PRODUCTS OF RAT MAMMARY ADENOCARCINOMA CELLS
AND THEIR RELEVANCE TO CANCER METASTASIS

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by

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DMBA 7,12-Dimethylbenz[a]anthracene  
EDTA ethylenediaminetetraacetate  
HEPES 4-(2-hydroxyethyl)-1-piperazine-ethanesulfonic acid  
HPLC high-performance liquid chromatography  
NIH thrombin reference standard (Bureau of Biologics)  
PA plasminogen activator  
SDS-PAGE sodium dodecyl sulphate/polyacrylamide gel electrophoresis  
t-PA tissue-type plasminogen activator  
u-PA urokinase-type plasminogen activator  
TRIS 2-amino-2-hydroxymethylpropane-1,3-diol  
Z-Lys-SBzl N-benzyloxy carbonyl-L-lysine thiobenzyl ester  

Enzymes: Plasmin (EC 3.4.21.7); urokinase (EC 3.4.21.31); thrombin (EC 3.4.21.5); trypsin (EC 3.4.21.4).  

DEFINITION: PU, Ploug unit, a unit of urokinase activity equivalent to approximately 1.4 Committee on Thrombolytic Agents (CTA) units, or to approximately 1 international unit (Barlow, 1976).
ABSTRACT

(1) The plasminogen activator (PA) of intact metastatic cells has been measured in vitro and the activity immediately available at the cell-surface was found to be of an equivalent amount to that which was secreted by the same cells over a period of 6h. Both cell-surface and cell-secreted PA are active under physiological salt conditions.

(2) An inhibitor of human urokinase and metastatic cell PA (both secreted and cell-surface) which is secreted by non-metastatic cells, has been purified to apparent homogeneity and characterized. This inhibitor binds very rapidly at low concentrations in a noncompetitive manner.

(3) A pro-enzyme form of PA has been discovered that can be activated very rapidly by trypsin and a substance secreted by metastatic cells but, unlike previously reported pro-PA enzymes, it is not activated by plasmin.

(4) Incubation (24h) of metastatic cells with the antibiotic tunicamycin prior to intravenous injection into rats, inhibited both PA production and metastatic potential. However, incubation (6h) of metastatic cells with dexamethasone inhibited PA production but had no effect on metastatic potential.

(5) Modification of an existing method of plasminogen isolation has resulted in the preparation of purified native plasminogen free of plasmin contamination which can be used at physiological concentrations in vitro.
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CHAPTER 1

THE POSSIBLE ROLE OF PROTEASES IN CANCER METASTASIS
1.1 INTRODUCTION

The malignant state is characterized by the ability of tumour cells to invade host tissue and to disseminate to other, sometimes distant sites to form secondary tumours or metastases.

The development of metastases is a multistep process. Some of the postulated steps involved are: uncontrolled cell proliferation and vascularization of the tumour; detachment of tumour cells and invasion of the surrounding host tissue, local lymphatics and blood vessels; transport of these cells to new sites; arrest and adherence of tumour cells at these sites; penetration of the adjacent tissue and proliferation of cells to form a new tumour (Poste & Fidler, 1980).

From these steps, it is apparent that the ability of cells to degrade tissue and infiltrate vessel walls is an essential requirement for metastasis to occur. There is circumstantial evidence to suggest that these processes involve secreted or cell-surface associated hydrolytic enzymes (Robbins & Cottram, 1979). Some of the steps in which such enzyme activities may be involved are illustrated in Figure 1.

1.2 PROTEASE ACTIVITIES

Two proteolytic enzymes which have been studied with respect to increased rates of activity in neoplasms are: plasminogen activator (PA), an enzyme which converts the circulating zymogen plasminogen into the active protease, plasmin; and collagenase, an enzyme which degrades
FIGURE 1. Diagram outlining some of the steps (?) in metastasis in which tumour cell enzymes may be involved.

This diagram has been adapted from that of Schirrmacher (1985).
metastatic neoplasm → primary neoplasm → white blood cell → proliferation and vascularization → invasion into tissue and bloodstream → thrombus formation → arrest → extravasation → degradation and invasion of new tissue → growth of metastases → degradation of basement membrane → cleavage of cell surface glycoproteins
collagen, a major component of connective tissues and capillary walls (Poste & Fidler, 1980).

1.2.1 Physiological role of collagenase and PA

Both collagenase and PA play an important role in normal physiological processes. In the mammal, tissue remodelling occurs at certain periods with a consequent increase in the proliferation and migration of specific cell types followed by a subsequent degradation of tissue. Examples of normal processes in which these two enzymes have been found to be present in large amounts are:

(i) In blastocyst implantation during early embryonic development, in which the time course of increased PA activity is closely correlated with the invasive period (Strickland et al., 1976);

(ii) during mammalian ovulation, PA production by granulosa cells is induced by hormones and reaches high levels immediately prior to the disintegration of follicle walls (Strickland & Beers, 1976);

(iii) in mammary gland involution following cessation of lactation, a transient increase in PA production parallels the initiation of involution in response to hormones (Ossowski et al., 1979);

(iv) during wound repair, a migration of epithelium and the formation of new collagen is followed by resorption of the newly formed collagen during wound contraction; a high level of collagenase activity has been observed during the latter phase (Grillo & Goss, 1967); and
increased collagenase activity has been observed during uterine resorption (Woessner, 1980). This evidence suggests that these two enzymes play an essential role in tissue remodelling and cell migration. The increases in enzyme activities are usually under hormonal control and, following remodelling, the enzymes levels return to normal (Ossowski et al., 1979).

However, some malignant cells have been found to have higher levels of these enzymes than normal cellular counterparts (Ossowski et al., 1979; Dresden et al., 1972; Tarin et al. 1982) and may be less responsive to hormonal control (Ossowski et al., 1979). It therefore seems likely that the ability of cells to metastasize may be derived from basic physiological processes which have escaped normal regulatory controls. This could occur if specific genes which are required for the normal control and synthesis of degradative enzymes, have suffered aberrant change(s) (Robbins & Cottrnan, 1979). These changes could result in a lack of expression, or a reduced amount of regulatory products, leading to an increased activity of these enzymes.

1.3 EVIDENCE FOR INCREASED PROTEASE ACTIVITY IN MALIGNANCY

1.3.1 Collagen degradation

In some experiments tumour tissue explants were found to lyse collagen (Ogilvie et al., 1985; Taylor et al., 1979); and in others, a high collagenolytic activity was observed in cultured tumour cells (Harris et al., 1972; Tane et al., 1978).
A recent study using murine mammary tumours, showed that secretion of collagenase was higher in tumours capable of colonizing lungs than in normal proliferating mammary tissue (Tarin et al., 1982). Further, a collagenase specific for Type IV collagen, the type of collagen found in basement membranes and capillary walls (Jackson, 1980), was found in the culture medium from a highly metastatic murine tumour (Liotta et al., 1979). A later study correlated basement membrane degradation with metastatic capacity (Liotta et al., 1980).

The basement membrane consists of a matrix of collagen, laminin and proteoglycans and separates endothelial or epithelial cells from connective tissue (Laurie, 1982). It has an important role in maintaining the orderly positioning of new cells within the correct tissue and organ (Vracko, 1974). If metastatic cells produce increased amounts of enzymes which degrade basement membrane components, then theoretically there is the opportunity for invasion into capillaries and surrounding tissues (Figure 2).

However, other studies have not observed any increased collagenolytic activity in the media of cells cultured from metastatic tissue (Recklies et al., 1980) although an increase in the activity of another proteolytic enzyme, cathepsin B, was observed in the media from tumour cell culture (Recklies et al., 1980; Poole et al., 1978).
FIGURE 2. Diagram showing the composition of basement membrane and the components which can be degraded by enzymes.

Enzymes

(1) Cathepsin B
(2) Cathepsin D
(3) Cathepsin G
(4) Lysosomal elastase
(5) Plasmin (can also activate pro-collagenase)
(6) Collagenase

This diagram is adapted from that of Stanley and workers (1982).
cardiovascular spaces

lamina lucida (10 50nm)

lamina densa (20 300nm)

basal cells

laminin (5)

proteoglycans (2) (3) (4)

collagen (Type IV) (1) (3) (4) (6)

proteoglycans (2) (3) (4)

connective and supporting tissue
1.3.2 Plasminogen activator

Experimental evidence exists which links increased PA production with malignant transformation (Moscatelli et al., 1980). The presence of elevated urokinase type PA (u-PA), either intracellular or secreted, has been observed in many transformed cells (Markus et al., 1983). It has been proposed that PA could play an important role in tumour angiogenesis (Berman et al., 1982) and metastasis (Rohrlick & Rifkin, 1979) through the activation of PA to plasmin, a protease with a wide substrate specificity. Plasmin has the ability to hydrolyze cell-surface glycoproteins and to digest hormones such as glucagon and ACTH (Castellino & Powell, 1981) and the capacity to activate latent enzymes such as pro-collagenase (Werb et al., 1977) and pro-PA (Wun et al., 1982). Plasmin can also cause the disappearance of internal actin-containing cables, a phenomenon which has been observed in transformed cells. Absence of these cables has been correlated with a loss of anchorage dependence (Pollack & Rifkin, 1975). In other studies plasmin was required for cell migration in artificial wounds (Ossowski et al., 1975). Therefore, plasmin formed as a result of increased PA production could potentially aid cancer cells in both motility and invasion.

In recent experiments, application of an anti-urokinase antibody delayed the onset of pulmonary metastases in chicken embryos following inoculation of human carcinoma cells onto the chorioallantoic membrane (Ossowski & Reich, 1983). This evidence suggests that
activity in tumour tissue cultures only during the first and second days of culture. Nevertheless a brief period of increased activity may be all that is necessary to begin the process of metastasis;

(b) substrate specificity which may be important. One group (Liotta et al., 1979) discovered that a tumour enzyme which degraded type IV collagen did not degrade collagens of types I, II, III or V. However, in a study in which no increased collagen degradation was observed (Recklies et al., 1980), the substrate was derived from guinea pig skin which contains primarily types I and II collagen (Duance & Bailey, 1981). It is possible that different tumours secrete different types of collagenase and whether they metastasize to bone (Type I) or liver (Type V) (Jackson, 1980) for example, may depend upon the specific collagenase which they secrete. Thus, a limited range of collagen substrates may not detect any apparent increased activity;

(c) if only one enzyme is being investigated, other enzyme activities with similar degradative properties may be overlooked. As has been proposed (Liotta et al., 1977), activation of more than one enzyme system may be necessary for the dissolution of basement membrane due to the low solubility of its structure which is caused by extensive crosslinking. Although the natural routes for collagen degradation are not yet completely understood, \textit{in vitro} results suggest that a combination of enzymes act synergistically.
PA may play a role in the early stages of metastasis.

However, although some reports have shown a correlation between high PA production and metastatic potential (Eisenbach et al., 1985; Wang et al., 1980), others could find no correlation (Wilson & Dowdle, 1978; Recklies et al., 1980). Using another approach, administration of protease inhibitors has had variable results, reducing metastases in some cases (Giraldi et al., 1977; Saito et al., 1980), but increasing metastases in other experiments (Clifton & Agostino, 1964; Turner & Weiss, 1981) so that the role of proteases in cancer metastasis is still unresolved.

1.4 POSSIBLE REASONS FOR CONFLICTING RESULTS

One of the most likely reasons for the variations in observed results is the apparent heterogeneity of tumour cells. Within a primary tumour, subpopulations of cells exist with differing metastatic capacities (Poste & Fidler, 1980), and in fact less than 0.1% of cells from a primary tumour are capable of metastasizing (Fidler et al., 1978). Consequently, in experiments employing tumour tissues, the enzyme activities of a small population of metastatic cells, may be masked by the products of the less malignant population of cells.

Other reasons which could account for apparent inconsistent results are:

(a) timing, which may be important in some systems to detect increased amounts of an enzyme. Harris and colleagues (1972) observed a large amount of collagenolytic
Whereas specific collagenases degrade certain types of collagen, other enzymes are also capable of non-specific collagenolysis. These enzymes include neutral proteinases such as cathepsin G and lysosomal elastase, and acidic proteinases such as cathepsin B (Baici, 1980). Two of these enzymes which can dissolve basement membrane collagen (Type IV), are cathepsin G and elastase.

1.5 CELL SURFACE PROTEINS

Alterations in certain enzyme activities which could lead to invasion have already been discussed. However, the importance of alterations in cell surface components leading to uncontrolled proliferation has been shown by the evidence that transformed cells lack certain cell surface glycoproteins (Nicolson, 1976; Hynes, 1976; Yamada et al., 1980).

It has been postulated that cell surface components lead to contact inhibition and cessation of growth in mitotic cells and that the loss of proteoglycans and glycoproteins through increased protease activity, may contribute to malignancy. Evidence to support this theory has come from the observation that addition of protease inhibitors and certain free sugars can cause the cessation of division and 'piling up' which has been observed in malignant cell cultures (Roth, 1973).

This implies that protease inhibitors may be doing the work of the gene products which are missing in transformed cells due to the alteration of regulatory genes. Potential mediators of proteoglycan degradation
in pathological circumstances are the cathepsins B, D and G, and lysosomal elastase (Christman et al., 1977). Some glycoproteins are also readily degraded by plasmin and elastase (Vaheri et al., 1980).

With dual roles affecting both cell surface components and collagen, the enzymes plasmin, elastase and cathepsin B are prominent candidates for effectors of metastasis. However, it is unclear whether one enzyme such as PA initiates a cascade of enzyme reactions leading to metastasis, or whether several enzymes act synergistically. If the important enzyme mechanism(s) governing metastasis could be elucidated then it might be possible to find some means of controlling and perhaps preventing metastasis.

1.6 PROPOSED MODEL FOR METASTASIS

There are at least two stages in the development of malignant tumours. The first stage involves transformation which allows cells to grow in an uncontrolled fashion. This can lead to tumorigenesis. In the second stage, further alterations in the genome occur allowing expression of the metastatic phenotype.

It appears that transforming genes or oncogenes may be involved in these phenomena. When activated oncogenes, originally detected in retroviruses, are transferred into normal cells, they induce transformation. A number of oncogenes have now been isolated from retroviruses which are closely related to normal
cellular genes (Spandkdas & Wilke, 1984). It seems that all vertebrate cells carry a number of proto-oncogenes, that is, genes which have the potential to become oncogenes (Hunter, 1984; Lebowitz, 1983). Thus, an oncogene is defined as a gene which is derived from a normal DNA sequence that has undergone a change enabling the gene to cause transformation when introduced into normal cells (Weinberg, 1982).

There are several mechanisms in which proto-oncogenes can be converted to active oncogenes:

1. acquisition of a novel transcriptional promoter following chromosomal translocation which leads to deregulation;
2. amplification of the gene which leads to overexpression of gene product;
3. alteration of the gene structure which leads to an altered structure of encoded protein; and
4. the contribution of an enhancer sequence which influences levels of transcription and therefore levels of gene products.

In 3T3 mouse fibroblasts transformed by a ras$^{Ha}$ oncogene, the metastatic phenotype could be conferred on these cells when they were transfected with DNA from a human metastatic tumour. But the introduced DNA segment did not appear to be closely related to known myc or ras oncogenes (Bernstein & Weinberg, 1985). Also, while the introduction of ras$^{H}$ cloned oncogenes alone induced metastatic behaviour in some
transformed cell lines, not all transfected cell
types displayed this capacity. So while transforming
genes may lead to tumorigenicity, they do not
necessarily lead to metastasis. The evidence suggests
that the interaction of oncogenes with at least one
other factor already present in cells is required
for expression of the metastatic phenotype (Muschel
et al., 1985). These other factors may be represented
by mutations in existing genes and therefore, the
combined effect of several genomic alterations is
required before metastasis become evident.

The chromosomal location of oncogenes may be
important. It has been suggested that in view of
the possible role of PA in cancer metastasis, there
may be some significance in the fact that two
oncogenes, c-mos and c-myc, are located on chromosome
eight (Neel et al., 1982), which is the chromosome to
which PA genes have also been mapped (Rajput et al.,
1985). Alterations in chromosome eight have been
linked to two types of cancer, namely Burkitt's
lymphoma and acute myelogenous leukaemia (Rowley, 1981;

Accordingly, the activation of several oncogenes
(Vousder & Marshall, 1984) could lead to a cascade
of cellular gene products which are normally only
expressed during physiological conditions of tissue
remodelling (Muschel et al., 1985). Some of these
events could lead to alteration of cell-surface
components and deregulation of PA activity thus
allowing cells to become metastatic.

1.7 CONCLUDING REMARKS

From the evidence presented it appears that increased activities in several proteolytic enzymes may be related to metastasis. These activities could be related to the loss, or alteration of genes involved in the regulation of these enzymes. However, several changes are required before the development of malignant tumours occurs.

As preliminary experiments using metastatic and non-metastatic cells derived from the same cell line, showed no difference in levels of activities of the enzymes collagenase and cathepsin B, but did show higher levels of PA in the metastatic cell line, this project focused on PA activity in relation to metastasis.

The main investigations involved the purification and characterization of a PA inhibitor secreted by non-metastatic cells. A pro-enzyme form of PA was discovered in the crude inhibitor preparation, and this was also examined. Another approach examined compounds which inhibited PA release from cancer cells. These studies were considered from the point of view of the possible inter-relationship of PA and inhibitors in cancer metastasis.
CHAPTER 2

GENERAL EXPERIMENTAL PROCEDURES
2.1 MATERIALS

Materials were obtained from the following sources:
bovine trypsin (Type X1), bovine thrombin (Grade 1),
bovine fibrinogen (Type 1-S), plasmin from porcine blood,
6-aminohexanoic acid, Coomassie Brilliant Blue R,
Coomassie Blue G250, Tris HCl, tunicamycin, dexamethasone
and molecular weight markers for SDS-PAGE from Sigma, U.S.A.;
Lysine sepharose 4B, DEAE- Sephacel, Polybuffer exchanger
PBE 94, Polybuffer 74 and Cytodex no. 2 from Pharmacia,
Sweden; piperazine, Fluka, Switz.; polyacrylamide and Affigel
Blue 100-200 mesh, Biorad, U.S.A.; Amicon concentrator
model 8200 and Amicon membranes, Amicon Corp. Australia;
HPLC Protein Pak ion-exchange column DEAE-5PW, Waters,
U.S.A.; RPMl 1640, Gibco, U.S.A.; foetal calf serum and
Linbro tissue culture plates, Dulbecco's Modified Eagle's
Medium (DMEM), Flow Lab., Australia; N-Benzzyloxycarbonyl-
L-Lysine thiobenzyl ester and human urokinase (lot 103285,
1700PU/vial) from Calbiochem-Behring Corp., U.S.A.; glycine
and imidazole, BDH Chem. Ltd., U.K.; tissue culture flasks,
Nunc, Australia; ampholytes and agarose H, LKB, Sweden;
bovine serum albumin (Fraction V) from Armour, U.K. and
Trasylol (0.8mg/ml), Bayer Pharm. Co., Australia.

The DMBA 8 cell line, which was derived from a solid
tumour and adapted to in vitro culture, and the Fischer
344 rat mammary adenocarcinoma cell lines MAT 13762 and
R3230, were obtained from EG & G Mason Research Institute,
U.S.A. Culture medium from the human melanoma cell line
(MM-170) was the gift of Dr H. Warren (Cancer Research Unit,
Woden Valley Hospital, Australia. L-[35S] methionine
(1160Ci/mmol), Amplify, Amersham International, Australia;
X-OMAT RP film (XRP-5), Kodak, Australia.
2.2 METHODS

2.2.1 Cell culture and collection of medium

Cell lines were cultured in RPMI 1640 with 10% heat-inactivated foetal calf serum and antibiotics (penicillin, 100IU/ml; streptomycin, 100µg/ml; and neomycin, 50µg/ml).

For collection of secreted PA, cells were washed and cultured in medium without serum for 18h at a density of 1x10⁶ cells/ml. For collection of inhibitor, 175cm² flasks of subconfluent cells (8.5x10⁵/ml in 30ml) were washed five times and incubated for 12-20h in medium serum. PA samples were stored with triton x-100 (0.1%).

2.2.2 Preparation of tumour tissue for assay of PA

Small pieces of tumour tissue were weighed then frozen at -70°C. After thawing, glycine buffer (50mM, ph 8.3) containing Triton X-100 (0.5%) was added (10µl/mg tissue) then left on ice for 1h before homogenizing. Samples were assayed for protein and diluted in AGTG buffer for PA assay (2.2.4).

2.2.3 Protein determination

Absorbance at 280nm was monitored during purification procedures; a simple and rapid assay using Coomassie Blue G250 (Rylatt & Parish, 1980) was used for estimation of specific activity. This method can measure as little as 0.1µg protein and is linear between 0-3µg protein (Figure 1). BSA was used as a standard.

SDS gels were stained for protein either with Coomassie Brilliant Blue R or with silver (Wray et al., 1981).
FIGURE 1. Standard curve for BSA.

Coomassie Brilliant Blue G250 (100µl of 0.06% w/v in 1.9% w/v perchloric acid) was added to protein (100µl in H2O) in 96 well tissue culture plates (U-shaped) and the absorbance read within 10min in a Dynatech microtiter plate reader (dual wavelength of 630nm, test; 410nm reference). For determination of inhibitor protein concentration, BSA was dissolved in the same buffer as the inhibitor. Values represent the mean ±S.D. (n=4-13).
2.2.4 Assay for plasminogen activator

**SDS-PAGE, native protein PAGE and zymograms**

PA samples mixed with electrophoresis sample buffer were added to wells in polyacrylamide vertical slab gels (10% resolving gels) which were electrophoresed at a constant voltage (50v, 16h, 25°C or 35v, 4h, 4°C) according to the method of Laemmli (1970) under non-reducing conditions.

Native protein polyacrylamide gels were run as in above with the following modifications: 7.5% resolving gels, separation buffer (pH9.5), and reservoir buffer (pH9.0) omitting SDS from all steps.

For zymograms, the gels were washed with gentle rocking in five changes of 2.5% Triton X-100 for 2h to remove SDS, followed by another 2 washes in Tris-HCl (50mM, pH8.0). The gels were then placed onto fibrin-plasminogen-agar matrices (Granelli-Piperno & Reich, 1970) consisting of: fibrinogen (20mg/ml), thrombin (0.2NIH units/ml), agarose (1%) and purified plasminogen (20µg/ml), and incubated at 37°C in a moist atmosphere. In this method, the opaque layer of agar contains the sequential substrates of plasminogen and fibrin which enables the conversion of plasminogen to plasmin by PA, and the detection of plasmin by lysis of fibrin. Clear zones localized in the opaque background demonstrate the position of enzyme activity following separation according to Mr in SDS-PAGE. This results in the production of a zymogram.
**Fibrin radial lysis assay**

A fibrin-plasminogen-agar matrix was poured into a plastic dish (10cm²) and wells were cut to enable the addition of PA. Dishes were incubated in a moist atmosphere (37°C). Enzyme activity produced radial zones of lysis by diffusion into the opaque background. A urokinase standard curve up to 20mPU was included.

**Colorimetric assay**

Fibrinolysis assays are frequently employed for the detection of PA. However, the activity of tissue-type PAs is strongly stimulated by fibrin (Camiolo et al., 1981) which may give results which do not accurately reflect the actual amount of PA in the sample. Another disadvantage in using fibrin substrate assays, is the difficulty in separating the two activities of PA and plasmin. Therefore the two-part colorimetric assay (Coleman & Green, 1981) which enables separation of activity due to plasmin from PA measurement, was used. This assay consists of two parts:

(Part 1) plasminogen \(\frac{\text{PA}}{}\) plasmin

(Part 2) plasmin buffer \(\frac{\text{plasmin}}{\text{mixed disulphide}}\) \(\text{DTNB} + \text{Z-Lys-S} \text{Bzl}\) + thiophenolate

Initial experiments using purified plasminogen (0.9µM) and incubations of 45min for Part 1 followed by 60min for Part 2, suggested the possibility of substrate depletion. Determination of the apparent \(K_m\) was carried out using urokinase (50mPU) and varying plasminogen concentrations. A plot of \(1/v\) against \(1/s\) showed an apparent \(K_m\) of 2.5µM (Figure 2). By increasing purified plasminogen to a physiological
FIGURE 2. Double reciprocal plot ($1/v$ versus $1/s$) using urokinase (50mPU) in the presence of varying concentrations of plasminogen where $v$=initial velocity and $s$=substrate ($\mu$M).
concentration of 2µM (Collen, 1980; Friberger, 1982),
the standard curve was linear up to at least 8mPU
urokinase with shortened assay times of 15min for each
incubation.

The greater sensitivity and shorter assay periods
reduced the possibility of plasmin degradation of
plasminogen, PA and plasmin itself (Dano & Reich, 1979)
and ensured prevention of substrate depletion.

Summary

AGTG buffer used in Part 1

Glycine (50mM, pH 8.3), gelatine (0.1%, boiled for
15min), Triton X-100 (0.1%), and AHA (5mM).

Plasmin substrate buffer used in Part 2

K₂HPO₄ (200mM, pH 7.5), KCl (200mM), Triton X-100
(0.01%), DTNB (220µM) and Z-Lys-S Bzl (200µM).

Method

In Part 1, PA samples (20µl diluted in AGTG buffer)
were added to AGTG buffer (60µl) and the reaction begun
with the addition of purified plasminogen (20µl, 910µg/ml)
prepared as in 2.2.5, and incubated for 15min at 37°C.
For Part 2, 950µl of the plasmin substrate buffer were
added and incubation continued for a further 15 min. The
reaction was stopped with Trasylol (20µl, 10⁵KIU/ml)
and samples placed on ice. Absorbance (412nm) was read
in a Varian spectrophotometer at 25°C. Plasminogen-free
samples for detection of plasminogen-independent activity
were included in each assay and samples diluted to fit
within a urokinase standard curve (0-4mPU).
Addition of the plasmin substrate buffer for the second incubation effectively reduced the activation of plasminogen to <1% by (a) alteration of pH optimum for PA (pH 8.3) to that for plasmin (pH 7.5), (b) an addition of chloride (200 mM) which depresses plasminogen activation and (c) a tenfold dilution of the first assay (Coleman & Green, 1981).

2.2.5 Plasminogen isolation

Plasminogen is the natural substrate for plasminogen activators and occurs in plasma at a concentration of 1.5-2 µM (Collen, 1980; Friberger, 1982). It has also been found extravascularly in locations such as follicular and uterine fluids, saliva and basal layers of epidermis (Danø et al., 1985).

The most frequently cited method for plasminogen purification is that of Deutsch & Mertz (1970). However, isolation of plasminogen from plasma at room temperature can lead to spontaneous activation of plasminogen to plasmin by plasma activators (Chibber et al., 1974). Also, the omission of protease inhibitors allows the release of an amino-terminal peptide from native Glu-plasminogen resulting in Lys-plasminogen as the partially degraded product (Castellino & Powell, 1981; Wallen & Wiman, 1970).

Another problem encountered when using this method is that of plasmin contamination due to autocatalytic degradation of plasminogen isolated in the absence of plasmin inhibitors (Rickli, 1971; Robbins & Summaria, 1970).
Although plasmin contaminated plasminogen has been employed as a means of activation of pro-PA (Stephens & Golder, 1984), the use of such contaminated plasminogen in physiological concentrations can lead to high background levels in sensitive PA assays and may result in degradation of other assay components such as plasminogen, PA and plasmin itself (Danø & Reich, 1979) especially if the assay is continued over several hours. These activities are difficult to quantify and may obscure the actual amount of PA being assayed (Skriver et al., 1982).

The following method of plasminogen purification is based on that of Danø & Reich (1979) with alterations which give a product of plasmin-free Glu-plasminogen.

A. Collection of plasma

Human plasma from volunteers was centrifuged immediately after collection (1500g, 20min, 4°C) to pellet red blood cells. Protease inhibitors (Trasylol, 20KIU/ml; LBTI 50µg/ml; NPGB 60µM) were added to the supernatant which was then centrifuged (10,000g, 15min, 4°C). Any floating lipids were removed and the plasma (up to 700ml) loaded at 4°C onto a lysine sepharose 4B column (2.5 x 20cm) equilibrated with K₂HPO₄ (100mM, pH7.4) and EDTA (3mM). The flow rate was 100ml/h.

B. Washing and elution

The column was washed for at least 6h at 100-150ml/h with K₂HPO₄ (300mM, pH7.4), EDTA (3mM) and the same protease inhibitors as those used in Step A. Partially purified plasminogen was eluted (60ml/h, 4ml fractions)
with K$_2$HPO$_4$ (100mM, pH7.4), AHA (200mM) and the same concentrations of EDTA and protease inhibitors as those used in step A. Absorbance (280nm) was monitored closely and immediately upon tailing, the collection tube was changed (Arrow, Figure 3A). This step is particularly important as low levels of plasmin can be detected in the elution volume immediately following the protein peak (Table 1). Chibber and colleagues (1974) have suggested that lysine agarose may act as an ion-exchange matrix causing retardation and elution of contaminants (including plasmin) along with plasminogen. These workers suggest using uncharged butyl-p-aminobenzoate agarose which selectively binds plasminogen while excluding plasmin, and elution of plasminogen with lysine. However, lysine can only be removed by extended dialysis against HCl or acetate, which may lead to altered forms of plasminogen (Chibber et al., 1974).

C. Dialysis and further purification

The peak fractions were pooled and dialyzed (100:1 v/v, 3 changes) against K$_2$HPO$_4$ (100mM, pH7.4), EDTA (3mM), NPGB (60µM), and Trasylol (20KIU/ml) over 24h (4°C). The same buffer was used to re-equilibrate the column after first washing the column with a further 200ml of the elution buffer containing AHA.

The partially purified plasminogen was loaded onto the column, washed and eluted as previously described in step B (omitting LBTI) paying particular attention to monitoring fraction collection as before. Purified plasminogen was then dialyzed for at least 24h against
FIGURE 3A  Elution profile of purified plasminogen from lysine sepharose 4B. Tubes were changed at the position of the arrow. The asterisk * indicates the fraction referred to in Table 1 (92.5-100ml).

FIGURE 3B  SDS gel of purified plasminogen (10µg) showing the closely spaced doublet (lane 2) which is typical of Glu-plasminogen. Lane 1 contains molecular weight standards: β-galactosidase, 116,000; phosphorylase B, 97,400; bovine albumin, 66,000; ovalbumin, 45,000; carbonic anhydrase, 29,000.
glycine (50mM, pH 8.3, 100:1, v/v, 3 changes 4°C). The protein concentration was determined using the value of $A_{280\text{nm}}^{1\%\text{cm}} = 16.8$ (Sjöholm et al., 1973); aliquots were frozen at -70°C.

The overall yield was $8\pm1\text{mg}$ (±S.D., n=6)/100ml fresh plasma which is about 10-20% less than previously reported (Deutsch & Mertz, 1970; Collen & deMaeyer, 1976; Wallen & Wiman, 1970). However, incubation of purified plasminogen (52µg) with the sensitive plasmin substrate Z-Lys-S Bzl (Coleman & Green, 1981) at 37°C for one hour, resulted in an absorbance of <0.001 (Table 1). Analysis in SDS-PAGE showed a closely spaced doublet between $M_r92,000-100,000$ (Figure 3B) which is typical of native Glu-plasminogen (Claeys & Vermylen, 1974; Summaria et al., 1976; Castellino & Powell, 1981). After electrophoresis, there was no evidence of lysis when the gel was placed on a fibrin-plasminogen-agar matrix used to detect plasmin activity.

Determination of the amino terminus of purified plasminogen (2nmol) was performed (by D. Shaw) using a Beckman microsequencer (890M-2) with identification of residues by HPLC (Appendix I). The first five NH$_2$-terminal amino acids were Glu-Pro-Leu-Asp-Asp which is in agreement with the structure of Glu-plasminogen (Wiman & Wallen, 1975), and there was <1% lysine at residue one. These results are consistent with a product of native plasminogen, which is in fact free of plasmin contamination.
TABLE 1

Plasmin contamination of plasminogen

<table>
<thead>
<tr>
<th>Elution Volume</th>
<th>Protein</th>
<th>Absorbance (412nm) with Z-Lys-SBz1 for 1h.</th>
</tr>
</thead>
<tbody>
<tr>
<td>80-92.5ml</td>
<td>52</td>
<td>0</td>
</tr>
<tr>
<td>92.5-100ml</td>
<td>9</td>
<td>0.25</td>
</tr>
</tbody>
</table>
Urokinase activity was determined by a colorimetric assay (Coleman & Green, 1981) which is sensitive to about 0.3 fmol urokinase. Instead of acidifying plasminogen, AHA (5 mM) was included in the first buffer to enhance the rate of urokinase activation of Glu-plasminogen. (Thorsen & Mullertz, 1977; Violand et al., 1978; Friberger, 1982). By increasing the plasminogen concentration to a physiological level (2 μM), an absorbance (412 nm) of 0.3 was generated from urokinase (1 mPU) with shortened incubation periods of 15 min for the plasminogen to plasmin step, followed by 15 min for the assay of formed plasmin. This compares with an absorbance of only 0.8 obtained with incubation times of 45 and 60 min in the original assay (Coleman & Green, 1981).

Discussion

There are several advantages in using pure plasminogen at physiological levels: higher plasminogen concentrations increase sensitivity and enable a shorter time for assay, thus reducing the possibility of formed plasmin degrading various proteins present in the assay (Danø et al., 1985); the use of higher plasminogen concentrations avoids substrate depletion which is important in studies of PA inhibitors where substrate depletion may mask effects which are due to inhibitors. However, to be able to use high concentrations of plasminogen, it needs to be free of plasmin contamination.

The important points in this method are:

(i) All steps should be carried out at 4°C.
(ii) protease inhibitors must be added immediately after removal of red blood cells;

(iii) plasmin inhibitors should be included in all but the last dialysis step;

(iv) particular attention should be paid to monitoring the absorbance (280nm) during elution and collection tubes changed immediately upon decline of absorbance to prevent collection of trailing plasmin contaminants;

(v) NPGB (stock of 60mM), due to ease of hydrolysis, should be prepared fresh in dimethylformamide and added dropwise to rapidly mixing buffers.

With attention paid to these precautions, a product of Glu-plasminogen free of plasmin contamination can be obtained. Plasma frozen at -70°C for one year can be used provided protease inhibitors are added as in (ii).

2.2.6 Preparation of fibrinogen

Fibrinogen was prepared by the modified method of Laki (Baughman, 1970). Bovine fibrinogen (5g) was dissolved in 250ml of K$_2$HPO$_4$ (100mM, pH6.4, 25°C). An equal volume of H$_2$O was added and the solution left overnight at 4°. The cold insoluble proteins were removed by centrifugation (1,500g, 20min, 25°C) and NH$_4$SO$_4$ (23% saturation, 25°C) was added to the supernatant. The precipitated fibrinogen was centrifuged (3,000g, 30min, 4°C) and the pellet dissolved in 60ml KCl (0.3M, pH7.4) and dialyzed (40:1, v/v) against three changes of KCl over 48h. Protein concentration was determined using the value of $A_{280nm}^{1%cm} = 6.4$ and aliquots frozen at -70°C.
For plasminogen-free fibrinogen, the above preparation was passed through a lysine sepharose 4B column (2.5x10cm, flow rate 34ml/h) equilibrated with Tris-HCl (50mM, pH8.0) and 4ml fractions collected and stored as above (Yamamoto et al., 1984).
CHAPTER 3

PRELIMINARY OBSERVATIONS OF AN INHIBITOR OF PLASMINOGEN ACTIVATOR
3.1 INTRODUCTION

Elevated levels of urokinase-type PA have been observed in physiological situations where tissue degradation and rearrangement occur (Rohrlich & Rifkin, 1979). In these circumstances PA activity is closely regulated, in part by inhibitors of its activity (Januszko & Buluk, 1966). However, malignant tissue may be less responsive than normal tissue to these control mechanisms (Ossowski et al., 1979).

Early studies into the possible role of PA in cancer metastasis using rat mammary adenocarcinoma cell lines, showed a positive correlation between PA activity and metastatic potential (Carlsen et al., 1984). Hybrid cells (Clone 44) formed from fusion of a highly metastatic, high-PA producing line (MAT 13762 TGO\textsuperscript{R}) with a non-metastatic, non-PA producing line (DMBA 8), were initially non-metastatic and produced little or no detectable PA (Ramshaw et al., 1983). As these hybrid cells contained chromosomes from both parents, they inherited characteristics from each cell line, although these would not necessarily be expressed in equal amounts. With further passaging and as chromosomes were lost, the hybrid cells became metastatic.

Because the original hybrid was non-metastatic, the question was raised as to whether this related to the lack of PA or to the production of a PA inhibitor, or both. The second possibility was confirmed (Ramshaw et al., 1983) and the inhibitor was examined.
to determine its possible role in the inhibition of PA produced by the metastatic cell line.

3.2 METHODS

3.2.1 Determination of inhibitor activity

Inhibitor activity was determined after preincubation of urokinase and inhibitor at 37°C for 10 min. The remaining urokinase activity was measured in the colorimetric assay (2.2.4). Inhibition is expressed as the percentage reduction compared with urokinase controls (100%).

3.2.2 Separation of PA using lysine sepharose chromatography

After concentration over an Amicon YM30 membrane, culture medium from DMBA 8 cells was passed through a lysine sepharose 4B column (15 x 0.5 cm) equilibrated with Tris-HCl (50 mM, pH 7.8) containing EDTA (1 mM). The column effluent was concentrated over a fresh membrane and dialyzed in the concentrator against NH₄HCO₃ (50 mM, pH 7.8) then lyophilized. Absorbed PA was eluted from the column with a buffer containing arginine (0.5 M, pH 4.2), acetate (10 mM), EDTA (1 mM) and Triton X-100 (0.01%, v/v).

3.2.3 Isoelectric focusing of inhibitor culture medium

DMBA 8 (3 mg protein dissolved in 3 ml H₂O) was focused on a flat bed (1.8 x 28 cm) of Sephadex G-75 containing LKB ampholytes (2%, pH 3-10) for 16 h at constant power (8 W, 4°C). Beds were sliced into thirty-six fractions. Each fraction was mixed with
1ml H₂O and centrifuged (1500g, 5min). The supernatants were measured for pH then dialyzed against three changes (100:1, v/v) of glycine (50mM, pH8.3), for 21h at 4°C, and tested for inhibition of urokinase (4mPU) in the colorimetric assay. To ensure that ampholytes did not inhibit urokinase, a bed loaded with H₂O and run in parallel with the test bed, was treated similarly.

Other methods are as described in Chapter 2.

3.3 RESULTS AND DISCUSSION

3.3.1 Interfering substances in crude inhibitor preparations

In initial experiments which involved incubation of Clone 44 cell culture supernatant (50µl) with urokinase (500mPU, 30min, 25°C), and assay of the residual urokinase activity in a [³H] fibrin assay, 52% inhibition was observed (Ramshaw et al., 1983).

However, when inhibition by Clone 44 cell supernatant was measured as a function of protein concentration in the colorimetric PA assay, it became clear that the pattern was not one of simple inhibition. Inhibition was observed over the range 0-0.3µg protein, but at higher protein concentrations, activity was recovered, increasing to 130% at 2.5µg (Figure 1). A similar pattern of inhibition followed by restoration of activity was observed using culture supernatant from the non-metastatic parent cell line DMBA 8, though higher protein concentrations were required (Figure 1).
FIGURE 1. Inhibition of urokinase as a function of concentration of DMBA 8 or Clone 44 protein.

Cell supernatant was concentrated (20-fold over an Amicon YM10 membrane, dialyzed for 36h (NH4HCO3 50mM, pH 7.8, 100:1 v/v) then lyophilized. Protein was resuspended in glycine (50mM, pH 8.3) and tested for inhibition of urokinase (4mPU) after preincubation (10min, 37°C). The residual urokinase activity was measured in the colorimetric assay. The value of 100% represents urokinase incubated in AGTG buffer.

Clone 44 (♦)

DMBA 8 (●)

DMBA 8 after passage through lysine sepharose (▲)

INSERT

PA eluted from the lysine sepharose column was dialyzed and resuspended as above. A sample was run in SDS-PAGE (10% acrylamide) prior to detection of PA activity on a zymogram (2.2.4). Mobilities of molecular weight standards (ovalbumin, 45,000; bovine albumin, 66,000; β-galactosidase, 116,000), run in the same gel are shown.
Investigations showed that at protein concentrations above 0.1 µg, a plasminogen-dependent (PA) activity could also be detected in both Clone 44 and DMBA 8 supernatants. This activity increased sharply above 0.3 µg and was more apparent in supernatant from Clone 44 cells (Figure 2).

Several reports have mentioned suppression of apparent PA activity by high ionic strength (Radcliffe & Heinze, 1980; Aggeler et al., 1981; Stephens & Golder, 1983). Was it possible that dialysis against a low ionic strength buffer had removed salt inhibition of an endogenous PA in cell supernatants? This might account for an apparent restoration of urokinase activity following inhibition as the concentration of cell-secreted protein (and therefore PA activity) increased. However, a similar pattern of inhibition followed by recovery of activity was observed when either high (200 mM glycine) or low (50 mM NH₄HCO₃) ionic strength buffer was used for dialysis of Clone 44 protein (Figure 3). Consequently, the reappearance of activity did not appear to be due to the removal of salt inhibition of endogenous PA in Clone 44 preparations. It was therefore concluded that both cell lines secreted a small amount of PA, while simultaneously producing an inhibitor of human urokinase.

Further experiments using Clone 44 cell culture supernatant showed a serine esterase (plasminogen-independent) activity which could be detected using the colorimetric assay, of which a small amount could
FIGURE 2. PA activity of Clone 44 and DMBA 8 cell culture supernatant as a function of protein concentration measured in the colorimetric assay over 15 min.

DMBA 8 (▲)

Clone 44 (●)
FIGURE 3. Clone 44 inhibition of urokinase as a function of ionic strength of dialysis buffer.

Concentrated Clone 44 protein after dialysis against glycine (200mM) or NH₄HCO₃ (50mM) for 36h (100:1, v/v), was diluted in AGTG buffer and tested for inhibition of urokinase (4mPU) after preincubation (10min, 37°C). The residual urokinase activity was measured in the colorimetric assay. The value of 100% represents the activity of urokinase incubated in AGTG buffer.

glycine (♦)
NH₄HCO₃ (♦)
be blocked by the addition of the protease inhibitor Trasylol (Table 1A). It was apparent that Clone 44 culture supernatant contained several activities which affected colour formation in the assay system: (1) an inhibitor of urokinase; (2) endogenous PA; and (3) a plasminogen-independent serine esterase activity. Relative contributions of these activities to total colour formation were dependent upon the amount of crude material assayed. Clearly, attempts to determine characteristics specific for the inhibitor were likely to be difficult under these conditions.

**Attempted isolation of inhibitory activity from endogenous PA**

3.3.2 Heat denaturation of endogenous PA

Denaturation of endogenous PA by heating DMBA 8 cell culture supernatant (56°C, 2h) decreased PA activity to barely detectable levels (Table 1B). Inhibition (89%) of urokinase (4mPU) was still apparent after heat treatment. However, as some proteases are active at high temperatures (Scopes, 1982) there was a chance of modifications occurring to the inhibitor during heat treatment which might not be detected.

3.3.3 Separation of PA by passage through lysine sepharose

Lysine sepharose chromatography has been employed as a means of PA purification (Radcliffe & Heinze, 1978). After passage of DMBA 8 concentrate through lysine sepharose, greater than 70% inhibition of urokinase (4mPU) was apparent (Figure 1). The PA eluted from the lysine
TABLE 1

Activities of concentrated cell supernatants detected in the colorimetric PA assay which interfered with the measurement of inhibitory activity against urokinase

<table>
<thead>
<tr>
<th></th>
<th>Absorbance (412nm)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>minus plasminogen</td>
</tr>
<tr>
<td><strong>A.</strong></td>
<td></td>
</tr>
<tr>
<td>Clone 44</td>
<td></td>
</tr>
<tr>
<td>(µg protein)</td>
<td></td>
</tr>
<tr>
<td>2.5</td>
<td>0.02</td>
</tr>
<tr>
<td>10</td>
<td>0.11</td>
</tr>
<tr>
<td>15</td>
<td>0.13</td>
</tr>
<tr>
<td><strong>B.</strong></td>
<td></td>
</tr>
<tr>
<td>DMBA 8 (10 µg)</td>
<td>plus plasminogen</td>
</tr>
<tr>
<td>before heat treatment</td>
<td>0.64</td>
</tr>
<tr>
<td>after heat treatment (56°C, 2h)</td>
<td>0.02</td>
</tr>
<tr>
<td><strong>C.</strong></td>
<td></td>
</tr>
<tr>
<td>DMBA 8 (7µg)</td>
<td>minus plasminogen</td>
</tr>
<tr>
<td>after passage through lysine sepharose</td>
<td>0.05</td>
</tr>
</tbody>
</table>

n.d. = not determined
*The extremely low yield of activity obtained by this procedure is open to criticism. Possible reasons for the low yield are: during the 16h period taken for focusing, the inhibitor protein may have remained at or near its isoelectric point for some hours leading to some loss of biological activity (Scopes, 1982, p175); prolonged dialysis (21h) of dilute material could further contribute to loss of activity. A similar experiment also gave poor recovery and therefore this method was discarded as being unsuitable for purification. However, as part of preliminary experiments, the results suggested that the isoelectric point of the inhibitor was in the vicinity of 5.0 and this information was utilized for ion-exchange chromatography and chromatofocusing. In the chromatofocusing step, the pH of fractions was adjusted to pH 7.4 within 1h of collection and dialysis was not required for removal of Polybuffer.
sepharose column showed several bands of activity between $M_r 48,000-50,000$ and $82,000-120,000$ after SDS-PAGE and zymography (Figure 1, insert). Nevertheless, there were still small amounts of both PA and plasminogen-independent activity evident in the preparation (Table 1C).

3.3.4 Other methods attempted for isolation of an inhibitor secreted by DMBA 8 cells

Gel filtration is a method commonly employed for separation of proteins according to their molecular size (Fischer, 1980). Gel filtration (3mg protein) using Sephacryl 300 (1m x 2.5cm) was tried but neither protein nor activity could be detected in collected fractions, implying that non-specific adsorption or dilution of inhibitor had occurred.

Isoelectric focusing (3mg protein) which separates proteins according to isoelectric points was attempted (with assistance from C. Claudianos), but recovery of activity was very low (about 0.5%), perhaps due to the length of time the inhibitor remained at its isoelectric point. However, the results showed 2 peaks of inhibitor activity at pH 4.9 and 5.4 (Figure 4). Increased PA activity was observed at about pH 3.0, 7.0 and 9.0, which agrees with isoelectric points reported for other PA enzymes (Wu et al., 1977; Barrett & McDonald, 1980). This suggested that inhibitor activity could be separated from PA by methods such as ion-exchange chromatography and chromatofocusing. The purification of inhibitor from DMBA 8 using these methods is detailed in Chapter 4.
FIGURE 4. Isoelectric focusing of DMBA 8 protein.

Fractions were tested for inhibition of urokinase (4mPU) after preincubation (10 min, 37°C). Enzyme activity was measured in the colorimetric assay. The value of 100% represents urokinase incubated in AGTG buffer.

percentage urokinase activity (●)

pH gradient
CHAPTER 4

PURIFICATION AND CHARACTERIZATION OF DMBA 8 INHIBITOR
4.1 INTRODUCTION

Plasminogen activators belong to a family of serine proteases which catalyze the conversion of plasminogen to the active protease, plasmin. There are two main types of PA: a urokinase-type, which has a $M_r$ of 55,000 in humans (Aoki & von Kaulla, 1971) and 48,000 in rodents (Marotti et al., 1982); and a tissue-type PA which has a $M_r$ of about 65,000 in humans (Aoki & von Kaulla, 1971) and 79,000 in rodents (Marotti et al., 1982).

As has already been mentioned, the importance of PA in cancer metastasis has been debated for some time. The presence of elevated levels of a urokinase-type PA, either intracellular or secreted in cancer cells, has been observed in many instances (Markus et al., 1983). Recently, it has been suggested that malignancy may be accompanied by a reduction in the net inhibitory activity of a proteinase inhibitor (Waxler et al., 1984). As discussed in Chapter 3, the non-metastatic DMBA 8 cell line produces a PA inhibitor and this chapter describes its purification and characteristics.

4.2 CELL LINES

The DMBA 8 cell line, which was derived from a solid tumour and adapted to in vitro culture, and the Fischer 344 rat mammary adenocarcinoma cell lines, MAT 13762 and R3230, were obtained from EG & G Mason Research Institute, U.S.A. The cell
line PU5-1.8, is a continuous macrophage line from BALB/c mice obtained from C.L. Geczy, Kolling Institute, Sydney. Culture medium from the human melanoma cell line (MM-170) was the gift of Dr H. Warren (Cancer Research Unit, Woden Valley Hospital, Australia).

The R3230 cell line is usually only metastatic in about 30% of rats when injected subcutaneously. The metastatic variants were obtained from 3/10 rats 8 weeks after injection of $2 \times 10^6$ cells into footpads. The pleural effusion line (PE) was obtained after intravenous injection of a large number ($5 \times 10^6$ cells, passage 36), of a pseudo-diploid clone selected from DMBA 8 cells (Ramshaw et al., in press).

4.3 METHODS

4.3.1 Determination of inhibitor activity

Inhibitor activity was routinely assayed after preincubation of enzyme and inhibitor at 37°C for 10 min. The remaining enzyme activity was measured as in section 2.2.4. Activity is expressed as percentage inhibition of PA or, for specific activity, as units per milligram of protein (unit/mg) where one unit is defined as the amount of inhibitor required to give a 50% decrease in the absorbance produced by urokinase (4mFU) in the colorimetric assay. At each purification step, urokinase was incubated with various amounts of inhibitor and the amount of the latter required for 50% inhibition
estimated from plots of percentage inhibition versus inhibitor concentration. One mPU of urokinase is equivalent to $2.7 \times 10^{-16}$ mol (Coleman & Green, 1981).

All buffers were tested for interference in the colorimetric assay. Routinely, a 1 in 20 dilution of fractions was tested for inhibition of urokinase and, at this dilution, none of the buffers caused interference. However, if a lower dilution of sample was required, then an equivalent buffer dilution was included in urokinase controls.

'Crude inhibitor' refers to the uncloned DMBA 8 cell culture supernatant which was passed through a column of Lysine Sepharose (1.5 x 9cm) equilibrated with Tris-HCl (50mM, pH 7.8), EDTA (1mM), and Triton X-100 (0.1%, v/v) at 4°C. Initial experiments using untreated supernatant showed a small amount of endogenous PA activity which was detectable in the sensitive colorimetric assay and also a source of interference at high concentrations of inhibitor (Section 3.3.1).

4.3.2 Procedure for isolation of inhibitor

4.3.2.1 Collection of secreted inhibitor

Clones of DMBA 8 isolated in soft agar were randomly selected and tested for inhibitor production. A clone ("Clone 1") which secreted 3200 unit/ml of culture medium (compared with 100 unit/ml for uncloned DMBA 8) was used as the source of inhibitor. Culture medium was centrifuged (1,500g, 20min, 4°C) in a Beckman TJ6 centrifuge, then concentrated 100-fold over an
Amicon YM30 membrane followed by dialysis in imidazole (20mM, pH 6.2) and centrifugation in a Sorvall SS34 rotor (12,000g, 30min, 4°C) to remove insoluble protein.

4.3.2.2 Ion-exchange chromatography and chromatofocusing

The supernatant from the previous step was applied to a column (2.5 x 18cm) of DEAE-Sephacel equilibrated in imidazole buffer and washed until the absorbance reached zero. Fractions of 4.5ml were eluted with a linear gradient of 0-0.2M NaCl. Conductivity was measured in a Philips meter (PQ9501, Holland). Fractions containing the highest activity were dialyzed against piperazine (25mM, pH 6.0) and added to a Pharmacia C40 column (1 x 40cm) packed with Polyexchanger 94 equilibrated with piperazine buffer. Immediately prior to sample addition, 5ml of eluant buffer (Polybuffer 74, 1 in 10 dilution, pH 4.0) were added to the column. The column was focused with 400ml of Polybuffer and fractions of 2.15ml were collected and tested for activity. The pH of fractions was adjusted with KOH to 7.4 at 25°C. Fractions with the highest activity were pooled and run in SDS-PAGE (10% polyacrylamide) under non-reducing conditions (Laemmli, 1970).

4.3.2.3 Alternative method of purification

Because the molecular weight of the inhibitor obtained from the chromatofocusing step was similar to that of BSA, an alternative method of purification
using Affigel Blue for the removal of BSA was also employed (Travis et al., 1976). Concentrated Clone 1 culture medium was added to an Affigel Blue column (1.5 x 20cm) equilibrated with a buffer of imidazole (50mM, pH7.0) and NaCl (100mM). Activity was found in the effluent volume between 55-77ml (flow rate 30ml/h). The active fractions were concentrated in Amicon CM30 microconcentrators and dialyzed against imidazole (20mM, pH6.2), then chromatographed on a Waters HPLC DEAE ion-exchange column. Fractions of 0.5ml were eluted with a linear gradient of 0-0.4M NaCl at room temperature. Those showing peak activity were run in SDS-PAGE as above.

4.3.3 Growth of cells on microcarriers

For production of large volumes of DMBA 8 cell culture supernatant for inhibitor purification, microcarriers were employed. Microcarriers (5g Cytodex, no.2) were swollen in phosphate buffered saline (300ml, 2h, 37°C). A quantity of beads (60ml) was washed twice in RPMI then added in RPMI, containing foetal calf serum (10%) and antibiotics to 6 culture flasks (175cm²) of confluent cells (10ml/flask). Flasks of cells and microcarriers were incubated for 2-3h and examined for migration of cells to microcarriers. When about 80% of microcarriers carried >1cell/microcarrier, the contents of the flasks were added to stirrer bottles (3 flasks/stirrer bottle) in RPMI (300ml), foetal calf serum
beads were added to the remaining cells in the culture flasks and the procedure repeated to provide a total of 1.2 $\times 10^9$ of cells in medium from 6 cell culture flasks. Stirrer bottles were incubated (37°C, 48h) then washed five times and incubated (18h) in RPMI without serum with the inclusion of Hepes (10mM, pH 7.3) and antibiotics. The total contents were poured out of the stirrer bottles and centrifuged (3,000g, 30min) and the supernatant then concentrated over an Amicon membrane (YM30) and processed as detailed for the alternative method of inhibitor purification (Section 4.3.2.3).

4.3.4 Radioactive labelling of secreted inhibitor

Five flasks (175cm$^2$) of subconfluent DMBA 8 Clone 1 cells were washed five times with RPMI then incubated in methionine-free, glutamine-free DMEM (20ml/flask) to which glutamine (2mM) and L-$[^{35}\text{S}]$ methionine (approximately 50$\mu$Ci/ml) were added. Cells were incubated (18h) then culture medium collected and centrifuged (1500g, 20min, 4°C). The supernatant was concentrated 30-fold over an Amicon YM30 membrane and added to an Affigel Blue column (1.5 x 15cm) equilibrated with NaCl (100mM/imidazole (50mM, pH 7.0). The flow rate was 30ml/h. Fractions (2ml) were collected and assayed for inhibition of urokinase (4mPU) in the colorimetric assay. Scintillation counting was carried out in xylene/Triton X-100/PPO scintillation fluid using $[^{14}\text{C}]$ settings in a Packard Tri-Carb liquid scintillation counter. Protein precipitated with trichloracetic acid
(7%, w/v) and neutralized with Tris-HCl, was added to SDS gels (10% polyacrylamide) and electrophoresed under non-reducing conditions at constant voltage (350V, 4h, 4°C). The gel was silver stained for protein, soaked in scintillant (Amplify) for 30min then dried under vacuum for fluorography. Gels were placed on pre-flashed X-ray film (XRP-5) and developed for 50 days.

4.3.5 Inhibition of cell-surface PA

Partially purified inhibitor from the ion-exchange step (section 4.3.2.2) was used to test the inhibition of cell-surface PA of MAT 13762 cells. Cells were washed and resuspended in Hank's buffered saline at 2 or 3 x 10^5/ml. Inhibitor (20 µl) or Hank's buffered saline and cells (100 µl) were added to a 96 well tissue culture plate and preincubated for 15min at 37°C. Plasminogen (20 µl of 910 µg/ml) or 50mM glycine (for plasminogen independent activity controls) were added to appropriate wells and incubated for 30min in a 5% CO₂ controlled incubator at 37°C. Plasmin generated during this incubation was assayed by the addition of the second buffer (100 µl, containing Z-Lys-S Bzl) as used in the colorimetric assay, and the incubation continued. Urokinase standards were included in each assay. The absorbance was read after 60min in a Dynatech microplate reader (MR 600), with dual wavelengths of 410nm (test) and 630nm (reference). Under these conditions,
secreted PA represented ≤20% of the total absorbance values.

4.3.6 Specificity of inhibitor for serine proteases

The esterolytic activities of trypsin, thrombin and plasmin were assayed in the colorimetric assay omitting plasminogen and using Z-Lys-S Bzl as substrate (Coleman & Green, 1981). For comparison with inhibition of urokinase, enzyme concentrations were chosen to give a similar absorbance to that obtained by activation of plasminogen by urokinase (4mPU).

4.3.7 Kinetic studies

Kinetic studies were performed using urokinase (2 or 4 mPU) and various concentrations of plasminogen and an inhibitor fraction from the ion-exchange step. Urokinase and inhibitor were mixed immediately prior to the addition of plasminogen and the residual enzyme activity measured in the colorimetric assay.

The time course of inhibition was performed using either a purified Clone 1 fraction from the chromatofocusing step or crude inhibitor. Enzyme and inhibitor were mixed together and aliquots removed at the specified times to tubes containing plasminogen, and the residual enzyme activity measured in the colorimetric assay.

Other methods are detailed in Chapter 2.
4.4 RESULTS

4.4.1 Purification of inhibitor

Isolation of inhibitor by ion-exchange chromatography and chromatofocusing resulted in a 1300-fold purification and an overall yield of 13\% (Table 1). Peak activity from the ion-exchange step eluted between 0.134-0.144M NaCl (Figure 1A). In the chromatofocusing step (Figure 1B), peak activity eluted at the isoelectric point (pH 4.5). The active fractions isolated by this method showed one main band at about $M_r$ 66,000 in an SDS gel after silver staining (Figure 2A). The fraction with highest activity isolated by HPLC, after Affigel Blue chromatography to remove BSA (Figure 2B), also showed a band in the same region under similar conditions suggesting that the inhibitor was not a small molecule attached to BSA. A small peak of much lower activity (Figure 1B) was observed at a pI of about 4.6 but there was very little of this material and it was not investigated further.

4.4.2 Inhibitor production by cells grown on microcarriers

Figure 3 shows microcarriers with adherent DMBA 8 cells after incubation in RPMI without serum. The DMBA 8 cells attached rapidly (2-3h), multiplied and remained adhered to microcarriers throughout incubation thus effectively increasing the total surface area of cells available for secretion of inhibitor. However, although large volumes of culture
supernatant were provided from cells on microcarriers in stirrer bottles (1 l/bottle compared with 1 l/25 flasks), it proved difficult to wash the cells free of foetal calf serum for incubation in RPMI alone. The specific activity of the dialyzed concentrate (5.6 x 10^3 unit/mg protein) was lower than that obtained from culture flasks (3.1 x 10^4 unit/mg protein) and isolation of inhibitor using Affigel Blue chromatography and HPLC did not succeed in isolating pure fractions of inhibitor (Figure 4). The lower specific activity may have been a result of stirrer culture conditions and impurity may have been due to inadequate removal of serum proteins in the washing stages.

CHARACTERISTICS OF INHIBITOR

4.4.3 Distribution of inhibitor

Supernatant fractions of homogenates obtained by differential centrifugation (100,000g, 90min) of uncloned DMBA 8 and clone 44 cells, (fraction 3) contained most of the inhibitory activity (Table 2) which suggests that the intracellular location of the inhibitor is in the cytoplasm (Quigley, 1976). However, as this fraction was not purified, it is not known whether it has similar characteristics to the secreted form of the inhibitor.

To determine whether the inhibitor was located at the cell surface, cells were preincubated for 30 min with urokinase (2-6mPU) and residual urokinase activity
*However, this is not conclusive evidence that the radioactively labelled 66,000 band is DMBA 8 inhibitor.*
measured (Section 4.3.5). There was no significant inhibition of urokinase (Table 3) which indicates that the inhibitor is a secreted product unlike the PA activity of MAT 13762 cells which is both secreted and located at the cell surface (Section 4.4.11.2 and 4.4.11.3).

As in the case of MAT 13762 cells (Table 5B), there is evidence of cell-surface, plasminogen-independent serine esterase activity. In contrast, there was very little PA activity in the case of DMBA 8 cells (Table 3).

4.4.4 Time course of inhibitor production

Secretion of inhibitor by DMBA 8 clone 1 (Figure 5) is rapid. By 5h, cell supernatant (10μl) inhibited urokinase (4μPU) by greater than 50%.

4.4.5 Inhibitor is a newly synthesized protein

Partially purified fractions from an Affigel Blue column (Section 4.3.2.3) which contained the highest specific inhibitory activity showed bands of radioactivity at Mr 66,000 in an SDS gel following incorporation of L-[35S] methionine and passage through Affigel Blue to remove serum albumin (Figure 6). This suggests that the DMBA 8 inhibitor is a newly synthesized protein.

4.4.6 Effects of preincubation temperature upon PA inactivation by 'crude' inhibitor

There was significant inhibition of urokinase activity (Figure 7A) at 0°C which increased only slightly with
increasing temperature which suggests that inactivation of PA occurs as a result of binding rather than from an enzyme reaction. A similar effect was observed with inhibition of MAT 13762 secreted PA although more DMBA 8 was required for an equivalent amount of inhibition (Figure 7B).

4.4.7 Effect of dexamethasone treatment on inhibitor production

In some studies, incubation of rat hepatoma cells (Coleman et al., 1982) or human fibroblasts (Crutchley et al., 1981) with the hormone dexamethasone not only decreased PA production but also resulted in the production of a PA inhibitor.

Incubation of uncloned DMBA 8 cells with dexamethasone did not cause a substantial increase in inhibitory activity (Figure 8) so it seems unlikely that dexamethasone can influence inhibitor production at concentrations equivalent to biogenic glucocorticoids (about $10^{-7}$M, Thompson, 1979).

4.4.8 Stability of inhibitor

Table 4 shows that 'crude' inhibitor was stable at 56°C but lost most of its activity in 30 min at 70°C. Acid conditions resulted in only a 46% loss of activity after 1h. Partially purified inhibitor from the ion-exchange step was stable to four cycles of freezing and thawing (pH 6.2). However, purified inhibitor from the chromatofocusing step lost 15% of its activity
when left at 0°C for 4 days at pH 7.4 and up to 73% if left at its isoelectric point (pH 4.5). After HPLC, purified inhibitor lost all its activity after two cycles of freezing and thawing (pH 6.2).

4.4.9 No evidence for inhibition continuing during the assay

To ensure that PA inhibition actually occurred completely during the 10min preincubation and did not continue throughout the 15min assay period, the following experiment was carried out. Urokinase and inhibitor were preincubated for 10min prior to addition of AGTG buffer and plasminogen. At various times (5-20min) the buffer for part 2 was added and the incubation continued for 15min. For each time point the residual activity from urokinase and inhibitor samples was compared with urokinase in AGTG. Figure 9 shows that following addition of buffer and plasminogen, inhibition did not continue throughout the assay.

4.4.10 Kinetics of inhibition of urokinase

From the linear results in the Dixon plot (Figure 10A), the inhibitor appears to function as a dead-end, rather than an allosteric, inhibitor (Segel, 1975). These results also show that one mole of inhibitor binds to one mole of enzyme (Webb, 1963).

From the Lineweaver-Burk plot (Figure 10B), it is apparent that inhibition is noncompetitive, and is not simply acting as a substrate analogue. Further
analysis of this inhibition data by non-linear regression analysis fitted to the function of:

\[ v = \frac{VA}{K_a(1 + I/K_{is}) + A(1 + I/K_{ii})} \]

(where \( V \)=maximum velocity, \( A \)=substrate concentration, \( I \)=inhibitor concentration, \( K_a \)=Michaelas constant for substrate, \( K_{is} \)=inhibition constant associated with the slope of the double reciprocal plot, and \( K_{ii} \)=inhibition constant associated with intercept of the double reciprocal plot)

gave values of \( K_i \) slope = 8.5ng and \( K_i \) intercept = 5.1ng. Based on an \( M_r \) of 66,000 as determined for purified protein, this yields \( K_{is} \) =1.3x10^{-9}M and \( K_{ii} \) =7.7x10^{-10}M.

A plot of \(-\ln E_r/E_t\) versus time of incubation, where \( E_r \)=residual enzyme activity and \( E_t \)=total enzyme activity in Figure 10C, shows the biphasic nature of the inhibition of urokinase (4mPU) which suggested that two different interactions were occurring. With about 2ng purified inhibitor, there was a rapid phase in which 50% inhibition of urokinase activity was reached at about 15min. With 'crude' inhibitor (5µg), 50% inhibition occurred at time zero, that is immediately after mixing urokinase and inhibitor together. In each case, the rapid phase was followed by a slower phase of inhibition. These results are consistent with the hypothesis that, with increasing time of preincubation, the initial enzyme-inhibitor complex undergoes transformation to a state from which the inhibitor does
4.4.11 Specificity of inhibitor

4.4.11.1 Effect on serine proteases

Partially purified inhibitor had no effect on thrombin or plasmin but did inhibit trypsin, however, only at an inhibitor concentration 100 times greater than that required to inhibit urokinase activity under similar conditions (Table 5).

4.4.11.2 Effect on cell-secreted PA

Table 6 demonstrates that crude inhibitor from uncloned DMBA 8 was very effective in inhibition of the PA secreted by several metastatic cell lines which all secreted a predominantly urokinase-type PA of approximately $M_r 48,000$ (Figure 11). The inhibitor from Clone 1 was also effective against another urokinase-type PA (Figure 12) secreted by the macrophage cell line PU5-1.8. However, it was far less effective in inactivating t-PA. In Figure 13, 10mPU urokinase was completely inhibited by 1.25 µg partially purified inhibitor but as much as 4.5 µg caused only 19% inhibition of 5mPU t-PA secreted by human melanoma cells ($M_r 66,000$, Golder & Stephens, 1983). It was necessary to use a fibrinolysis assay as t-PA requires fibrin for the expression of activity (Hoylaerts et al., 1982).

4.4.11.3 Effect on cell-surface PA

It has been suggested that cell-associated PA, reported to be located on the plasma membrane (Quigley, 1976) and associated with the outer surface (Lemaire
While the kinetic experiments were carried out with both pure and partially purified inhibitor, only partially purified material was used for the remaining steps involving characterization and specificity due to the limited amount of purified material.
et al., 1983), may be more effective in providing an immediate reservoir for conversion of plasminogen to plasmin than secreted PA (Lemaire et al., 1983). In Table 7, it is apparent that partially purified inhibitor had no effect on plasminogen-independent activity. After correction for this absorbance, it is evident that cell-surface PA was inhibited by 17% (0.5µg inhibitor) to 97% (5µg inhibitor). Absorbance was linear up to 90min and cells appeared viable during the assay as determined by trypan blue exclusion for up to 4h.

4.4.12 Co-culture of cells

When equal numbers of inhibitor-secreting DMBA 8 Clone 1 and PA-secreting MAT 13762 cells (5x10^5 or 1x10^6) were incubated together, PA activity was reduced by 56±12%. When twice the number of DMBA 8 cells were incubated with MAT 13762 cells, PA activity was reduced by 77±13%.

4.5 SUMMARY

An inhibitor of plasminogen activator (PA) secreted by a tumorigenic, but non-metastatic, rat mammary adenocarcinoma cell line has been purified to apparent homogeneity and characterized. It strongly inhibited human urokinase, but was 100 times less potent in inhibiting bovine trypsin and had no effect on plasmin or thrombin. Several secreted, urokinase-type PAs (M_r 48,000) and a cell-surface PA from a metastatic rat adenocarcinoma cell line were also strongly inhibited. In contrast, a tissue-type PA (M_r 66,000)
secreted by human melanoma cells was only slightly inhibited. Purified inhibitor showed a band of M_r 66,000 in SDS-PAGE and an isoelectric point of 4.5 after chromatofocusing. The inhibition of human urokinase was noncompetitive.

4.6 DISCUSSION

The DMBA 8 inhibitor has been purified by a simple method of concentration, ion-exchange chromatography and chromatofocusing, resulting in a 1300-fold purification. Few kinetic studies of PA inhibitors have been reported (Christensen et al., 1982). The data show that, without preincubation, the inhibitor is classically noncompetitive and binds to urokinase in a ratio of 1:1. The K_i of about 10^{-10}M is evidence of a strong interaction between enzyme and inhibitor. However, if inhibitor and urokinase are preincubated, inactivation occurs in a biphasic manner leading to an apparently irreversible inactivation (Williams & Morrison, 1979). A biphasic mode of PA inactivation has also been observed with a placental inhibitor (Aokie & Kawano, 1972) and a macrophage inhibitor (Golder & Stephens, 1983).

Several fibrinolytic inhibitors have been isolated but most of these, particularly the inhibitors found in plasma, inhibit several proteases and few are specific for urokinase-type PA (Table 8). The DMBA 8 inhibitor has an apparent M_r of 66,000 and a pI of 4.5. These characteristics resemble those of a PA inhibitor isolated from pig leucocyte nuclei (M_r 68,000, pI
4.4-4.5, Kopitar, 1981), but the DMBA 8 inhibitor differs in its ability to inhibit trypsin and in being a secreted product. It is also similar in $M_r$ to minactivin ($M_r$66,000), an inhibitor secreted by human macrophages, but minactivin does not inhibit MAT 13762 PA (Golder & Stephens, 1983). It is clearly different from two other constitutively secreted inhibitors, namely protease nexin ($M_r$53,000, pI 7.4, Eaton & Baker, 1983) which is secreted by a variety of cells including tumour cells, and an inhibitor secreted by endothelial cells ($M_r$55,000, pI 4.5-5.0) which is completely acid stable (Loskutoff et al., 1983). It differs from other plasma protease inhibitors either in $M_r$ or enzyme specificity and by being a noncompetitive inhibitor (Travis & Salvesen, 1983). Thus, the DMBA 8 inhibitor appears to be distinct from other reported PA inhibitors (Table 8).

Although co-culture of DMBA 8 and MAT 13762 cells in a 2:1 ratio did not completely abolish secreted MAT 13762 PA over a period of 18h, total neutralization of secreted PA may not be necessary to prevent localized proteolysis. In physiological situations, threshold levels of PA and inhibitor are probably required for the regulation of tissue degradation and, similarly, threshold levels of PA and/or inhibitor may also be necessary before metastatic invasion can occur. In studies with MAT 13762 cells (Carlsen et al., 1983) it was demonstrated that,
above a threshold level of PA secretion, the number of experimental metastases rose considerably (>200%) and, in experiments using urokinase antibodies, it was found that amounts of antibody below a certain level did not inhibit metastasis in chicken embryos (Ossowski & Reich, 1983), again suggesting that a threshold level of inhibitor is required to modulate PA activity.

Undoubtedly, several factors are involved in metastasis and the hypothesized role of PA is still unresolved. The high specificity of the DMBA 8 inhibitor towards urokinase-type PA rather than plasmin, and its ability to inhibit both the secreted and cell-surface PA of metastatic tumour cells, means that it may prove a useful tool in determining the possible role and levels of PA and/or inhibitors in cancer metastasis.
FIGURE 1. Profile of activity from chromatography steps

A. Ion exchange chromatography

Supernatant after centrifugation (12,000g, 30min, 4°C) was added to a 2.5x18cm DEAE-Sephasel column equilibrated in imidazole (20mM, pH6.2) and washed until absorbance (280nm) reached zero. Fractions (4.5ml) were eluted with a linear NaCl gradient (0-0.2M NaCl) at a flow rate of 10ml/hr and those showing peak activity were dialyzed in piperazine (25mM, pH6.0) prior to the next step.

Protein concentration

Peak activity

NaCl gradient

B. Chromatofocusing

The dialyzed sample from A was added to a C40 (Pharmacia) column of Polyexchanger 94, equilibrated in piperazine (25mM pH6.0), immediately following the addition of 5ml eluant buffer (Polybuffer 74, pH4.0). Fractions (2.15ml) were collected at a flow rate of 8ml/h.

Peak activity (●)

pH gradient (◆)
FIGURE 2. SDS-PAGE profiles of inhibitor purified by different methods

A. Lanes 2,3,4 represent increasing amounts of inhibitor from the chromatofocusing step.
FIGURE 2B. Lanes 1 and 2 represent inhibitor obtained from HPLC following Affigel Blue chromatography. Lanes 1A and 3B represent mobilities of molecular weight standards: carbonic anhydrase, 29,000; ovalbumin, 45,000; bovine albumin, 66,000; phosphorylase B, 97,400.
FIGURE 3. Attachment of DMBA 8 cells to microcarriers.

The photograph shows that many cells were still attached to microcarriers following incubation (18h) in RPMI (without foetal calf serum) in stirrer bottles. After incubation, the contents of stirrer bottles were centrifuged (3000g, 30min) and the supernatant used for purification of inhibitor.

Stirrer bottles were similar to those shown in Figure 20C in the Pharmacia booklet entitled 'Microcarrier cell culture, principles and methods'.
FIGURE 4. Showing SDS-PAGE profile of inhibitor purified from stirrer culture medium.

Fractions (Lanes 1 and 2) from HPLC showing inhibitory activity were electrophoresed then stained with silver. The mobilities of molecular weight standards (Lane 3) are as shown: carbonic anhydrase, 29,000; ovalbumin, 45,000; bovine albumin, 66,000; phosphorylase B, 97,400; and β-galactosidase, 116,000.
FIGURE 5. Time course of inhibitor production.

DMBA 8 Clone 1 cells were seeded in plastic dishes (60mm) in 2ml (0.5x10^6 cells/ml) and cultured in RPMI containing acid treated (a) foetal calf serum (10%) for 24h. The adhered cells were washed 5 times in RPMI and incubated without serum. At specified time intervals, culture medium was removed from duplicate dishes and centrifuged (1500g, 10min). Supernatants were assayed for inhibitory activity after 10min preincubation with urokinase (4mPU) at 37°C, in the colorimetric assay.

(a) Acid treatment of foetal calf serum (2h, pH 3.2, 25°C then addition of 1M NaOH (pH 7.0) is used to destroy serum PA inhibitors (Unkeless et al., 1974). Experiments in which serum was omitted gave similar results.
FIGURE 6. Fluorograph of $^{35}$S methionine labelled fractions from an Affigel Blue column.

Fractions of $^{35}$S methionine labelled inhibitor containing the highest specific activity (2.5-3.5x10$^5$ units/mg) obtained after passage through Affigel Blue, were electrophoresed, silver stained then fluorographed after soaking in Amplify. The dried gel was placed on a pre-flashed X-ray film and developed for 50 days. Lanes 2 and 3 represent fractions containing inhibitor showing strong bands of labelled protein at about Mr 66,000. Lane 1 shows the mobilities of silver stained molecular weight standards run in the same gel: carbonic anhydrase, 29,000; ovalbumin, 45,000; bovine albumin, 66,000; phosphorylase B, 97,400; and β-galactosidase, 116,000.
FIGURE 7. Effect of preincubation temperature upon PA inactivation by crude inhibitor.

'Crude' DMBA 8 inhibitor at various concentrations (values at the side of curves) and (A) urokinase (4mPU) or (B) MAT 13762 PA (3mPU) were preincubated for 10min at various temperatures then residual PA activity was measured in the colorimetric assay. The value of 100% represents the activity of PA incubated in AGTG buffer.
FIGURE 8. Effect of dexamethasone on inhibitor production by DMBA 8 cells.

Uncloned DMBA 8 cells were incubated in serum-free RPMI containing dexamethasone for 17h. The culture medium was removed and centrifuged (1500g, 15min). The supernatant was tested for inhibitory activity after preincubation with urokinase (4mPU) for 10min at 37°C. Residual urokinase activity was measured in the colorimetric assay. Urokinase in AGTG buffer equals 100% activity. The value for 0 dexamethasone represents the amount of activity present when supernatant from untreated cells was preincubated with urokinase.
Percentage urokinase activity

Dexamethasone concentration (M)
FIGURE 9. Inhibition of urokinase does not continue during the colorimetric assay.

Urokinase (4mPU) and 'crude' inhibitor (0.8µg) were preincubated (10min, 37°C). Residual urokinase activity was measured in the two part colorimetric assay. AGTG and plasminogen (Part 1) were added at time 0. Part 2 buffer was added at the times shown and the incubation continued for a further 15min. Percentage urokinase activity measured the residual activity of samples containing urokinase and inhibitor relative to urokinase in AGTG controls (100%) assayed under the same conditions.
FIGURE 10. Kinetics of urokinase inhibition

A. Inhibition of urokinase as a function of inhibitor concentration

Partially-purified inhibitor from the ion-exchange step (4.9x10^5 unit/mg) and urokinase (2mPU) were mixed immediately prior to addition of plasminogen (18 µg) and residual urokinase activity measured in the colorimetric assay. Velocity (10^-3) represents percentage activity of controls incubated (15 min, 37°C) in buffer (AGTG) without inhibitor.

B. Reciprocal plot of substrate versus velocity

This point shows the noncompetitive inhibition of urokinase (2mPU) with increasing concentrations (0-10 ng) of partially purified inhibitor from the ion exchange column (4.9x10^5 unit/mg) with variable substrate concentrations (2.25-22 µg plasminogen). Urokinase and inhibitor were mixed and plasminogen added immediately. Samples were then assayed by the colorimetric method.

C. Inhibition of urokinase as a function of time of preincubation

Purified inhibitor (~2 ng) from the chromatofocusing step or 'crude' inhibitor (5 µg), was mixed with urokinase (4mPU) and incubated at 37°C. Aliquots were removed to tubes containing plasminogen (18 µg) at specified times and the assay continued as above. Results are plotted as -ln residual enzyme activity (E_r)/total enzyme activity (E_t).
FIGURE 11. Zymogram of cell-secreted PA.

PA samples were electrophoresed in SDS gels (10% polyacrylamide) and placed on zymograms (Section 2.2.4). Mobilities of molecular weight standards run in the same gel are shown: ovalbumin, 45,000; bovine albumin, 66,000.

Lane 1, R3230; Lane 2, MAT 13762; Lane 3, PE.
FIGURE 12. Inhibition of PA secreted by the continuous macrophage cell line PU5-1.8.

Concentrated DMBA 8 Clone 1 (0.1-1.25 μg) protein cell supernatant was preincubated with PU5-1.8 PA from cell supernatant (3μPU) for 10min at 37°C then assayed for residual PA activity in the colorimetric assay.

INSET. Sketch of location of PU5-1.8 PA in a zymogram after SDS-PAGE. Mobility of molecular weight standards are as shown: ovalbumin, 45,000; bovine albumin, 66,000. This zymogram is sketched as incomplete removal of SDS resulted in clearing of the fibrin-agar matrix before photography could be arranged.
FIGURE 13. Effect of inhibitor on t-PA

Partially purified inhibitor (10µl) from the ion-exchange step (4.9x10^5 unit/mg) and 10µl urokinase (10mPU) or t-PA (5mPU) secreted by human melanoma cells (MM-170) in buffer (AGTG), were preincubated for 10min and then a 10µl sample was added to the wells of a fibrin-plasminogen matrix and incubated for 12h at 37°C in a moist atmosphere.

Row (a) contained t-PA and row (b), urokinase. Inhibitor concentrations (µg) were: 1a, 1b = 0; 2a, 2b = 1.25; 3a, 3b = 2.5; and 4a = 4.5. Well 4b contained inhibitor only.
TABLE 1. PURIFICATION OF DMBA 8 INHIBITOR

a One unit of activity is defined as the amount of inhibitor required to inhibit urokinase (4mPU) by 50% after preincubation (10min) at 37°C.

b This is an upper estimate of recovered protein. The high absorbance of Polybuffer in colorimetric assays precluded an absolute assessment of protein concentration. Various methods used to separate inhibitor from Polybuffer resulted in a large loss of activity. The actual amount of protein recovered was probably less, and therefore the specific activity higher.

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<th>PURIFICATION STEP</th>
<th>PROTEIN (mg)</th>
<th>INHIBITOR ACTIVITYa (total units)</th>
<th>SPECIFIC ACTIVITYb (units/mg)</th>
<th>YIELD %</th>
<th>PURIFICATION (FOLD)</th>
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<td>$2.40\pm0.86 \times 10^7$</td>
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<td>1300</td>
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</table>

(±S.D., n=4)
TABLE 2. INTRACELLULAR DISTRIBUTION OF INHIBITORY ACTIVITY IN CLONE 44 AND DMBA 8 CELLS

Washed cells were frozen (-70°C) and thawed (37°C) 3 times then homogenized and centrifuged (1,000g, 10min). The pellet was retained and resuspended in 50mM glycine (fraction 1).

The supernatant was centrifuged (100,000g, 90min) to give a pellet which was resuspended in 50mM glycine (fraction 2) and supernatant (fraction 3). Aliquots (10µg protein) were preincubated with urokinase (4mPU) and the residual enzyme activity assayed in the colorimetric assay.

<table>
<thead>
<tr>
<th>FRACTION</th>
<th>PERCENTAGE DECREASE IN ACTIVITY COMPARED WITH UROKINASE CONTROL (100%)</th>
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</tr>
<tr>
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<td>89</td>
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**TABLE 3. LACK OF DMBA 8 INHIBITOR AT THE CELL-SURFACE**

DMBA 8 Clone 1 cells were plated (2.5x10⁴/well) into 96 well trays and incubated for 24h. Adherent cells were washed 3 times and preincubated in Hank's buffered saline (100µl) with or without urokinase for 30min at 37°C. Then either glycine (20µl, 50mM, pH8.3) or plasminogen (20µl, 910µg/ml) was added. Cells were incubated for 30min then 100µl of Part 2 buffer used in the colorimetric assay were added for measurement of plasmin formed by urokinase activation of plasminogen and the incubation continued for 30min. Absorbance (630nm reference, 410nm test) was read in a Microplate reader. Absorbance represents activity. Values represent the mean ± S.D. (n=4).

The expected absorbance was calculated by adding the absorbance due to DMBA 8 cells plus plasminogen and the absorbance due to urokinase activity.

The percentage change between the observed absorbance and expected absorbance was calculated as follows:

\[
\text{Percentage Change} = \frac{\text{Expected Ab.} - \text{Observed Ab.}}{\text{Expected Ab.}} \times 100
\]

where Ab = absorbance

<table>
<thead>
<tr>
<th>DMBA 8 cells minus plasminogen</th>
<th>Absorbance (410nm)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0.413 ± 0.05</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>DMBA 8 cells plus plasminogen</th>
<th>Absorbance (410nm)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0.422 ± 0.07</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>UROKINASE (mPU)</th>
<th>PLUS DMBA 8 CELLS (OBSERVED Ab.)</th>
<th>EXPECTED (Ab.)</th>
<th>PERCENTAGE CHANGE</th>
</tr>
</thead>
<tbody>
<tr>
<td>2</td>
<td>0.038</td>
<td>0.455 ± 0.03</td>
<td>0.460</td>
</tr>
<tr>
<td>4</td>
<td>0.084</td>
<td>0.464 ± 0.01</td>
<td>0.506</td>
</tr>
<tr>
<td>6</td>
<td>0.126</td>
<td>0.486 ± 0.05</td>
<td>0.548</td>
</tr>
</tbody>
</table>
TABLE 4. STABILITY OF INHIBITOR TO HEAT AND ACID MEDIUM

'Crude' inhibitor (20 µg/ml) in AGTG buffer was used. Heat treatment consisted of heating inhibitor for 30 min then assaying 0.4 µg in the colorimetric assay with urokinase (4 mPU) without preincubation. Control samples were left on ice for the same period.

Acid stability of the same preparation of inhibitor was tested by the addition of concentrated HCl to lower the pH to 2.5 for 60 min at 25°C. The pH was then raised to that of buffer (pH 8.25) by the addition of KOH (6M). Control samples received the appropriate volume of H2O. Residual urokinase activity was measured as above.

<table>
<thead>
<tr>
<th>TREATMENT</th>
<th>% INHIBITION BEFORE TREATMENT</th>
<th>% INHIBITION AFTER TREATMENT</th>
</tr>
</thead>
<tbody>
<tr>
<td>Experiment 1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>None</td>
<td>47</td>
<td>47</td>
</tr>
<tr>
<td>56°C for 30 min</td>
<td>47</td>
<td>47</td>
</tr>
<tr>
<td>70°C for 30 min</td>
<td>47</td>
<td>9</td>
</tr>
<tr>
<td>Experiment 2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>None</td>
<td>52</td>
<td>52</td>
</tr>
<tr>
<td>acidification</td>
<td></td>
<td></td>
</tr>
<tr>
<td>for 60 min</td>
<td>52</td>
<td>28</td>
</tr>
</tbody>
</table>
Partial-purified inhibitor (4.9x10^5 unit/mg) from the ion-exchange step was used at various concentrations to test the specificity of inhibition. Enzyme and inhibitor were preincubated (10 min) and then the residual activity was measured in the second part of the colorimetric assay omitting plasminogen (with the exception of urokinase), using Z-Lys-SBzI as substrate.

<table>
<thead>
<tr>
<th>ENZYME</th>
<th>ENZYME CONCENTRATION</th>
<th>AMOUNT OF INHIBITOR REQUIRED FOR 50% INHIBITION</th>
</tr>
</thead>
<tbody>
<tr>
<td>urokinase</td>
<td>4mPU</td>
<td>1.45 x 10^{-2}µg</td>
</tr>
<tr>
<td>trypsin</td>
<td>40 ng</td>
<td>1.45 µg</td>
</tr>
<tr>
<td>plasmin</td>
<td>500 ng</td>
<td>no inhibition (up to 5 µg)</td>
</tr>
<tr>
<td>thrombin</td>
<td>0.5 NIH</td>
<td>no inhibition (up to 12 µg)</td>
</tr>
</tbody>
</table>
Inhibition of urokinase and plasminogen activators secreted by metastatic cell lines after incubation with 'crude' DMBA 8 inhibitor for 10min. at 37°C. Residual PA activity was measured in the colorimetric assay.

<table>
<thead>
<tr>
<th>PA SOURCE</th>
<th>EQUIVALENT mPU UROKINASE</th>
<th>INHIBITOR (µg)</th>
<th>AVERAGE % INHIBITION</th>
<th>No. EXPERIMENTS</th>
</tr>
</thead>
<tbody>
<tr>
<td>human urokinase</td>
<td>4</td>
<td>3</td>
<td>92 ± 0.94</td>
<td>3</td>
</tr>
<tr>
<td>MAT 13762</td>
<td>3</td>
<td>3</td>
<td>78 ± 1.71</td>
<td>6</td>
</tr>
<tr>
<td>PE</td>
<td>3</td>
<td>3</td>
<td>65</td>
<td>2</td>
</tr>
<tr>
<td>R3230</td>
<td>3</td>
<td>3</td>
<td>39 ± 4.62</td>
<td>4</td>
</tr>
</tbody>
</table>
TABLE 7. INHIBITION OF MAT 13762 CELL-SURFACE PA

Cells (100 µl) were preincubated with inhibitor for 15 min in Hank's buffered saline prior to the addition of plasminogen (18 µg). Incubation was continued for 30 min then plasmin substrate (Z-Lys-S Bzl) was added and further incubated for 60 min. Absorbance (410 nm) was read in a microplate reader. This experiment was repeated with cells of four different passages. The results from a typical experiment are presented, and are expressed as the average of 4-8 samples ± S.D. Those without S.D. are the average of duplicate samples.

<table>
<thead>
<tr>
<th></th>
<th>MINUS INHIBITOR</th>
<th>PLUS INHIBITOR (0.5 µg)</th>
<th>PLUS INHIBITOR (5 µg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>2 x 10^4 cells</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>minus plasminogen</td>
<td>0.087 ± 0.003</td>
<td>0.089</td>
<td>0.081</td>
</tr>
<tr>
<td>plus plasminogen</td>
<td>0.197 ± 0.013</td>
<td>0.178 ± 0.013</td>
<td>0.090 ± 0.006</td>
</tr>
<tr>
<td>3 x 10^4 cells</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>minus plasminogen</td>
<td>0.070 ± 0.005</td>
<td>-</td>
<td>0.070</td>
</tr>
<tr>
<td>plus plasminogen</td>
<td>0.358 ± 0.040</td>
<td>0.273 ± 0.026</td>
<td>0.181 ± 0.024</td>
</tr>
</tbody>
</table>

Plasminogen, inhibitor or cells in buffer alone gave an absorbance of < 0.01
<table>
<thead>
<tr>
<th>TYPE OF INHIBITOR</th>
<th>SOURCE</th>
<th>APPARENT Mr and pI</th>
<th>SPECIFICITY</th>
<th>REFERENCE</th>
</tr>
</thead>
<tbody>
<tr>
<td>$a_1$ proteinase inhibitor</td>
<td>human plasma</td>
<td>Mr53,000 pI 4-6</td>
<td>elastase, trypsin, chymotrypsin, thrombin, plasmin acrosin</td>
<td>Travis et al., 1975</td>
</tr>
<tr>
<td>$a_2$ plasmin inhibitor</td>
<td>rat plasma</td>
<td>Mr63,000 pI4.3-4.5</td>
<td>plasmin</td>
<td>Kumada &amp; Abiko, 1984</td>
</tr>
<tr>
<td>$a_2$ plasmin inhibitor</td>
<td>human plasma</td>
<td>Mr67,000 pI4.4-4.5</td>
<td>plasmin, urokinase, trypsin</td>
<td>Moroi &amp; Aoki, 1976</td>
</tr>
<tr>
<td>PA inhibitor</td>
<td>pig leucocytes (nuclei)</td>
<td>Mr68,000 pI4.4-4.5</td>
<td>urokinase</td>
<td>Kopitar, 1981</td>
</tr>
<tr>
<td>PA inhibitor</td>
<td>human placenta</td>
<td>48,000</td>
<td>u-PA, t-PA</td>
<td>Lecander et al., 1984 Holmberg et al., 1978</td>
</tr>
<tr>
<td>minactivin</td>
<td>human macrophages</td>
<td>Mr66,000</td>
<td>u-PA</td>
<td>Golder &amp; Stephens, 1983</td>
</tr>
<tr>
<td>PA inhibitor</td>
<td>bovine endothelial cells</td>
<td>55,000 pI 4.5-5.0</td>
<td>urokinase, t-PA</td>
<td>van Mourik et al., 1984</td>
</tr>
<tr>
<td>protease nexin</td>
<td>fibroblasts, myotubes, epithelial, muscle and carcinoma cells</td>
<td>53,000 pI 7.4</td>
<td>urokinase, plasmin trypsin thrombin</td>
<td>Eaton &amp; Baker, 1983</td>
</tr>
</tbody>
</table>
CHAPTER 5

PLASMINOGEN ACTIVATOR PRODUCTION BY TUMOUR CELLS AND THE EFFECTS OF DEXAMETHASONE AND TUNICAMYCIN ON MAT 13762 CELLS
5.1 INTRODUCTION

The metastatic spread of cancer cells occurs as a result of several steps which involve interactions between tumour cells and those of the host (Eisenbach et al., 1985). Tumour cell surfaces are probably important determinants of metastatic potential: studies with Bl6 mouse melanoma cells have shown that fusion of plasma membranes from highly metastatic cells with intact cells of low metastatic potential increased the frequency of experimental metastases by the membrane-fused cells (Poste & Nicolson, 1980).

Glycoproteins on cell surfaces may play a role in metastatic events as they are important in cell adhesion and cell-cell recognition (Frazier & Glaser, 1979) and because many antigenic determinants on cells are composed of carbohydrate structures (Feizi & Childs, 1985). Thus, expression of specific cell-surface glycoproteins may determine the ability of tumour cells to evade recognition and destruction by host immune cells (Thompson et al., 1973; Alexander, 1974; Kim et al., 1975; Markus et al., 1980) and determine the site of lodgement for secondary tumours (metastases). Studies in which tumour cell-surface glycoproteins were modified by enzymatic treatment with neuraminidase (Gasic & Gasic, 1962; Weiss et al., 1974) or trypsin (Sinha et al., 1974; Fidler, 1978) showed an alteration in the organ distribution of metastases after intravenous infusion of treated cells into animals. It has been suggested that proteases such as plasmin, formed as a result of tumour cell PA production, may modify the
surface of tumour cells themselves, without affecting
their viability (Nicolson, 1984).

Another factor which has been linked with
metastases is thromoplastin or procoagulant activity,
that is, the ability of cells to attract the formation
of a microthrombus or clot consisting of platelets
and fibrin (Warren & Vaes, 1972). Intravenous injection
of Walker 256 rat tumour cells into tail veins,
resulted in rapid platelet aggregation and fibrin
deposition in pulmonary arterioles within 5 min of cell
infusion (Chew & Wallace, 1976). Procoagulant activity
appears to be associated with cell membranes (Dvorak
et al., 1983) as both non-viable tumour cells and
tumour cell membranes were equally effective in causing
clot formation (Hilgard, 1973). It has been hypothesized
that a thrombus may protect tumour cells from mechanical
injury within blood vessels (Roos & Dingemans, 1979)
and that the host reaction of thrombus formation may be
in response to procoagulant activity produced by tumour
cells.

From studies of 10 rat tumour cell lines in which
the line demonstrating the highest metastatic potential
also showed both the highest procoagulant activity and
the highest fibrinolytic activity, it was inferred
that tumour cell PA, together with endothelial cell
PA, may be important in facilitating release of tumour
cells from the fibrin clot (Kohga, 1978) thus aiding
the extravasation of tumour cells at the site of
lodgement (Kodama & Tanaka, 1978; Markus, 1984).
However the fibrinolytic action may arise solely from host endothelial cells via the action of t-PA (Markus, 1984).

Administration of anticoagulants has frequently decreased the number of metastases in animals when these developed after intravenous infusion of tumour cells (Fisher & Fisher, 1967; Markus, 1984) but this has not always been the case (Glaves & Weiss, 1978). As anticoagulants have diverse effects the reduction in metastases may not necessarily have been due to their anticoagulant activity (Gastpar, 1982; Markus, 1984).

A compound that influences experimental metastasis and which has a more specific effect on cell surfaces than anticoagulants is the antibiotic tunicamycin, isolated from *streptomyces lisosuperificus*. Incubation of B16 melanoma cells with tunicamycin prior to injection decreased their metastatic potential (Irimura *et al.*, 1981). This antibiotic inhibits the synthesis of lipid-linked oligosaccharides by blocking the transfer of N-acetylglucosamine 1-phosphate from UDP-N-acetylglucosamine to dolichol phosphate, thereby blocking the synthesis of N-linked oligosaccharide chains of glycoproteins (Mahoney & Duksin, 1979). Tunicamycin is not metabolized either *in vivo* or *in vitro* (Kuo & Lampen, 1976) but has the capacity to modify cell surface glycoproteins, possibly at the post-transcriptional level (Butters *et al.*, 1980). However, although tunicamycin has a specific effect on glycoprotein synthesis it may, as a result of this
action, affect other cell functions which arise as a consequence of protein glycosylation (Lennarz, 1980). When tunicamycin treated Bl6 melanoma cells were injected intravenously, the number of experimental metastases was reduced (Irimura & Nicolson, 1981; Irimura et al., 1981) and it was inferred that changes to cell-surface glycoproteins may have prevented tumour cell-host tissue interactions which lead to the formation of metastases. Evidence to support this theory comes from studies in which incubation with tunicamycin led to decreased agglutination of some transformed cells by the lectin Concanavalin A (Duksin & Bornstein, 1977) and a lowered rate of adhesion of melanoma cells to endothelial cells (Irimura & Nicolson, 1981), suggesting that alteration of cell-surface properties had occurred.

Most tumours produce urokinase-type PA (Duffy & O'Grady, 1984; Marcus, 1983). U-PA is a glycoprotein containing asparagine-linked oligosaccharides (McLellan et al., 1980), the formation of which is dependent upon the first step in the lipid-linked glycosylation pathway which is inhibited by tunicamycin (Michaels, 1980). It is therefore possible, that tunicamycin may interfere with the formation of metastases by affecting the synthesis of PA (given that PA activity is important in metastasis). Several studies on the effect of tunicamycin on tumour cell-secreted tissue-type PA have been reported: two reported no effect on functional t-PA (Rijken, 1983; Grabel & Martin, 1983), whereas another found that
incubation of cells with high levels of tunicamycin (10 or 50μg/ml) inhibited t-PA secretion by Bowes melanoma cells (Little et al., 1984). There have been no reports on the effect of tunicamycin on u-PA. Therefore, although tunicamycin has been found to inhibit metastatic potential, an interrelationship between tunicamycin treatment, u-PA levels and metastasis has not been reported.

Another agent frequently used in studies on PA production is dexamethasone, which is a synthetic glucocorticoid analogue that consistently inhibits u-PA production while having variable effects on t-PA (Danø et al., 1985). Incubation of cells with dexamethasone inhibited the production of PA by macrophages (Vassalli et al., 1976), stimulated fibroblasts (Hamilton et al., 1981), glioblastoma cells (Danø et al., 1982), human lymphoblasts (Littlefield et al., 1985) and rat hepatoma cells (Seifert & Gelehrter, 1978). Several studies have reported that decreased PA production by human fibroblasts (Crutchley et al., 1981) and rat hepatoma cells (Coleman et al., 1982) was accompanied by the synthesis of a dexamethasone-induced PA inhibitor. However, biogenic glucocorticoids have a wide range of effects. They are anti-inflammatory hormones which are secreted by the adrenal cortex and act by reversibly binding to intracellular protein receptors. The hormone-receptor complex then binds to chromatin, thus leading to modulation of gene expression (Baxter, 1979). The only evidence that dexamethasone may have an effect
on cancer metastasis comes from a study in which daily treatment of rats with dexamethasone resulted in a reduction of the weights of hepatomas whilst having only a small effect on bowel tumours (Huang et al., 1984). Effects on the metastatic potential of tumour cells incubated with dexamethasone have not been reported.

Highly metastatic MAT 13762 cells have both high u-PA activity and procoagulant activity (Badenoch-Jones & Ramshaw, 1985). Investigations were carried out to determine the effects of tunicamycin on the PA activity, procoagulant activity and metastatic potential of these cells. Similarly the effects of dexamethasone on PA production and metastatic potential in MAT 13762 cells was examined.

5.2 METHODS

5.2.1 Dissociation of tumours and harvesting of cells

Tumour tissue was removed from Fischer 344 rats under aseptic conditions and finely chopped. Small quantities (about 100mg) of tissue were added to approximately 10ml of phosphate buffered saline (without Ca$^{2+}$ and Mg$^{2+}$), containing trypsin (2.5%, w/v) and EDTA (0.25%, w/v) and stirred gently for 15min at 37°C. The upper solution was poured off and centrifuged (500g, 5min) to pellet debris, then further centrifuged (1500g, 5min) to pellet cells which were finally resuspended in RPMI containing foetal calf serum (10%) and antibiotics. Fresh trypsin solution was added to the remaining tissue and the
process repeated twice.

5.2.2 Assay for experimental (blood-borne) metastasis

Fischer rats were inoculated via the tail vein with tumour cells ($1-2 \times 10^5$) which had been resuspended without the use of trypsin. After 12 days the rats were killed and their lungs removed and fixed in Bouin's fluid. The number of metastatic foci on the surface of the lung tissue was used as the indicator of the metastatic potential of the tumour cells.

5.2.3 Assay for spontaneous metastasis

Tumour cells ($1 \times 10^6$) were injected into rats in the hind footpad or subcutaneously into the mammary gland. Animals were killed 3-4 weeks later and examined for metastases. The tumours which arise at some distance from the primary tumour are defined as spontaneous metastases.

5.2.4 Procoagulant assay

Rats were bled via cardiac puncture, 9 volumes of blood being collected into one volume of sodium citrate (3.8%, w/v) to chelate Ca$^{2+}$ and prevent clotting. The blood was centrifuged (10,000g, 30min, 4°C) in a Sorval SS34 rotor and the platelet-free plasma was collected. Plasma was stored at -70°C for not more than 4 weeks. For assay of recalcification time (clotting) of plasma, tumour cells ($1 \times 10^4$ or $4 \times 10^4$) or cell culture supernatant ($100 \mu l$) were added to plasma ($100 \mu l$) and warmed at 37°C for 1min. CaCl$_2$ ($100 \mu l$, 30mM) was added and the time taken for plasma
to clot measured in a BBL fibrinometer. Procoagulant activity represents a shortening of the clotting time of plasma.

5.2.5 Incubation of cells with dexamethasone and tunicamycin

Subconfluent MAT 13762 cells were washed, resuspended in medium (without the use of trypsin) and incubated with the agent being tested. Dexamethasone was dissolved in ETOH (95% w/v) to provide a stock solution \(1 \times 10^{-2} \text{M}\) and tunicamycin was dissolved in NaOH \(10\text{mM}\) at a concentration of 3mg/ml. Freshly prepared stock solutions of dexamethasone and tunicamycin were filtered (Millipore, 0.2µm membrane) then diluted in RPMI. ETOH or NaOH in equivalent dilutions were added to control cell cultures.

Other experimental methods are as detailed in Chapter 2.

5.3 RESULTS

5.3.1 PA content of tumour tissues

Tumour tissues obtained from animals 2-4 weeks after subcutaneous injection of tumour cells were measured for PA activity to determine whether primary tumours and metastatic tumours contained similar levels of PA. Metastatic tissue which appeared after injection of MAT 13762 cells showed significantly higher levels of PA activity (920-1261 mPU/mg protein) than primary tumour tissue from the same animal (29-249 mPU/mg protein). R3230 tumour tissue contained almost as much PA activity (53-146 mPU/mg protein) as MAT 13762 primary tissue. Primary tumours arising
from injection of DMBA 8 cells showed very little PA activity (7-9 mPU/mg protein, Table 1).

5.3.2 PA production by tumour cells

Levels of PA secreted by tumour cells cultured from tissues, derived from spontaneously formed tumours and metastases, were compared. Cells cultured from primary tumours resulting from subcutaneous injection of cells from the non-metastatic cell lines DMBA 8 and R3230 secreted little or no PA (Table 2A), whereas cells cultured from either primary tumours or metastases resulting from subcutaneous injection of cells from the metastatic cell line MAT 13762 secreted high levels of PA (Table 2B). The cell line R3230 is usually non-metastatic and does not secrete PA (Table 2A) but metastases may arise in about 30% of animals following subcutaneous injection of tumour cells (Hoon et al., 1983). When cells were cultured from tumours in these animals, both primary tumours and metastases secreted high levels of PA (Table 2B). There was no consistent difference between the secreted PA levels of cells from either primary tumours or metastases derived from animals in which metastases arose for both R3230 and MAT 13762 cells (Table 2B).

Cells derived from pulmonary metastases which formed after intravenous injection of a specially selected DMBA 8 clone (Ramshaw et al., 1985) also secreted high levels of PA and actually showed the highest PA levels of all the tumour cells (Table 3).
Cells (1x10⁶) were injected subcutaneously into either a mammary gland or footpad of rats and tumours removed after 2-6 weeks. Tumour tissue was homogenized in glycine (50mM, pH 8.3) containing Triton X-100 (0.5%, v/v) in a ratio of 10µl/mg tissue. After protein determination, the homogenates were measured for PA activity in the colorimetric assay over 30min. (b) is significantly higher than (a) P<0.02 (Wilcoxon Rank test)

Each line represents an individual rat. A dash indicates that a metastatic tumour was not detected in that animal.

<table>
<thead>
<tr>
<th>ORIGINATING CELL LINE</th>
<th>PRIMARY TUMOUR</th>
<th>METASTATIC TUMOUR</th>
</tr>
</thead>
<tbody>
<tr>
<td>R3230</td>
<td>53</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>146</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>123</td>
<td>-</td>
</tr>
<tr>
<td>DMBA 8</td>
<td>7</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>8</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>9</td>
<td>-</td>
</tr>
<tr>
<td>MAT 13762</td>
<td>249)</td>
<td>1413)</td>
</tr>
<tr>
<td></td>
<td>619)</td>
<td>1261)</td>
</tr>
<tr>
<td></td>
<td>414) a</td>
<td>920) b</td>
</tr>
<tr>
<td></td>
<td>29)</td>
<td>2137)</td>
</tr>
<tr>
<td></td>
<td>57)</td>
<td>1173)</td>
</tr>
</tbody>
</table>
TABLE 2. COMPARISON OF SECRETED PA FROM EARLY PASSAGE CELLS CULTURED FROM TUMOUR TISSUES

PA activity of cell culture supernatants was measured in the colorimetric assay over 30min (A) or 15min (B) incubation with plasminogen. Values represent the average of samples collected from 1-4 separate passages. Each line represents an individual rat. A dash indicates that a metastatic tumour was not detected in that animal.

<table>
<thead>
<tr>
<th>ORIGINATING CELL LINE</th>
<th>PA ACTIVITY (mPU/10^6cells)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>PRIMARY TUMOUR</td>
</tr>
<tr>
<td>A. Non-metastic:</td>
<td></td>
</tr>
<tr>
<td>R3230</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>3.0</td>
</tr>
<tr>
<td>DMBA 8</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>0</td>
</tr>
<tr>
<td>B. Metastatic:</td>
<td></td>
</tr>
<tr>
<td>R3230</td>
<td>800</td>
</tr>
<tr>
<td></td>
<td>780</td>
</tr>
<tr>
<td></td>
<td>1180</td>
</tr>
<tr>
<td>MAT 13762</td>
<td>3990</td>
</tr>
<tr>
<td></td>
<td>1830</td>
</tr>
<tr>
<td></td>
<td>3590</td>
</tr>
</tbody>
</table>
TABLE 3. PA SECRETED BY CELLS DERIVED FROM EXPERIMENTAL METASTASES

DMBA 8 cells were cloned and one (Clone 25) selected for its pseudo-diploid chromosomal characteristics, was passaged to level 36 then injected in large numbers (5x10^6) via the tail vein, into rats. Some rats produced pulmonary metastases and one rat, a pleural effusion (PE). Cells were cultured from metastases and supernatants collected from early passages. PA activity was measured over 15min in the colorimetric assay.

<table>
<thead>
<tr>
<th>CELL LINES DERIVED FROM METASTASES AND PE</th>
<th>PA (mPU/10^6 cells)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Clone 4</td>
<td>8940</td>
</tr>
<tr>
<td>Clone 6</td>
<td>10960</td>
</tr>
<tr>
<td>PE</td>
<td>7240</td>
</tr>
</tbody>
</table>
PA is secreted by MAT 13762 cells at an early time period and PA levels rise exponentially (Figure 1). However, although PA is secreted as a function of time, the amount of PA present at the cell surface (56 mPU/2x10^4 cells) is similar to that secreted by the same cells (65 mPU/2x10^4 cells) after a period of 6h in vitro (Table 4) which supports the theory that cell-surface PA may be more effective in providing an immediate reservoir for conversion of plasminogen to plasmin than secreted PA (Lemaire et al., 1983).

5.3.3 Effect of dexamethasone treatment on PA production by MAT 13762 cells

Incubation (15-20h) of MAT 13762 cells with dexamethasone resulted in a dose-dependent inhibition of secreted PA maximal (93% reduction) at 10^{-8} M dexamethasone (Figure 2). Incubation (6h) of cells with dexamethasone significantly decreased both secreted PA and cell-surface PA to a similar extent (about 80%) and the effect began to plateau at 10^{-8} M dexamethasone (Figure 3).

Several studies have reported that a decrease in PA production following incubation of cells with dexamethasone was accompanied by the induction of a PA inhibitor (Crutchley et al., 1981; Coleman et al., 1982). Supernatants obtained after incubation (15h) of MAT 13762 cells with dexamethasone (10^{-6} or 10^{-7} M), which showed a maximum reduction in PA activity (Figure 1) compared to untreated cells, were tested for inhibition of urokinase to determine whether, as
TABLE 4. COMPARISON OF SECRETED AND CELL-SURFACE PA PRODUCED BY MAT 13762 CELLS

Cells (2x10^4/well) were incubated (6h) in 96 well trays then supernatants (50µl) removed to separate wells in fresh trays. The cells, still in the original wells, were centrifuged (1500g, 10min) and the remaining medium tipped off. Cells were washed twice and resuspended in Hank's buffered saline (100µl). The PA activity of cells was measured in the same manner, over 30min as described in Section 4.3.5. Results are expressed as mean ± S.D. (n=4) in mPU/2x10^4 cells. Two experiments in which cells were incubated in Petri dishes gave similar results.

<table>
<thead>
<tr>
<th></th>
<th>SECRETED PA</th>
<th>CELL-SURFACE PA</th>
</tr>
</thead>
<tbody>
<tr>
<td>Experiment 2</td>
<td>65 ± 10</td>
<td>56 ± 15</td>
</tr>
</tbody>
</table>
FIGURE 1. Secretion of PA by MAT 13762 cells as a function of time.

Cells (1x10^6 ml in 6 well trays) were incubated in RPMI containing antibiotics and, at appropriate times, supernatants were removed and centrifuged (1500g, 10min) then assayed for PA activity. Values represent the amount of PA as measured over 30min in the colorimetric assay compared with a urokinase standard curve.
FIGURE 2. Decreased production of secreted PA after incubation of MAT 13762 cells with dexamethasone.

Cells (1x10^6/ml) were incubated (15-20h) with various concentrations of dexamethasone in RPMI without serum then centrifuged (1500g, 10min) and supernatants collected for measurement of PA activity over 15min in the colorimetric assay. The control activity was measured in supernatants obtained from cells incubated in the absence of dexamethasone. Results are expressed as the mean ± S.D. of 2-5 experiments.
FIGURE 3. Comparison of the effect of dexamethasone treatment on cell-surface and secreted PA production by MAT 13762 cells.

Cells (1x10^6/ml) were incubated (6h) in RPMI (without serum) with various concentrations of dexamethasone then centrifuged (1500g, 10min). Cells were washed and resuspended in Hank's buffered saline and measured for cell-surface PA (quadruplicate wells) over 30min as described in Section 4.3.5. Secreted PA activity was measured using cell culture supernatants (in duplicate) in the colorimetric assay over 30min. The control activity was measured using cells, and supernatants from cells, incubated in the absence of dexamethasone. For each dexamethasone concentration the contents of 5 Petri dishes were pooled.

Secreted PA (●)

Cell-surface PA (▲)
Dexamethasone concentration (M)
well as PA suppression, a PA inhibitor had been produced. The results were inconclusive (Table 5). However, when the data were grouped according to the PA levels of control cultures, a pattern emerged. In experiments in which control cells secreted 3.4 PU/10^6 cells, inhibition (37%) of urokinase activity was observed, whereas in experiments in which only 10% inhibition of urokinase activity was apparent, the control cells secreted 60% more PA (5.5 PU/10^6 cells). The percentage inhibition of urokinase observed in experiments 3 and 4 is equivalent to that obtained when supernatant from uncloned DMBA 8 cells was tested for inhibitor production (Section 4.3.2.1). This suggests that treatment of cells with dexamethasone may induce an inhibitor under certain conditions and that the observed response may depend upon the levels of PA being produced by the cells at time of treatment. Dexamethasone itself did not affect the colorimetric assay.

5.3.4 Effect of dexamethasone treatment on the metastatic potential of MAT 13762 cells

Incubation (6h) of dexamethasone with cells prior to intravenous injection into rats showed no significant effect on experimental metastases (Table 6). The reduction in PA activity of supernatants secreted by treated cells was 50% or 68%. As discussed in more detail later, this may be insufficient reduction to prevent the formation of metastases or the cells may
Table 5. Possible induction of a secreted PA inhibitor by incubation of MAT 13762 cells with dexamethasone

Cells (1 × 10^6/ml) were incubated (15-20h) with dexamethasone then centrifuged (1500g, 10min) and supernatants retained for measurement of PA activity in the colorimetric assay.

Aliquots of supernatants from dexamethasone treated cells were preincubated (10min, 37°C) with urokinase (4mPU) and the residual urokinase activity measured in the colorimetric assay. The percentage change in urokinase activity between samples containing supernatants from dexamethasone treated cells (Dx + U) and urokinase controls in AGTG (U) was calculated as follows:

\[
\frac{\text{Ab (Dx + U)} - \text{Ab (Dx)}}{- \text{Ab (U)}} \times 100
\]

where absorbance (Ab) represents activity. Ab (Dx) equals the absorbance of supernatants from dexamethasone treated cells incubated with plasminogen.

<table>
<thead>
<tr>
<th>Experiment</th>
<th>MAT CONTROL (PU/ml)</th>
<th>DEXAMETHASONE CONCENTRATION (M)</th>
<th>PERCENTAGE CHANGE IN UROKINASE ACTIVITY</th>
</tr>
</thead>
<tbody>
<tr>
<td>Experiment 1</td>
<td>5.7</td>
<td>10^{-7}</td>
<td>-11</td>
</tr>
<tr>
<td></td>
<td></td>
<td>10^{-6}</td>
<td>-11</td>
</tr>
<tr>
<td>Experiment 2</td>
<td>5.22</td>
<td>10^{-7}</td>
<td>-10</td>
</tr>
<tr>
<td>Experiment 3</td>
<td>3.43</td>
<td>10^{-6}</td>
<td>-36</td>
</tr>
<tr>
<td>Experiment 4</td>
<td>3.35</td>
<td>10^{-7}</td>
<td>-38</td>
</tr>
</tbody>
</table>

Duplicate samples were assayed from pooled cell supernatants (5 Petri dishes)
Cells (1x10^6/ml) were incubated (6h) in RPMI and dexamethasone (10^{-6}M) then centrifuged (1500g, x 10min) and resuspended in RPMI containing foetal calf serum (5%) prior to tail vein inoculation (1x10^5 cells, experiment 1; 2x10^5 cells, experiment 2). Lungs were removed after 12 days and metastatic lung foci counted. Percentage decrease in PA activity was determined by comparison of the PA activity of cell culture supernatants from dexamethasone treated cells with those of untreated cells as measured in the colorimetric assay.

<table>
<thead>
<tr>
<th>METASTASES MEAN ± S.D.</th>
<th>PERCENTAGE DECREASE IN PA ACTIVITY</th>
</tr>
</thead>
<tbody>
<tr>
<td>MINUS DEXAMETHASONE</td>
<td>PLUS DEXAMETHASONE</td>
</tr>
<tr>
<td>Experiment 1 55±24 (n=5)</td>
<td>54±34 (n=5)</td>
</tr>
<tr>
<td>Experiment 2 69±35 (n=6)</td>
<td>89±11 (n=6)</td>
</tr>
</tbody>
</table>
rapidly recover in vivo from the short term incubation with dexamethasone.

5.3.5 Effect of incubation of MAT 13762 cells with tunicamycin on PA production and metastatic potential

Incubation of MAT 13762 cells with tunicamycin (1-2µg/2x10^5 cells) greatly decreased both PA production (72-78%) and metastatic potential (77-86%, Table 7). However, incubation of cells with a lower amount of tunicamycin (1µg/10^6 cells) decreased PA production by only 48% and had little effect on metastatic potential which suggests that a minimum level of tunicamycin may be required for suppression of PA production and that a threshold level of PA may be linked with metastatic potential. Tunicamycin did not affect growth rates of MAT 13762 cells under these conditions and did not interfere with the colorimetric PA assay.

5.3.6 Effect of tunicamycin treatment on the procoagulant activity of MAT 13762 cells

Incubation of cells with tunicamycin had no apparent effect on the procoagulant activity of MAT 13762 cells or cell supernatants (Table 8).

5.4 DISCUSSION

Although the number of tumour lines studied was small the data (Tables 1, 2 and 3) suggest that a correlation exists between PA production and the metastatic ability of rat mammary adenocarcinoma cells as previously suggested by Carlsen and colleagues (1983). All metastatic tumours and cells produced
TABLE 7. EFFECT OF TUNICAMYCIN ON PA PRODUCTION AND THE METASTATIC POTENTIAL OF MAT 13762 CELLS

Cells \((2 \times 10^5 \text{ or } 1 \times 10^6)\) were incubated \((24h)\) in RPMI \((2ml)\) containing foetal calf serum \((5\%)\) then centrifuged \((1500g, 10\text{min})\) and resuspended in RPMI containing foetal calf serum \((5\%)\) prior to tail vein inoculation \((2 \times 10^5\text{cells})\). Lungs were removed after 12 days and metastatic lung foci counted. Percentage decrease in PA was determined by comparison of the PA activity of cell culture supernatants from tunicamycin treated cells with those of untreated cells as measured in the colorimetric assay. The values are from 5 separate experiments. A dash in the column for metastases indicates that these cells were not injected into rats.

PA activity was determined in duplicate from pooled cell supernatants \((5\text{ Petri dishes})\).

<table>
<thead>
<tr>
<th>TUNICAMYCIN ((\mu g/ml))</th>
<th>NUMBER OF CELLS/ml INCUBATED</th>
<th>PERCENTAGE DECREASE IN PA ACTIVITY</th>
<th>METASTASES MEAN ± S.D.</th>
<th>PERCENTAGE DECREASE IN METASTASES</th>
</tr>
</thead>
<tbody>
<tr>
<td>none</td>
<td>(2 \times 10^5)</td>
<td></td>
<td>(146 ± 38\text{(n=4)})</td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>(2 \times 10^5)</td>
<td>76</td>
<td>(21 ± 6\text{(n=4)})</td>
<td>86</td>
</tr>
<tr>
<td>none</td>
<td>(2 \times 10^5)</td>
<td></td>
<td>(218 ± 57\text{(n=4)})</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>(2 \times 10^5)</td>
<td>72</td>
<td>(51 ± 14\text{(n=4)})</td>
<td>77</td>
</tr>
<tr>
<td>none</td>
<td>(2 \times 10^5)</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>(2 \times 10^5)</td>
<td>78</td>
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<td></td>
</tr>
<tr>
<td>none</td>
<td>(1 \times 10^6)</td>
<td></td>
<td>(64\text{(n=2)})</td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>(1 \times 10^6)</td>
<td>48</td>
<td>(54\text{(n=2)})</td>
<td>16</td>
</tr>
<tr>
<td>none</td>
<td>(1 \times 10^6)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>(1 \times 10^6)</td>
<td>75</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
TABLE 8. PROCOAGULANT ACTIVITY OF MAT 13762 CELLS AFTER INCUBATION WITH TUNICAMYCIN

Cells (in 4 or 5 Petri dishes) were incubated with tunicamycin (1µg/10^6 cells, 18h, experiment 1; 1µg/2x10^5 cells, 24h, experiments 2 and 3) then centrifuged (1500g, 10min). The supernatants from each Petri dish were kept separate and 100µl from each were measured for secreted procoagulant activity. Cells were pooled and assayed for cell procoagulant activity. Procoagulant activity was determined as a decrease in the clotting time of recalcified rat plasma measured in a fibrinometer.

Percentage change in clotting time was determined by comparison with the clotting time (sec) of plasma, RPMI and CaCl2 by the following formula:

\[
\text{Clotting time (RPMI)} - \text{Clotting Time (test sample)} \times 100
\]

\[
\text{Clotting time (RPMI)}
\]

Results are the mean ± S.D. (n=2-4, cells; n=4-8, supernatants).

<table>
<thead>
<tr>
<th>TREATMENT</th>
<th>CLOTTING TIME (sec)</th>
<th>PERCENTAGE CHANGE IN CLOTTING TIME</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Experiment 1</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>RPMI control</td>
<td>145</td>
<td></td>
</tr>
<tr>
<td>Cells (1x10^4) none</td>
<td>35 ± 2</td>
<td>-76</td>
</tr>
<tr>
<td>treated</td>
<td>35 ± 3</td>
<td>-76</td>
</tr>
<tr>
<td>Supernatant none</td>
<td>32 ± 0</td>
<td>-78</td>
</tr>
<tr>
<td>treated</td>
<td>32 ± 1</td>
<td>-78</td>
</tr>
<tr>
<td><strong>Experiment 2</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>RPMI control</td>
<td>246</td>
<td></td>
</tr>
<tr>
<td>Cells (4x10^4) none</td>
<td>63</td>
<td>-74</td>
</tr>
<tr>
<td>treated</td>
<td>49</td>
<td>-80</td>
</tr>
<tr>
<td>Supernatant none</td>
<td>91 ± 4</td>
<td>-63</td>
</tr>
<tr>
<td>treated</td>
<td>83 ± 3</td>
<td>-66</td>
</tr>
<tr>
<td><strong>Experiment 3</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>RPMI control</td>
<td>200</td>
<td></td>
</tr>
<tr>
<td>Cells (4x10^4) none</td>
<td>54</td>
<td>-73</td>
</tr>
<tr>
<td>treated</td>
<td>38</td>
<td>-81</td>
</tr>
<tr>
<td>Supernatant none</td>
<td>101 ± 3</td>
<td>-50</td>
</tr>
<tr>
<td>treated</td>
<td>96 ± 1</td>
<td>-52</td>
</tr>
</tbody>
</table>
large amounts of PA, whereas cells from animals in which there was no evidence of metastases produced little or no detectable PA. The main type of PA secreted by the metastatic cells of these cell lines is of urokinase-type ($M_r 48,000$, Section 4.4.11.2), the type of PA which is most frequently found in tumour cells (Duffy & O'Grady, 1984). A study of colorectal cancers and adjacent normal tissues showed that u-PA specific immunoperoxidase staining was restricted to cancer cells and that t-PA was only localized in the endothelium of blood vessels (Kohga et al., 1985).

The larger amount of PA present in metastases compared with primary tumours derived from the same animal, is in agreement with a study of human prostatic carcinomas and their respective bone metastases (Kirchheimer et al., 1985). However, in culture there was no consistent difference between secreted PA levels of primary tumour cells and metastatic cells derived from the same animals (compare first 3 rats for MAT 13762, Table 1 with MAT 13762 cells in Table 2). This differs from the results of Markus and colleagues (1983) who found in short-term organ culture experiments, that metastatic colon tumour tissue secreted PA at a much lower rate than primary tumours. As metastatic tumours and primary tumours were not measured from the same patients their results may only reflect variation between patients rather than between metastatic and primary tumours. However, another possibility is that macrophages were present in the metastatic tumours
(Talmadge, Key & Fidler, 1981) and that macrophage u-PA (Vassalli et al., 1976; Golder & Stephens, 1983) contributed to the total PA of the metastatic tissue samples (Table 1). As histology was not carried out this theory cannot be substantiated. The amount of PA in R3230 primary tumours (Table 1) may reflect the presence of macrophage PA and/or may be due to the population of cells which under certain conditions lead to metastases. The former seems more likely as cells cultured from R3230 primary tumour tissues secreted barely detectable PA (Table 2A).

The metastatic cell line MAT 13762 secretes PA rapidly in vitro but also has a similar amount of PA at the cell surface to that which is secreted into culture medium by 6h. This suggests that if high levels of PA are important for metastatic cell invasion, then cell-surface PA may be of more importance than secreted PA in localized proteolysis (Hoylaerts et al., 1983).

Although apparent PA activity can be masked by physiological salt concentrations (Stephens & Golder, 1984), it is evident that MAT 13762 PA (both cell-secreted and cell-surface) is very active at physiological salt levels (Table 2). Cell-secreted PA is of the urokinase type but the form of cell-surface PA is not known. Because it was readily detected in the colorimetric PA assay, it is unlikely to be t-PA which requires fibrin for appearance of activity (Hoylaerts et al., 1982). As both secreted
PA and cell-surface PA were inhibited by DMBA 8 inhibitor (Sections 4.4.11.2 and 4.4.11.3) they may be of similar type but further experiments are required to confirm this possibility.

Tunicamycin (1-2µg/2x10^5 cells) effectively suppressed both metastatic potential and PA production. The reduced metastatic potential may be the result of twin effects: decreased PA production due to impairment of glycosylation leading to non-functional PA, and alterations in cell-surface glycoproteins which are important in both evasion of destruction by immune cells and in location of metastases. However, after incubation of cells with tunicamycin for 24h, little effect on cell surface procoagulant activity was detected.

This is the first time that inhibition of u-PA activity by tunicamycin treatment has been demonstrated. In the only previous report in which secretion of t-PA was suppressed, a large amount of tunicamycin was used (10 or 50µg/ml) and at these concentrations protein synthesis may be inhibited (Keenan et al., 1981). The suppression of PA production may mean that the glycoprotein portion of u-PA is important for activity, although in vitro experiments with glycosidase treated t-PA have suggested that this may not be true for t-PA (Little et al., 1984). However, whether tunicamycin suppression of PA was relevant to suppression of metastases or whether other glycoprotein changes were involved has not
been defined by these experiments.

The circulating concentration of glucocorticoids in humans ranges diurnally between $5 \times 10^{-9}$ M and $4 \times 10^{-8}$ M but rises to about $10^{-7}$ M after ACTH stimulation (Thompson, 1979). Dexamethasone at similar concentrations ($10^{-8}$ and $10^{-7}$ M, Figures 2 and 3) significantly decreased both secreted and cell-surface PA. Induction of a PA inhibitor was not clearly shown but detection may depend upon the amount of PA synthesis occurring in the cells at the time of treatment. Incubation (6h) of cells with dexamethasone had no effect upon metastatic potential in experimental metastases in which the PA levels of treated cells were suppressed by 50% and 68%. This could mean that insufficient suppression of PA activity occurred to prevent metastasis formation (compare with 48% inhibition of PA by tunicamycin resulting in no reduction of metastatic potential). However, 72% inhibition of MAT 13762 PA by tunicamycin had a dramatic effect on metastatic potential. This may indicate that suppression of PA activity alone is insufficient to inhibit metastasis via this route. Another possibility is that, as suppression of PA activity by dexamethasone is rapidly reversible (within 2-4h) after short term incubation with dexamethasone (Rifkin, 1978), MAT 13762 cells may quickly recover after treatment with dexamethasone for only 6h. Continuous dexamethasone treatment of an animal after injection of treated cells may be more effective in preventing
metastasis because, as observed in one tumour system, continuous (3 weeks) dexamethasone treatment of rats reduced the weight of hepatomas by 64–90% (Huang et al., 1984).

As dexamethasone and tunicamycin both inhibit u-PA production, further experiments are required to determine the effects of these compounds on PA levels and metastatic potential in both experimental and spontaneous metastasis. These experiments could include continued infusion of the drugs after injection of treated cells and subsequent monitoring of PA levels in both primary tumours and metastases.
CHAPTER 6

PRO-ENZYME ACTIVITY IN INHIBITOR CULTURE MEDIUM
6.1 INTRODUCTION

Many enzymes are synthesized as inactive precursors or zymogens which are converted to active forms by limited proteolysis (Neurath & Walsh, 1976). These are frequently referred to as pro-enzymes. Serine proteases such as chymotrypsin, trypsin and elastase are released from the pancreas as zymogens and proteolytic cleavage of one or more peptide bonds is required to produce the active enzyme (Palmer, 1981).

The zymogen form of another protease, collagenase, can be activated by incubation with trypsin (Shinkai & Nagai, 1977) or activators from human skin and rat uterus (Tyree et al., 1981). Collagenase also exists in a latent form which can be activated by brief exposure to the detergent SDS, or by freezing and thawing (Stricklin et al., 1978), procedures which suggest an essential change in conformation rather than activation by proteolytic cleavage (Stadtman, 1970).

Both t-PA (Andreasen et al., 1983) and u-PA (Skriver et al., 1982) have been found in pro-enzyme forms which can be activated by incubation with plasmin (Wun et al., 1982a) or trypsin (Nolan et al., 1977).

Other reports have observed complexes consisting of latent t-PA linked to t-PA inhibitors and these complexes could be dissociated by treatment with SDS and hydroxylamine (Levin, 1983) or by treatment with NH₄OH/SDS (Thorsen et al., 1984).
Mixing experiments in which concentrated, dialyzed DMBA 8 inhibitor was added to PA in the form of culture supernatants from metastatic cell lines, suggested the existence of an inactive form of PA in the DMBA 8 inhibitor preparations. Instead of inhibition, an enhanced activity was observed. However, the same inhibitor preparation strongly inhibited urokinase. When culture supernatants from the metastatic cell lines were mixed together, no enhancement was evident. This implied the presence of an inactive enzyme in the inhibitor preparation which was activated by some substance(s) in the culture supernatants from metastatic cell lines.

In other experiments, zymograms of PA and DMBA 8 inhibitor prepared from SDS gels, initially showed complete inhibition of PA activity. However, following prolonged incubation of gels on fibrin-agar overlays, 'flares' of activity became apparent in the lanes containing inhibitor and PA. These 'flares' appeared in a $M_r$ range which was higher than that of enzyme alone which suggested the presence of a latent PA in the inhibitor preparation, perhaps as part of an enzyme-inhibitor complex, that had been dissociated by SDS.

It was therefore likely that the crude dialyzed DMBA 8 inhibitor preparation contained:

(a) a precursor PA which required activation before it could be detected or,

(b) an enzyme-inhibitor complex consisting of an endogenous PA linked to a PA inhibitor which could be
separated by SDS. The effect of this separation would be to allow expression of enzyme activity.

In view of the potential role of PA in cancer metastasis, the nature of this activity was examined.

METHODS

Assay for inhibitory activity is detailed in section 3.2.1. Other methods are as described in Chapter 2.

RESULTS AND DISCUSSION

6.2 ACTIVITIES OBSERVED IN DMBA 8 PREPARATIONS BEFORE DIALYSIS

6.2.1 Secreted PA

Very low amounts of secreted PA were detected in DMBA 8 inhibitor preparations. Most importantly, this activity was only evident when crude culture supernatant was concentrated >20 fold (<2mPU/µg) whereas PA secreted by the metastatic cell line, MAT 13762 was present in much higher amounts (>120mPU/µg) and the latter could be readily detected in diluted culture supernatant. Therefore, PA secreted by DMBA 8 equalled less than 2% of that secreted by the metastatic cell line.

6.2.2 Inhibition of urokinase and PA secreted by metastatic cells

Table 1 shows that concentrated DMBA 8 cell supernatant is an effective inhibitor of human urokinase and crude PA secreted by rat metastatic cells.
TABLE 1

Inhibition of crude PA secreted by metastatic cells from the rat adenocarcinoma cell lines, MAT 13762, PE and R3230 by DMBA 8 protein (0.5 µg) before dialysis

Absorbance reflects PA activity measured in the colorimetric assay after 10 min preincubation of DMBA 8 with crude PA at 37°C. The percentage change was calculated from the following formula allowing 0.07 for the absorbance observed for DMBA 8 plus plasminogen.

\[
\frac{\text{Ab(}\text{PA+DMBA 8}) - \text{Ab(DMBA 8)} - \text{Ab(PA Control)}}{\text{Ab(PA Control)}} \times 100
\]

where \(\text{Ab} = \text{absorbance which represents PA activity.}\)

<table>
<thead>
<tr>
<th>PA Source</th>
<th>Absorbance 412mm</th>
<th>% change</th>
</tr>
</thead>
<tbody>
<tr>
<td>Source</td>
<td>PA Control</td>
<td>PA+DMBA 8</td>
</tr>
<tr>
<td>urokinase (4 mPU)</td>
<td>0.95</td>
<td>0.26</td>
</tr>
<tr>
<td>MAT 13762</td>
<td>0.75</td>
<td>0.26</td>
</tr>
<tr>
<td>PE</td>
<td>0.51</td>
<td>0.28</td>
</tr>
<tr>
<td>R3230</td>
<td>0.84</td>
<td>0.35</td>
</tr>
</tbody>
</table>
6.2.3 Activation of pro-PA by trypsin

When trypsin was added to increasing amounts of concentrated DMBA 8 cell supernatant and assayed for PA activity (without preincubation) an increase in activity was observed (Figure 1) which was maximal at 1.25µg DMBA 8 protein. This suggests that trypsin activated a pro-PA in DMBA 8 material as activity was dependent upon plasminogen (section 6.3.2). Why activation by trypsin declined as the DMBA 8 concentration increased is unclear. This may be due to the presence of other factors in the preparation which interfere with trypsin activation of pro-PA.

6.3 ACTIVITIES OBSERVED IN DMBA 8 PREPARATIONS AFTER DIALYSIS

6.3.1 Pro-PA activated by addition of metastatic cell culture supernatants

Table 2 demonstrates the effect of adding concentrated and dialyzed (3 changes, 100:1, v/v, 50mM NH₄HCO₃, pH 7.8 over 36h) DMBA 8 inhibitor to the same crude PA samples from metastatic cell lines which were inhibited by non-dialyzed DMBA 8 (Table 1). In column 3 the percentage change in absorbance compared with PA controls is listed. It is evident that while still able to inhibit urokinase, enhanced activity was observed when DMBA 8 was mixed with metastatic cell supernatants.

That the increased activity was due to a component(s) within the DMBA 8 material is evident in Table 3. When crude PA samples from metastatic cells were added together, the resulting absorbance equalled the sum of each
FIGURE 1. Effect of trypsin on non-dialyzed, concentrated DMBA 8 culture supernatant.

Trypsin (10µg) and varying amounts of DMBA 8 protein were mixed together and immediately measured for PA activity (with plasminogen) in the colorimetric assay. Time of second incubation was 9min.

Trypsin plus DMBA 8 (●)

DMBA 8 (▲)
Enhancement of PA activity when analysed in DMBA 8 (µg) was carried out with crude PA (cell supernatant) obtained from the rat sarcoma cell lines MAT-276, 245 and 220. Absorbance of crude PA activity measured in the spectrophotometric assay after 4 h of preincubation of DMBA 8 with cell supernatant at 37°C. The percentage change was calculated after subtraction for the absorbance of control DMBA 8 plus pleomorphic rat liver cell line samples.

Absorbance 412 nm

DMBA 8 (µg protein)
TABLE 2

Enhancement of PA activity when dialyzed DMBA 8 (0.6 µg) was mixed with crude PA (cell supernatant) secreted by the metastatic rat cell lines MAT 13762, PE and R3230.

Absorbance reflects PA activity measured in the colorimetric assay after 10 min preincubation of DMBA 8 with cell supernatant at 37°C. The percentage change was calculated after correction for the absorbance due to DMBA 8 plus plasminogen (0.04) by the following formula.

\[
\frac{Ab(PA+DMBA 8) - Ab(DMBA 8) - Ab(PA \text{ Control})}{Ab(PA \text{ Control})} \times 100
\]

where \( Ab = \) absorbance which represents PA activity

<table>
<thead>
<tr>
<th>Sample</th>
<th>Absorbance 412mm</th>
<th>% change</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>PA Control</td>
<td>PA+DMBA 8</td>
</tr>
<tr>
<td>Urokinase</td>
<td>1.08</td>
<td>0.54</td>
</tr>
<tr>
<td>MAT 13762</td>
<td>0.95</td>
<td>1.32</td>
</tr>
<tr>
<td>PE</td>
<td>0.71</td>
<td>1.04</td>
</tr>
<tr>
<td>R3230</td>
<td>0.39</td>
<td>0.96</td>
</tr>
</tbody>
</table>
TABLE 3

Effect of mixing crude PA from metastatic rat cell lines MAT 13762, PE and R3230.

Crude PA samples were preincubated either in AGTG buffer (PA control) or together (observed absorbance) for 10 min at 37°C prior to assay for PA in the colorimetric assay. Absorbance reflects PA activity. The expected absorbance was determined by adding the separate PA control absorbances for respective pairs of samples.

<table>
<thead>
<tr>
<th>PA source</th>
<th>PA control</th>
<th>Observed Ab.</th>
<th>Expected Ab.</th>
</tr>
</thead>
<tbody>
<tr>
<td>MAT 13762</td>
<td>0.65</td>
<td></td>
<td></td>
</tr>
<tr>
<td>PE</td>
<td>0.51</td>
<td></td>
<td></td>
</tr>
<tr>
<td>R3230</td>
<td>0.84</td>
<td></td>
<td></td>
</tr>
<tr>
<td>MAT 13762+PE</td>
<td></td>
<td>1.18</td>
<td>1.16</td>
</tr>
<tr>
<td>MAT 13762+R3230</td>
<td></td>
<td>1.47</td>
<td>1.49</td>
</tr>
<tr>
<td>PE + R3230</td>
<td></td>
<td>1.35</td>
<td>1.35</td>
</tr>
</tbody>
</table>
separate activity. These results suggested the existence of a pro-enzyme in DMBA 8 material. This activation was dependent upon the concentration of DMBA 8 protein and increased with increasing concentrations of MAT 13762 supernatant protein (Figure 2).

Incubation of DMBA 8 with metastatic cell supernatants without plasminogen did not lead to increased activity which suggests that the observed enzyme activity is a form of PA (Figure 3).

Activation was rapid (Figure 4A) as significant activity was observed immediately upon mixing dialyzed DMBA 8 material with MAT 13762 culture supernatant. A similar profile was observed when the same inhibitor preparation was preincubated with a smaller amount of MAT 13762 supernatant (Figure 4B).

The speed of this process suggested that the activation may not be a simple proteolytic cleavage. To test whether this could be a binding phenomenon rather than the result of proteolytic activation, varying concentrations of DMBA 8 were mixed with MAT 13762 supernatant and incubated at either 0°C or 37°C for 10 min. No difference in activation was found except for a small temperature dependence at a concentration of 16 ng DMBA 8. These observations suggest that either the activation occurs as a result of a rapid binding of an activator, or the enzyme activation is so rapid that under these conditions
FIGURE 2. Concentration dependence of pro-PA activation.

Varying amounts of dialyzed DMBA 8 protein (0-1.25µg) were mixed with MAT 13762 cell supernatant protein (3, 4 and 6ng) and preincubated for 10min at 37°C prior to addition of plasminogen and measurement of PA in the colorimetric assay. The ordinate axis represents the increase in PA activity (mPU) above that of MAT 13762 control.

MAT 13762

3ng (■)
4ng (○)
6ng (△)
FIGURE 3. Incubation of DMBA 8 with metastatic cell supernatants omitting plasminogen.

Dialyzed DMBA 8 protein was mixed with cell supernatant from metastatic cell lines (A) MAT 13762 & (B) PE, and preincubated for 10 min at 37°C. Samples were then assayed without plasminogen in the colorimetric assay. Absorbance (412 nm) represents activity measured by the ester substrate Z-lys-S Bzl.

DMBA 8 (▲)

(A) MAT 13762+DMBA 8 (●)

(B) PE + DMBA 8 (●)
FIGURE 4. Preincubation of dialyzed DMBA 8 protein with MAT 13762 cell supernatant as a function of time.

100 µl DMBA 8 (50 µg protein/ml) was mixed with 100 µl MAT 13762 cell supernatant A (1.0 µg/ml) or B (400 ng/ml) and aliquots removed at specified times to tubes containing ACTG and plasminogen and PA activity measured in the colorimetric assay. Incubation time of second part of assay was 15 min (Part A) and 10 min (Part B). Absorbance represents PA activity.

DMBA 8  ------

MAT 13762  ------

DMBA 8 plus MAT 13762  (●)
the rate could not be measured (Table 4).

Although increased activity rather than inhibition was apparent after the inhibitor was dialyzed and preincubated with crude PA from metastatic cell culture supernatants, this enhancement was no longer evident after the inhibitor was passed through a lysine sepharose column (Table 5) and only inhibition was observed. This suggests that both pro-PA and PA bound to lysine sepharose.

6.3.2 Pro-PA activation by trypsin

When trypsin was added to dialyzed DMBA 8 protein, enzyme activity increased linearly with increasing concentrations of DMBA 8 (Figure 5A), above the maximum increase at 1.25µg observed with non-dialyzed material (Figure 1). This suggests that the increased absorbance was due to activation of pro-PA which was present in non-dialyzed material combined with another pool of pro-PA which became available for activation after dialysis or, that substances which interfered with the activation process had been removed by dialysis. Again the enzyme activated by trypsin is plasminogen-dependent which confirms the activation of a PA enzyme (Figure 5B).

Table 6 compares the effect of mixing dialyzed DMBA 8 protein with either active trypsin or heat-treated trypsin. With heat-treated trypsin, there was less activation (33%) compared with active trypsin (81%). These results are consistent with a proteolytic activation of a pro-PA catalyzed by trypsin which is
TABLE 4

Effect of preincubation temperature on the appearance of pro-PA activity

Dialyzed DMBA 8 protein and MAT 13762 cell supernatant (6ng protein) were mixed together and preincubated (10min) at 0°C or 37°C before measurement of PA activity in the colorimetric assay. The percentage increase in PA was determined by the following formula:

\[
\frac{\text{Ab(DMBA 8+MAT 13762)} - \text{Ab(MAT 13762)} - \text{Ab(DMBA 8)}}{\text{Ab(MAT 13762)}} \times 100
\]

where \text{Ab} = \text{absorbance}

\text{Ab(DMBA 8)} for 1.25\mu g protein = 0.03 and at lower concentrations \text{Ab(DMBA 8)} = 0

<table>
<thead>
<tr>
<th>DMBA 8 protein</th>
<th>Temperature of preincubation(°C)</th>
<th>Ab. MAT 13762</th>
<th>Ab. MAT 13762 +DMBA 8</th>
<th>Percentage increase in PA</th>
</tr>
</thead>
<tbody>
<tr>
<td>16ng</td>
<td>0</td>
<td>0.25</td>
<td>0.27</td>
<td>8</td>
</tr>
<tr>
<td>16ng</td>
<td>37</td>
<td>0.2</td>
<td>0.25</td>
<td>25</td>
</tr>
<tr>
<td>40ng</td>
<td>0</td>
<td>0.26</td>
<td>0.35</td>
<td>35</td>
</tr>
<tr>
<td>40ng</td>
<td>37</td>
<td>0.24</td>
<td>0.32</td>
<td>33</td>
</tr>
<tr>
<td>1.25\mu g</td>
<td>0</td>
<td>0.25</td>
<td>0.98</td>
<td>188</td>
</tr>
<tr>
<td>1.25\mu g</td>
<td>37</td>
<td>0.23</td>
<td>0.75</td>
<td>165</td>
</tr>
</tbody>
</table>
TABLE 5

Lack of appearance of pro-PA activity after passage of dialyzed DMBA 8 protein through lysine sepharose

'Crude' DMBA 8 inhibitor (3µg) was preincubated (10min, 37°C) with PA (3mPU) secreted by metastatic cells from rat cell lines MAT 13762, PE and R3230 and the residual PA activity measured in the colorimetric assay. The percentage inhibition was determined by the following formula:

\[
\frac{Ab(\text{PA}) - Ab(\text{PA+DMBA 8})}{Ab(\text{PA})} \times 100
\]

where \( Ab = \) absorbance which represents PA activity

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Percentage inhibition mean ± S.E.M.</th>
<th>Number of experiments</th>
</tr>
</thead>
<tbody>
<tr>
<td>MAT 13762</td>
<td>78 ± 1.7</td>
<td>6</td>
</tr>
<tr>
<td>PE</td>
<td>65</td>
<td>2</td>
</tr>
<tr>
<td>R3230</td>
<td>39 ± 4.6</td>
<td>4</td>
</tr>
</tbody>
</table>
FIGURE 5. Effect of trypsin on dialyzed DMBA 8 protein.

(A) Trypsin (10ng) was mixed with dialyzed DMBA 8 (0 - 2.5µg protein) and immediately measured for PA activity (with plasminogen) in the colorimetric assay. Time of second incubation was 7min. With 10min second incubation the absorbance was off-scale (>2.0) for 1.25µg DMBA 8. Absorbance represents PA activity.

DMBA 8 (▲)
DMBA 8 plus trypsin (●)

(B) The same conditions as above except that plasminogen was omitted and the time of second incubation was 18min. Absorbance represents activity measured by the ester substrate Z-Lys-S Bzl.

DMBA 8 (▲)
DMBA 8 plus trypsin (●)
Comparison of the effects of heat-treated or active trypsin on dialyzed DMBA 8 protein

Trypsin (10ng) either heated (56°C, 2h) or kept at 0°C, was mixed with DMBA 8 (2µg protein) and preincubated for 15min at 37°C prior to the addition of plasminogen and measurement of PA activity in the colorimetric assay. Time of incubation for part 2 equalled 15 min. Absorbance represents activity. Percentage change was calculated after correction for the absorbance due to DMBA 8 plus plasminogen (0.07) by the following formula:

$$\frac{Ab(\text{trypsin }+ \text{ DMBA 8}) - Ab(\text{DMBA 8}) - Ab(\text{trypsin})}{Ab(\text{trypsin})} \times 100$$

<table>
<thead>
<tr>
<th>Absorbance (412nm)</th>
<th>Percentage change</th>
</tr>
</thead>
<tbody>
<tr>
<td>trypsin</td>
<td>1.0</td>
</tr>
<tr>
<td>trypsin plus DMBA 8</td>
<td>1.88</td>
</tr>
<tr>
<td>heat-treated trypsin</td>
<td>0.12</td>
</tr>
<tr>
<td>heat-treated trypsin plus DMBA 8</td>
<td>0.23</td>
</tr>
</tbody>
</table>

Ab = absorbance
Nevertheless, in view of the data in Table 4 (discussed on page 118) an enzymatic conversion of pro-PA to PA cannot be conclusively inferred.
not seen when the latter is denatured.

Figure 6 shows the effect of time of preincubation of dialyzed DMBA 8 material with trypsin. Maximum activation by trypsin occurred immediately upon mixing similarly to the activation which occurred when MAT 13762 supernatant was mixed with DMBA 8 protein. Thus, no time dependence was evident under these experimental conditions.

6.3.3 Effect of plasmin

Pro-PA is usually activated by preincubation with plasmin (Skriver et al., 1982; Wun et al., 1982a; Eaton et al., 1984). Pro-PA in HEp3 human carcinoma cells (0.12µg) was activated by preincubation with plasmin (100ng) in 3-5min (Wun et al., 1982a). However, incubation of DMBA 8 (0.08-5µg protein) with plasmin (500ng) did not result in activation of pro-PA (Figure 7).

6.4 POSSIBLE EFFECTS OF DIALYSIS

Although dialysis allowed activation of pro-PA by metastatic cell culture supernatant, simple dilution (1 in 12750) of a concentrated preparation (before dialysis) did not enable activation but rather inhibition was observed (arrow, Figure 8). Dialysis may remove small molecules such as ions from DMBA 8 material, thus altering the conformation and consequently, the state of PA enzyme activities (Carafoli & Penniston, 1985). Therefore removal of these species by dialysis could then allow activation of pro-PA.
FIGURE 6. Preincubation of trypsin and dialyzed DMBA 8 protein as a function of time.

100µl trypsin (500ng/ml) and 100µl DMBA 8 (50µg/ml) were mixed together and aliquots removed at specified times to tubes containing AGTG buffer and plasminogen, and PA activity measured in the colorimetric assay. Incubation time of second part of assay was 10min.

DMBA 8

Trypsin (■)

DMBA 8 plus trypsin (●)
FIGURE 7. Lack of effect of plasmin on dialyzed DMBA 8 protein.

Plasmin (500ng) and DMBA 8 (0-2.5µg protein) were preincubated for 10min, 37°C then measured for PA activity (with plasminogen) in the colorimetric assay. Absorbance represents PA activity.

DMBA 8 (▲)

DMBA 8 plus plasmin (●)
FIGURE 8. Inhibition of MAT 13762 PA by non-dialyzed DMBA 8.

A freshly prepared sample of concentrated DMBA 8 cell supernatant (11.4mg/ml) was diluted in AGTG buffer and tested for inhibition of MAT 13762 cell supernatant as a source of crude PA. Samples of DMBA 8 and PA were preincubated for 10min, at 37°C then assayed for residual PA activity in the colorimetric assay. Absorbance represents PA activity (MAT 13762 Control = 1.4mPU). Arrow indicates the DMBA 8 concentration which is equivalent to a 1/12750 dilution of the original preparation.
The increased enzyme activity apparent after addition of hypoxanthine to DMBA 8 (µg protein) suggests that the enzyme is primarily activated by a result of the activation of a reaction which probably required protection. A decrease in the activity was likely to be due to an increase in inhibitor as a result of dialysis. Inhibition of the reaction during purification procedures, the absorption of the inhibitor increased rather than decreased. The activity of the enzyme was measured and compared with the enzyme activity obtained after the addition of an enzyme-inhibitor peptide for an enzyme-kinetic reaction.
Since addition of plasminogen to dialyzed DMBA 8 material resulted in little or no increase in PA activity, it follows that (i) dialysis did not separate a latent PA-inhibitor complex and (ii) the increased enzyme activity apparent after addition of trypsin is in fact due to the activation of a pro-enzyme, which probably requires proteolytic cleavage, rather than the presence of a latent PA.

The increase in PA activity was unlikely to be due to denaturation of inhibitor as a result of dialysis because during purification procedures, the specific activity of inhibitor increased rather than decreased following concentration and dialysis (Table 1, Chapter 4).

6.5 NO EVIDENCE FOR DISSOCIATION OF AN ENZYME-INHIBITOR COMPLEX OR ACTIVATION OF PRO-PA FOLLOWING SDS-PAGE

Some workers have suggested that trace amounts of plasmin in zymograms (Vassalli et al., 1983) or SDS in electrophoresis gels (Levin, 1983) can separate endogenous PA-inhibitor complexes leading to the appearance of activity in zymograms. Other reports have shown activation of pro-PA in zymograms after SDS-PAGE (Wun et al., 1982a; Eaton et al., 1984). However, there was no evidence of activity in zymograms when DMBA 8 preparations were run alone in SDS gels (5-13 µg, n=9). This suggests that 'flares' of activity in zymograms (lane 6, Figure 9A; lanes 2 and 4, Figure 10B) were due neither to the separation of an enzyme-inhibitor complex nor to activation of pro-PA. Nevertheless, the 'flares' of lysis of these zymograms
remained unexplained.

Inhibition of urokinase (Figure 9A) or MAT 13762 PA (Figure 9B) was concentration dependent and was evident in zymograms when SDS gels were incubated on fibrin agar overlays for 5-10h; however, after prolonged incubation or at higher concentrations of urokinase (lane 6, Figure 9A) or MAT 13762 (lanes 2 and 4, Figure 10) 'flares' of lysis sometimes appeared. This activity was only apparent in lanes which contained both urokinase and DMBA 8 inhibitor or secreted PA and DMBA 8 inhibitor.

6.6 PLASMIN SEPARATION OF COMPLEXES FORMED BETWEEN INHIBITOR AND EXOGENOUS PA

One possibility which could account for the appearance of 'flares' of activity in zymograms was that SDS had denatured the inhibitor and prevented apparent inhibition of added PA (Granelli-Piperno & Reich, 1978). To determine whether SDS had this effect, 'native' protein gels were run in the absence of SDS. One gel was run which contained 30, 50, and 100 mPU urokinase with and without inhibitor (10µg). After electrophoresis, this gel was sliced in half and each half placed on a separate overlay and incubated (6h) for the production of zymograms. The overlay which contained 100mPU urokinase (Figure 10A) showed complete inhibition by inhibitor whereas the overlay containing 30 and 50mPU showed only partial inhibition (Figure 10B). The difference lay in the fibrin-agar overlays. The overlay onto which the
FIGURE 9. Zymogram showing inhibition of urokinase by 'crude' inhibitor.

Urokinase plus 'crude' inhibitor (after passage through lysine sepharose) were preincubated for 15min at 37°C then loaded onto SDS gels and placed on zymograms as detailed in Section 2.2.4.

<table>
<thead>
<tr>
<th>Line number</th>
<th>PA mpu (µg protein)</th>
<th>Time of incubation on zymogram (h)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>30</td>
<td>5</td>
</tr>
<tr>
<td>2</td>
<td>30</td>
<td>5</td>
</tr>
<tr>
<td>3</td>
<td>50</td>
<td>5</td>
</tr>
<tr>
<td>4</td>
<td>50</td>
<td>5</td>
</tr>
<tr>
<td>5</td>
<td>100</td>
<td>5</td>
</tr>
<tr>
<td>6</td>
<td>100</td>
<td>5</td>
</tr>
</tbody>
</table>

Fig. 9A Urokinase

Mobilities of molecular weight standards run in the same gel are shown: carbonic anhydrase, 29,000; ovalbumin, 45,000; bovine albumin, 66,000; β-galactosidase, 116,000.
FIGURE 9. Zymograms showing inhibition of MAT 13762 PA by 'crude' inhibitor.

PA plus 'crude' inhibitor (after passage through lysine sepharose) were preincubated for 15 min at 37°C then loaded onto SDS gels and placed on zymograms as detailed in Section 2.2.4.

<table>
<thead>
<tr>
<th>Lane Number</th>
<th>PA mPU</th>
<th>Inhibitor (µg protein)</th>
<th>Time of incubation on zymogram (h)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fig. 9B. MAT 13762</td>
<td>1</td>
<td>13</td>
<td>0</td>
</tr>
<tr>
<td>MAT 13762 plus inhibitor</td>
<td>2</td>
<td>13</td>
<td>7</td>
</tr>
<tr>
<td>MAT 13762</td>
<td>3</td>
<td>20</td>
<td>0</td>
</tr>
<tr>
<td>MAT 13762 plus inhibitor</td>
<td>4</td>
<td>20</td>
<td>10</td>
</tr>
</tbody>
</table>

MAT 13762 PA was determined in the colorimetric assay.

Mobilities of molecular weight standards run in the same gel are as shown: carbonic anhydrase, 29,000; ovalbumin, 45,000; bovine albumin, 66,000.
FIGURE 10A. Zymogram prepared from native protein polyacrylamide gels.

Urokinase plus or minus 'crude' DMBA 8 inhibitor were preincubated for 15 min at 37°C prior to loading onto native protein gels (7.5% acrylamide) and electrophoresed in the absence of SDS. Zymograms were prepared as in Section 2.2.4.

<table>
<thead>
<tr>
<th>Urokinase (mPU)</th>
<th>Lane number</th>
<th>Inhibitor (µg)</th>
<th>Time of incubation of zymogram</th>
</tr>
</thead>
<tbody>
<tr>
<td>100</td>
<td>1</td>
<td>0</td>
<td>6</td>
</tr>
<tr>
<td>100</td>
<td>2</td>
<td>5</td>
<td>6</td>
</tr>
</tbody>
</table>
FIGURE 10B. Zymogram prepared from native protein polyacrylamide gels.

Urokinase plus or minus 'crude' DMBA 8 inhibitor were preincubated for 15 min at 37°C prior to loading onto native protein gels (7.5% acrylamide) and electrophoresed in the absence of SDS. Zymograms were prepared as in Section 2.2.4.

<table>
<thead>
<tr>
<th>Urokinase (mPU)</th>
<th>Lane number</th>
<th>Inhibitor (µg)</th>
<th>Time of incubation of zymogram</th>
</tr>
</thead>
<tbody>
<tr>
<td>30</td>
<td>1</td>
<td>0</td>
<td>6</td>
</tr>
<tr>
<td>30</td>
<td>2</td>
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</tr>
<tr>
<td>50</td>
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<td>6</td>
</tr>
<tr>
<td>50</td>
<td>4</td>
<td>5</td>
<td>6</td>
</tr>
</tbody>
</table>
FIGURE 10C. Zymogram prepared from native protein polyacrylamide gels.

Urokinase plus or minus 'crude' DMBA 8 inhibitor were preincubated for 15 min at 37°C prior to loading onto native protein gels (7.5% acrylamide) and electrophoresed in the absence of SDS. Zymograms were prepared as in Section 2.2.4.

<table>
<thead>
<tr>
<th>Urokinase (mPU)</th>
<th>Lane number</th>
<th>Inhibitor (µg)</th>
<th>Time of incubation of zymogram</th>
</tr>
</thead>
<tbody>
<tr>
<td>100</td>
<td>1</td>
<td>0</td>
<td>12.5</td>
</tr>
<tr>
<td>100</td>
<td>2</td>
<td>5</td>
<td>12.5</td>
</tr>
</tbody>
</table>
100mP.U. urokinase gel slice had been placed was prepared from plasminogen-free fibrinogen to which purified plasminogen had been added, whereas the gel slice containing 30 and 50 mP.U. urokinase had been placed on an overlay prepared from plasminogen-rich fibrinogen. When the 100mP.U. urokinase gel was placed on a fresh overlay prepared with plasminogen-rich fibrinogen, 'flares' of lysis appeared in the lane containing urokinase and inhibitor (Lane 2; Figure 10C).

Small areas of lysis throughout the overlay indicated protease contamination, probably caused by autocatalytic degradation of plasminogen when plasminogen-rich fibrinogen was maintained at 46°C prior to mixing the overlay reagents. This was more apparent when the fibrinogen had been frozen and thawed more than once.

'Flaring' in lanes containing MAT 13762 PA and inhibitor was less apparent after a longer period of preincubation prior to SDS-PAGE. In Figure 11 which shows partial inhibition of MAT PA (30mP.U.) by DMBA 8 (10µg), there was less evidence of 'flaring' after 2h preincubation (Lane 2) compared with 30min preincubation (Lane 4). This observation is in agreement with the kinetics of inhibition; the latter was consistent with tighter binding of inhibitor to urokinase which occurs with increasing time of preincubation (Section 4.4.9).

From these results, it was concluded that the 'flares' of enzyme activity were due to the separation
FIGURE 11. Zymogram showing reduction of 'flaring' as a function of preincubation time of MAT 13762 PA with 'crude' DMBA 8 inhibitor.

PA plus crude inhibitor (10µg) were preincubated for 2h (Lane 2) or 30min (Lane 4) at 37°C then loaded onto SDS gels and placed on zymograms as detailed in Section 2.2.4. MAT 13762 PA (30mPU) in ACTG buffer only was run in Lane 1 (2h preincubation) and Lane 3 (30min preincubation). Mobilities of molecular weight standards run in the same gel are shown:

ovalbumin, 45,000; bovine albumin, 66,000; β-galactosidase, 116,000.
of loosely-bound exogenous PA-inhibitor complexes by a protease (probably plasmin) rather than to SDS denaturation of inhibitor, and that separation was dependent upon the amount of protease contamination present in the overlay.

6.7 TYPE OF ENZYME ACTIVATED

There appeared to be an optimum ratio of metastatic cell secreted protein to crude inhibitor concentration for maximum activation of pro-PA (Figure 2). While the increase in absorbance and therefore the appearance of this activity was apparent in the sensitive colorimetric assay, the actual amount of activated enzyme was small, \(<2\text{mPU/µg} \text{ crude DMBA 8 inhibitor and would be difficult to detect in a zymogram after SDS-PAGE in which a minimum of } 10\text{mPU} \text{ is required. However the amount of activity liberated by trypsin was } >4\text{mPU/µg and a zymogram of trypsin and inhibitor showed bands of lysis at about } M_\text{r} 45,000 \text{ and } 30,000 \text{ (Figure 12).}

6.8 SUMMARY AND CONCLUSIONS

The activities observed in DMBA 8 concentrated preparations before and after dialysis are represented diagrammatically in Figure 13.

There was a small amount of endogenous PA present in crude inhibitor preparations which could be detected in the sensitive colorimetric assay. There was no evidence for separation of a latent PA-inhibitor complex by dialysis.

While rapid activation of pro-PA was apparent
FIGURE 12. Zymogram showing Mr species of pro-PA activated by trypsin.

Trypsin (20ng) plus non-dialyzed, concentrated DMBA 8 (5µg) were mixed together then loaded onto an SDS gel and placed on a zymogram as detailed in Section 2.2.4. The zymogram was stained with Coomassie Blue. Lane 1 contains inhibitor in AGTG buffer, Lane 2 contains inhibitor plus trypsin.

Mobilities of molecular weight standards run in the same gel are as shown: carbonic anhydrase, 29,000; ovalbumin, 45,000.
FIGURE 13. Diagram summarizing the activities of DMBA 8 concentrated cell supernatant before and after dialysis.
DMBA 8 culture supernatant

- PA
  - Pro-PA
    - inhibitor of: urokinase, metastatic cell PA from:
      - MAT 13762, PE, R3230

Dialysis

- PA

Pro-PA

- trypsin
  - Pro-PA
    - little effect
      - heat-treated trypsin
    - no effect
      - plasmin

- metastatic cell secreted activator from:
  - MAT 13762, PE, R3230

PA
when the culture supernatant obtained as a source of crude PA from metastatic cells was added to dialyzed DMBA 8 protein, urokinase itself did not lead to activation so it seems unlikely that a urokinase-type PA is responsible. Also, pro-PA was not activated either by added plasmin or by the plasmin generated from urokinase or metastatic cell PA. Since trypsin, a serine protease, is a very effective activator, it is possible that a plasminogen-independent serine esterase secreted by metastatic cells may be involved (Table 7; Chapter 4). However, the exact nature of the metastatic cell-secreted activator has yet to be determined.

A pro-enzyme form of PA in DMBA 8 could be activated by trypsin. Activation was rapid (<1 min) being evident immediately after mixing trypsin and inhibitor together. After dialysis activation by trypsin was greatly enhanced which suggests that dialysis enabled another form or more of the same pro-PA to become accessible to trypsin activation, perhaps by removal of interfering substances which inhibited activation of pro-PA.

Pro-PA activity in dialyzed DMBA 8 material was detected after incubation with metastatic cell culture supernatant. The reaction was concentration dependent but independent of time and temperature of preincubation. Dialysis of DMBA 8 material was required for the appearance of this activity before which only inhibition was apparent.
The pro-PA was concomitantly secreted with the urokinase inhibitor and these activities appeared to be separate. There was no evidence for SDS activation of latent PA or separation of a pro-PA-inhibitor complex from zymogram data.

Dialysis was necessary to enable activation of pro-PA by cell-secreted activator as dilution alone was insufficient.

DMBA 8 pro-PA appears different from previously reported pro-enzyme forms of PA as it was not activated by plasmin. Also, it is activated very rapidly at low concentrations by both trypsin and some substance in metastatic cell supernatants so a binding phenomenon rather than an enzyme activation cannot be excluded.

Although the data suggest that pro-PA is present in DMBA 8 preparations, the results do not positively preclude the possibility that some substance in DMBA 8, which is active only after dialysis, activates pro-PA in metastatic cell culture supernatants. However, this seems less likely for the following reasons:

(1) all metastatic cell supernatants would have to contain a pro-PA which was activated by the same substance present in DMBA 8. Implicit in this argument are: (a) the metastatic cell supernatants all have the same type of pro-PA or (b) the DMBA 8 substance has a wide specificity;

(2) If (1) occurs, then it is still a very rapid process of pro-PA activation, a phenomenon not previously
reported in the literature;

(3) assuming that all metastatic cell lines secrete a pro-PA, then unless all contain the same atypical pro-PA type, one would expect that some of the plasmin generated in mixing experiments would lead to an increased activity above that of the separate activities added together;

(4) if the activator of pro-PA is present in DMBA 8, then it is apparently absorbed to lysine sepharose.

Clearly, an attempt to define the exact mechanism of pro-PA activation is difficult while using crude cell culture supernatants. However, the experiments described here have uncovered an interesting phenomenon which requires further study. Experiments mixing purified fractions from each cell supernatant may determine the precise location of pro-PA and readdition of various ions may help to define the manner in which pro-PA is maintained in its inactive form.

6.9 POTENTIAL ROLE OF PRO-PA IN CANCER METASTATIS

Although dialysis was the method used here which uncovered the presence of pro-PA, there are probably physiological mechanisms which would achieve the same effect. For example, metal ion fluxes occur in and around cells as a result of hormonal stimulation (Williamson et al., 1985). Ions or other diffusible molecules, may act as allosteric inhibitors (Palmer, 1981; Neurath & Walsh, 1976) which bind to and maintain DMBA 8 pro-PA in its inactive form. Accordingly, an event such as hormonal stimulation which can lead to
the removal of small molecules from proteins could be one step in a cascade required for enzyme activation.

PA levels in the mammal may be regulated by several mechanisms:

(a) PA which is secreted by normal cells including activated macrophages (Reich, 1975; Hamilton, 1976);

(b) the activation of latent u-PA (apparent only after dialysis), which has been reported in human macrophages (Stephens & Golder, 1984) and in human plasma (Wun et al., 1982b);

(c) pro-PA that can be activated by proteases, which is secreted by normal cells such as macrophages (Vassalli et al., 1984) fibroblasts (Scott et al., 1983), and human embryonic kidney cells (Bernik et al., 1974; Nolan et al., 1977). Pro-PA may provide a ready pool for localized proteolysis by these cells;

(d) plasma (Moroi & Aoli, 1976; Kruithof et al., 1984) and cell-secreted inhibitors (McCabe & Evans, 1984; Knauer & Cunningham, 1984) which also serve to regulate PA levels.

These mechanisms may therefore serve to control PA levels and consequently plasmin activity, thereby preventing uncontrolled degradation of surrounding tissues.

The importance of pro-PA in cancer metastasis is a matter of conjecture. If metastatic cells secreting a substance which activates pro-PA are in the vicinity of normal cells which are producing pro-PA, then the relative levels of PA inhibitors, pro-PA, activators
of pro-PA and PA will determine which activity predominates. For example, as some metastatic cells have a large amount of active PA at their cell surface (Table 4, Chapter 5), this may be more than sufficient to bind all PA inhibitors. Accordingly, activation of the pro-PA of normal circulating host cells such as macrophages, by metastatic cell-secreted activator could result in amplification of the metastatic cells' PA. Thus, pro-PA produced by normal cells could be activated by substances released from tumour cells.

There is another mechanism by which interactions with host cells could enhance the PA activity of metastatic cells. For example, pro-PA is secreted by carcinoma cells (Wun et al., 1982a) and transformed murine cells (Skriver et al., 1982). There is no evidence so far to suggest that a metastatic cell-secreted activator of pro-PA can activate its own pro-PA. However, if plasmin formed from PA released by activated macrophages cleaves the pro-PA of metastatic cells to form PA, then the presence of plasminogen would lead to an amplification of plasmin levels. Thus pro-PA produced by tumour cells could be activated by host cells.

The resultant plasmin activity might be of sufficient quantity to allow metastatic cells the necessary access into adjacent tissue, blood vessels or lymphatic channels to begin the metastatic process. Other feed-back activations and interactions could
also occur. A scheme of possible interactions between host cells and metastatic cells is sketched in Figure 14. In this manner, the combined degradative enzyme activities of normal and metastatic cells could lead to local tissue degradation enabling invasion and migration by the cancer cells resulting in the formation of metastases.
FIGURE 14. Diagram showing some of the possible interactions between host cells and metastatic cells leading to amplification of PA and plasmin activities thus causing tissue degradation.

- Pro-PA
- PA
- Inhibitor of PA
- PA/inhibitor complex
- Pro-collagenase
- Collagenase
- Activation +
- Suppression −
- Tissue degradation
CHAPTER 7

CONCLUDING DISCUSSION AND FUTURE DIRECTIONS
7.1 CONCLUDING DISCUSSION

Modification of an existing method of plasminogen isolation has resulted in a purified native plasminogen preparation, free of plasmin contamination, which can be used at physiological concentrations for in vitro studies. The PA activity of intact metastatic cells has been measured in vitro and the activity immediately available at the cell-surface was found to be of an equivalent amount to that which was secreted by the cells over a period of 6h. An inhibitor of PA secreted by non-metastatic cells has been purified and characterized. This inhibitor is noncompetitive and inhibits human urokinase as well as u-PA produced by several metastatic cell lines. A pro-enzyme form of PA has been discovered, which differs from previously reported zymogen PA in that it is not activated by plasmin, but which can be rapidly activated by trypsin and a substance(s) secreted by metastatic cell lines. Incubation of metastatic cells with the antibiotic tunicamycin can inhibit both PA production and blood-borne metastases.

The most important aspect of this thesis has been the purification of a PA inhibitor. Investigations into the possible relationship of PA and cancer metastasis have been hindered by the lack of specific and potent inhibitors which are non-toxic. The DMBA 8 inhibitor's high specificity for urokinase-type PA, unlike the majority of inhibitors so far reported (Chapter 6, Table 8), means that an early step in an enzyme cascade which leads to tissue
destruction can be examined more closely.

Inhibitors of PA from a variety of tissues and cells have been reported but very few have been observed in tumour cell systems. Two studies have reported an induction of inhibitor production in response to hormone treatment. In one study, dexamethasone treatment which suppressed PA activity in rat hepatoma cells also resulted in the production of a PA inhibitor (Coleman et al., 1982). In a second study, treatment of rats with LHRH (luteinizing hormone releasing hormone) led to a decrease in the PA content of prostate tumours and the production of a cytosolic inhibitor of both u-PA and t-PA was also observed. Hierowski & Schally (1985) deduced that the increased inhibitor activity may have been important in inhibiting the growth and invasiveness of prostate tumours. The DMBA 8 inhibitor differs from the observed results in these tumour systems in that it is a constitutively secreted product.

Few cell and tissue PA inhibitors have been purified to apparent homogeneity and fully characterized; even fewer kinetic studies have been reported. Kinetic studies of the DMBA 8 inhibitor show that it differs from other known serine protease inhibitors in that it is noncompetitive. Perhaps the role of such a cell-secreted inhibitor is to protect the inhibitor-secreting cells from the degradative actions of extracellular PA. In this
context, a noncompetitive inhibitor would be more useful to a cell because it can inactivate the specific enzyme independently of substrate concentration. With competitive inhibitors, inhibition can be overcome by a sufficiently large amount of substrate. The inhibitor's tight-binding and essentially irreversible mechanism of enzyme inactivation is also in keeping with a defensive role.

The importance of the purification of plasminogen free of plasmin contamination should not be underestimated. It has been reported that plasminogen preparations always contain plasmin activity (Robbins & Summaria, 1970). In fact, some workers utilize this contamination to activate pro-PA (Stephens & Golder, 1983). Under these conditions, results which are difficult to define may result from interactions between various assay components. An alternative is to use a chromogenic substrate for PA which circumvents the need for plasminogen. However, plasminogen which is free of plasmin contamination can be used as a natural PA substrate at physiological concentrations. Apart from the practical advantage of increasing sensitivity thus enabling more samples to be assayed within a short period, the validity of assaying for properties specific to PA is strengthened.

Many studies have linked the production of u-PA with tumorigenicity (Corosanti et al., 1980; Markus
et al., 1983; Danø et al., 1985) but few have linked u-PA with cancer metastasis (Wang et al., 1982; Kramer et al., 1985). The data in this thesis support a correlation of u-PA production with the ability of tumour cells to form metastases. The cell-associated PA activity of tumour cells is usually determined from cell lysates. Cell fractionation studies have determined that the location of cell-associated PA is at the outer surface of the plasma membrane (Lemaire et al., 1983). Although one report has measured macrophage PA in vitro (Weinberg et al., 1984), this is the first time that PA production by intact metastatic cells has been demonstrated in the presence of physiological salt concentrations and also compared with the amount of PA which is secreted by the same cells over a period. Several reports have shown that PA can be inhibited by physiological (0.15M NaCl) salt concentrations (Aggeler et al., 1981; Stephens & Golder 1984). One can then ask whether these enzymes would play an active role in vivo. The fact that both cell-secreted and cell-surface PA are active at physiological salt concentrations suggests that the PA produced by these metastatic cells (MAT 13762) may be of significance in vivo. The relevance of cell-surface PA to cancer metastasis may be that it provides an immediate and localized supply of tumour cell enzyme for conversion of plasminogen to plasmin. Such an enzyme activity could therefore aid tumour cells in the degradation and invasion of
surrounding tissue and perhaps lead to alteration of the tumour cell's own surface. Modification of cell-surface components such as fibronectin and antigenic determinants by protease activity, could alter both the adhesion properties (Vaheri & Mosher, 1978) and the immunogenicity of tumour cells (Thompson et al., 1973).

Preliminary investigations have led to the discovery of a pro-PA secreted by DMBA 8 cells. The fact that some conformational change is apparently required before activation can occur suggests that DMBA 8 cells in fact secrete a pro-pro-PA, that is a zymogen which requires several intermediate steps before the active enzyme form becomes apparent. From the aspect of metastasis and the role of PA, activation of pro-PA may be of significance in determining which cells exhibit a metastatic phenotype. For example, non-metastatic DMBA 8 and metastatic variants of DMBA 8 cells produce similar quantities of mRNA-PA as determined by hybridization with a PA-specific probe (Ramshaw & Badenoch-Jones, 1985) which suggests that the apparent quantity of secreted PA may be due to a difference in post-transcriptional events. This difference may allow the metastatic variants to secrete PA in an active form, rather than in a pro-enzyme form as seems to occur in the case of non-metastatic DMBA 8 cells. Perhaps substances
(such as dexamethasone) which suppress PAs production may in fact be acting at the genetic site of a PA regulator rather than acting on PA synthesis per se, thereby maintaining synthesized PA in an inactive form. The possible relevance of pro-PA in cancer metastasis has already been postulated in Section 6.4.

7.2 FUTURE DIRECTIONS

Further studies are required to purify and characterize both the cell-secreted and cell-surface PA of MAT 13762 cells and to determine whether other metastatic cells similarly produce PA at the cell surface. An important factor of cell-surface PA is that it is readily accessible to inhibitors such as that secreted by DMBA 8 cells. However, the difficulty in carrying out further studies with the DMBA 8 inhibitor has been in obtaining sufficient quantities of pure material.

There are several strategies which could be employed to increase the quantity of inhibitor for future experiments once the inhibitor sequence has been determined (a sample is at present awaiting analysis). One method would be to raise monoclonal antibodies to the purified inhibitor and use these to prepare affinity columns for inhibitor purification. Another method would be to isolate the gene for the DMBA 8 inhibitor which could then be expressed in vectors carrying cDNA. Linkage of inhibitor cDNA to genetic promoters may also increase efficiency of production
(Travis et al., 1985). A third method and the one most suitable for providing inhibitor for clinical use, would be to synthesize peptide analogues from imitotypes derived from the inhibitor sequence (Geysen et al., in press).

Transfer of inhibitor genes to metastatic cells may help to determine whether the PA production of metastatic cells is simply a matter of gene levels of PA and inhibitors or whether regulators at other sites on the genome are involved in the 'fine tuning' regulation of PA activities. The results observed with metastatic DMBA 8 variants which produce as much mRNA-PA as the non-metastatic cells (Ramshaw & Badenoch-Jones, 1985) would, at present, suggest the latter. Nevertheless a threshold level of PA production may be important in the initial steps of metastatic cell invasion into adjacent tissue (Ossowski & Reich, 1983) and a certain level of PA suppression by inclusion of inhibitor genes may be sufficient to prevent an event such as spontaneous metastasis from occurring.

Hormones have been found to influence PA and inhibitor production in several systems. Progesterone treatment induced a plasmin/trypsin inhibitor in pregnant porcine uteri (Fazleabas et al., 1982) and murine mammary cell PA levels can be regulated by hormones such as prolactin and oxytocin during lactation and involution events (Ossowski et al., 1979).
Synthesis of PA in DMBA induced rat mammary tumours was stimulated both in vivo and in cultured cells by administration of estrogen (Yamashita et al., 1984). As MAT 13762 metastatic mammary cells appear to be responsive to the synthetic hormone, dexamethasone, both in decreased PA production and perhaps by synthesis of a PA inhibitor, it would be useful to test the effects of a variety of naturally occurring hormones in this system.

Because of its high specificity, potency and low toxicity, this PA inhibitor should provide a useful tool for in vivo studies. Once sufficient quantities of purified inhibitor are available, incubation of metastatic cells with the inhibitor prior to injection of treated cells both intravenously and subcutaneously, may help to determine at which steps PA may be important in cancer metastasis. Experimentally the methods used to assay for metastasis may reflect different characteristics of tumour cells. For example, in experimental metastasis by intravenous injection of tumour cells, the cells have bypassed the initial steps such as escape from the primary tumour and invasion and infiltration of the blood supply. Thus, experimental metastasis may merely demonstrate the ability of tumour cells to survive in the circulation and implant in specific locations whereas spontaneous metastasis may reflect in addition, the capacity of tumour cells for invasion. Experiments of this type may help to determine whether PA is required for angiogenesis or
perhaps for induction of thrombus formation, bearing in mind that PA is a glycoprotein which is located outside the cell in at least some metastatic cells. These are steps in which the role of PA and PA inhibitors have not yet been investigated.

Protease inhibitors may play a significant role in preventing the initial tissue invasion by PA-producing tumour cells. Recent studies have shown that tissue destruction by human fibrosarcoma cells was significantly inhibited when the cells were given daily supplements of the purified protease inhibitor, nexin (Bergman et al., 1986; and, see Table 8, Chapter 6). Continuous infusion of the inhibitor into animals via an osmotic pump at the site of subcutaneous metastatic cell implantation, may provide valuable information on the importance of PA in primary tumour growth as well as metastatic invasion.

Monoclonal antibodies to this inhibitor could also be used to determine its location in various organs of normal rats as distribution of inhibitors in the intact organism is not known (Danø et al., 1985). As PA and inhibitor activities are important in mammary gland functions, one might expect to observe high concentrations of inhibitor in normal mammary gland tissue.

Because DMBA 8 inhibitor is very potent in its action towards human urokinase, radioactively labelled inhibitor peptides may be of clinical use as
probes to detect areas of increased u-PA activity such as in metastatic lesions which are not readily detected by other measures. Some immunocytochemical studies have observed strong staining for u-PA in the areas of invasive growth by Lewis lung carcinoma (Skriver et al., 1984).

This thesis has added support to the hypothesis that u-PA may be involved in the metastatic process. As PA activity is an early step in an enzyme cascade which leads to a wide variety of degradative events, it is important to be able to investigate PA and its regulation rather than the events which appear as an end result of the cascade. The experiments outlined above using a PA specific inhibitor which interrupts this cascade at an early step, and others which will no doubt be stimulated by the results of those experiments, should help to provide an answer to the actual role(s) of PA in cancer metastasis.
APPENDIX

Amino acid sequence analysis of purified plasminogen.

A 200µg sample of plasminogen, after precipitation with methanol (to 90%) from glycine (50mM) buffer, was subjected to automated amino acid sequencing for 10 residues in a Beckman 890M-2 sequencer. Quantitative identification of the amino acid residues was by HPLC on a Hewlett Packard Model 1084B with an Altex 165 variable wavelength detector; the column was a Beckman/Altex Ultrasphere ODS (25x0.46cm I.D.) with gradient elution by sodium acetate/acetonitrile.

The sequence was determined to be:
Glu-Pro-Leu-Asp-Asp-Tyr-Val-Asn-Thr-Glu......,
which agrees completely with that reported by Sottrup-Jensen and workers (1978). The absolute yield for the first residue was 44% (885pmol), while the repetitive yield for the sequence was approximately 96%. There were 94pmol of glycine at residue one from incomplete removal of the buffer but no other sequence was detected. There was no detectable lysine (<5pmol) at residue one. While there were 16-21pmol of valine at residues one and two, indicating the possibility of ca 2% plasmin being present, this level of valine remained fairly constant and there were no other residues indicative of a plasmin or 'Lys' plasminogen sequence (that is both <0.5%). Therefore it can be concluded that the sample was in fact Glu-plasminogen (>99%).


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