was to :

Examine the effects of several hormones and anti-hormones including oestrogen, progesterone and tamoxifen, together with different growth factors like TGFβ1, EGF and TGFα on the components of the uPA system in relation to invasive potential.

Specific questions were:

1) Were there any significant differences between the regulation of secretion of members of the urokinase family when cells were grown on either plastic or EHS (Extracellular matrix).

2) To what extent was modification of uPA secretion reflected in change in invasive potential?

3) Was the regulation of these potential metastatic enzymes at transcriptional and/or translational levels?

4) Did oestrogen alter sensitivity of the cells to exogenous factors?

5) How did PAI-1 levels relate to uPA levels relative to invasive potential?

CHAPTER 2

MATERIALS AND METHODS

2.1 Materials

2.1.1 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

2.1.2. Tissue Culture Medium:

Gibco/BRL	RPMI-1640 medium with 25mM HEPES	Paisley, UK
	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.	

2.1.3 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)
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
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	17-β Oestradiol
Poole, Dorset, U.K.	Progesterone
	MTT
	Plasminogen
	BSA

	Tamoxifen
	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

Nijmegen, Netherland	Sheep anti-chicken IgG
	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. Collin Wilde	EHS
Hannah Research Institute,	
Ayr Scotland	

2.1.4 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

2.1.5 Equipment:

Progene	PCR amplification machines
Cambridge, U.K.	
Labsysytems Multiscan	MCC/340 ELISA plate reader
UK	
LKB Biocrom ltd	Ultraspectrophotometer 4050
UK	
Hitachi-Perkin Elmer	MPF-2A fluorescent spectrophotometer
UK	
Bio-Rad	MRC 600 Confocal illumination unit attached to a Nikon Diaphot inverted microscope

2.2 General and Tissue Culture Solutions

Phosphate Buffered Saline (PBS), pH 7.2

170 mM NaCl

3.4 mM KCl

10 mM Na_2HPO_4

2 mM KH_2PO_4

Adjust to pH 7.2 with H_2SO_4

HE buffer

20 mM HEPES; pH 7.4

1.5 mM EDTA

10% glycerol

ETN Buffer

10 mM Tris-HCl; pH 7.0

10 mM EDTA

100 mM NaCl

Hank's Modified Buffer (pH 7.2)

1.3 mM $CaCl_2$

5.4 mM KCl

0.5 mM $MgCl_2$

0.5 mM $MgSO_4$

1.37 mM NaCl

4 mM Na_2HCO_3

0.4 mM $Na_2HPO_4.2H_2O$.

Tris Buffer (pH 8.5)

10 mM Tris-HCl

50 mM NaCl

Glycine Buffer (pH 10.5)

0.1 M glycine

0.1 M NaCl

Triton X-100 Solution

1% (v/v) Triton X-100 in Tris-buffer

Versene

125 mM NaCl

2.7 mM KCl

6.3 mM Na_2HPO_4

3.2 mM KH_2PO_4

0.5 mM EDTA

0.0015% (w/v) phenol red

Trypsin-Versene

40 mls Versene

10 mls 0.25% (w/v) trypsin

Cell Freezing Medium

90% (v/v) FCS

10% (v/v) dimethyl sulphoxide

Dextran Coated Charcoal

0.5% (w/v) sieved, prewashed charcoal 0.005% (w/v) dextran; suspended in HE buffer and stirred continuously for 30 minutes at room temperature.

Dialysed Heat-Inactivated Dextran-Coated Charcoal Stripped Fetal Calf Serum (DHIDCCFCS)

100 ml of FCS was dialysed against four 1 litre changes of Hank's modified buffer over 48 hours at 4°C. The serum was then heat inactivated by incubating at 56°C for 45 minutes. The serum was then cooled and a pellet of dextran coated charcoal (derived from 12.5ml of solution. See 2.2.7) was added and this mixture was stirred at 4°C for 30 minutes before centrifuging at 10,000 rpm for 30 minutes at 4°C to pellet the charcoal. Subsequently, the supernatant was filter sterilised through a 0.2 mm filter.

Experimental Medium (MCF-7 and MDA-MB-231 Cells)

RPMI-1640 with L-glutamate, without phenol red and sodium bicarbonate. This was prepared from powdered stock with the addition of 23.8 mM sodium bicarbonate and 25 mM HEPES. pH was adjusted to 7.2-7.4

Routine Sub-Culture Medium for MCF-7 and MDA-MB-231 Cells.

RPMI-1640 medium supplemented with L-glutamine and 25 mM HEPES; pH 7.4, 10% fetal calf serum. This was further supplemented if required with penicillin (100 units/ml) and streptomycin (50 mg/ml) .

Solutions for SDS-PAGE Electrophoresis

Protein Sample Buffer (2X)

60 mM Tris-HCl; pH 6.8

2% (w/v) SDS

10% glycerol

100 mM dithiothretol

0.05% (w/v) bromophenol blue

Electrophoresis Running Buffer

25 mM Tris-HCl; pH 8.3

192 mM glycine

0.1% (w/v) SDS

Coomassie Blue Stain

0.25% (w/v) Coomassie Brilliant Blue R-250

40% (v/v) methanol

10% (v/v) glacial acetic acid

Lysis Buffer

1% Triton-X100 in Tris buffered saline (TBS); pH 8.5

Solutions and Buffers for ELISA

Coating Buffer pH 9.6

15 mM Na_2CO_3

35 mM $NaHCO_3$

Phosphate Buffered Saline (PBS); pH 7.4

14 mM NaCl

2.7 mM KCl

1.5 mM KH_2PO_4

8.1mM Na_2HPO_4

Blocking Buffer

1% BSA in PBS

Washing Buffer

0.1% Tween-20 in PBS

Dilution Buffer

1% BSA in washing buffer

Colour Buffer; pH 5.0

76 mM $NaH_2PO_4.H_2O$

35 mM citric acid

Substrate Solution

4 mg OPD + 10 µl 30% H_2O_2 in 11 ml colour buffer

Antibodies for ELISA

Coating Ab

Sheep anti chicken; SO2; # 91/99

Catching Ab

Chicken anti uPA; chrm # 93/100

Chicken anti PAI-; chrm # 94/101

Chicken anti tPA; chrm # 95/102

Tagging Ab

Rabbit anti uPA; chrm # 96/103

Rabbit anti PAI-1; chrm # 97/104

Rabbit anti tPA; chrm 98/105

Detecting Ab

Goat anti Rabbit/Peroxidase conjugate;

2.3 Cell Culture Methods

2.3.1 Cell Lines

Two breast cancer cell lines, namely MCF-7 and MDA-MB-231, used for these studies were obtained from the European Collection of Animal Cell Cultures (Porton Down, Sailsbury, UK).

The MCF-7 cell line was derived from a pleural effusion from a 69 year old woman. The line has been characterised as being oestrogen receptor positive and so is responsive to steroid hormones. It exhibits some of the characteristics of differentiated mammary epithelium including oestrogen metabolism.

The MDA-MB-231 cell line was also generated from a pleural effusion and is oestrogen receptor negative and therefore is not responsive to steroid hormones.

2.3.2 Maintenance of Cell Lines

Subculture Techniques

All cells were routinely grown in a 37°C incubator with atmospheric air enriched with 5% CO_2. Experimental plates and dishes were grown in a humid environment to prevent media evaporation. Flasks of various surface areas were used depending on the mass of the cells required: 25cm^2 containing 5ml of medium, 75cm^2 containing 10 ml of medium and 150cm^2 containing 25ml of medium. Fresh culture media were generally renewed every 48 hours. All manipulations were performed aseptically within the confines of a clean laminar airflow.

All the solutions were prewarmed to 37°C prior to use. Cells were passaged when the surface area was about 80-90% confluent. The medium was decanted aseptically and the cellular monolayer washed twice with approximately 5 mls of sterile PBS. Subsequently, 0.05% trypsin, prepared in a Versene solution, was added to each flask (1ml for a 25cm^2 flask; 3ml for a 75cm^2 flask, and 5ml for a 150cm^2 flask). The flasks were incubated at 37°C for 2-3 minutes, then checked under the microscope to make sure that the cells were rounded. This was followed by removing most of the trypsin-Versene and the flasks incubated for a further 2-3 minutes to allow the cells to detach completely. Trypsin was neutralised by adding fresh culture medium (2-3 volumes) and the cells evenly dispersed by rapid pipetting. This suspension was then dispensed into new culture flasks containing appropriate amounts of fresh growth medium. For routine subculturing the cells were split in a ratio of 1:3. To plate cells for experiments the above procedure was modified slightly as follows. The cells were trypsinised and resuspended as described previously and counted using a haemocytometer. After making appropriate dilutions to obtain the desired cell density the cell suspension was plated on different experimental plates. For instance, 2 ml of the required dilution was plated in 6-well plate, 1ml per well for a 24-well plate and 200µl per well for a 96-well plate.

2.3.3 Mycoplasma Testing

Cell lines were routinely screened for the presence of contaminating mycoplasma because it causes serious intracellular infection which results in aberrant cell growth. In order to screen for this infection, which cannot be detected with the naked eye, the cells were fixed and stained with a fluorescent dye. This involved growing cells on a sterile coverslip or a glass slide under

standard tissue culture conditions. The cells were allowed to reach approximately 50-60% confluence and the medium was aspirated and the cells washed with three changes of PBS. The cells were then fixed with freshly prepared Carnitov's fixative (75% methanol; 25% glacial acetic acid for 5 minutes). After air drying, the cells were stained with a 10ng/ml solution of Hoechst 33258 and left for 15-20 minutes at room temperature. After washing the cells thrice with PBS, the cover slips or slides were inverted onto a drop of mounting fluid (90% glycerol; 10% PBS). The cells were viewed under a fluorescent microscope for visual evidence of any infection. Presence of mycoplasma was indicated by the presence of extranuclear DNA which stained with the dye. Any contaminated cell line was destroyed and new stocks from the negative test date were retrieved from the frozen stock or new stocks were ordered from the European Animal Cell Culture Collection.

2.3.4 Cryopreservation of Cell Lines

Frozen stocks of cells were prepared while the cell line was still at the early passage number. This meant that all the cells used in experiments were of a similar passage number. To keep the stock of same passage number going new stocks were grown every few months from the frozen stocks. In order to prepare frozen stocks, the cells were grown in 150cm^2 flasks in RPMI-1640 (routine culture medium) supplemented with 10% FCS until they were approximately 80-90% confluent. Cells were trypsinised and resuspended as described previously, and the cell suspension was transferred to a sterile Universal container and centrifuged for 3-4 minutes at 500rpm. The supernatant was aspirated and the pellet resuspended in a freshly prepared solution of 10% DMSO in FCS. Aliquots of 1ml were then transferred to sterile Cryo tubes. These were initially slowly frozen at -70^{o}C in a polystyrene box overnight but once frozen were transferred to liquid nitrogen vats.

2.3.5 Rejuvenation of Frozen Cells

To rejuvenate cells, the frozen vials were removed from the liquid nitrogen and thawed rapidly in a waterbath at 37°C. The vials were removed allowing few ice crystals to remain in order to minimise the cytotoxic action of DMSO. The vials were sterilised by spraying then with some 70% ethanol and the semi-frozen cell suspension was transferred to a Universal container to which 10ml routine medium had been added. This was centrifuged for 5 minutes at 500rpm. The supernatant was discarded and the pellet resuspended in approximately 10 ml of prewarmed medium. Subsequently, the cells were transferred to a new tissue culture flask ($25cm^2$ or $75cm^2$) containing medium. The flasks were then incubated in a 37°C incubator. The medium was replaced with fresh medium after 24 hours.

2.3.6 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.

2.4 Colorimetric Assays for Quantitation of Cell Number

2.4.1 MTT Assay

Cellular proliferation and viability was determined by MTT (3-(4, 5-dimethylthiazol-2-yl)-2,5-diphenyltetrazoliumbromide) which is a yellow water soluble tetrazolium salt which is reduced by the hydrogenase enzymes in viable cells into an insoluble purple formazan product. This was dissolved in dimethylsulphoxide and the absorbance read at 540nm. The assay was carried out by subculturing cells followed by the addition of 50µl of MTT (5mg/ml in PBS) into each well. The plates were wrapped in an aluminium foil and incubated for 4 hours at 37°C in a humidified environment. After incubation with MTT, the medium was replaced by 200µl of DMSO in order to dissolve the MTT-formazan crystals. Finally 25µl of glycine buffer was added to each well and the absorbance read at 540nm immediately using a plate reader. One column was used as a blank .

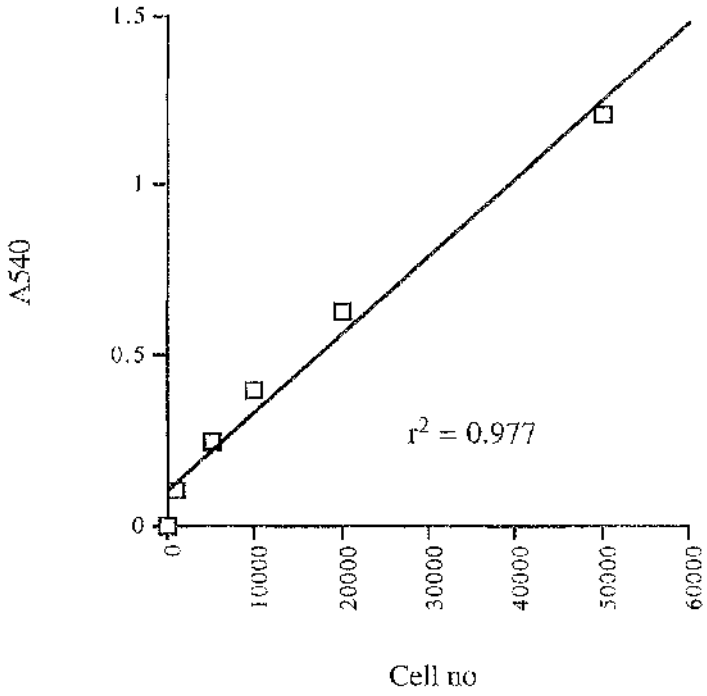


Figure 3 Cell number versus absorbance at 540 nm reading in MTT assay.

A range of numbers of cells were plated onto 96 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. Absorbence was read at 540nm, subsequently adding the glycine buffer, in the plate reader. These data are the mean values of three separate experiments.

2.4.2 XTT Assay

This assay was used instead of MTT because the XTT was more efficient. It involves fewer steps than MTT and did not involve the use of irritating chemicals, like DMSO. This colorimetric assay was used to quantitate cell proliferation and viability. The assay is based on the hydrolysis of the yellow tetrazolium salt XTT (sodium 3'-[1-(phenylaminocarbonyl)-3,4-tetrazolium]-bis(4-methoxy-6-nitro) benzene sulphonic acid hydrate) to form an orange formazan product by the action of the dehydrogenases of the viable cells. The formazan dye formed is soluble in aqueous solutions and is directly quantified using a scanning multiwell spectrophotometer at 540 nm. Cells from a 60-70% confluent flask were trypsinised, centrifuged for 5 minutes at 1000rpm and resuspended in the medium. Subsequently, the cells were counted in a hemocytometer and $1x10^5$ cells/ml were used. The cell suspension was plated down in 96 well microtitre flat bottomed plates in a final volume of 200µl culture medium per well. The first column had growth medium only to be used as a blank. Plates were incubated for 24 hours in a humidified incubator at 37°C. After 24 hours the medium was aspirated from the wells with a sterile hypodermic needle attached to a suction pump and replaced with experimental medium containing the appropriate growth factors and drugs. The plates were then incubated from 24 to 72 hours depending on the aims of the experiment. After the incubation period, 50µl of XTT prepared according to manufacturer's instructions (final XTT concentration 0.3mg/ml) was added to each well. The plates were wrapped in aluminium foil and incubated for a further 2-8 hours according to the need of the experiment and the absorbance of the samples were determined at 540nm.

2.4.3 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 3ml ETN buffer, Hoechst 33258 (100ng/ml) and RNase (5µg/ml). The mixture was vortexed and incubated in the dark for 30 min at room temperature. The fluorescence enhancement at 450nm was measured using the fluorescent spectrophotometer with an excitation wavelength of 360nm.

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 0-60µg/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 540nm (A540), using MTT assay, is shown in (Fig 4).

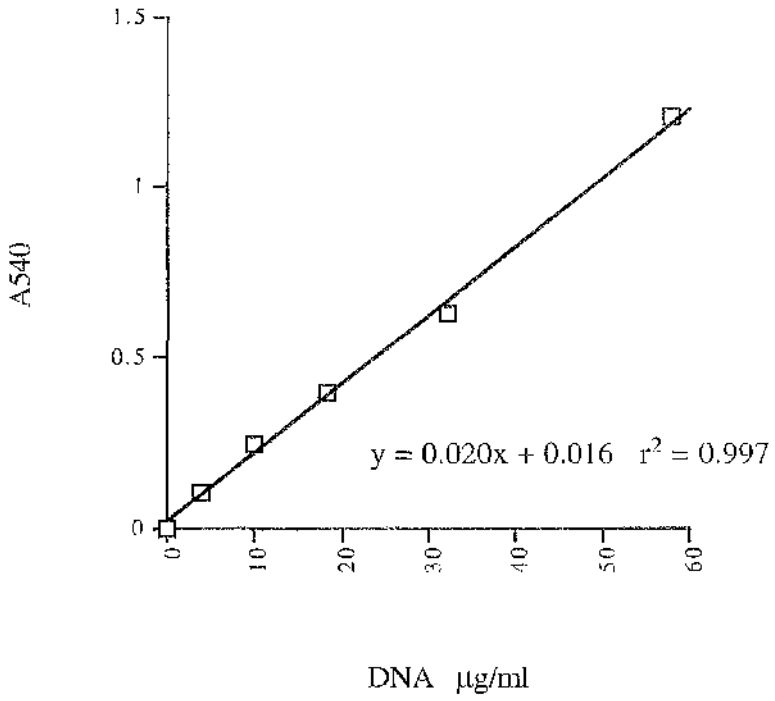


Figure 4 Total cellular DNA counting versus absorbance at 540 nm reading in MTT assay.

MTT readings of the increasing number of cells were obtained as described in Fig 3. 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. 100µl aliquots of samples were added into RT-30 tubes containing 3 ml ETN buffer, Hoechst 33258 (100ng/ml), RNase (5µg/ml). The mixture was vortexed and incubated in the dark for 30 min. at room temperature. The fluorescence enhancement at 450nm was measured with an excitation wavelength of 360nm. These data are the mean values of three separate experiments.

2.4.5 Protein Determination

Protein was routinely measured by the method of Bradford (1976) with bovine serum albumin (BSA) as a standard. Standard curve was constructed using 0-20 µg 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 = A595) (Fig 5).

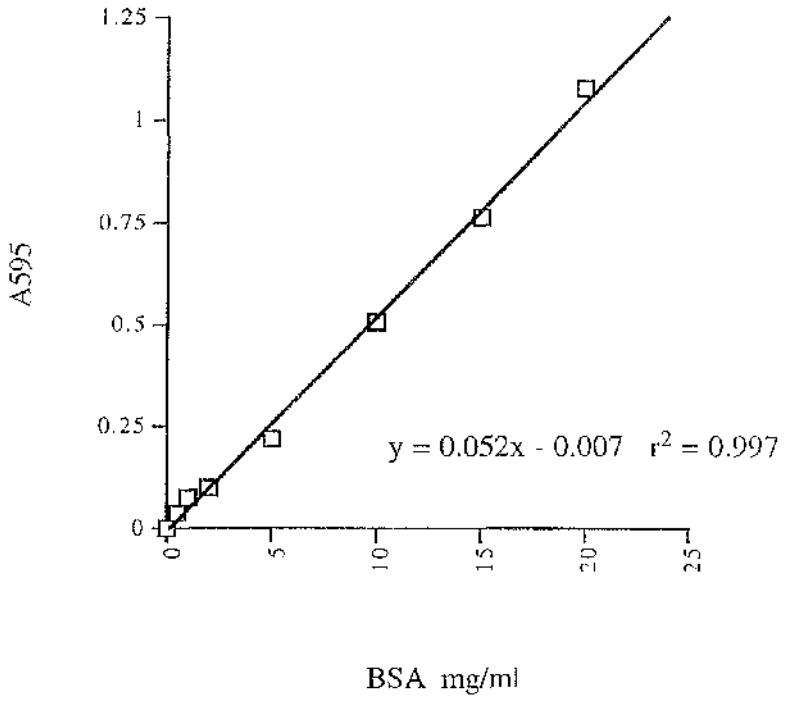


Figure 5. Standard curve for a Bradford protein assay

The samples for the standard curve were prepared as described in section 2.4.5. Bovine serum albumin concentrations of 0-20µg/ml were used as indicated and the absorbance was read at 595nm. 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.

2.5 SDS-Polyacrylamide Gel Electrophoresis

Separation of proteins by SDS-discontinuous polyacrylamide gel electrophoresis (SDS-PAGE) was essentially as described by (Laemmli, 1970). Generally the Bio-Rad Mini-Protein II Electrophoresis system was used with 0.75 mm spacers and combs. A large and a small plate separated by spacers was assembled in the clamp assemblies in the gel casting apparatus as directed by Bio-Rad. Depending on the molecular weight of the proteins to be analysed, the appropriate separating gel mix (see table 1) was prepared and poured in between the glass plates, leaving about a 1.5 cm space at the top for the stacking gel. Subsequently, water-saturated butanol (1 ml) was poured on top of the separating gel before allowing it to polymerise.

Table 1: Composition of SDS-PAGE gel mixes

	Percent Gel (30 mls)			
Solution Components	**8%**	**10%**	**12%**	**15%**
dH_2O	13.9	11.9	9.9	6.9
30% Acrylamide	8.0	10.0	12.0	15.0
1.5 M Tris-HCl; pH 8.8	7.5	7.5	7.5	7.5
10% SDS	0.3	0.3	0.3	0.3
10% Ammonium persulfate	0.3	0.3	0.3	0.3
TEMED	15 ul	15 ul	15 ul	15 ul

Preparation of 10 ml of a 5% stacking gel (5%) was carried out by mixing the following:

dH_2O	6.8 ml
30% acrylamide	1.7 ml
1.0 M Tris-HCl; pH 6.8	1.25 ml
10% SDS	0.1 ml
10% Ammonium persulfate	0.1 ml
TEMED	10 ul

For both separating and stacking gels, all reagents except ammonium persulfate (APS) and TEMED were combined and the monomer solution degassed under vacuum for 5 minutes followed by the addition of APS and TEMED to initiate polymerisation.

After washing the butanol away with dH_2O, the stacking gel was poured and the comb put into position. The comb was removed, gel assembled into the electrophoresis apparatus and the reservoirs filled with protein running buffer (0.192 M glycine, 25 mM Tris, and 0.1% SDS). Protein samples were boiled in an equal volume of Laemmli sample buffer (containing 0.062 M Tris-HCl; pH 6.8, 2% (w/v) SDS, 10% (w/v) sucrose, and 10 mM DTT) for 3 minutes before loading onto the gel. Samples and standards (20-30 ml per well) were loaded into the wells with a microsyringe (Hamilton). Electrophoresis was carried out at a constant voltage of 200 Volts using a power supply (Biorad; Model 200/2.0) until the bromophenol blue tracker dye reached the bottom of the gel. The gel was then removed and either electroblotted onto a

nitrocellulose membrane for Western blotting or directly stained with Coomassie blue.

2.5.1 Staining and Destaining Gels

Staining was carried for 1 hour at room temperature using 0.1% (w/v) Coomassie Brilliant Blue dissolved in 10% (v/v) acetic acid, 40% (v/v) methanol. Gels were destained by washing several times with gentle stirring between 2-3 hours or overnight in 20% (v/v) methanol, 10% (v/v) acetic acid.

2.6 Western Blotting and Immunological Detection of Specific Proteins by Enhanced Chemiluminescence (ECL)

Following separation by gel electrophoresis, proteins were electrophoretically transferred onto Hybond-C nitrocellulose paper (Amersham) in the presence of a buffer containing 25mM Tris-HCl; pH 8.3, 192mM glycine, 0.02% (w/v) SDS and 20% (v/v) methanol as described by Towbin *et al.*, (1979) and Batteiger *et al.*, (1982). This was carried out in a BioRad Trans-Blot cell either at 10mA overnight or at 250mA for 1.5 hours. The nitrocellulose was briefly stained with Ponceau S solution which allowed visualisation of transferred protein bands. The membrane was washed free of this stain with distilled water and subsequently blocked by immersing it for 1-2 hours on a rotatory shaker in a solution containing TBS (20mM Tris-HCl; pH 7.2, 15mM NaCl, 5% (w/v) non-fat dried milk and 0.2% Tween- 20 (v/v). Excess blocking reagent was then washed with TBS, containing 20mM Tris-HCl: pH 7.2, 15mM NaCl and 1% (w/v) non-fat dried milk. Washing was carried out twice briefly, once for 15 minutes and then twice for 5 minutes using 200 ml of wash buffer each time. The membrane was then incubated for 1-2 hours at

room temperature or overnight at 4°C in a 100ml solution containing 20mM Tris-HCl; pH 7.2, 1% (w/v) non-fat dried milk, 0.1% (v/v) Tween-20 and the appropriate dilution of the primary antibody. The membrane was washed again as detailed above and incubated for 2 hours at room temperature with a 1:1000 dilution of horseradish peroxidase conjugated secondary antibody. This was carried out in approximately 10ml of buffer containing 20mM Tris-HCl; pH 7.2, 150mM NaCl and 1% (w/v) non-fat dried milk. In order to keep the volumes of the antibody solutions as small as possible, incubation was sometimes carried out in a sealed bag. Following incubation in secondary antibody the membrane was given three, 30 min washes in wash buffer and one 30 min wash with a buffer containing 20mM Tris-HCl; pH 7.2 and 150mM NaCl. The detection steps were carried out in a dark room following the protocol outlined in the ECL kit manual. Exposure times were typically 15 seconds to 1min.

2.7 Enzyme-Linked Immunosorbent Assay (ELISA)

Assay Principle

Four-span ELISA is an enzyme linked immunoassay for the determination of human uPA, PAI-1 and tPA in monolayer cultures and tissue extracts. The method was developed by Grebenshikov *et al.*. (1997). The assay detects latent, active and complexed forms of uPA, PAI-1 and tPA.

Antibodies (Ab)

The four-span ELISA comprised four antibodies and a substrate: sheep anti-chicken as the coating antibody (1st Ab), chicken anti-analyte as the catching antibody (2nd Ab), rabbit anti-analyte as the tagging antibody (3rd Ab) and

goat anti-rabbit conjugated horseradish peroxide (HRP) as the detecting antibody (4th Ab).

2.7.1 Sample Preparation

uPA or PA1-1 Extraction from Human Cell Lines

Cells were grown in 25 cm^2 flasks in routine culture medium RPMI-1640, 10% FCS and incubated at 37°C in a humidified incubator for 24 hours. The medium was then replaced by experimental medium with or without growth factors and steroid hormones and further incubated, for time course studies, from 48 and 72 hours. At the end of each time point the medium was collected and snap frozen until assay at -70°C. The cells were then washed with PBS. Subsequently, 1ml of 1% Triton X-100 in TBS was added to the cells and incubated for 2-4 hours at 4°C with constant stirring. The cell suspension was then centrifuged at 100,000rpm for 60 minutes at 4°C to pellet cell membranes and other debris. The supernatant was collected and the total protein quantitated using the (Bradford, 1976) protein assay. The supernatant was aliquoted into 200 µl portions and stored at -70°C. For immediate use the samples were diluted 1:100 for PAI-1, and 1:50 for uPA for MDA-MB-231 cell line. MCF-7 cell line was used without further dilution.

2.7.2 Assay Procedure

The 96-well microtitre plate was coated using 100µl of the coating antibody (dilution 300-fold) per well and incubated overnight at 4°C. After 24 hours the plates were washed for 5 minutes, 4 times in a washing buffer (PBS+ 1% Tween-20) and were blocked at 37°C for 2 hours with blocking buffer (PBS+ 1% Tween-20+ 1% BSA). The plates were again washed with washing buffer

as before. Standards were prepared in a range of 0.1-1ng/ml in a dilution buffer. Where required, samples were diluted and 100µl of the standard/control and samples were added to each well and incubated overnight at 4°C. All measurements were performed in duplicate. After washing four times with washing buffer, the catching antibody was diluted (600-fold uPA and 475-fold PAI-1). 100µl per well each was added and incubated at room temperature for 2 hours. The plates were then washed as before with washing buffer to remove any unbound antibody and incubated further with biotinylated tagged antibody diluted (300X uPA and 150X PAI-1). 100 µl per well and incubated at ambient temperature for 2 hours. The plates were washed four times and incubated with the freshly prepared substrate OPD (0-phenylenediamine) and H_2O_2 wrapped in an aluminium foil and incubated in the dark for 30 minutes. The enzymatic reaction was stopped by adding 50 µl of 1 M H_2SO_4. Yellow colour was developed and the absorbance read at a wavelength of 492 nm in an ELISA plate reader. The background average of the blanks was deducted from the standard and sample readings. The standard curve was constructed from the standards and the values of the unknown samples determined (Fig 6).

The sample values were multiplied by the dilution factor in the assay and divided by the protein concentration (mg/ml) of the cell extracts to convert ng/ml values of uPA/PAI-1 to ng uPA/PAI-1/ mg protein.

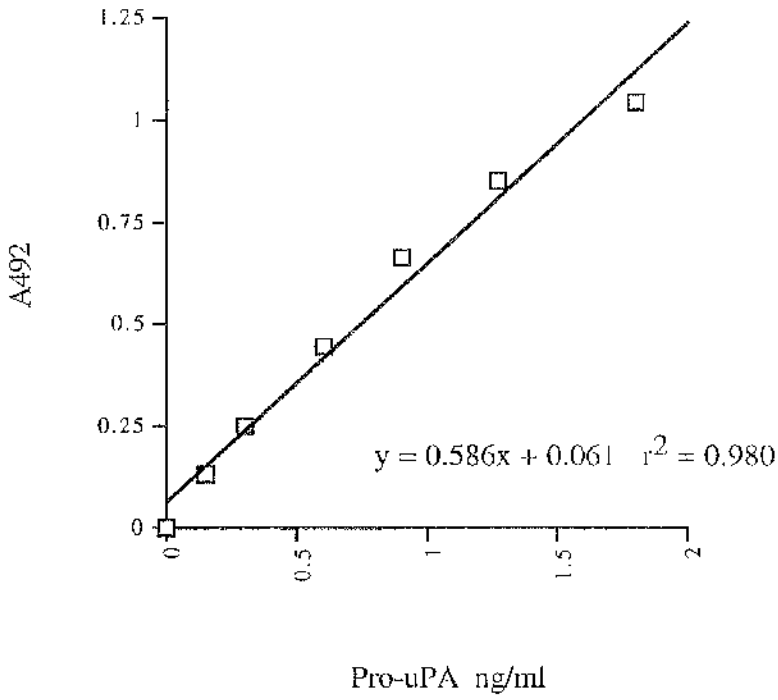


Figure 6. Standard curve for an ELISA assay

The samples for the standard curve were prepared as described in section 2.7.1. 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.

2.8 Enzyme Activity Assay

Plasminogen Activator Assay

The cells were plated at a density of $5x10^4$ cells per well in a 6-well plate with growth medium containing 10% fetal calf serum in it. After a 24 hour incubation time the cultures were washed twice with PBS and incubated for an additional period of 8-10 hours with experimental medium supplemented with 2.5% FCS. This medium was then replaced with control medium or medium supplemented with treatments and the plates incubated for further 48 and 72 hours. At each time point conditioned medium (CM) was collected and the cells were lysed with 1ml of 1% Triton X-100 for 2 hours at 4°C. The supernatant (SN) was collected after centrifugation at 1000 rpm for 30 minutes. Both CM and SN were snap frozen and stored at -70°C until assayed for uPA activity.

The activity of the samples was determined using the chromogenic substrate for urokinase pyro-Glu-Gly-Arg-pNA (S-2444, Kabi, Stockholm, Sweeden). 30µl of the standard or sample was incubated with 120 ml of the buffer containing 110µl of 10mM Tris-HCl; pH 7.5. 10µl of 1.5mg/ml of the substrate S-2444 and 1.5µl of plasminogen 10 U/ml. All the incubations were performed at 37°C in microtitre plates for 2 hours. The activity was measured with a Titertek Multiscan spectrophotometer (Flow Laboratories). Activity expressed as the change in the absorbance at 405 nm. Urokinase (UK) activity was obtained by comparison with the WHO International standard for UK.

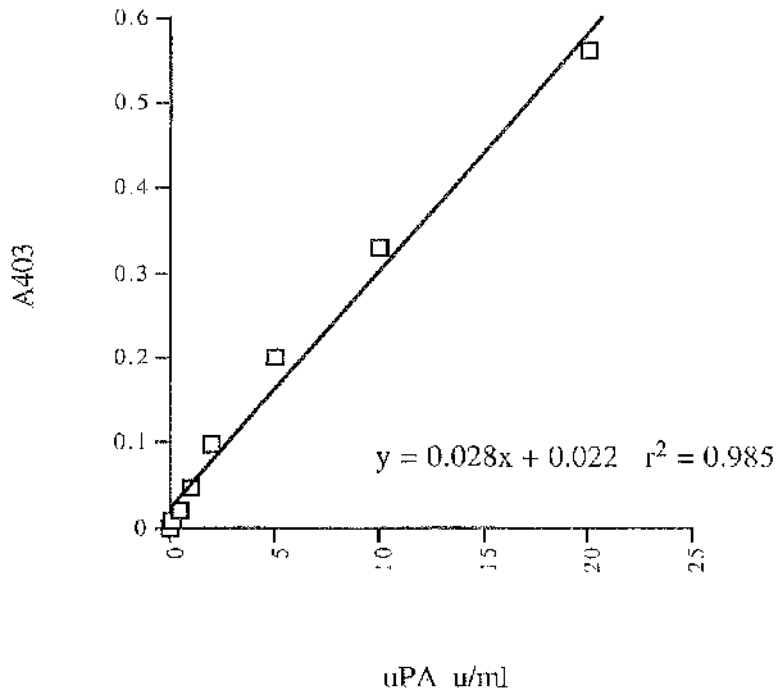


Figure 7. Standard curve for an uPA enzyme activity assay

The samples for the standard curve were prepared as described in section 2.8. Active-uPA concentrations of 0-20µg/ml were used as indicated and the absorbance was read at 403nm. 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 chromogenic substrate S-2444 was changed to a more specific substrate S-2251 (Quadratech) 5mg/ml for urokinase. Because of the sensitivity of the assay the amount of the standard/ sample and the substrate was reduced to 10µl and 4µl respectively. while the assay buffer mix was increased from 120µl to 190µl. All the other measurement steps in the assay performed were the same as described above

2.9 Invasion Assays

Principle of the Assay

An *in vitro* invasion assay was carried out to quantify the invasive potential of tumour cells after different treatments. In this assay filter membranes were used to support the artificial basement membrane and to discriminate between cells that have traversed from those which have not.

2.9.1 Cell Harvest for Invasion Experiments

Cells were harvested from 70-80% confluent culture flasks with 0.02% EDTA, counted and resuspended in experimental medium, and seeded in the upper chamber onto the lower side of the membrane of Boyden blind-well apparatus for 6 to 8 hours at 37°C in 5% CO_2 in air (Fig 8).

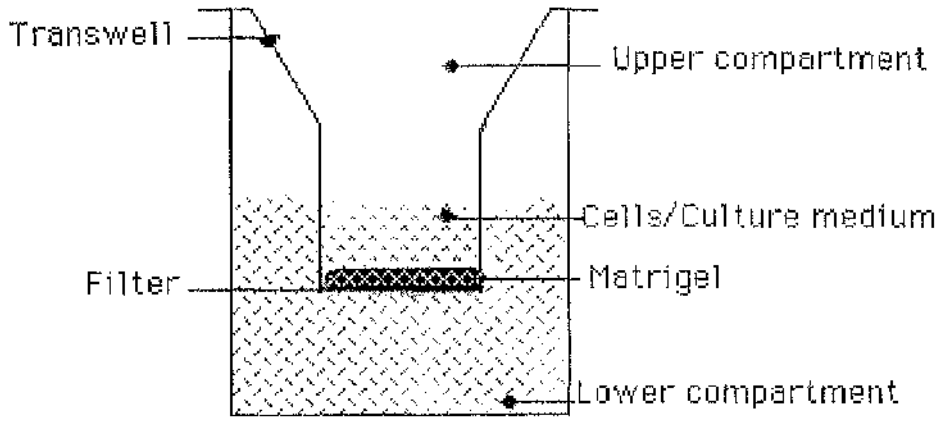


Figure.8 Schematic description of *In vitro* invasion assay.

For experimental details see section 2.9.3.

2.9.2 Invasion Assay Procedure

The Boyden blind-well chamber invasion assay was performed, as described by (Albini *et al.*, 1986). Transwell chambers (Costar) with 6.5mm diameter, 8μm pore size polycarbonate filters were pre-coated with matrigel for the invasion assays. Each 24-well plate contains 12 removable inserts having the carbonate filter and a corresponding lower well. Twelve separate wells without inserts could be used for positioning the inserts during experimental manipulations.

Each filter was coated with 80μl of matrigel diluted to a final concentration of 1mg/ml in experimental serum free RPMI 1640 medium to form a continuous barrier on top of the filter. The filter was left at 37°C for an hour to allow an even gel layer to form on the filter. The lower Boyden blind well chamber was filled with either 780μl experimental medium or where indicated an experimental medium plus 2-5 mg/ml matrigel was used. Filters coated on the upper side with matrigel were placed in the chamber and 5 x 10^5 cells/ml resuspended in the experimental medium. 100μl out of this stock was placed on top of the matrigel layer and incubated at 37°C in 5% CO_2 in air. After 72 hours the percentage of invading cells was determined as described below. All experiments were performed in duplicate.

2.9.3 Evaluation of Invasion Using the MTT Assay

The viability testing MTT assay was used to measure quantitatively the proportion of invasive cells. 200μl experimental medium either alone (control) or with treatments were added into the chamber. The cells were incubated for three days. After 72h, 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. At the end of the incubation period medium was gently aspirated from the upper chambers. The upper chambers were removed and the filters containing the cells, were transferred to a 24-well culture plate. Subsequently, after aspiration of the MTT medium cells which had not traversed the filter and remained on the top or in the layer of matrigel were removed together with the matrigel with a cotton swab. The cotton swab and the remaining filter-containing cells which had traversed to the lower side of the filter were placed in separate wells of a 24-well culture plate. To release the formazan from the cells , 800µl DMSO was added to each well and the plates were gently shaken for 5 minutes. 200µl glycine buffer was then added per well and absorbance at 540nm was read immediately thereafter in a spectrophotometer.

Assessment of Invasive Capacity of Cells

The percentage of cells capable of traversing through the matrigel layer and attaching to the lower side of the filter was expressed as a percentage of the total number of cells present following the 72 hours incubation period in an experimental medium:

% Invasion = Number of cells attached to the lower side of the filter (A540) / total number of cells, i.e. sum of cells attached to the lower filter side and cells still present in the matrigel (A540) x 100%.

2.9.4 Evaluation of Invasion by Confocal Microscopy

Approximately $5x10^5$ cells/ml were prepared from the stock and 100µl of this dilution was plated on the bottom of the filter and placed in a humidified incubator at 37°C for 4-5 hours in order to attach the cells to the filter. The inserts were then inverted upside down and 200µl of the respective drug was prepared in the experimental medium and added on top of the filter which acts as a chemoattractant. Then, 750µl of the experimental medium was added in the well and the plate incubated for 5 days at 37°C in a humidified incubator to prevent the medium from evaporation.

Fixation of the Cells

The inserts from the wells were removed and washed four times with sterile PBS (5 minutes per wash). The cells were then fixed with methanol (100% or 70%) for 20 minutes at room temperature. After washing four times with PBS the cells were stained with propidium iodide-50µl per insert (5 mg/ml). 1:1000 dilution was used to stain the cells for 30 minutes at room temperature in the dark. After thorough washing with PBS the cells were read under confocal microscope (Confocal Bio-Rad illumination unit attached to a Nikon diaphot inverted Microscope).

2.10 Immunofluorescence

Having been seeded at a cell density of $2x10^4$ cells per well, cells were plated down overnight into 8-well glass slide/tissue culture vessels. Following this the wells were aspirated and washed three times with PBS and 300µl of RPMI-1640 with 2.5% DHIDCCFCS +/- growth factors or hormones were

then added to the wells. After 48 hours the wells were aspirated and washed twice with PBS.

The cells were fixed by the addition of 200µl acetone:methanol (1:1) at -20°C per well for 5 minutes and allowed to air dry. 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 the wells were treated with primary mouse monoclonal antibody (200µl/well) uPA/PAI-1 (1:50 in PBS + 1% BSA) added. The wells were then incubated for 1 hour at room temperature in a humidified chamber. The slides were then washed three times with PBS. Fluorescent labelled goat antimouse IgG (200µl/well) 1:50 in dilution buffer (PBS + 1% BSA) was then added and incubated for one hour as before. Following incubation the wells were washed three times with PBS and then mounted with DPX mountant and immunofluorescence examined using a fluorescent microscope (Leitz).

2.11 RNA Isolation

Total RNA was isolated using the Ultrasoec-II RNA Isolation System (Biotecx) according to manufacturers' instructions. Essentially, cells were pelleted and resuspended in three volumes of lysis buffer (provided in the kit). This mixture was left on ice for five minutes to allow complete dissociation of nucleoprotein complexes. Subsequently, 0.2 volumes of chloroform were added and the mixture vortexed vigorously for 15 seconds and left on ice for 5 minutes. After centrifugation at 14,000 rpms at 4°C for 15 minutes 4/5th volume of the aqueous phase was transferred to a fresh tube and to this 0.5 volume of isopropanol and 0.05 volume RNATack Resin was added and the mixture was vortexed for 30 seconds. The tube was then centrifuged for 1 minute and the supernatant was discarded. The pellet was washed twice with 1

ml 75% ethanol by vortexing and spinning for about 30 seconds. The pellet was dried *in vacuo*. To elute RNA 0.1 volume of DEPC treated water was added to the dried pellet and the mixture vortexed for 30 seconds and centrifuged for 1 minute. The supernatant containing total RNA was collected and stored at -70°C.

2.11.1 Reverse Transcriptase-Polymerase Chain Reaction

For the synthesis of cDNA, 10µg of total RNA was used and 50ng of the appropriate reverse oligonucleotide (for oligonucleotide sequences see below) annealed in a 19.25µl reaction consisting of AMV reaction buffer for 30 minutes at 42°C. This was followed by the addition of 0.5µl of 20mM dNTPs and 0.25µl (5 units) of avian myeloblastosis virus (AMV)-reverse transcriptase (Boehringer Mannheim) and the reaction was incubated for 30 minutes at 42°C.

From the 20µl, 5µl were used in a polymerase chain reaction (100µl total volume) which contained 500ng of each of the oligonucleotides, 0.2mM dNTPs, 10µl 10X reaction buffer and 0.25µl (1U) Taq DNA polymerase (Boehringer Mannheim). PCR was carried out on a Perkin Elmer thermocycler using the following program: 94°C 4 minutes (one cycle); 94°C 2 minutes, 55°C 1 minute, and 72°C 1 minute (30 cycles); 72°C 5 minutes (one cycle). DNA sequence of the respective pairs of oligonucleotides for the amplification of uPA, uPAR, PAI-1 and actin cDNAs were as following:

uPA: 5' - AGAATTCACCACCATCGAGA - 3' (Forward)

5' - ATCAGCTTCACAACAGTCAT - 3' (Reverse)

PAI-1 5' - ATGGGATTCAAGATTGATGA - 3' (Forward)

5' - TCAGTATAGTTGAACTTGTT - 3' (Reverse)

uPAR 5' - TTACCTCGAATGCATTTCCT - 3' (Forward)

5' - TTGCACAGCCTCTTACCATA - 3' (Reverse)

β-Actin 5' - TTGAAGGTAGTTTCGTGGAT - 3' (Forward)

5' - GAAAATCTGGCACCACACCTT - 3' (Reverse)

DNA Cloning

In order to unequivocally establish the identity of the obtained PCR products with the four different sets of primers the four fragments were cloned into the vector pGEM-T Easy (Promega). This was carried out by ligating each of the fragments and 50 ng of the vector in a 10 µl reaction as follows:

1µl PCR Fragment (≈100 ng)

1µl pGEM-T Easy(50 ng)

1µl 10x Ligase Buffer

6µl dH_2O

1µl T4 DNA Ligase (Boehringer Mannheim; 5 units)

The reaction was incubated at 15°C overnight. For bacterial transformation, 100µl of $CaCl_2$ competent *E. coli* strain XL1-Blue (Stratagene) was added to the ligation reaction and the mixture left in ice-water for 20 minutes. Subsequently, the tube was transferred to a 42°C waterbath for 2 minutes (in

order to induce heat shock) prior to plating the whole reaction mixture on a prewarmed LB/ampicillin plate. Plates were left over night in a 37°C incubator.

In order to characterise the clones, bacterial colonies from the plates were picked and grown in 3 mls of liquid culture in the presence of ampicillin. Plasmid DNA from the turbid cultures was isolated by the alkaline lysis method using the Qiagen miniprep kit. Recombinant clones were identified by digesting approximately 500ng of plasmid DNA with the restriction endonuclease EcoRI and electrophoresing the products on a 1.2% agarose gel prepared and run in Tris-acetate EDTA buffer (TAE)

DNA Sequencing

Recombinant plasmids were sequenced with Sequenase (USB) using the T7 primer. Five micrograms of plasmid DNA was denatured at room temperature for 5 minutes in a 20µl reaction containing 200mM NaOH. Subsequently, 4µl of 10M ammonium acetate together with 100µl ice-cold 95% ethanol was added and the mixture left on ice for 10 minutes. The precipitate was harvested by centrifugation at 14,000 rpm for 10 minutes and the supernatant discarded. The pellet was washed with 0.5 ml of 75% ethanol and then dried *in vacuo*. Annealing of the primer to the template, labelling and termination reactions were carried out according to the manufacturer's instructions. The reactions were heat denatured prior to loading on a 6% sequencing gel. Once the bromophenol blue dye had run off, the gel was fixed for 10 minutes in a 1 litre solution containing 10% methanol and 10% glacial acetic acid prior to drying. The dried gel was then exposed to autoradiographic film (Fuji) and developed after 12-15 hours.

2.12 Statistical Analysis

Data from individual experiments are expressed as the mean ± standard deviation (SD) and data pooled from at least two experiments, each performed in duplicate. To test for differences between groups, the student's t-test was used, with $p < 0.001$ considered as statistically very significant. The student t-test was performed using Macintosh computer programme "instat".

CHAPTER 3

RESULTS

Regulation of The Growth of MCF-7 and MDA-MB-231 Cell Lines

Background

The ultimate focus of this work was to study the invasive potential of two cell lines, MDA-MB-231 and MCF-7, the former hormone insensitive and the latter oestrogen sensitive, in response to various growth factors and steroid hormones. As a first step therefore, it was imperative to observe how these growth factors and hormones effected the proliferative behaviour of the two cell lines. The growth factors and hormones used in this study were, EGF, TGFα, TGFβ, progesterone, tamoxifen and oestrogen. These were each used at five different concentrations with each of the two cell lines. Subsequently, the effects of these exogenously added compounds were monitored by counting cells after 24, 48 and 72 hours. Charcoal stripped serum was used for these studies to estimate the effects of (most) serum growth factors.

The roles of the growth factors EGF, TGFα, and TGFβ in breast cancer *In vitro* invasion remains controversial. The following experiments addressed this question.

The growth and function of epithelia in tissue culture are significantly influenced by the nature of substratum. To our knowledge this is the first comparison of the rate of proliferation on plastic with that on a matrigel from mouse sarcoma, which is a complex mixture of extracellular proteins including type-IV collagen, laminin, fibronectin and glycosaminoglycans.

3.1 PROLIFERATION

3.1.1 Transforming Growth Factor α (TGFα)

TGFα, a stimulatory growth factor that mediates its effect via the epidermal growth factor receptor (EGFR), was tested on cell growth for both MDA-MB-231 and MCF-7 cells. Different concentrations of TGFα were used in this study, ranging from 0.1-10 ng/ml. This dose range was selected since previous experiments in our laboratory had shown that this range covered both subtle and maximum growth stimulation. To ascertain the optimum mitogenic concentrations the MTT/XTT assay was employed. It was found that exposure of low doses (i.e. 0.1 ng/ml) of TGFα, resulted in a slight reduction of proliferation after 24, 48 and 72 hours for the MDA cell line on EHS (Fig 9). EHS is a matrigel derived from Engelbreth-Holm-Swarm murine tumour. Treatment with higher doses of TGFα resulted in a gradual increase in proliferation. Stimulation of growth became significant ($p< 0.01$) at 3.0 ng/ml TGFα after 24 hours in comparison to the control. The MDA cell line grown on plastic showed a similar behaviour to that which was observed when these cells were grown on EHS in that an initial decrease in growth with lower concentrations of TGFα was observed (Fig 10). This decrease was consistent with time period. However, a gradual increase in proliferation, with increasing dose was observed. The maximum increase observed was about 20% with the highest dose (10 ng/ml).

The effect of TGFα on the growth of the MCF-7 cells grown on EHS and plastic were also studied. With MCF-7 cells, TGFα augmented the growth rate in a concentration and time dependent manner on EHS. Maximum growth (50%) was observed at TGFα 1 ng/ml (Fig 11). Maximum stimulation of 75 % at 3 ng/ml was observed after 72 hours on plastic (Fig 12).

Conclusions

Figures 9 (EHS) and 10 (plastic) show dose response curves for MDA-MB-231 cells in response to TGFα. MDA growth was stimulated by TGFα with maximum stimulation at 3ng/ml on EHS and 10ng/ml on plastic. Lower dose inhibited the growth at 0.1ng/ml on both EHS and plastic.

Figures 11 (EHS) and 12 (plastic) show dose response for MCF-7 cells in reponse to TGFα. The growth of MCF-7 cells was stimulated by TGFα with maximum stimulation at lower doses on EHS and higher doses on plastic.

This shows that growth responses of both cell lines in response to TGFα. were only slightly altered by nature of the substratum.

Figure 9 Cell proliferation under varying stimulation of TGFα on EHS in MDA-MB-231 cells.

MDA cells (2000 per well) were plated in 96 well plates. After overnight incubation, the routine medium was replaced with experimental medium with 1% FDDDCS only (control) or supplemented with doses of TGFα (0.1, 0.3, 1.0, 3.0 and 10 ng/ml). After 24, 48 and 72 hours proliferation was evaluated by MTT as described in "materials and methods" section 2.4.1. Results are expressed in percent of control value. *$p < 0.001$ and •$p < 0.05$. The experiments were performed in triplicate 8-96 wells. Results are mean ± SD.

Figure 10 Cell proliferation under varying stimulation of TGFα on plastic in MDA-MB-231 cells.

MDA cells (2000 per well) were plated in 96 well plates. After overnight incubation, the routine medium was replaced with experimental medium with 1% FDDDCS only (control) or supplemented with doses of TGFα (0.1, 0.3, 1.0, 3.0 and 10 ng/ml). After 24, 48 and 72 hours proliferation was evaluated by MTT as described in "materials and methods" section 2.4.1. Results are expressed in percent of control value. *$p < 0.001$ and •$p < 0.05$. The experiments were performed in triplicate 8-96 wells. Results are mean ± SD.

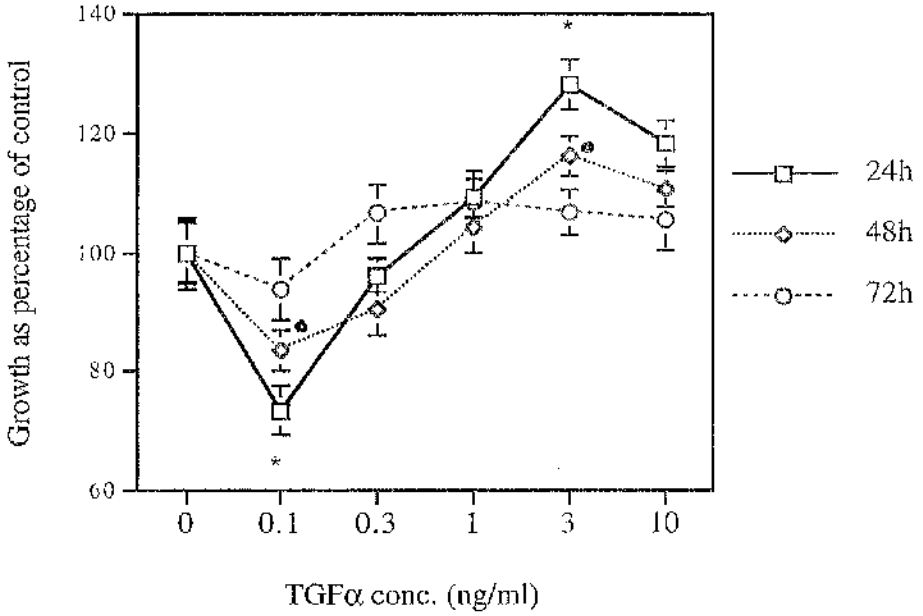


Fig: 9 MDA proliferation on EHS in response to TGFα

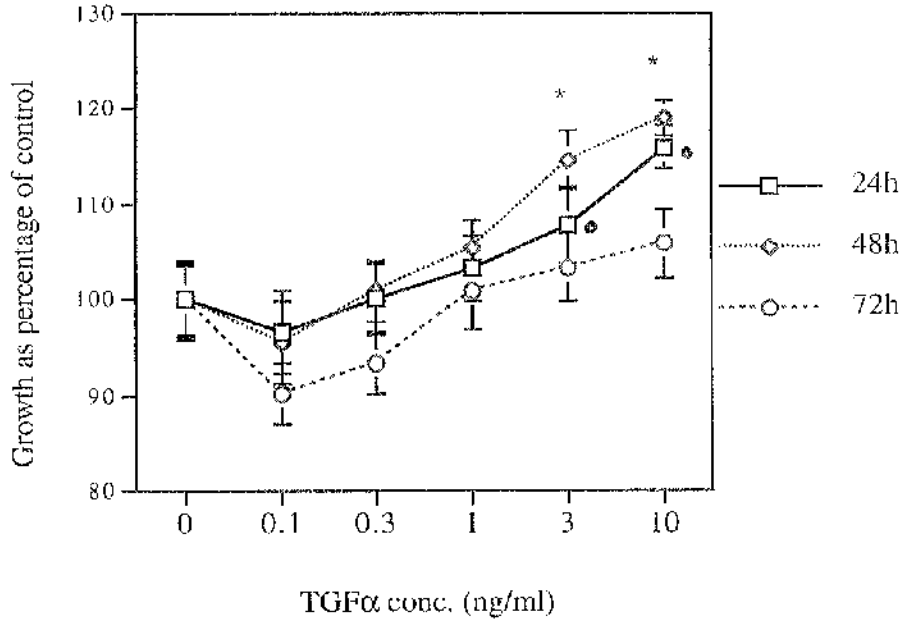


Fig: 10 MDA proliferation on plastic in response to TGFα

Figure 11 Cell proliferation under varying stimulation of TGFα on EHS in MCF-7 cells.

MCF-7 cells (2000 per well) were plated in 96 well plates. After overnight incubation, the routine medium was replaced with experimental medium with 1% DHIDCCFCS only (control) or supplemented with doses of TGFα (0.1, 0.3, 1.0, 3.0 and 10 ng/ml). After 24, 48 and 72 hours proliferation was evaluated by MTT as described in "materials and methods" section 2.4.1. Data is expressed as a percentage of the control of untreated cells. *$p < 0.001$ and •$p < 0.05$. The experiments were performed in triplicate 8-96 wells. Results are mean ± SD.

Figure 12 Cell proliferation under varying stimulation of TGFα on plastic in MCF-7 cells.

MCF-7 cells (2000 per well) were plated in 96 well plates. After overnight incubation, the routine medium was replaced with experimental medium with 1% DHIDCCFCS only (control) or supplemented with doses of TGFα (0.1, 0.3, 1.0, 3.0 and 10 ng/ml). After 24, 48 and 72 hours proliferation was evaluated by MTT as described in "materials and methods" section 2.4.1. Data is expressed as a percentage of the control of untreated cells. *$p < 0.001$ and •$p < 0.05$. The experiments were performed in triplicate 8-96 wells. Results are mean ± SD.

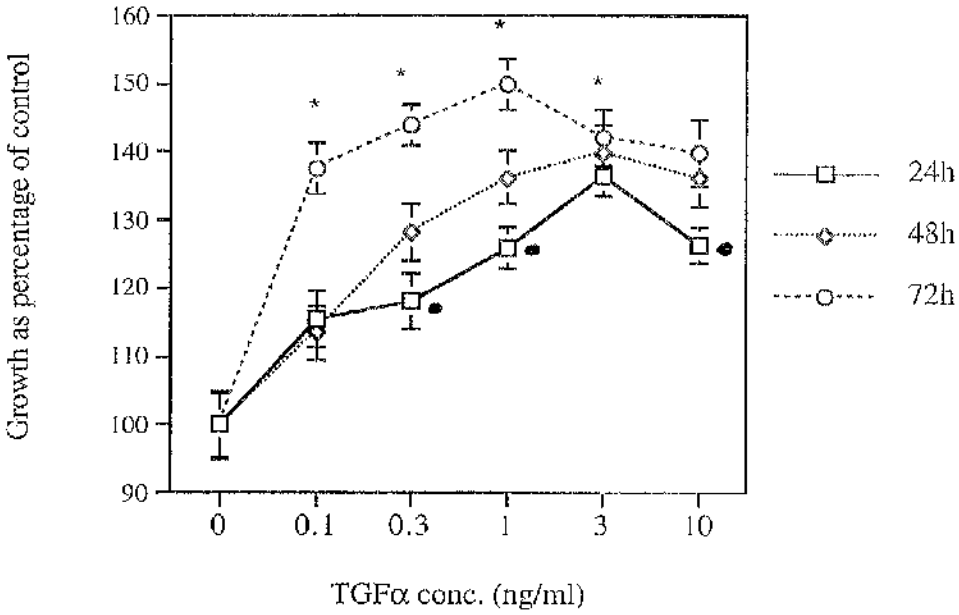


Fig.11 MCF-7 proliferration on EHS in response to TGFα

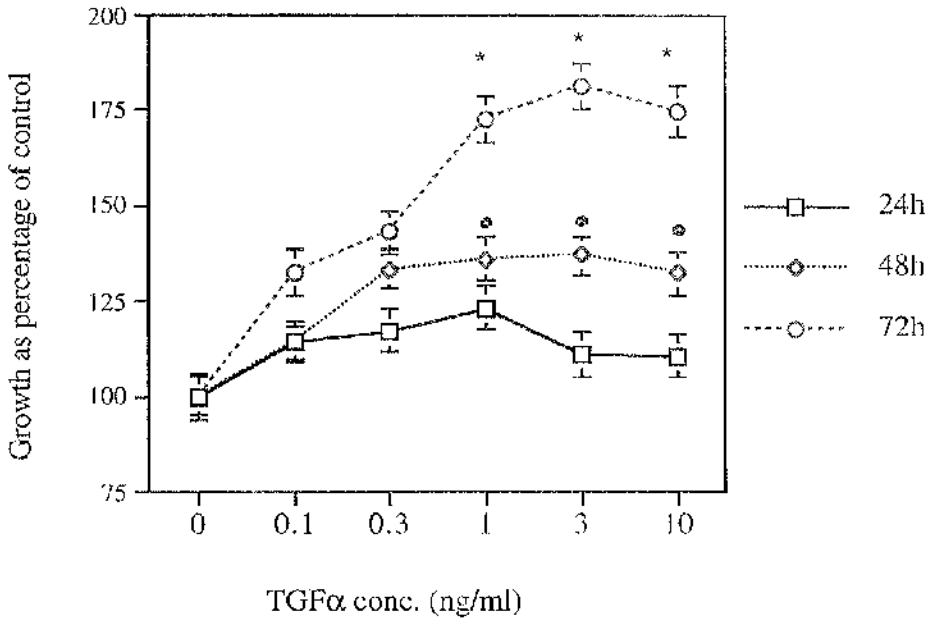


Fig.12 MCF-7 proliferation on Plastic in response to TGFα

3.1.2 Epidermal Growth Factor (EGF)

Next, treatment of the MDA and MCF-7 cell lines with varying amounts of EGF was studied on either plastic or EHS.

The effect of EGF on MDA cells, on EHS was found to be slightly inhibitory after 48 and 72 hours, when exposed to 0.63 ng/ml which lead to a 10% decrease in growth rate (Fig 13). This agrees with the growth inhibition seen with low doses of TGFα and the stimulation is sustained in that higher amounts of EGF overcame this inhibition and induced gradual increase of growth. Analysis of cells after 24 hours showed a slight increase in growth rate which continued with the increasing concentrations. The MDA cell line on plastic (fig 14), showed a different behaviour. A gradual increase in the growth rate was observed with increasing concentrations of EGF, to about 15% (Fig 9). A further increase in the concentration of EGF resulted in a sharp decrease in growth rate. However stimulation with EGF on plastic was short-lived and fell after 48h or 72h.

When grown on EHS, MCF-7 cells showed a time dependent increase in their growth rate, which was quite dramatic after 72h with 0.63 ng/ml reaching a maximum of 58% above control (Fig 15). A gradual increase in proliferation was observed with increasing concentrations after 24 and 48 hours. On plastic, MCF-7 cells exposed to 0.63 and 1.89 ng/ml of EGF showed a gradual increase in proliferation at all time points reaching maximum (100%) after 48h (Fig 16). Higher concentrations of EGF subsequently decreased proliferation.

Conclusions

Figures 13 (EHS) and 14 (plastic) show the dose response curve for MDA cells to EGF. On EHS, high doses of EGF induced slight growth stimulation at all time points, whereas, on plastic, any effect of higher dose was inhibitory.

Figures 15 (EHS) and 16 (plastic) show the dose response curve for MCF-7 cells. On both substrata EGF showed growth stimulation but the difference was that, on EHS, a dose-related increased growth was observed whilst that on plastic was bell-shaped.

The overall results shows that the growth response to EGF, like TGFα, was slightly altered by the nature of the substratum.

Figure 13 Cell proliferation under varying stimulation of EGF on EHS in MDA-MB-231 cells.

MDA cells (2000 per well) were plated in 96 well plates. After overnight incubation, the routine medium was replaced with experimental medium with 1% DHIDCCFCS only (control) or supplemented with doses of EGF (0.63, 1.89, 6.3, 18.9 and 63 ng/ml). After 24, 48 and 72 hours proliferation was evaluated by MTT as described in "materials and methods" section 2.4.1. Results are expressed in percent of control value. *$p < 0.001$ and •$p < 0.05$. The experiments were performed in triplicate 8-96 wells. Results are mean ± SD.

Figure 14 Cell proliferation under varying stimulation of EGF on plastic in MDA-MB-231 cells.

MDA cells (2000 per well) were plated in 96 well plates. After overnight incubation, the routine medium was replaced with experimental medium with 1% DHIDCCFCS only (control) or supplemented with doses of EGF (0.63, 1.89, 6.3, 18.9 and 63 ng/ml). After 24, 48 and 72 hours proliferation was evaluated by MTT as described in "materials and methods" section 2.4.1. Results are expressed in percent of control value. *$p < 0.001$ and •$p < 0.05$. The experiments were performed in triplicate 8-96 wells. Results are mean ± SD.

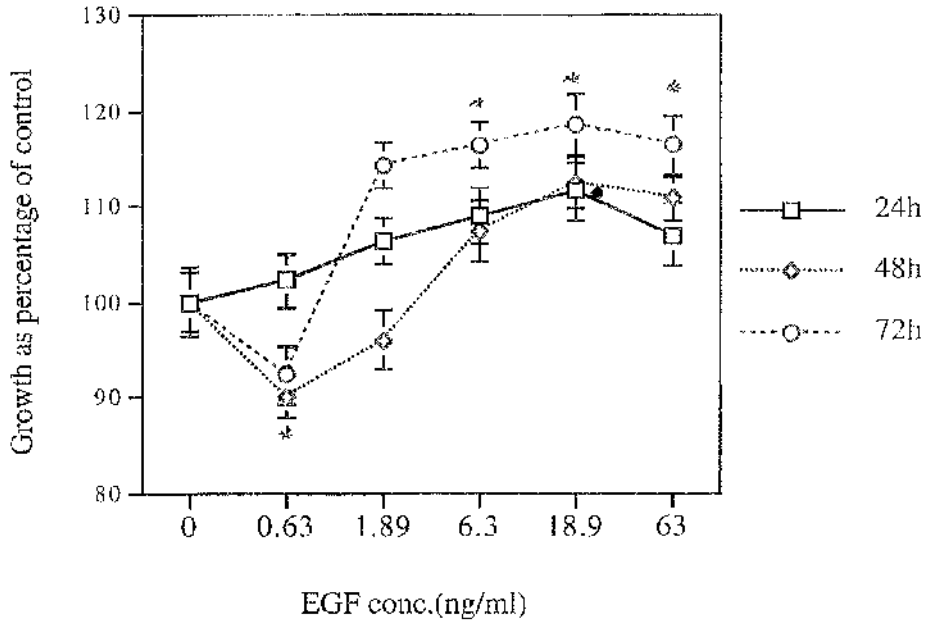


Fig: 13 MDA proliferation on EHS in response to EGF

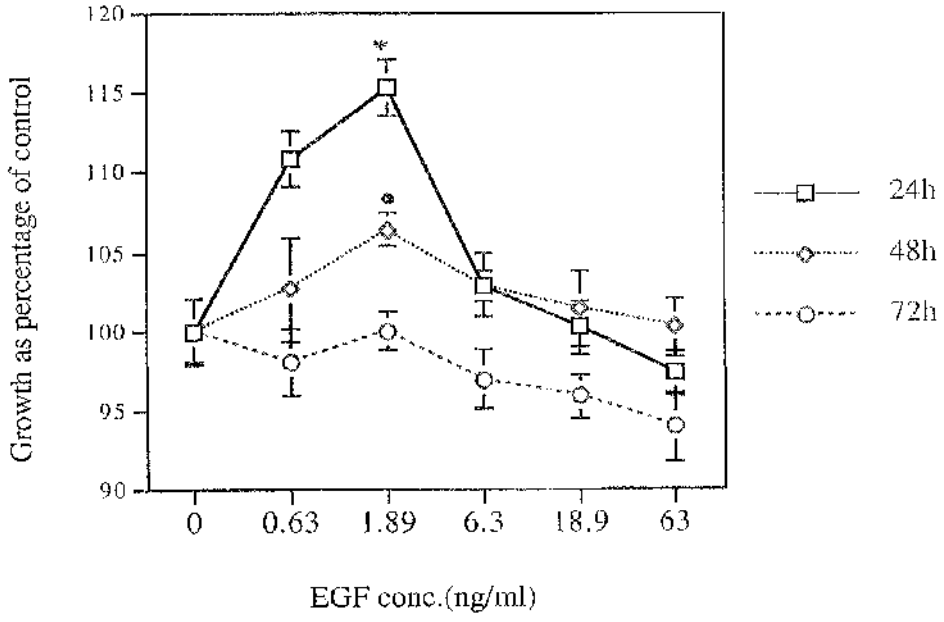


Fig: 14 MDA proliferation on plastic in response to EGF

Figure 15 Cell proliferation under varying stimulation of EGF on EHS in MCF-7 cells.

MDA cells (2000 per well) were plated in 96 well plates. After overnight incubation, the routine medium was replaced with experimental medium with 1% DHIDCCFCS only (control) or supplemented with doses of EGF (0.63, 1.89, 6.3, 18.9 and 63 ng/ml). After 24, 48 and 72 hours proliferation was evaluated by MTT as described in "materials and methods" section 2.4.1. Results are expressed in percent of control value. *$p < 0.001$ and •$p < 0.05$. The experiments were performed in triplicate 8-96 wells. Results are mean ± SD.

Figure 16 Cell proliferation under varying stimulation of EGF on plastic in MCF-7 cells.

MCF-7 cells (2000 per well) were plated in 96 well plates. After overnight incubation, the routine medium was replaced with experimental medium with 1% DHIDCCFCS only (control) or supplemented with doses of EGF (0.63, 1.89, 6.3, 18.9 and 63 ng/ml). After 24, 48 and 72 hours proliferation was evaluated by MTT as described in "materials and methods" section 2.4.1. Results are expressed in percent of control value. *$p < 0.001$ and •$p < 0.05$. The experiments were performed in triplicate 8-96 wells. Results are mean ± SD.

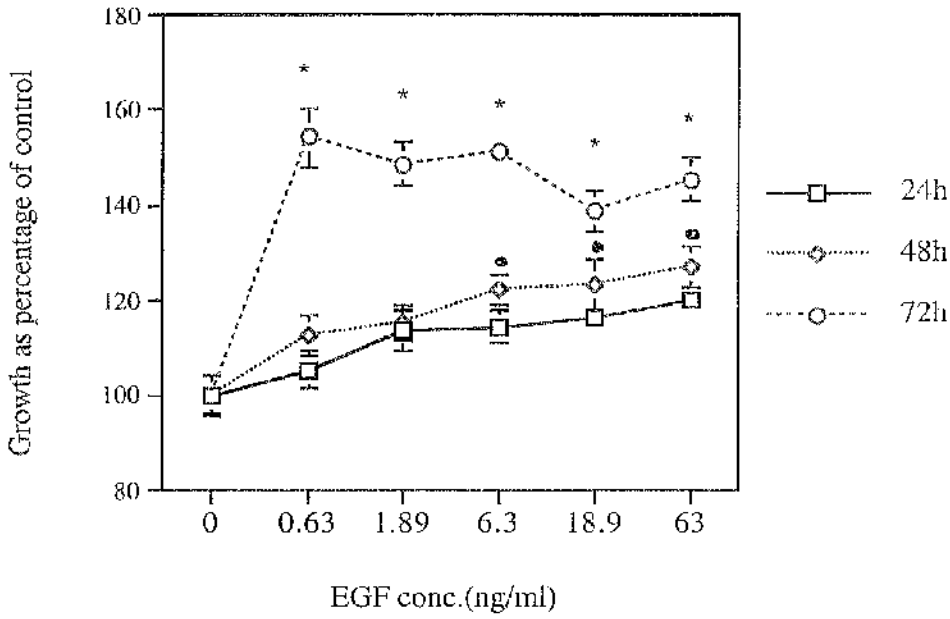


Fig: 15 MCF-7 proliferation on EHS in response to EGF

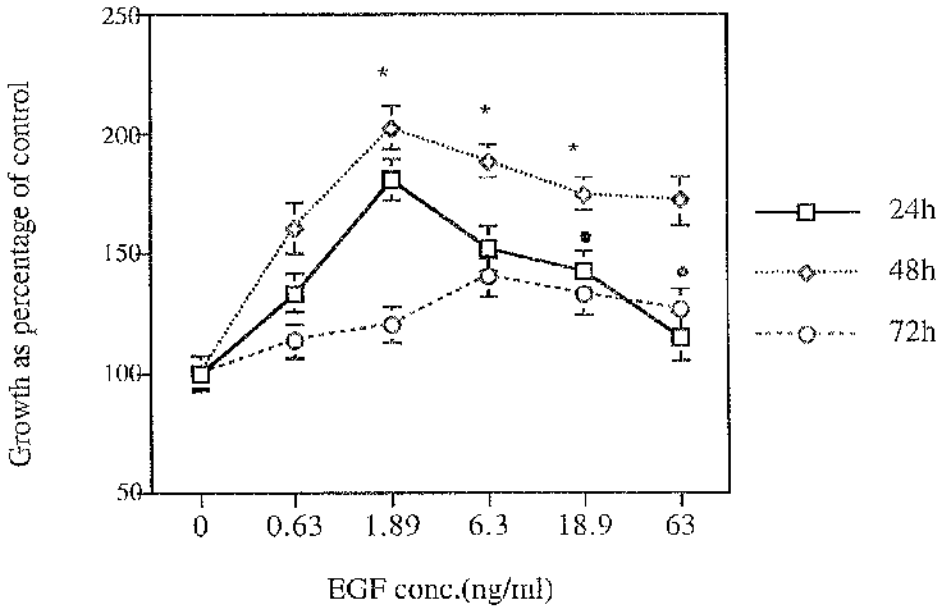


Fig: 16 MCF-7 proliferation on plastic in response to EGF

3.1.3 Transforming Growth Factor β (TGFβ)

It was important to find the effect of TGFβ (a growth factor known for its diverse functions under normal and malignant conditions) on the growth response of the two cell lines on both plastic and EHS. A time course experiment was performed with varying concentrations of TGFβ 0.01-1.0 ng/ml. MDA cells grown on EHS (Fig 17) showed an inhibition which varied with time and concentrations. In the presence of the lowest concentration (0.01ng/ml) inhibition reached about 20% after 24 and 48 hours. The observed effect approaches a bell-shaped curve because the inhibitory effects were decreased with increased doses. When cells were exposed to 1ng/ml, only 5% inhibition was observed after 48 hours. The same dose after 72 hours did not show any inhibitory effect. When grown on plastic, the MDA cell line showed the concentration dependent inhibition over the time (Fig 18). The most pronounced decrease in growth rate (about 25%) was observed after 24 hours in the presence of 1 ng/ml. It is not known whether use of higher doses of TGFβ would have led to another bell-shaped curve. Careful monitoring of the cells showed that the inhibitory effects were lost after 48 and 72 hours with 0.1 ng/ml as compared to control values though the significance of this is not clear. The higher concentrations 0.3-1.0 ng/ml remained inhibitory.

In contrast, MCF-7 cells, on EHS, (Fig 19) showed a time and concentration dependent small increase in the growth rate in response to TGFβ. The maximum increase of only 15% in proliferation was observed in the presence of 0.3 ng/ml, after 24 hours. The later time points showed an inhibitory effect on plastic of TGFβ on MCF-7 cells at lower concentrations. MCF-7 cells, on plastic, surprisingly showed a time dependent increase in proliferation with even the lowest concentration of 0.01 ng/ml. This increase rose to about 2 fold after 24 hours (Fig 20). When cells were exposed to higher concentration of

1ng/ml, there was a reduction in the growth rate but it remained 50% above control. After 48 and 72 hours the growth rate dropped.

Conclusions

Figures 17 (EHS) and 18 (plastic) show the dose response curve for MDA cells in response to TGFβ. MDA cells were growth inhibited by TGFβ with maximum inhibition at 0.01ng/ml on EHS. The higher doses showed little inhibition on EHS but significantly inhibited growth on plastic.

Figures 19 (EHS) and 20 (plastic) show the dose response curve of MCF-7 cells in response to TGFβ. MCF-7 cells initially showed a growth stimulation (at 24h) but there was a little inhibition on EHS at the lowest doses of TGFβ at later time points. Whereas growth stimulation was observed with all doses on plastic.

The effects of TGFβ on MDA cells reflected those of TGFα in that effects were seen at lower doses on EHS compared with those cells grown on plastic. On EHS, the inhibition curve approached the expected bell shape. However the stimulatory effect of TGFβ on MCF-7 cells, especially over 24h on plastic, was most unexpected and although completely reproducible in these studies, contrasts with previous observations in the literature.

Figure 17 Cell proliferation under varying stimulation of TGFβ on EHS in MDA-MB-231 cells.

MDA cells (2000 per well) were plated in 96 well plates. After overnight incubation, the routine medium was replaced with experimental medium with 1% DHIDCCFCS only (control) or supplemented with doses of TGFβ (0.01, 0.03, 0.1, 0.3 and 1 ng/ml). After 24, 48 and 72 hours proliferation was evaluated by MTT as described in "materials and methods" section 2.4.1. Results are expressed in percent of control value. *$p < 0.001$ and •$p < 0.05$. The experiments were performed in triplicate 8-96 wells. Results are mean ± SD.

Figure 18 Cell proliferation under varying stimulation of TGFβ on plastic in MDA-MB-231 cells.

MDA cells (2000 per well) were plated in 96 well plates. After overnight incubation, the routine medium was replaced with experimental medium with 1% DHIDCCFCS only (control) or supplemented with doses of TGFβ (0.01, 0.03, 0.1, 0.3 and 1 ng/ml). After 24, 48 and 72 hours proliferation was evaluated by MTT as described in "materials and methods" section 2.4.1. Results are expressed in percent of control value. *$p < 0.001$ and •$p < 0.05$. The experiments were performed in triplicate 8-96 wells. Results are mean ± SD.

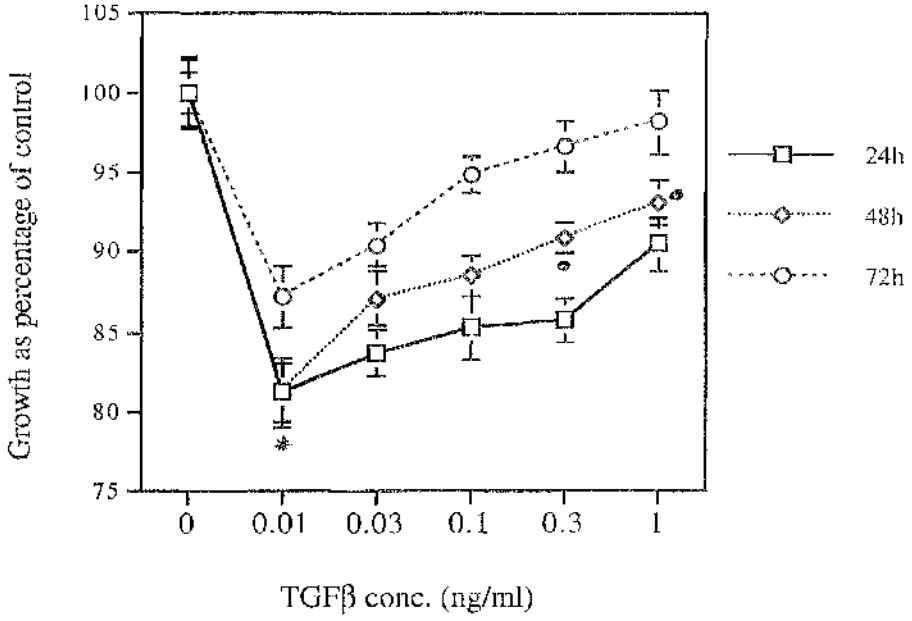


Fig: 17 MDA proliferation on EHS in response to TGFβ

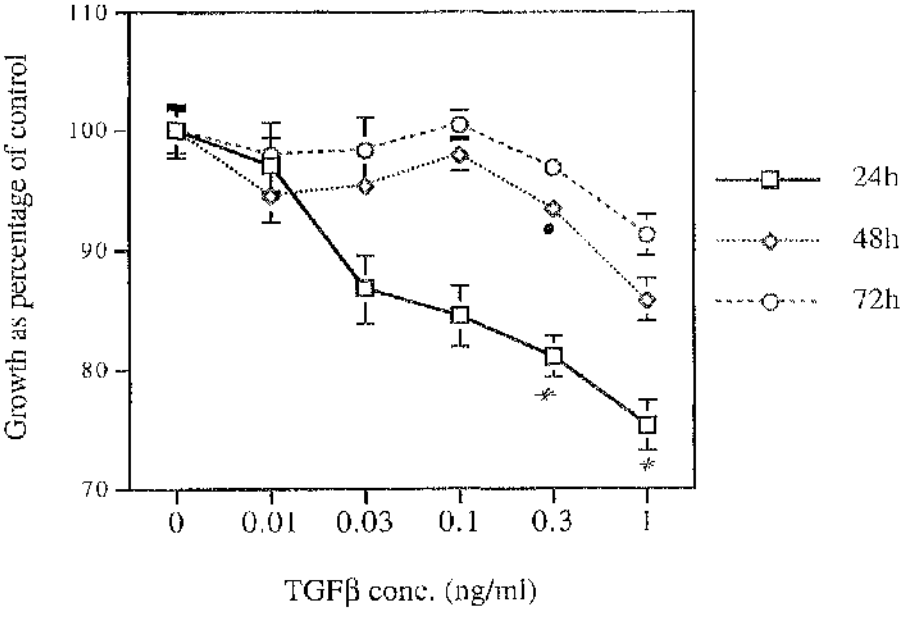


Fig: 18 MDA proliferation on plastic in response to TGFβ

Figure 19 Cell proliferation under varying stimulation of TGFβ on EHS in MCF-7 cells.

MCF cells (2000 per well) were plated in 96 well plates. After overnight incubation, the routine medium was replaced with experimental medium with 1% DHIDCCFCS only (control) or supplemented with doses of TGFβ (0.01, 0.03, 0.1, 0.3 and 1 ng/ml). After 24, 48 and 72 hours proliferation was evaluated by MTT as described in "materials and methods" section 2.4.1. Results are expressed in percent of control value. *$p < 0.001$ and •$p < 0.05$. The experiments were performed in triplicate 8-96 wells. Results are mean ± SD.

Figure 20 Cell proliferation under varying stimulation of TGFβ on plastic in MCF-7 cells.

MCF cells (2000 per well) were plated in 96 well plates. After overnight incubation, the routine medium was replaced with experimental medium with 1% DHIDCCFCS only (control) or supplemented with doses of TGFβ (0.01, 0.03, 0.1, 0.3 and 1 ng/ml). After 24, 48 and 72 hours proliferation was evaluated by MTT as described in "materials and methods" section 2.4.1. Results are expressed in percent of control value. *$p < 0.001$ and •$p < 0.05$. The experiments were performed in triplicate 8-96 wells. Results are mean ± SD.

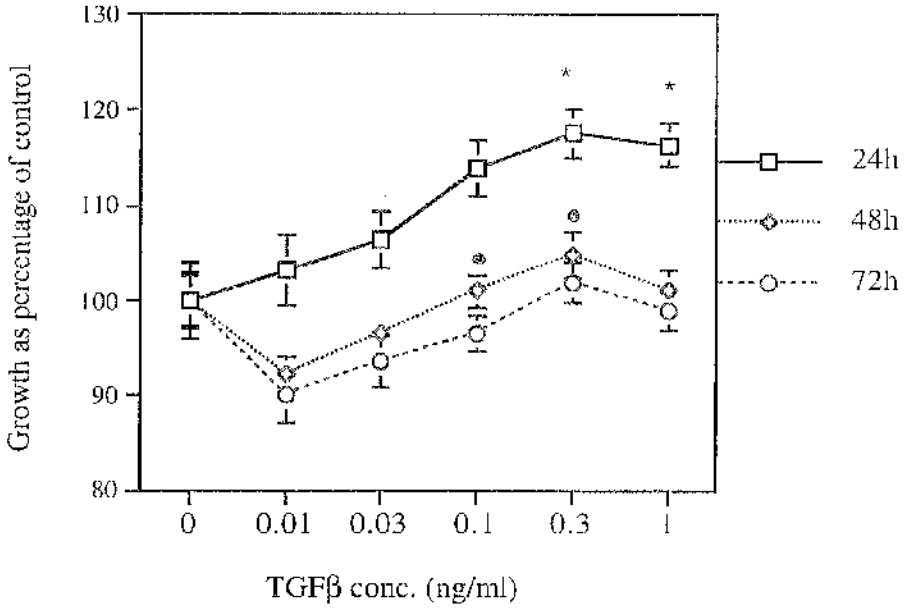


Fig: 19 MCF-7 proliferation on EHS in response to TGFβ

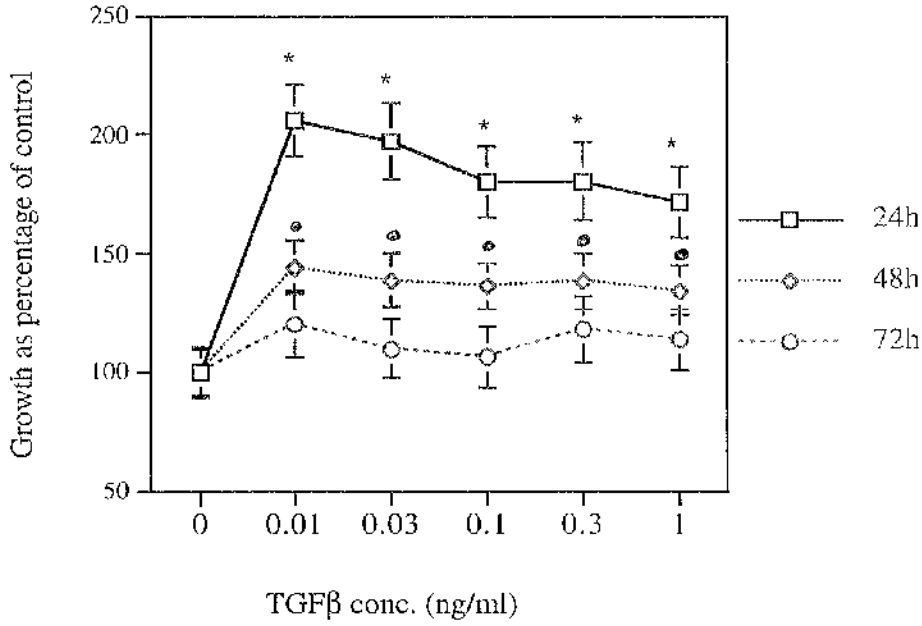


Fig: 20 MCF-7 proliferation on plastic in response to TGFβ

3.1.4 Estradiol (E2)

It has been shown previously that E2 stimulates the growth of ER^+ cell lines on plastic (Dickson *et al.*, 1990). In order to compare its effect on the growth rate of the MCF-7 cell line on plastic and matrigel (EHS), experiments were carried out under identical experimental conditions. Concentrations of E2 from10^{-12} to 10^{-6}M, were used. As expected, E2 increased the rate of proliferation with increased doses. On EHS (Fig 21), E2 appeared to stimulate the growth in a similar fashion to that on plastic though the absolute level of stimulation was less than that observed on plastic (Fig 22). The increase in growth rate was concentration dependent with a significant increase in growth (23%) being observed with 10^{-12} M estradiol on both substrata. Higher doses (10^{-8} M and greater) reduced the growth rate on both EHS and plastic. The overall effect of E2 on the rate of proliferation was stimulatory.

Conclusions

Figures 21 (EHS) and 22 (plastic) show the dose response curve for MCF-7 cells. MCF-7 cells were growth stimulated by E2 with maximum stimulation at 10^{-10}-10^{-9}M concentrations on both EHS and plastic, though the magnitude of stimulation on plastic was about 3 times that on EHS.

Thus the nature of the substratum had no effect on the type of response to oestradiol but the extent of growth stimulation was much greater on plastic than EHS.

Figure 21 Cell proliferation under varying stimulation of estradiol (E2) on EHS in MCF-7 cells.

MCF cells (2000 per well) were plated in 96 well plates. After overnight incubation, the routine medium was replaced with experimental medium with 1% DHIDCCFCS only (control) or supplemented with doses of E2 (10^{-12}, 10^{-10}, 10^{-9}, 10^{-8} and 10^{-6} M). After 24, 48 and 72 hours proliferation was evaluated by MTT as described in "materials and methods" section 2.4.1. Results are expressed in percent of control value. *$p < 0.001$ and •$p < 0.05$. The experiments were performed in triplicate 8-96 wells. Results are mean ± SD.

Figure 22 Cell proliferation under varying stimulation of estradiol (E2) plastic in MCF-7 cells.

MCF cells (2000 per well) were plated in 96 well plates. After overnight incubation, the routine medium was replaced with experimental medium with 1% DHIDCCFCS only (control) or supplemented with doses of E2 (10^{-12}, 10^{-10}, 10^{-9}, 10^{-8} and 10^{-6} M). After 24, 48 and 72 hours proliferation was evaluated by MTT as described in "materials and methods" section 2.4.1. Results are expressed in percent of control value. *$p < 0.001$ and •$p < 0.05$. The experiments were performed in triplicate 8-96 wells. Results are mean ± SD.

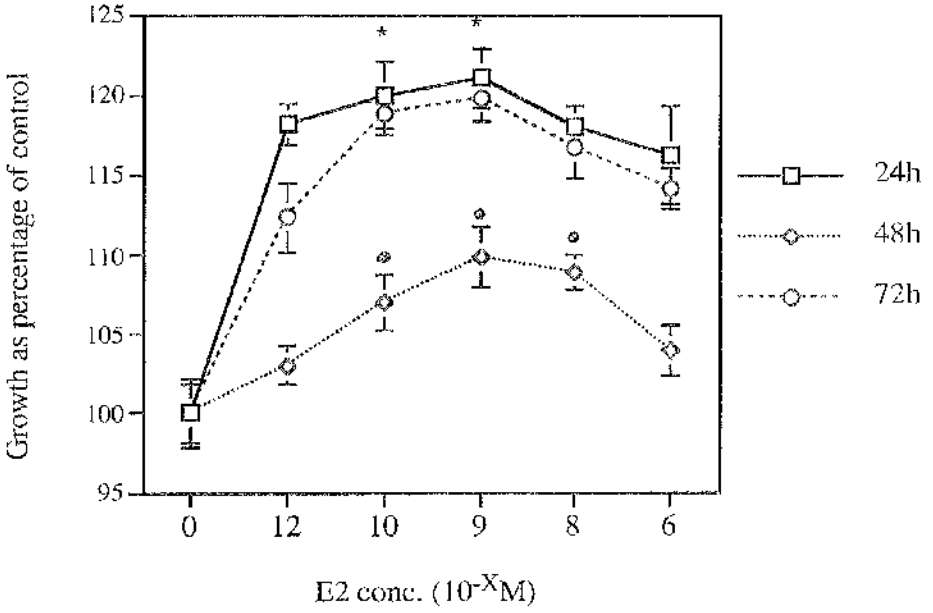


Fig: 21 MCF proliferation on EHS in response to Estradiol

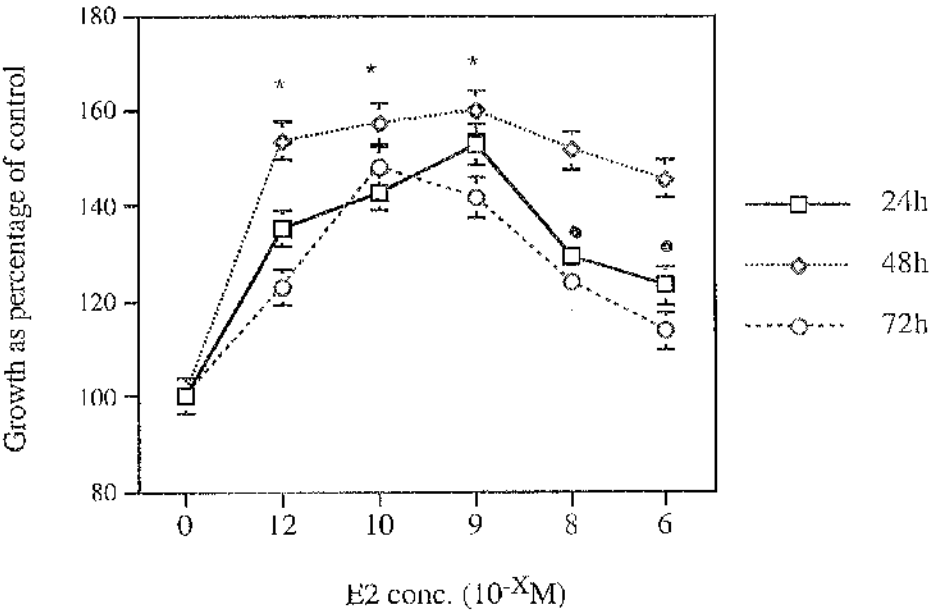


Fig: 22 MCF proliferation on plastic in response to Estradiol

3.1.5 Progesterone (Pg)

Progesterone mediates its effect through its receptor, PgR. Like E2, it is also known to stimulate the growth of hormone responsive breast cancer cell lines when cultured on plastic substratum. In this study, the effects on MCF-7 cell line were studied in response to varying amounts of Pg on either EHS or plastic.

When grown on EHS, the MCF-7 cells showed a time dependent increase in their growth. The most dramatic stimulation was observed after 72 hours (34%) at 10^{-12} M concentration, as compared to the control values (Fig 23). When higher amounts were used a gradual decrease in growth rate was observed at all time points.

On plastic, MCF-7 cells showed a gradual increase in proliferation after 24, 48 and 72 hours. The most dramatic increase by about 100% was observed after 48 hours (Fig 24). Increasing the concentration of Pg to 10^{-8} M or higher caused a slight decrease in proliferation. Thus no major differences were observed between the growth response curves to progesterone when MCF-7 cells were grown on plastic or EHS.

Conclusions

Figures 23 (EHS) and 24 (plastic) show the dose response curve of MCF-7 cells in response to progesterone. The growth reponse of MCF-7 cells towards progesterone was similar on EHS and plastic in that both were growth stimulated at lower doses and the magnitude of stimulation observed on EHS was less than that on plastic.

Figure 23 Cell proliferation under varying stimulation of progesterone (Pg) on EHS in MCF-7 cells.

MCF cells (2000 per well) were plated in 96 well plates. After overnight incubation, the routine medium was replaced with experimental medium with 1% DHIDCCFCS only (control) or supplemented with doses of Pg (10^{-12}, 10^{-10}, 10^{-9}, 10^{-8} and 10^{-6} M). After 24, 48 and 72 hours proliferation was evaluated by MTT as described in "materials and methods" section 2.4.1. Results are expressed in percent of control value. *$p < 0.001$ and •$p < 0.05$. The experiments were performed in triplicate 8-96 wells. Results are mean ± SD.

Figure 24 Cell proliferation under varying stimulation of progesterone (Pg) plastic in MCF-7 cells.

MCF cells (2000 per well) were plated in 96 well plates. After overnight incubation, the routine medium was replaced with experimental medium with 1% DHIDCCFCS only (control) or supplemented with doses of Pg (10^{-12}, 10^{-10}, 10^{-9}, 10^{-8} and 10^{-6} M). After 24, 48 and 72 hours proliferation was evaluated by MTT as described in "materials and methods" section 2.4.1. Results are expressed in percent of control value. *$p < 0.001$ and •$p < 0.05$. The experiments were performed in triplicate 8-96 wells. Results are mean ± SD.

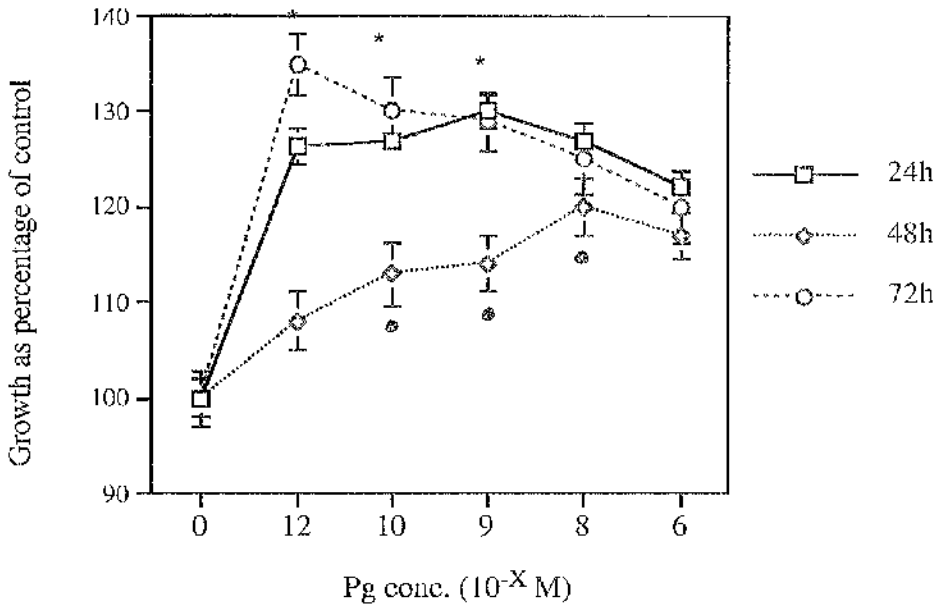


Fig: 23 MCF proliferation on EHS in response to Progesterone

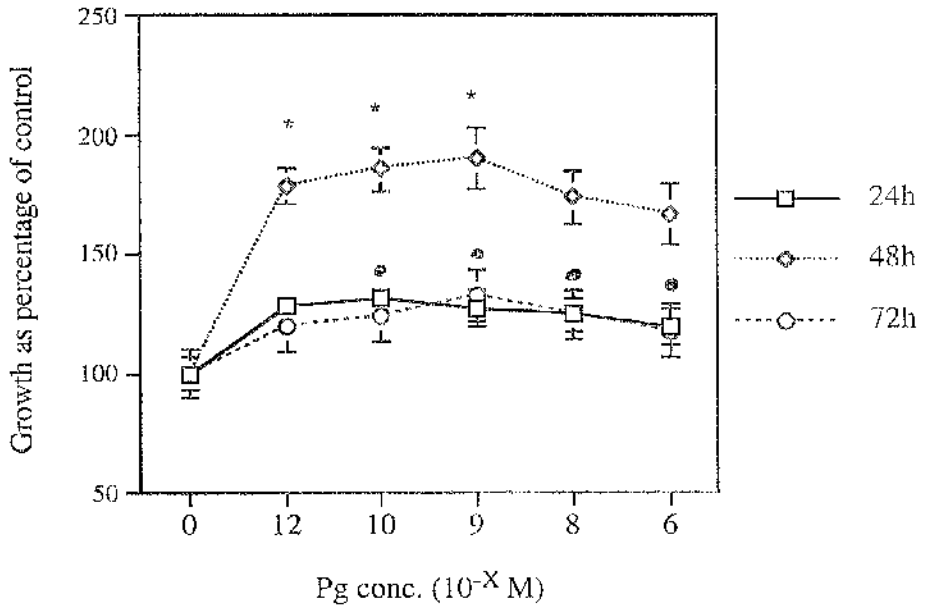


Fig: 24 MCF proliferation on plastic in response to Progesterone

3.1.6 Tamoxifen (TAM)

The "antiestrogen" tamoxifen is known to be a weak oestrogen agonist for breast epithelial cells though it will act as an antagonist in the presence of estradiol. The effect of TAM on MCF-7 was analyzed on both EHS and plastic. Different doses of TAM ranged from 10^{-12}-10^{-6}M.

On EHS, MCF-7 cells when exposed to 10^{-9}M tamoxifen showed an increase of about 60% after 72 hours as compared to the control values (Fig 25). Overall, TAM stimulated the growth rate in a concentration dependent manner. On plastic, MCF-7 showed a similar concentration dependent increase in proliferation (Fig 26). A significant increase was observed after 24 hours (30%) and reached a peak around 48 hours at all doses tested. A maximum increase of 72% was observed when exposed to 10^{-8}M.

Conclusions

Figures 25 (EHS) and 26 (plastic) show the dose response of MCF-7 cells to tamoxifen. As observed with estradiol and progesterone, tamoxifen stimulated the growth of MCF-7 cells. A similar dose response curve on both substrata was observed, though the difference in maximum stimulation between the two surfaces was much less marked with tamoxifen.

The growth studies *in vitro* may somehow correspond to *in vivo* like situation but caution may be taken in *In vitro* studies with growth factors since changes in their environment may affect their behaviour.

Figure 25 Cell proliferation under varying stimulation of tamoxifen (TAM) on EHS in MCF-7 cells.

MCF cells (2000 per well) were plated in 96 well plates. After overnight incubation, the routine medium was replaced with experimental medium with 1% DHIDCCFCS only (control) or supplemented with doses of TAM (10^{-12}, 10^{-10}, 10^{-9}, 10^{-8} and 10^{-6} M). After 24, 48 and 72 hours proliferation was evaluated by MTT as described in "materials and methods" section 2.4.1. Results are expressed in percent of control value. *$p < 0.001$ and •$p < 0.05$. The experiments were performed in triplicate 8-96 wells. Results are mean ± SD.

Figure 26 Cell proliferation under varying stimulation of tamoxifen (TAM) on plastic in MCF-7 cells.

MCF cells (2000 per well) were plated in 96 well plates. After overnight incubation, the routine medium was replaced with experimental medium with 1% DHIDCCFCS only (control) or supplemented with doses of TAM (10^{-12}, 10^{-10}, 10^{-9}, 10^{-8} and 10^{-6} M). After 24, 48 and 72 hours proliferation was evaluated by MTT as described in "materials and methods" section 2.4.1. Results are expressed in percent of control value. *$p < 0.001$ and •$p < 0.05$. The experiments were performed in triplicate 8-96 wells. Results are mean ± SD.

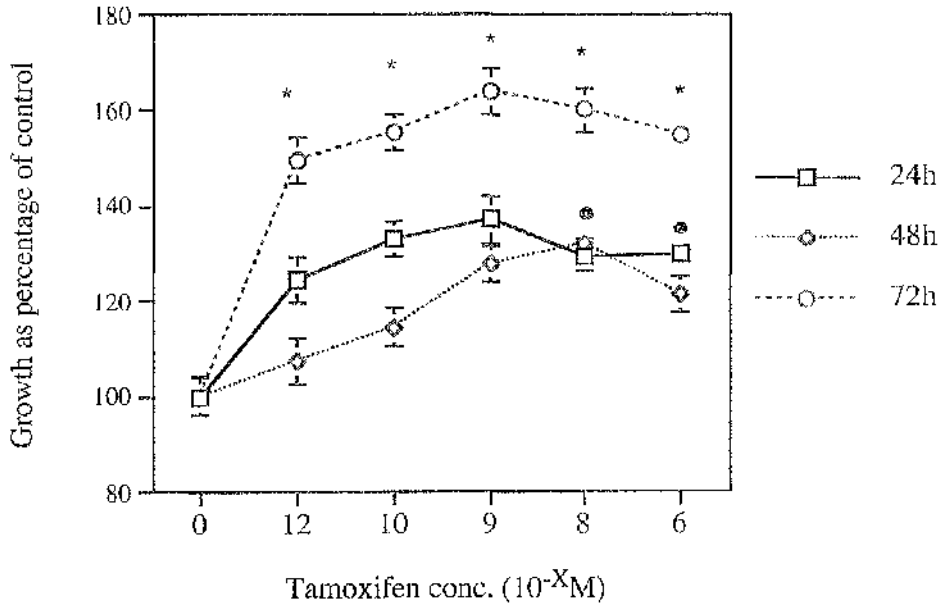


Fig: 25 MCF proliferation on EHS in response to Tamoxifen

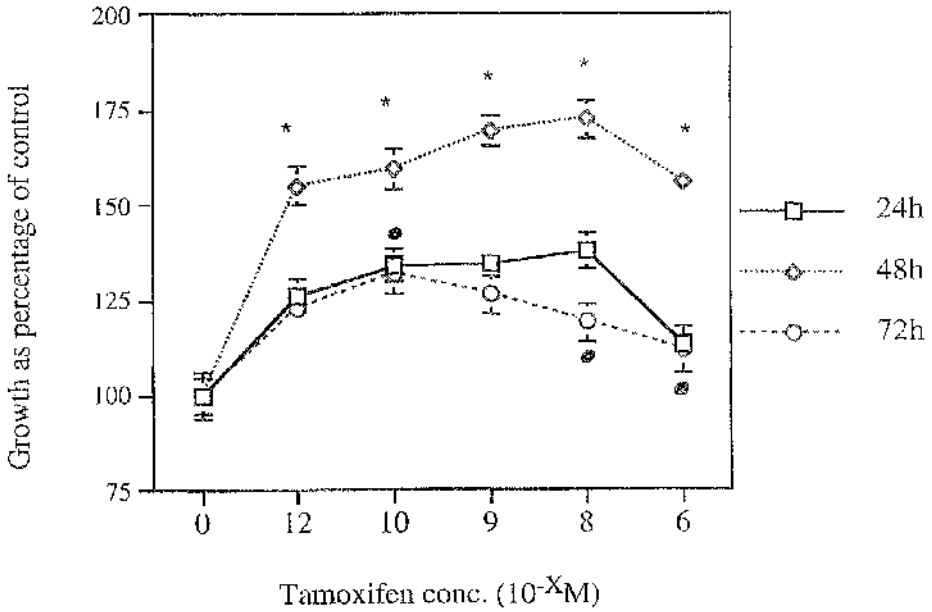


Fig: 26 MCF proliferationon plastic in response to Tamoxifen

3.1.7 Discussion

Effect of Growth Factors on the Growth of MCF-7 and MDA-MB-231 Breast Cancer Cell Lines

Breast cancer cell proliferation and differentiation are associated directly or indirectly with several growth factor families including the epidermal growth factor family (EGF), the fibroblast growth factor (FGF) family, insulin like growth factors (IGF) and transforming growth factors β, TGFβ. The present study was undertaken to determine the effect of different doses of TGFα, EGF and TGFβ on cell growth in hormone dependent and hormone insensitive epithelial cell system using both EHS and plastic.

TGFα, like EGF, is mitogenic for normal and malignant breast epithelial cells. The growth of MDA-MB-231 cells on EHS, slightly inhibited by TGFα at low doses. MDA cells were, otherwise, gently stimulated by higher doses of TGFα, irrespective of substratum. In this study TGFα was found to have a mitogenic influence on MCF-7 cells in culture, irrespective of the nature of substratum though lower doses may be more effective on EHS.

EGF treated MDA cells had an opposite effect of doses when grown on EHS as compared to plastic. On EHS, a low concentration of EGF was inhibitory. Gradual increase of EGF dosage however led to an increase in growth which reached a maximum of 17%. Maximum stimulation was observed on plastic at low concentrations whereas no significant effect, as compared to the control, was observed at higher EGF concentrations.

It had previously been observed that EGF at low or very high concentration had only minor effects on the growth of MDA-MB-231 cells on plastic. Murayama & Mishima (1995) have also confirmed the inhibitory action of high concentration of EGF on breast cancer, oesophageal and gastric cancer cells. Long & Rose (1996) have shown no effect of EGF on MDA-MB-231

cell growth using the same range of concentrations as were used in our experiments. Further Veber Nelal also showed that under standard culture conditions MDA cells, which possess large amounts of EGFR on the cell surface, do not respond to EGF (Verber *et al.*, 1994; Jarrard *et al.*, 1994) Carruba *et al.*, (1994) showed that EGF did not influence the growth of prostate epithelial carcinoma cell line PC3.

The growth response of MCF-7 to low concentrations of EGF was stimulatory both on EHS and plastic with the maximum stimulation being 1.6 fold on EHS (0.63 ng/ml) and 2 fold on plastic (at 1.89 ng/ml). This observation is keeping with (Cappelleti *et al.*, 1993) who reported a 1.6-1.8 fold increase in growth of MCF-7 cells on plastic although at an EGF concentration that was 5 times higher than that used here. Surprisingly exposure of MCF-7 cells to gradually higher doses of EGF (i.e. from 1.89 to 63 ng/ml, in three increments) led to a linear decrease in growth on plastic but had the opposite effect on EHS. In this experiment, there was no significant difference in the maximum stimulation in growth of MCF-7 cells by EGF whether they were grown on EHS or plastic.

Transforming growth factor β (TGFβ) is a potent growth inhibitor for normal epithelial cells. Both ER positive and negative cells contain TGFβ receptors and are growth inhibited by both TGFβ1 and TGFβ 2 (Dickson *et al.*, 1990). The escape of malignant transformed cells from regulation by TGFβ may be an important stage in tumour development. The inhibitory effect of TGFβ on the growth of MDA cell line, has been shown previously by other groups (Desruisseau *et al.*, 1996). However, the effect of TGFβ on cells grown on matrigel has not been studied. MDA cells grown on EHS displayed the classic inverse bell-shaped curve in response to treatment of TGFβ after 24, 48, and 72 hours. Maximum decrease was seen at 0.01 ng/ml at all three time points while higher concentrations did not appear to have much inhibitory effect on

growth on EHS. On plastic, these cells underwent growth inhibition at high concentrations but no effect was observed at lower concentrations.

Unexpectedly, MCF-7 cells on the other hand, showed a dose-dependent increase in growth on EHS over 24 hours which was much more pronounced with cells grown on plastic. The fact that TGFβ stimulates growth of MCF-7 cells is really surprising given the plethora of literature which indicates without exception that this molecule has an inhibitory role in cell proliferation (Ying & Zhang, 1996; Welch *et al.*, 1990; Dickson *et al.*, 1990). However, the TGFβ preparations used were the same as those which growth inhibition in MDA cells and the sub-clone of MCF-7 used has given expected results in other conditions. Some recent studies have shown a cell-density and serum dependent sensitivity to TGFβ in MATLyLu (Morton & Barrack, 1995). The authors have shown that at high density and in the presence of serum (0.5%), cell growth was insensitive to TGFβ while in the absence of serum and low density, growth was inhibited in response to TGFβ. The escape of inhibitory action of MCF-7 cells in reponse to TGFβ may therefore be due to the presence of serum in the medium.

Growth Response of Breast Cancer Cells to Steroid Hormones and Anti-Oestrogens

Steroid hormones play an important role in the control of growth and development of normal mammary gland and breast cancer progression. Therefore, the growth response of hormone receptor positive MCF-7 cell line to estradiol, progesterone and an anti-oestrogen tamoxifen was evaluated on both plastic and EHS. It is known that oestrogen stimulates the growth of oestrogen receptor positive breast cancer cells like MCF-7 and ZR75 (Zajchowski *et al.*, 1993) (Dickson *et al.*, 1989). However, these early

observations were achieved on plastic only. In the current studies the effect of estradiol (E2) was observed on both kinds of substrata. The growth rate on the two surfaces were comparable. Oestrogen, as expected, showed stimulation in the proliferation of MCF-7 cells both on plastic and EHS. The dose response curve was similar on both the surfaces. On plastic, significant stimulation of about 1.6 fold was observed with 10^{-9}M concentration, while the same dose showed about 1.25 fold stimulation on EHS indication of nearly 3 fold difference in response. Others e.g. (Cappelleti *et al.*, 1993) have found an increase in proliferation of MCF-7 cells on plastic. Similarly, (Masamura *et al.*, 1995) have shown that wild type MCF-7 cells respond maximally to 10^{-8} M $E2$, while Cappelleti *et al.* (1993) have observed a 1.5-fold increase in proliferation with 10^{-8} M E_2. Progesterone also increased the growth rate of MCF-7 cells on both substrata. 10^{-12} M progesterone gave maximum stimulation of about 1.7 and 1.3 fold after 48 hours on plastic and EHS, respectively.

TAM is known to display both agonistic and antagonistic effects (Katzenellenbogen *et al.*, 1995) and exert its agonistic effect through the oestrogen receptor (ER). Bliss has shown that the agonistic effect of TAM may be due to the ability of this anti-oestrogen to dimerize the oestrogen receptor necessary for gene transcription (Bliss *et al.*, 1996). In our experiments, TAM showed a dose dependent stimulatory effect on MCF-7 cells on both plastic and EHS. 10^{-8} and 10^{-9} M TAM gave the maximum growth between 1.7-1.65 fold on plastic and EHS, respectively. These results are corroborated by (Cabot *et al.*, 1995) and (Dickson *et al.*, 1990) who have described an increase in proliferation with TAM. (Grondahl-Hansen *et al.*, 1988) also have shown no inhibition of growth by anti-oestrogens. TAM has no growth effect on the MDA-MB-231 cells (data not shown).

3.1.8 Summary

In this chapter we have examined the effects of various growth factors and hormones on the two cell lines, MDA and MCF-7, plated on both EHS and plastic. The aforegoing results unequivocally demonstrate that the growth of cells in response to certain growth factors or hormones vary depending on the substratum. For instance, as discussed above, when MDA and MCF-7 cells were treated with EGF and TGFα, on either plastic or EHS, the pattern of proliferation was different. MCF-7 cells were more responsive to lower doses of growth factors on EHS. TGFβ showed significant differences in the growth pattern, depending on the concentrations used, between the two substrata.

In contrast to growth factors, steroid hormones used in this study, had comparable effects on both plastic and EHS. It is clear therefore, from this and other examples, that the growth of a given cell line can either be enhanced or inhibited depending upon the surface on which it is grown. These results suggest that in addition to hormones and growth factors the surface is also a co-determinant of a cell line's potential for growth.

3.2 PLASMINOGEN ACTIVATORS

3.2.1 Determination of uPA, PAI-1 and uPAR mRNA levels in MDA and MCF-7 cells exposed to various hormones and growth factors

In order to monitor the respective amounts of the uPA, PAI-1 and uPAR mRNA from MDA and MCF-7, cells treated with TGFα or TGFβ, and EGF were then processed using reverse transcriptase polymerase chain reaction (RT-PCR) (Tables 2 & 3). Since it is technically very difficult to use this technique quantitatively, it has been used in this study as a qualitative yardstick for the presence or absence of a certain mRNA species. Total RNA was isolated from each of the cell lines which had been treated with various doses of hormones and growth factors and subsequently converted into cDNA. Primers specific for each of the three genes, as well as that for β-actin, which was used as a positive control, were used in a PCR reaction and the products analysed on an agarose gel. It should be noted that the identity of all four PCR products was established by cloning and analysis of the recombinant clones by DNA sequencing (data not shown).

The MDA cell line has been well characterised by several groups and it has been shown to be highly invasive *in vitro* (Long & Rose, 1996). This is most likely due to the fact that it expresses all the components essential for invasiveness. RT-PCR analysis of total RNA obtained from untreated MDA cells showed that, in contrast to the MCF-7 cells, it contained some uPAR and larger amounts of uPA and PAI-1 mRNAs. Exposure of MDA cells to 1 ng/ml of EGF, TGFα or β, had no net effect on the levels of uPA and PAI-1 mRNA relative to the control. Treatments with growth factors, however, increased the uPAR transcript as compared to the control (Fig. 27B).

In spite of numerous attempts no uPA, PAI and uPAR mRNA could be detected in the untreated MCF-7 cell line (Fig. 28A). The possibility that the

three mRNAs were not found because of RNA degradation can be eliminated given that the mRNA for β-actin, which was used as a positive control, was detected successfully using this protocol. As a further control, PCR primers for β-actin failed to produce a band of the expected size for amplified genomic DNA when the purified RNA alone was used in a PCR reaction, suggesting that the purified RNA had not been contaminated with genomic DNA.

Upon exposure of MCF-7 cells to 1 ng/ml of TGFβ, no uPA was found but PAI-1 and uPAR mRNA were found after 48 hours of incubation (Fig.28B). In EGF 1 ng/ml treated cells, no uPA mRNA could be found, as had been observed with the other growth factor. A considerable amounts of uPAR and PAI-1 was observed at 48 hours after EGF addition.

Figure 27 The effect of growth factors on uPA, PAI-1 and uPAR transcripts in MDA-MB-231 cells at different time periods

MDA cells were treated with growth factors TGFα (1 ng/ml), EGF (10 ng/ml) and TGFβ (1 ng/ml) and were incubated for 48h. After exposure to these growth factors, RNA was extracted as described in the methods section 2.11. It was followed by RT-PCR using the primers specific for uPA, PAI-1 and uPAR with the expected 460, 452 and 455 bps, respectively. β-actin was 560 bps The PCR reaction employed 1 minute at 95 ^{O}C, 1 minute at 50 ^{O}C, and 1.5 minute at 72 ^{O}C for 30 cycles. The products were electrophoresed on 1.5 % agarose gel and visualised with ethidium bromide under UV light. The results are representative of three different experiments using RNA from different extractions.

Figure A: Control Cells

Time period	Lane numbers
24h	1, 2, 3, 4
48h	5, 6, 7, 8
72h	9, 10, 11, 12
Marker	M
β-actin	4, 8, 12

Figure B: Treated Cells (48h)

Treatment	Lane numbers
TGFβ	1, 2, 3
EGF	5, 6, 7
TGFα	9, 10, 11
β-actin	4, 8, 12

The lanes in figures (A and B) represent transcripts in the following order:

uPA	1, 5, 9
PAI-1	2, 6, 10
uPAR	3, 7, 11
β-actin	4, 8, 12

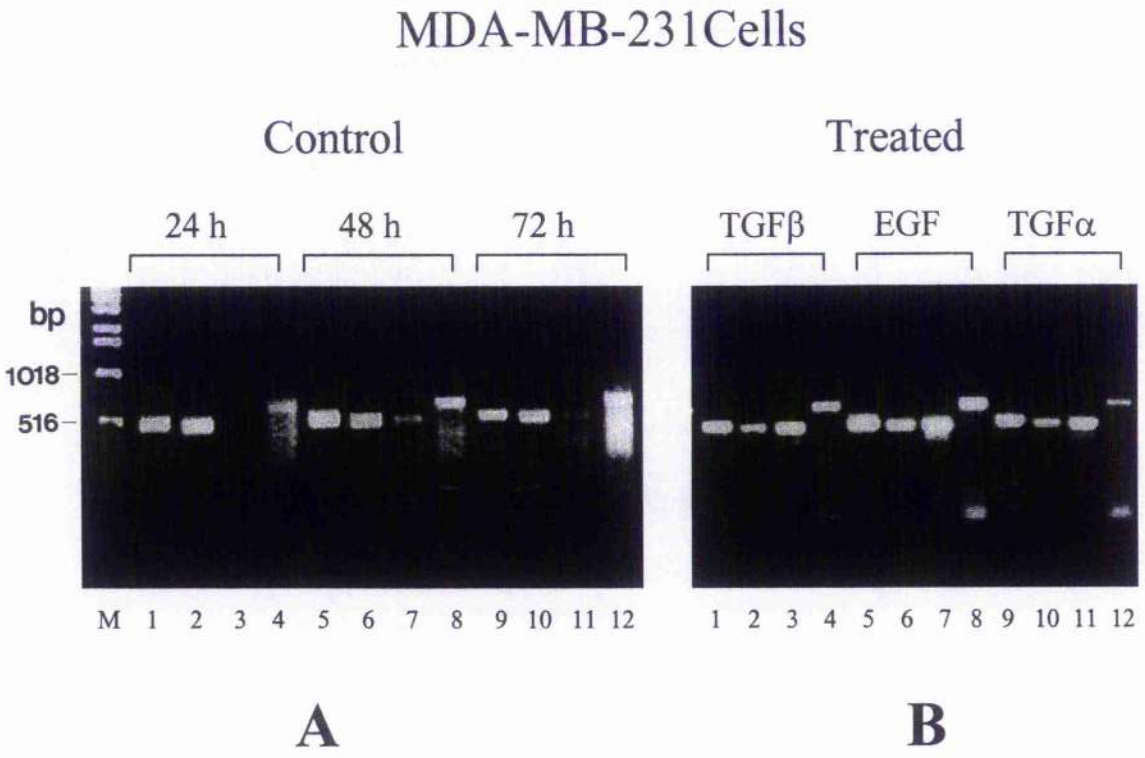

MDA-MB-231Cells
Control
Treated
24 h
48 h
72 h
TGFβ
EGF
TGFα
bp
1018
516
M 1 2 3 4 5 6 7 8 9 10 11 12
1 2 3 4 5 6 7 8 9 10 11 12
A
B

Table 2 The effect of growth factors on mRNA in MDA-MB-231 cells

The effect of growth factors on mRNA in MDA-MB-231 cells are presented in +/- symbols. This table shows the qualitative determination of the message in MDA cells in response to growth factors (ng/ml), TGFβ, EGF, TGFα, as compared to control. Results are the mean of three independent experiments.

Key to symbols: + =present, ++ = abundant

Doses	uPA	PAI-1	uPAR	β-actin
	48h	48h	48h	48h
control	++	++	+	+
TGFβ 1 ng/ml	++	++	++	+
EGF 10 ng/ml	++	++	++	+
TGFα 1 ng/ml	++	++	++	+

Figure 28 The effect of growth factors on uPA, PAI-1 and uPAR transcripts in MCF-7 cells at different time periods

MCF-7 cells were treated with growth factors EGF (10ng/ml) and TGFβ (1 ng/ml) and were incubated for48h. After exposure to these growth factors, RNA was extracted as described in the methods section 2.11. It was followed by RT-PCR using the primers specific for uPA, PAI-1 and uPAR with the expected 460, 452 and 455 bps, respectively. β-actin was 560 bps The PCR reaction employed 1 minute at 95 ^{o}C, 1 minute at 50 ^{o}C, and 1.5 minute at 72 ^{o}C for 30 cycles. The products were electrophoresed on 1.5 % agarose gel and visualised with ethidium bromide under UV light. The results are representative of three different experiments using RNA from different extractions.

Figure A: Control Cells

Time period	Lane numbers
24h	1, 2, 3, 4
48h	5, 6, 7, 8
72h	9, 10, 11, 12
Marker	M
β-actin	4, 8

Figure B: Treated Cells (48h)

Treatment	Lane numbers
TGFβ	1, 2, 3
EGF	5, 6, 7
β-actin	4, 8

The lanes in figures (A and B) represent transcripts in the following order:

uPA	1, 5, 9
PAI-1	2, 6, 10
uPAR	3, 7, 11
β-actin	4, 8, 12

MCF-7 Cells

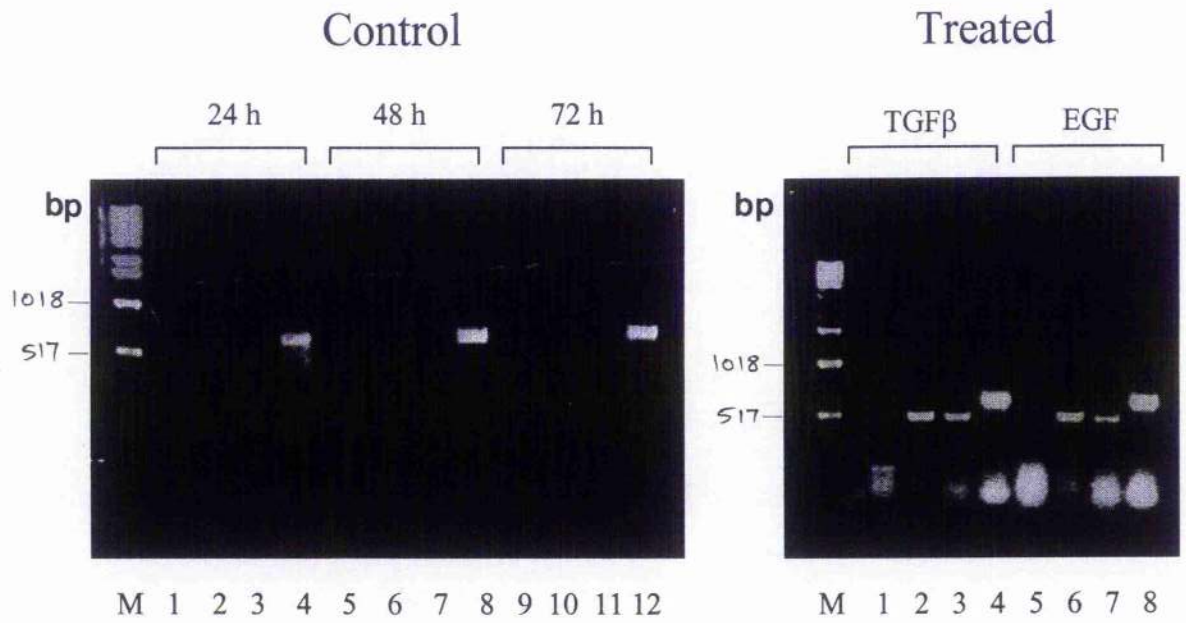

Control
24 h
48 h
72 h
bp
1018
517
M 1 2 3 4 5 6 7 8 9 10 11 12
Treated
TGFβ
EGF
bp
1018
517
M 1 2 3 4 5 6 7 8

Table 3 The effect of growth factors on mRNA in MCF-7 cells are presented in +/- symbols. This table shows the qualitative determination of the message in MCF-7 cells in response to growth factors (ng/ml), TGFβ, EGF as compared to control. Results are the mean of three independent experiments.

Key to symbols: + =present, ++ = abundant

Doses	uPA	PAI-1	uPAR	β-actin
	48h	48h	48h	48h
control	-	-	-	+
TGFβ 1 ng/ml	-	+	++	+
EGF 10 ng/ml	-	+	++	+

3.3 Secreted and Cell Associated Plasminogen Activator Levels

3.3.1 The effect of growth factors on secretion of uPA antigen by the MDA-MB-231 cell line

Transforming Growth Factor α (TGFα)

After treating the cells with 1 ng/ml and 0.1 ng/ml TGFα on EHS and plastic, the amount of secreted uPA was measured in the conditioned medium (CM). On EHS, uPA secretion was not altered by incubation of cells with high or low amounts of TGFα during all time points (Figs 29A, 30A).

On plastic, an initial increase of about 60-70% observed in uPA levels with both 0.1-1 ng/ml TGFα, (Fig 29B) subsequently reduced to control values or showed slight inhibition in uPA levels after 72 hours (Fig 30B).

Epidermal Growth Factor (EGF)

EGF is known to induce the expression of uPA in a culture system on plastic. To compare this with uPA secretion on EHS, MDA cells were exposed to 10 ng/ml and 1 ng/ml of EGF. On EHS, uPA secretion remained lower than the control values, after treating the cells with high or low doses of EGF (Figs 29A, 30A).

On plastic, EGF 1 ng/ml significantly increased uPA secretion up to 104% above control after 48 hours (Fig 29B). The same dose after 72 hours decreased the uPA levels. EGF treatment for longer time did not increase the amounts of uPA, instead uPA levels were reduced by 30% with 1 ng/ml EGF, as compared to control values (Fig 30B).

Taken together the results suggest that the effects of TGFα and EGF on secretion of uPA into the conditioned medium were similar on EHS and plastic; in that both did not effect the antigen amounts on EHS.and increased uPA levels after 48 hours on plastic. The nature of the substratum showed a small transient effect on the secreted uPA levels.

Transforming Growth Factor β (TGFβ)

MDA cells were treated with TGFβ at 1 ng/ml and 0.1 ng/ml. On EHS, a dramatic time and concentration dependent effect was observed when the cells were treated with this growth factor for 48 and 72 hours. TGFβ 1 ng/ml significantly increased uPA levels by 330% after 48 hours and about 250% after 72 hours. (Figs 29A, 30A). The lower concentration of TGFβ 0.1 ng/ml also increased uPA levels by about 105% after 48 and 72 hours (Figs 29A, 30A).

On plastic, when incubated with 1 ng/ml or 0.1 ng/ml for 48 hours, uPA levels increased but still only above control values 58% and 86%, respectively (Fig. 29B). TGFβ did not increase the levels when the cells were treated for 72 hours. The amounts of proteins secreted are shown in the tables 4 and 5.

Taken collectively the results suggest that, whereas on EHS TGFβ increased uPA levels after 48 hours by a very large amount. Further incubation of 72 hours decreased the levels significantly. On plastic, TGFβ potentiates a small increase in uPA levels secreted into CM after 48 hours.

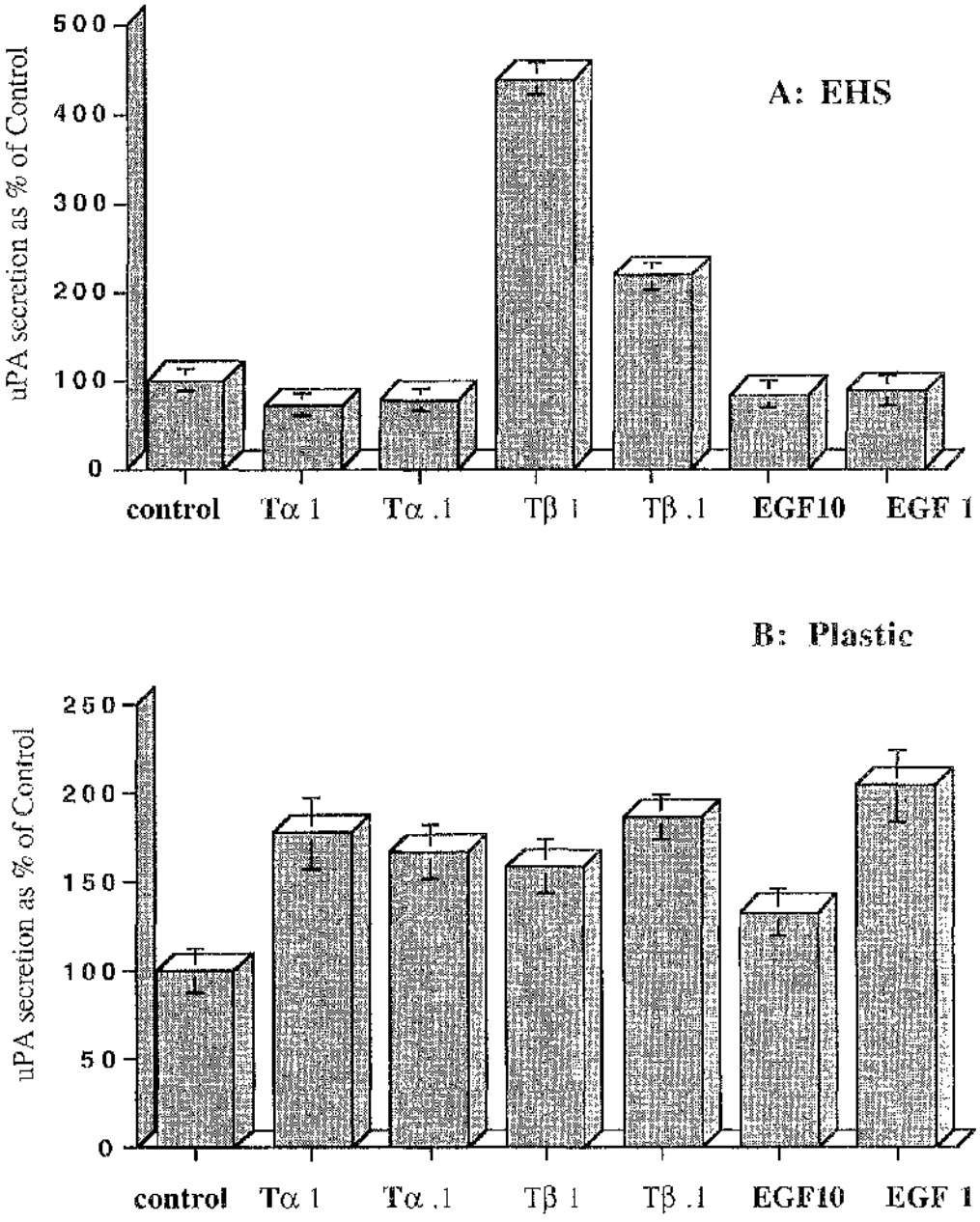


Figure 29 The effect of growth factors on uPA secretion in MDA cells, on both EHS (A) and plastic (B). (48h)

TGFα (1 and 0.1 ng/ml), TGFß (1 and 0.1 ng/ml) and EGF (10 and 1 ng/ml) were used for 48h. uPA levels in the CM. in all experiments were assayed in duplicate using 4-span ELISA (see section 2.7.2). The average of three independent determinations ± SD is presented.

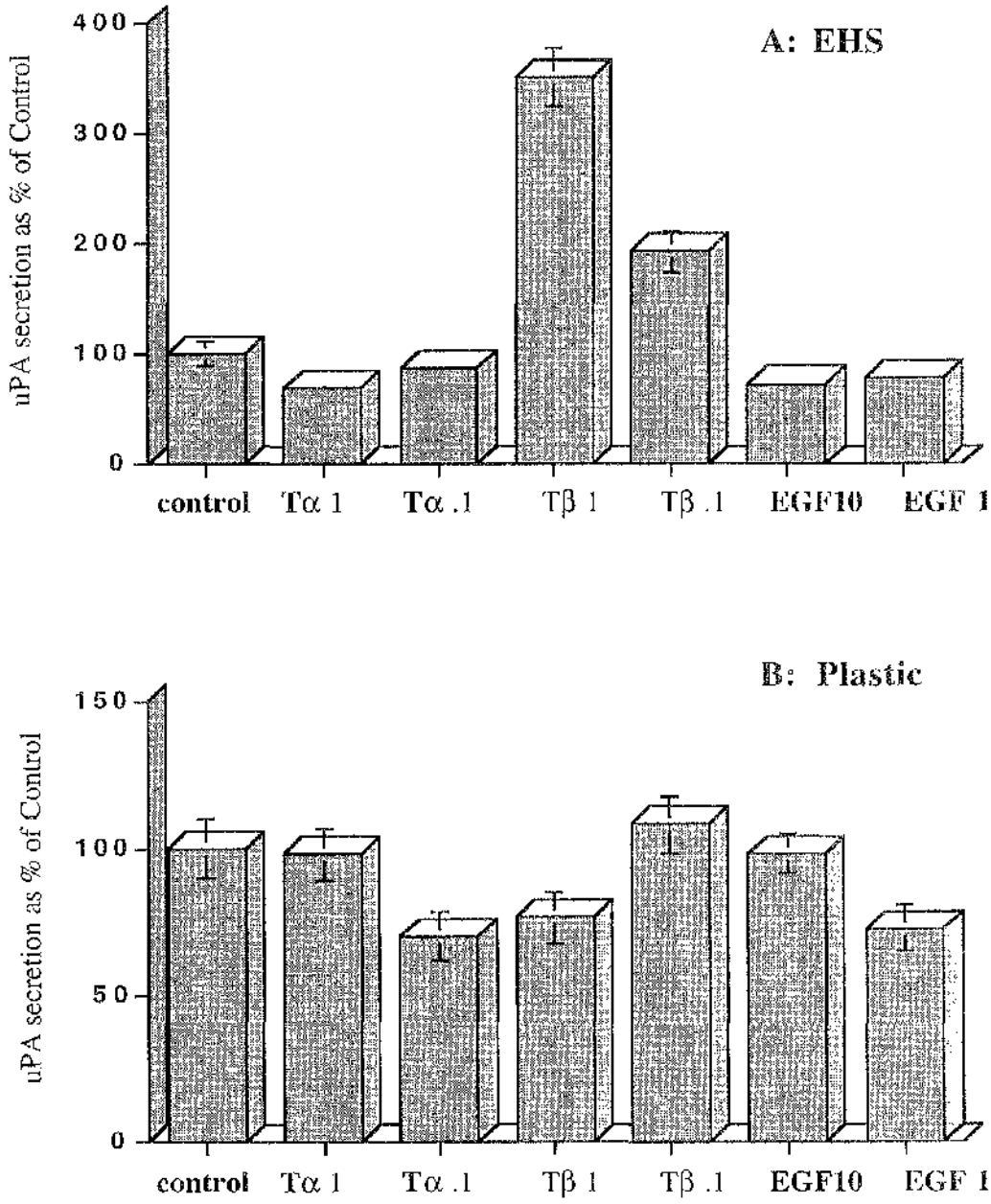


Figure 30 The effect of growth factors on uPA secretion in MDA cells, on both EHS (A) and plastic (B). (72 h)

TGFα (1 and 0.1 ng/ml), TGFß (1 and 0.1 ng/ml) and EGF (10 and 1 ng/ml) were used for 72h. uPA levels in the CM, in all experiments were assayed in duplicate using 4-span ELISA (see section 2.7.2). The average of three independent determinations ± SD is presented.

Table 4 Secreted uPA protein in MDA-MB-231 cells on EHS.

The amounts of uPA protein was measured in the conditioned medium (CM), on EHS in MDA-MB-231 cell line. Values (ng/ml) represents the mean of three independent experiments and each experiment was carried out in duplicate.

Time	Control CM	TGFα 1ng/ml	EGF 1ng/ml	TGFß 1 ng/ml
48h	10.4	8	9.1	45.8
72h	16.6	14.4	12.8	58.4

Table 5 Secreted uPA protein in MDA-MB-231 cells on plastic.

The amounts of uPA protein was measured in the conditioned medium (CM), on plastic in MDA cell line. Values (ng/ml) represent the mean of three independent experiments and each experiment was carried out in duplicate.

Time	Control CM	TGFα 1ng/ml	EGF 1ng/ml	TGFß 1 ng/ml
48h	14	10	9.3	23.1
72h	11.5	11.4	18.1	24.8

3.3.2 The effect of growth factors on cell associated uPA antigen in MDA-MB-231 cell line in cell extracts

uPA is active when bound to its receptor, uPAR on a cell membrane. To find the effects of growth factors on cell associated uPA levels, cells, after removing medium, were washed with PBS and treated with 1% Triton X-100. Cell extracts were prepared as described in method section 2.7.1. Cell supernatants (SN) were then measured for the amounts of uPA antigen in the presence of different concentrations of growth factors at different time points.

TGFα

On EHS, uPA levels remained unchanged after 48 hours when cells were exposed to both doses of TGFα (1 and 0.1ng/ml) as compared to the control values (Fig 31A)) whereas about 70% stimulation was observed with 0.1 ng/ml after 72 hours (Fig 32A). The higher concentration of TGFα did not effect uPA levels at any time point. On plastic, uPA levels remained unchanged after 48 hours with TGFα whereas a small increase, about 26% was observed with TGFα 1 ng/ml (Fig 31B).

Overall, MDA cells treated with 1 ng/ml or 0.1 ng/ml of TGFα showed little change of uPA levels in cell supernatants at either concentration of growth factor.

EGF

EGF, like TGFα, also stimulated uPA levels after longer incubation hours but to a greater extent. On EHS, a concentration and time dependent increase was observed after 48 and 72 hours. EGF 1 ng/ml and 10 ng/ml showed a gradual increase in uPA levels by 39% and 44% after 48 hours (Fig. 31A). The increase (123%) was observed in the presence of 1 ng/ml EGF after 72 hours (Fig 32A).

On plastic, MDA cells in the presence of EGF 10 ng/ml and 1 ng/ml increased uPA levels in cell extracts to 85% and 140% after 72 hours, respectively (Fig 32B). uPA levels remained below the control values in the presence of the same concentrations after 48 hours.

Conclusions

The general effect of EGF, on the levels of uPA antigen, was stimulatory on both the surfaces. However, different time periods were observed for the mediation of induction of uPA. Although an early uPA induction was observed on EHS, a longer time was required by EGF to induce the cell associated uPA on plastic.

It is interesting to note that on EHS lower doses of both TGFα (0.1ng/ml) EGF (1ng/ml) inhibited cellular proliferation, but at the same time increased the amount of uPA.

TGFβ

TGFβ increased both secreted and cell associated uPA levels quite significantly. On EHS, cell associated uPA showed time and concentration dependent stimulation. MDA cells in the presence of 1 ng/ml TGFβ showed significant stimulation by 151% after 48 hours (Fig 31A). This increase remained the same after 72 hours (Fig 32A). On the other hand, treatment of cells with 0.1 ng/ml TGFβ showed a little increase in uPA levels by 36% after 48 hours.

On plastic, MDA cells when incubated with 1 ng/ml and 0.1 ng/ml TGFβ showed significant increase in uPA levels with the greater stimulation at the lower concentration. 0.1 ng/ml TGFβ increased uPA levels by 90% after 48 hours (Fig 31B). The antigen levels were subsequently increased much significantly to 360% after 72 hours incubation (Fig 32B).

Conclusions

The aforementioned results suggest that TGFβ modulates uPA levels in cell extracts according to the nature of the substratum. On EHS, the low dose could only marginally increase uPA antigen but the high dose gave large increase in cell associated uPA levels. On plastic, the lower dose was quite enough to stimulate uPA levels very significantly.

This observation shows that lower concentrations of TGFβ remained either inert or slightly inhibited the growth on plastic, whereas lower doses of TGFβ increased uPA levels significantly. This may be due to the fact that TGFβ may follow a different pathway to stimulate uPA levels than proliferation.

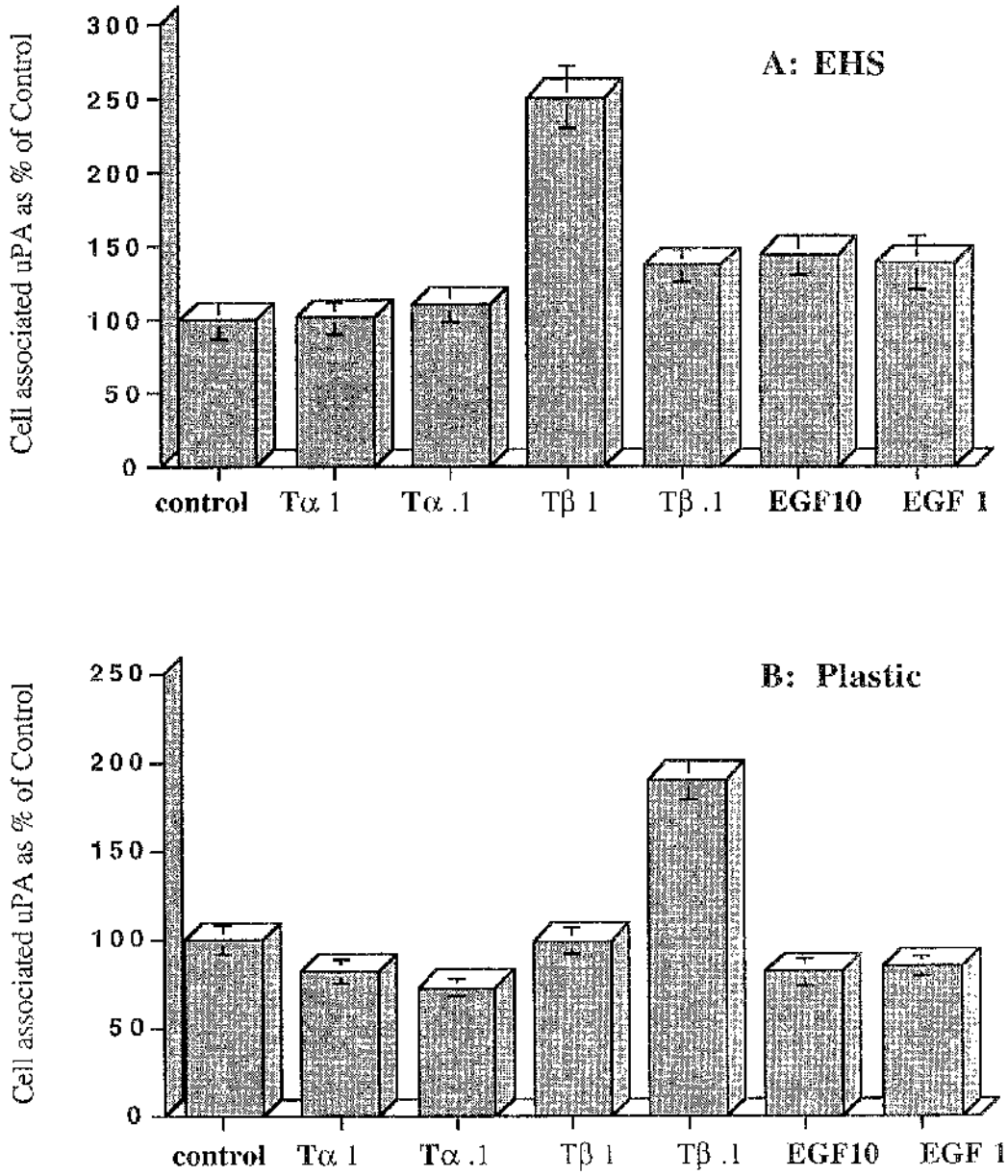


Figure 31 The effect of growth factors on uPA in cell cytosol (SN) in MDA cells, on both EHS (A) and plastic (B). (48 hrs)

TGFα (1 and 0.1 ng/ml), TGFß (1 and 0.1 ng/ml) and EGF (10 and 1 ng/ml) were used for 48h. uPA levels in the SN, in all experiments were assayed in duplicate using 4-span ELISA (see section 2.7.2). The average of three independent determinations ± SD is presented.

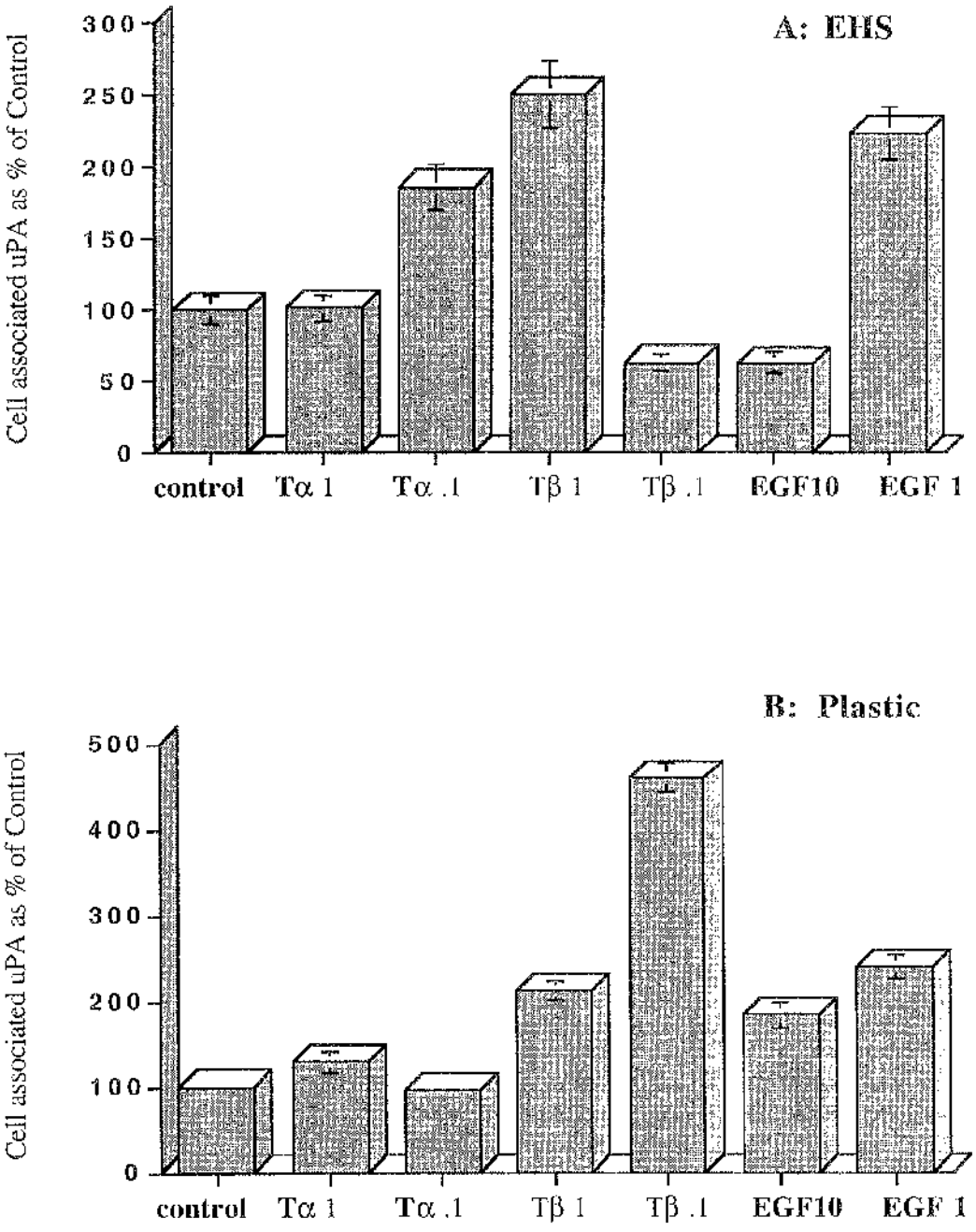


Figure 32 The effect of growth factors on uPA in cell cytosol (SN) in MDA cells, on both EHS (A) and plastic (B). (72 hs)

TGFα (1 and 0.1 ng/ml), TGFβ (1 and 0.1 ng/ml) and EGF (10 and 1 ng/ml) were used for 72h. uPA levels in the SN, in all experiments were assayed in duplicate using 4-span ELISA (see section 2.7.2). The average of three independent determinations ± SD is presented.

Table 6 uPA protein in cell cytosol in MDA cells on EHS.

The amounts of uPA protein was measured in the cell cytosol (SN), on EHS in MDA-MB-231 cell line. Values (ng/mg protein) represents the mean of three independent experiments and each experiment was carried out in duplicate.

Time	Control SN	TGFα 1ng/ml	EGF 1 ng/ml	TGFß 1 ng/ml
48h	13	14.4	18.1	32.7
72h	21.7	40.4	48.6	54.4

Table 7 uPA protein in cell cytosol in MDA cells on plastic.

The amounts of uPA protein was measured in the cell cytosol (SN), on plastic in MDA-MB-231 cell line. Values (ng/mg protein) represents the mean of three independent experiments and each experiment was carried out in duplicate.

Time	Control SN	TGFα 1ng/ml	EGF 1 ng/ml	TGFß 1 ng/ml
48h	21.3	15.4	18	21.1
72h	14.4	16.9	34.6	30.8

3.3.3 The effect of growth factors on secretion of PAI-1 antigen in MDA-MB-231 cell line

uPA may be regulated by many inhibitors, but the most established inhibitor is the plasminogen activator inhibitor type-1 (PAI-1) (see introduction section 1.13.1). Therefore, it was also important to measure this inhibitor in response to various growth factors.

Transforming growth factor α (TGFα)

MDA cells grown on EHS revealed that both doses of TGFα (1 and 0.1 ng/ml) showed no significant alteration of secreted PAI-1 levels at any time points (Figs 33A, 34A). When the same experiment was carried out on plastic, PAI-1 levels were stimulated by about 30% after 48 hours and 67% after 72 hours with 0.1 ng/ml TGFα (Figs 33B, 34B).

TGFα did not alter the secreted PAI-1 levels on EHS but did increase the levels on plastic.

Epidermal growth factor (EGF)

PAI-1 levels were also measured in the MDA cells in response to 10 ng/ml and 1 ng/ml EGF. On EHS, cells exposed to EGF, showed no stimulation of secreted PAI-1 after 48 hours, (Fig 33A) and the levels actually dropped after 72 hours with the 1 ng/ml . On plastic, the high dose of EGF 10 ng/ml slightly increased PAI-1 levels by 18% after 48 hours (Fig 33B). No significant effect was observed at further time point.

The results suggest that EGF, like TGFα, did not have significant effect on secreted PAI-1 levels, on both EHS and plastic.

Transforming growth factor β (TGFβ)

Experiments were designed to determine whether cells cultured on plastic or EHS altered the sensitivity. MDA cells were exposed to 1 ng/ml and 0.1 ng/ml TGFβ. On EHS, the high dose of TGFβ showed a dramatic increase in PAI-1 levels by 379% (Fig 33A) after 48 hours. Susequently the levels were reduced to 105% after 72 hours (Fig 34A). Lower amounts of TGFβ also significantly increased the secretion of PAI-1 levels, although to lesser extent by 123% after 48 hours. On plastic, TGFβ 1 ng/ml showed an increase in PAI-1 levels by 147% (Fig 34B) and 123% with TGFβ 0.1 ng/ml, after 48 hours. This was followed by a subsequent reduction in PAI-1 levels after 72 hours.

The foregoing results showed that the secretion of PAI-1 levels into the conditioned medium in response to TGFβ was concentration and time dependent on both plastic and EHS. On EHS, the high dose stimulated the secretion much more significantly than the lower dose. The extent of secretion was higher on EHS than on plastic. The secretion of PAI-1 levels was reduced with longer incubation time.

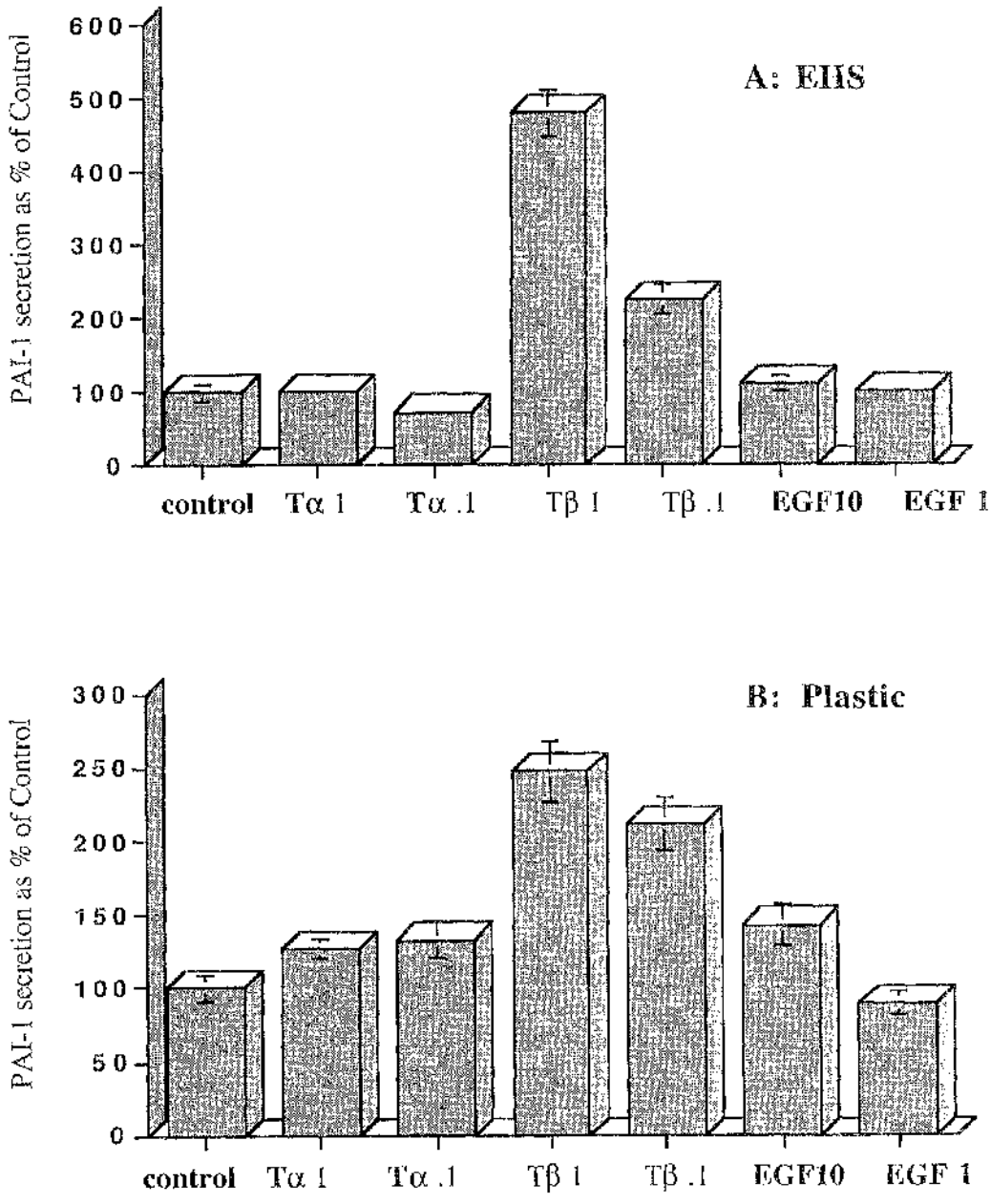


Figure 33 The effect of growth factors on PAI-1 secretion in conditioned medium (CM) in MDA cells, on both EHS (A) and plastic (B). (48 h)

TGFα (1 and 0.1 ng/ml), TGFβ (1 and 0.1 ng/ml) and EGF (10 and 1 ng/ml) were used for 48h. PAI-1 levels in the CM, in all experiments were assayed in duplicate using 4-span ELISA (see section 2.7.2). The average of three independent determinations ± SD is presented.

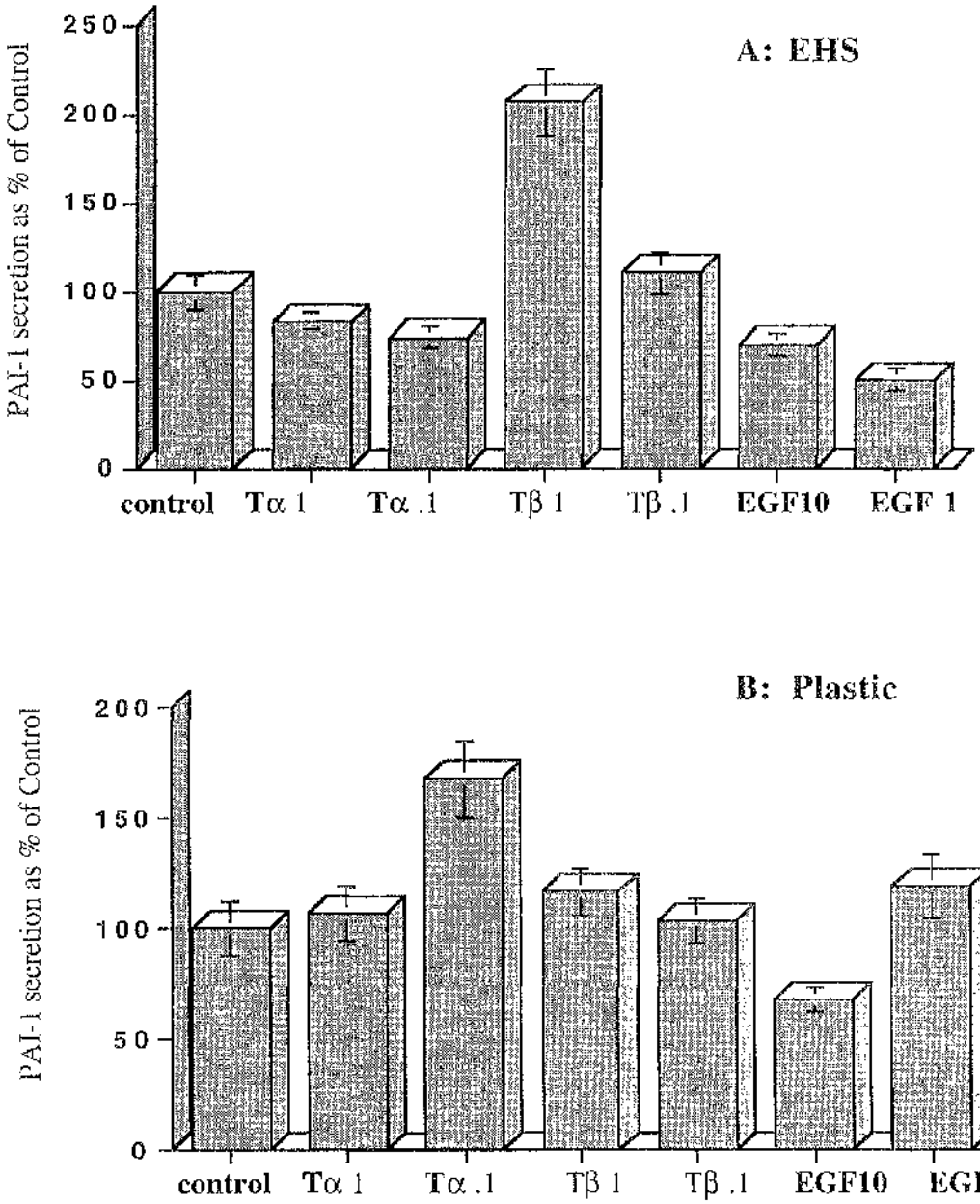


Figure 34 The effect of growth factors on PAI-1 secretion in conditioned medium (CM) in MDA cells, on both EHS (A) and plastic (B). (72 h)

TGFα (1 and 0.1 ng/ml), TGFß (1 and 0.1 ng/ml) and EGF (10 and 1 ng/ml) were used for 72h. PAI-1 levels in the CM, in all experiments were assayed in duplicate using 4-span ELISA (see section 2.7.2). The average of three independent determinations ± SD is presented.

Table 8 Secreted PAI-1 in MDA cells on EHS.

The amounts of PAI-1 protein measured in the conditioned medium (CM), on EHS in MDA-MB-231 cell line. Values (ng/ml) represents the mean of three independent experiments and each experiment was carried out in duplicate.

Time	Control CM	TGFα 1ng/ml	EGF 1ng/ml	TGFß 1 ng/ml
48h	135.4	96.9	95.6	647.6
72h	293.8	216.5	145.7	604.9

Table 9 Secreted PAI-1 in MDA cells on plastic.

The amounts of PAI-1 protein measured in the conditioned medium (CM), on plastic in MDA-MB-231 cell line. Values (ng/ml) represents the mean of three independent experiments and each experiment was carried out in duplicate.

Time	Control CM	TGFα 1ng/ml	EGF 1ng/ml	TGFß 1 ng/ml
48h	363	385	236	602
72h	399	424	558	459

3.3.4 The effect of growth factors on cell associated PAI-1 antigen in MDA-MB-231 cell line in cell extracts

PAI-1 has been shown to bind to the uPA/uPAR complex which is then internalised and degraded. Therefore, PAI-1 complexed to membrane bound uPA was measured in cell extracts in response to various growth factors.

TGFα

MDA cells when exposed to 1 ng/ml and 0.1 ng/ml showed a time dependent increase of cell associated PAI-1 antigen. On EHS, though a slight increase of 10% was observed after 48 hours in the presence of 0.1ng/ml. The same dose significantly stimulated PAI-1 levels by 91% after 72 hours (Fig 36A).

On plastic, however the effect of TGFα was inhibitory over the time period. Both, TGFα 0.1 and 1.0 ng/ml slightly inhibited the growth after 48h. At 72h, TGFα reduced PAI-1 levels by about 40-50% (Fig 36B).

The overall effect of TGFα on PAI-1 levels was very different on both the substrata i.e. stimulatory on EHS and inhibitory on plastic. This stimulation was time dependent which increased with time.

EGF

On EHS, EGF showed increased PAI-1 levels by 46% and 41% when the cells were treated with 10 ng/ml and 1 ng/ml after 48 hours (Fig 35A) and by 132% in the presence of 0.1 ng/ml EGF after 72 hours (compared with stimulation of 91% by 0.1 ng/ml TGFα).

The effect of EGF, like TGFα, was also inhibitory on plastic. Incubation with either 10 or 1 ng/ml reduced PAI-1 levels by about 10%. Further incubation over a time period of 72 hours, reduced the PAI-1 levels by about 30-50% (Fig 36B).

Conclusions

The overall effects of TGFα and EGF showed how selection of substratum is critical to establish the physiological role of these growth factors *In vitro*.

TGFβ

The effects of TGFβ on PAI-1 levels in the cell extracts were as dramatic as they were in conditioned medium. On EHS, TGFβ 1 ng/ml, was more effective in increasing PAI-1 by 180% after 48 hours (Fig 35A) in contrast to plastic. This level was more or less maintained after 72 hours in the presence of the same dose. TGFβ at 0.1 ng/ml slightly increased PAI-1 levels by 38% after 48 hours.

On plastic, MDA cells showed a dramatic increase of about 260% in PAI-1 levels with a low dose of 0.1 ng/ml after 48 hours (Fig 35B), whereas only 34% increase was observed after 72 hours (Fig 36B). At higher concentration of TGFβ 1 ng/ml, antigen levels after 48 hours were only 20% above control which was further reduced after 72 hours.

Conclusions

Considering the overall effect of TGFβ on PAI-1 levels in cell extracts of MDA cell line, the results suggest that much greater PAI-1 levels were observed on plastic with TGFβ 0.1 ng/ml as compared to EHS. General effect of TGFβ observed on both the substrata, was the induction of PAI-1 levels, though different concentrations were required for the inductions.

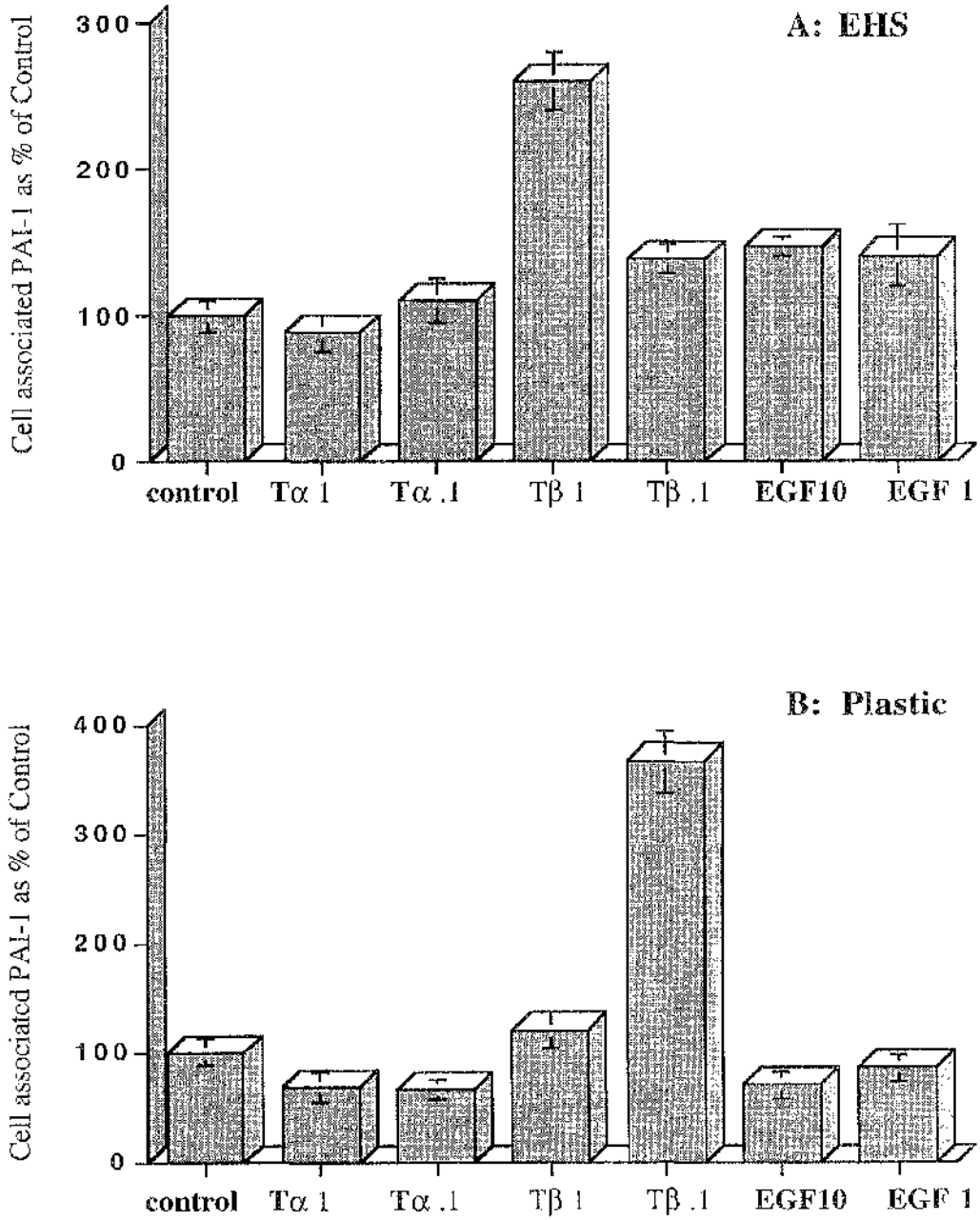


Figure 35 The effects of growth factors on PAI-1 in cell cytosol (SN) in MDA cells, on both EHS (A) and plastic (B).(48h)

TGFα (1 and 0.1 ng/ml), TGFß (1 and 0.1 ng/ml) and EGF (10 and 1 ng/ml) were used for 48h. PAI-1 levels in the SN, in all experiments were assayed in duplicate using 4-span ELISA (see section 2.7.2). The average of three independent determinations ± SD is presented.

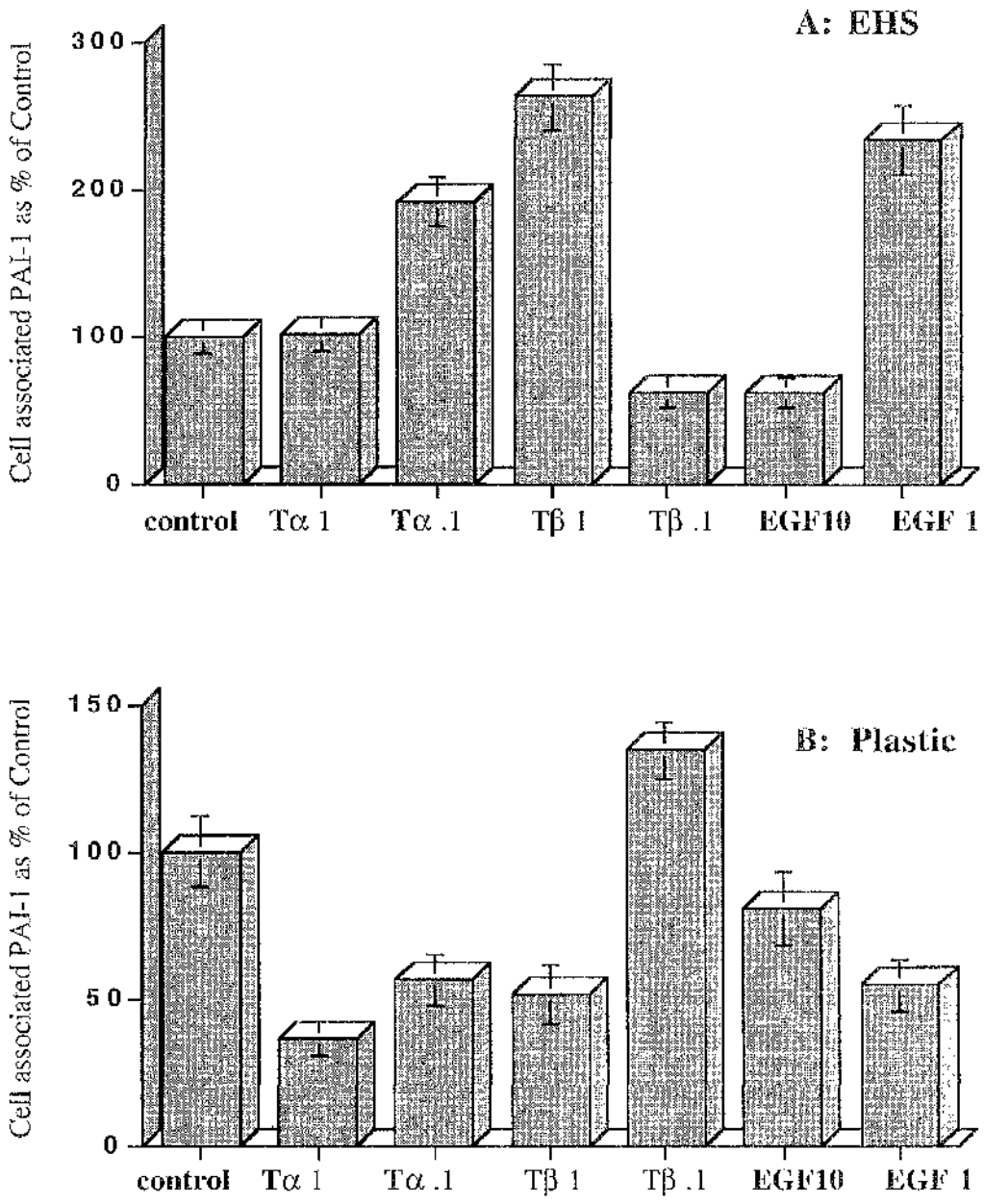


Figure 36 The effect of growth factors on PAI-1 in cell cytosol (SN) in MDA cells, on both EHS (A) and plastic (B). (72 h)

TGFα (1 and 0.1 ng/ml), TGFß (1 and 0.1 ng/ml) and EGF (10 and 1 ng/ml) were used for 72h. PAI-1 levels in the SN, in all experiments were assayed in duplicate using 4-span ELISA (see section 2.7.2). The average of three independent determinations ± SD is presented.

Table 10 PAI-1 Protein in SN in MDA cells on EHS.

The amounts of PAI-1 protein measured in the cell cytosol (SN), on EHS in MDA-MB-231 cell line. Values (ng/mg protein) represents the mean of three independent experiments and each experiment was carried out in duplicate.

Time	Control SN	TGFα 1ng/ml	EGF 1 ng/ml	TGFß 1 ng/ml
48h	51	50.4	72.1	133.7
72h	86.7	90.7	201.6	227.8

Table 11 PAI-1 Protein in SN in MDA cells on plastic.

The amounts of PAI-1 protein measured in the cell cytosol (SN), on plastic in MDA-MB-231 cell line. Values (ng/mg protein) represents the mean of three independent experiments and each experiment was carried out in duplicate.

Time	Control SN	TGFα 1ng/ml	EGF 1 ng/ml	TGFß 1 ng/ml
48h	265	182	249.1	319
72h	583	213	291.6	380

3.3.5 Dose response curve of TGFβ on uPA levels

Effect of different doses of TGFβ on secretion of uPA into the culture medium and on the cell associated uPA levels

It has been observed in the previous experiments that the MDA-MB-231 cell line when exposed to 1 ng/ml and 0.1 ng/ml TGFβ showed an enormous increase in the levels of uPA antigen both on plastic and EHS but with different dose dependence. An experiment was performed to determine the dose response to TGFβ over a range of doses, 0.01, 0.1, 0.3 and 1 ng/ml.

Dose response of TGFβ on secreted uPA levels in conditioned medium:

A time course experiment was performed with varying amounts of TGFβ 0.01 to 1 ng/ml. MDA cells when incubated with these doses showed a concentration dependent increase in uPA levels. The most significant increase of over 300% was observed in the presence of 1 ng/ml TGFβ after 48 hours. Lower doses gave the expected smaller stimulation (Fig 37A). uPA levels significantly dropped after 72 hours.

Dose response of TGFβ on uPA levels in the cell extracts:

Cell lysates were prepared as described in method (2.7.1) and uPA levels were measured in the presence of different doses of TGFβ 0.01-1 ng/ml for 48 and 72 hours. It was observed that uPA amounts varied with TGFβ concentrations and also with length of time. As the concentration increased from 0.01-1 ng/ml, uPA levels increased to about 100% above control, after 48 hours. After further incubation the level of stimulation was reduced although the antigen levels retained a dose response curve (Fig 37B). Taken together the results suggest that TGFβ maintained uPA levels in the conditioned medium and cell extracts in a concentration dependent manner.

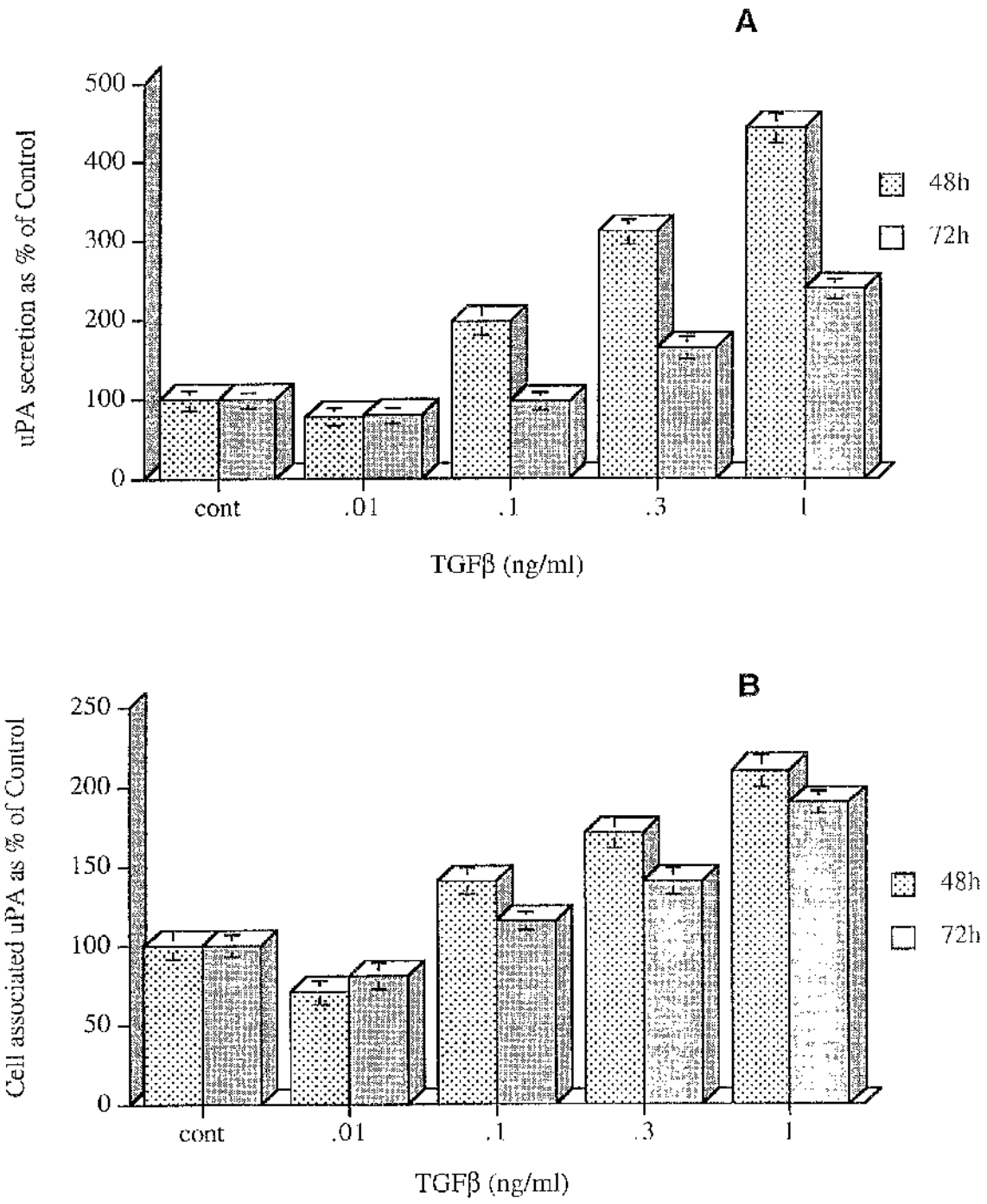


Figure 37 Dose response of TGFβ on the amounts of uPA in MDA cells in conditioned medium (A) and cell cytosol (B)

uPA protein in response to different doses of TGFß (ng/ml) were assayed for different time points, by ELISA in duplicates. The average of three independent determinations + is presented.

3.3.6 Dose response curve of TGFβ on PAI-1 levels

TGFβ increased PAI-1 levels, even more than uPA levels, in MDA-MB-231 cell line in the presence of 1 ng/ml and 0.1 ng/ml. Therefore cells were exposed to different concentrations (0.1, 0.1, 0.3 and 1.0 ng/ml) of TGFβ to determine dose response at different time points.

Dose response of TGFβ on secreted PAI-1 levels in conditioned medium:

MDA cells were incubated with different doses of TGFβ ranged from 0.01-1 ng/ml. As the concentration of TGFβ increased PAI-1 levels increased correspondingly. The most remarkable stimulation 350% was observed in the presence of 1 ng/ml TGFβ (Fig 38A). The lowest concentration 0.01 ng/ml also showed increased PAI-1 levels by 50%. The levels of stimulation reduced significantly after 72 hours.

Dose response of TGFβ on PAI-1 levels in cell extracts:

TGFβ increased PAI-1 levels in the cell extracts of MDA cells when exposed to 0.01-1 ng/ml TGFβ amounts. Maximum increased levels of PAI-1 (125%) were observed with 1 ng/ml after 48 hour (Fig 38B). The levels of PAI-1 fell after 72 hours, though not significantly.

These results suggest that exposure of cells to increasing concentrations of TGFβ causes a linear increase in the relative amounts of uPA and PAI-1 (i.e., the higher the concentraton of TBFβ, the higher the levels of uPA and PAI-1) The extent of stimulation was similar for both uPA and PAI-1.

3.3.7 The effect of Growth factors and hormones on PAI-1 antigen secretion into conditioned medium by the MCF-7 cell line

TGFα

A time course experiment was performed with MCF-7 cells in the presence of 1 ng/ml and 0.1 ng/ml TGFα. Both concentrations of TGFα increased PAI-1 levels after 72 hours (Fig 39A).

EGF

A time and concentration dependent increase was observed in the presence of 10 ng/ml and 1 ng/ml EGF. Both doses increased PAI-1 levels after 48 hours which were substantially increased (17 fold) after 72 hours (Fig 39A).

TGFβ

MCF-7 cells when treated with TGFβ at 1 ng/ml and 0.1 ng/ml, showed a remarkable increase over a time period 48 and 72 hours. Maximum stimulation of about 19 fold was observed in the presence of 1 ng/ml TGFβ after 72 hours (Fig 39A). The effect of 0.1 ng/ml TGFβ could only increase the PAI-1 levels to about 5 fold.

Oestradiol

MCF-7 cell line is oestrogen receptor positive and therefore various steroid hormones were used to check their effects on the PAI-1 secretion into the medium. Cells were incubated with 10^{-9}M and 10^{-12}M E2. Neither concentrations of E2 stimulated any PAI-1 into the medium after 48 hours. High concentrations of estradiol (10^{-9}M) did increase the antigen levels by about 3 times as compared to control values, after 72 hours (Fig 39B). The lower concentration remained ineffective.

Progesterone

MCF-7 cells when exposed to 10^{-8}M and 10^{-12}M showed an increase of PAI-1 levels The hormone only slightly increased PAI-1 antigen after 48 hours with 10^{-8}M. This increase remained high after 72 hours with 10^{-12}M (Fig 39B).

Tamoxifen

Tamoxifen is an anti-oestrogen and is used as a first line endocine therapy for breast cancer patients. To find its effect on the secretion of PAI-1 levels into the conditioned medium, the drug was incubated at a concentration of 10^{-9}M and 10^{-12}M. Both doses increased the antigen levels in a concentration and time dependent manner (Fig39B).

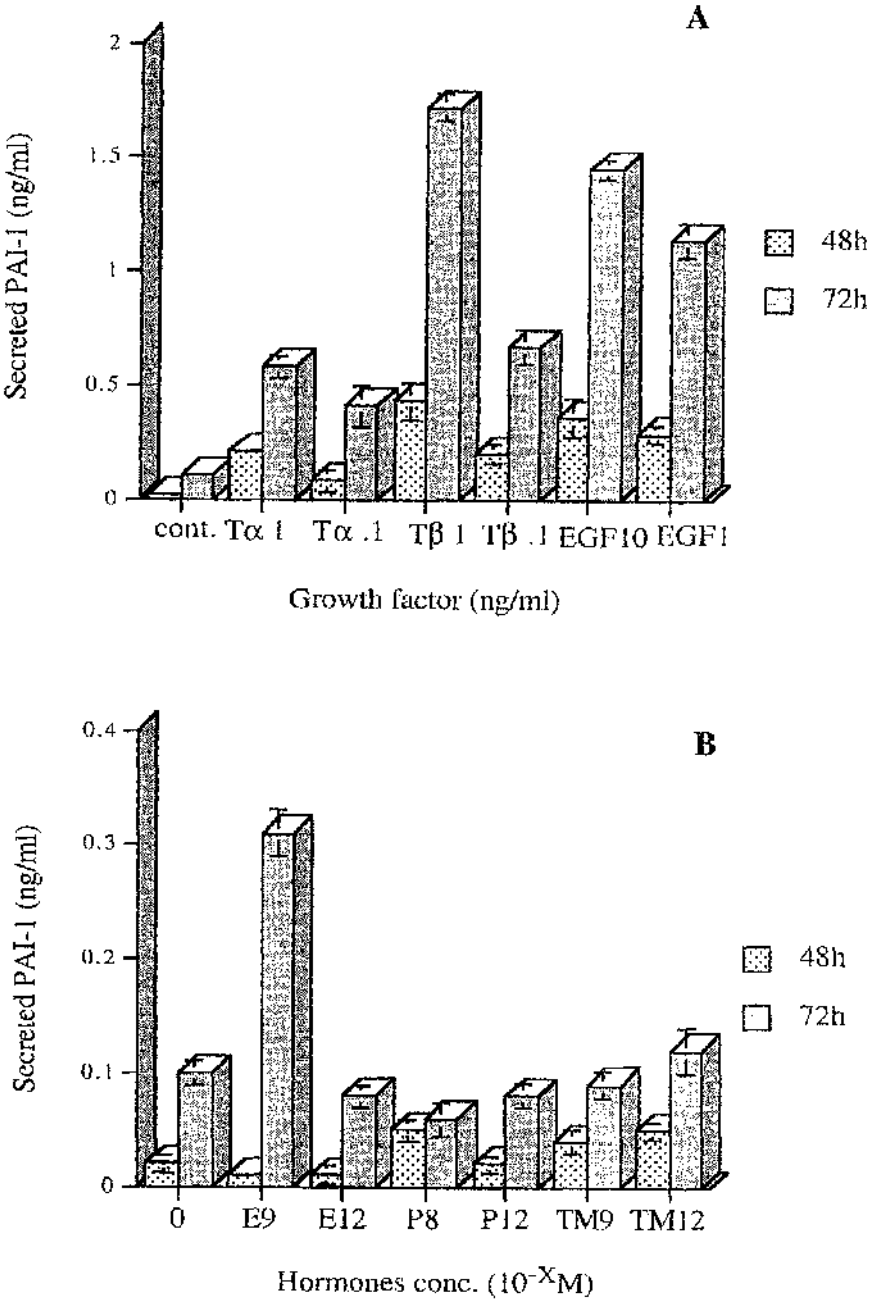


Figure 39 Effect of growth factors (ng/ml) (A) and steroid hormones (Molar conc.) (B) on PAI-1 levels in conditioned medium (CM) by MCF-7 cells.

Total PAI-1 in the CM of MCF-7 cells was determined by ELISA. The samples were collected for 48 and 72 hours. The results (mean±SD) are for three separate experiments.

3.3.8 The effect of growth factors and hormones on PAI-1 antigen levels in cell cytosols of MCF-7 cells

TGFα

This molecule has been known to have a mitogenic affect on various cell lines. When used at a concentration of 1, 10 and 100 ng/ml it stimulated cell growth. The results of its effects on PAI-1 levels in the MCF-7 cell line are depicted in Fig 40A. It should be noted that a time and concentration dependent increase was observed when MCF-7 cells were incubated with TGFα (1 and 0.1 ng/ml). Maximum increase of 7.5 fold was observed after 72 hours.

EGF

EGF 10 and 1 ng/ml concentrations were used in the study to determine its effects on PAI-1 levels in MCF-7 cell cytosols. The higher dose of EGF increased PAI-1 levels by about 4 fold which remained high 5 fold, after 72h (Fig 40A). Lower dose remained ineffective.

TGFβ

As with other growth factors, TGFβ also increased the PAI-1 levels. The higher dose 1 ng/ml increased PAI-1 levels by about 4 folds after 48h whereas TGFβ 0.1ng/ml increased PAI-1 levels by about 17.5 folds (Fig 40B).

Steroid Hormones

The effects of lower concentrations of hormones E2, Pg remained ineffective over a time period of 48-72h. The only significant increases of 4.5 and 3.5 fold, respectively, were observed with E2 (10^{-9}M) and TAM (10^{-12}M) after 72h, respectively (Fig 40B)

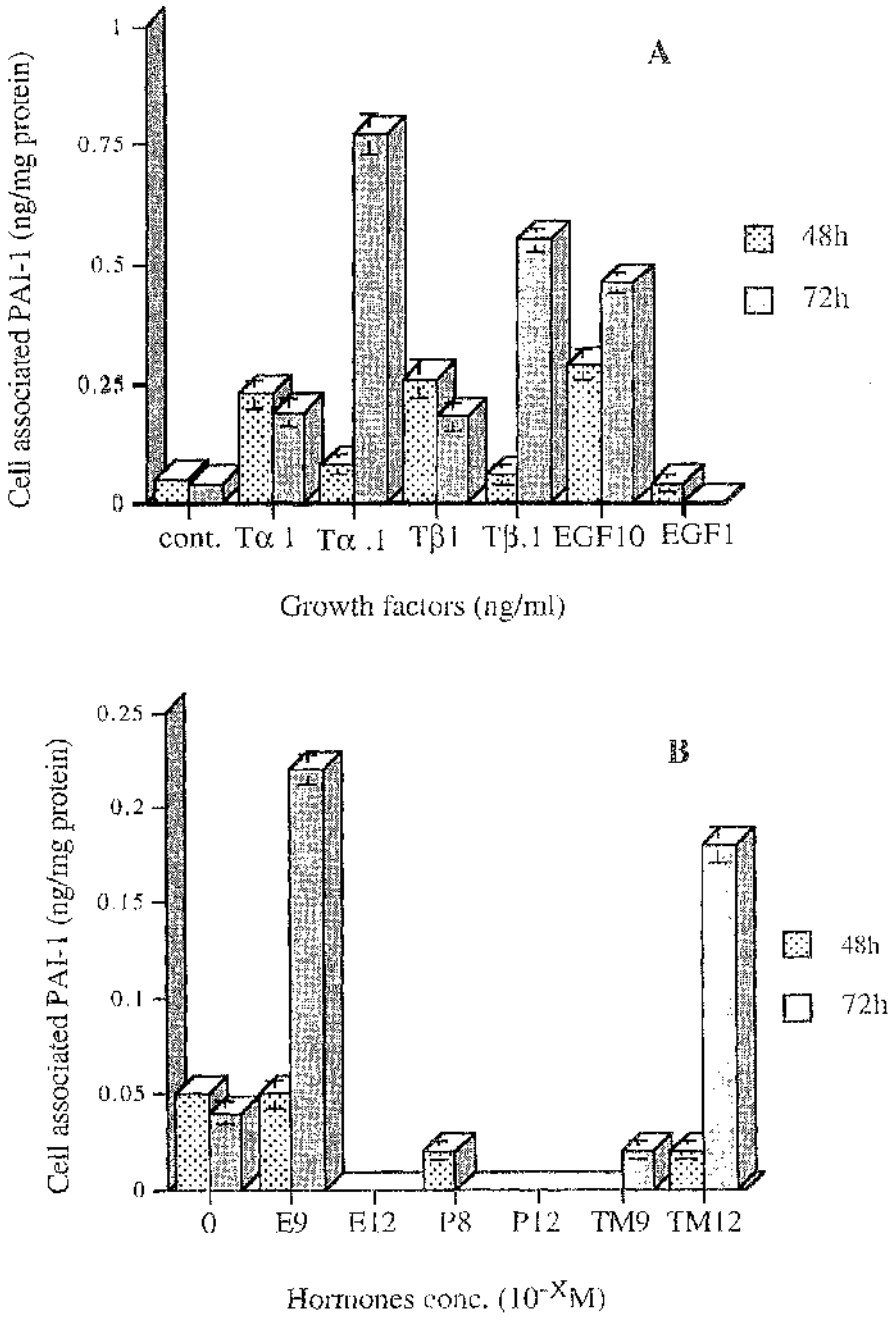


Figure 40 Effect of growth factors (ng/ml) (A) and steroid hormones (Molar conc.) (B) on PAI-1 levels in cell cytosol (SN) by MCF-7 cells.

Total PAI-1 in the SN of MCF-7 cells was determined by ELISA. The samples were collected for 48 and 72 hours. The results (mean±SD) are for three separate experiments.

3.3.9 Analysis of uPA and PAI-1 Levels in the MDA and MCF-7 Cell Lines by Western Blotting

In order to determine the presence of uPA and PAI-1 and to find its apparent molecular weight, a Western blotting was carried out. Extracts from both the normal and hormone/growth factor treated MDA and MCF-7 cell lines were prepared, electrophoresed on a 10% SDS-PAGE, electroblotted onto a membrane and subsequently probed with the mouse anti-uPA (Fig 41) and PAI-1 mAb (Fig 42). Surprisingly but consistent with the ELISA results, no detectable uPA was found in the control and treated MCF-7 cells. However, PAI-1 protein was detected in both MCF-7 and MDA cells, both in control and TGFβ treated cells (Fig 42). In the control MDA cells, uPA was detected as a single band of about 55 kDa. The amount of this protein increased about 3-fold in the cell cytosol when the cells were exposed to TGFβ (1 and 0.1 ng/ml) and EGF 1 ng/ml (compare lanes 2, 3 and 4 to lane 1). The same cells with TGFβ showed uPA antigen in the conditioned medium also (compare lane 6 and 7 and 8 to control lane 5) However, TGFβ treated MCF-7 cells showed no uPA (lane 10) as compared to control cells (lane 9) (Fig 41A). To ensure that equal amount of protein was loaded in all the lanes a separate gel containing the same amount of protein as was used in the gel which was electroblotted was run and subsequently stained with silver. This showed that the relative increase or decrease in uPA levels is real, since all lanes contained approximately equal amounts of the samples (Fig 41B).

Figure 41 Immunological identification of uPA protein in MDA-MB-231 and MCF-7 cells

Control and growth factor treated MDA and MCF-7 cells were collected after 48h. The cell extract prepared was resuspended in Laemmli buffer. The samples were then separated by 10% (w/v) SDS-PAGE and transferred to Hybond C membrane. ECL detection of the protein was performed with uPA mAb (1/50 dilution) as described in materials and methods (section 2.6). Silver staining was performed on the same samples run separately on 10% SDS-PAGE to show equal loading (Fig B). Both CM and SN were analysed for MDA cell line. Only CM was tested for MCF-7 cells.

Blot A: The lane numbers correspond to the following in MDA cells

Control	TGFβ 1ng/ml	TGFβ 0.1ng/ml	EGF 1ng/ml
1, 5	2, 6	3, 7	4, 8

MCF-7 lane numbers

Lane 9 is control and lane 10 is TGFβ 1 ng/ml.

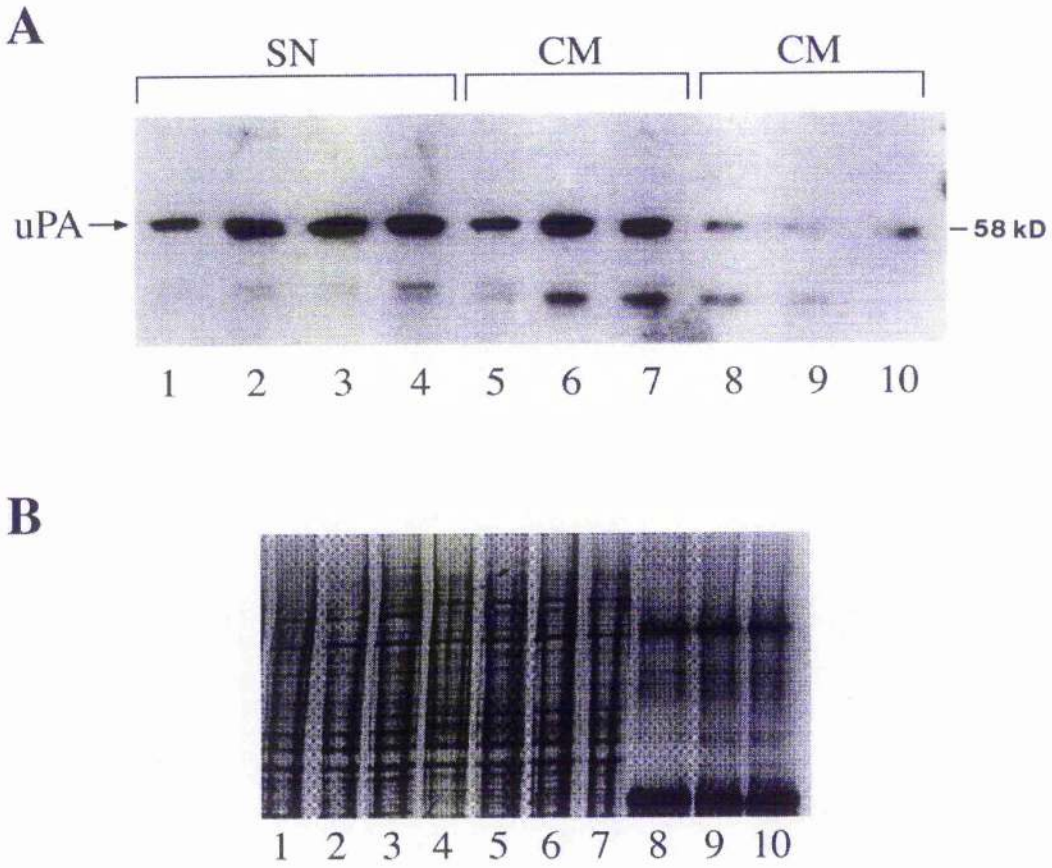

A
SN
CM
CM
uPA
58 kD
1 2 3 4 5 6 7 8 9 10
B
1 2 3 4 5 6 7 8 9 10

Figure 42 Immunological identification of PAI-1 in MDA-MB-231 and MCF-7 cells

Control and growth factor treated MDA and MCF-7 cells were collected after 48h. The cell extract prepared was resuspended in Laemmli buffer. The samples were then separated by 10% (w/v) SDS-PAGE and transferred to Hybond C membrane. ECL detection of the protein was performed with anti-PAI-1 mAb (1/200 dilution) as described in materials and methods (section 2.6). All the samples were treated with TGFβ 1 ng/ml.

Blot A: The lane numbers correspond to the following in MDA cells.

Lane 1, marker; lane 2 and 7, control; lane 3 and 6, CM; lane 4 and 5, SN.

PAI-1
MDA-MB-231
MCF-7
1 2 3 4 5 6 7
55 kD

3.3.10 Regulation of uPA Enzyme Activity

Effect of growth factors on the activity of uPA in conditioned medium (CM) and cell cytosols (SN) of MDA-MB-231 cell line on both EHS and plastic

uPA directed invasion and metastasis depend on the presence of active uPA in the system although uPA is initially secreted as an inactive form. uPA activity in response to various growth factors was analysed by using a chromogenic substrate specific for uPA as described in the method (2.8). A time course experiment was carried with different amounts of growth factors on both EHS and plastic.

TGFα

MDA cells were treated with TGFα 1 ng/ml and 0.1 ng/ml, as in ELISA, to find its effect on the activity of uPA over a length of 48 and 72 hours. On EHS TGFα showed no increase in uPA activity in conditioned medium (CM) (Fig 43A) whereas in the cell extract (SN) uPA activity increased significantly by about 40% after 72 hours, with TGFα 1ng/ml (Fig 44A). On plastic, TGFα showed no effect on the uPA activity levels either in CM (Fig 43B) or SN (Fig 44B).

EGF

The effect of EGF on uPA activity on both EHS and plastic was observed. When uPA activity was analyzed in the CM of MDA cells, an increased activity levels of 10% were obtained with EGF 10 ng/ml after 72hours (Fig 43A). In the cell extracts also initially EGF 10 ng/ml showed increased uPA activity after 48 hours (35%) which was subsequently increased by about 50% (Fig 44A).

On plastic, initially EGF showed no effect on the uPA activity levels either in CM or SN. After 72 hours incubation a higher dose (10ng/ml) showed a marginal increase of 10% in CM and about 50% in SN. EGF 1 ng/ml also stimulated uPA activity after 72 hours by 65% (Fig 44B).

TGFβ

The dramatic increase in the uPA activity was obtained with TGFβ 1 ng/ml and 0.1 ng/ml in a time dependent manner. On EHS, TGFβ both doses inhibited the activity levels by about 50% in CM. When CM on plastic was analyzed in the presence of 1 ng/ml TGFβ, a huge amount of active uPA was observed. This stimulation was 280% as compared to the control values (Fig 43B)) after 48 hours. This increase was further enhanced to 320% during the course of 72 hours. TGFβ 0.1 ng/ml initially showed a significant increase of uPA activity by 135% after 48 hours(Fig 43B). This increase in the active uPA remained the same after 72 hours.

In cell extracts the levels of active uPA on EHS, were higher as compared to the levels in the CM. TGFβ 1 and 0.1 ng/ml showed an increase of (45-55%) in uPA activity on EHS. Whereas, on plastic a remarkable increase in uPA activity by 280% was observed at both time points in the presence of TGFβ (45-55%) 1ng/ml (Fig 44B). TGFβ at 0.1 ng/ml also showed stimulation, though to a lesser extent, showing an increase of 150% after 48 hours which was further enhanced to 180% after 72 hours (Fig 44B).

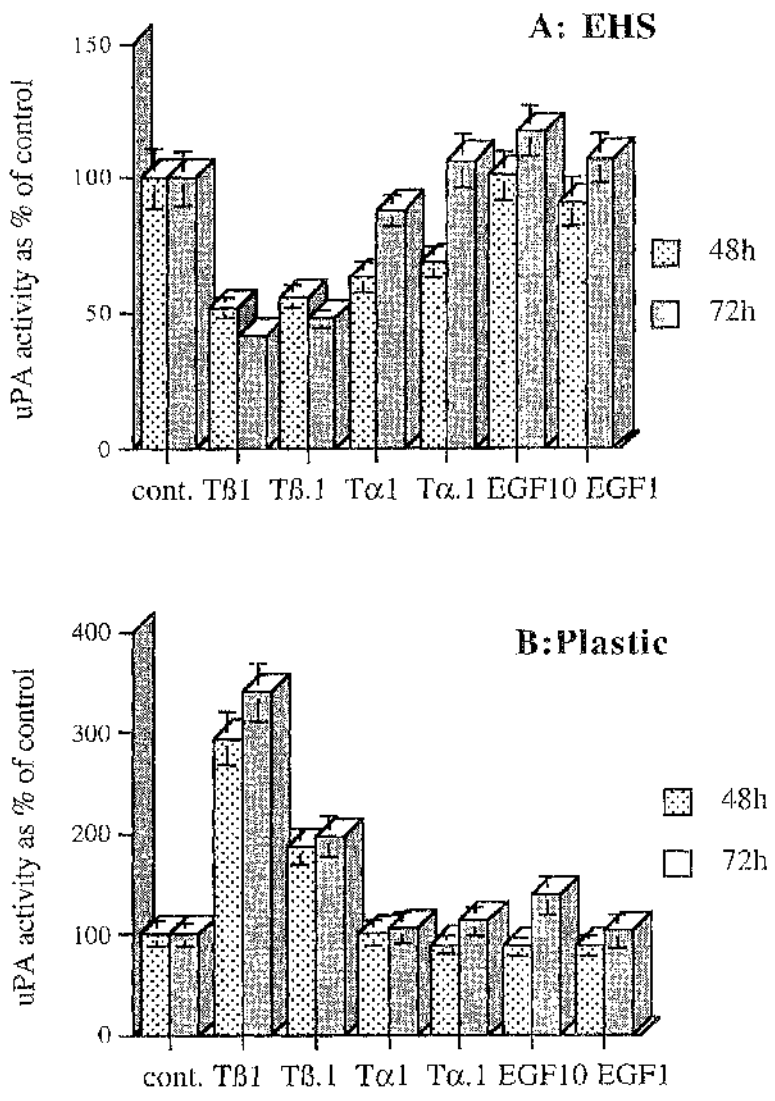


Figure 43 uPA enzyme activity in conditioned medium (CM) on EHS (A) and plastic (B) in MDA cells in reponse to different doses of growth factors at different time points.

Effects of TGFß (1 and 0.1 ng/ml),TGFα (1 and 0.1 ng/ml) and EGF (10 and 1 ng/ml) on the levels of uPA in CM is shown as percentage of control. Activity was measured by using S-2224 in duplicates in all the samples. The results (mean + SD) are for three seperate experiments.

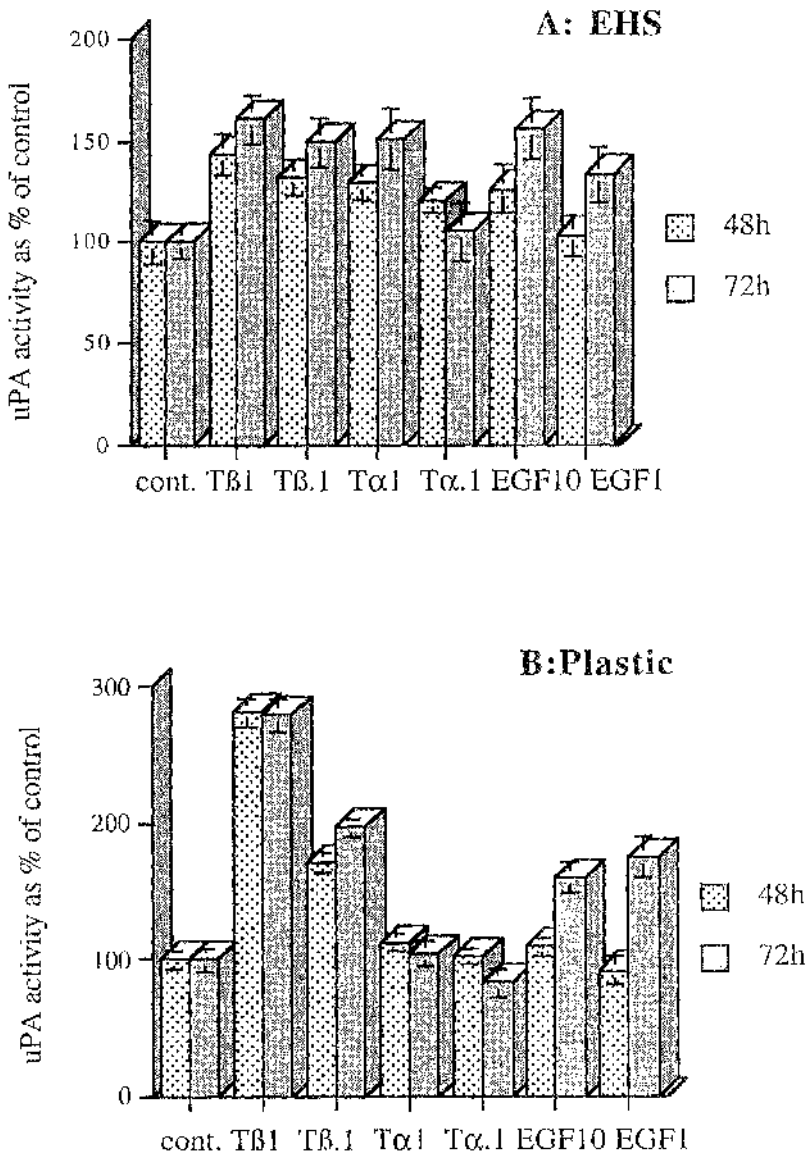


Figure 44 uPA enzyme activity in cell cytosols (SN) on EHS (A) and plastic (B) in MDA cells in reponse to different doses of growth factors at different time points.

Effects of TGFß (1 and 0.1 ng/ml),TGFα (1 and 0.1 ng/ml) and EGF (10 and 1 ng/ml) on the levels of uPA in SN is shown as percentage of control. Activity was measured by using S-2224 in duplicates in all the samples. The results (mean + SD) are for three seperate experiments.

3.3.11 Discussion

Effect of Growth Factors on the Expression of Plasminogen Activators and Plasminogen Activator Inhibitor in Human Breast Cancer Cells

The rate of protease and inhibitor secretion by MCF-7 and MDA-MB-231 cell lines, under various treatments and at different time points were investigated. The levels of plasminogen activators and their inhibitors were initially determined by western blotting and then later on quantified by enzyme-linked-immunosorbent assays (ELISAs).

Using the techniques described herein, although ample uPA was found in the MDA cells, surprisingly no uPA could be detected in the MCF-7 cells. In contrast, PAI-1 was found in both cell lines.

Effect of TGFα and EGF on uPA and PAI-1 protein levels

When cells were grown on EHS, TGFα initially decreased the levels of both secreted and cell associated uPA and PAI-1. However, after 3 days both uPA and PAI-1 levels were increased in cell extracts with lower dose of TGFα i.e. 0.1 ng/ml.

The effect of TGFα on plastic was different to that on EHS, in that TGFα initially increased the secreted forms of both uPA and PAI-1 levels in CM while the cell associated levels of uPA and PAI-1 levels were inhibited in cell extracts.

The effect of EGF on the secretion of uPA and PAI-1 followed the same pattern as TGFα. EGF also had no detectable effect on uPA and PAI-1 levels in CM on EHS, but both uPA and PAI-1 levels were increased by about 40-46% after 48 hours, on EHS. It was interesting to note that unlike TGFα, after

72 hours uPA and PAI-1 levels increased by nearly 130% in the cell extract, with EGF 1 ng/ml. On plastic however the general trend of increase in uPA and PAI-1 levels was only 30-40% in the presence of various doses of EGF.

In MDA cells higher concentrations of EGF and TGFα stimulated the growth of rate whereas the same growth factors did not alter the plasminogen activator and inhibitor levels significantly. It may be that EGF and TGFα follows different a different pathway to influence proliferation and/or plasminogen activaor levels.

In MCF-7 cells, TGFα and EGF increased the PAI-1 levels in a concentration and time dependent manner. Higher concentrations increased PAI-1 levels in CM whereas in cell extracts the same dose was not much more effective as compared with the lower dose. Maximum stimulation (7.5 fold) was achieved with TGFα 0.1 ng/ml. Like TGFα, EGF also increased both secreted and cell associated PAI-1 levels and the extent of stimulation was more in CM about 8 fold with highest dose 10 ng/ml. However the absolute levels remained very low in this cell line.

In MCF-7 cells, increased growth rate and also increased PAI-1 secretion was observed in the presence of higher doses of TGFα and EGF

Effect of TGFβ1 on uPA and PAI-1 Protein Levels

The levels of uPA in MDA cells were significantly elevated in response to transforming growth factor β (TGFβ) on EHS and plastic, in both the conditioned medium (CM) and in cell cytosol (SN). The increased levels of uPA and PAI-1 in response to TGFβ were time and dose dependent. The doses required for the stimulation on both the surfaces were different. For instance, on EHS high dose 1.0 ng/ml TGFβ increased the uPA levels to maximum

whereas on plastic, the lower dose was more effective in increasing the antigen levels. The amount of stimulation observed also varied in CM and SN on the same surface. On EHS, TGFβ at 1 ng/ml gave maximum stimulation of uPA by 330% in CM which decreased with time, whereas the increase in cell extracts was only 150-160% in response to the same dose. The effect of TGFβ on cells grown on plastic was dose dependent. Lower doses of TGFβ, i.e. 0.1 ng/ml, did not have any effect on uPA levels in the CM after 72 hours but increased the amount of uPA by 360% in cell extracts after 72 hours. There is no obvious explanation for this contrast. The only reason may be that EHS is more a physiological substratum than plastic and therefore the cell interactions with extracellular matrix modulates the functions of the cells, thereby requiring higher doses for the maximum stimulation. However, it is interesting to note the remarkable contrasting differences in the secreted and cytosolic amounts of uPA in cells grown on plastic and EHS in response to TGFβ.

This stimulatory effect of TGFβ on PAI-1 levels was even more pronounced on both EHS and plastic. TGFβ at 1 ng/ml increased PAI-1 levels after 24 hours by 415% and about 300% in the CM on both EHS and plastic as compared to the untreated cells, respectively. After 48 and 72 hours the amount of PAI-1, however, gradually decreased substantially on both substrata. At ten-fold lower concentration TGFβ, increased PAI-1 levels in the CM by 136% after 24 hours on EHS. On plastic the increase in PAI-1 levels was initially 226% after 24 hours but later dropped off after 48 and 72 hours.

In the cytosol, 1 ng/ml increased PAI-1 by 186% on EHS, while on plastic the increase was 198% after 24 hours. On both substrata a decrease in the amount of PAI-1 was noted although this decrease was more substantial on plastic. At lower doses (0.1 ng/ml) TGFβ increased PAI-1 by a modest 38% on EHS

while it dramatically increased PAI-1 levels by 414% on plastic after 24 hours. After 48 hours, the PAI-1 levels decreased substantially.

The overall effect of TGFβ was stimulatory on uPA and PAI-1 levels, both on EHS and plastic. The effect was more dramatic on PAI-1 levels in the CM of EHS. The same effect was maintained in SN on plastic. The relative amounts in both these cases were similar.

In MCF-7 cells, modest amounts of PAI-1 already present were substantially increased by TGFβ in a dose and time dependent manner. Though TGFβ mediates its effect by a different receptor than EGF and TGFα, it showed the same pattern of PAI-1 stimulation in MCF-7 cells, in that the lower doses increased PAI-1 levels in SN whereas a higher concentration was more effective in stimulating PAI-1 levels in CM. The extent of stimulation was greater in CM (17.5 fold) than SN (12 fold). The absolute amounts of PAI-1, however remained very low. The growth rate was however, stimulated with all doses.

3.4 INVASION

In vitro tumour cell invasion was assessed by the microinvasion Boyden chamber invasion assay as described in methods section 2.9.2.

3.4.1 Assessment of Invasive Capacity of the Control Versus Treated MCF-7 and MDA Cell-lines

Since metastasis is the principal life threatening event in the process of carcinogenesis, it is important to understand the effect(s) that certain hormones or growth factors have in bringing about certain biochemical changes in a group of cells which enhance their invasive ability. The next series of experiments were, therefore, designed to address this question.

Untreated, as well as oestrogen, EGF, TGF-α and β treated MDA and MCF-7 cells were used in an invasion assay and the invasion was measured by confocal microscopic analysis as well as by the MTT assay.

Consistent with the aggressive nature of MDA cells, they were found to be more intrinsically invasive than MCF-7 cells (Hachiya *et al.*, 1995). In the initial phase of the study, different experiments were performed in order to optimise the conditions for invasion as well as to compare different methods available for its measurement. Different amounts of matrigel/well were tested. 80 μg matrigel/well was found to be the optimal amount to discriminate the invasive potential of both the cell lines.

Fetal calf serum (FCS) and invasion

The effect of fetal calf serum (FCS) on the invasive ability of MDA and MCF-7 cells was studied. This experiment demonstrated that in the presence of FCS, invasiveness of both cell lines was inhibited, compared to those which had never been exposed to FCS. The effect of FCS on the invasion was measured by both confocal microscopy (Fig 45) and the MTT assay (Fig 46). It was found that the two methods showed approximately the same level of inhibition in their invasive potential (11% for the MDA cells which had been exposed to 2.5% FCS for 72 hours). A gradual increase in the amount of FCS (0, 2.5. 5.0 and 10%) resulted in a gradual decrease in the invasive capacity of both cell lines after 72 hours as measured by the MTT assay (Fig 46).

Interestingly MCF-7 cells after incubation with 2.5% FCS showed a 34% decrease in invasive capacity whereas in the MDA cell line a drop of only 11% was observed. This suggests that the MCF-7 cells are much more sensitive to serum and this may reflect the fact that they are better differentiated cells. At higher concentrations of FCS, the relative decrease in the percent inhibition for the two cell lines became less marked, 41% and 46% decrease in inhibition for MCF-7 cell line (Fig 46A), to 21% and 34% for MDA cells (Fig 46B), in the presence of 5 and 10% FCS, respectively.

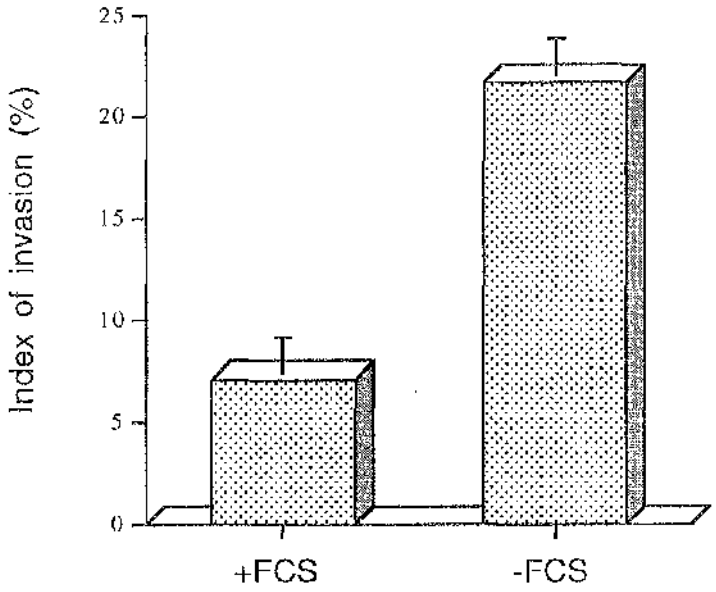


Figure 45 Comparison of invasion across a matrigel, in control MDA-MB-231 cells in the presence or absence of serum as determined by confocal microscopy.

Transwell filters were coated with 80µg matrigel/well and 5 x 10^5 cells were plated per well. After 72h, the invasion of matrigel by cells, was determined by confocal microscope as described in methods (2.9.4). The effect of FCS on the in vitro invasion was studied. The results (mean ± SD) are from three separate determinations.

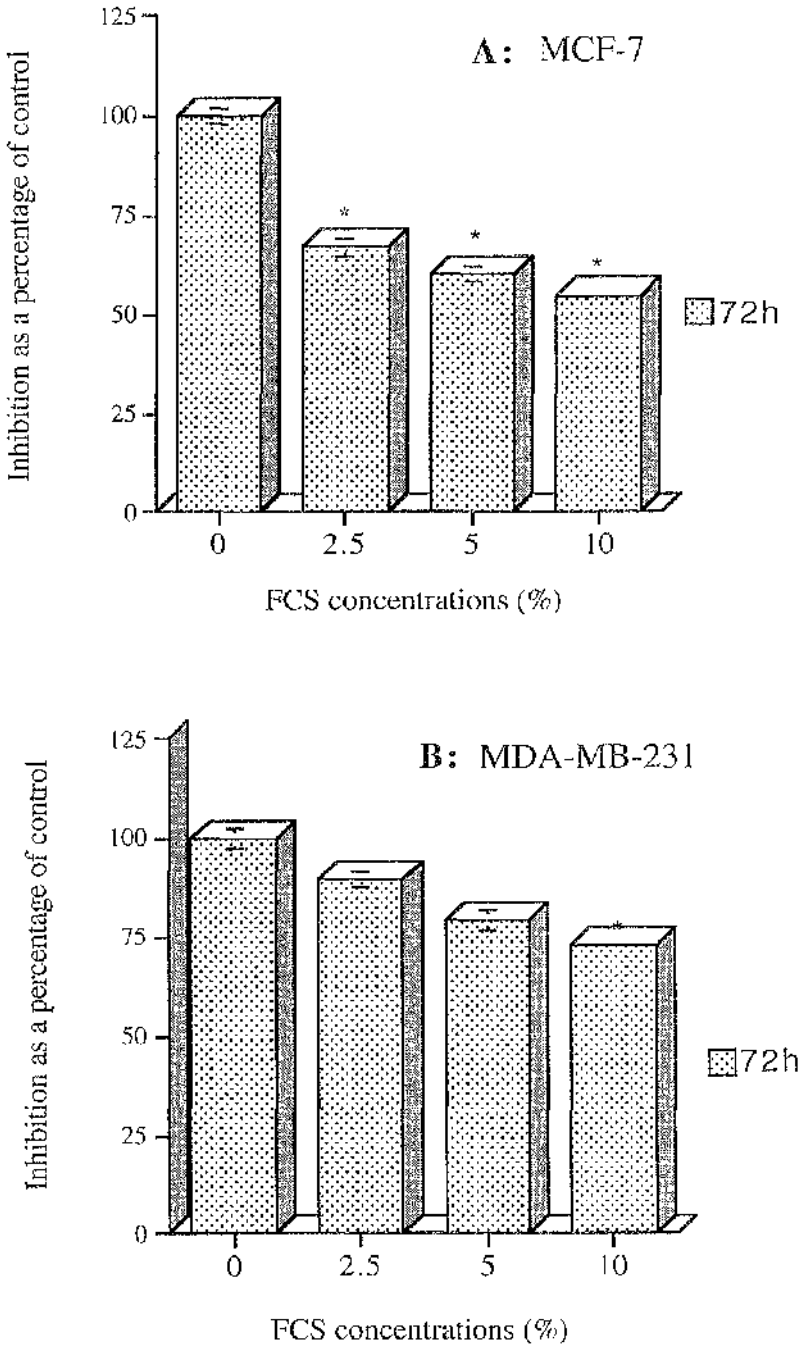


Figure 46 Percent inhibition in in vitro invasion of MCF-7 and MDA-MB-23 cell in response to FCS.

Transwell filters were coated with 80µg matrigel/well and 5 x 10^5 cells were plated per well. After 72h, the invasion of MCF-7 (A) and MDA (B) cells across a matrigel was measured by MTT as described in methods (2.9.3). Different amounts of fetal calf serum (FCS) (2.5-10%) were used. Each sample was measured in duplicates and the mean of three different experiments ± SD is presented.

Effects of matrigel use as a chemoattractant on invasion

In vitro invasion is affected by many factors. After determining the concentration of matrigel and concentration of FCS, the next study was medium with/without matrigel (as a chemoattractant) (Fig 47). It had been observed that the presence of a chemoattractant (100µg/ml matrigel and 2µg/ml plasminogen) placed in the lower compartment of the chamber, only increased the percentage of invasion by about 2% as compared to the wells where no matrigel was added in the lower chambers (Fig 47). This shows that chemoattractant in the lower compartment of the chamber only slightly influence the invasive ability of the cells.

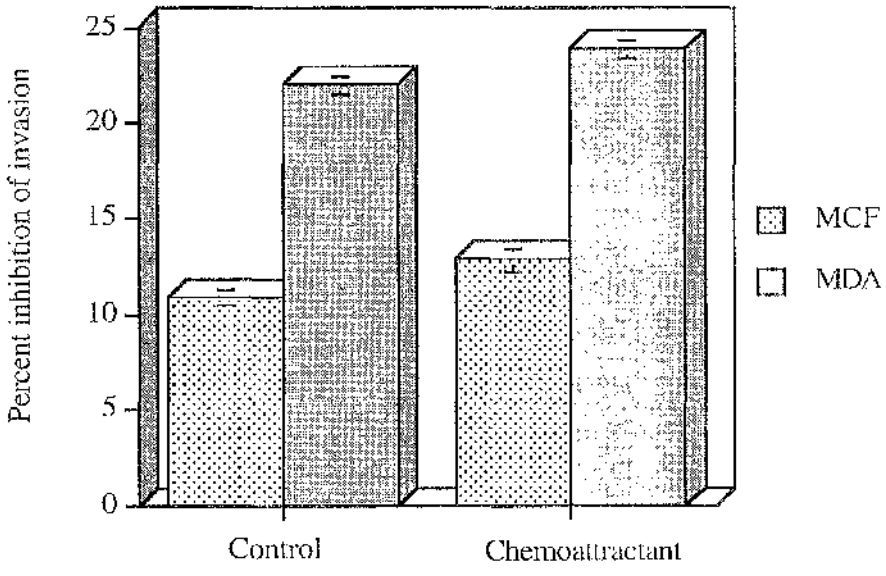


Figure 47 Effect of chemoattractant on the percentage of invasion

The invasive potential of both MCF-7 and MDA cells were determined. 5×10^5 cells were plated in the wells. The medium in the lower chamber was supplemented with matrigel (100µg/ml) as a chemoattractant. The values are expressed as a mean ± SD for triplicate experiments

Effect of plasminogen on invasion

Effect of plasminogen on invasion with or without FCS is shown in (Fig 48). Plasminogen is the direct substrate of uPA. Therefore, its effect on invasion was determined by mixing plasminogen with matrigel. Plasminogen increased the invasiveness of MDA cells by only 6%. In the previous experiments it was shown that FCS inhibited the invasion of these cells. So, to find if FCS inhibits the increased invasion by plasminogen, FCS was added to the medium. It was interesting to see again that FCS could even reduce the plasminogen mediated increase in invasion (Fig 48).

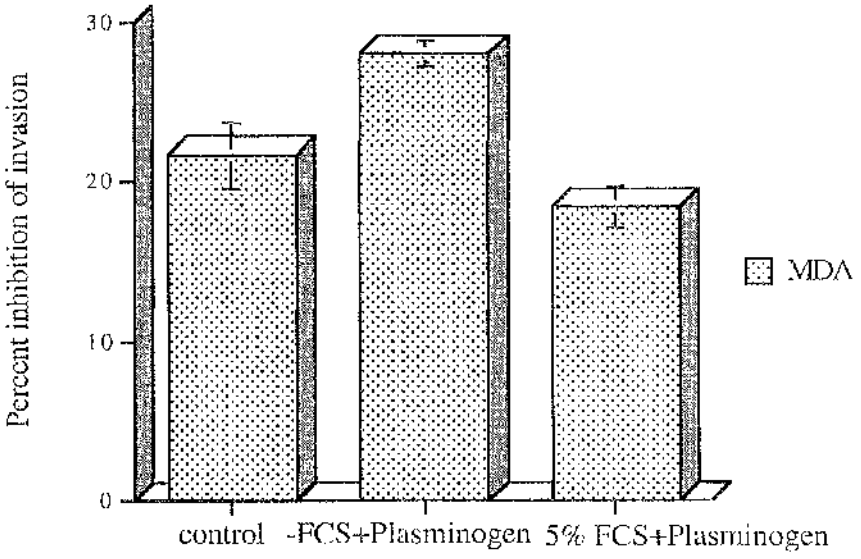


Figure 48 Effects of plasminogen on the invasion of MDA cells

The invasive potential of MDA cells were determined. 5 x10^5 cells were plated in the wells. The matrigel in the insert was supplemented with plasminogen (2ug/ml) and the medium used was with or without FCS. The values are expressed as a mean ± SD for triplicate experiments.

3.4.2 Effect of Growth Factors on Invasion

Evaluation of invasion by confocal laser microscope

After optimising all the necessary conditions for invasion, the effects of growth factors on invasion was determined. Both MDA-MB-231 and MCF-7 cells were cultured in a growth medium containing EGF (10 ng/ml), TGFα (1 ng/ml) or TGFβ (1 ng/ml), on matrigel coated filters. Control cells were cultured only in the presence of culture medium. The percentage of invasion is expressed as the number of cells that migrate above a minimum distance from the original layer, in comparison with the number of cells invading under control conditions. Percentage of invasion was measured under the confocal laser microscope after fixing the cells on matrigel as described in methods section 2.9.4.

It was observed that after five days of incubation, EGF increased the invasive ability of the MDA cell line by 10%. Exposure of cells to TGFα did not result in any significant change in their invasive ability as compared to the control. Incubation of cells with TGFβ, which has been shown to be an inducer of uPA gene expression, increased invasiveness by about 30%. (Fig 49).

Evaluation of invasion using MTT

MDA cells were cultured in the same concentration of EGF, TGFβ and TGFα for 5 days. Invasion was defined as the proportion of cells that crossed the matrigel and filter in each compartment as determined by MTT assay. It was observed that EGF induced invasion by 10% whereas TGFβ increased the invasion by about 40%. TGFα, as observed in confocal experiment, remained ineffective in increasing the invasive properties of cell (Fig 49).

Taken together these data suggest that both methods, confocal and MTT, showed comparable results in that TGFβ stimulated invasion by 30% as assessed by confocal microscopy, and 40% as assessed by MTT assay. TGFβ showed maximum invasion as compared to EGF or TGFα.

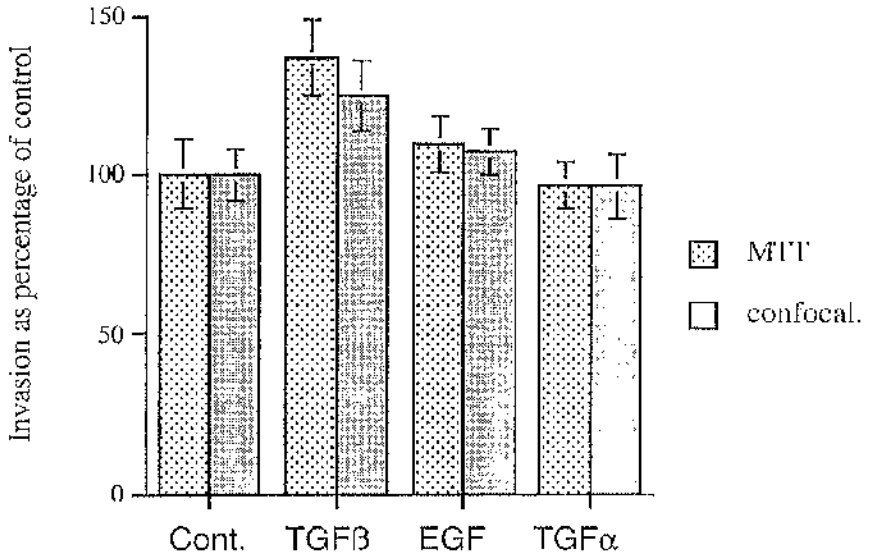


Figure 49 Comparison of the percentage of invasion between confocal and MTT in MDA cells in response to growth factors.

5×10^5 cells were left for 5 days on the bottom of the filter for confocal microscopy and inside the insert on top of the matrigel for MTT assay as described in methods section 2.9.3. TGFß 1 ng/ml, EGF 10 ng/ml, TGFα 1 ng/ml were used in the study. The results (mean ± SD) are the average of three separate experiments. Each experiment was performed in duplicate.

Table 12 Growth factor modulation of invasion of MDA-MB-231 cells into matrigel evaluated by confocal microscopy and MTT.

MDA cells (5x 10^5) were plated in the Boyden chamber microinvasion assay. Different growth factors (TGFβ, TGFα, EGF) were used in the study. The percentage of invasion was compared by both confocal microscopy and MTT.

Results (mean) are from three independent experiments.

Doses	% of invasion confocal	% of invasion MTT
control	67.4	31
TGFβ 5 ng/ml	87	42.5
EGF 10 ng/ml	72	34
TGFα 1ng/ml	65	30

Time course invasion

In the next experiment the effect of growth factors and hormones on the invasive ability of both MDA and MCF-7 cells was observed over 48 and 72 hours in order to study the kinetics of invasive capacity of cells. To this end, both cell lines were treated with the EGF (10 ng/ml), TGFβ (1 ng/ml), TGFα (10 ng/ml) or tamoxifen (10^{-8}M); since the MDA cell line is oestrogen insensitive, only MCF-7 cells were exposed to oestrogen (10^{-9} M) and progesterone (10^{-8}M) (Fig 50A). Because both methods, confocal and MTT were comparable as shown in section 3.9.2 therefore invasion was evaluated by using MTT alone.

Figure 50A show that MCF-7 cells treated with oestrogen were found to be 60% more invasive after 72 hours as compared to the non-oestrogen treated cells. Initially progesterone slightly inhibited the invasiveness after 48 hours which reached to the level of control after 72h. Tamoxifen inhibited invasion by 25% after 48 hours but the inhibition was much more pronounced, about 40% after 72 hours. TGFβ had a stimulatory effect on invasion with an increase of 27% and 75% after 48 and 72 hours, respectively.

When the same experiment was done in the presence of a monoclonal anti-uPA antibody invasive capacity was not completely reduced to control levels implying that TGFβ stimulated invasion cannot be ascribed entirely to increased uPA secretion. Treatment of cells with EGF did not appear to have any effect after 48 hours. After 72 hours, however, it increased the invasive capacity of the MCF-7 cells by 55%. Initially TGFα inhibited invasion by about 35%. This inhibition overcame after 72h (30% stimulation in invasion was observed).

For the MDA cell line TGFβ stimulated invasiveness by 22% after 48 hours and 40% after 72 hours. The anti-uPA antibody when used together with TGFβ reduced the invasive ability of cells to control levels.(Fig 50). EGF stimulated invasion by about 20% after 72h. TGFα and tamoxifen both failed to influence invasion by MDA cells.

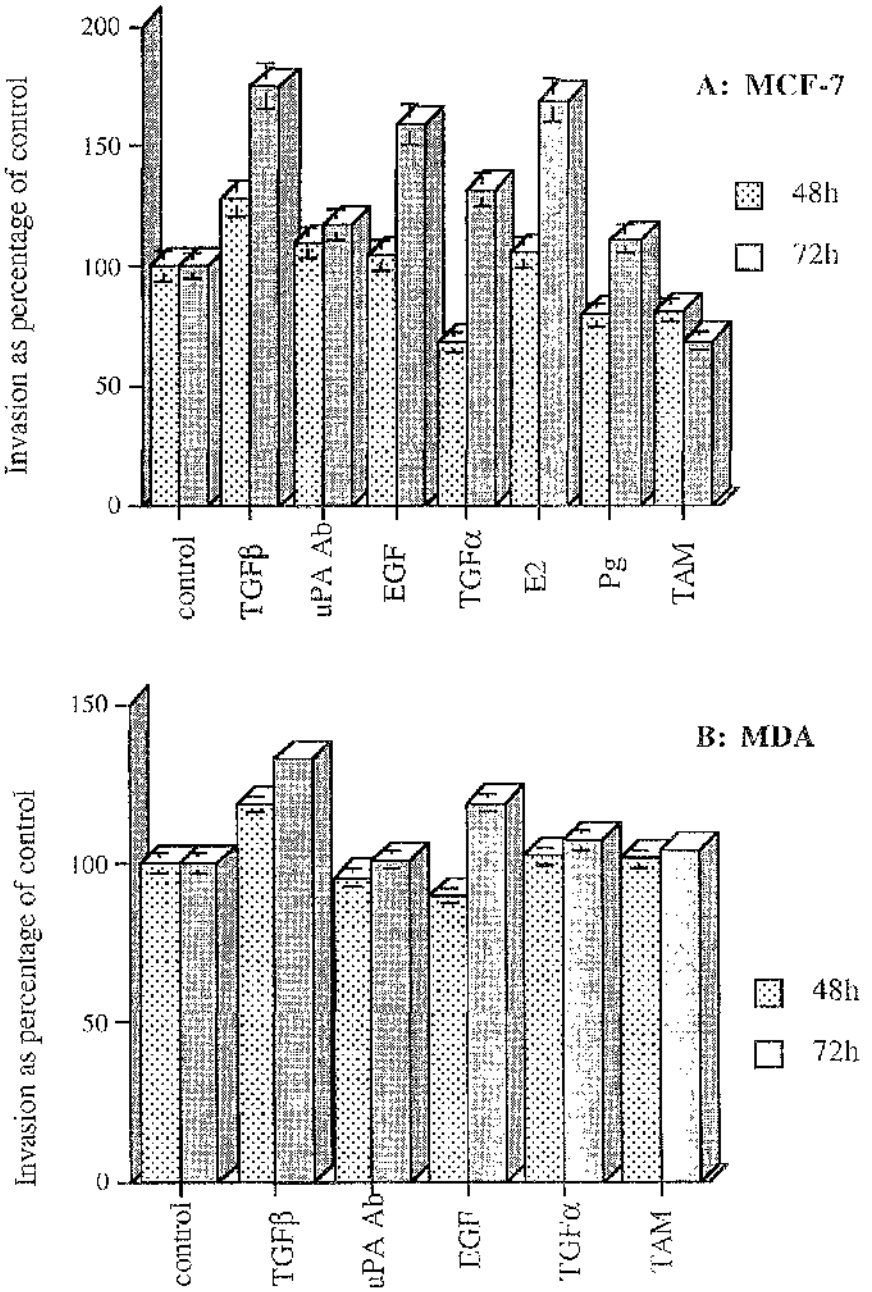


Figure 50 Time course effects of growth factors on the invasion of MCF-7 (A) and MDA (B) cells.

Effects of growth factors TGFβ 1ng/ml, TGFα 1ug/ml, EGF 10 ng/ml were used for both MCF-7 and MDA cells in the Boyden chamber invasion for 48 and 72 hours. uPA antibody (1 in 200) dilution was used to check the uPA dependent invasion. Also in MCF-7 cells steroid hormones were used to determine its effects on the invasion under the same experimental conditions.
The results (mean ± SD) represents the average of three separate determinations.

3.4.3 Discussion

Growth factors, steroid hormones and invasion

It has been known for sometime that the invasive potential of a given cell line is related to the amount of active uPA it synthesizes and secretes. It is also clear that when bound to its receptor, uPAR, uPA catalyzes the conversion of plasminogen to plasmin which in turn activates the proteases required for the breakdown of the extracellular matrix. As discussed previously (section 1.13.1), an important molecule which is responsible for inhibiting the action of uPA is its inhibitor PAI-1. When bound to uPA, PAI-1 masks its catalytic site and renders uPA inactive. When this is the case then the cell does not have much invasive ability. Yet, paradoxically, high levels of PAI-1 within the tumour are associated with poor prognosis. In this study, uPA and its associated molecules were measured both by ELISA and in the case of uPA, enzyme activity.

Further experiments were designed to determine the effect of growth factors and steroid hormones on the invasive ability of MCF-7 and MDA-MB-231 cells. It is known that *In vitro* invasion is affected by many factor e.g. number of cells per well, the amount of matrigel used in the inserts of Boyden chambers, the amount of fetal calf serum and plasminogen (the only substrate for uPA) the length of time and the filter size. Therefore, it was necessary to optimise the conditions for the cell lines that were used in this study. In doing so, we found that 80µg matrigel per well was a good concentration to demonstrate the relative invasive potential between the cell lines. Furthermore, it was observed that FCS inhibits invasion of both cell lines used but had a much more dramatic effect on MCF-7 cells at lower concentrations. This may reflect the relative agressiveness and differentiation of the two cell lines. MCF-7 cells showed inhibition (34%) in the presence of 2.5% FCS, while a drop of only 11% was observed in MDA cells.

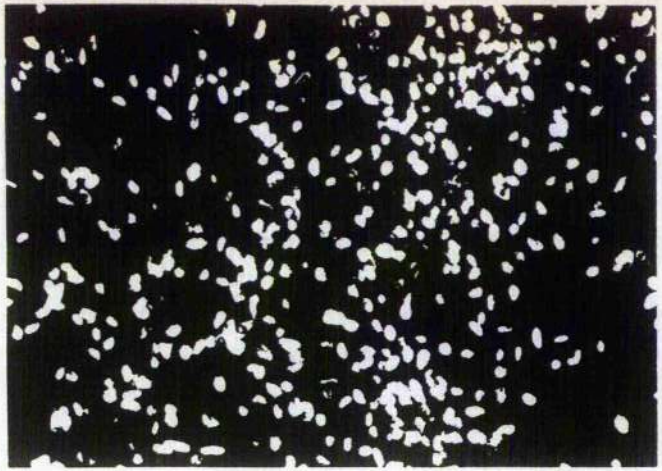
It is widely accepted that plasminogen increases the invasiveness of cells. However, in this study addition of plasminogen (2ug/ml) had little effect implying that our EHS preparations already contained adequate plasminogen. Furthermore, it was interesting to note that when plasminogen was added in the presence of FCS, it did not increase the invasive ability of the cells. In addition to the above mentioned factors, we tried to compare the two available methods for the determination of invasion, confocal laser microscopy and MTT. The results showed that both the methods were comparable, as can be seen in Fig. 49 although confocal microscopy normaly gave a higher value than MTT. This may be because confocal microscopy is less invasive than MTT and therefore the percentage of manual error is minimum. The effect of growth factors after 5 days in culture, were evaluated by both confocal and MTT. TGFβ showed maximum stimulation of invasion as compared to EGF and TGFα. TGFβ 1 ng/ml stimulated invasion of MDA cells by about 40% as assessed by MTT as compared to about 30% as determined by confocal microscope.

Since the results were comparable in reflecting TGFβ effect on urokinase components, the subsequent time course experiments with growth factors were determined by MTT only. These experiments showed a different pattern of invasion for MCF-7 and MDA cells. Invasion in MDA cells only slightly increased with time while MCF-7 cells showed a significant increase in invasion with time. In MDA cells, TGFβ 1 ng/ml increased invasion after 48 and 72 hours by about 25 and 30%, respectively, although the same dose increased invasion in MCF-7 cells, with time. The maximum stimulation observed in MCF-7 cells, however was greater (75% after 72h) than MDA cells. EGF 10ng/ml also increased invasion in MCF-7 by 60% after 72h. The effect of EGF and TGFα were barely significant in MDA cells. Importantly, TAM inhibited invasion in MCF-7 cells significantly by about 32% after 72 hours. This implies a full anti-oestrogenic effect of TAM on invasion not the

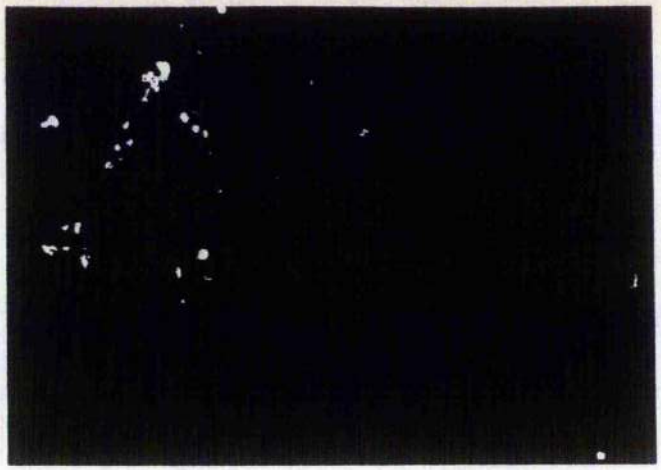
usual weak agonist effect. TAM did not effect the levels of invasion in MDA cells. To determine if the invasion was uPA dependent, uPA antibody was used. It was interesting to note that uPA antibody 1/500 dilution reduced activity of MDA cells to control levels implying that TGFβ-induced invasion was due to uPA but that the endogenous level of invasion was due to some other mechanisms.

Figure 51 A photograph of invading cells across a matrigel as seen under the confocal microscope.

This fig illustrates the use of confocal microscopy in the assessment of invasive potential. MDA cells were plated into the boyden chamber under control conditions and then confocal microscopy was used to visualise cells at the surface and at 10, 20 , 30 and 40 μm into the gel as shown in figure. Cells were counted at each depth and relative migration assessed under different conditions of stimulations.



A



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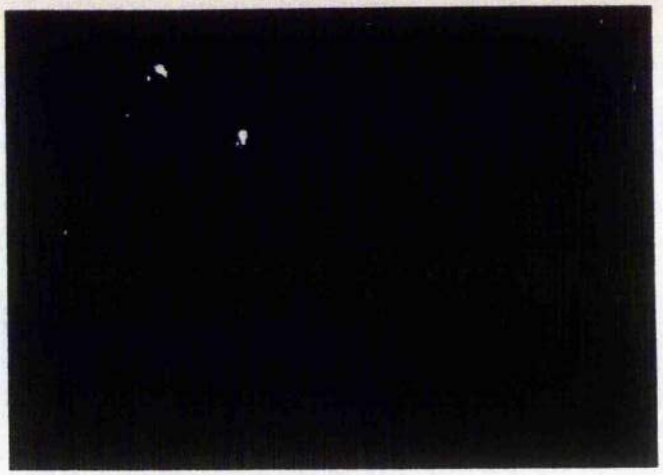

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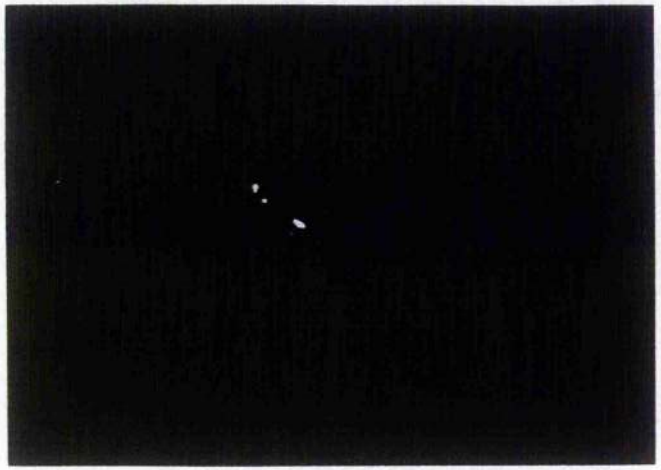

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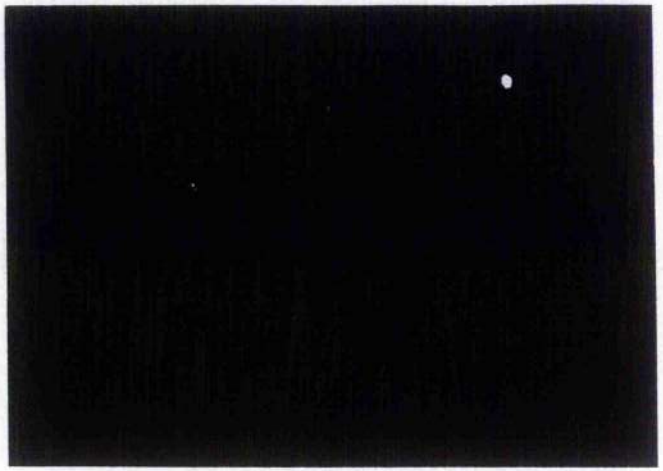

E

3.5 Immunofluorescence

Having used ELISA to quantitate the respective amounts of uPA and PAI-1 in MCF-7 and MDA cel lines, we wanted to determine the localization of these proteins in both MDA and MCF-7 cells. For this, anti-uPA and anti-PAI-1 antibodies were used in immunofluorescence studies to visualise the two antigens and their patterns of distribution. The uPA and PAI-1 antibodies as described in the Materials and Methods (section 2.10) were used at a dilution of 1:200. Negative controls had only normal goat serum at the same dilution. The number of stained cells were counted under the immunofluorescence microscope and the photographs were taken to show the distribution of both the PAI-1 and uPA in untreated and TGFβ treated MDA cells (Figs 52 and 53). The pattern of distribution could not be clearly distinguished. However, the micrographs clearly show an increase in staining in the treated cells (Figs 53 and 55) confirming the ELISA data. On the other hand, MCF-7 cells showed no staining for uPA but did show some PAI-1 antigen. The level of distribution was not much different both in control and TGFβ treated cells (data not shown), again confirming the ELISA data.

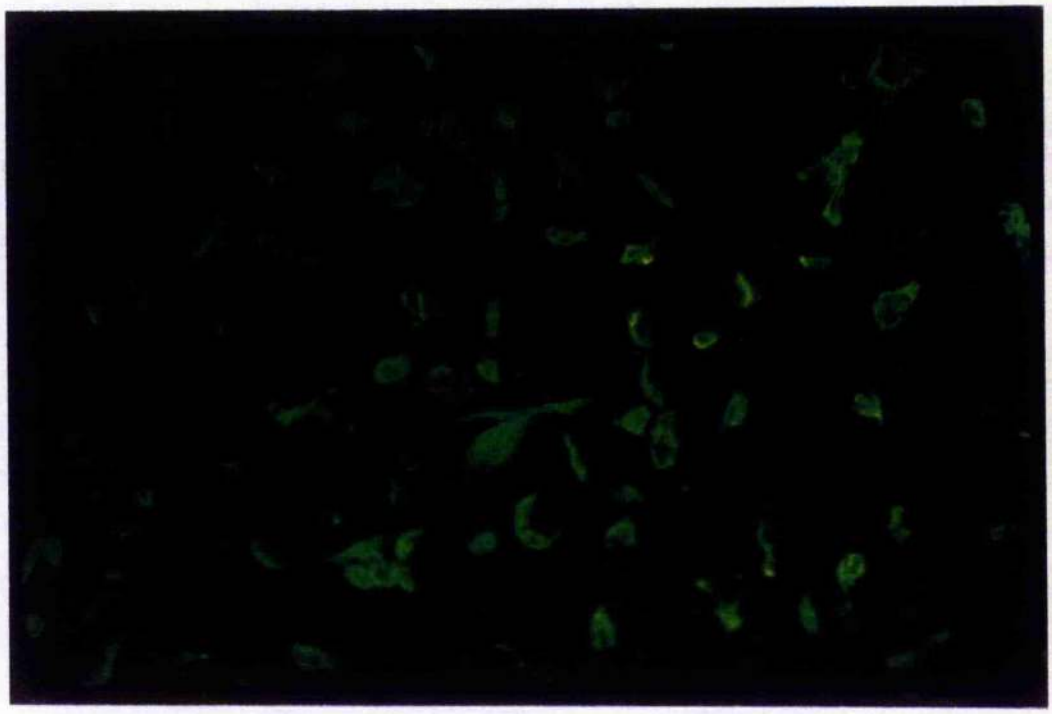

Figure 52 PAI-1 staining in control MDA cells.

The cells were incubated with anti-PAI-1 antibody for 2 hours after fixation as described in methods (section 2.10) The cells showed diffused staining throughout.

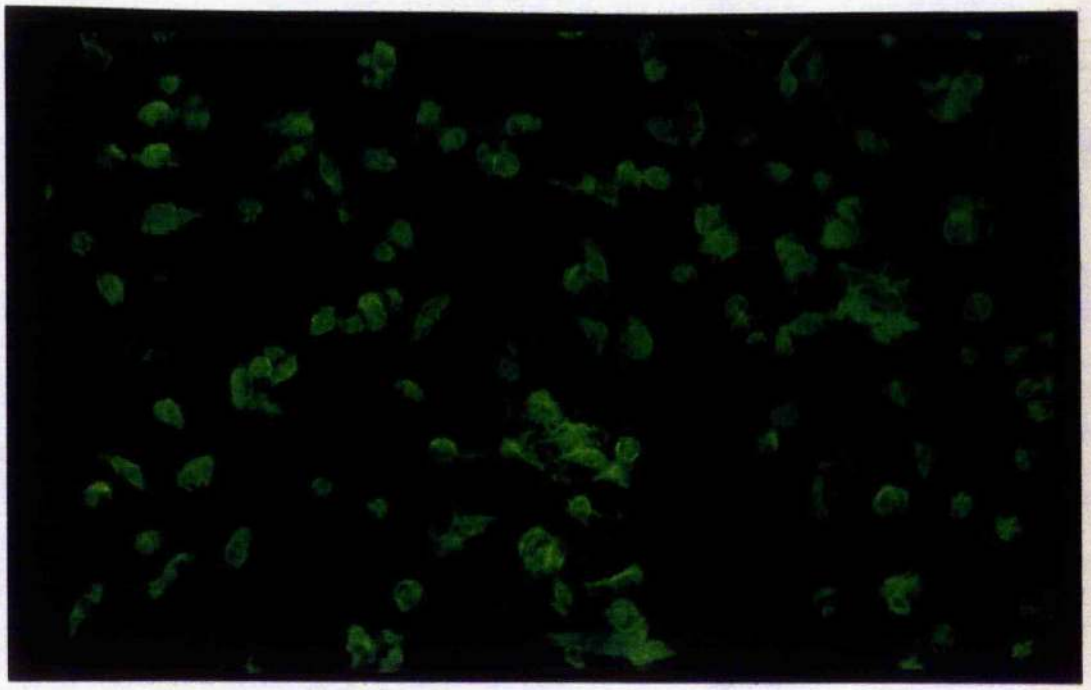

Figure 53 PAI-1 staining in TGFβ treated MDA cells.

The cells were pretreated with TGFβ (1ng/ml) for 48 hours, and then incubated with anti-PAI-1 antibody for 2 hours after fixation as described in methods (section 2.10) The cells showed diffused staining.

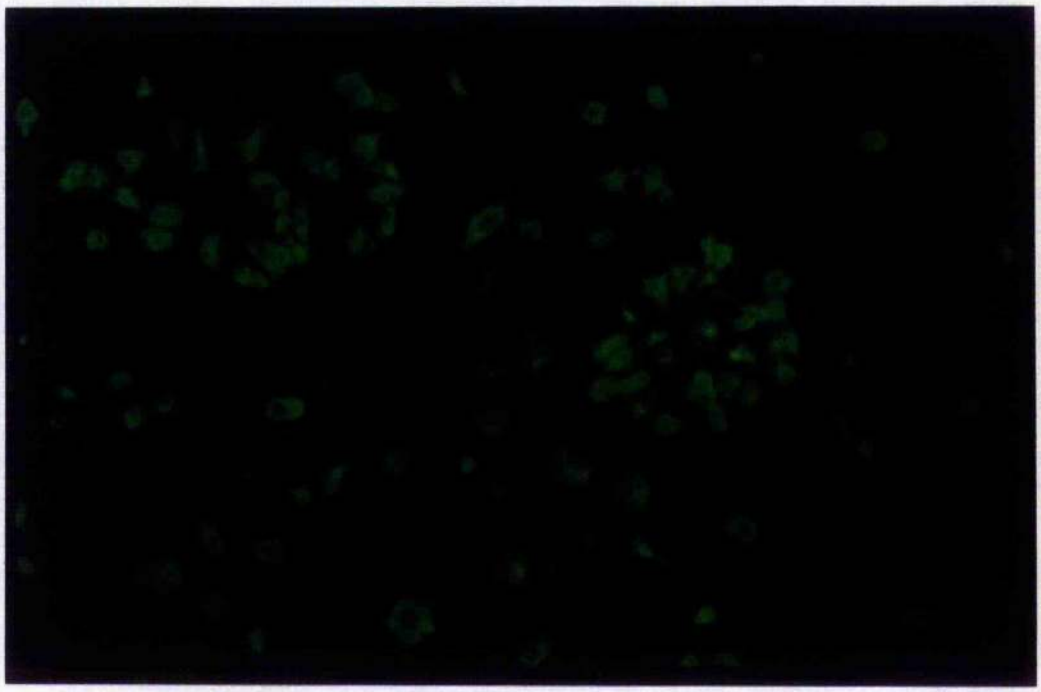

Figure 54 uPA staining in control MDA cells

The cells were incubated with anti-uPA antibody for 2 hours after fixation as described in methods (section 2.10) The cells showed diffused staining.

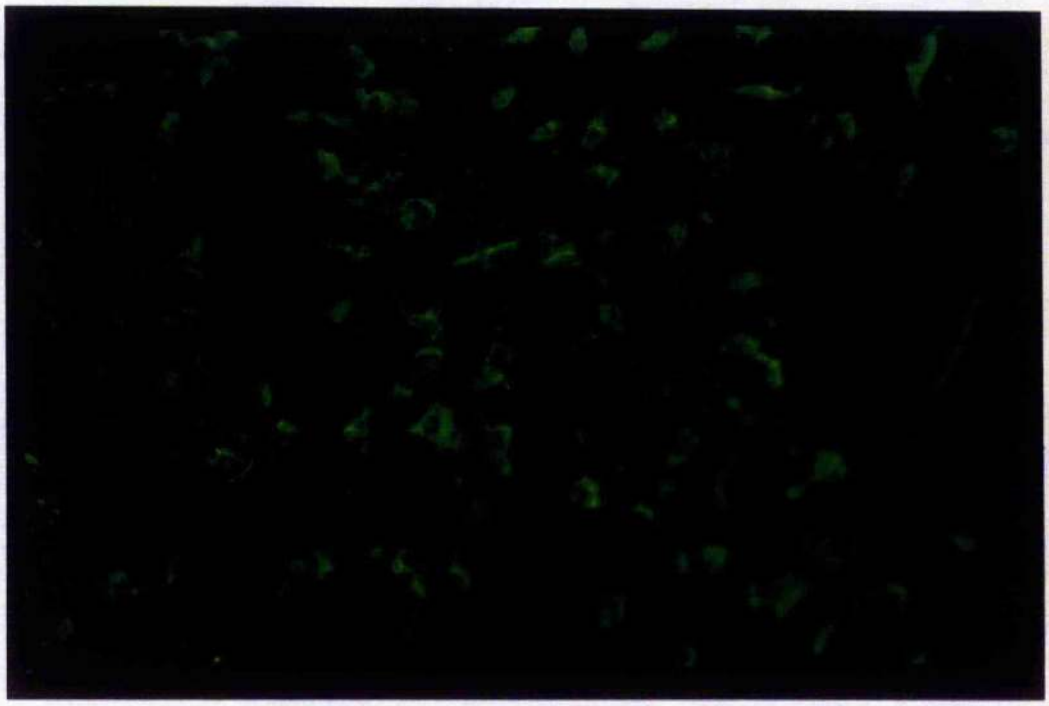

Figure 55 uPA staining in TGFβ treated MDA cells

The cells were pretreated with TGFβ (1 ng/ml) for 48 hours and then incubated with anti-uPA antibody for 2 hours after fixation as described in methods (section 2.10). The cells showed diffused staining.

3.6 Effect of Different Surfaces on the Shape and Morphology of the MDA and MCF-7 Cells

Since the nature of the substratum has been shown to effect the morphology of the plated cells, we compared the shapes of both MCF-7 and MDA cells after plating them on plastic and EHS. When MCF-7 cells are plated on plastic (Fig 56), they are bigger in diameter than on EHS where they form aggregates. This aggregations starts 5-6 hours after plating the cells on EHS (Fig. 57). With time they grow and fold the matrigel on themselves and form a pseudo-mammosphere structure (Fig. 58). Upon changing the medium or after 96 hours the cells detach themselves from the matrigel and begin to grow on plastic.

With MDA cells (Fig. 59), they are smaller in size than MCF-7 cells and are more epidermoid in shape. They start making "communication lines" or pseudopodia like structures within 4-6 hours of their plating on EHS (Fig. 60). On EHS, these pseudopodia like structures that appear from one cell to another were more defined after 48 hours and appeared like a network under a microscope (Fig. 61).

Figure 56 Morphology of MCF-7 cells on plastic.

5 x 10^5 cells were cultured in 6-well plates. The cells were left overnight and the photographs were taken.

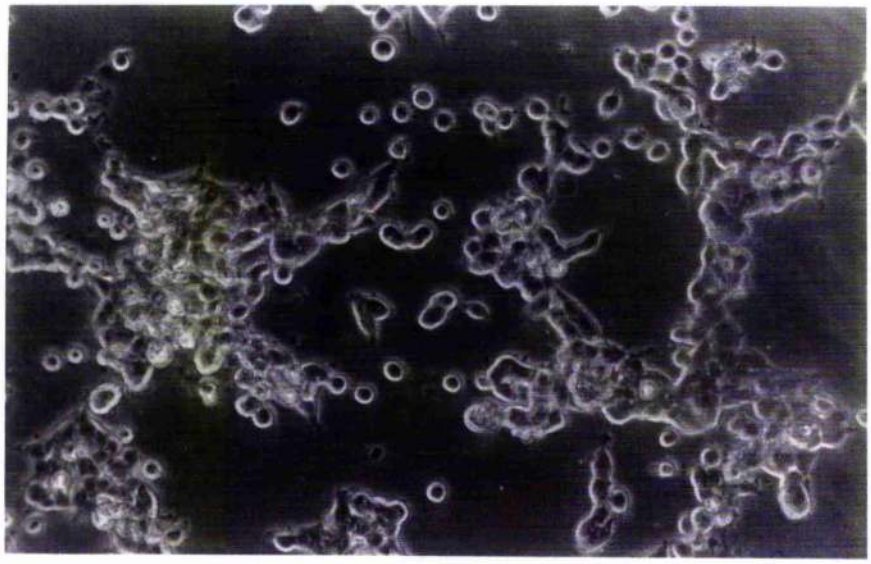

Figure 57 Morphology of MCF-7 cells on EHS.

5 x 10^5 cells were cultures in 6-well plates precoated with matrigel. The photographs were taken within 4-6 hours. Magnification used was x4 x 1.5.

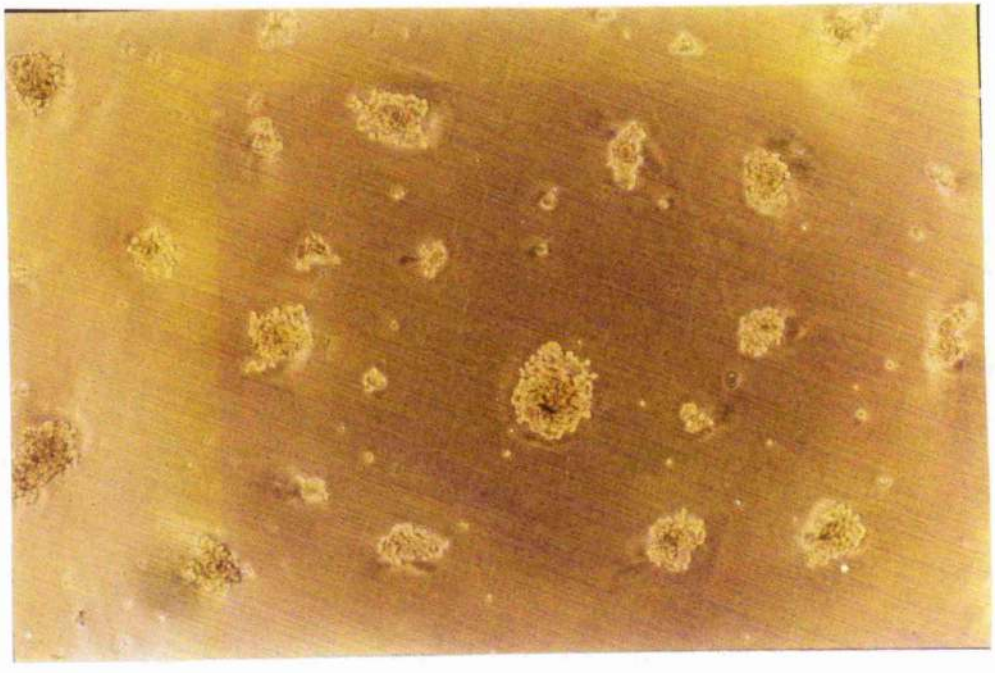

Figure 58 Morphology of MCF-7 cells on EHS.

5 x 10^5 cells were cultures in 6-well plates precoated with matrigel. The photographs were taken after 48h. The magnification used was 4 x1.5.

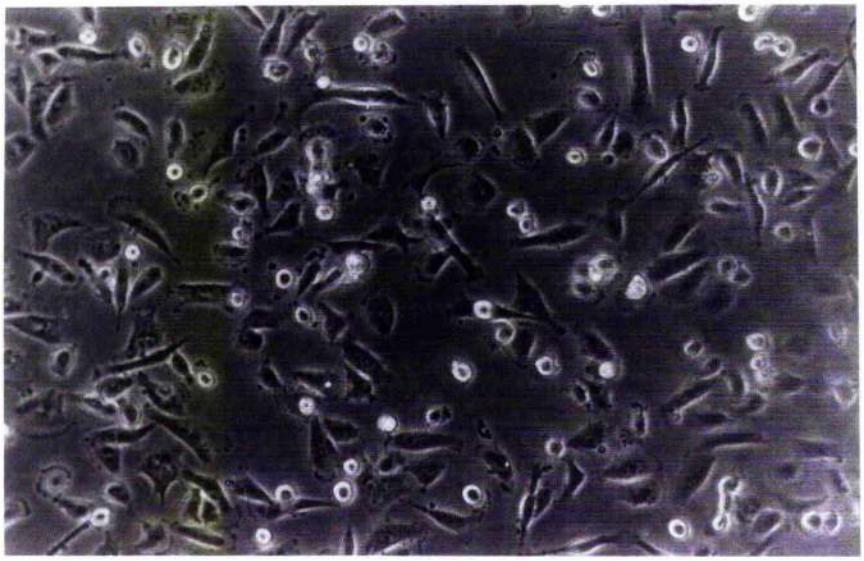

Figure 59 Morphology of MDA cells on plastic.

5 x 10^5 cells were cultures in 6-well plates tissue culture plates. The cells were left overnight and photographs were taken.

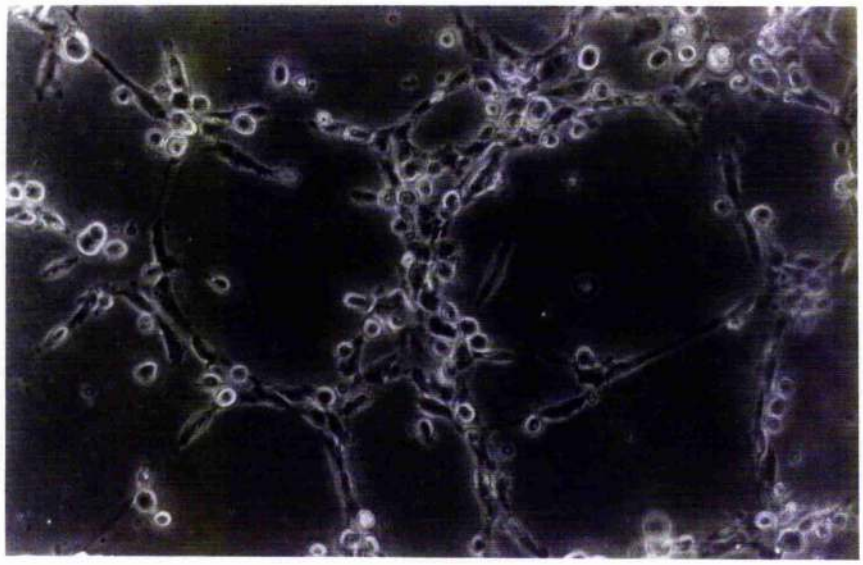

Figure 60 Morphology of MDA cells on EHS.

5 x 10^5 cells were cultures in 6-well plates precoated with matrigel. The photographs were taken after 4-6 hours. Magnification used was 4 x 1.5.

The arrow shows the pseudopodia like structure that originate from one cell towards the other

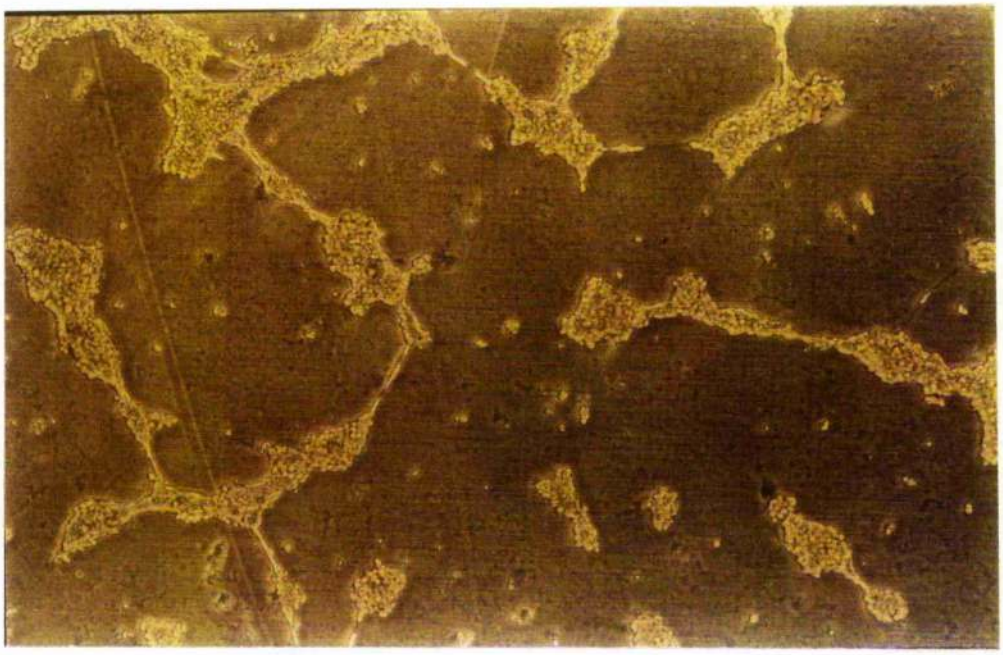

Figure 61 Morphology of MDA cells on EHS.

5 x 10^5 cells were cultures in 6-well plates precoated with matrigel. The photographs were taken after 48 hours. The magnification used was 4 x 1.5.

CHAPTER 4

DISCUSSION

DISCUSSION

Many biological processes involve a precise and co-ordinated network of cellular proliferation and differentiation. The development and progression of cancer is believed to involve altered events in the regulation of these fundamental processes. In cancer the balance of cells entering S-phase to those going into apoptosis changes. Breast cancer, like other tumours of epithelial origin including ovary, endometrium and prostate, belongs to a group of cancers that may be hormone dependent. These tumours, however, contain both hormone dependent and independent cells.

Steroid hormones, peptide hormones and growth factors play a critical role in the control of normal growth and development of the breast and also play a pivotal role in breast cancer progression. Previously, a plethora of reports has shown the role of these effectors of cell growth on plastic only. Clearly, the results of these studies should be taken with caution since growing cells on plastic is far from the real *in vivo* scenario. It is known that growth factors influence the growth characteristics of cells also by affecting the structure of the extracellular matrix and may therefore disturb or enhance the interactions of the cells with their growth substratum (Laiho & Oja, 1989). In the light of this, it was important to compare the behaviour of cells on plastic and EHS (Engelbreth Holm Swarm, a tumour basement membrane) in parallel, when treated with different growth factors and hormones. The present study was conducted in order to specifically address three questions of fundamental biological importance: (a) what effect, if any, do the various hormones have on the growth properties of breast cancer cells when grown on different substrata (i.e. EHS and plastic)? (b) are there changes in the expression of uPA, uPAR and PAI-1 gene products when cells are exposed to steroids and

growth hormones? and (c) is there is a correlation between the amount of uPA, uPAR and PAI-I with invasive ability of a cell line? (In other words, can certain hormones or growth factors alter the invasive potential of breast cancer cells?).

Proliferation Studies

Breast epithelium is directly under hormonal control and these hormones play an important role in regulating proliferation and protein synthesis of target cells. Oestrogen hormones also control the regulation of locally acting growth factors (Dickson *et al.*, 1990), thus steroid hormones, including oestrogen, progesterone and the anti-oestrogen, tamoxifen, were used in this study. The effect of growth factors was also analysed on the two human breast cancer cell lines, MCF-7 (hormone responsive) and MDA-MB-231 (hormone independent).

Proliferation results presented in this thesis showed that EGF and TGFα increased the growth rate of MCF-7 cells on both plastic and EHS. Stimulation of growth on either substratum was relatively small but cells grown on EHS showed growth stimulation at lower concentrations of TGFα than those grown on plastic. It may be the result of interactions of cells with the extracellular matrix (EHS) which may modify or alter the receptor levels in a three dimentional growth thereby making the cells more responsive to lower doses of growth factors. This response may vary from cell line to cell line and may alter according to different conditions. This can be seen in MDA cells on EHS where lower doses of EGF and TGFα decreased the growth. The actual mechanism of action of these growth factors on EHS is complex and remains to be further elucidated. By contrast, the growth of MDA cells on plastic was

stimulated by EGF at the lower concentrations though only a maximum of 15% increase above control was observed. It should be noted that (Murayama & Mishima, 1995) have shown that whereas lower concentrations of EGF support cell proliferation, higher concentrations cause growth inhibition. Similarly, studies of (Imai *et al.*, 1982) (Dong *et al.*, 1991) on human breast cancer cells, corroborate the EGF results presented in this study on plastic, where higher concentrations are inhibitory. TGFα increased MDA cell growth at higher concentrations on either substratum.

TGFα, like EGF, exerts its effect via the EGF receptor (EGFR). In spite of this, the MDA cell line responded differently to the two growth factors. It should be noted, however, that the percentage of increase in growth of MDA cells, as compared to MCF-7 cells, was small, about 20% (Fig 9 and 10), as compared to MCF-7 cells (about 60%). In other words, it appears that the exogenously added growth factors had little or no effect on MDA proliferation. This lack of response might be due to the large amounts of EGF or TGFα that is produced endogenously by the cells which masks the effect of the exogenously added growth factors. Cappelleti *et al.* (1993) have estimated the amount of EGFR to be 3 fmol/mg of total protein in MDA cells as compared to 60 fmol/mg in MCF-7. It is likely that the differences in EGFR levels shown by various groups may be due to different experimental conditions. The literature also suggests that co-expression of the receptor and the growth factor leads to the cytoplasmic activation of the receptor without involvement of the cell surface membrane. This may create an autocrine loop between the endogenous growth factors and their receptors leaving only a few receptors accessible to exogenously added EGF to stimulate growth (Long & Rose, 1996; Verber *et al.*, 1994).

effect on MDA cells in this study. All these different observations show the complexity of TGFβ and also that different cell lines and different experimental conditions can alter results for the same cell line and same drug used under different laboratory conditions. The status becomes even more complex when cells interact with EHS components and also in the presence of growth factors. Because both growth factors and EHS components (collagen, fibronectin, laminin etc.) affect and alter each other's functions. e.g. TGFβ regulates the ECM remodelling and functions such as immunosuppresion may help in tumour progression.

MCF-7 cells showed a similar growth pattern towards oestrogen, progesterone or anti-oestrogen, tamoxifen on either plastic or EHS. Concentrations required for proliferation were similar but the extent of growth between the two substrata were consistently different. The extent of stimulation was 2 to 3 times more on plastic than EHS. However, the extent of stimulation of MDA cells in response to growth factors remained similar. Albini and associates showed an increased growth of MCF-7 cells on reconstituted basement membrane (ECM/EHS) and or laminin (component of ECM) but they have not compared the same on plastic (Albini *et al.*, 1986).

It can be concluded from the proliferation studies that EHS has no significant effect on the growth of MCF-7 cells in response to both hormones and growth factors. Whereas, MDA-MB-231 cells showed a significant difference on both the substrata in response to growth factors, especially TGFβ. The restricted growth on EHS may be because the cells are relatively differentiated when in association with extracellular matrix.

Plasminogen Activators

It is known that the inoculation of matrigel together with human tumour cells MDA-MB-435 (Bao *et al.*, 1994) MDA-MB-231 or MCF-7 cells (Noel *et al.*, 1993) into nude mice has increased the formation and growth of the tumour but the exact mechanism is unknown. They showed differences in growth rates, MCF-7 being slower in tumour formation than MDA, in spite of the same conditions. No work has been done so far to find the effect on inoculation of TGFβ and other growth factor treated cells and matrigel. As matrigel effected the growth and and tumour formation ability of the cells *in vivo*, it should influence the invasive behaviour of MCF-7 and MDA cells *in vitro* also.

It is clear from this as well as other studies that hormones and growth factors effect not only the biosynthesis and subsequent secretion of a number of different proteins, but also the proliferative and invasive potential of both ER^+ and ER^- cells (Tedone *et al.*, 1996) . Although it is still unclear how hormones and growth factors alter the invasive properties of tumour cells it is widely accepted that the process of invasion impinges upon the activities of the various components of the plasminogen activator system.

In this study the relative amounts of urokinase (uPA), its inhibitor PAI-1 and uPAR were quantitated in two well characterised breast cancer cell lines in order to understand how their levels are effected in response to various hormones and growth factors on different substrata and whether there exists a linear correlation between the levels of these three molecules to the invasive potential of a cell line.

uPA, PAI-1 and uPAR transcripts and protein expression were measured in MDA and MCF-7 cells. Although uPA was detected in the MDA cell line in

large quantities, no uPA could be detected in the MCF-7 cells in spite of numerous attempts throughout the course of this study. This is surprising given that other groups have documented the presence of uPA in the MCF-7 cell line. It should be noted that the same antibody which detected uPA in MDA cells failed to find its antigen in the MCF-7 cells. Moreover, no uPA mRNA could be detected using RT-PCR analysis carried out on total RNA isolated from untreated MCF-7 cells. The same set of PCR primers however successfully detected large amounts of the uPA mRNA in the MDA cell lines. Remarkably, neither uPA mRNA nor its protein product were detected even after 72 hours of treatment with growth factors in MCF-7 cells. The situation is specially conflicting since in this study control MCF-7 cells retain their ability to invade, although with much less efficiency than MDA cell line. Does this mean that proteins other than uPA are able to activate plasmin or the proteases which directly breakdown the BM?

In the MDA cells, ELISA showed large amounts of uPA. More importantly activity assays revealed an active uPA and it is not surprising therefore, that this cell line is much more aggressive in the invasion assay than MCF-7 cells. Although the levels of uPA are thought to dictate whether a cell line is invasive, the amount of PAI-1 is also an important co-determinant of the invasive capacity of a cell line. It has been shown that it is the relative amounts of uPA and PAI-1 which determine whether a cell line would be invasive or not.

The MDA-MB-231 and MCF-7 cells have been shown to produce and secrete mainly uPA or tPA and PAI-1. The synthesis of uPA, its inhibitor, PAI-1 and receptor, uPAR is known to be controlled by a variety of hormones, growth factors and cytokines (Andreasen *et al.*, 1990; Dano *et al.*, 1985; Saksela, 1985). Growth factors especially TGFβ have been implicated in invasion and metastasis in some malignant cells. However, the importance of EGF and

TGFα in the process of invasion remains to be elucidated. TGFβ is involved in the metastatic cascade in some cells, including MCF-7 and MDA-MB-231 (Thompson *et al.*, 1990) and TGFβ mRNA levels have also been found to be elevated in malignant human breast tumour biopsy specimens. The work describe here shows that TGFβ induces an increase in cell-associated uPA expression, but has little effect on uPA secretion. These results are in line with those of Arnoletti *et al.* (1995) and Keski-Oja *et al.* (1988) who demonstrated that TGFβ1 induced an increase in cell-associated uPA expression and uPAR biosynthesis in the A549 cell line. Lund *et al.* (1987) have shown that TGFβ strongly decreases the PA production in W1-38 human lung fibroblasts. In contrast, Hachiya *et al.* (1995) have reported that exogenous TGFβ1 suppresses both PA production and *in vitro* invasiveness of MDA-MB-231 cells. In MCF-7 cell line, however, they found that TGFβ increased PA and also *In vitro* invasiveness. In contrast, our results show an induction in the cell associated uPA levels by 2-4 fold in MDA-MB-231 cells, whereas levels of uPA in the CM were either decreased or slightly affected by TGFβ in MDA-MB-231 cells and therefore demonstrates the importance of assessing extracellular plasminogen activator levels as well as cell associated factors.

Our results showed that TGFβ also induced a remarkable elevation of cell secreted PAI-1 (250-300%) as compared to (100-150%) increase in the cell secreted PAI-1 (in the CM of MDA-MB-231 cells). Arnoletti *et al.* (1995) have also shown increased cell secreted PAI-1 levels in CM of breast cancer cells in response to thrombospondin, an adhesive glycoprotein known to be involved in the mechanism of tumour progression and TGFβ1. TGFβ also increased the amounts of PAI-1 and uPA secreted from cultured adult skin fibroblasts Lund *et al.* (1987; Desruisseau *et al.* (996) further confirmed that TGFβ increases the levels of secreted PAI-1 by about 700% in a dose dependent manner in human prostatic cancer cell line PC3.

In some studies decreased uPA levels and increased PAI-1 levels are translated into decreased plasmin activity, measured by chromogenic assay, and ultimately decreased invasion. However this work shows that elevated levels of uPA and PAI-1 in breast cancer cells results in increased proteolytic activity. The levels of uPA and PAI-1 both were up-regulated in MDA-MB-231 cell line whereas in MCF-7 cells, a significant PAI-1 increase was observed. The increase in uPA is associated with malignancy, while the role of PAI-1 is still conflicting. Duffy *et al.*, (1998) has proposed that the levels of the two proteins, uPA and PAI-1, are inversely correlated with patient prognosis. Others have argued that high levels of PAI-1 leads to decreased invasion by inhibiting uPA proteolytic activity. Several authors including (Pappot & Brunner, 1995) have demonstrated that high levels of PAI-1 propagate tumour progression by increasing the motility of the cells, which is a pre-requisite of tumour invasion. The results in this thesis are in agreement with the latter hypothesis.

This simultaneous up-regulation of uPA and PAI-1 in association with increased invasion confirms the hypothesis that both uPA and PAI-1 are strong and independent prognostic factors in breast cancer patients (Duffy *et al.*, 1990; Janicke *et al.*, 1993; Schmitt *et al.*, 1990; Schmitt *et al.*, 1994; Grondahl-Hansen *et al.*, 1993; Janicke *et al.*, 1991; Bouchet *et al.*, 1994; Blasi, 1993; Brunner *et al.*, 1994; etc). Both the enzyme and its inhibitor are thought to be required for cell migration, adhesion and invasion. uPA is localised at the cell surface and at the invasive foci whilst PAI-1 is distinctly localised in the ECM. Receptor bound uPA remains available to PAI-1. Internalisation and degradation of the uPA-PAI-1 complexes enables cells to migrate through the recycled receptor, uPAR. Increased levels of PAI-1 restricts invasion (Mignatti & Rifkin, 1993). Thus PAI-1 has a role in new tumour stroma formation and also protects cancer tissue against proteolytic degradation. This is obviously important in the formation of distant metastasis. Grondahl-Hansen *et al.*

(1993) and Janicke *et al.* (1993) have shown that high levels of PAI-1 are present in distant metastasis. PAI-1 synthesis by breast cancer cells is influenced by activation of the protein kinase C and protein kinase A pathways (Knudsen *et al.*, 1993).

Cell associated uPA can be considered partially equivalent to receptor bound uPA and therefore can be used as an index of cell surface proteolytic activity. When cells were cultured with TGFβ, it promoted cell expression of uPA and thus mediated tumour cell invasiveness by increasing proteolytically active areas of the cell surface. Welch *et al.* (1990) also showed that TGFβ can stimulate invasion and metastatic potential for mammary adenocarcinoma cells.

The inhibition of proliferation by TGFβ in MDA-MB-231 cell line and stimulation of uPA/PAI-1 system indicate that proliferation and plasminogen activator system are regulated by two distinct mechanisms independent of each other. This has been suggested by Lambrecht and colleagues during their study on the regulation of proliferation and uPA/PAI-1 system in breast cancer cell lines, MCF-7 and MDA-MB-231 (Lambrecht *et al.*, 1998).

It is important to note that the anti-uPA Ab abolished TGFβ-induced invasion but not the level of invasion by control cells. This suggests that TGFβ-induced invasion is directly due to secreted uPA but the basal level is due to other proteases.

It is also important to draw attention to the fact that tamoxifen, usually considered as a weak agonist did increase proliferation, but inhibited invasion by MCF-7 cells. This observation strongly supports the anti-invasive ability of tamoxifen and underline its use as an effective anticancer drug in breast cancer patients.

SUMMARY

MDA is more invasive than MCF-7. This is reflected in uPA and PAI-1 contents. The effects of various exogenous factors in the levels of these critical components of the invason system were suprisingly variable. TGFα and EGF showed no exciting effects on the uPA and PAI-1 levels. Interestingly, TGFβ showed huge effects on the plasminogen activator and inhibitor levels, proliferation. This study has shown that the *in vitro* invasive potential of human breast cancer cell line MDA-MB-231, across a reconstituted basement membrane (matrigel) correlated with the synthesis of both uPA and PAI-1 which were effected by growth factors EGF, TGFα and TGFβ. Matrigel, an important component of invasion assay has been used as a substratum, in the present study, to determine its effects on the growth potential and plasminogen activator secretion from epithelial cells

This study has clearly demonstrated that the nature of the substratum is critical for physiological experiments. Comparitive study on EHS and plastic of the two breast cancer cell lines, MDA-MB-231 and MCF-7, showed that the level of stimulation in growth and secretion of secreted and cell associated plasminogen activators, uPA and its inhibitor PAI-1 responded differently to growth factors and steroid hormones, on EHS and plastic.

Low concentrations of TGFα and EGF demonstrated an increase in the cell associated uPA and PAI-1 levels on EHS and inhibition on plastic, under same experimental conditions. Similarly, the activity of cell associated uPA in response to TGFα slightly increased on EHS and remained unaltered on plastic. Growth studies showed that cells on EHS responded to high doses and thus stimulated the rate of proliferation while low doses slightly inhibited the growth. On plastic, however, higher doses were inhibitory.

Invasion assays also showed that EGF and TGFα did not increase invasiveness of MDA cells. As the MDA cells were intrinsically highly invasive, it is perhaps not surprising that exogenous factors had little effect in increasing invasive activity. This correlated to the fact that uPA levels and uPA activity only slightly changed in response to these growth factors, and therefore played no role in the process of invasion. However, the anti-uPA antibody used in the invasion studies did not completely inhibit the *in vitro* invasion of cells. Thus it showed that uPA may not be the only enzyme necessary for invasion. Other metalloproteases may be involved in the process.

The effects of TGFβ on PA production and *in vitro* invasiveness was examined. The studies showed that TGFβ stimulated both uPA, PAI-1 production on both EHS and plastic by about 350%. The level of secreted proteins were high on EHS than on plastic. On the other hand, the production of cell associated proteins were higher on plastic than on EHS.The effects were clearly dose dependent on both EHS and plastic. Higher doses stimulated uPA and PAI-1 expression on EHS but on plastic much lower doses were needed to achieve the same level of stimulation. As a potent growth inhibitor, TGFβ inhibited the growth of MDA cells on EHS at a low concentration but on plastic a much higher dose is needed to inhibit cellular proliferation. All these observations point to the fact that the mechanisms of regulations of growth are different from those of invasion.

It was surprising to see that MCF-7 cells showed invasion with EGF, E2 and much enhanced with TGFβ, although no detectable uPA could be observed both at the transcriptional or translational levels. The uPA antibody used also did not inhibit the invasion completely but did decrease it slightly. This shows

that the invasion may not only be uPA dependent but other proteases may be similarly able to drive this process.

It is clear from these studies that changes in the growth and invasive potential of a cell line are not only dependent on the type and concentration of a given growth factor or a steroid, but are also dependent on the nature of the substratum and the nature of the cells themselves.

Future Work

The work reported herein shows the effects of different growth factors and hormones on the proliferation and invasive potential of two breast cancer derived cell lines, MDA and MCF-7. Although, the biochemical features of the MDA-MB-231 cells are consistent with those that have been reported previously, the MCF-7 cells appear to be completely distinct from the conventional MCF-7 cell-line. It is important to further charcaterise this cell line in order to understand and define its salient features which make it different from other MCF-7 cells. For instance, the TGFβ stimulation of growth of this sub-clone of MCF-7 cells was unexpected and while MCF-7 cells have been shown by others to express uPA, neither the mRNA nor the uPA protein could be detected in the MCF-7 cell-line reported here. Since the same anti-uPA antibody could successfully detect uPA in MDA cells, it is unlikley that failure to detect uPA in MCF-7 cells on several occasions was due to technical reasons. It is interesting to note however, that in spite of the absence of uPA, MCF cells scored positively, albeit to a much lesser extent than the MDA cells, in invasion assays. This observation, together with the fact that anti-uPA antibody brought invasion to the levels of control but control continued to invade that was independent of uPA is indicative of the fact that proteins other than uPA are also capable of triggerring a cascade of events that lead to metastasis. Identifying such factor(s) in the future is of obvious importance since they have the potential to be used as targets for therapeutic intervention.

It has also become clear from this work that the proliferative behaviour of cells in response to various growth factors and hormones is affected by the nature of the substratum. Although EHS, which is a mixture of various proteins, was

used for these studies it would be interesting to identify specific proteins whose presence or absence affects the growth of cells. It would also be interesting to mix the stromal fibroblasts from normal or tumour cells in matrigel and find their effects on the functional modification of these cell lines in term of invasion rates.

In order to monitor cellular invasion a new strategy that is more sensitive than that used here needs to be adopted. Such a novel and cost-effective method employing the use of eggs from chickens was in fact reported recently and appears to be very promising. It would be useful in future to rely on such methods in order to make the data more quantitative.

Tamoxifen inhibited invasion in the ER+ MCF-7 cell line, whereas it showed an agonistic effect on the growth of the same cells. Therefore, it will be interesting to find the mechanisms of this differential behaviour of tamoxifen in the regulation of growth and invasion.

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