Assessment of plasminogen activator inhibitor type 2 (PAI-2) as an imaging and therapeutic agent of human cancer

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ASSESSMENT OF PLASMINOGEN ACTIVATOR INHIBITOR TYPE 2 (PAI-2) AS AN IMAGING AND THERAPEUTIC AGENT OF HUMAN CANCER

A thesis submitted in fulfilment of the requirements for the award of the degree

PhD

from

UNIVERSITY OF WOLLONGONG

by

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DEPARTMENT OF BIOLOGICAL SCIENCES

2001

STATEMENT OF ORIGINALITY

I, Minh-Thu N. Hang, declare that this thesis contains no material which has been accepted for the award of any degree or diploma in any University, and to the best of my knowledge contains no material which has been previously published or written by another person except where due reference is made in the text of this thesis.

Minh-Thu N. Hang

ACKNOWLEDGEMENTS

My gratitude goes out to quite of a lot of people who have helped and supported me throughout my PhD. Thank-you to my supervisors Clive Bunn and Mark Baker for giving me an interesting project to work on and especially Clive, for sharing with me his enthusiasm for science. Thank-you to my two advisors Marie Ranson and Xiao-Ming Liang for the guidance and technical assistance they provided. A special thanks to Marie for all the encouragement and positive attitude when experiments were not working. Thank-you to my friends from the Department of Biological Sciences and students from lab112 for their friendship and humour. Thank-you to Professor Rob Whelan, for his support and words of advice throughout.

Special thanks to the people at Biotech Australia for all the PAI-2 preparations and numerous reagents and supplementing my PhD scholarship. Thank-you to Southern Pathology and the people in the Department of Histology at North Wollongong Hospital for histological sectioning and staining.

Most importantly, the achievement of my PhD is due to the love and support from my family, Cha (dad), Me (mom), Quynh-Thu, Anh-Thu, and my husband, Michael. I am deeply greatful to my family for always being there for me and believing in me. Thank-you to Cha and Me, through the sacrifices they made to provide me a good education. Thank-you Michael for being so patient with all the late nights in the lab, the cranky moods and the instant noodle dinners and for proof reading this thesis.

ABSTRACT

The plasminogen activation cascade is an important proteolytic pathway involved in the growth and spread of cancer. Potentially, an inhibitor of plasminogen activation could make an excellent cancer imaging agent or cancer treatment. The aim of this thesis was to assess whether plasminogen activator inhibitor 2 (PAI-2) can image or treat colorectal cancer. The first part of this thesis examined the ability of PAI-2 to bind specifically to the human colorectal cancer cell line HCT116. These experiments involved confirmation of u-PA expression by HCT116 cells and cell binding studies with ^{125}I-PAI-2. The second part was examining the biodistribution and kinetics of ^{125}I-PAI-2 in nude mice bearing tumour xenografts derived from HCT116 cells. The final part involved examining the effect PAI-2 treatment had on mice bearing HCT116 tumour xenografts.

PAI-2 was found to bind specifically to u-PA on HCT116 cells. There appeared to be a high turnover rate of bound PAI-2 because it was difficult to detect ^{125}I-PAI-2/u-PA complexes by autoradiography. ^{125}I-PAI-2 had a biphasic distribution in the bloodstream of control mice (distribution phase ($T_{1/2\alpha}$) 12.5min, elimination phase ($T_{1/2\beta}$) 342min) and mice bearing tumour xenografts ($T_{1/2\alpha}$ 1.4min, $T_{1/2\beta}$ 29min). Approximately 1% of ^{125}I-PAI-2 localised to the tumour xenograft after a single intravenous injection. However, more ^{125}I-PAI-2 could be localised to the tumour by multiple intravenous injections. From three separate therapy experiments with PAI-2, there did not appear to be any effect on relatively large tumours. However, in one

experiment PAI-2 injections did cause two 1mm tumours to disappear. In conclusion, PAI-2 does bind to u-PA on HCT116 cells *in vitro*. *In vivo*, injected PAI-2 appeared unsuitable for the imaging of tumours or metastasis. However preliminary data from this thesis suggest that PAI-2 may have therapeutic potential against smaller tumours.

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ABBREVIATIONS

#387	t-PA monoclonal antibody
#394	u-PA monoclonal antibody
#3750	PAI-2 monoclonal antibody
#3936	u-PAR monoclonal antibody
AP1	Activator protein 1
ATTC	American Tissue Type Collection
B428	4-iodobenzo[b]thiopene-2-carboxamidine, a synthetic u-PA inhibitor
bFGF	Basic fibroblast growth factor
BSA	Bovine serum albumin
CEA	Carcinoembryonic antigen
DNP-9	IgG1 isotype control antibody
DTNB	5,5'-dithiobis (2-nitrobenzoic acid)
ECM	Extracellular matrix
EDTA	Ethylenediaminetetraacetic acid
EGF	Epidermal growth factor
EGR-CMK	Glu-Gly-Arg chloromethyl ketone
ELISA	Enzyme linked immunosorbent assay
ER	Estrogen receptor
FBS	Fetal bovine serum
FITC	Fluorescein isothiocyanate
G155-78	IgG2a isotype control antibody
GFD	Growth factor domain
GM-CSF	Granulocyte-macrophage colony stimulating factor
gp330	Glycoprotein 330
GPI	Glycosylphosphatidylinositol
H&E	Hemotoxylin & Eosin
hPAI-2	Human plasminogen activator inhibitor type 2
HUVEC	Human umbilical vein endothelial cells
i.p.	Intraperitoneal
i.v.	Intravenous
IgG	Immunoglobulin G
IL-1	Interleukin-1
IL-2	Interleukin-2
IUGR	Intra-uterine growth retardation
K_d	Dissociation constant
LPS	Lipopolysaccharide
LRP	Low density lipoprotein receptor-related protein
M-CSF	Macrophage colony stimulating factor
MMP-2	Matrix metalloproteinase 2
mPAI-2	Mouse plasminogen activator inhibitor type 2
M_r	Molecular weight

OD	Optical density
OPD	o-Phenyldiamine
PA	Plasminogen activator
PAI	Plasminogen activator inhibitor
PAI-1	Plasminogen activator inhibitor type 1
PAI-2	Plasminogen activator inhibitor type 2
PAI-3	Plasminogen activator inhibitor type 3
PBS	Phosphate buffered saline
PEG	Polyethylene glycol
PGE_2	Prostaglandin E_2
PI	Propidium iodide
PKC	Protein kinase C
PLD	Phospholipase D
PMA	Phorbol myristate acetate
PMSF	Phenylmethylsulfoxide fluoride
PN-1	Protease nexin 1
r^2	Correlation coefficient
RAP	Receptor associated protein
RIA	Radioimmunoassay
RIGS	Radioimmunoguided surgery
RT	Room temperature
s.c.	Subcutaneous
sc-tPA	Single chain tissue type plasminogen activator
sc-uPA/pro-u-PA	Single chain urokinase type plasminogen activator
SCID	Severe combined immunodeficient
SD	Standard deviation
SDS-PAGE	Sodium dodecyl sulphate polyacrylamide gel electrophoresis
SERPIN	Serine protease inhibitor
SLE	Systemic lupus erythematosus
TAG	Tumour associated glycoprotein
TBS	Tris buffered saline
tc-tPA	Twin chain tissue type plasminogen activator
tc-uPA	Twin chain urokinase type plasminogen activator
TCA	Trichloroacetic acid
TGF-α	Transforming growth factor α
TGF-β1	Transforming growth factor β1
TNF-α	Tumor necrosis factor alpha
TNP	Trinitrophenol
t-PA	Tissue type plasminogen activator
TSP-1	Thrombospondin 1
u-PA	Urokinase type plasminogen activator
u-PAR	Urokinase type plasminogen activator receptor
UV	ultra-violet
VEGF	Vascular endothelial growth factor

VLDL	Very low density lipoprotein receptor
ZLS	Z-lysine thibenzyl ester

TABLE OF CONTENTS

1. THE PLASMINOGEN ACTIVATION CASCADE, CANCER IMAGING AND THERAPY

1.1 INTRODUCTION

Cancer is a pathological process that claims the lives of millions world wide. The causes of most cancers is still not known. Various factors have been implicated and still more are being elucidated, from oncogenic transformation to cancer-causing agents. The fatalities in cancer patients increases with the transformation of the tumour from benign to malignant, which involves spread of the cancer from the site of origin to secondary sites in the body, known as metastasis. The process of cancer metastasis is discussed below in greater detail. Cancer research consists of studies into the multiple aspects of this disease including; factors that may have lead to transformation of a normal cell into a cancerous cell, biology of the different cancers, cancer growth and cancer metastasis. The answers from the previously mentioned studies facilitates subsequent research. Strategies include development of prognostic indicators to allow for detection of patients that are at risk for the development of cancer, early detection of the development of cancer or cancer recurrence, agents that can prevent cancer metastasis and finally agents that will cure patients of the cancer.

From research documented throughout the literature it is known that to facilitate cancer metastasis, individual cancer cells need to escape the surrounding environment to reach distant site. Barriers such as the basement membrane and extracellular

matrix (ECM) need to be "passed". This involves degradation of basement membrane components and the ECM. Several families of enzymes have been implicated in this process. One such family of enzymes is the plasminogen/plasmin system. Members of the plasminogen/plasmin system, which will be discussed below, consists of the pro-enzyme, plasminogen; the active enzyme, plasmin; plasminogen activators; and inhibitors of both plasmin and the plasminogen activators. The interaction between the activators, plasminogen and the inhibitors serves to regulate the generation of proteolytic activity required for the degradation of the basement membrane and ECM, facilitating the spread of cancer. This chapter will review available evidence implicating the involvement of members of the plasminogen/plasmin system in the process of cancer metastasis. To a lesser extent, some of the available agents for the imaging and treatment of cancer will also be reviewed with emphasis on agents that abolish the activity of members of the plasminogen/plasmin system.

1.2 CANCER INVASION AND METASTASIS

1.2.1 The Metastatic Process

Cancer metastasis is a complex multistep process that involves interaction between host tissues and cancer cells. The process of metastasis begins with the invasion of

the primary tumour into the host stroma accompanied by angiogenesis. Invasion is followed by intravasation which involves entry of the tumour cells into the host's circulation or into the lymphatics. Once the cancer cells are within the circulation, the host's defence mechanisms must be evaded so the cells can become arrested at a capillary bed. The tumour cells then extravasate by leaving the blood vessel and migrating into the parenchyma of the target organ. At the secondary site, to survive and form a permanent metastasis, tumour cells must proliferate and form new blood vessels. A simplified schematic representation of the complex process of cancer metastasis is shown in Figure 1. For more information refer to the following references: Zeidman, 1957; Fidler, 1977; Weiss, 1990; Fidler, 1990.

1.2.2 Cellular Movement

Complex biochemical interactions between the tumour cells and the host's normal tissue are required at each step of the metastasis. For movement to occur cells require membrane receptors for attaching and detaching to components of the membrane. Cancer cells must be able to degrade components of the basement membrane to form a path into the interstitial connective tissue. Several co-ordinated steps are required for cell movement/mobility. Namely; 1) the cell must form an adhesion with the underlying substratum, 2) the cell must protrude beyond the boundaries of the site of attachment and form new adhesions, and finally 3) the old adhesions must be broken.

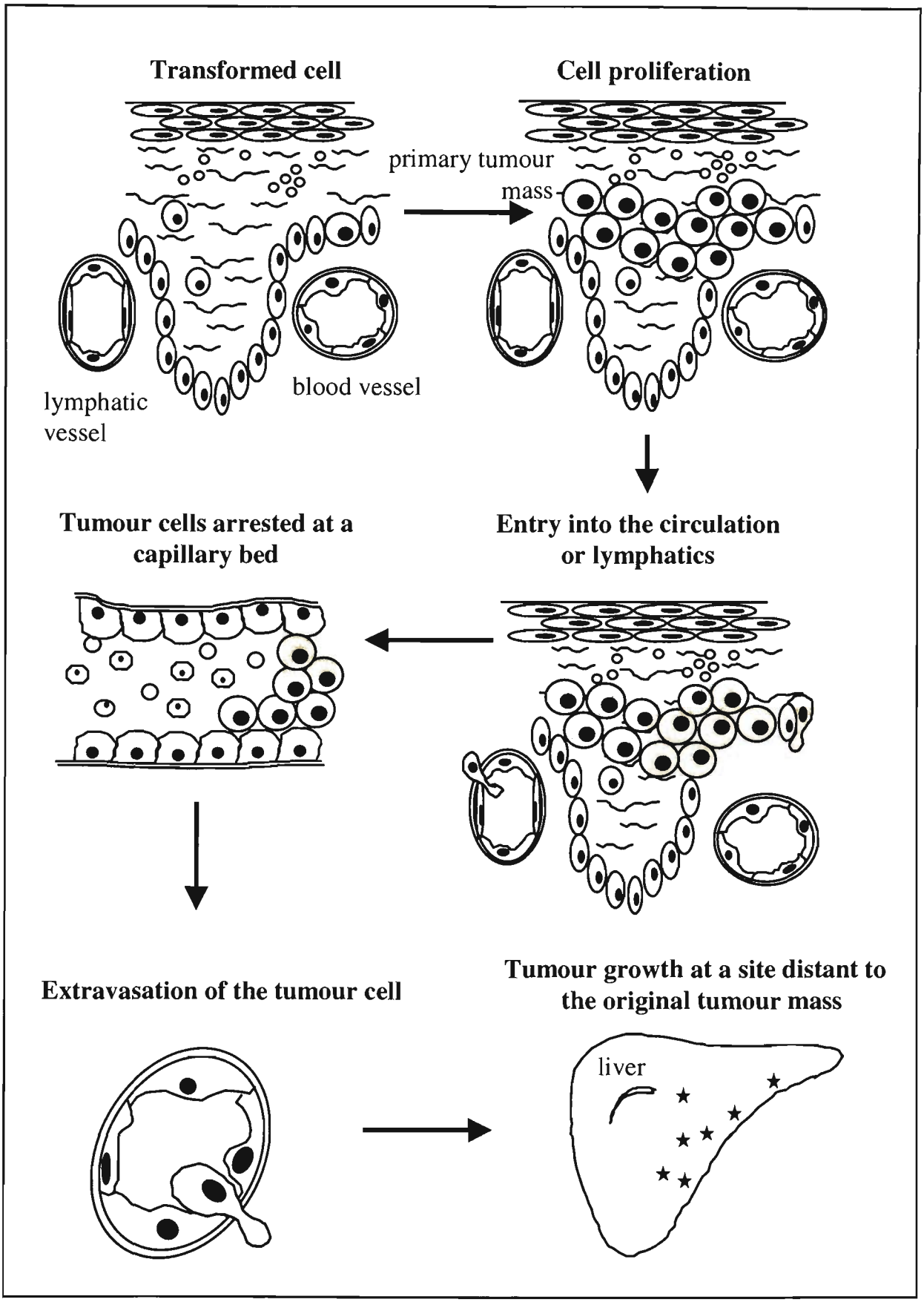


Figure 1. A simplified schematic diagram representing the steps of cancer metastasis. Modified from Fidler, 1990.

The above steps are repeated over and over again for the process of movement to occur.

1.2.3 Routes of Cancer Spread

There are various routes different types of cancer may take to reach distant sites to form secondary metastasis. For colorectal cancer there are four different ways in which the cancer can spread. 1) local spread from the primary site, 2) invasion of the lymphatics system and lymph nodes, 3) invasion into the circulation and subsequent distant metastases and 4) direct implantation of the tumour on the peritoneum (Thomas, 1993). The liver and lungs are the two major targets for distant metastasis in colorectal cancer patients (Weiss, 1985).

1.2.4 Involvement of proteolytic enzymes in cancer metastasis

The degradation of the basement membrane and ECM by tumour cells is essential for the progression of the metastatic process. Numerous proteolytic enzymes and enzymatic cascades have been implicated in the process of metastasis. The actions of these proteolytic enzymes facilitate degradation of the basement membrane and other barriers, to allow the migration of cancer cells. The group of enzymes responsible for degrading the ECM are proteinases (also known as endopeptidases) that can hydrolyse bonds in the primary amino acid sequence of polypeptides. Proteinases have been

found and studied in a variety of tissues, under normal and pathological conditions. The proteinases are subdivided into five classes; the metalloproteinases, cysteine proteinases, threonine proteinases, aspartic proteinases and serine proteinases (Mignatti & Rifkin, 1993). Classification of the proteinases are carried out according to the presence of a metal atom or an amino acid residue in the active site of the enzyme. The introduction will be limited to descriptions of u-PA and PAI-2. Members of the serine proteinase family, contain the amino acid serine at the catalytic site.

1.3 PLASMINOGEN/PLASMIN SYSTEM

As mentioned previously, the components of this system are plasminogen, plasmin, two plasminogen activators (PAs) and various inhibitors of plasmin and plasminogen activators. The two activators are tissue-type plasminogen activator (t-PA) and urokinase-type plasminogen activator (u-PA). The main inhibitor of plasmin activity is α_2-antiplasmin. The inhibitors that are known to regulate the activities of t-PA and u-PA are plasminogen activator inhibitor 1 (PAI-1), plasminogen activator inhibitor 2 (PAI-2), plasminogen activator inhibitor 3 (PAI-3) and protease nexin 1 (PN-1). A schematic diagram of components of the plasminogen/plasmin system is depicted in Figure 2.

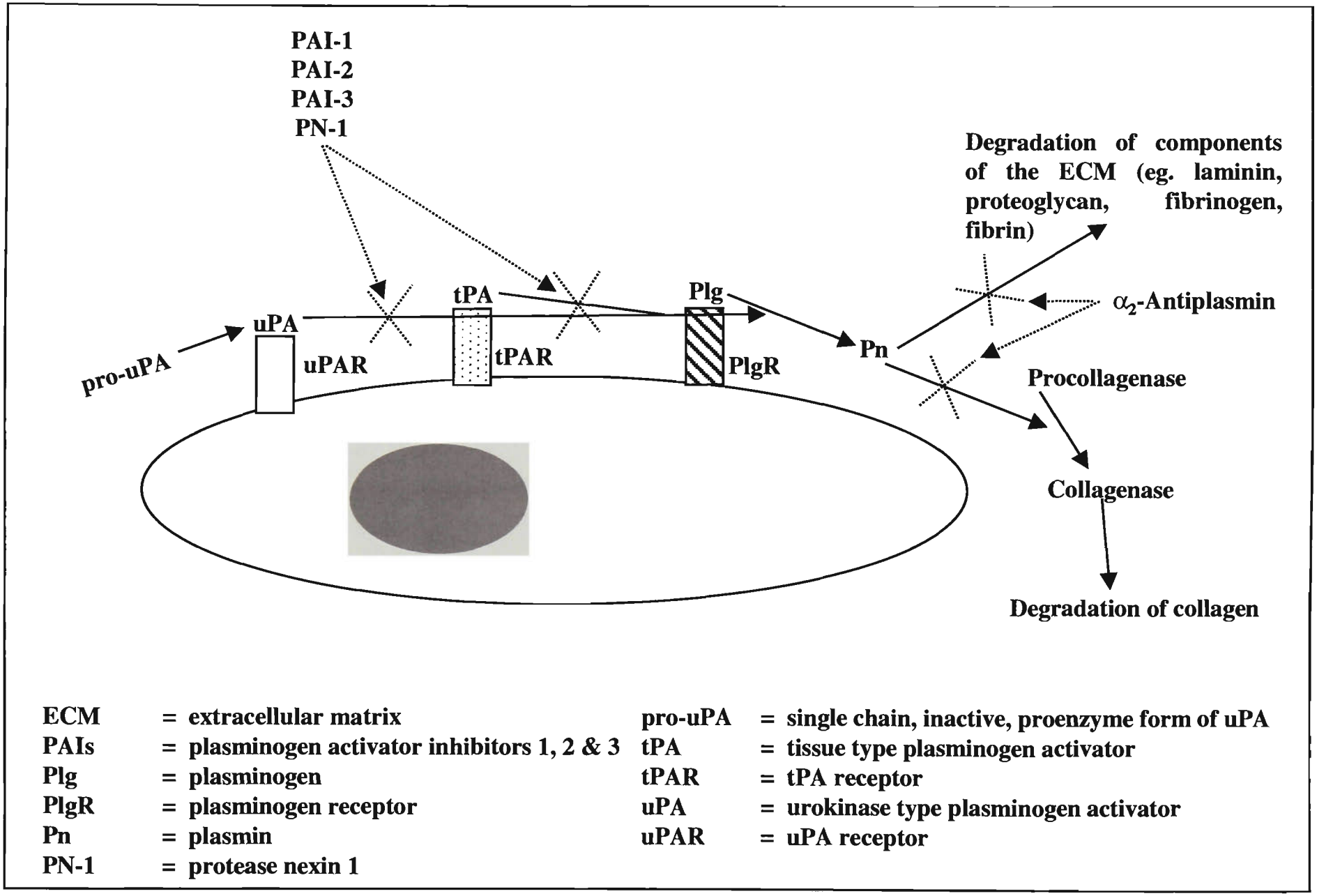


Figure 2. A simplified schematic diagram of the components of the plasminogen/plasmin system.

1.3.1 Plasminogen and Plasmin

1.3.1.1 Synthesis

Plasmin, a neutral serine proteinase, is secreted as an inactive zymogen, plasminogen, which is synthesised primarily by the liver (Raum *et al.*, 1980). Plasminogen is found in the circulation at a relatively high concentration of 1-2μM (Robbins & Summaria, 1970; Lijnen & Collen, 1982; Plow, 1986; Kwaan, 1992). In humans, approximately 40% of plasminogen is located in the extravascular sites (Collen *et al.*, 1972; Mignatti & Rifkin, 1993). Plasminogen has also been demonstrated in various locations including in human epidermis (Saito *et al.*, 1980; Gissler *et al.*, 1993; Schaefer *et al.*, 1996), rat liver (Saito *et al.*, 1980), human saliva (Moody, 1982) and in human uterine fluid (Casslen & Ohlsson, 1981). The native plasminogen consists of a single polypeptide chain with a M_r of ~92kDa (Lijnen & Collen, 1982). Plasminogen is converted to the active enzyme plasmin by cleavage of the Arg_{560}-Val_{561} peptide bond, either by t-PA (Hoylaerts *et al.*, 1982) or u-PA by limited proteolysis (Robbins *et al.*, 1967).

1.3.1.2 Proteolytic Activity of Plasmin

The generation of plasmin plays key roles not only in the proteolytic breakdown of fibrin clots but also in extracellular matrix degradation and cell migration (Bonnar *et*

al., 1990). In addition to this, plasmin also plays a role in the degradation of the extracellular matrix proteins in various physiological and pathological processes including implantation (Zini *et al.,* 1992; Feinberg *et al.,* 1989; Jensen *et al.,* 1989; Jonasson *et al.,* 1989), cervical ripening (Stys, 1986; Vasilenko & Mead, 1987; Lee *et al.,* 1992) and tumour invasion (O'Grady *et al.,* 1981; Liotta *et al.,* 1981; Baker, M.S. *et al.,* 1990; Vassalli *et al.,* 1991). Involvement of the plasminogen/plasmin system with the process of cancer metastasis in various cancer types is well documented throughout the literature (Danø *et al.,* 1985; Vassalli *et al.,* 1991; Quax *et al.,* 1992; Andreasen *et al.,* 1997).

Plasmin consists of two polypeptide chains held together by disulfide bonds. The active site of plasmin is located on the light chain with a M_r of ~25kDa (Danø *et al.,* 1985). Plasmin has a broad substrate specificity, being able to degrade various components of the ECM which include fibronectin, fibrinogen, laminin, proteoglycan, vitronectin and type V collagen (Mochan & Keler, 1984; Collen, 1988a; Schlechte *et al.,* 1989; Matrisian & Hogan, 1990; Mignatti & Rifkin, 1993; Tran-Thang *et al.,* 1994). Plasmin can activate metalloproteinases, like collagenases and gelatinases that have been shown to play a role in tumour invasion (Werb *et al.,* 1977; Mignatti *et al.,* 1986; Gavrilovic & Murphy, 1989; Fisher *et al.,* 1989; Mignatti & Rifkin, 1993). Collagenases are responsible for the degradation of various subtypes of collagen molecules, which are integral components of the basement membrane (Liotta *et al.,* 1981). Recently it has been found that plasmin may have a role in the activation of

maxtrix metalloproteinase 2 (MMP-2) and MMP-9 (Baramova *et al.,* 1997). Plasmin is also responsible for the activation of pro-u-PA into the active u-PA (Kasai *et al.,* 1985; Ellis *et al.,* 1987; Matrisian & Hogan, 1990; So *et al.,* 1992), and the conversion of single-chain t-PA into two-chain t-PA (Andreasen *et al.,* 1984; Matsuo *et al.,* 1989). The expression of plasminogen at the cell surface, which in turn is activated by the action of the PAs to plasmin, allow for the precise localisation of ECM protein proteolysis (Vaheri *et al.,* 1990; Plow & Miles, 1990).

1.3.1.3 Inhibitors of Plasmin

The proteolytic activity of plasmin is regulated at several levels and by various factors. It is also dependent upon the presence of enzymes and activators, and on the balance between active enzyme and it's inhibitor (Hamilton & Millis, 1990). Plasmin is inhibited by several proteinase inhibitors such as α_2-antiplasmin, α_2-macroglobulin, antithrombin III, α_1-antitrypsin and C1-inhibitor. Among these inhibitors, α_2-antiplasmin, a fast-acting plasmin inhibitor, is believed to be the most important *in vivo* (Levi *et al.*, 1993). α_2-antiplasmin consists of a single-chain glycoprotein with a M_r of 70kDa and is found in the circulation at a concentration of ~1μM (Wiman & Collen, 1977). Complex formation between plasmin and α_2-antiplasmin in a 1:1 ratio, occurs on the light chain of both protease and inhibitor and is strongly dependent on the availability of free lysine-binding sites and a free active site in the plasmin molecule (Collen & Wiman, 1978; Lijnen & Collen, 1982). The lysine

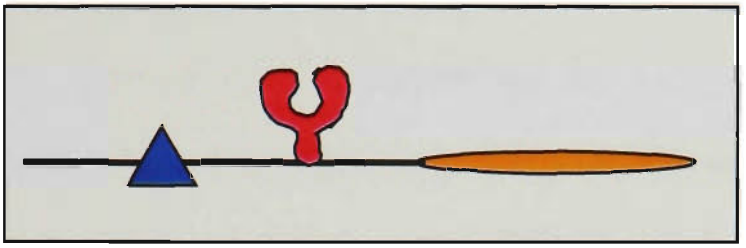
binding sites are also involved in the binding of plasminogen and plasmin to fibrin (Collen and Wiman 1978). ECM and cell surface bound plasmin has been found to be protected from inhibition by α_2-antiplasmin (Ellis *et al.*, 1991; Hall *et al.*, 1991).

1.3.2 Plasminogen Activators (PAs)

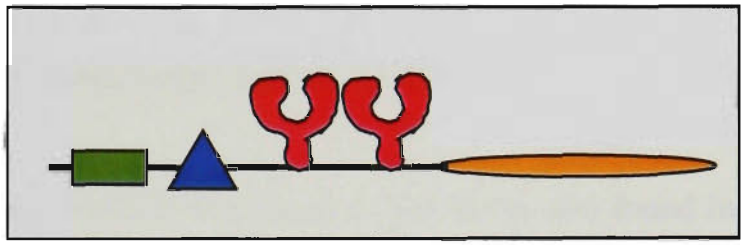
The PAs, u-PA and t-PA catalytically convert the inactive plasma zymogen, plasminogen, into the active proteinase, plasmin (Koelbl *et al.,* 1989; Matrisian & Hogan, 1990; Zini *et al.,* 1992). Human tissue-cultured trophoblast, macrophages and endothelial cells also have been found to synthesise PAs (Sharrow *et al.,* 1989). The structure of active t-PA and u-PA are similar, although they are the product of two different genes (Steffens *et al.,* 1982; Pennica *et al.,* 1983; Heyneker *et al.,* 1983). Both PAs consists of two polypeptide chains, the A and B chain, joined together by a disulphide bond. The A chain, also known as the light chain, forms the amino-terminal end. The A chain of u-PA contains only one kringle region, while t-PA has two kringle regions. Both PAs also have a growth factor domain (GFD) in the A chain. The B chain, also referred to as the heavy chain, forms the carboxy-terminal end and contains the catalytic active site of the PAs (Figure 3, Andreasen *et al.,* 1994). The main differences between t-PA and u-PA is differences in their physiological roles (Duffy, 1993). t-PA is found in the circulation where it is primarily involved in clot lysis. Meanwhile u-PA is located in the extracellular space, where it mediates tissue-remodelling in processes such as cancer metastasis, implantation and wound healing

Figure 3. Modified schematic representation of uPA and tPA structure and the binding of uPA to it's specific receptor, uPAR (Andreasen *et al.,* 1994). **A.** uPA has an EGF domain and one kringle at the N-terminal. **B.** tPA contains the fibronectin-type III domain, an EGF domain and two kringles at the N-terminal. **C.** uPA binds to uPAR via the uPAR binding site found at the N-terminus. The uPA binding site of uPAR is also located on the N-terminus.

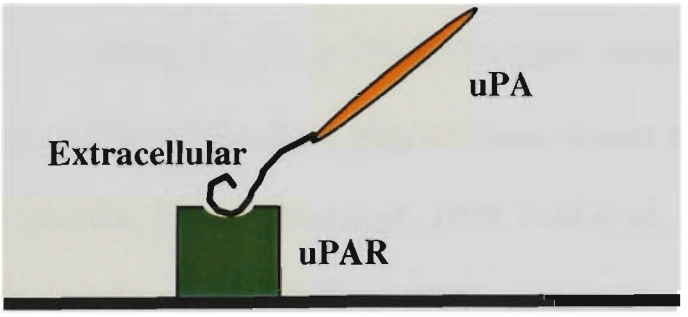
A. urokinase-type plasminogen activator



B. tissue-type plasminogen activator

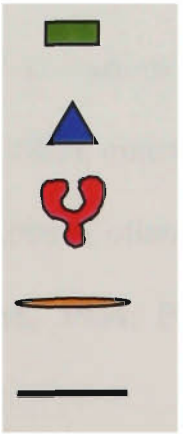


C.



plasma membrane

Intracellular



fibronectin-type III domain

epidermal growth factor domain

kringle domain

C-terminal/B-chain

N-terminal/A-chain

(Mignatti & Rifkin, 1993). Regulation of plasminogen activator activity is a complex process and can occur at different levels. These include the cellular production and release of activators, conversion of the activators from inactive to active state, the stimulation of enzyme activities by extracellular factors or other enzymes and the inhibition of activity by specific inhibitors. u-PA, t-PA (to a lesser extent), and the role of u-PA in cancer metastasis is reviewed in the following sections.

1.3.2.1 Tissue-type plasminogen activator (t-PA)

t-PA, predominantly found in the blood circulation is also found in the pericellular environment. t-PA is produced by endothelial cells of the vascular wall (Levin, 1983; Shimonaka *et al.,* 1984), as well as other cells types including smooth muscle cells and fibroblasts. t-PA has also been purified from various sources including, human uterine tissue (Soszka, 1977; Rijken *et al.,* 1979; Pohl *et al.,* 1984), kidney (Sueishi *et al.,* 1982), plasma (Thorsen & Philips, 1984), organ cultures of human ureter cells (Ljungnaer *et al.,* 1984), as well as porcine heart tissue (Wallaen *et al.,* 1982), 2-day-old rat epidermis (Nakagawa *et al.,* 1989) and HeLa cells (Ossowski, 1988). t-PA has also been observed in various cancers such as prostate (Strickland *et al.,* 1983; Kirchheimer *et al.,* 1984), colon carcinoma (Tran-Thang *et al.,* 1994) and melanoma (Rijken & Collen, 1981; Collen *et al.,* 1982; Wallaen *et al.,* 1983; Andreasen *et al.,* 1984; Heussen *et al.,* 1984; Pohl *et al.,* 1984). However, t-PA has been found

predominantly associated with melanomas more than any other cancer types (Rijken & Collen, 1981; Wagner & Binder, 1986; Bizik *et al.,* 1990; Tran-Thang *et al.,* 1994).

t-PA is secreted as a single-chain pro-enzyme (sc-tPA) which is proteolytically cleaved at the C-terminal side of Arg275 to generate the active two-chain form (tc-tPA), with the subunits held together by a single disulfide bond (Andreasen *et al.,* 1984, Boose *et al.,* 1989). The molecular weight of the purified t-PA analysed by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) has been found to range from 60kDa up to >80kDa (Rijken *et al.,* 1979; Thorsen & Philips, 1984; Heussen *et al.,* 1984; Shimonaka *et al.,* 1984; Andreasen *et al.,* 1984). Analysis of t-PA purified from human melanoma cell-conditioned tissue culture medium gave rise to predominantly a 72kDa doublet with lesser amounts of 68kDa and 115kDa proteins (Heussen *et al.,* 1984). Human umbilical vein endothelial cells (HUVEC) released t-PA with a molecular weight of 60kDa in cultured media (Levin, 1983). An 80kDa t-PA molecule was purified from cultured rat prostate adenocarcinoma cells (Strickland *et al.,* 1983).

t-PA has been purified from human uterine tissue as two chains with a molecular weight of 31kDa and 38kDa, connected by disulphide bridges. The 31kDa chain was found to contain the serine residue at the active site (Rijken *et al.,* 1979). In contrast t-PA purified from human melanoma cells consisted predominantly of a single polypeptide chain form, with lower amounts of the two polypeptide chains form held

together by one or more disulphide bridges (Andreasen *et al.,* 1984). Only the two-chain form of t-PA purified from human melanoma cells was found to have activity (Andreasen *et al.,* 1984). A latent form of t-PA associating with inhibitor secreted from endothelial cells was found to be inactive and did not bind to fibrin clots (Levin, 1983). Similar results were obtained by Thorsen & Philips (1984), where t-PA purified from human plasma complexed with the inhibitor, was also enzymatically inactive. Analysis by SDS-PAGE under both reducing and non-reducing conditions of sc-tPA purified from culture medium of a cell line established from human uterine muscle revealed a single band with a molecular weight of 70kDa (Matsuo *et al.,* 1989). Electrophoretic enzymography demonstrated a single lytic zone at M_r of 70kDa indicating that, to a lesser extent, sc-tPA was active (Matsuo *et al.,* 1989). However the 70kDa species was predominantly without activity (Matsuo *et al.,* 1989).

In contrast to the above findings other researchers have found the zymogen form of t-PA to contain activity (Ranby *et al.,* 1982; Tate *et al.,* 1987; Boose *et al.,* 1989). Single chain t-PA was found to contain the same amount of activity as the two chain form of t-PA study under physiological conditions (Tate *et al.,* 1987). It was also observed that single chain t-PA bound fibrin significantly more than two chain t-PA (Tate *et al.,* 1987). by Boose *et al.,* (1989) showed that wild-type single chain t-PA and mutant form of t-PA, which could not be cleaved into two-chain form, had similar Vmax and Km values. For further discussions on the activity of single chain t-PA refer to review by Stubbs *et al.,* (1998).

The enzymatic activity of t-PA is largely dependent on its binding to fibrin (Zamarron *et al.,* 1984; Collen, 1988b; Fischer, 1992) with which it has high binding affinity. The t-PA/fibrin binding is mediated by the second kringle domain and fibrin binding finger domain on t-PA (van Zonneveld *et al.*, 1986a; van Zonneveld *et al.*, 1986b; Verheijen *et al.*, 1986). This affinity of t-PA for fibrin makes it a major candidate for uses as a thrombolytic agent. t-PA is available as a biotechnologically manufactured recombinant protein for therapeutic use in the treatment of thrombolytic diseases (Kessler *et al.,* 1988; Collen *et al.,* 1989; Krause & Tanswell, 1989). In another study by Machovich and Owen (1997), it was observed that denatured proteins expressed similar cofactor activity to that of fibrin. Denatured proteins such as, apoferritin, antithrombin, alpha1-protease inhibitor, alpha2-macroglobulin, and albumin were shown to increase plasminogen activation by tPA with similar rate as that of fibrin fragments (Machovich and Owen, 1997). More recently, Lawrence *et al.,* (2000) observed a 32-fold increase in plasminogen activation when t-PA was bound to the G_1 subdomain of the laminin-5α_3 subunit. For reviews on t-PA refer to the following (van Zonneveld *et al.,* 1986c; Rijken, 1988; Rijken, 1995; Ghosh and Stack, 2000).

1.3.2.2 Urokinase-type plasminogen activator

1.3.2.2.1 Synthesis

u-PA is the other serine proteinase responsible for the conversion of plasminogen to plasmin (Robbins *et al.,* 1967; Saksela, 1985; Pöllänen *et al.,* 1991; Ellis *et al.,* 1991). u-PA, originally identified in human kidney (Sueishi *et al.,* 1982) has also been found in human blood plasma (Tissot *et al.,* 1982), human serum (Wun *et al.,* 1982), human colon tissue (Grøndahl-Hansen *et al.,* 1991), human lung tissue (Nagayama *et al.,* 1994) and 2-day-old rat epidermis (Nakagawa *et al.,* 1989). It has been found associated with a wide range of cancers including, breast (Janicke *et al.,* 1994), gastric (Maeda *et al.,* 1996), colon (Pyke *et al.,* 1991; de Bruin *et al.,* 1989; Grøndahl-Hansen *et al.,* 1991), prostate (Kirchheimer *et al.,* 1984), ovarian (Pujade-Lauraine *et al.,* 1993) and endometrial cancer (Gleeson *et al.,* 1992). SDS-PAGE analysis of u-PA secreted by subcultured normal human endothelial cells revealed a single protein band with M_r of 54kDa (Booyse *et al.,* 1984; Booyse *et al.,* 1988) which was similar to urinary urokinase. A protein purified from skin lesions with allergic vasculitis with a molecular weight of 110kDa was confirmed to be immunologically identical to human urokinase (Toki *et al.,* 1982). In contrast, u-PA purified from 2-day-old rat epidermis had a molecular weight of 44kDa (Nakagawa *et al.,* 1989).

u-PA has the ability to degrade the ECM protein fibronectin (Quigley, 1979; Gold *et al.,* 1989; Strickland & Richards, 1992). It also appears to have an important role in the

activation of pro-collagenase into the active collagenase, because it was observed that the presence of u-PA was required for local production of collagenase (O'Grady *et al.*, 1981; So *et al.*, 1992; Reith & Ruckling, 1992).

1.3.2.2.2 Pro-u-PA

Bernik (1973), was first to suggest that u-PA may exist in a pro-enzyme form and that it's activity was enhanced by treatment with trypsin. It has since been confirmed that u-PA exists primarily as a single-chain zymogen, pro-u-PA (or sc-uPA) (Eaton *et al.*, 1984; Ellis *et al.*, 1987; Stephens *et al.*, 1987; Booyse *et al.*, 1988; Lijnen *et al.*, 1990), transcribed from mRNA 2.5kb in length (Verde *et al.*, 1984; Duffy, 1993). The human u-PA gene is 6.4kb in length and located on chromosome 10 (Rajput *et al.*, 1985; Tripputi *et al.*, 1985). Sc-uPA has a molecular weight of 50kDa under both reducing and non-reducing SDS-PAGE conditions (Mangel *et al.*, 1991).

After secretion sc-uPA is thought to bind to its specific receptor, urokinase-type plasminogen activator receptor (u-PAR), on the plasma membrane where it is activated by proteolytic cleavage at Lys158-Ile159 giving rise to active two-chain u-PA (tc-u-PA) (Vassalli *et al.*, 1991; Duffy, 1993; Mignatti & Rifkin, 1993). Proteases that can activate u-PA include: plasmin (Wun *et al.*, 1982; Blasi *et al.*, 1987), trypsin, plasma kallikrein (Eaton *et al.*, 1984; Ichinose *et al.*, 1986), cathespin B, cathepsin L (Schmitt *et al.*, 1992), cathespin G (Drag and Petersen, 1994) bacterial thermolysin (Marcotte

and Henkin, 1993) and tryptase (Stack and Johnson, 1994). Unlike sc-tPA, sc-uPA has little to no enzymatic activity (Kasai *et al.,* 1985; Booyse *et al.,* 1988). However, while some have found that sc-uPA is inactive, others have shown that sc-uPA is capable of activating plasminogen, albeit not as efficiently as tc-uPA (Skriver *et al.,* 1982; Eaton *et al.,* 1984; Ellis *et al.,* 1987; Ellis *et al.,* 1989). It has also been observed that the affinity of sc-uPA for fibrin in clots was greater than that of tc-uPA (Kasai *et al.,* 1985; Booyse *et al.,* 1988).

As stated above plasmin is one of the enzymes responsible for the activation of sc-uPA to tc-uPA. Treatment of sc-uPA secreted by cultured human kidney cells with catalytic amounts of plasmin (0.4μg/ml) resulted in approximately a 300-fold increase in u-PA activity compared to the untreated control (Kasai *et al.,* 1985). CD spectra analysis indicated that cleavage of pro-u-PA by plasmin resulted in a conformational change in the pro-enzyme giving rise to a new structure in the active tc-uPA (Kasai *et al.,* 1985). Tc-uPA can be further cleaved by plasmin giving rise to a 30-33kDa form of tc-uPA (often referred to as low-molecular weight u-PA). The low-molecular weight form of tc-uPA contains the complete B chain but lacks most of the A chain including the receptor-binding domain. Therefore, this low molecular weight form of tc-uPA cannot associate with u-PAR (Danø *et al.*, 1985; Sim *et al.*, 1986; Pöllänen *et al.*, 1991). The catalytic active site of tc-uPA is located on the B chain and is formed by the amino acids His204, Asp255 and Ser356 (Figure 3, Duffy, 1993). For reviews refer to the following (Blasi *et al.,* 1987; Kruithof, 1988).

1.3.2.2.3 Regulation of u-PA

There are a variety of factors known to modulate the expression of u-PA. These include growth factors, cytokines, hormones, oncogenes, chemical carcinogens and tumour promoters (Matrisian & Hogan, 1990; Miller *et al.,* 1991). Factors that regulate the expression of cell-surface u-PAR will also subsequently govern the proteolytic activity of u-PA at the cell surface (for review see Besser *et al.,* 1996). The effects of some of these factors seem to vary with cell type and cellular conditions (Tran-Thang *et al.,* 1996). The extracellular activity of u-PA is also finely regulated by the plasminogen activator inhibitors (Figure 2).

1.3.2.2.3.1 Growth Factors

The various growth factors responsible for regulation of u-PA expression include vascular endothelial growth factor (VEGF), epidermal growth factor (EGF), transforming growth factors α and β1 (TGF-α, TGF-β1), (Boyd, 1989; Long & Rose, 1996; Li *et al.,* 1997; Albo *et al.,* 1997; Graham, 1997; Mandriota & Pepper, 1997). The addition of EGF into the media resulted in a 50% reduction of u-PA secreted by a serum-free adapted colon cancer cell line, CBS_{sf}, as measured via enzyme linked immunosorbent assay (ELISA) (Boyd, 1989). In contrast, EGF was found to increase u-PA secretion from both MDA-MB-231 human breast cancer cells and a transfectant (S30) stably expressing estrogen receptor (ER), as determined by ELISA and zymography (Long & Rose, 1996). This increase in u-PA protein levels correlated

with increased expression of u-PA mRNA (Long & Rose, 1996). The addition of EGF to primary cultures of human endometrial cells stimulated the release of u-PA (Miyauchi *et al.,* 1995). Both EGF and FBS treatment of the human prostate cancer cell line, PC-3, resulted in a dose-dependent increase in u-PA expression (Liu & Rabbani, 1995).

u-PA concentrations in conditioned-medium of primary cultures of endometrial stromal cells were increased by TGF-β1 (Casslen *et al.,* 1998a). Upregulation of u-PA and t-PA expression by cultured bovine endothelial cells by VEGF is dependent upon endogenous basic fibroblast growth factor (bFGF) (Mandriota & Pepper, 1997). TGF-α was found to upregulate the u-PA gene at both the transcriptional and translational levels, resulting in increased mRNA and protein levels, with subsequent increase in u-PA activity in hen granulosa cells (Li *et al.,* 1997). Treatment of human synovial fibroblast cultures with TGF-β resulted in stimulation of both u-PA activity and DNA synthesis (Hamilton *et al.,* 1991). Treatment of cultured human cytotrophoblasts with TGF-β1 resulted in a 65% reduction in u-PA activity in conditioned media compared to untreated cells (Graham, 1997). The reduction of u-PA activity observed with TGF-β1 treatment was due to reduced secretion of u-PA and increased PAI-1 production and secretion. Therefore TGF-β may have an indirect role in the regulation of uteroplacental blood flow by down-regulating trophoblast-derived PA activity (Graham, 1997).

Thrombospondin-1 (TSP-1), through activation of TGF-β1, was observed to up-regulate u-PA and u-PAR expression in MDA-MB-231 human breast cancer cells (Albo *et al.*, 1997). In ASPC1 human pancreatic adenocarcinoma cells, TSP-1 and TGF-β1 induced only a slight increase on total u-PA. However, a two-fold increase on u-PAR expression was observed with TSP-1 and TGF-β1 treatment compared to untreated cells (Albo *et al.*, 1998).

1.3.2.2.3.2 Cytokines

The expression of u-PA is also known to be regulated by various cytokines including interleukin 1 and 2 (IL-1 & IL-2, Gyetko *et al.*, 1993). Treatment of human monocytes with both IL-1 and IL-2 increased steady-state u-PA mRNA. IL-1 appears to increase mRNA for u-PA to a greater extent than does IL-2 (Gyetko *et al.*, 1993). Recombinant IL-1α, recombinant IL-1β and lipopolysaccharide (LPS) increased levels of both u-PA mRNA and protein in RC-K8 human pre-B lymphoma cells (Niiya *et al.*, 1995). The macrophage colony stimulating factor (M-CSF) increased the number of endogenously bound u-PA on A549 and Calu-1 cells leading to a subsequent increase in the invasive potential of these cell lines (Bruckner *et al.*, 1992).

1.3.2.2.3.3 Hormones

The hormones known to regulate u-PA expression include; prostaglandins, hydrocortisone and dexamethasone (Miller *et al.,* 1991; Bator *et al.,* 1998; Liu & Rabbani, 1995). Treatment of primary cultures of endometrial stromal cells with a combination of estradiol and progesterone resulted in a decrease in secreted u-PA levels in conditioned-medium (Casslen *et al.,* 1998a). Meanwhile, the addition of prostaglandin E_2 (PGE_2) to primary cultures of rat neonatal osteoblast-like cells was found to enhance both the mRNA and activities of u-PA (Allan & Martin, 1995). The steroid hormone hydrocortisone significantly reduced u-PA activity in conditioned medium of primary cultures of murine keratinocytes (Bator *et al.,* 1998). The glucocorticoid dexamethasone, was observed to down regulate u-PA mRNA expression in human prostate cancer cells (Koutsilieris *et al.,* 1997). Treatment of the human prostate cancer cell line, PC-3, with dexamethasone resulted in a dose-dependent decrease in u-PA expression (Liu & Rabbani, 1995). Treatment of HT-1080 cells with dexamethasone resulted in a significant reduction in u-PA gene expression at a transcriptional level.

1.3.2.2.3.4 Others

Incubation of cultured HUVECs at 37°C with low ethanol (0.1% v/v) resulted in approximately 6- to 7-fold increases in the transcription of new u-PA mRNAs (Grenett *et al.,* 1998). In contrast, treatment of murine mammary adenocarcinoma

cells with n-butanol resulted in approximately a 50% reduction in u-PA secreted into the culture medium compared to untreated cells (Aguirre Ghiso *et al.*, 1997). Miralles *et al.*, (1998) found that endogenous murine u-PA gene product was transcriptionally upregulated by ultra-violet (UV) radiation in NIH 3T3 fibroblast and F9 teratocarcinoma cells. This induction required an activator protein 1 (AP1) enhancer element. Peptides derived from the α1 chain of laminin stimulated u-PA expression in a dose- and time-dependent manner by monocytes/macrophages. The increase in activity was preceded by an increase in levels of u-PA mRNA (Khan & Falcone, 1997). Trans retinoic acid treatment of the human prostate cancer cell line, PC-3 resulted in a dose-dependent increase in u-PA mRNA and protein levels (Liu & Rabbani, 1995). Treatment of murine mammary adenocarcinoma cells with the phorbol myristate acetate (PMA) or EGF, strongly increased u-PA production (Aguirre Ghiso *et al.*, 1997). Meanwhile, co-treatments with n-butanol and PMA resulted in slight inhibition of u-PA production by murine mammary adenocarcinoma cells. These results suggests that u-PA production is regulated by phospholipase D (PLD) and protein kinase C (PKC) signal transduction pathways in murine mammary adenocarcinoma cells (Aguirre Ghiso *et al.*, 1997). The expression of PU.1, a member of the Ets family of transcription factors, can stimulate u-PA gene expression at the post-transcriptional level (Kondoh *et al.*, 1998).

1.3.2.2.4 Urokinase-type Plasminogen Activator Receptor (u-PAR)

u-PAR, a single chain glycoprotein, was first found on human blood monocytes and on the monocyte-like cell line U937 (Vassalli *et al.,* 1985). The molecular weight of u-PAR has been found to be around 55-60kDa (Vassalli *et al.,* 1985; Nielsen *et al.,* 1988 Estreicher *et al.,* 1989). The human u-PAR gene is located on chromosome 19 (Vagnarelli *et al.*, 1992) and consists of 3 repeated domains with approximately 90 amino acids each (Duffy, 1993). u-PAR is attached to the cell membrane via a glycosylphosphatidylinositol (GPI) anchor (Ploug *et al.,* 1991). Refer to Figure 3 for a simple schematic diagram of u-PAR/u-PA on the cell surface. The binding of u-PA to u-PAR is of high-affinity (dissociation constant (K_d) 0.1-1nM) and does not require the catalytic site of u-PA (Vassalli *et al.*, 1985; Vittoria *et al.*, 1986). The region of u-PA that binds to u-PAR is located between residues 13-30 of human u-PA in the GFD (the N-terminal domain) (Appella *et al.,* 1987). The binding region of u-PAR to u-PA is also found in the N-terminal domain of u-PAR. u-PAR has been identified on the surface of other cell types, including a variety of cells of neoplastic origin (Vassalli *et al.*, 1991; Hollas *et al.*, 1991; Glass *et al.*, 1993). For reviews refer to the following (Blasi *et al.,* 1990; Blasi, 1996).

1.3.2.2.5 u-PA and cancer

Accumulated evidence exists implicating the involvement of u-PA in tumour invasion and metastasis (Andreasen *et al.,* 1997). These evidence includes; inhibition of

cancer spread after abolition of u-PA activity with u-PA antibodies, PAI-1 or PAI-2 (Evans & Lin, 1995); the metastatic phenotype was conferred or enhanced in a range of different cell types transfected with the u-PA gene (Achbarou *et al.,* 1994); and a correlation between shortened disease-free interval as well as survival in patients with different cancers and high levels of u-PA (Evers *et al.,* 1982; Duffy *et al.,* 1988; Duffy *et al.,* 1990; Jänicke *et al.,* 1991; Reilly *et al.,* 1991; Spyratos *et al.,* 1992; Foekens *et al.,* 1992; Grøndahl-Hansen *et al.,* 1993; Maeda *et al.,* 1996; Cantero *et al.,* 1997). Human cancers including breast (Reilly *et al.,* 1991; Sumiyoshi *et al.,* 1992; Del Vecchio *et al.,* 1993), lung (Skriver *et al.,* 1994), colonic (Corasanti *et al.,* 1980; Miseljic *et al.,* 1995), gastric (Cho *et al.,* 1997), pancreatic (Cantero *et al.,* 1997) and urinary (Hasui *et al.,* 1992) have been shown to have increased levels of u-PA. In human breast cancer the ability of cancer cells to generate plasmin, and thus the invasive properties of cancer cells were dependent on the amount of u-PA expression (Holst-Hansen *et al.,* 1996). Harvey *et al.,* (1997) observed that various human cancer cell lines including lung, ovarian, and epidermoid, as well as human embryonic lung cell lines secreted u-PA in the form of large molecular weight multiprotein oligomeric complexes. Immunocytochemistry analysis of two human colon carcinoma cell lines and a human melanoma cell line found intermediate u-PA immunoreactivity compared to normal human skin fibroblasts which had a weak expression (Buø *et al.,* 1994).

An excellent correlation exists between elevated levels of u-PA and higher incidences of breast cancer relapse, shorter overall survival and increased degree of invasion (Bouchet *et al.*, 1994). In lung cancer tissue, u-PA levels were significantly higher than those in normal tissue (Nagayama *et al.*, 1994). Gleeson *et al.*, (1992) found that the level of u-PA in malignant endometrium was greatly increased compared to the normal endometrium. Significantly higher levels of u-PA were found in tumour tissue from both colorectal carcinoma and adenocarcinoma of the stomach, compared to the normal tissues (de Bruin *et al.*, 1989). Examination of tissue extracts from human epithelial ovarian tumours revealed more u-PA antigen in malignant compared to benign tumours, and more u-PA antigen in stage III cancers compared to stage I and II (Pujade-Lauraine *et al.*, 1993). Hewin *et al.*, (1996) observed higher levels of u-PA in tumour samples in both the adenocarcinoma and squamous cell carcinoma compared to the normal mucosa. Enhanced secretion of u-PA was observed in plasma and urine of patients with carcinomas of the pancreas, colorectum and stomach (Markus *et al.*, 1983; Kirchheimer *et al.*, 1987; Nishino *et al.*, 1988).

The increase in u-PA may be a result of malignant cells utilizing the activator to initialize the proteolytic cascade, which culminates in the breakdown of ECM components and facilitates the spread of cancer cells. In the Lewis lung tumour model the highest level of u-PA was found associated with areas of invasive growth (Skriver *et al.*, 1984). A positive correlation was found to exist between u-PA content in human colon cancers and their stage of invasiveness (Mulcany *et al.*, 1994).

Postive staining for u-PA was greater in patients with liver metastases compared to those without (Maeda *et al.*, 1996). Significantly higher levels of u-PA were found in tumours with axillary lymph-node involvement than those without lymph-node involvement in breast cancer (Sumiyoshi *et al.*, 1992).

1.3.2.2.6 Localisation of u-PA in cancerous tissues and cells

Conflicting results have been found by different groups as to the biological location of u-PA. u-PA was mostly confined to malignant cells in human melanomas (Markus *et al.*, 1984) and colonic cancers (Markus *et al.*, 1983). In Lewis lung cancers the presence of u-PA was demonstrated in the cytoplasm of tumour cells and in the extracellular spaces (Skiver *et al.*, 1984). Grøndahl-Hansen *et al.*, (1991) showed with three different monoclonal antibodies and a polyclonal antibody that the majority of u-PA was located in fibroblast-like cells and endothelial cells in the tumour stroma. Meanwhile, no staining was detected in the malignant cancer cells. Sappino *et al.*, (1991) found mRNA for u-PA in neoplastic cells of squamous cancer of the skin. In contrast to this, using *in situ* hybridisation Pyke *et al.*, (1991) found that mRNA for u-PA was only present in fibroblast-like cells in human colonic cancers. No staining for either u-PA or PAI-2 was obtained in normal gastric mucosa, while both antigens were predominantly found in the cytoplasm or the membrane of carcinoma cells (Maeda *et al.*, 1996). u-PA has also been observed associated with renal tumour cells near focal areas, and u-PA was induced in the tumoural capillary bed and tumour

associated macrophages (Xu *et al.*, 1997). These differences in location of u-PA protein and mRNA may be reflective of differences in the specificity of the antibodies used, or perhaps differences in different types of malignant cancers. For reviews refer to the following (Duffy, 1993; Andreasen *et al.*, 1997).

1.3.2.2.7 The interaction of u-PA with u-PAR in cancer metastasis

The existence of u-PAR is of major importance to the generation of plasmin activity at the cell surface (Stoppelli *et al.*, 1986; Ellis and Danø 1991; Pöllänen *et al.*, 1991; Vassalli *et al.*, 1992). The interaction of u-PA with u-PAR at the cell surface on several human cancer cells may serve several purposes. These include focalisation of enzymatic activities at the cell surface, greater activation of pro-u-PA, accelerating plasminogen activation and enhancing u-PA-mediated invasion (Estreicher *et al.*, 1990; Pöllänen *et al.*, 1991; Mignatti & Rifkin, 1993). Ellis *et al.*, (1989) observed that activation of pro-u-PA was enhanced when bound to u-PAR. Also, the activation of plasminogen was greatly enhanced when u-PA was bound to u-PAR compared to u-PA in solution (Plow *et al.*, 1986; Ellis *et al.*, 1989). An increase in u-PAR levels was observed in both the adenocarcinoma and squamous cell carcinoma compared to the normal mucosa (Hewin *et al.*, 1996). Breast carcinomas contained 5 times more u-PAR located at the periphery of the tumour than benign breast lesions (Del Vecchio *et al.*, 1993). u-PAR was detected only in cancer cells at the periphery in esophageal squamous cell carcinoma (Shiomi *et al.*, 2000). Northern blot analysis revealed a 4-

fold increase in levels of u-PAR mRNA in pancreatic cancers compared with normal controls (Cantero *et al.,* 1997). u-PAR is capable of binding free or complexed u-PA. Pre-formed ^{125}I-u-PA/PAI-2 complexes were found to bind to THP-1 cells with slightly less efficiency than active u-PA (Estreicher *et al.,* 1990).

In the absence of u-PAR the production of u-PA did not lead to invasion of chorioallantoic membranes by tumour cells (Ossowski, 1988). In a separate experiment colonic cancer cells required the presence of u-PA bound to u-PAR for degradation of laminin (Schlechte *et al.,* 1989). Colon cancer cell lines that contained greater numbers of u-PAR invaded the extracellular matrix more than those cells lines with lower numbers of u-PAR (Hollas *et al.,* 1991). In patients with colorectal carcinoma higher levels of u-PAR was related to poor prognosis (Abe *et al.,* 1999). In certain lung cancer cell lines the binding of u-PA to u-PAR was also necessary for invasion (Bruckner *et al.,* 1992). Immunohistochemical staining for both u-PA and u-PAR were strongest in cancer lesions with signs of invasion (Cantero *et al.,* 1997). In the highly metastatic human melanoma cell line, M24met, the binding of u-PA to u-PAR facilitated invasion of cells through a reconstituted basement membrane (Stahl & Mueller, 1994). Patients with concomitant over-expression of u-PA and u-PAR had a shorter post-operative survival than patients in whom only u-PA or u-PAR or neither factors were over expressed (Cantero *et al.,* 1997). These and such evidence indicate that u-PA binding to u-PAR is necessary to initiate the process of cancer cell invasion.

1.3.3 Plasminogen Activator Inhibitors

Another level of regulation of plasmin generation is through the regulation of u-PA and t-PA activities by known physiological plasminogen activator inhibitors (PAIs) belonging to the serine protease inhibitor (SERPIN) super family. Currently there are five known plasminogen activator inhibitors. These are PAI-1, PAI-2, plasminogen activator inhibitor 3 (PAI-3), PN-1 (Kirchheimer & Remold, 1989) and neuroserpin (Hastings *et al.,* 1997). Both PAI-1 and PAI-2 are known to specifically inhibit the activities of u-PA and t-PA, while the other PAI-3 and PN-1 have broader protease inhibitor activities. PAI-3 has been reported to be identical to plasma protein C inhibitor (Heeb *et al.,* 1987). PN-1 has been reported to inhibit plasmin, thrombin and other serine proteases (Scott *et al.,* 1985). Neuroserpin found to be predominantly expressed in human brain and spinal cord is more effective with inhibiting the activity of t-PA compared to u-PA (Osterwalder *et al.,* 1996; Hastings *et al.,* 1997; Osterwalder *et al.,* 1998). The specific properties of each PAI are given in Table 1. For reviews refer to the following (Sprengers & Kluft, 1987; Saksela & Rifkin, 1988; Hart & Rehemtulla, 1988; Mikus *et al.,* 1993; Reilly *et al.,* 1994; Kruithof *et al.,* 1995; Dear & Medcalf, 1995). The following sections will focus on PAI-2.

	PAI-1	PAI-2	PAI-3	Protease nexin I	Neuroserpin
Synthesised by	endothelial cells, fibroblasts, vascular smooth muscle cells, hepatocytes, hepatoma cells, mammary cells, melanoma cells, fibrosarcoma cells, monocytes/macrophages & platelets, as well as in plasma (Loskutoff & Edgington, 1977; Andreasen *et al.*, 1990; Castellote *et al.*, 1990)	placenta, human peripheral blood monocytes, keratinocytes, U937, THP-1, K562, HT1080, (Kawano *et al.*, 1970; Chapman & Stone, 1985; Wohlwend *et al.*, 1987; Lyons-Giordano *et al.*, 1994; Kruithof *et al.*, 1986; Rehemtulla *et al.*, 1990)	Male reproductive tract, spermatazoa, human kidney tubular cells, human heart, human liver, human pancreas, mouse ovary, platelets, megakaryocytes, Hep G2 cells, rat seminal vesicles and testis (Morito *et al.*, 1985; Fair & Marlar, 1986; Radtke *et al.*, 1994; Zheng *et al.*, 1994; Geiger *et al.*, 1996; Zechmeister-Machhart *et al.*, 1996; Hayashi *et al.*, 1998; Nishioka *et al.*, 1998; Wakita *et al.*, 1998)	fibroblastic cells, fibrosarcoma cells, heart muscle cells, kidney epithelial cells, epidermal carcinoma cells, endothelial cells, glial cells & activated platelets (Baker 1980; Eaton & Baker, 1983; Guenther, 1985; Gronke, 1987, 1989)	Brain, spinal cord, ocular vitreous fluid of chicken embryos, pituitary gland, medullary cells in the adrenal gland, (Osterwalder *et al.*, 1996; Hastings *et al.*, 1997; Osterwalder *et al.*, 1998; Hill *et al.*, 2000)

Table I. Properties of PAI-1, PAI-2, PAI-3, PN-1 and Neuroserpin.

	PAI-1	PAI-2	PAI-3	Protease nexin I	Neuroserpin
M_r (Da)	46,000 - 54,000 (Erickson *et al.*, 1984; Andreasen *et al.*, 1986a)	47,000 (non-glycosylated) (Kruithof *et al.*, 1986b; Collen *et al.*, 1986) 60,000 (glycosylated) (Genton *et al.*, 1987; Medcalf *et al.*, 1988)	50,000 (Stump *et al.*, 1986c) 57,000 (Suzuki *et al.*, 1989)	38,000-52,000 (Baker, J. *et al.*, 1986; Gloor *et al.*, 1986; McGrogan *et al.*, 1988)	50,000-55,000 (Schrimpf *et al.*, 1997; Osterwalder *et al.*, 1998)
Amino acid residues	379 (Ny *et al.*, 1986; Pannekoek *et al.*, 1986; Ginsburg *et al.*, 1986; Andreasen *et al.*, 1986c)	415 (Webb *et al.*, 1987; Ye *et al.*, 1987; Antalis *et al.*, 1988)	405 (mouse) (Zechmeister-Machhart *et al.*, 1997) 387(human) (Meijers & Chung, 1991)	392 (Gloor *et al.*, 1986; McGrogan *et al.*, 1988)	410 (Schrimpf *et al.*, 1997)
mRNA	2.0-3.0kB (Ny *et al.*, 1986; Ginsburg *et al.*, 1986)	2.0kB (Antalis *et al.*, 1988)	2.2kB (Meijers & Chung, 1991)	1.6kB (Gloor *et al.*, 1986)	1.8kB (Hastings *et al.*, 1997)
Chromosomal localization	chromosome 7 (Loskutoff *et al.*, 1987; Klinger *et al.*, 1987)	chromosome 18 (Webb *et al.*, 1987)	chromosome 14 (Meijers & Chung, 1991)	chromosome 2 (Carter *et al.*, 1995)	Chromosome 3 (Schrimpf *et al.*, 1997)
Principle target enzyme	sctPA, tctPA and tcuPA (Erickson *et al.*, 1984; Colucci *et al.*, 1986)	tcuPA (Åstedt *et al.*, 1985; Kruithof *et al.*, 1986)	activated protein C (Heeb *et al.*, 1987; Stief *et al.*, 1987), scuPA, tcuPA (Geiger *et al.*, 1989; Schwartz, B. & Espana, 1999)	thrombin (Wagner *et al.*, 1989)	t-PA (Hastings *et al.*, 1997; Osterwalder *et al.*, 1998)

1.3.3.1 PAI-2

As stated previously the two main inhibitors of PAs are PAI-1 and PAI-2. PAI-1 is of endothelial origin (Loskutoff & Edgington, 1977). PAI-2 was originally isolated from the placenta and cell lines of monocyte/macrophage lineages (Kawano *et al.*, 1970; Åstedt *et al.*, 1985; Stephens *et al.*, 1985) and found to be almost identical at the amino acid sequence level from both sources (Webb *et al.*, 1987, Ye *et al.*, 1989). The human PAI-2 gene is located on the long arm of chromosome 18 and consists of 8 exons (Webb *et al.*, 1987). PAI-2 exists in two isoforms. These are, a predominantly intracellular non-glycosylated form of about 43-47kDa and an extracellular glycosylated form of about 60kDa (Kruithof *et al.*, 1986; Åstedt *et al.*, 1987; Genton *et al.*, 1987; Belin *et al.*, 1989, Vassalli *et al.*, 1992). A single 2kb mRNA encodes both the secreted and intracellular forms of PAI-2 (Antalis *et al.*, 1988). The PAI-2 mRNA produces a 415 amino acid peptide with 3 potential N-glycosylation sites and a reactive center located at Arg380 and Thr381 (Kiso *et al.*, 1988).

1.3.3.1.1 Biokinetics

PAI-2 is an efficient inhibitor of active tc-uPA and tc-tPA with rate constants of $10^6 M^{-1}s^{-1}$ and $2x10^5 M^{-1}s^{-1}$, respectively (Christensen *et al.*, 1982; Åstedt *et al.*, 1985; Kruithof *et al.*, 1986). Since fibrin-bound t-PA is protected from inhibition by PAI-2 (Leung *et al.*, 1987) and is ten thousand times slower than PAI-1 inhibiting sc-tPA

(Åstedt *et al.,* 1985; Kruithof *et al.,* 1986), PAI-2 is probably less likely to be involved in the physiological regulation of t-PA. Therefore PAI-2 is thought to be the main inhibitor of u-PA activity since the association rate between PAI-2 and tc-uPA is 5 times greater than that of PAI-2 and tc-tPA (Thorsen *et al.,* 1988; Noguchi-Takino *et al.,* 1996). PAI-2 inhibits tc-uPA (33kDa and 55kDa) in solution by forming slowly dissociable and SDS-stable complexes with u-PA in a 1:1 ratio by binding to the u-PA active site (Åstedt *et al.,* 1985; Stephens *et al.,* 1985; Kruithof *et al.,* 1986). Both PAI-1 and PAI-2 have been shown to inhibit the activity of u-PA bound to u-PAR. However the association rate constants for both inhibitors binding to receptor bound u-PA was approximately 40% lower than for u-PA in solution (Ellis *et al.,* 1990). The most important distinction between the two inhibitors relates to the specificity of PAI-2 for tc-uPA, while PAI-1 has a broader inhibitory capacity which rapidly inhibits both forms of t-PA as well as tc-uPA (Pöllänen *et al.*, 1991).

1.3.3.1.2 Localisation

Since it was first isolated from placental tissue, PAI-2 has also been found in other tissues and cell types including the monocyte/macrophage (Chapman & Stone, 1985; Wohlwend *et al.,* 1987), keratinocyte (Hibino *et al.,* 1986; Hashimoto *et al.,* 1989; Lyons-Giordano *et al.,* 1994; Reinartz *et al.,* 1996) and syncytiotrophoblast (Hofmann *et al.,* 1994). It has also been found in cancer cell lines such as U937 (Kruithoff *et al.,* 1986), human glioblastoma U138 (Murphy *et al.,* 1993) and K562 (Rehemtulla *et al.,*

1990). PAI-2 has also been identified in breast cancer tissue (Umeda *et al.*, 1997). A study by Jensen *et al.*, (1989) demonstrated that PAI-2 may be found on or near the surface of trophoblasts. Using ELISA PAI-2 was detected intracellularly in cultured normal human epidermal keratinocytes, the human keratinocyte cell line (HaCaT), and a Ha-ras transfected HaCaT variant (HaRas) (Reinartz *et al.*, 1996). However, only the normal human epidermal keratinocytes and the HaCaT were found to secrete PAI-2 into the cultured medium. Flow cytometric analysis showed that HaCaT cells expressed high amounts of PAI-2 while HaRas cells expressed only low levels. Buø *et al.*, (1994) determined that u-PA, t-PA, u-PAR and PAI-1 were on the cell surface of a human melanoma cell line, while PAI-2 was believed to be intracellular.

1.3.3.1.3 Regulation

Like u-PA, the expression of PAI-2 is regulated by various agents including growth factors, cytokines and hormones. The effects of the regulating agent can also vary according to the cell that expresses PAI-2.

1.3.3.1.3.1 Growth factors

TGF-β did not increase synthesis of PAI-2 levels in human synovial fibroblasts, instead it inhibited the IL-1-mediated stimulation of PAI-2 expression by acting at the mRNA level (Hamilton *et al.*, 1993). However, PAI-2 expression was increased by

EGF I in A431 epidermoid carcinoma (George *et al.*, 1990) and human granulosa luteal cells (Piquette *et al.*, 1993).

1.3.3.1.3.2 Cytokines

IL-1 was found to enhance PAI-2 mRNA and protein levels in human and fetal synovial fibroblasts (Michel & Quertermous, 1989; Hamilton *et al.*, 1992; Hamilton *et al.*, 1993). Both IL-1 and IL-2 significantly up-regulated PAI-2 activity in human monocyte-conditioned medium in a dose-dependent manner. This up-regulation of activity was accompanied by a significant increase in PAI-2 mRNA levels (Gyetko *et al.*, 1993). It was concluded that both IL-1 and IL-2 modulate monocyte proteolytic activity by increasing expression of u-PA and PAI-2. However, up-regulation of PAI-2 expression was greater than that of u-PA expression (Gyetko *et al.*, 1993). IL-1 was also found to increase the expression of PAI-2 in human bone marrow stromal cells (Hannocks *et al.*, 1992), human endothelial cells (Zoellner *et al.*, 1993) and granulosa luteal cells (Piquette *et al.*, 1994). An increase in the secretion of PAI-2 from monocytes/macrophages was observed with treatment with macrophage colony stimulating factor (M-CSF) and granulocyte-macrophage colony stimulating factor (GM-CSF) (Wohlwend *et al.*, 1987; Hamilton *et al.*, 1993).

1.3.3.1.3.3 Hormones

Dexamethasone caused a decrease in PAI-2 mRNA levels in the human fibrosarcoma cell line HT1080 while increasing transcription of PAI-1 (Medcalf *et al.,* 1988). A similar decrease in PAI-2 antigen levels was observed when macrophages were treated with dexamethasone (Wohlwend *et al.,* 1987). In contrast, dexamethasone increased PAI-2 production by the human promyelocytic leukemia cells, PL-21 (Niiya *et al.,* 1991).

1.3.3.1.3.4 Others

Both LPS and PMA increase PAI-2 mRNA levels in cultures of differentiating U937 cells (Schleuning *et al.,* 1987; Genton *et al.,* 1987). Thrombin stimulated production of both intracellular and secreted PAI-2 by monocytes from human peripheral blood (Ritchie *et al.,* 1995). Both LPS and thrombin treatment of the monocytes resulted in similar increases in PAI-2 levels, with a 6-fold and 5-fold increase in secreted and intracellular PAI-2, respectively. This increase in PAI-2 was also observed at the mRNA level, where treatment with either thrombin or LPS resulted in a 3-fold increase in PAI-2 mRNA in monocytes (Ritchie *et al.,* 1995). Stimulation of U937 cells with thrombin also resulted in significant increases in both secreted and intracellular PAI-2 levels compared to untreated cells (Ritchie *et al.,* 1995). Levels of intracellular PAI-2 was much higher than levels of secreted PAI-2 in LPS- and thrombin-stimulated monocytes from human peripheral blood (Ritchie *et al.,* 1995).

This was also true for U937 cells (Ritchie *et al.,* 1995). LPS also stimulates PAI-2 expression in human mesothelial (Van Hinsbergh *et al.,* 1990) and endothelial cells (Webb *et al.,* 1987). PMA was also found to induce PAI-2 expression in various myelo-monocytic cell lines such as HL-60, K562 and PL-21 (Alving *et al*, 1988; Arndt *et al*, 1989; Oliver *et al*, 1989; Antalis & Dickinson 1992; Dickinson *et al*, 1993; Niiya *et al*, 1994).

A 24h incubation with Linomide resulted in a dose-dependent increase in PAI-2 antigen production by human peripheral blood monocytes (Billström *et al.,* 1996). Linomide (roquinimex), which is a quinoline-3-carboxamide, has been shown to regulate immune activities as well as exert anti-tumor effects, by inhibition of angiogenesis, in several animal models (Ichikawa *et al.,* 1992; Borgström *et al.,* 1995). Suramin, a polysulfonated naphthylurea compound was found to enhance PAI-2 production by the human renal cell carcinoma cell line, SN12C-PM6 (Marutsuka *et al.,* 1995). Tumor necrosis factor-alpha (TNF-α) has been found to stimulate PAI-2 expression in various cell types including monocyte-like cells (Medcalf, 1992; Gyetko *et al.,* 1992), fibroblast-like cells (Pytel *et al.,* 1990; Kumar & Baglioni, 1991) and melanoma cells (Pytel *et al.,* 1990; Johnson & Baglioni, 1990). Recently TNF-α treatment resulted in increased levels of PAI-2 mRNA and protein by cultured human keratinocytes (Wang and Jensen, 1998).

1.3.3.1.4 Interaction between PAI-2 and u-PA

Proteinase inhibitors of the SERPIN superfamily can exist in one of three distinct conformations: the native form which is a fully active protein with an intact reactive site loop; a proteolytically modified form in which the inhibitory activity is lost; and the protease/complexed form in which the inhibitor forms an equimolar complex with a target protease (Mast *et al.,* 1991). Plasminogen activation by u-PA occurs preferentially at the cell surface, where u-PAR-bound u-PA is accessible to inhibition by PAI-2 (Ellis *et al.,* 1990; Baker, M.S. *et al.,* 1992). The reaction between PAI-2 (45kDa) and high (54kDa) and low (33kDa) M_r u-PA resulted in complex bands of 82kDa and 62kDa, respectively (Kiso *et al.,* 1988). Dissociation of u-PA/PAI-2 complexes by treatment with ammonia resulted in intact u-PA. However, there were two PAI-2 fragments, a 40kDa and 4kDa components (Kiso *et al.,* 1988). The 4kDa component was thought to correspond to the C-terminal part of PAI-2 (Kiso *et al.,* 1988; Huber & Carrell, 1989), while the 40kDa component corresponded to the N-terminal part of PAI-2. A single cleavage at the Arg380-Thr381 bond of PAI-2 occurs when the inhibitor reacts with the activator. Identical results were obtained for PAI-2 preparations from human placenta and U937 cells. The 35-residue C-terminal peptide of PAI-2 is also released on reaction with u-PA in the absence of a reducing agent. The amino acid sequence of PAI-2 at the cleavage site was determined to be Met377-Thr378-Gly379-Arg380|Thr381-Gly382-His383-Gly384 (Kiso *et al.,* 1988).

1.3.3.1.5 Cellular processing of PAI-2

Little is known of the cellular processing of PAI-2, while more is known about the processing of PAI-1. Various cell-associated receptors including, low density lipoprotein receptor-related protein (LRP), glycoprotein 330 (gp330), very low density lipoprotein receptor (VLDL), and α_2-macroglobulin receptor, have been found to bind, internalize and degrade u-PA/PAI-1 and t-PA/PAI-1 complexes (Orth *et al.*, 1992; Strickland *et al.*, 1994; Bu *et al.*, 1994 Argraves *et al.*, 1995; Casslen *et al.*, 1998b). Jensen *et al.*, (1990) found that uncomplexed u-PA, ^{125}I-u-PA/PAI-1 complexes and ^{125}I-u-PA/PAI-2 complexes bound with similar affinities to u-PAR on JAR cells at 4°C. When cells were transferred from 4°C to 37°C ~50% of cell-bound ^{125}I-u-PA/PAI-1 and ^{125}I-u-PA/PAI-2 complexes were degraded while ~15% of cell-bound uncomplexed u-PA were degraded. Thus, degradation of cell-bound u-PA/inhibitor complexes is more efficient than uncomplexed u-PA, independent of whether complexes were formed before or after association with u-PAR. u-PAR was also shown to participate in the clearance of u-PA/inhibitor complexes in JAR cells (Jensen *et al.*, 1990).

Incubation of THP-1 cells with PAI-2 at 37°C after the cells were incubated with ^{125}I-u-PA resulted in loss of cell associated radioactivity at a much faster rate compared to cells not incubated with PAI-2 (Estreicher *et al.*, 1990). PAI-2 treatment had no effect on the cell associated radioactivity when cells were pre-treated with DFP-inactivated ^{125}I-u-PA (Estreicher *et al.*, 1990). Therefore, the addition of PAI-2 to

THP-1 cells resulted in formation of ^{125}I-u-PA/PAI-2 complexes which were rapidly cleared from the cell surface (Estreicher *et al.,* 1990). A 70kDa radioactive species found on the cell surface was thought to be the earliest detectable evidence of degradation (Estreicher *et al.,* 1990). In addition, the binding of preformed ^{125}I-u-PA/PAI-2 complexes by THP-1 cells at 37°C resulted in the disappearance of cell-associated radioactivity and the appearance of degradative intermediates with time (Estreicher *et al.,* 1990; Ragno *et al.,* 1993; Ragno *et al.,* 1995).

In a study by Estreicher *et al.,* (1990), ^{125}I-u-PA/PAI-2 complexes with M_r of 95kDa and 70kDa were found in the trypsin-treated samples of THP-1 cells. In another study, Ragno *et al.,* (1993) found preformed ^{125}I-u-PA/PAI-2 complex binding to u-PAR on THP-1 cells at 4°C resulted in rapid cleavage of the complex into two fragments of 70 and 22kDa. The 70kDa fragment was endocytosed or released, while the 22kDa fragment remained bound to u-PAR preventing the binding of new active u-PA. The 70kDa fragment was found to contain the active site of both u-PA and PAI-2. The 22kDa fragment was identified as the N-terminal fragment of u-PA (Ragno *et al.,* 1993). Cross-linking of the u-PA/PAI-2 complex to THP-1 cells at 4°C gave rise to a 130kDa product which was found to co-migrate with u-PA/PAI-2 complexes bound to u-PAR in solution. This was confirmed because the 130kDa fragments were immunoprecipitated out from THP-1 cell lysates using a monoclonal antibody specific for u-PAR (Ragno *et al.,* 1995). Evidence indicates that u-PAR serves to accumulate u-PA/PAI-2 complexes at the cell surface, which is then cleared

by clearance receptors such as LRP for u-PA/PAI-1 complexes (Estreicher *et al.*, 1990; Ragno *et al.*, 1995).

1.3.3.1.6 Functions/roles of PAI-2

The function of PAI-2 is still speculative. However the reported localisation and properties of PAI-2 are suggestive of its role in the regulation of inflammatory processes (Wohlwend *et al.*, 1987; Schwartz, B. & Bradshaw, 1992), maintenance of haemostasis during pregnancy (Åstedt *et al.*, 1986) and protection from cytolysis (Kumar & Baglioni, 1991). The involvement of PAI-2 in a range of physiological and pathological events in which u-PA-mediated proteolysis plays important roles are such as keratinocyte differentiation, wound healing, inflammation, pregnancy, pre-eclampsia, intrauterine growth retardation, leukocyte migration, apoptosis and tumour invasion (Lecander & Åstedt, 1986; Lecander & Åstedt, 1987; Kruithof *et al.*, 1988; Mignatti and Rifkin, 1993; Jensen *et al.*, 1994; Bouchet *et al.*, 1994; Nagayama *et al.*, 1994; Danø *et al.*, 1985). Some of these events and the roles of PAI-2 in these events is reviewed in the following sections.

1.3.3.1.6.1 Keratinocyte differentiation

Keratinocyte-derived PAI-2, which was immunologically identified as placental-type PAI-2, is the main inhibitor of PA activity in normal human epidermis (Hibino *et al.*,

1988; Gissler *et al.,* 1993; Lyons-Giordano *et al.,* 1994; Reinartz *et al.,* 1996). PAI-2 mRNA was detected by *in situ* hybridization, throughout the normal epidermis, with the most intense signal located in the granular layers at the cell periphery, as well as in the hair follicles. PAI-2 antigen was also most prominent in the more differentiated keratinocytes in the granular layers (Lyons-Giordano *et al.,* 1994). Extracts of normal epidermis also contained PAI-2 activity as confirmed by immunoprecipitation with a PAI-2 specific polyclonal antibody (Lyons-Giordano *et al.,* 1994). Hibino *et al.,* (1988) localized PAI-2 to the granular layer as well as to the stratum corneum in human plantar epithelium. However, in contrast to the findings by Lyons-Giordano *et al.,* (1994) no PAI-2 was detected in the basal layer (Hibino *et al.,* 1988). These discrepancies may be due to differences in the characteristics of the epidermis from different parts of the body. Hibino *et al.,* (1988) examined plantar skin while Lyons-Giordano *et al.,* (1994) studied skin from the abdomen, face and breast. In addition, PAI-2 has been identified in the cornified envelopes of keratinocytes (Jensen, P.J. *et al.,* 1995; Robinson *et al.,* 1997).

It has been reported that with increasing concentrations of calcium in the media, the cultured human epidermal keratinocytes stratify and become more differentiated (Boyce & Ham, 1983). Meanwhile, keratinocytes cultured in media with low levels of calcium morphologically resembled keratinocytes in the basal layer of the normal epidermis. Lyons-Giordano & Lazarus (1994) found that increasing the concentration of calcium in cultured media of human keratinocytes resulted in approximately a 90%

and 25% reduction of u-PA and PAI-2 mRNA levels, respectively. However, even though a reduction in PAI-2 was observed, the reduction of u-PA was much greater, hence the ratio of PAI-2 to u-PA was increased. It was suggested that the ratio of PAI-2 to u-PA increased with keratinocyte differentiation. In contrast, Wang and Jensen (1998) found that calcium increases the steady-state levels of PAI-2 mRNA and protein. With longer incubations in calcium the cultured cells became more differentiated, with the glycosylated form of PAI-2 preferentially elevated. Both the glycosylated and non-glycosylated PAI-2 remained predominantly cell-associated and were thought to be associated with the intracellular aspect of cells (Wang and Jensen, 1998).

Systemic lupus erythematosus (SLE) is a disease in which epidermal differentiation is disturbed. PAI-2 antigen was found in epidermis from both normal and SLE epidermis (Bechtel *et al.,* 1996). PAI-2 staining was most prominent in the granular layer of normal epidermis. In the lesional epidermis of SLE an increase in PAI-2 antigen was observed. In both cases PAI-2 was located at the cell periphery, indicating an association with the cornified envelope. No u-PA or t-PA was detected in either the normal epidermis or lesional epidermis of SLE. It was speculated that PAI-2 from the human epidermis may have functions other than the regulation of PA activity (Bechtel *et al.,* 1996).

Psoriatic epidermis is a condition in which the process of continuous cellular proliferation and differentiation are aberrant compared to normal epidermis. In psoriatic epidermis PAI-2 mRNA and antigen were also found predominantly in the more superficial layers beneath the cornified cells like normal epidermis. However, unlike normal epidermis which showed diffuse staining for PAI-2 in the relatively undifferentiated basal area, psoriatic epidermis did not show staining of PAI-2 in this area (Lyons-Giordano *et al.,* 1994). Keratinocytes in the basal layers have been found to synthesize u-PA as shown by *in situ* hybridization and immunohistochemistry (Chen *et al.,* 1993). In another study using *in situ* zymography u-PA was also localized to the basal area of normal human epidermis (Spiers *et al.,* 1994). u-PA activity has also been detected in the basal area of normal epidermis (Lyons-Giordano *et al.,* 1993). Therefore, the localisation of PAI-2 in the basal area of normal epidermis may function to regulate the activity of u-PA.

1.3.3.1.6.2 Inflammation, wound healing and apoptosis

Bullous pemphigoid is an autoimmune inflammatory skin disease characterised by subepidermal blisters. PAI-2 staining was observed in all lesional and non-lesional epidermis. However the staining pattern between the two differed. In lesional skin PAI-2 was detected intracellularly as well as cell border-associated. Meanwhile, in non-lesional epidermis staining for PAI-2 was only observed along cell borders in keratinocytes of the stratum spinosum and stratum granulosum. In both non-lesional

and normal epidermis PAI-2 staining was restricted to the stratum granulosum and diffusely in basal cells. Co-localization of PAI-2 and u-PA was observed both intracellularly and along the cell border in keratinocytes surrounding the blister cavity. Co-localization of PAI-2 and u-PA was observed only along the cell border and not intracellularly in keratinocytes at the bottom of the blister. Co-localization of PAI-2 and u-PA suggest the regulation of u-PA activity by PAI-2 in lesional epidermis (Schaefer *et al.,* 1996). Thus the upregulation of u-PA, u-PAR and PAI-2 in keratinocytes of bullous pemphigoid lesions initiates epidermal repair in response to dermo-epidermal dyshesion.

Elevation in PAI-2 levels was found in the suprabasal layer of an organotypic skin culture system which resembles wound healing (Chen *et al.,* 1993). PAI-2 mRNA was found in healing human skin wounds (Schaefer 1996). A strong expression of PAI-2 was observed in keratinocytes that re-epithelialized dermal burn wounds or lesions caused by the autoimmune blistering disease pemphigus vulgaris (Bechtel *et al.,* 1998). PAI-2 along with α_2-antiplasmin were found in the primary matrix and the granulation tissue in healing human skin wounds by immunohistology. The PAI-2 was mainly associated with monocytes/macrophages and fibroblasts (Bechtel *et al.,* 1998).

Super-expression of the intracellular form of PAI-2 was found to inhibit the TNF-mediated killing of a fibrosarcoma cell line, HT-1080 (Kumar & Baglioni, 1991). A

cleaved form of PAI-2 (a reduction of 10kDa in the M_r of PAI-2), which was functionally active, was consistently detected in cell homogenates of the human promyelocytic leukaemic NB_4 cell undergoing apoptosis compared to normal cells (Jensen *et al.,* 1994). The cleaved PAI-2 isoform was also present in leukaemic cells from patients with acute and chronic myeloid leukaemia, with greater frequency occurring among patients with chronic myeloid leukaemia than among the acute myeloid leukemia patients. Thus it was suggested that the cleaved PAI-2 isoform may be a biochemical marker of apoptosis (Jensen *et al.,* 1994).

1.3.3.1.6.3 Pregnancy

Elevated levels of PAI-2 are observed with increasing gestational period in pregnant women, while no PAI-2 has been detected in non-pregnant women (Lecander & Åstedt, 1989). PAI-2 was thought to have a role in protecting the newborn from bleeding during parturition (ie. intraventricular haemorrhage) (Lecander & Åstedt, 1987). Throughout normal pregnancy there is a progressive increase in PAI-1 and PAI-2 antigen levels in plasma (Kruithof *et al.,* 1987; Halligan *et al.,* 1994; Nakashima *et al.,* 1996). A slight increase in PAI-1 activity was observed in the first 3 months of pregnancy compared to plasma from non-pregnant women. From 3 months to term an almost linear increase in PAI-1 activity was observed (Kruithof *et al.,* 1987). Similar trends were observed for PAI-1 antigen levels, which were found to be approximately 3-fold higher between weeks 32 and 36 compared to non-

pregnant women. A sharp decrease in both PAI-1 antigen and activity levels were observed 1h after delivery and returned to normal 3 to 5 days after delivery.

PAI-2 antigen was below the detection limit (<10ng/ml) in plasma from non-pregnant women (Kruithof *et al.*, 1987). From the 8th week until the 32nd week of pregnancy the level of PAI-2 antigen was observed to increase almost linearly. PAI-2 levels peaked and stabilized around the 32nd week and at term had increased more than 25-fold. Unlike PAI-1, PAI-2 levels did not decrease 1h after delivery. Five days after delivery PAI-2 levels were still 8-fold higher compared to levels in non-pregnant women. Similar trends were observed for PAI-2 activity. Due to the high levels of PAI-2 observed after delivery it was thought that the placenta was not the main site of inhibitor synthesis. Another explanation was that PAI-2 may have a longer (several days as opposed to hours) half-life *in vivo* (Kruithof *et al.*, 1987). In contrast to this, a rapid decrease in PAI-1 levels indicate that the placenta is the source of PAI-1 production, and PAI-1 may have a short half-life *in vivo* (Colucci *et al.*, 1985).

Ekelund *et al.*, (1970), using a fibrin clot method, observed greater inhibition of u-PA activity by serum from newborn infants compared to serum from non-pregnant adults. Two forms of PAI-2 were thought to exist in plasma obtained from pregnant women in the third trimester, but only one form of PAI-1 was evident (Kruithof *et al.*, 1987). A complex of 95kDa resulted when ^{125}I-u-PA was added to plasma from non-pregnant women, which was determined to be ^{125}I-u-PA/PAI-1 complexes (Kruithof

et al., 1987). In contrast to this, when ^{125}I-u-PA was added to plasma from the last trimester of pregnancy, three bands in the molecular weight region of 95-110kDa were observed. The smallest (95kDa) and largest (115kDa) complexes were determined to be complex formation between ^{125}I-u-PA and PAI-2. Meanwhile the middle band was identified as ^{125}I-u-PA/PAI-1 complexes (Kruithof *et al.,* 1987).

ELISA was used to measure u-PA, t-PA and PAI in plasma samples from 44 women with normal pregnancy near term (Koelbl *et al.,* 1989). These values were compared with values obtained from a healthy age-matched non-pregnant group. Plasma samples were collected before the onset of labour and 1, 2, 3, 4 and 5 days after delivery. The PAI measured may either be PAI-1 or PAI-2 since the assay implemented was incapable of distinguishing between the two. No significant differences were observed for u-PA and t-PA antigen levels compared to the control group before the onset of labour. In the days following delivery, the levels for both u-PA and t-PA remained the same. There was no influence of birth weight and placental weight on the plasma levels of both u-PA and t-PA. An increase in PAI activity was observed in women before delivery compared to the non-pregnant control group. One day after delivery the PAI level returned back to levels observed in the non-pregnant control group and remained the same up to day 5 after delivery. This suggests that high PAI levels are responsible for maintaining levels of t-PA, which were similar to that in non-pregnant control subjects. The decrease in PAI activity was thought to result from removal of the placenta (Koelbl *et al.,* 1989). Thus, during

pregnancy there are changes in levels and activities of PA and PAIs. However, these changes appear to have no effect on the overall fibrinolytic activity throughout pregnancy and postpartum (Kruithof *et al.*, 1987).

Lecander and Åstedt (1987) were the first to demonstrate PAI-2 in amniotic fluid and cord blood from women during normal labor. Using ELISA, the highest concentration of PAI-2 was found in amniotic fluid, followed by maternal blood, with the lowest levels of PAI-2 found in cord blood. In another study a 2.4-fold and 3.3-fold increase in PAI-2 and PAI-1 levels, respectively, was observed in amniotic fluid between the first and the third trimester. This was accompanied by a decrease in t-PA antigen levels (Estelles *et al.*, 1990). Immunohistochemical localisation using polyclonal antibodies resulted in the staining for PAI-2 throughout the epithelial trophoblast of formalin-fixed placental tissue (Åstedt *et al.*, 1986). By immunohistochemistry (Nakashima *et al.*, 1996) and *in situ* hybridization (Grancha *et al.*, 1996) PAI-2 antigen was localized in syncytiotrophoblasts of the placenta. It is not known whether the trophoblast is the source of PAI-2. However, the presence of PAI-2 in the trophoblasts indicate that these cells may be a source of mRNA coding for placental source of PAI-2. It was not ruled out that the trophoblast may only be storing PAI-2 (Åstedt *et al.*, 1986). Immunohistochemical studies also showed strong staining for PAI-2 in decidual cells of postpartum fetal membranes (Watanabe *et al.*, 1993).

The purification of PAI-2 from human placenta has resulted in identification of various forms of PAI-2 with different molecular masses. Purification of placental PAI-2 by affinity chromatography resulted in a single protein band with a molecular weight of approximately 48kDa as seen by SDS-PAGE (Åstedt *et al.,* 1985). Meanwhile Nielsen *et al.,* (1987) purified PAI from human placental tissues and observed three protein bands using Coomassie blue stained non-reducing SDS-PAGE. It was confirmed that the purified antigen was placental PAI by immunoblotting and using placental PAI specific monoclonal antibodies. However, under reducing conditions placental PAI showed only one band with M_r of ~50kDa. This was thought to be the monomeric form of purified PAI from the human placenta (Nielsen *et al.,* 1987). Analysis of PAI-2 purified from amniotic fluid, cord blood and maternal blood found a high molecular weight 60kDa form and a low molecular weight 48kDa form (Lecander & Åstedt, 1987). Both the 60kDa and 48kDa were present in approximately equal amounts in amniotic fluid. Cord blood contained more of the 48kDa form, with only a weak band of 60kDa. Meanwhile, in maternal blood the majority of PAI-2 was found as 60kDa, with only little amounts of the 48kDa form. The 60kDa protein was found to exist in a single-chain structure that could not be reduced (Lecander & Åstedt, 1987). Analysis by SDS-PAGE and zymography found two forms of PAI-2 with a M_r of approximately 75 and 130kDa detected in plasma at 12 weeks gestation and which persisted in the maternal circulation for up to 7 days after delivery (Booth *et al.,* 1988). It was thought that

these two forms, which had a molecular weight much higher than purified PAI-2 (M_r of 47-60kDa), may represent complexes or aggregates of PAI-2.

Analysis of placental tissue by non-denaturing SDS-PAGE and Western blotting identified a 76kDa and 80kDa protein band. Both the 80kDa and 76kDa bands reacted to monoclonal antibodies directed against PAI-2 and vitronectin. From this it was suggested that PAI-2 is predominantly found in high molecular weight forms covalently bound to vitronectin via the disulfide bridges in placental extracts. Purified PAI-2 from human placenta and pregnancy plasma with M_r of 45kDa under reducing SDS-PAGE conditions (which cross-reacted with monoclonal antibodies against PAI-2) was able to inhibit the activity of u-PA (Radtke *et al.*, 1990).

A 15min incubation of placental PAI-2 with both 31kDa and 54kDa u-PA and tc-tPA resulted in abolishing the proteolytic activity of the plasminogen activators. In contrast, for the same incubation period only approximately 30% of the activity of sc-t-PA was inhibited by PAI-2. Reaction of PAI-2 with the activators occurred in a 1:1 ratio with the loss of a peptide from the inhibitor during complex formation (Åstedt *et al.*, 1985). The inhibitory activity of PAI-2 against u-PA was approximately 15-fold higher in amniotic fluid and maternal plasma than in cord blood. Treatment of PAI from the placenta with SDS resulted in a decrease of inhibitor activity by approximately 70% (Nielsen *et al.*, 1987).

1.3.3.1.6.4 Pre-eclampsia

Pre-eclampsia is a condition occurring during pregnancy and is characterised by various factors, including maternal hypercoagulable state and impairment of uteroplacental circulation. Excessive fibrin deposition occurs in the placenta, suggesting that disorders of placental coagulation and fibrinolysis physiologic systems may have a role in hemostasis activation (Kanfer *et al.*, 1996). Both PAI-2 antigen and activity levels were markedly reduced in women with severe pre-eclampsia (Estelles *et al.*, 1989; Reith *et al.*, 1993; Halligan *et al.*, 1994; He *et al.*, 1995; Nakashima *et al.*, 1996; Schjetlein *et al.*, 1997). PAI-2 antigen and activity levels were significantly reduced in patients with either severe pre-eclampsia or chronic hypertension with superimposed severe pre-eclampsia in their third trimester compared with normal third-trimester pregnant women (Estelles *et al.*, 1989; Nakashima *et al.*, 1996). In another study, PAI-2 antigen in placental tissue did not stain as clearly in severe pre-eclampsia, compared to during the 3rd trimester of normal pregnancy (Nakashima *et al.*, 1996). In contrast to this the antigen and activity levels of PAI-1 in severe pre-eclampsia patients were significantly increased compared with normal pregnant women (Estelles *et al.*, 1989; Reith *et al.*, 1993; He *et al.*, 1995). In the established pre-eclamptic group PAI-2 antigen levels were significantly lower compared to the normal control group. The decrease in PAI-2 may be a useful marker of placental function in pre-eclampsia (Gilabert *et al.*, 1995; Nakashima *et al.*, 1996) while increased levels of t-PA reflected the severity of the condition (Halligan *et al.*, 1994). In contrast to all the above studies, Kanfer *et al.*,

(1996) observed an increase in PAI-2 antigen levels in placentas collected at term from hypertensive/pre-eclamptic women compared to placentas from normal pregnancies.

1.3.3.1.6.5 Intrauterine fetal growth retardation (IUGR)

As with pre-eclampsia, a reduction of PAI-2 antigen level in plasma was observed from IUGR pregnancy compared to normal pregnancy (Schjetlein *et al.,* 1997). The significant decrease in levels of PAI-2 in plasma and the placenta observed in pregnancies complicated with IUGR was found to be due to a decreased expression of PAI-2 mRNA in the placenta (Grancha *et al.,* 1996). In contrast to the conditions of pre-eclampsia and IUGR, it was observed that plasma levels of PAI-2 of patients who had early recurrent miscarriages of unknown origin were increased rather than decreased (Gris *et al.,* 1993).

1.3.3.1.6.6 Other disease states

PAI-2 has been observed associated with other disease states, and can be used for the treatment of certain diseases. Immunohistochemical analysis detected the presence of PAI-2 in brain microglia in postmortem brain tissue of control subjects and patients with Alzheimer's disease. PAI-2 expression was found to be upregulated in Alzheimer disease lesions compared to control subjects, suggesting it has a role in the

regulation of the plasminogen activators and plasmin system (Akiyama *et al.*, 1993). Leiper *et al.*, (1994) observed significant quantities of PAI-2 in alcoholic cirrhosis, primary biliary cirrhosis and metastatic liver disease. The presence of PAI-2 was thought to be one of two reasons; 1) a response to u-PA present in the plasma in some forms of liver disease and is often present in malignant cells; or 2) depressed hepatic function may have reduced the clearance mechanism for PAI-2, resulting in the accumulation of PAI-2 produced by the monocyte in plasma. Treatment of ophthalamic wounds in rabbit eyes with PAI-2 inhibited neovascularization, suppressed inflammation of the cornea and stroma, and prevented ulceration, restoring the transparency of the eyes (Schuler *et al.*, 1993).

1.3.3.1.6.7 PAI-2 and cancer

PAI-2 has been found to be either elevated or reduced with certain cancers. The human glioblastoma U138 cells which are known to produce a non-invasive tumour, and does not readily form tumours in the nude mouse model, were found to produce PAI-1, PAI-2 and PN-1, but no PA activity (Murphy *et al.*, 1993). In contrast the more aggressive U373 glioblastoma-astrocytoma constitutively produces PA but must be induced *in vitro* to produce PAI (Murphy & Hart, 1992). Thus the production of PAIs and no PA activity may result in the lack of invasive properties of the U138 glioblastoma cells.

A number of observations have suggested a link between PAI-2 and reduced metastatic potential in cancerous tissues. An inverse correlation between PAI-2 levels and the metastatic potential of breast cancer tissues was observed, where significantly higher levels of PAI-2 antigen were found in carcinomas without lymph-node involvement (Sumiyoshi *et al.*, 1992; Ishikawa *et al.*, 1996). Furthermore, a positive expression of u-PA, u-PAR and PAI-1 significantly correlated with negative expression of PAI-2 (Ishikawa *et al.*, 1996). PAI-2 levels were significantly lower in adenocarcinomas compared to the normal mucosa (Hewin *et al.*, 1996). Nagayama *et al.*, (1994) found that in non-small human lung cancer tissues significantly lower PAI-2 antigen levels were observed in tumors that had metastasized to lymph nodes. Similarly, Robert *et al.*, (1999) observed an inverse relationship between PAI-2 expression and tumour progression in lung cancer. In contrast, non-metastasising gastric cancers have increased levels of tumour-associated PAI-2 (Nakamura *et al.*, 1992). Reduced expression of PAI-2 in pancreatic carcinomas was found to be associated with metastasis to the peritoneum (Takeuchi *et al.*, 1993).

Approximately 80% of primary lung cancers (total of 105 cases) were found to express u-PA, u-PAR and PAI-1, while PAI-2 expression was observed in only half of the overall cases (Yoshino *et al.*, 1998). A significant correlation was observed between diminished expression of PAI-2 with increased lymph node metastasis and a poor prognosis for primary lung cancer patients (Yoshino *et al.*, 1998). Low levels of PAI-2 in breast tumours were also found to correlate with shorter metastasis-free

survival (Bouchet *et al.*, 1994). Furthermore, high levels of PAI-2 of tumour origin were associated with relapse-free survival, metastasis-free survival and overall survival in patients with breast carcinoma (Foekens *et al.*, 1995). Meanwhile, over-expression of PAI-2 in human melanoma cells inhibits spontaneous metastasis in severe combined immunodeficient (SCID) mice (Mueller *et al.*, 1995). Evans and Lin (1995) showed that pre-treatment of MATB rat mammary cancer cells with recombinant human PAI-2, or slow infusion of the inhibitor with osmotic pumps led to a significant decrease in lung metastasis post intravenous administration. It has also been found that introduction of the PAI-2 gene into HT1080 tumour cells reduced their ability to form metastasis in a nude mouse model (Praus *et al.*, 1999).

In contrast to the above findings, Kruithof *et al.*, (1988) reported high levels of PAI-2 in a patient with hepatocellular carcinoma. Significantly higher levels of PAI-2 were found in the more advanced stages of endometrial cancer compared to the normal endometrium (Gleeson *et al.*, 1992). PAI-2 levels, as well as levels of other plasminogen activation components were also found elevated in ovarian cancer, which indicated that the fibrinolytic system contributed to the potential of cancer cells (Schmalfeldt *et al.*, 1995). However, high levels of PAI-2 in ovarian cancer (Chambers *et al.*, 1997) and breast cancer (Duggan *et al.*, 1997) have been associated with a good prognosis. It has been suggested that PAI-2 expression is an indice of the risk of recurrence of gastric cancer after curative operation (Kammori *et al.*, 1999). In studies by Tran-Thang *et al.*, (1989) it was suggested that PAI-2 may be an onco-fetal

marker in the diagnosis of hepatocellular carcinoma. However, this was disputed by studies carried out by Özyilkan *et al.*, (1992), which found no correlation between α-fetoprotein, an onco-fetal diagnostic marker of hepatocellular carcinoma (Bates and Longo, 1987), and PAI-2 expression.

Strong evidence exists supporting the role of PAI-2 in modulating u-PA-mediated tumour breakdown of the extracellular matrix in carcinomas of the lung, pancreas and breast (Gris *et al.*, 1993; Nagayama *et al.*, 1994; Mueller *et al.*, 1995; Evans & Lin, 1995; Ishikawa *et al.*, 1996; Yoshino *et al.*, 1998). Clinical studies have demonstrated an association between high levels of PAI-2 and favourable outcome (Bouchet *et al.*, 1994; Foekens *et al.*, 1995). In addition, Shiomi *et al.*, (2000) speculated that the expression of PAI-2 in the fibroblasts surrounding esophageal squamous cell carcinoma is protective. Thus, it appears that through regulation of u-PA-mediated proteolysis, PAI-2 restricts the metastatic potential of a variety of cancers.

PAI-2 has also been used to modify the behaviour of cancer cells which could lead to it's use as a cancer therapeutic agent. Baker, M.S. *et al.*, (1990) using COLO394 and LIM1215 cells found that recombinant PAI-2 inhibited the activity of u-PA resulting in subsequent inhibition of extracellular matrix degradation by cells. Similar results were observed with M24met melanoma cells. The inhibition of u-PA activity by PAI-2 decreased the invasiveness of M24met melanoma cells in a dose-dependent manner

(Stahl & Mueller, 1994). The expression of recombinant PAI-2 by the human HT1080 fibrosarcoma cell line resulted in decreased degradation of extracellular matrix proteins compared to cells which did not express PAI-2 (Laug *et al.,* 1993). Pre-incubation of the cells with a 20 to 40-fold excess of fluid phase PAI-2 prior to incubation in CSF-1, which lead to an increase in the number of endogenously bound u-PA on A549 and Calu-1 cells, resulted in inhibition of cancer cell invasiveness as determined by amnion invasion assay (Bruckner *et al.,* 1992). They also found that PAI-1 in the fluid phase did not abolish the invasiveness of the A549 and Calu-1 cells, but PAI-1 binding to vitronectin resulted in reduction in the invasiveness of the cells.

1.4 ANIMAL MODELS IN CANCER RESEARCH

Most model system in cancer research are based on animals carrying a tumour, often localized subcutaneously. Initial studies were carried out on animals in which the tumour was transplanted in regions of the body where the immune response was not so strong or delayed. These areas included the anterior chamber of the eye, the brain and cheek pouch in the hamster (Giovanella & Fogh, 1985). However the immune response eventually developed and the xenograft was rejected. With the discovery of genetically immunosuppressed animals, research of human cancer in an animal model became possible (Flanagan, 1966). The use of immunosuppressed animals improved tumour take (Rygaard & Povlsen, 1969). The growth of human tumours in such

animals allowed for studies into tumourgenicity of human cancer cell lines (Iwamura *et al.,* 1987; Gamboa *et al.,* 1995; Yoshikawa *et al.,* 1997), tumour biology, the metastatic potential of cancer cells lines as well as analysis of the process of cancer metastasis (Naito *et al.,* 1986; Schackert & Fidler, 1989; Price, 1994; Giavazzi *et al.,* 1986a; Giavazzi *et al.,* 1986b; Price, 1996), the role of the immune system in cancer (Goldrosen, 1980; Dinney *et al.,* 1991) and experimental therapeutic agents for the treatment of human cancers (Rygaard & Povlsen, 1982; Fidler, 1991; Shinkfield *et al.,* 1992; Jankun *et al.,* 1997).

1.4.1 The use of nude mouse models in cancer research

The congenitally athymic nude mouse, which lack functional T lymphocytes (Pantelouris, 1968; Sousa *et al.,* 1969), is the most widely used animal model in cancer research. Phenotypically, nude mice are distinct from conventional mice due to the absence of body hair. The nude mice although lacking in mature T lymphocytes, however, are not completely deprived of an immune response. Nude mice produce B cells, which produce antibodies allowing the mice to mount a humoral immune response (Bankhurst & Warner, 1972; Weisz-Carrington *et al.,* 1979; Rygaard & Povlsen, 1982). Natural killer cells, known to have a major role in cancer immunity (Herberman *et al.,* 1975; Kyriazis *et al.,* 1979; Hanna & Schneider, 1983; Jacubovich *et al.,* 1984), are present at higher levels in nude mice compared to the conventional mouse (Kiessling *et al.,* 1975; Herberman, 1978; Lozzio *et al.,*

1982). Adult nude mice have higher levels of natural killer cell activity than young nude mice, therefore the young mice are more susceptible to metastasis (Hanna, 1980). The incidence of cancer metastasis was found to be higher in mice with low natural killer cell activity and vice versa (Hanna & Fidler, 1980; Hanna, 1982a). Nude mice have normal numbers of functional macrophages which were found to be more active compared to macrophages from normal mice (Sharp & Colston, 1984). Therefore even though nude mice are thymic deficient, they are still able to mediate immune responses through the functions of B-lymphocytes, macrophages and natural killer cells (Rygaard & Povlsen, 1982; Hanna, 1982a; Jacubovich *et al.,* 1984).

The main advantages of nude mice were that these animals accept a wide range of human tumours. The resultant xenograft retains a lot of the characteristics of the original tumour allowing for the study of cancer in a biological system (Giovanella & Stehlin, 1974; Giavazzi, 1986a). However, because of their lack of T-lymphocyte mediated immunity the nude mice were susceptible to viral and parasitic infections and were not surviving very long when maintained under the same conditions as the conventional mice. When placed in carefully controlled pathogen-free conditions, the life span of nude mice were greatly increased to the extent that they were living as long as conventional mice. It has also been reported that higher rates of tumour take were achieved when nude mice were kept in a specific pathogen-free environment compared to nude mice housed in conventional facilities (Kameya *et al.,* 1976). Thus, a disadvantage of using nude mice is that, due to their need to be housed in a specific

pathogen-free environment, the keeping and maintenance of nude mice are much more costly compared to conventional mice. Rygaard and Povlsen (1969) were the first to successfully transplant xenografts of human adenocarcinoma tumours into athymic nude mice. Since that observation, nude mice models have been used extensively in studies ranging from studies of the immune system to studies of human cancer biology, the mechanisms of metastasis and potential therapeutic agents. For review refer to the following (Fidler & Hart, 1982; Hanna, 1982b; Sharkey & Fogh, 1984; Fidler, 1989; Price *et al.,* 1990; Price, 1994; Price 1996; Clarke, 1996; Wenisch, 1996; Culp *et al.,* 1998; Svane *et al.,* 1999).

1.4.1.1 Transplantation of human tumours into nude mice

Tumour take, growth and subsequent metastasis of human tumours transplanted into nude mice is dependent on various host and tumour-related properties such as the origin and type of tumour, the route of inoculation and the age, strain, gender and state of health of the recipient animals (Hart, 1982; Hanna 1982b; Rygaard & Povlsen, 1982; Giovanella & Fogh, 1985). The growth rate, invasiveness and metastatic properties of the tumour was also found to be dependent on the route of transplantation (Kyriazis & Kyriazis, 1980). Tumour lines derived from metastases grew more readily and showed higher incidences of metastasis in nude mice than those derived from primary tumours (Hajdu *et al.,* 1981; Fogh *et al.,* 1982;

Kozlowski, *et al.*, 1984a; Giavazzi *et al.*, 1986a). The advantages and the limitations of some of the routes of transplantation are reviewed in the following sections.

1.4.1.1.1 Subcutaneous inoculation

Subcutaneous inoculation of solid tumour blocks or tumour cell suspensions is relatively easy to accomplish and allows for direct observations of tumour xenografts. The subcutaneous route allowed for propagation of larger tumour masses (Gasdar *et al.*, 1981). However, human tumours transplanted subcutaneously are often encapsulated and rarely metastasize (Fidler, 1986; Morikawa *et al.*, 1988). When tumours were subcutaneously implanted into the anterior lateral thoracic wall of nude mice tumour take and incidences of metastasis were higher than implantation into the caudal flank region (Kyriazis & Kyriazis, 1980; Kozlowski, *et al.*, 1984a). A number of cancer types have been shown to spread readily after subcutaneous inoculation into nude mice such as breast carcinoma (Giovanella and Fogh, 1985), prostate cancer (Crowley *et al.*, 1993) and melanoma (Giovanella *et al.*, 1973; Quax *et al.*, 1991). In contrast, pancreatic adenocarcinoma (Kajiji *et al.*, 1982), renal-cell carcinoma (Kozlowski *et al.*, 1984b; Straroselsky *et al.*, 1992) and human colon cancer (Fidler, 1990) rarely metastasize after subcutaneous transplantation.

1.4.1.1.2 Intravenous injection

Intravenous injection of human tumour cells into nude mice have resulted in higher incidences of metastasis when compared to subcutaneous injection (Giavazzi *et al.*, 1986a; Quax *et al.*, 1991). Intravenous injection of human melanoma (Quax *et al.*, 1991) and colorectal carcinoma (Giavazzi *et al.*, 1986a) cell lines into nude mice produced lung metastasis compared to subcutaneous injection which produced no metastasis. However, the whole process of metastasis is not represented with intravenous injection of tumour cells. The initial steps of detachment from the primary tumour and invasion into blood vessels are bypassed (Hanna, 1982b). It does however, allow for the study of the later steps of metastasis, namely passage of tumour cells in the circulation, arrest of tumour cells in capillary beds, extravasation of tumour cells into the target organ and the growth of cells in the target organ (Zeidman, 1957; Weiss, 1990). Thus, intravenous injection of tumour cells allows for seeding of cells within the circulation to increase the incidences of metastases.

1.4.1.1.3 Orthotopic transplantation

The implantation of human tumour tissue from surgical specimens into the appropriate organ sites is termed orthotopic implantation. Orthotopic implantation produces higher incidences of tumour take than subcutaneous injection and can also lead to a higher incidence of metastasis (Fidler, 1986; Price *et al.*, 1990; Manzotti *et al.*, 1993). A variety of cancer types including lung (Wang *et al.*, 1992), colon

(Fidler, 1986; Bresalier *et al.,* 1987; Morikawa *et al.,* 1988; Rashidi *et al.,* 2000), breast (Price *et al.,* 1990), bladder (Aherling *et al.,* 1987), gastric (Furukawa *et al.,* 1993), pancreatic (Vezeridis *et al.,* 1988; Fu *et al.,* 1992b) and prostate cancer (Fu *et al.,* 1992a) have been shown to exhibit higher incidences of metastasis from the primary tumours after orthotopic implantation. Subsequent metastatic behaviour of the tumours in nude mice after orthotopic transplantation of intact human tumours also closely resembled the course of tumours in human patients (Furukawa *et al.,* 1993; Kubota, 1994; Rashidi *et al.,* 2000).

1.5 IMAGING AGENTS FOR HUMAN CANCERS

Early detection of cancer recurrence is an important step in the management of patient care. Early identification of patients at risk allows for appropriate and more aggressive treatment to ensure remission and longer periods of disease-free survival. The ability to detect labeled antibody is dependent on the proportion of label that is attached to the tumour as opposed to surrounding tissues (Spar, 1976). Histological grading and hormone receptor status are the two routinely used markers that have shown prognostic significance in breast cancer patients (Sigurdsson *et al.,* 1990). In order to be a successful imaging agent certain criterias must be met.

The target protein of the imaging agent must be located on the surface of the tumour cell to be available for binding allowing detection of the tumor mass. The half-life of

the target protein at the cell surface is also important. A longer half-life would provide time for binding and accumulation of the signal on the tumour cell. With a short half-life the amount of radioactivity accumulating on the tumour may be minimal and may not permit detection. The half-life of the imaging agent is also important for similar reasons. Most importantly the target protein must be tumour specific and not present on normal tissues (Spar, 1976). The available research on possible imaging agents for cancer prognosis is very extensive, hence only a small aspect of this area will be reviewed. For reviews refer to the following (Spar, 1976; Goldenberg *et al.,* 1989; Goldenberg, 1989; Schwartz, M. 1992).

1.5.1 Imaging agents

Serum enzymes such as alkaline phosphatase or acid phosphatase have been used to confirm the presence of cancer to assist in the staging of cancer, or to monitor the presence of metastases (Schwartz, M., 1989). Elevated levels of enzymes such as lactate dehydrogenase, thymidine kinase or cathepsin D in the serum have been found to be good prognostic markers in various cancers including melanoma, adenocarcinoma of the lung, chronic lymphocytic leukemia and breast cancer (Finck *et al.,* 1983; Kallander *et al.,* 1984; Thorpe *et al.,* 1989; Tandon *et al.,* 1990; Schwartz, M., 1992). In the case of cathepsin D, monoclonal antibodies have been developed, for the detection of the enzyme (Schwartz, M., 1992).

The use of isotype labelled antibodies for imaging purposes has been widely documented (Goldenberg *et al.,* 1978; Goldenberg *et al.,* 1980; Mach *et al.,* 1980; Farrands *et al.,* 1982; Epenetos *et al.,* 1982; Buchsbaum *et al.,* 1988). The same criteria for an imaging agent apply for the use of isotype labelled antibodies. In addition, the antigen should be found on all tumours of the same cancer type. If the antigen on the tumour were different for a specific cancer type then different antibody preparations would be required to treat individual tumours (Spar, 1976). Antibodies directed against human carcinoembryonic antigen (CEA), which is found in human colonic tumours, was used as an imaging agent (Reif *et al.,* 1974). These attempts were unsuccessful due to several reasons. One was the presence of substantial amounts of CEA antigen in the circulation, which ended up reacting with injected labeled antibody. Another was that the antibody produced was not absolutely specific for CEA and in this case was also found to react with erythrocytes (Reif *et al.,* 1974).

Since then successes in cancer imaging have been detailed. The monoclonal antibody 791T/36 preferentially localized in the primary and recurrent ovarian cancer after intravenous administration (Perkins *et al.,* 1989). Intra-peritoneal administration of radiolabeled monoclonal antibody also allowed for the localization of solid tumours via the circulation (Perkins *et al.,* 1989). Positive correlation between immunohistochemical staining of colorectal tumour-associated antigens using various monoclonal antibodies was found to correlate with uptake of radiolabeled antibody by tumours in patients (Chatal *et al.,* 1984; Beatty *et al.,* 1986; Leyden *et al.,* 1986).

Clinical trials have been reported for the monoclonal antibody B72.3 directed against tumour associated glycoprotein (TAG) in colon cancer patients (Esteban *et al.,* 1987; Divgi *et al.,* 1995; Mulligan *et al.,* 1995; Meredith *et al.,* 1996; Triozzi *et al.,* 1997).

u-PA was found to be more sensitive in localising cancer while the two established markers, carcinoembryonic antigen (CEA) and CA 19-9, were more sensitive in patients with metastases. It has been suggested that u-PA may be of relevance for screening asymptomatic patients at risk for the development of colorectal cancer (Huber *et al.,* 1993). It has also been suggested that the expression of PAI-2 may be useful as a marker for evaluating the prognosis of lung cancer (Yoshino *et al.,* 1998).

1.6 THERAPEUTIC STRATEGIES FOR THE TREATMENT OF HUMAN CANCERS

The heterogeneity of cell subpopulations within a tumour cell line with regards to metastatic capacity, hormone receptors, enzyme markers, antigenicity or immunogenicity and response to various therapeutic agents presents a challenge in finding the appropriate therapy. There are extensive studies on potential new therapeutic agents documented throughout the literature. This section will review the available studies examining therapeutic agents of cancer metastasis that have focused on the abolition of u-PA-mediated metastasis.

u-PA is a potential prognostic marker for a wide range of cancer types including lung (Oka *et al.,* 1991), bladder (Hasui *et al.,* 1992), colorectum (Mulcahy *et al.,* 1994), stomach (Nekarda *et al.,* 1994), kidney (Hofmann *et al.,* 1996), cervix (Kobayashi *et al.,* 1994a), pancreatic (Cantero *et al.,* 1997) and most promising, breast cancer (Duffy *et al.,* 1990; Janicke *et al.,* 1991; Reilly *et al.,* 1991). Therefore, the inactivation of u-PA activity abolishing subsequent u-PA-mediated metastasis, may be a possible strategy for cancer therapy. Strategies implemented to interfere with the expression or activity of u-PA at the gene or protein level includes antisense oligonucleotides, antibodies, enzyme inhibitors or synthetic u-PA analogues.

Some therapeutic strategies include inhibition of u-PA activities with antibodies. While tumour growth was not inhibited, inhibition of lung metastasis by human tumour cells was achieved by antibodies directed against human u-PA (Ossowski and Reich, 1983a,b). Similar results were obtained using a murine melanoma experimental metastasis model (Hearing *et al.,* 1988). In another study, antibodies directed against human u-PA prevented local invasion by LB6 cells (Ossowski *et al.,* 1991). An inhibitory antibody directed against high molecular weight u-PA was shown to inhibit the formation of pulmonary metastasis by murine Lewis lung carcinoma cells, 3LL, in C57BL/6 mice (Kobayashi *et al.,* 1994).

The process of neovascularization is an essential step in the development and growth of solid tumours. Intraperitoneal injection of amiloride, an inhibitor of u-PA activity

(Vassalli & Belin, 1987), for 5 consecutive days resulted in a 55% decrease of induced neovascularization in rabbit corneas (Avery *et al.,* 1990). Amiloride given to Fisher 344 rats, after tail vein injection of R3230AC rat mammary adenocarcinoma cells, in drinking water over a period of 24 days prevented formation of lung metastasis compared to control animals which developed multiple lung foci 12-18 days after injection of cells (Kellen *et al.,* 1988). Using Matrigel invasion chambers, two inhibitors of u-PA activity, amiloride and B428 significantly suppressed the invasive capacity of human breast cancer cells (Evans & Sloan-Stakleff, 2000a). The addition of amiloride to R3230AC rat mammary adenocarcinoma cell cultures *in vitro* resulted in a slight inhibition of urokinase activity in the cytosolic fraction of cells compared to untreated cells (Kellen *et al.,* 1988). Amiloride was observed to inhibit the proliferation of H6 cells. Amiloride given in a series of injections over a 5-6 day period inhibited the growth of both H6 hepatoma and DMA/J mammary adenocarcinoma in a dose-dependent manner *in vivo* (Sparks *et al.,* 1983). One of the side effects observed in the amiloride treated group was a 7% loss of total body weight, thought to be due to the diuretic effects of amiloride (Avery *et al.,* 1990). In addition, a slight hepatotoxicity of amiloride was observed in treated rats (Kellen *et al.,* 1988).

In a more recent study, inhibitors of u-PA activity were found to reduce tumour size in experimental animals (Jankun *et al.,* 1997). Amiloride and *p*-aminobenzamidine were given to mice with established tumors of human prostate cancer via their

drinking water (200mg/kg of body weight). PAI-1 was injected by tail vein (2.28mg/kg of body weight) every 5 days. Tumour volume (height x length x width) was measured twice a week. *p*-Aminobenzamidine treatment resulted in complete remission of tumors in some animals. Amiloride caused a reduction in tumor size, but was toxic to the animals. *p*-aminobenzamidine and amiloride bind to u-PA, which is then internalized into the cancer cells. This results in accumulation of the inhibitors at a concentration that is toxic to the cancer cells. Treatment of mice with two mutant forms of PAI-1 with an extended half-life (160h and 6h), amiloride and *p*-aminobenzamidine diminish tumour growth by decreasing the activity of u-PA. Using the non-u-PA expressing human prostate cancer cell line, Ln CaP, the u-PA inhibitors were found to be interacting with ECM components that express u-PA rather then directly with the cancer cells. Small molecule inhibitors and protein u-PA inhibitors reduce tumor growth by inhibition of u-PA activity (blocking the S1 active site of u-PA) in the ECM (Jankun *et al.,* 1997).

At the molecular level, antisense oligonucleotide inhibition of u-PA or u-PAR expression resulted in a decrease in invasiveness in three esophageal cancer cell lines, OC1, OC3 and the OC2 clones transfected with the u-PA cDNA (Morrissey *et al.,* 1999). A SERPIN derived from myoepithelial cells from the mammary gland that shares 58% sequence simliarity with PAI-2 was used to treat breast cancer (Xiao *et al.,* 1999). Transfection of breast cancer cells with this SERPIN lead to a significant

inhibition of primary tumor volumes, axillary lymph node metastasis, and lung metastasis.

The role of PAI-1 in cancer invasion and metastasis is still unclear, while in most studies consisting of both *in vitro* assays and *in vivo* animal model systems, PAI-2 was found to inhibit cancer invasion (Andreasen *et al.*, 1997). Although PAI-1 is an efficient inhibitor of u-PA correlations have been found between PAI-1 levels and poor prognosis in patients with breast cancer (Janicke *et al.*, 1991; Knoop *et al.*, 1998). An antibody directed against PAI-1 was found to decrease pulmonary metastases of human fibrosarcoma cell line, HT-1080, in athymice mice (Tsuchiya *et al.*, 1995). PAI-2 was thought to be more effective in regulating u-PA activity in human cancers than PAI-1 (Noguchi-Takino *et al.*, 1996). It has been postulated that PAI-2 may play a critical role in the regulation of ECM degradation during tumor cell invasion and metastasis, and the expression of PAI-2 may be useful as a marker to evaluate the prognosis of breast cancers (Ishikawa *et al.*, 1996). The use of PAI-2 as a therapeutic agent has been examined. Intraperitoneal injection of recombinant PAI-2 over a period of 18 days (100 units/day in 0.5ml PBS) produced significant reduction in tumour mass (human tumour cell line, A431) compared to the PBS-injected control group (Shinkfield *et al.*, 1992). Billström *et al.*, (1996) examined the strategy of stimulating PAI-2 production by macrophage/monocytes as an anti-tumor treatment. As mentioned previously (section 1.3.3.1.6.6), a preliminary study examining the efficacy of PAI-2 in treatment of inflammation and wounds found that PAI-2

treatment suppressed inflammation and prevented ulceration aiding in the healing process (Schuler *et al.*, 1993).

1.7 FOCUS OF THE PROJECT

With the available technology allowing for production of large quantities of recombinant proteins, human recombinant PAI-2 was assessed for its potential use as an imaging and/or therapeutic agent of u-PA-mediated human cancer. Initially studies will examine the cell surface expression of u-PA on two human colorectal cancer cell lines to verify which cell line of the two expresses more u-PA. The cell line expressing higher levels of cell-surface u-PA will be used in further studies. Human recombinant PAI-2 kindly provided by Biotech Australia will be radiolabelled and cell binding studies will be carried out to assess the ability of the inhibitor to bind to u-PA found on the surface of cells. The labelling of PAI-2 will allow for detection of binding as well as information regarding number of PAI-2 binding sites and K_d value. Once it is established that PAI-2 can bind to colorectal cancer cells, a nude mouse model will be implemented to determine if PAI-2 can also bind to colorectal cancer cells in tumour xenografts *in vivo*. The clearance and biodistribution of PAI-2 *in vivo* will also be examined. The mouse model will also be used to examine the efficacy of PAI-2 as a therapeutic agent of human colorectal cancer.

2. *IN VITRO* STUDIES OF RECOMBINANT HUMAN PAI-2 USING THE HUMAN COLORECTAL CANCER CELL LINE HCT116

2.1 INTRODUCTION

Elevated levels and activity of u-PA are consistent with neoplastic growth and metastatic spread in adenocarcinomas (Sier *et al.*, 1991). The basic genetic mechanisms involved in the genesis of colorectal cancer are the activation of oncogenes, inactivation of tumor suppressor genes and inactivation of DNA repair system (Boland, 1996). The human colon carcinoma cell line HCT116, is a prime example of this. The HCT116 cell line genome exhibits high levels of microsatellite instability, which are characterized by somatic genomic instability at simple repeated sequences (ie. mutational hot spots). The major class of mutation found in HCT116 cells are transition mutations. However, approximately 35% of mutations are caused by frameshifts, while a small proportion are caused by deletions. The alterations in HCT116 cells may have affected the repair of both mismatches and frameshifts (Bhattacharyya *et al.*, 1995). All these genomic mutations subsequently led to the tumourogenic phenotype of the HCT116 cells.

Phenotypically, HCT116 cells have been documented to be a highly invasive and metastatic cell line. HCT116 cells secrete minimal amounts of metalloproteinases

(Naito *et al.,* 1994). Hence a potential mechanism by which these cells are invasive may be via the u-PA system. The expression of u-PA would suggest that there are potential binding sites for PAI-2, the physiological inhibitor of u-PA activity, on these cell lines.

PAI-2 has been found to inhibit the activity of u-PA expressed by different cell lines. Baker, M.S. *et al.,* (1990) using COLO394 and LIM1215 cells, found that recombinant PAI-2 inhibited the activity of u-PA resulting in subsequent inhibition of extracellular matrix degradation by the cells. The expression of recombinant PAI-2 by the human HT1080 fibrosarcoma cell line resulted in decreased degradation of extracellular matrix proteins compared to cells which did not express PAI-2 (Laug *et al.,* 1993). PAI-2 does not appear to have a role in cancer spread while a correlation was found between PAI-1 levels and poor prognosis in breast cancer patients (Janicke *et al.,* 1991; Tsuchiya *et al.,* 1995; Knoop *et al.,* 1998; Kawasaki *et al.,* 1998).

Human recombinant PAI-2 preparations, a kind gift from Biotech Australia, was purified from one of two different sources. The first preparation of PAI-2 used was purified from an *Escherichia coli* (*E. coli*) expression system while the second was from a *Saccharomyces cerevisiae* (*S. cerevisiae*) expression system. Both sources of PAI-2 are biochemically and physiologically similar (as tested at Biotech Australia). Initial experiments were carried out using the *E. coli* source of PAI-2. However, the

majority of experiments including those in this chapter and the remaining two chapters were carried out on yeast PAI-2.

This project set out to assess the potential use of human recombinant PAI-2 as an imaging and/or therapeutic agent using a nude mouse model with xenografts of human colorectal cancer. With the overall aim of this project in mind, initial studies to assess the ability of exogenously added human recombinant PAI-2 binding to HCT116 cells were implemented. The radio-nuclei ^{125}Iodine (^{125}I) was chosen as the label for the detection of PAI-2 binding to HCT116 *in vitro* before assessing this in a mouse model *in vivo*.

^{125}I is widely chosen for labeling of proteins used in sensitive immunoassays and other procedures for the detection, localization and quantitation of substances in biological samples. ^{125}I is a gamma (γ) ray emitter with a half-life of 59.6 days. Although there are more recent methods now available with relatively high sensitivity in detection, such as immunofluorescence and biotinylation, radio-iodine is still the most sensitive label. Therefore ^{125}I-labeled PAI-2 was used for the detection of protein binding to cells as well as for the quantitation of binding sites on the cell surface.

The most widely used methods in radio-iodination involves oxidation of Na^{125}I or Na^{131}I in the presence of a protein or other molecule. There must be a tyrosine

residue or tyrosine residue derivative present in the molecule as it is the major amino acid where the radio-iodine is incorporated. Iodine atoms substitute ortho to the hydroxyl group in the phenol ring of tyrosine. The greater the incorporation into a compound, the higher the specific activity of the resulting labeled preparation. The specific activity will vary depending on the molecular weight of the protein (ie. μCi/μg).

As stated previously in the literature review, PAI-2 has been shown to inhibit the activity of u-PA both in solution and receptor bound activator. Ellis *et al.,* (1990) found both PAI-1 and PAI-2 binding to receptor bound u-PA with an association rate constants that was approximately 40% lower than for u-PA in solution. This study set out to examine: (a) whether human recombinant PAI-2 can inhibit u-PA activity of the human colon cancer cell line HCT116; (b) if PAI-2 can bind to HCT116 cells *in vitro*; and (c) determine if there are other protein(s) apart from u-PA responsible for binding PAI-2 on HCT116 cells.

2.2 MATERIALS AND METHODS

2.2.1 Reagents

Recombinant human non-glycosylated PAI-2 (47kDa) was from Biotech Australia, Roseville, Australia. ^{125}I-Na was obtained either from ICN Biomedicals, NSW,

Australia or from Amersham Ltd., UK. Iodo-beads^{R} and Disuccinimidyl suberate (DSS) were obtained from Pierce Chemical Co., Rockford, IL, USA. Bovine serum albumin (BSA), 5,5'-dithiobis (2-nitrobenzoic acid) (DTNB), 6-aminocaproic acid, amiloride, phenylmethylsulfonyl fluoride (PMSF) and propidium iodide (PI) were obtained from Sigma Chemical Co., St Louis, MO, USA. NAP-10 columns (containing Sephadex G-25) and pre-stained high and low molecular weight standards were obtained from Pharmacia, Uppsala, Sweden. Reagents and standards for SDS-PAGE and the DC protein assay kit were from Bio-Rad Laboratories, North Ryde, NSW, Australia. Seeblue SDS-PAGE prestained molecular weight standards and Mark 12 SDS-PAGE molecular weight standards were from Novex Australia Pty. Ltd., Terrey Hills, NSW, Australia. Human urokinase containing both the 33kDa and 55kDa form of u-PA were obtained from Serono Australia Pty. Ltd., Frenchs Forest, NSW, Australia. L-Glutamyl-L-Glycyl-L-Arginyl chloromethyl ketone (EGR-CMK) was purchased from Calbiochem-Novabiochem, Sydney, Australia. The plasminogen, urokinase high molecular weight (used only as standards when measuring u-PA activity) and antibodies directed against human u-PA (#394 and #3986), u-PAR (#3936) and t-PA (#387) were purchased from America Diagnostica Incorporated, Greenwich, CT, USA. The hybridoma DNP-9 (immunoglobulin G1 (IgG1) isotype control) was a kind gift from Dr. Mark Wilson, University of Wollongong, Wollongong, NSW, Australia. The IgG2a isotype control (G155-78) was from Pharmingen, San Diego, CA, USA and was directed against trinitrophenol (TNP). Fluorescein isothiocyanate (FITC) labelled affinity isolated $F(ab)'_2$ fragments

of anti-mouse immunoglobulin was obtained from Silenus, Hawthorn, VIC, Australia. High performance autoradiography film (Hyperfilm) was from Amersham Ltd., UK. Fetal bovine serum (FBS), was purchased from CSL Limited, VIC, Australia. RPMI 1640 culture media and trypsin/ ethylenediaminetetraacetic acid (EDTA) were from Trace Bioscientific, Ryde, NSW, Australia. Z-lysine thiobenzyl ester (ZLS) was from Peninsula Laboratories, Belmont, CA. All optical density (OD) readings were made using the BioRad Plate Reader from Bio-Rad Laboratories, North Ryde, NSW, Australia. Flow cytometric readings were carried out using the FACSorter from Becton Dickinson, Australia. All radioactivity measurements were made using a gamma counter, Auto Gamma 5000R, Packard Instrument Co., VIC, Australia.

2.2.2 Cell culture

The human colon cancer cell lines, HCT116 and LIM1215 were obtained from the American Type Tissue Collection (ATTC). The cell lines were free of mycoplasma as judged by the Hoechst stain #33258 (Hu *et al.,* 1995). Both cell lines were cultured in RPMI 1640 media containing 10% (v/v) heat inactivated FBS, 4mM L-glutamine, 10mM HEPES and 24mM $NaHCO_3$. Cells were incubated in a humidified incubator at 37°C in 5% (v/v) CO_2.

Cells were grown until approximately 100% confluent and then passaged. Passaging of the two cells types, which were both adherent cell lines, consisted of incubation

with 5ml trypsin/EDTA for 3min at 37°C. The flasks were then shaken to remove the cells off the bottom. The activity of trypsin was neutralised by the addition of RPMI+10% (v/v) FBS. The cell suspensions were then transferred to a tube and centrifuged at 200g for 3min at room temperature (RT) to pellet cells. The supernatant was discarded and cells resuspended in RPMI+10% (v/v) FBS. Cell viability and numbers were assessed using a haemocytometer and the trypan blue exclusion method. The cells (10^5) were placed into new 75cm^2 flasks and RPMI+10% (v/v) FBS was added. Culture media was changed as required. The cells were left to grow to approximately 100% confluency before use in all subsequent experiments.

2.2.3 Flow cytometric analysis of cell surface u-PA and u-PAR on viable HCT116 and LIM1215 cells

2.2.3.1 Detection of cell surface u-PA and u-PAR

Confluent HCT116 and LIM1215 cells were harvested by the addition of trypsin/EDTA for 3min at 37°C. RPMI+10% (v/v) FBS was added and the cells transferred to a tube and centrifuged at 200g for 3min at RT. The supernatant was discarded and the cells were resuspended in RPMI+0.1% (w/v) BSA and centrifuged again at 200g for 3min at RT. The supernatant was discarded as before and the cells

resuspended again in RMPI+0.1% (w/v) BSA. Cell number and viability were then determined with trypan blue staining and the use of the hemocytometer.

Using sterile pipettes cells ($2x10^6$/well) were placed into vee-bottomed 96-well polystyrene plates. The cells were then pelleted by centrifugation at 200g for 2min at 4°C. The supernatant was discarded and the cell pellets resuspended in 50µl of varying concentrations (2.5, 5, 10, 20 & 100µg/ml) of antibodies, directed against human u-PA and u-PAR, diluted in RPMI+0.1% (w/v) BSA and left for 30min at 4°C. After 30min, RPMI+0.1% (w/v) BSA was added to each well (total volume of 200µl) then cells were pelleted by centrifugation at 200g for 2min at 4°C and washed. The washes consisted of resuspending the cells in 200µl of RPMI+0.1% (w/v) BSA and then pelleting the cells by centrifugation at 200g for 2min at 4°C. This was repeated twice. Following the washes, FITC labeled affinity isolated F(ab)'2 fragments of anti-mouse immunoglobulin diluted 1:50 in RPMI+0.1% (w/v) BSA was added to the cells and left at 4°C for 30min. The cells were then washed three times as before and resuspended in 200µl RPMI+0.1% (w/v) BSA.

The cell suspensions were then transferred to tubes for use with the FACSort. Prior to determining the cell-associated fluorescence, PI was added to the cells to give a final concentration of 5µg/ml. Cells were then left for 5min at 4°C in the dark. PI is a fluorochrome which stains DNA, hence the addition of PI allowed for determination of the cell viability by flow cytometry (Wrobel *et al.*, 1996; Deere *et al.*, 1998).

Viable cells exclude PI while non-viable cells become stained. A total of either 20,000 or 50,000 events were acquired in each experiment.

2.2.3.2 Effects of plasminogen treatment on cell surface u-PA and u-PAR levels

The two cell types were harvested as above (section 2.2.3.1). Following centrifugation after trypsin/EDTA treatment the cells were divided into two separate tubes and resuspended in RPMI+0.1% (w/v) BSA in either the presence or absence of 400μg/ml plasminogen (final concentration of 20μg/ml) and then placed in a 37°C water bath for 15min. Cells were then pelleted with centrifugation at 200g for 3min at RT. The pellets were resuspended in RPMI+0.1% (w/v) BSA and cell viability and number were determined. Using sterile pipettes, cells ($2x10^6$/well) were placed into vee-bottomed 96-well polystyrene plates then pelleted by centrifugation at 200g for 2min at 4°C. The supernatant was discarded and cell pellets resuspended in 50μl of either antibodies against u-PA (#3689) or u-PAR (#3936) (20μg/ml, diluted in RPMI+0.1% (w/v) BSA). Cells were left with the antibodies for 30min at 4°C. After 30min, cells were treated as above for the rest of the experiment leading to subsequent determination of cell-associated fluorescence by flow cytometry using the same settings from the above experiments (section 2.2.3.1).

2.2.3.3 Data analysis

From the mean fluorescence reading of the flow cytometer, the cell-associated fluorescence was calculated for each of the two test antibodies used. Cell-associated fluorescence was calculated by dividing the mean fluorescence obtained from the test antibodies by the mean fluorescence obtained in the presence of the specific isotype for each antibody. Values were presented as the mean ± standard deviation (SD) from at least two separate experiments.

2.2.4 u-PA activity of HCT116

2.2.4.1 Lysis of HCT116 cells

Five 75cm^2 flasks containing confluent HCT116 cells were rinsed three times with 20ml ice cold phosphate buffered saline (PBS). After the final rinse, 5ml of 50mM glycine, pH 8, 0.5% (v/v) Triton X-100 (lysis buffer) was added to each flask. The flasks were then placed in a -20°C freezer and left until the buffer had frozen. The flasks were then removed and placed at 4°C until the buffer thawed. This freeze and thaw cycle was carried out a total of five times. After the final cycle, cells were transferred to 50ml tubes and any cellular debris present were pelleted with centrifugation at 1000g for 15min at 4°C. The supernatant was collected after centrifugation. The protein contents of the supernatant was measured using the Bio-

Rad DC kit. The supernatant was then transferred to tubes, in 500µl volumes, and stored at -20°C for later use.

2.2.4.2 Measurement of u-PA activity of HCT116

u-PA activity of HCT116 cells were measured using the Coleman and Green assay (1981). u-PA standards and HCT116 cell lysates were diluted in 50mM glycine, pH 9. The u-PA standard (0 to 50mIU) and cell lysates (7.5mg of total protein) were placed in 96 well plates (total volume of 20µl) in two sets of duplicates. HCT116 cells lysates were either added alone, with 10µg/ml PAI-2, or with 10µg/ml inhibitory monoclonal antibody against active human u-PA (#394). To one set of duplicate, 20µl of 100µg/ml plasminogen which was diluted in GTGE buffer (see Appendix) was added. To the other set of duplicate u-PA standards and cell lysates, 20µl of GTGE buffer only was added. The plate was then covered and placed on a shaker in an incubator set at 37°C and left for 30min with gentle shaking. Prior to the end of the 30min, under the fume hood, 20µl of the plasmin substrate, ZLS, was added to 20ml of PLA buffer (see Appendix). The ZLS mixture (160µl/well) was then added and the plate was left to shake again at 37°C for 15min. The absorbance was then measured at 415nm using a BioRad plate reader. The u-PA activity of HCT116 cell lysates were calculated from the u-PA standard curve. The samples containing no plasminogen were used as the background, hence this was subtracted from

corresponding samples with plasminogen to obtain the corrected absorbance values. The data is presented as the mean ± SD from two separate experiments.

2.2.5 Iodination of recombinant human PAI-2 and characterisation of ^{125}I-PAI-2

2.2.5.1 Iodination

Human recombinant PAI-2 was radiolabeled with Na-^{125}I using the Iodo-bead procedure (Markwell, 1982). Three iodobeads were placed in a 1.5ml tube and washed using iodination buffer (0.1M sodium phosphate buffer pH 7.2). The beads were then air-dried on a tissue for 5min at RT. All iodination reaction were carried out in 1.5ml tubes placed in lead containers. With the beads in a dry tube iodination buffer was added. To this 0.5 mCi Na-^{125}I was then added and left for 5min at RT. Following this, 80μg of PAI-2 was added and the reaction continued at RT for 15min, with gentle shaking every three minutes. The total reaction volume was 500μl. The reaction was stopped by removing the iodination mix from the beads and placing into a new 1.5ml tube. The beads were then washed with 500μl of iodination buffer, and the wash pooled with the iodination mix.

^{125}I-PAI-2 was separated from free ^{125}I on a NAP-10 column previously equilibrated with elution buffer (50mM sodium phosphate buffer pH 7.4, 0.1% (w/v) BSA). This

was achieved by passing three bed volumes of elution buffer through the column. Prior to addition of the iodination mix (total volume of approximately 1ml) to the column, three 2µl samples were taken for measurement of radioactivity. The iodination mix was then added to the column and allowed to drain through. This was all collected as the first fraction. Then 200µl elution buffer was added and collected as one fraction. This step was then repeated. After this, more elution buffer was added and eleven 500 µl fractions were collected. A total of 14 fractions were collected for each iodination procedure. Two 2µl samples were taken from each fraction, transferred to radioimmunoassay (RIA) tubes and the radioactivity measured. The two fractions (fractions 4 & 5) containing ^{125}I-PAI-2 were pooled and stored at 4°C. All ^{125}I-PAI-2 preparations were used within 3 weeks after iodination either for experiments carried out in this chapter or the next chapter.

2.2.5.2 Characterisation of ^{125}I-PAI-2

The inhibitory activity of each ^{125}I-PAI-2 preparation was examined by incubating with u-PA or EGR-CMK-inactivated u-PA. u-PA (500ng) was inactivated with 0.5mM EGR-CMK for 20min at RT. ^{125}I-PAI-2 (50ng) was added to either 500ng of active or inactivated u-PA and left for 40min at RT (total reaction volume was 15µl). The reactions were stopped by the addition of 5µl of 4x non-reducing SDS-PAGE sample buffer. The 12% SDS-PAGE gels were prepared according to the method of Laemmli *et al.*, (1970). Samples were then loaded into the wells and the gel was

conducted at 200V in electrophoresis buffer (3.0g/l Tris base, 14.4g/l glycine, 1.0g/l SDS). Following this, the gel was fixed by placing in 50% (v/v) methanol and 10% (v/v) acetic acid for 30-60min. The gel was then dried for 3hrs using a Bio-Rad gel dryer. Dried gels were then exposed to Hyperfilm and left at -70°C overnight for autoradiography. Either prestained (Bio-Rad low or high molecular weight standards or Seeblue standards) or unstained (Mark 12) molecular weight protein standards were used. When Mark 12 molecular weight protein standards were used, the gels were stained with 0.2% (w/v) Coomassie Blue R250, 50% (v/v) methanol and 10% (v/v) acetic acid for 2-12hr at RT. To remove the excess stain the gels were then by placed in 50% (v/v) methanol and 10% (v/v) acetic acid for 3-12h at RT. The gels were then dried and exposed to film as previous stated above.

To determine the molar ratio at which human recombinant ^{125}I-PAI-2 would react with u-PA, 50ng ^{125}I-PAI-2 was incubated with varying concentrations of u-PA (10, 20, 40, 200 and 400ng) for 40min at RT. As controls ^{125}I-PAI-2 was also incubated with inactivated u-PA at a ratio of 1:1. u-PA (50ng) was inactivated with either 0.5mM EGR-CMK or 10μg/ml u-PA inhibitory monoclonal antibody #394 for 20min at RT. Complex formation was examined after separation on 12% SDS-PAGE and autoradiography. Analysis and quantification of resulting protein bands were carried out using densitometry. This was repeated another two times on different preparations of ^{125}I-PAI-2.

2.2.6 ^{125}I-PAI-2 binding assay

2.2.6.1 Optimisation of plasminogen concentration

Once the HCT116 cultures were approximately 100% confluent, cells were left in the old media and were not used until 1 to 2 days later. The purpose of this was to deplete all traces of plasmin activity from the FBS. The media was discarded and the cells rinsed with 5ml of RPMI+0.1% (w/v) BSA. The cells were then harvested and cell viability determined as described in section 2.2.3.

Various concentrations of plasminogen (1, 10, 20, 60 & 100μg/ml) were added to cells ($2x10^6$) and incubated in a 37°C water bath for 15min. Three washes were carried out after 15min. The washes consisted of resuspending cells in 1ml RPMI+0.1% (w/v) BSA then pelleting the cells by centrifuging at 3000rpm for 2min at RT. The supernatant was then discarded and the wash step repeated. ^{125}I-PAI-2 (1.3μg) was added to cells (in RPMI+0.1% (w/v) BSA, final volume of 500μl) and left at 4°C for 6h. Three washes, as described above, were carried out before transferring the cells to RIA tubes for measurement of cell-associated radioactivity. This experiment was repeated another three times with either duplicates or triplicates of samples per experiment. After determination of cell-associated radioactivity cell viability was determined using the trypan blue exclusion method for alternate cell binding experiments performed throughout this chapter.

2.2.6.2 Time course of ^{125}I-PAI-2 binding to HCT116 cells

HCT116 cells were harvested as described above (section 2.2.6.1). Cells were then placed in a 37°C water bath either in the presence or absence of 20μg/ml plasminogen (in RPMI+0.1% (w/v) BSA) for 15min. After 15min, cells were pelleted by centrifugation at 200g for 3min at RT and resuspended in RPMI+0.1% (w/v) BSA. Cells were then washed three times. Washes consisted of resuspending cells in 5ml RPMI+0.1% (w/v) BSA followed by centrifugation at 200g for 3min at RT. After the final wash, cells ($2x10^6$ cells) were left in ^{125}I-PAI-2 either in the absence or presence of a 50-fold excess of unlabelled PAI-2 (total volume of 200μl) for various times (2, 4, 6, 8, 10, 20 & 24h) at 4°C. At the end of the time period, cells were washed three times with RPMI+0.1% (w/v) BSA as described above (section 2.2.6.1). Cells were then transferred to RIA tubes for radioactivity measurement. Experiments from cells not treated with plasminogen were repeated three times. Experiments from cells treated with plasminogen were repeated four times. In all experiments triplicates of samples were carried out for each time point. Measurements of radioactivity was determined at different times, so to account for possible decay of radioactivity and instrumental errors, duplicates of the ^{125}I-PAI-2 source that was used in the experiment were also counted with the cells at each time point.

2.2.6.3 Optimisation of ^{125}I-PAI-2 concentration

The cells were harvested and were either treated or not treated with plasminogen as described above (section 2.2.6.2). Cell viability and number were then determined for

both groups. Cells (2-8x10^6 cells/tube) were placed into 1.5ml tubes and pelleted with centrifugation at 3000rpm for 2min at RT. To determine the specific binding of ^{125}I-PAI-2 by the HCT116 cells, prior to the addition of ^{125}I-PAI-2, a 50-fold excess of unlabelled PAI-2 was added for each concentration of ^{125}I-PAI-2. The concentrations of ^{125}I-PAI-2 added were; 62.5, 125, 250, 500 and 750ng/ml. The reaction volume was made up to a total of 200µl with RPMI+0.1% (w/v) BSA. Cells were then left at 4°C for 6h. Following this the cells were washed three times as described previously (section 2.2.6.1) and transferred to RIA tubes and the cell-associated radioactivity measured. This experiment was repeated another two times with either duplicates or triplicates of each concentration in each experiment. The specific binding of ^{125}I-PAI-2 to HCT116 was calculated by subtracting the non-specific binding (binding in the presence of unlabelled PAI-2) from the total binding (binding in the absence of unlabelled PAI-2) for both plasminogen treated and untreated cells.

2.2.6.4 Scatchard analysis of ^{125}I-PAI-2 binding to HCT116 cells

Scatchard analysis (Scatchard, 1949) was used to calculate the number of ^{125}I-PAI-2 binding sites and K_d value, on the HCT116 cells for individual experiments. The data are expressed as the mean ± SD of values obtained from three experiments.

2.2.6.5 Optimisation of inhibitors of u-PA activity

To determine whether ^{125}I-PAI-2 bound specifically to active u-PA on HCT116 inhibitors of u-PA were used in competition assays. The u-PA inhibitors used were a u-PA monoclonal antibody (#394), EGR-CMK and amiloride (Vassalli & Belin, 1987). Varying concentrations of inhibitors were used to determine optimal concentrations at which these inhibitors would maximally inhibit ^{125}I-PAI-2 binding. Cells were harvested, treated with plasminogen, washed and cell viability and number determined as stated previously (section 2.2.6.2). Cells were then aliquotted into 1.5ml tubes (2-6x10^6 cells/tube).

To each tube varying concentrations of inhibitors were added prior to the addition of ^{125}I-PAI-2 (500ng/ml). The concentrations used for monoclonal antibody #394 were 1, 5, 10, 20 and 40μg/ml. The concentrations of EGR-CMK were 0.1, 0.25, 0.5, 1, 2 and 8mM. The concentrations tested for amiloride were, 0.1, 0.5, 1, 2, 4, 8 and 10mM. In each experiment, two controls were used. The first control consisted of cells without inhibitors present. While a 50-fold excess of unlabelled PAI-2 was added to cells in the second control. The cells were left in ^{125}I-PAI-2 for 6h at 4°C. This was followed by three washes as described above (section 2.2.6.1). The cell-associated radioactivity was then measured. This experiment was carried out twice with either duplicates or triplicates of each concentration in each experiment.

2.2.6.6 Specific inhibition of ^{125}I-PAI-2 binding to HCT116 cells

Using the optimal concentrations of inhibitors obtained from the above experiments, the binding of ^{125}I-PAI-2 to component(s) of the plasminogen activation cascade was elucidated. Inhibitors of u-PA (10μg/ml #394, 1mM amiloride, 0.5mM EGR-CMK and a 50-fold excess of unlabelled PAI-2), an inhibitory monoclonal antibody against the activity of human t-PA (10μg/ml #387) and an inhibitor of plasmin activity (1mM aprotinin) (Cajot *et al.*, 1989) were used. All methods up to cells being placed into tubes were carried out as described above (section 2.2.6.1).

The various inhibitors were added (2-8x10^6 cells/eppendorf) at the stated concentrations, followed by addition of 500ng ^{125}I-PAI-2 and cells were left at 4°C for 6h. As controls, cells were either treated or not treated with plasminogen with no inhibitors present. After 6h, cells were washed three times (section 2.2.6.1) and the cell-associated radioactivity measured. This experiment with all the inhibitors tested was carried out two times with triplicates of each sample per experiment.

2.2.7 SDS-PAGE analysis of ^{125}I-PAI-2 binding to HCT116 cells

HCT116 cells were harvested (section 2.2.6.1) and all the cells were treated with 20μg/ml plasminogen (section 2.2.6.2). Using pipettes and sterile tips, cells were placed in 1.5ml tubes (4x10^6 cells/tube). ^{125}I-PAI-2 (500ng) was then added and the

cells left for various times (5, 10, 30 and 90min) at 4°C. After each time point, the cells were washed three times as described previously (section 2.2.6.1). Following this, each cell pellet was then resuspended in 50µl of 4x non-reducing SDS-PAGE sample buffer and boiled for 10min. The samples were then centrifuged at 14,000rpm for 5min at RT and 20µl of the supernatant loaded into the wells of a 12% SDS-PAGE. Gels were conducted and dried as in section 2.2.4. Gels were then exposed to Hyperfilm and stored at -70°C for autoradiography. This experiment was carried out three times.

In a separate experiment HCT116 cells (10^7) were either treated or not treated with plasminogen (section 2.2.6.2). To some of the cells, a 5-fold excess of unlabelled PAI-2 was added. ^{125}I-PAI-2 (5µg) was then added to all the cells and left at 4°C for 6h. After 6h, cells were washed three times with RPMI + 0.1% (w/v) BSA (section 2.2.6.1). Following the final wash, cells were resuspended in 500µl 4x non-reducing sample buffer and boiled for 10min. This was followed with a centrifugation at 14,000rpm for 10min at RT to pellet down cellular DNA. The samples (40µl) were then separated on a 12% SDS-PAGE. The gel was stained, then placed in destaining solution and subsequently dried for 3h. The dried gel was exposed to a phosphor screen for phosphor imaging using the Storm system.

2.2.8 Cross-linking of ^{125}I-PAI-2 to HCT116

2.2.8.1 Purification of plasma membrane from HCT116 cells

A two-phase polymer system was implemented to purify membrane preparations of HCT116 cells. The two polymers used were dextran and polyethylene glycol (PEG), which must be prepared prior to the day of purification of the plasma membrane. This involved dissolving 5.5g dextran and 4.2g PEG in 100ml 0.1M phosphate buffer, pH6.5. The mixture was placed in a separating funnel and left overnight at 4°C. The upper (PEG) and lower (dextran) phases were collected in separate bottles and stored at 4°C.

HCT116 cells were grown to 100% confluency in 125cm^2 tissue culture flasks (a total of 5 flasks). Prior to the purification procedure cells were rinsed three times with ice-cold PBS, then scraped off the flask using a cell rake. The cell suspension was transferred to a centrifuge tube and pelleted by centrifugation at 500g for 5min at 4°C. Cells were then resuspended and left in ice-cold homogenization buffer (5mM Tris-HCl pH7.6, 1mM PMSF) at 4°C for 10min. PMSF, a serine protease inhibitor, was added prior to use, as it has a half life of 100min in aqueous solutions. A glass dounce homogenizer was used to break-up cells (cell damage was checked regularly under the light microscope).

The cellular suspension was transferred to Corex glass tubes and centrifuged (Sorvall HB-4 swing out rotor) at 5000rpm for 10min at 4°C. The supernatant was discarded and the pellet was resuspended in 10ml PEG. Then 10ml of dextran was added and the suspension mixed thoroughly. This was followed by centrifugation at 6000rpm for 10min at 4°C. The plasma membrane enriched fraction (found at interface of upper and lower phases) was aspirated into another clean glass tube and resuspended in another 10ml PEG. Then 10ml dextran was added and thoroughly mixed again. The suspension was then centrifuged at 6000rpm for 10min at 4°C. After this centrifugation the resulting plasma membrane enriched fraction was aspirated and resuspended in ice cold distilled water. This suspension was centrifuged again at 6000rpm for 10min at 4°C. The supernatant was discarded and the pellet resuspended in 1ml of ice-cold distilled water. It was then centrifuged at 14,000rpm for 10min at 4°C in a microfuge. The supernatant was discarded and the plasma membrane enriched fraction was resuspended in RIPA solubilization buffer (10mM Tris-HCl, pH 7.4; 1% (v/v) Triton X-100; 1% (w/v) deoxycholate). The protein concentration was determined and the plasma membrane enriched fractions stored at -20°C in small aliquots for later use.

2.2.8.2 Cross-linking with DSS

HCT116 cells were harvested and both cells and the plasma membrane enriched fractions were treated with plasminogen (2.2.6.2). ^{125}I-PAI-2 (500ng) was added to

HCT116 cells ($4x10^6$ cells) or 2mg of total protein from the HCT116 plasma membrane enriched fractions in a reaction volume of 200µl. The cells and plasma membrane fractions were then left for 6h at 4°C. The cells and plasma membrane enriched fractions were then pelleted by centrifugation at 2000rpm for 3min at 4°C and the supernatant discarded.

The cross-linker, 0.1mM DSS in reaction buffer (20mM $NaHPO_4$, 0.15M NaCl, pH 7.5) in a volume of 200µl was added and left for 1h at 4°C. 0.1M Tris solution, pH 5 (final concentration of 0.01mM) was added after 1h to stop the reaction. The cells and plasma membrane enriched fractions were then washed 3 times as previously described (section 2.2.6.1). Then 100µl of 4x non-reducing sample buffer was added. The samples were boiled for 5min and 20µl of the samples loaded into the wells of a 12% SDS-PAGE gel. The gels were then conducted, stained, dried and exposed to Hyperfilm for autoradiography as described in section 2.2.4.2.

2.2.9 ^{125}I-PAI-2 binding and degradation at 37°C

A flask of confluent HCT116 was passaged (section 2.2.2) and cells (10^5 cells/well) were placed in a 24-well plate. The cells were left to grow to confluency with changes of media as required. Once approximately 100% confluent, the media was changed and the cells were used 1-2 days later for the same reason as in section

2.2.6.1. The media was removed and the cells were rinsed three times with 3ml of RPMI+0.1% (w/v) BSA. Plasminogen (10μg/ml in RPMI+0.1% (w/v) BSA) was added to cells and incubated at 37°C for 15min. The cells were then washed 3 times. Washes consisted of the addition of 1ml RPMI+0.1% (w/v) BSA to the wells and then gently swirling the plate. The RPMI+0.1% (w/v) BSA was then aspirated and discarded.

After washing, to some of the cells either 10μg/ml a u-PA inhibitory monoclonal antibody (#394) or 10μg/ml aprotinin were added. ^{125}I-PAI-2 (500ng) was then added to all cells (final incubation volume of 500μl) and incubated at 37°C for 8h. After 8h, adherent cells were dissociated from the bottom of the wells by pipetting the solution up and down several times. The cellular suspension was then transferred to 1.5ml tubes and pelleted with centrifugation at 2000rpm for 3min at RT. A trichloroacetic acid (TCA) precipitation was then carried out on the supernatants. This involved taking 100μl volume of supernatant from the cellular suspension and adding it to 300μl of 20% (w/v) TCA. The samples were mixed thoroughly and then left at RT for 15min with constant shaking to precipitate any proteins in solution. Samples were then centrifuged at 14,000rpm in a microfuge for 10min at RT to pellet any precipitated protein. The 50μl volumes of the supernatant were placed into RIA tubes and the free ^{125}I in solution determined. TCA precipitation was also carried out on the ^{125}I-PAI-2 source (in the absence of cells) incubated at 37°C for 8h.

2.2.10 Statistical analysis of data

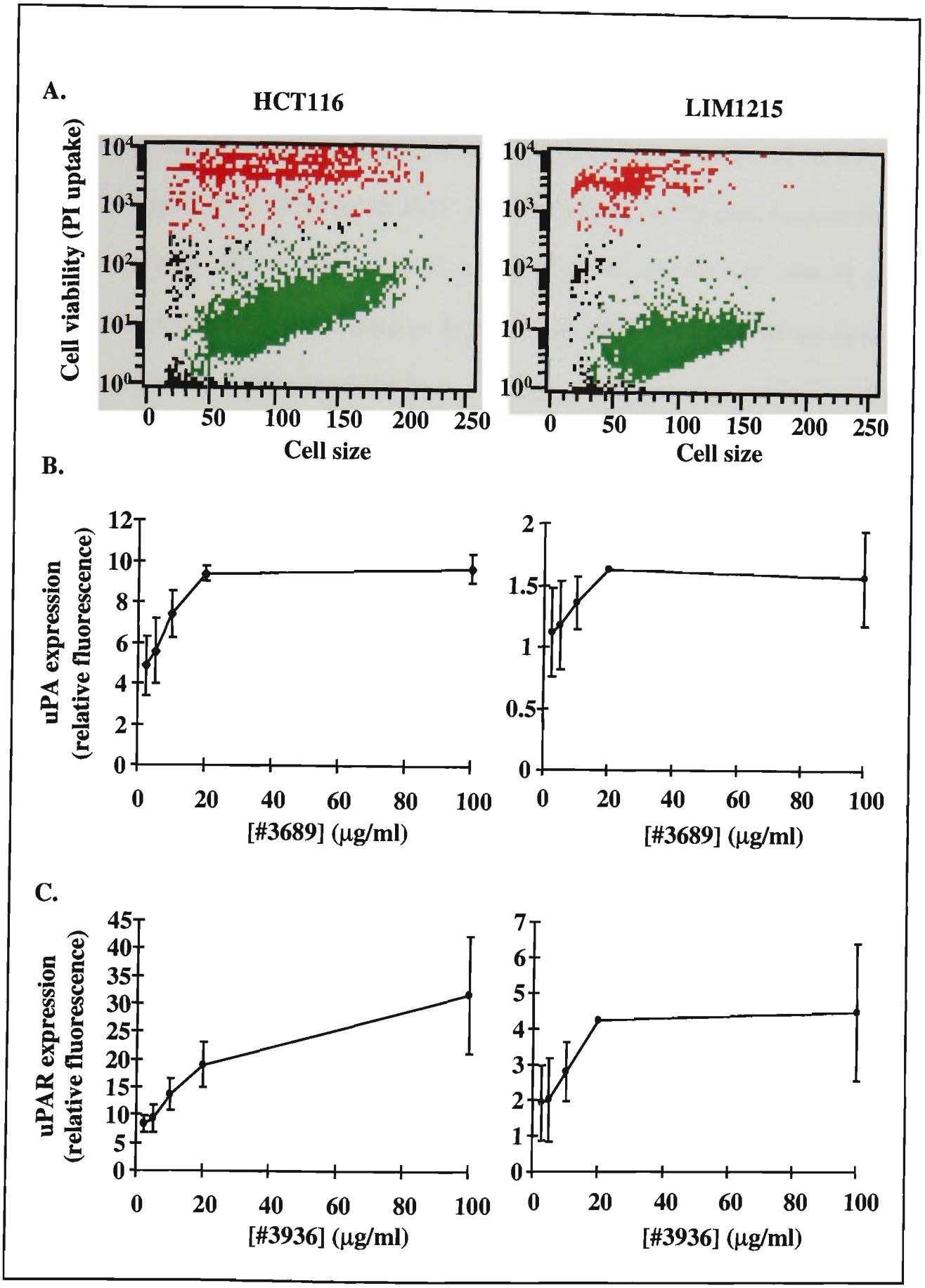
For all ^{125}I-PAI-2 binding experiments the data were expressed as the cell-associated radioactivity. This was calculated from the mean cpm from either duplicate or triplicate samples per 10^6 cells ± SD. Due to the varying specific activities of different batches of ^{125}I-PAI-2 used for some repeated experiments, a representative experiment was chosen to depict the overall results from repeats of each set of different experiments performed. The mean, SD values and all calculations were carried out using the program Microsoft Excel.

2.3 RESULTS

2.3.1 Cell-surface expression of u-PA and u-PAR on HCT116 and LIM1215 cells

The DNA stain, propidium iodide, was used to gate out the viable (green dots) from non-viable (red dots) cells as seen from the dot plots of HCT116 (Figure 1A, left plot) and LIM1215 (Figure 1A, right plot) cells. u-PA and u-PAR associated fluorescence was measured on viable cells using specific monoclonal antibodies directed against the two antigens. With increasing concentration of antibodies, the relative fluorescence increased. For the u-PA antibody, #3689, the relative fluorescence peaked and reached a plateau between 20μg/ml and 100μg/ml in both the HCT116

Figure 1. Flow cytometric analysis on the expression of uPA and uPAR on the cell surface of HCT116 and LIM1215 cells. The top panels (A.) are dot plots of cell viability vs cell size of the two cell types. Viable cells are green while non-viable cells are red. B. Graphical representation of the expression of uPA when HCT116 (on the left) and LIM1215 (on the right) cells were incubated with increasing concentrations of the uPA monoclonal antibody, #3689. C. These graphs represent the expression of uPAR with increasing concentrations of the uPAR monoclonal antibody, #3936. The values are expressed as mean ± SD of three separate experiments for HCT116 cells and two separate experiments for LIM1215 cells.



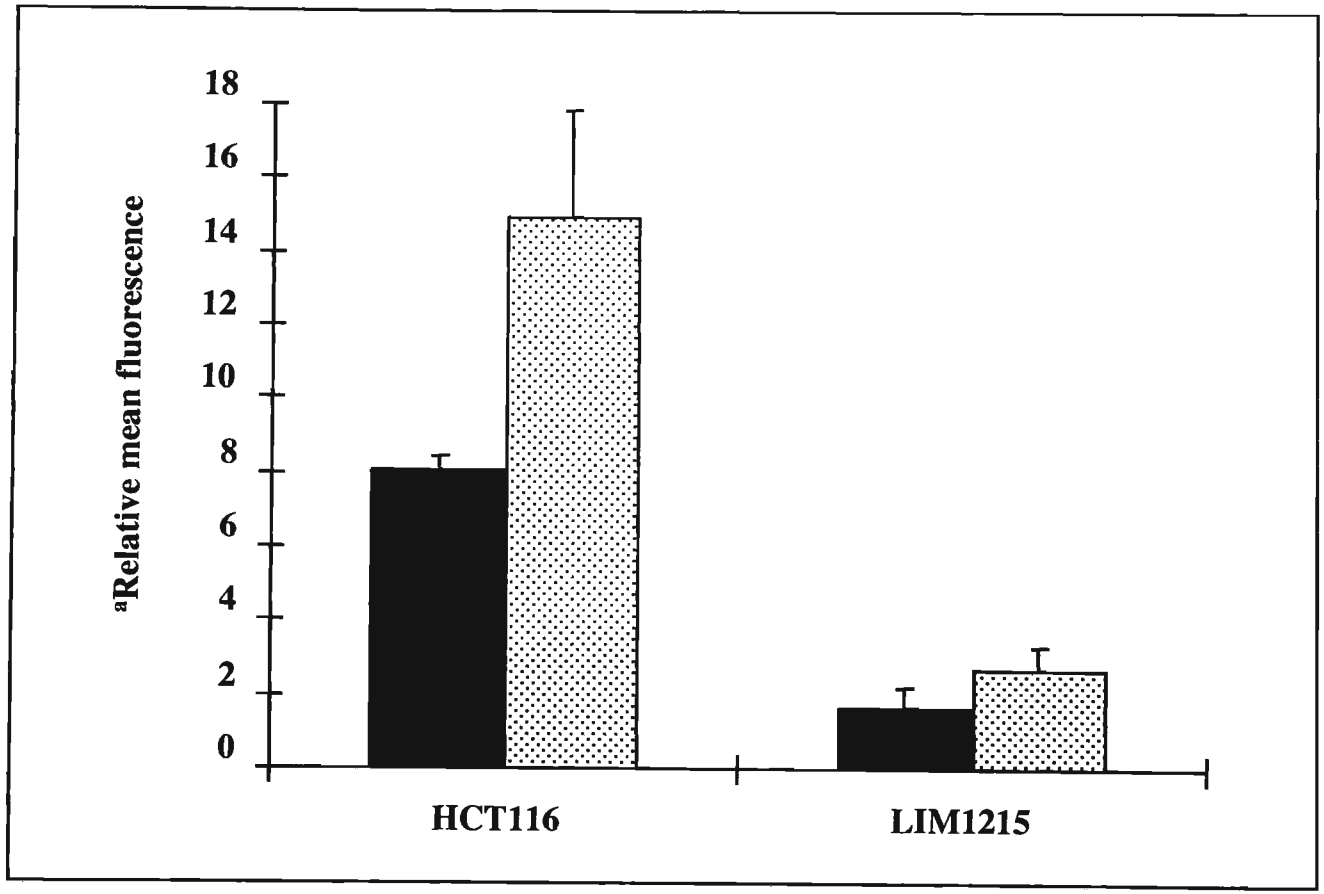
Relative fluorescence: the fluorescence of cells incubated with the monoclonal antibody divided by the fluorescence from cells incubated with the iostype control for each concentration of antibody used.

and LIM1215 cells (Figure 1B). The antibody for u-PAR, #3936, appeared to have peaked and reached a plateau in LIM1215 cells at a similar concentration as the u-PA antibody (Figure 1C). However, in HCT116 cells this was not the case, because the u-PAR antibody did not seem to reach a plateau at the highest concentration of 100μg/ml. A concentration of 20μg/ml for both antibodies was chosen for use in all subsequent flow cytometry experiments.

At 20μg/ml of u-PA antibody and u-PAR antibody, viable HCT116 cells expressed 5-fold and 5.5-fold more cell surface u-PA and u-PAR, respectively, than LIM1215 cells (Figure 2). Both HCT116 (1.8 times) and LIM1215 (1.6 times) cells expressed approximately twice the amount of cell surface u-PAR than u-PA. When both cell lines were treated with plasminogen prior to staining with the monoclonal antibodies, a 3-fold and 5.5-fold increase in cell surface u-PA (Figure 3A.) was observed in HCT116 and LIM1215 respectively, as compared to cells that were not treated. In contrast, plasminogen treatment had no effect on cell surface u-PAR (Figure 3B.) in both cell lines.

2.3.2 u-PA activity of HCT116

An indirect u-PA activity assay which measures plasmin generation was implemented to determine whether PAI-2 can also inhibit the u-PA activity of HCT116 cells, since flow cytometric analysis has confirmed that HCT116 cells used in this study express

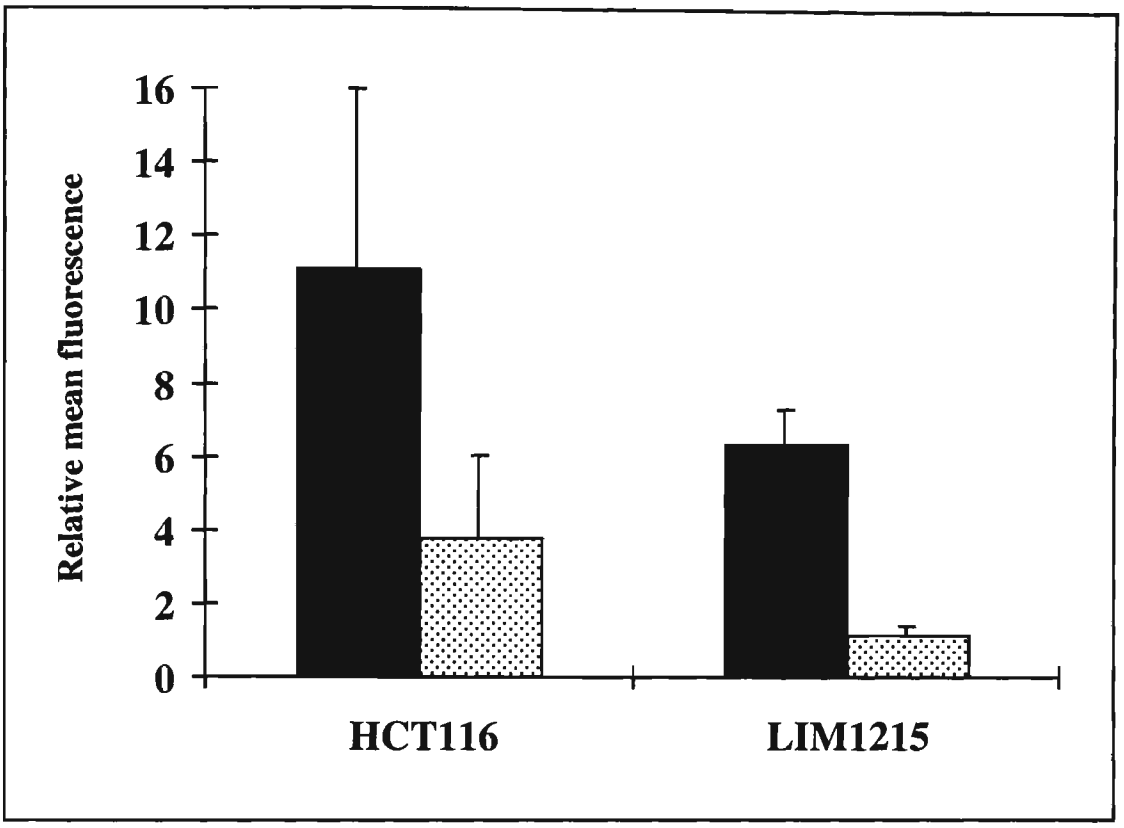


[a]Relative mean fluorescence: These values were obtained by dividing the mean fluorescence from cells stained with antibodies against uPA and against uPAR by the mean fluoresence of the isotype control for each antibody.

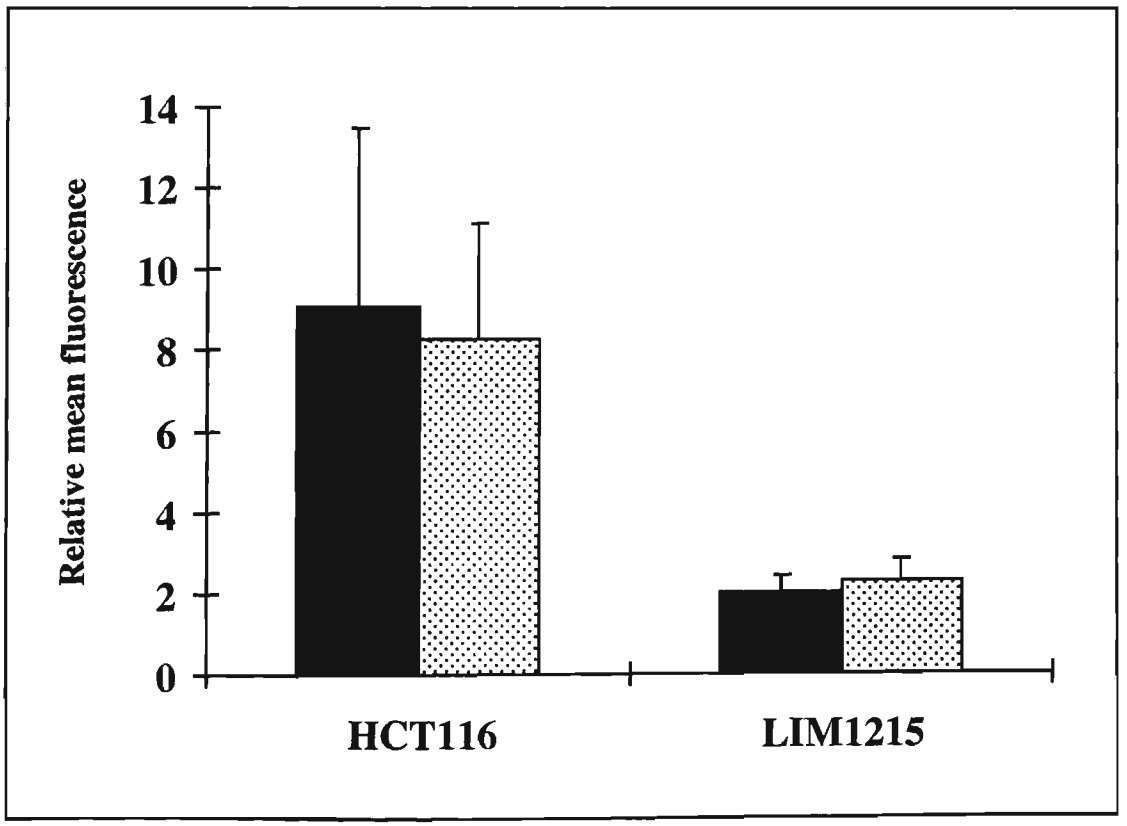
Figure 2. Cell surface expression of uPA and uPAR on HCT116 and LIM1215 cells. Using monoclonal antibodies , flow cytometric analysis was carried out examining the expression of uPA (■) and uPAR (⊡) on viable (as judged by exclusion of propidium iodide) HCT116 and LIM1215 cells. The data is presented as the mean ± SD of three separate experiments for HCT116 and two separate experiments for LIM1215 cells.

Figure 3. The effect of plasminogen treatment on cell surface expression of uPA and uPAR by HCT116 and LIM1215 cells. Using monoclonal antibodies the effects of plasminogen treatment (■) versus control (no treatment, ⚄) on cell-surface expression of uPA (graph A.) and uPAR (graph B.) on viable HCT116 and LIM1215 cells (as judged by the exclusion of propidium iodide) were examined by flow cytometric analysis. The data are presented as the mean ± SD of two experiments.

A.


Relative mean fluorescence
16
14
12
10
8
6
4
2
0
HCT116
LIM1215

B.


Relative mean fluorescence
14
12
10
8
6
4
2
0
HCT116
LIM1215

cell surface u-PA. HCT116 cell lysates alone, or in the presence of either recombinant human PAI-2 or an inhibitory monoclonal antibody directed against human u-PA (#394), were measured for u-PA activity (Figure 4). A reduction of approximately 90% in the activity of u-PA was observed in the presence of both PAI-2 and the u-PA monoclonal antibody as compared to HCT116 cell lysate without any inhibitors present. This indicates that both PAI-2 and the monoclonal antibody were capable of inhibiting the u-PA activity of HCT116 cells.

2.3.3 Active ^{125}I-PAI-2

Both preparations of ^{125}I-PAI-2 (*E. coli* and *S. cerevisiae*) gave similar banding patterns by SDS-PAGE. The specific radioactivity of the preparations ranged from 4 to >10x10^3 cpm/ng protein. Free ^{125}I remaining in protein preparations was ~10% of the total as determined by TCA precipitation.

The activity of ^{125}I-PAI-2 was analysed by incubation with a 10-fold excess of purified human u-PA or EGR-CMK-pre-inactivated u-PA (< 1% original activity). ^{125}I-PAI-2 alone (Figure 5A, lane 1) gave rise to two major bands at 46kDa and 118kDa and a very minor band at 208kDa. These bands probably represent the monomeric, dimeric and trimeric forms of PAI-2, respectively, since polymerisation of this serpin has been observed previously (Mikus *et al.*, 1993). From the density of these bands, the monomer and dimer constitute about 60% and 27% respectively of the ^{125}I-PAI-2 preparation. Incubation with active u-PA (33/55kDa) (lane 2) resulted

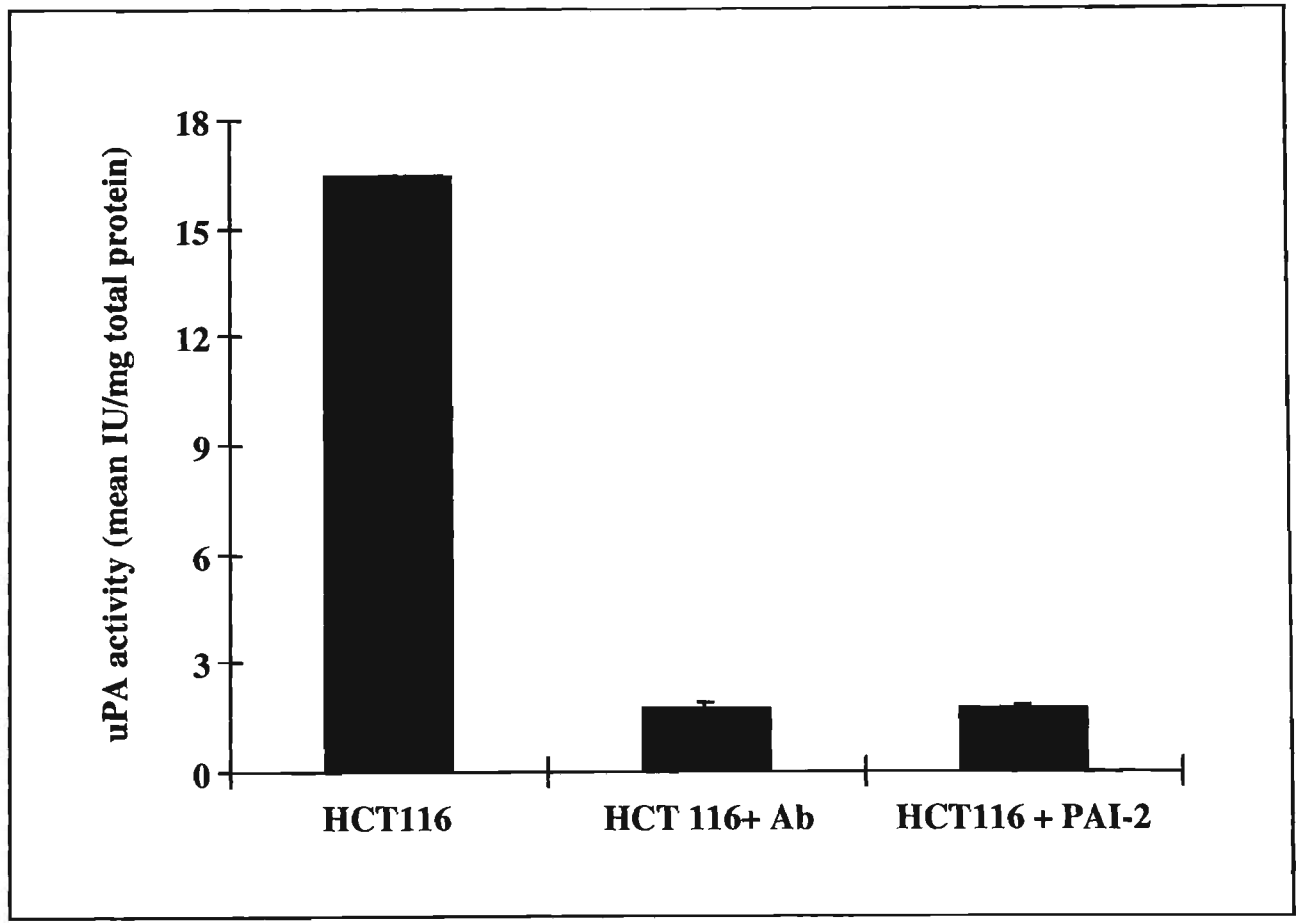
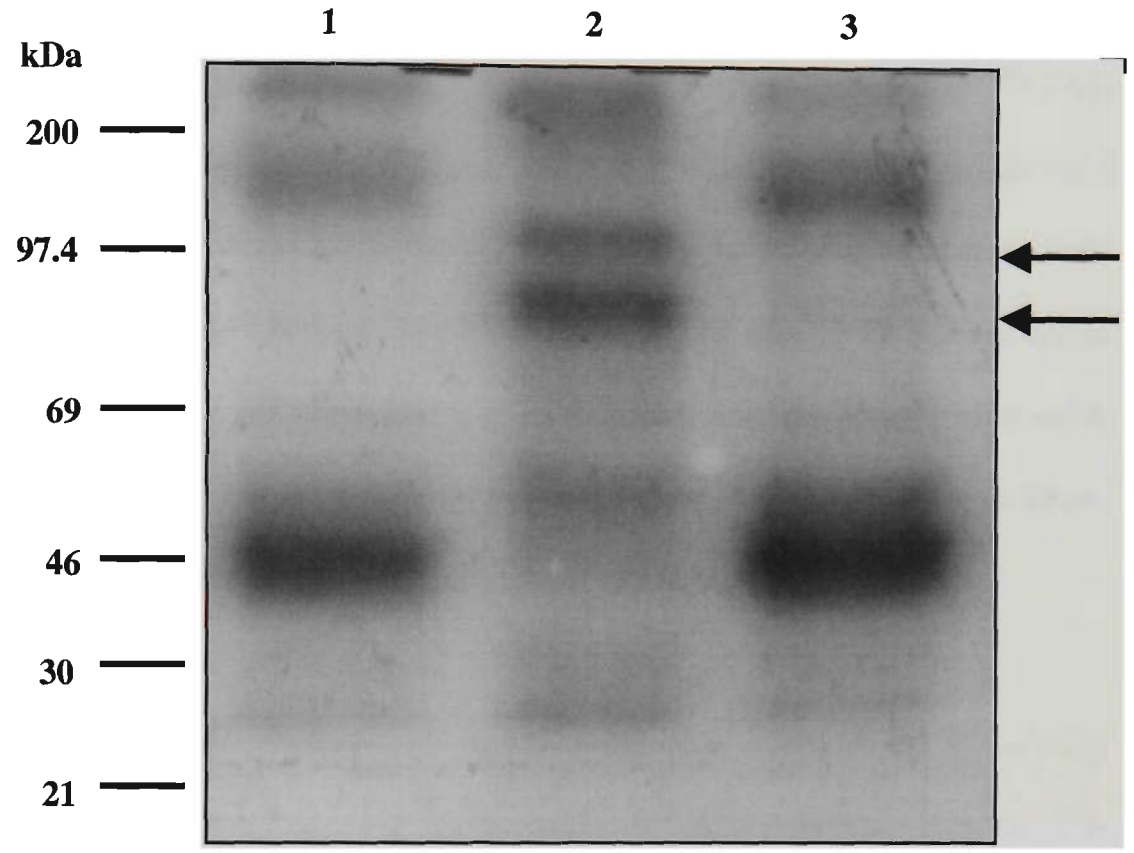


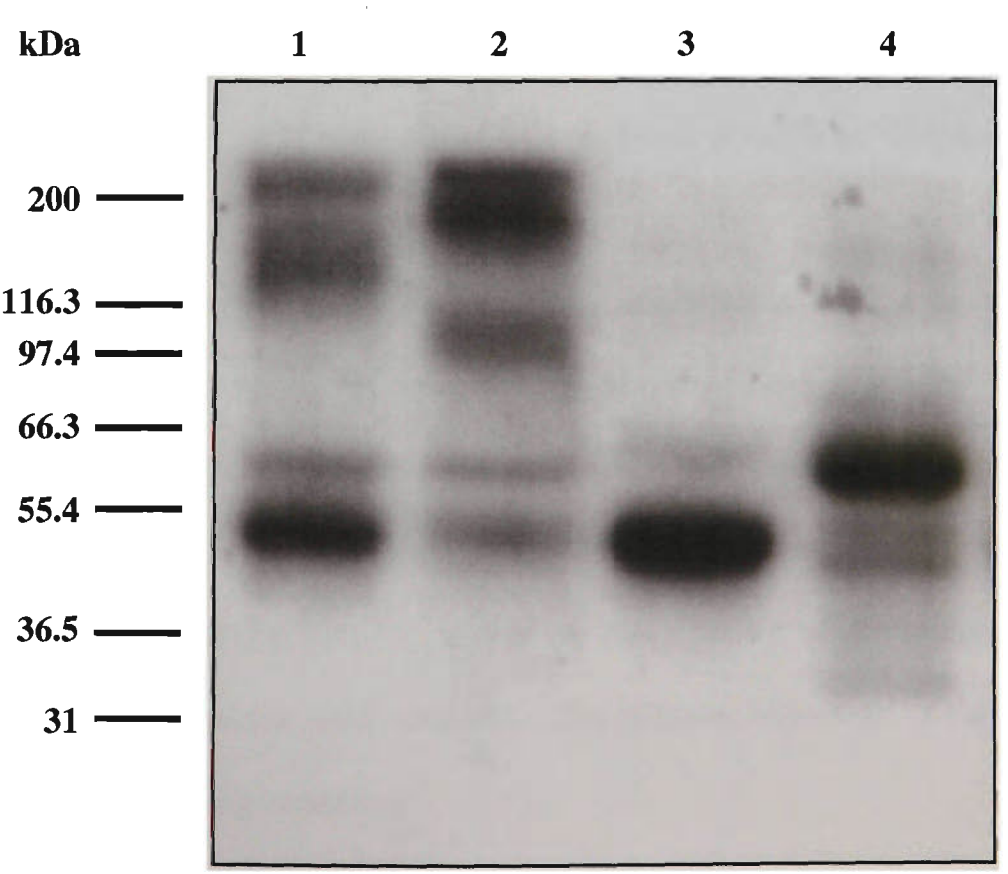
Figure 4. The effect of human recombinant PAI-2 and uPA inhibitory monoclonal antibody on uPA activity of HCT116 cell lysates. The uPA activity of HCT116 cell lysates alone, HCT116 cell lysates in the presence of an uPA inhibitory monoclonal antibody (#394) and HCT116 cell lysates in the presence of recombinant PAI-2 were indirectly measured by measuring the generation of plasmin. The data is presented as the mean ± SD from two experiments.

Figure 5. Characterisation of ^{125}I-PAI-2. A: Lane 1 is ^{125}I-PAI-2 alone. Lane 2 is ^{125}I-PAI-2 complexed with uPA in a ratio of 1:10. Lane 3 is ^{125}I-PAI-2 and EGR-CMK-inactivated uPA. All samples in this autoradiography were analysed under non-reducing conditions. The arrows on the right indicate the ^{125}I-PAI-2/uPA complexes of 82kDa and 98kDa respectively. B: Lanes 1 and 3 is ^{125}I-PAI-2 alone, while lanes 2 and 4 is ^{125}I-PAI-2 and uPA. Samples from lanes 1 and 2 were analysed under non-reducing conditions, while samples from lanes 3 and 4 were analysed under reducing conditions. The samples were separated on a 12% SDS-PAGE and subsequent autoradioagraph obtained. The ^{125}I-PAI-2 from each autoradiography were from two different iodination procedures.

A.



B.



in the appearance of new bands at 82kDa and 98kDa and a significant reduction in density of the 46kDa and 118kDa bands. This indicated the formation of ^{125}I-PAI-2/u-PA complexes with the low (33kDa) and high (55kDa) molecular weight (M_r) forms of u-PA. The density of the 46kDa and 118 kDa bands were reduced by 70% and 90%, respectively, indicating that more than 70% of the ^{125}I-PAI-2 was active after iodination. The 82kDa and 98kDa complex bands were not present when u-PA was inactivated by exposure to the peptidyl inhibitor EGR-CMK (Kettner & Shaw, 1979), which irreversibly blocks the active site of u-PA (lane 3).

Another preparation of ^{125}I-PAI-2 (Figure 5B) was also examined by SDS-PAGE under both non-reducing (lanes 1 & 2) and reducing conditions (lanes 3 & 4). ^{125}I-PAI-2 alone under non-reducing conditions gave rise to 3 distinct protein bands at 48kDa, 128kDa and 184kDa, which were also thought to be monomeric, dimeric and trimeric forms (lane 1). A minor band was observed at 62.5kDa which was also found in lanes 2 and 3. It is not known what this band may be because it was also observed weakly in some, but not all preparations of ^{125}I-PAI-2. One possibility is that it may be iodinated BSA as the molecular weight of BSA is 60kDa. It is not known how BSA may have been labelled, since it was only present in the elution and not in the iodination buffer. Analysis of ^{125}I-PAI-2 under reducing conditions resulted in a major band at 48kDa (lane 3) as well as the presence of the minor band at 62.5kDa. No others bands were observed. The polymer forms of ^{125}I-PAI-2 were abolished under reducing conditions.

In the presence of u-PA under non-reducing conditions, two new bands with M_r of 98kDa and 160kDa, which were not present in the sample of ^{125}I-PAI-2 alone, were observed (Figure 5B, lane 2). These bands were thought to be ^{125}I-PAI-2 forming complexes with u-PA. The 98kDa band is similar to ^{125}I-PAI-2/u-PA complex observed from the first preparation (Figure 5A). The 160kDa may be polymers of ^{125}I-PAI-2 complexing with u-PA. Under reducing conditions, a major band of 63kDa (Figure 5B., lane 4) was observed. This was also thought to be ^{125}I-PAI-2/u-PA complexes. Under both non-reducing and reducing conditions, the appearance of the ^{125}I-PAI-2/u-PA complex was associated with >80% reduction of the 48kDa monomeric ^{125}I-PAI-2 band.

While Kirchheimer and Remold (1989) have observed that purified u-PA interacts with PAI-2 in a 2:1 molar ratio in the fluid phase and 20:1 molar ratio cell-surface bound to u-PAR, it also has been found that u-PA and PAI-2 forms 1:1 molar ratio complexes (Thorsen *et al.,* 1988). To characterise the interaction of recombinant human ^{125}I-PAI-2 with u-PA, complex formation between the inhibitor and activator was examined in the presence of varying concentrations of u-PA, giving rise to different molar ratios of ^{125}I-PAI-2 to u-PA (Figure 6). ^{125}I-PAI-2 only (lane 8) and ^{125}I-PAI-2 to u-PA in a molar ratio of 1:0.2 (lane 7) gave rise to two distinct protein bands of 47kDa and 98kDa. The 47kDa and 98kDa species were thought to be monomers and polymers of ^{125}I-PAI-2, respectively. Doubling the amount of u-PA (ratio of 1:0.4) resulted in a new band with M_r of 84kDa (lane 6), which was not

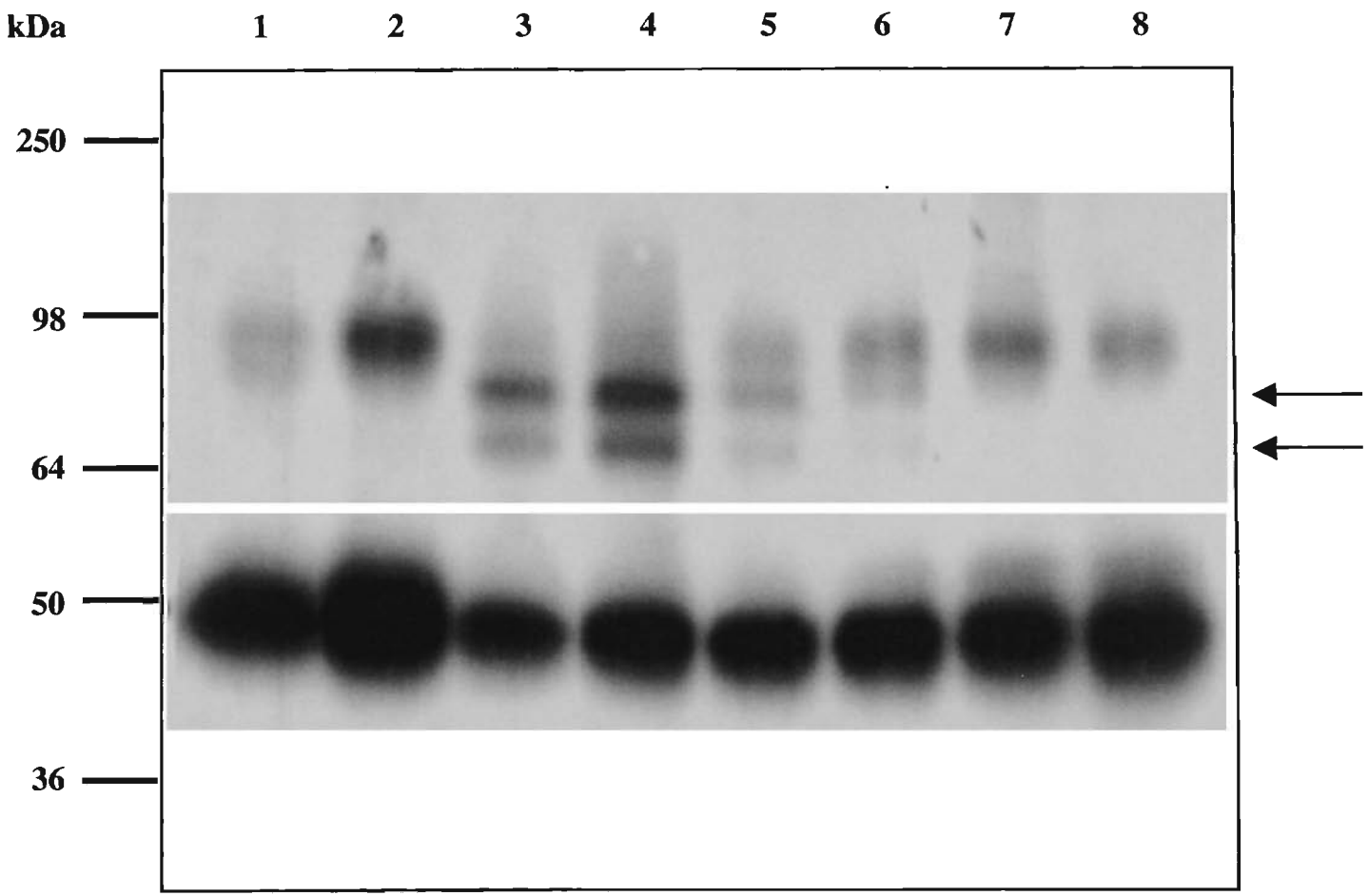


Figure 6. ^{125}I-PAI-2 binding to varying concentrations of uPA analysed by autoradiography. Lane 8 is ^{125}I-PAI-2 only. Lane 7 is ^{125}I-PAI-2/uPA complex in a 1:0.2 molar ratio. Lane 6 is ^{125}I-PAI-2/uPA complex in a 1:0.4 molar ratio. Lane 5 is ^{125}I-PAI-2/uPA complex in a 1:0.8 molar ratio. Lane 4 is ^{125}I-PAI-2/uPA complex in a 1:4. molar ratio. Lane 3 is ^{125}I-PAI-2/uPA complex in a 1:8. molar ratio. Lane 2 is ^{125}I-PAI-2 in the presence of EGR-CMK inactivated uPA. Lane 1 is ^{125}I-PAI-2 in the presence of #394 inactivated uPA. The arrows on the right indicate the ^{125}I-PAI-2/uPA complexes of 84kDa and 71kDa respectively.

present in the two previous samples (lanes 7 & 8). Doubling the u-PA content again (molar ratio of 1:0.8) resulted in the appearance of the 84kDa band as well as a new band with M_r of 71kDa (lane 5). Both the 84kDa and 71kDa bands were thought to be ^{125}I-PAI-2/u-PA complexes as they were not present in the ^{125}I-PAI-2 only sample (lane 8). These two bands were also absent when ^{125}I-PAI-2 was reacted with u-PA that had been inactivated with either EGR-CMK (lane 2) or u-PA inhibitory monoclonal antibody, #394 (lane 1). The intensity of the two complex bands were greatly increased when one part of ^{125}I-PAI-2 and four parts of u-PA were used (ie. 1:4 molar ratio), compared to the intensity of these same bands in lanes 3, 5 and 6. A molar ratio of 1:8 (^{125}I-PAI-2:u-PA) also resulted in the complex bands. However a decrease in intensity of the complex bands were observed when compared to the same bands in lane 4. With increasing amounts of u-PA, the intensity of the ^{125}I-PAI-2 monomer and polymer bands were observed to decrease, with the decrease in the polymer being more pronounced.

2.3.4 Optimisation of plasminogen concentration for ^{125}I-PAI-2 binding to HCT116 cells

These experiments were performed to determine the optimal concentration of plasminogen that would result in maximal binding of ^{125}I-PAI-2 to HCT116 cells. At 20μg/ml of plasminogen the binding of ^{125}I-PAI-2 to HCT116 cells increased by

approximately 5-fold as compared to no plasminogen treatment (Figure 7). Beyond 20μg/ml of plasminogen the amount of cell-associated radioactivity was found to decrease. At the highest concentration of plasminogen used (100μg/ml) the cell-associated radioactivity was approximately 70% of the value obtained at 20μg/ml. One reason for this may be that at higher concentrations of plasminogen, cells were being lysed (ie. the cloudy cell suspension became more viscous with increasing concentrations of plasminogen). As a control, the cells were not treated with plasminogen and the cell binding assay carried out in a 50-fold excess of unlabelled PAI-2. The cell-associated radioactivity of cells not treated with plasminogen and in the absence of unlabelled PAI-2 was approximately 2.5 times greater than the radioactivity obtained in the presence a 50-fold excess of unlabelled PAI-2.

2.3.5 Time course of ^{125}I-PAI-2 binding to HCT116 cells at 4°C

Cells were either treated or not treated with plasminogen and either in the presence or absence of a 50-fold excess of unlabelled PAI-2. The total binding of ^{125}I-PAI-2 to plasminogen treated cells increased with time and reached a maximum amount of cell-associated radioactivity around 6h (Figure 8A). Longer incubations of ^{125}I-PAI-2 (> 6h) resulted in a reduction of total binding to cells. The total binding of ^{125}I-PAI-2 to HCT116 cells at 20h was greater than was observed at any other times. One reason

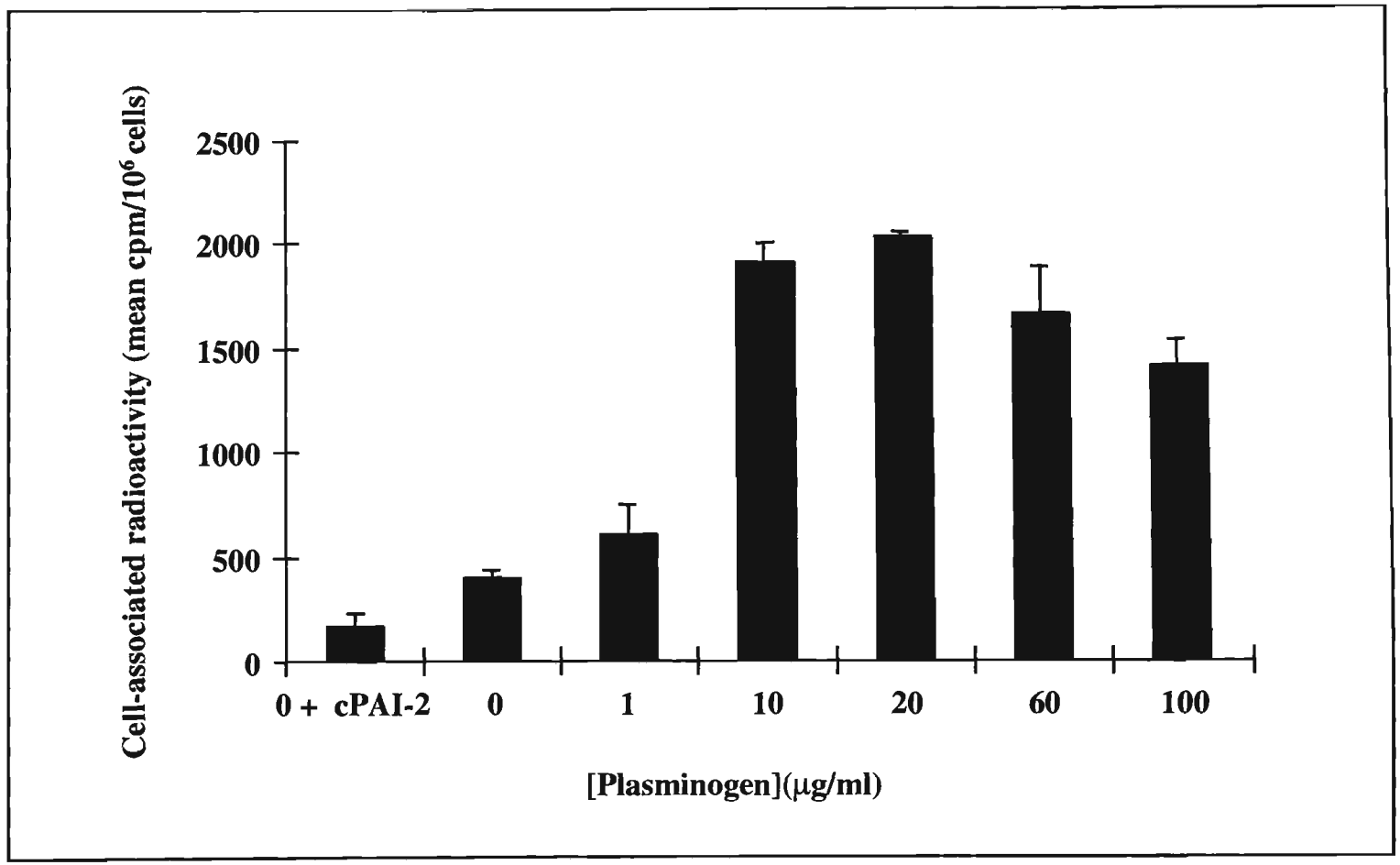
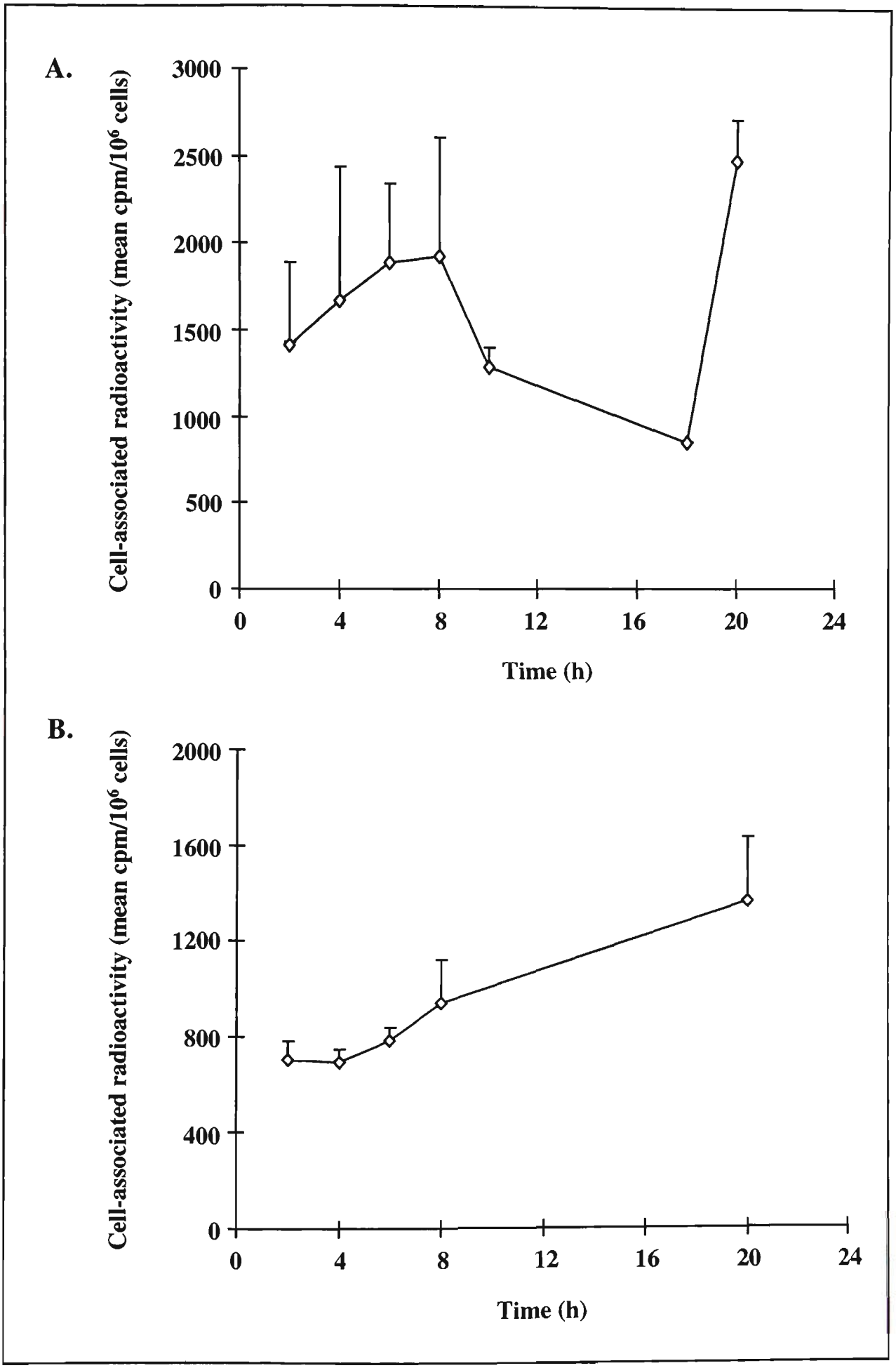


Figure 7. Optimisation of plasminogen concentration used in the ^{125}I-PAI-2 cell binding studies. HCT116 cells were pretreated with various concentrations of plasminogen prior to the addition of ^{125}I-PAI-2. Each concentration was carried out in triplicate. A 50-fold excess of unlabelled PAI-2 (cPAI-2) was included as a control. This result is a representative of two separate experiments.

Figure 8. Time course for ^{125}I-PAI-2 binding to HCT116 cells at 4°C. A constant amount of ^{125}I-PAI-2 was added to HCT116 cells and left for various lengths of time at 4°C. The cells were either treated (A.) or not treated (B.) with plasminogen prior to incubation of ^{125}I-PAI-2 and the total binding of ^{125}I-PAI-2 measured. The data from cells treated with plasminogen are a representative from four separate experiments with either duplicates or triplicates at each time point per experiment. The data from cells not treated with plasminogen are a representative from three separate experiments with duplicates or triplicates at each time point. The data are presented as the mean ± SD of triplicates.


A.
Cell-associated radioactivity (mean cpm/10⁶ cells)
3000
2500
2000
1500
1000
500
0
0
4
8
12
16
20
24
Time (h)
B.
Cell-associated radioactivity (mean cpm/10⁶ cells)
2000
1600
1200
800
400
0
0
4
8
12
16
20
24
Time (h)

for this may be that leaving cells at 4°C for 20h caused the majority of cells to die or undergo apotosis, hence increasing the number of non-specific binding sites. The specific activity (cpm per μg of protein) of the different ^{125}I-PAI-2 batches used in the individual experiments were not accounted for, and may attribute to the large standard deviation in the results of the total and non-specific binding. The results are represented as the mean ± SD from four different experiments with either duplicates or triplicates of each sample. From these results, future cell binding experiments at 4°C were carried out for a duration of 6h.

The opposite was observed when examining the total binding curve for cells not treated with plasminogen as compared to treated cells. The results show that the total PAI-2 binding curve for untreated cells remained stable between 2h and 4h and then increased after 6h to 20h (Figure 8B). The results from cells not treated with plasminogen are presented as mean ± SD of triplicates from a representative of three separate experiments.

The plasminogen treatment of cells (Figure 8A) resulted in approximately twice the amount of cell-associated radioactivity compared to untreated cells at each time point (Figure 8B). When determining the cell-associated radioactivity, an aliquot of the ^{125}I-PAI-2 source that was added to the cells at the beginning of the experiment was also counted with the cells to ensure that differences in cell-associated radioactivity was not due to radioactive decay of ^{125}I-PAI-2. The radioactivity measured for the

^{125}I-PAI-2 source at 20h was similar to that at 2h, hence no significant radioactive decay of ^{125}I-PAI-2 was observed for the times used in these experiments.

2.3.6 The effect of increasing concentrations of ^{125}I-PAI-2 binding to HCT116 cells

These experiments consisted of four main groups (Figure 9). The cells were either treated (filled symbols) or not treated with plasminogen (open symbols) in the presence (broken lines) or absence (solid lines) of a 50-fold excess of unlabelled PAI-2. An increase in cell-associated radioactivity was observed with increasing concentrations of ^{125}I-PAI-2 in both plasminogen treated and untreated cells. However, there was more cell-associated radioactivity observed with plasminogen treatment compared to untreated cells as was also seen in section 2.3.4. A reduction in cell-associated radioactivity was observed in the presence of a 50-fold excess of unlabelled PAI-2 in both plasminogen treated and untreated cells compared to cells without unlabelled PAI-2 (Figure 9).

The specific binding curves obtained for cells treated with plasminogen (Figure 10A) were greater then that obtained for cells not treated with plasminogen (Figure 10B). For plasminogen treated cells, the specific binding curve (Figure 10A, filled triangle) was found to increase at a faster rate with increasing concentrations of ^{125}I-PAI-2 and eventually reached maximum cell-associated radioactivity at a concentration of

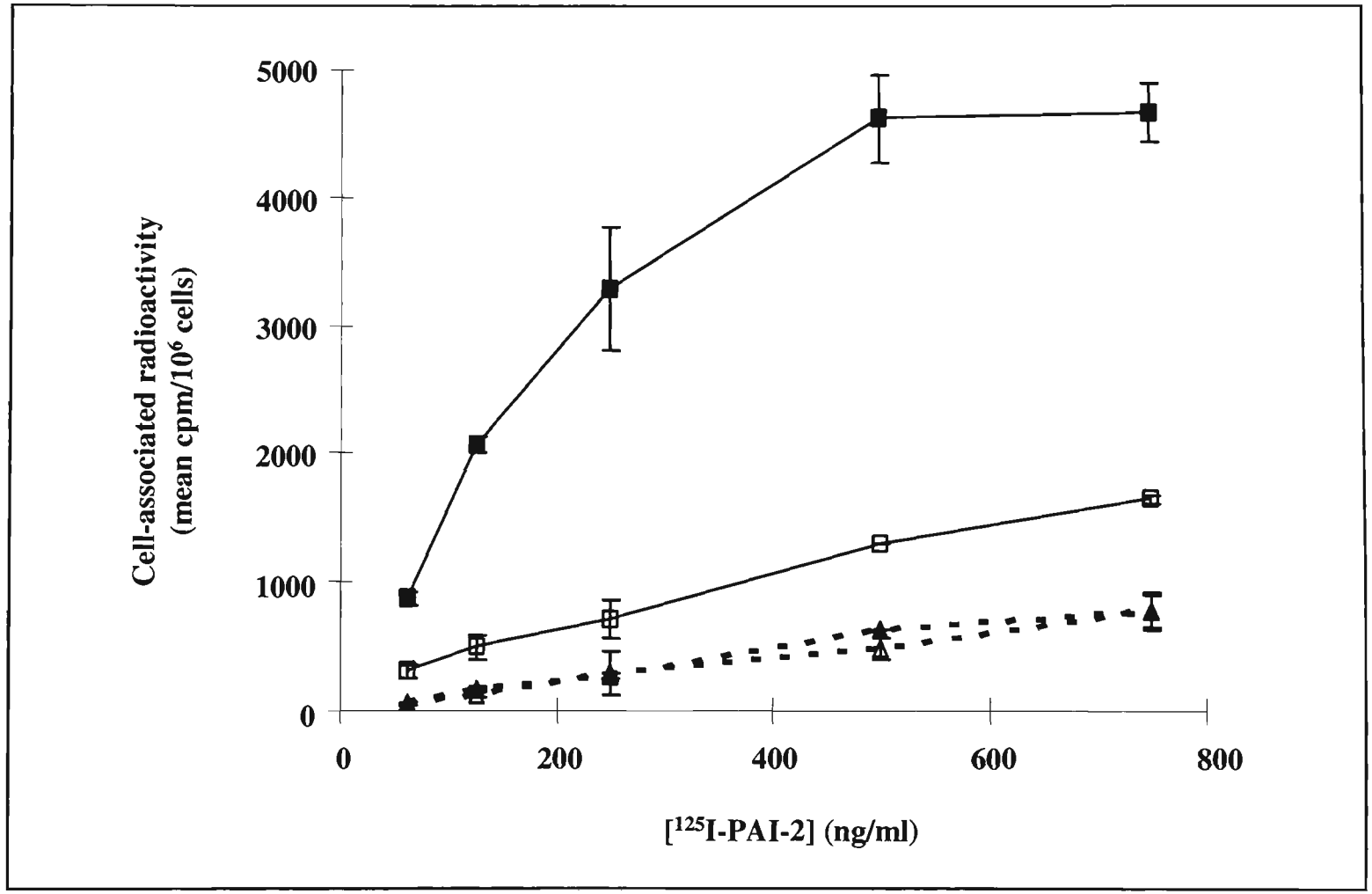
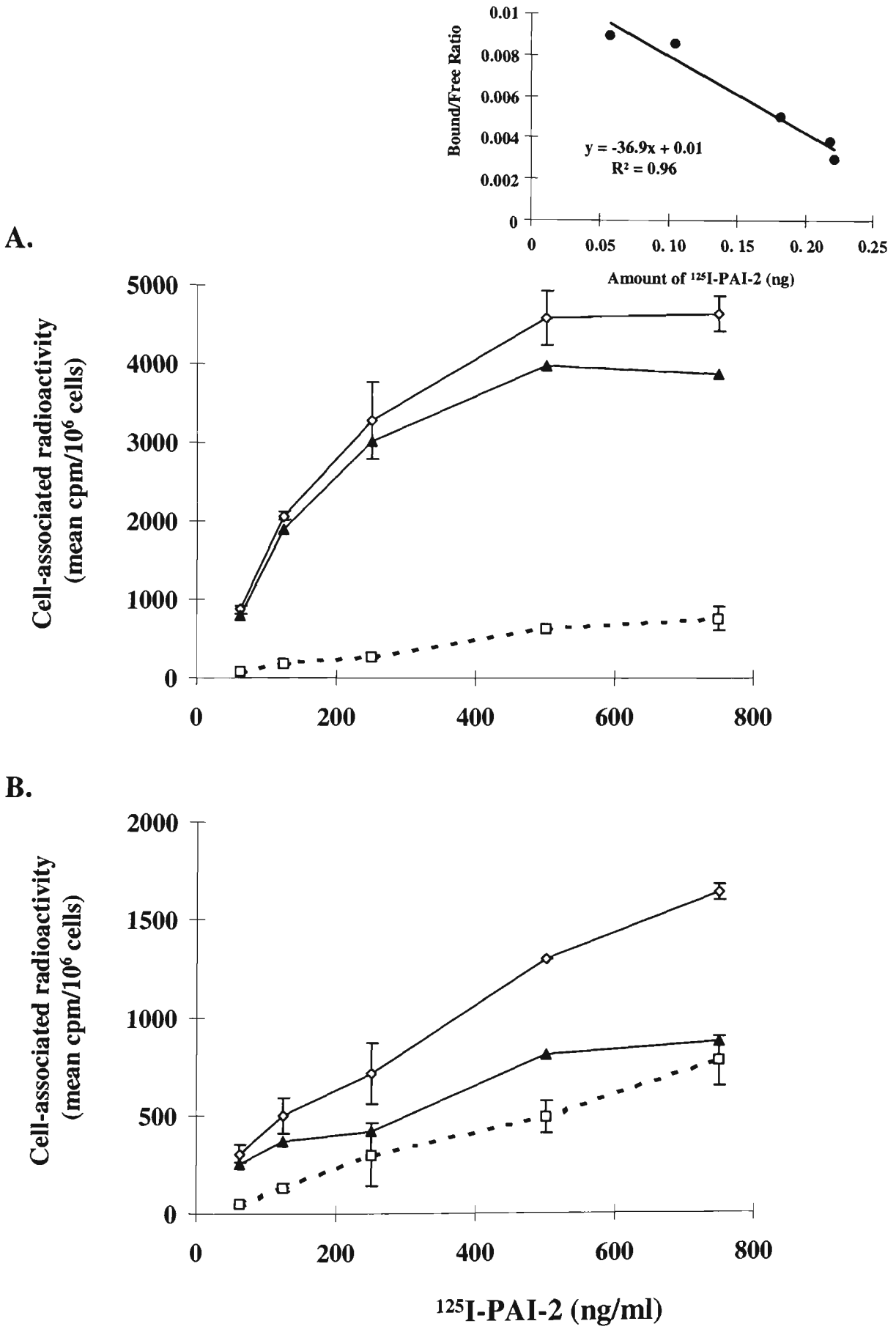


Figure 9. ^{125}I-PAI-2 binding to HCT116 cells. Varying concentrations of ^{125}I-PAI-2 were added to HCT116 cells which were either treated (filled symbols; ■ , ▲) or not treated (unfilled symbol; □ , Δ) with plasminogen and either in the presence (broken line) or absence (solid line) of a 50-fold excess of unlabelled PAI-2. The cells were left in ^{125}I-PAI-2 for 6h, washed and the amount of cell-associated radioactivity determined. The data from this graph was a representative from five different experiments with duplicates at each concentration. The results are expressed as the mean ± SD of the duplicates.

Figure 10. The specific binding of ^{125}I-PAI-2 to plasminogen treated (graph A) and untreated (graph B) HCT116 cells. The specific binding curve (▲) was obtained by subtracting the non-specific binding of ^{125}I-PAI-2 (□ , binding in the presence of unlabelled PAI-2) from the total binding (◇ , binding in the absence of unlabelled PAI-2) of ^{125}I-PAI-2 to cells. Calculations of the number of PAI-2 binding sites per cell and the dissociation constant was carried out using the method of the Scatchard plots (inset graph). Values obtained from the linear regression equation were used to calculate the number of binding sites per cell and the dissociation constant. The data presented is the mean ± SD from a representative of three experiments with duplicates or triplicates of each sample point per experiment.


A.
Bound/Free Ratio
0.01
0.008
0.006
0.004
0.002
0
y = -36.9x + 0.01
R² = 0.96
0
0.05
0.10
0.15
0.20
0.25
Amount of 125I-PAI-2 (ng)
Cell-associated radioactivity
(mean cpm/10⁶ cells)
5000
4000
3000
2000
1000
0
0
200
400
600
800
B.
Cell-associated radioactivity
(mean cpm/10⁶ cells)
2000
1500
1000
500
0
0
200
400
600
800
125I-PAI-2 (ng/ml)

500ng/ml. Increasing the concentration of ^{125}I-PAI-2 to 750ng/ml did not result in any further increase in cell-associated radioactivity. In comparison, the slope of the specific binding curve from cells not treated with plasminogen (Figure 10B, filled triangle) increased at a slower rate with increasing concentrations of ^{125}I-PAI-2, and did not appear to reach a plateau at the concentrations used. The maximum cell-associated radioactivity of cells not treated with plasminogen was approximately 25% of that obtained from cells treated with plasminogen.

2.3.7 Scatchard analysis

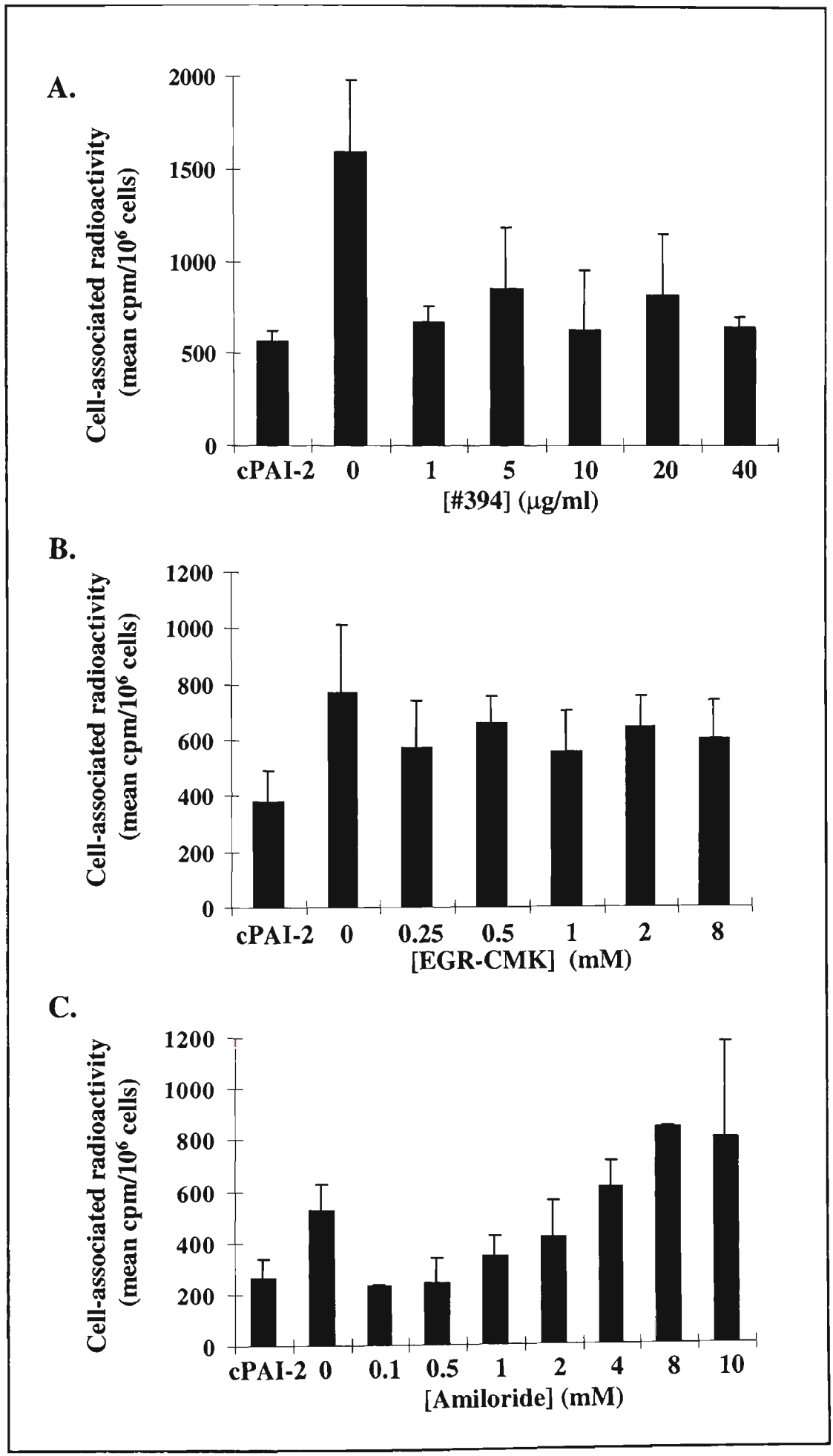
A linear regression was obtained from Scatchard plot for individual experiments. The values obtained from the linear regression equation were used to calculate the number of ^{125}I-PAI-2 binding sites per cell and the K_d value. A representative Scatchard plot from one representative experiment is depicted in Figure 10A (inset graph). For HCT116 cells treated with plasminogen the number of ^{125}I-PAI-2 binding sites per cell was $4.1 \times 10^3 \pm 5.5 \times 10^2$. The K_d value was 2.8nM ± 0.4nM. Calculations of PAI-2 binding sites and dissociation constants for cells not treated with plasminogen could not be calculated. This is because it was not possible to obtain a linear regression with a r^2 value of greater than 0.5 from those curves.

2.3.8 Optimal concentration of u-PA inhibitors

To determine the concentration at which maximal inhibition of ^{125}I-PAI-2 binding to HCT116 cells was achieved, a range of concentrations of u-PA inhibitors were tested (Figure 11A-C). The following results are from one representative experiment of at least three different experiments for each inhibitor, with duplicates or triplicates of each concentration per experiment. At the lowest concentration of u-PA inhibitory monoclonal antibody tested (#394, 1μg/ml), the cell-associated radioactivity obtained was 42% of the value obtained in the absence of antibody (Figure 11A). Maximal inhibition was obtained when 10μg/ml #394 was used, with a 61% reduction in cell-associated radioactivity observed when compared to cell-associated radioactivity in the absence of antibody. A concentration of 10μg/ml was used in subsequent competition assays.

In this representative concentration course for EGR-CMK (Figure 11B.) maximal inhibition was around the concentration of 1mM which inhibited cell-associated radioactivity by 27% compared to the absence of inhibitors. However at 0.25mM EGR-CMK, a 26% inhibition was achieved. These two concentration (0.25 and 1mM) resulted in relatively similar inhibition percentages. Taken together with results from another experiment where maximal inhibition of cell-associated radioactivity was observed to be between 0.5mM and 1mM of EGR-CMK, a concentration of 0.5mM was chosen to be used in subsequent competition assays. At

Figure 11. Concentration courses for the inhibitors of uPA activity. Concentration courses were carried out for the three known inhibitors of uPA activity; A: An inhibitory monoclonal antibody directed against human uPA, #394 (ADI), B: The peptidyl inhibitor EGR-CMK and C: The inhibitor of Na^+ transport, amiloride. The optimal concentrations of all three inhibitors were used in subsequent binding studies. The data are presented as the mean ± SD from a representative experiment from three different experiments with each inhibitor concentration carried out in either duplicates or triplicates in each experiment.


A.
Cell-associated radioactivity
(mean cpm/10⁶ cells)
2000
1500
1000
500
0
cPAI-2
0
1
5
10
20
40
[#394] (μg/ml)
B.
Cell-associated radioactivity
(mean cpm/10⁶ cells)
1200
1000
800
600
400
200
0
cPAI-2
0
0.25
0.5
1
2
8
[EGR-CMK] (mM)
C.
Cell-associated radioactivity
(mean cpm/10⁶ cells)
1200
1000
800
600
400
200
0
cPAI-2
0
0.1
0.5
1
2
4
8
10
[Amiloride] (mM)

higher concentrations of 2mM and 8mM EGR-CMK, a 14% and 22% inhibition of cell-associated radioactivity was obtained, respectively, compared to the absence of any inhibitors.

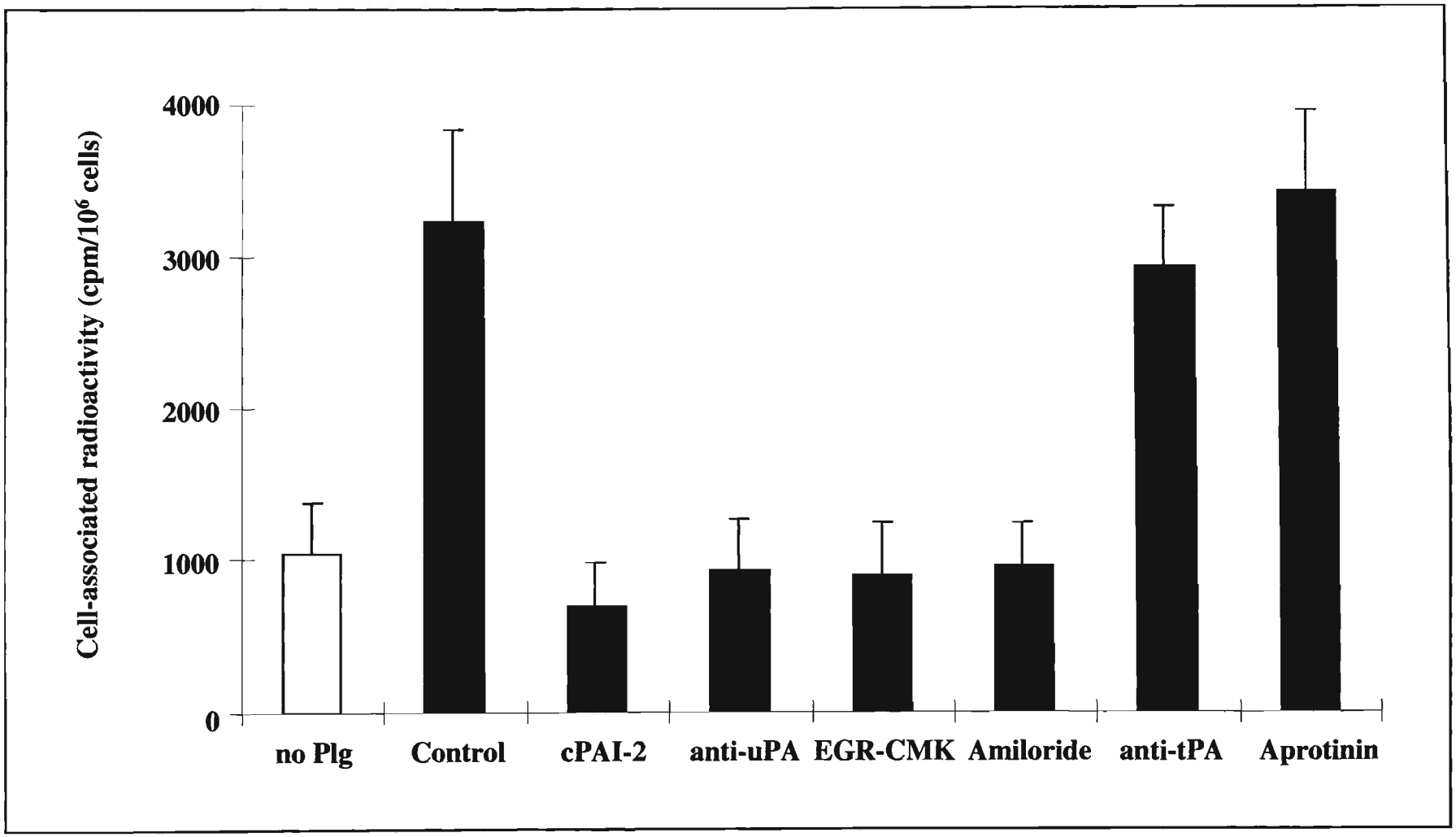
With the amiloride concentration course (figure 11C) maximal inhibition was obtained around concentrations of 0.1 and 0.5mM. The higher concentrations of amiloride (4, 8 and 10mM) were very difficult to dissolve and tended to precipitate. Thus, amiloride concentrations of 4, 8 and 10mM were not effective in the inhibition of ^{125}I-PAI-2 binding to uPA.

In all the concentration courses of the three u-PA inhibitors, a control consisting of a 50-fold excess of unlabelled PAI-2 was included (Figure 11A-C) to ensure the integrity of the experiment. The addition of unlabelled excess PAI-2 has already been shown to compete with ^{125}I-PAI-2 binding to cells (section 2.3.5). In these sets of experiments, a 50-55% reduction of cell-associated radioactivity was observed in all experiments in the presence of unlabelled PAI-2 compared to cells without any inhibitors present.

2.3.9 ^{125}I-PAI-2 binds specifically to active u-PA on HCT116 cells

The amount of cell-associated radioactivity per 10^6 cells was greater in cells treated with plasminogen (Figure 12, control) as compared to untreated cells (no Plg). The

Figure 12. ^{125}I-PAI-2 binding to active uPA on HCT116 cells. Cells were either treated (filled bars) or not treated (open bar) with plasminogen prior to the addition of the inhibitors and ^{125}I-PAI-2. ^{125}I-PAI-2 was added to cells in the presence of various inhibitors of uPA, an inhibitor of t-PA, and an inhibitor of plasmin. The inhibitors of uPA included, a 50-fold excess of unlabelled PAI-2 (cPAI-2), an inhibitory monoclonal antibody #394 (anti-uPA), EGR-CMK and amiloride. An inhibitory monoclonal antibody directed against human tPA, #387 (anti-tPA), was tested. Aprotinin, an inhibitor of plasmin activity was also used. The data is presented as the mean ± SD from two separate experiments with the samples carried out in triplicates in each experiment.


Cell-associated radioactivity (cpm/10^6 cells)
4000
3000
2000
1000
0
no Plg
Control
cPAI-2
anti-uPA
EGR-CMK
Amiloride
anti-tPA
Aprotinin

inhibitors of u-PA activity including the inhibitory monoclonal antibody #394 (anti-u-PA), EGR-CMK and amiloride resulted in approximately a 71%, 73% and 71% decrease in the cell-associated radioactivity of plasminogen treated HCT116 cells, respectively, compared to the plasminogen treated control. A 50-fold excess of unlabelled PAI-2 (cPAI-2) also resulted in a 78% reduction of the cell-associated radioactivity compared to the control cells. In the presence of the inhibitory antibody directed against human t-PA (#387, anti-tPA) and aprotinin ^{125}I-PAI-2 was still able to bind to HCT116 cells, with no significant decrease in cell-associated radioactivity. These results indicate that active u-PA was responsible for binding ^{125}I-PAI-2 on HCT116 cells, as only the u-PA inhibitors resulted in reducing ^{125}I-PAI-2 binding to cells.

2.3.10 Analysis of ^{125}I-PAI-2 binding to HCT116 cells by SDS-PAGE

The ^{125}I-PAI-2 standard gave rise to three protein bands of 47, 60 and 118kDa (Figure 13, lane 1). The 47kDa band is thought to be the monomeric forms of ^{125}I-PAI-2, while the other two bands are dimers and trimers of ^{125}I-PAI-2, respectively. ^{125}I-PAI-2 complexed with u-PA resulted in five bands (33, 69, 87, 101 & 154kDa) which were not present in the ^{125}I-PAI-2 only standard. The 87kDa and 101kDa bands were thought to be ^{125}I-PAI-2 forming complexes with high and low molecular weight u-PA. The 33kDa band was thought to be a degradative product of ^{125}I-PAI-2. The

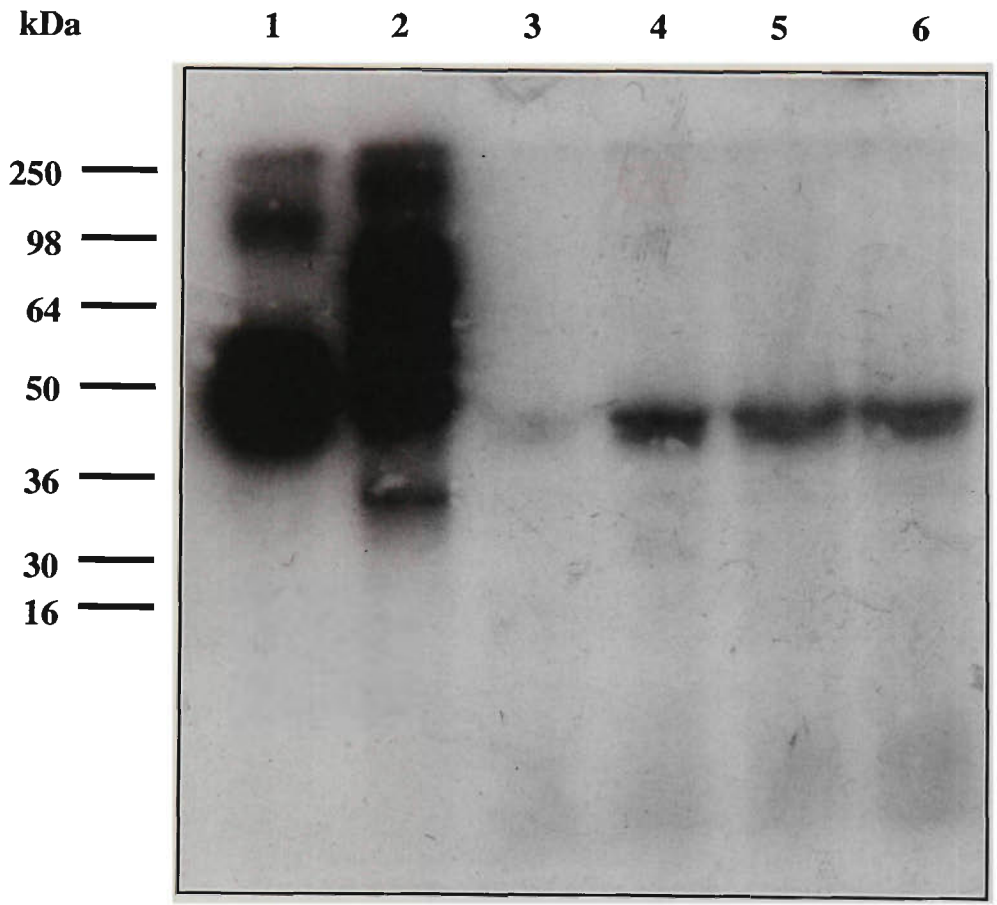
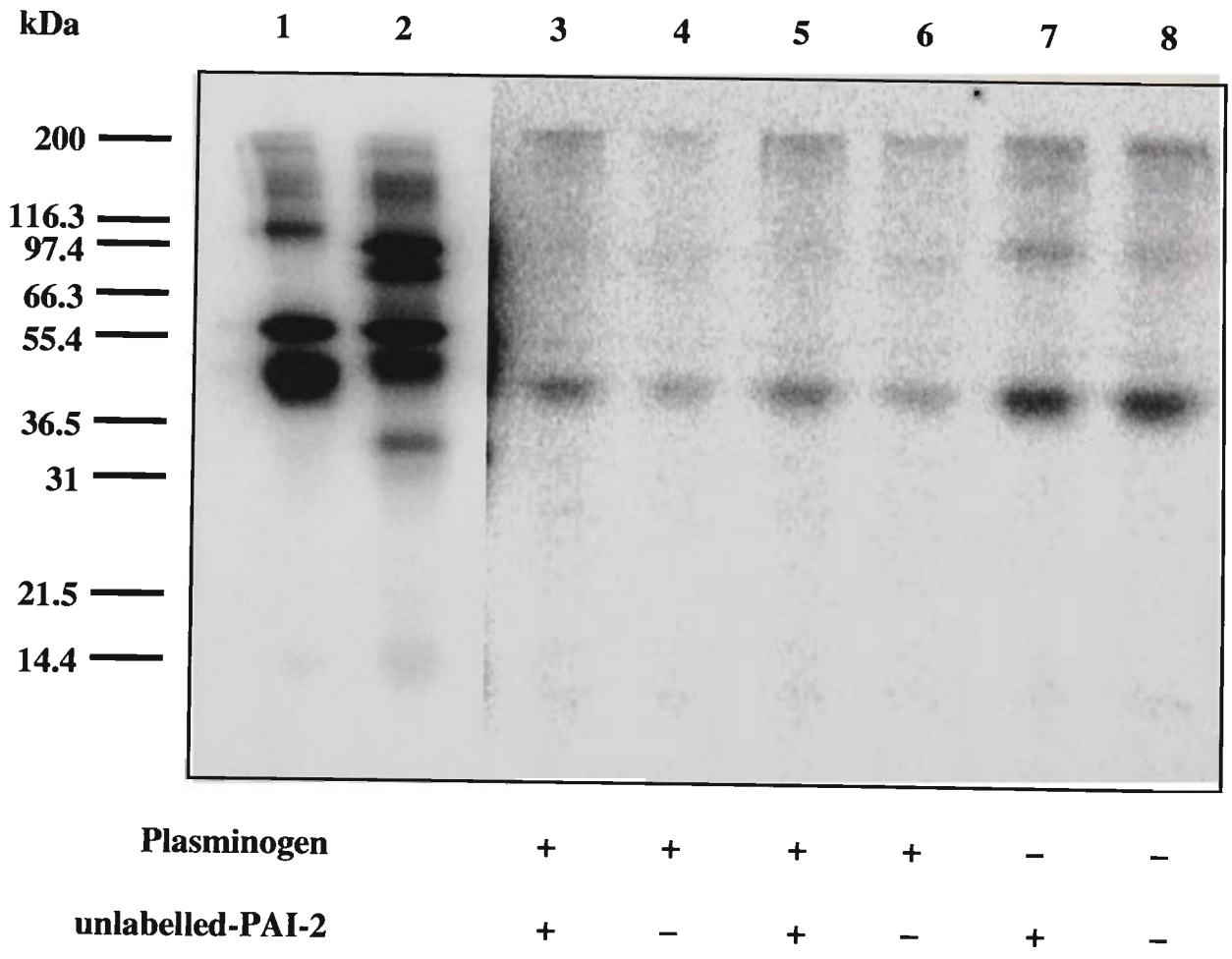


Figure 13. ^{125}I-PAI-2 binding to HCT116 cells for various times analysed by autoradiography. The cells were left with ^{125}I-PAI-2 for 5min (lane 3), 10min (lane 4), 30min (lane 5) and 90min (lane 6) at 4°C. ^{125}I-PAI-2 alone (lane 1) and ^{125}I-PAI-2 complexed with uPA (lane 2) standards were also included. The cells were washed then lysed in non-reducing SDS-PAGE sample buffer and separated on a 12% SDS-PAGE gel. The gel was dried and exposed to Hyperfilm for autoradiography.

other bands are probably complexes formed between degradation products of ^{125}I-PAI-2 with u-PA. Incubation of HCT116 cells with ^{125}I-PAI-2 resulted in a protein band with molecular weight of 43kDa (lanes 3-6) a difference of 4kDa compared to the standards (lanes 1 & 2). The intensity of the monomeric band of ^{125}I-PAI-2 increased significantly from 5min to 10min (lanes 3-6), indicating more ^{125}I-PAI-2 bound to cells. After 10min no further increase in the intensity of the monomeric ^{125}I-PAI-2 was observed (lanes 5 & 6).

In a separate experiment, cells were either treated or not treated with plasminogen prior to being left in ^{125}I-PAI-2 for 6h at 4°C before being lysed and analysed by SDS-PAGE. The gel was exposed to a phosphor-imaging screen, which is more sensitive than Hyperfilm, because levels of radioactivity in the samples in the gel after separation was low. The ^{125}I-PAI-2 only standard (Figure 14, lane 1) gave rise to two bands of similar molecular weights as the standard in the previous figure. The molecular weight of the third band (101kDa) was less than that of the standard (118kDa) in the previous figure (Figure 13, lane 1). This may be due to degradation of ^{125}I-PAI-2 during storage. The ^{125}I-PAI-2/u-PA complex standard (lane 2) also gave rise to similar banding patterns seen in the previous figure. The molecular weights of ^{125}I-PAI-2/u-PA complexes were 79kDa and 94kDa (Figure 14, lane 2), slightly lower than observed previously (Figure 13, lane 2). The 33kDa degradative product was also evident.

Figure 14. ^{125}I-PAI-2 binding to HCT116 cells after being left for 6h at 4°C analysed by autoradiography. In this figure the image of lanes 1 to 8 were obtained from the phosphor-imaging screen at the same time. However, due to the weak intensity of the bands in lanes 3 to 8, these lanes were darkened using the Imagequant software. The ^{125}I-PAI-2 only (50ng, lane 1) and ^{125}I-PAI-2/uPA complex (in 1:10 ratio, lane 2) standards did not require darkening. The cells were either treated (+) or not treated (-) with 20μg/ml plasminogen for 15min at 37°C prior to incubation with ^{125}I-PAI-2. A 50-fold excess of unlabelled PAI-2 was also added to some (+) cells prior to the addition of ^{125}I-PAI-2. The cells were lysed with non-reducing sample buffer and separated using 12% SDS-PAGE. After being dried, the gel was exposed to a phosphor-imaging screen for autoradiography.

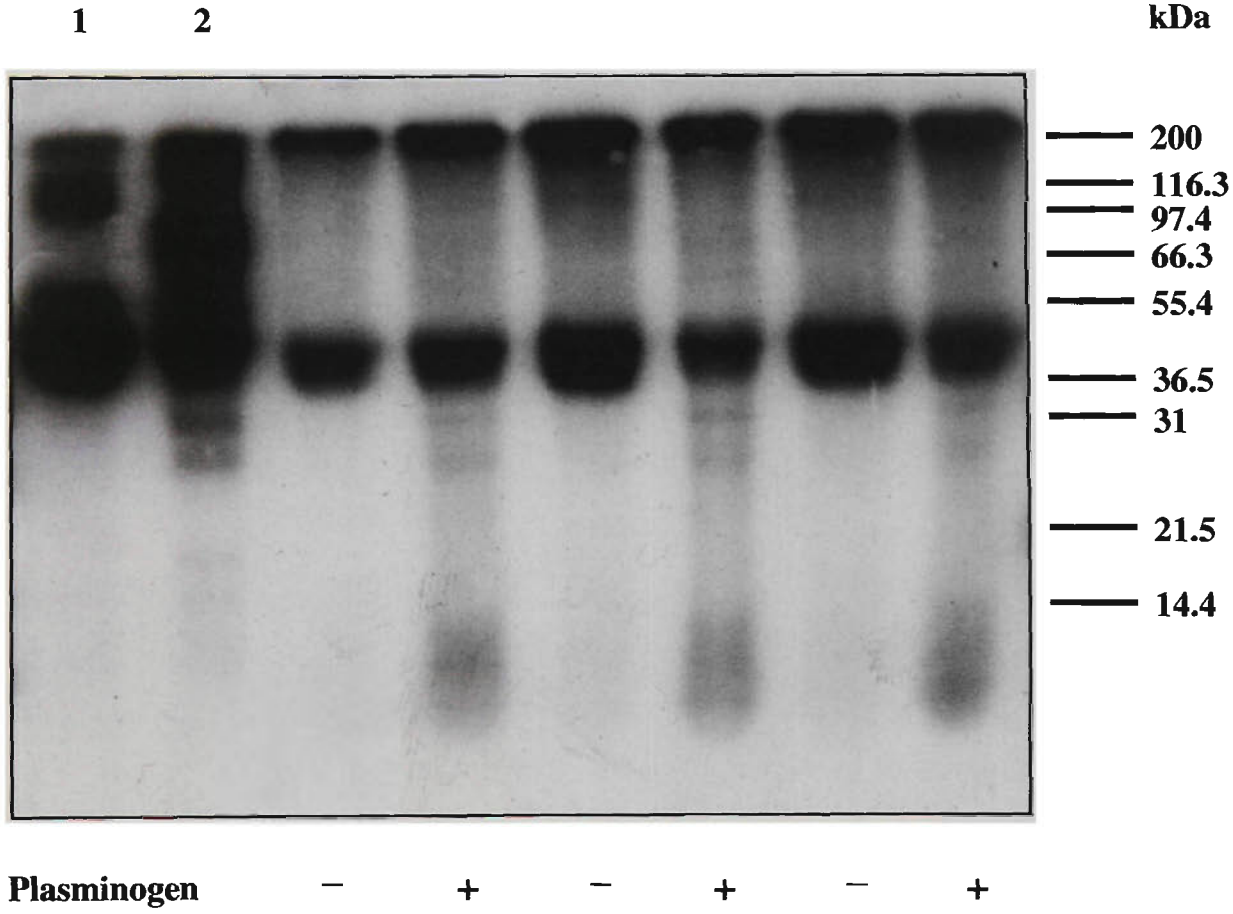

kDa
1
2
3
4
5
6
7
8
200
116.3
97.4
66.3
55.4
36.5
31
21.5
14.4
Plasminogen + + + + – –
unlabelled-PAI-2 + – + – + –

In the presence of plasminogen treatment, (lanes 3-8) the intensity of the monomeric ^{125}I-PAI-2 band was less than that of the untreated cells. There also appeared to be a reduction of the monomeric ^{125}I-PAI-2 in the presence of unlabelled PAI-2 (lanes 4, 6 and to a lesser extent 8). In all lanes of HCT116 cell samples (lanes 3-8) two bands with molecular weight of 47kDa and >200kDa were evident. Plasminogen treatment (lanes 3-6) resulted in the appearance of a very weak 94kDa band, which was thought to be complexes between ^{125}I-PAI-2 and u-PA. In contrast, a 101kDa band was observed in untreated cells (lanes 7 & 8) which was also present in the ^{125}I-PAI-2 alone standard.

2.3.11 Crosslinking of ^{125}I-PAI-2 to HCT116

Although the ^{125}I-PAI-2 only and ^{125}I-PAI-2/u-PA complex standard shown in Figure 15 (lanes 1 & 2) were over-exposed, determination of molecular weights of the protein bands present in the standards was possible from the original autoradiography. Present across all the lanes was a high molecular weight band which had just migrated into the gel and is parallel to the 250kDa myosin band of the molecular weight markers. These large complexes may represent cross-linked ^{125}I-PAI-2/u-PA/u-PAR complexes. However, it cannot be ruled out that these high molecular weight bands are polymers of ^{125}I-PAI-2, since the ^{125}I-PAI-2 standard (lane 1) also contains large molecular weight bands.

Figure 15. The effects of plasminogen treatment on ^{125}I-PAI-2 cross-linked to HCT116 cells. The cells were either treated (+) with or not treated (-) with plasminogen then left in ^{125}I-PAI-2 for 6h at 4°C. Lane 1 is a ^{125}I-PAI-2 standard and lane 2 is a ^{125}I-PAI-2/uPA standard. After 6h the crosslinker DSS, was added to all the cells and left overnight at 4°C. Samples were then analysed by non-reducing 12% SDS-PAGE followed by autoradiography.


1
2
kDa
200
116.3
97.4
66.3
55.4
36.5
31
21.5
14.4
Plasminogen
−
+
−
+
−
+

Three protein bands of 115, 58 and 46kDa were obtained for the ^{125}I-PAI-2 only standard (lane 1). The last two bands were also observed in the ^{125}I-PAI-2/u-PA complexes standards (lane 2) along with five other bands with M_r of 26, 30, 76, 92 and 167kDa. In both cases, the 46kDa is thought to be monomeric PAI-2 (lanes 1 & 2), while the higher M_r bands were thought to be polymers of PAI-2 in the ^{125}I-PAI-2 only standard (lane 1). The 167kDa band is thought to be u-PA forming complex with polymeric ^{125}I-PAI-2 (lane 2). The 92 and 76kDa bands were thought to result from complex formation between monomeric ^{125}I-PAI-2 and u-PA (33kDa and 55kDa). While the 30 and 26kDa bands were thought to be degradative products as a result of complex formation between ^{125}I-PAI-2 and u-PA.

Membrane samples which were not treated with plasminogen contained greater amounts of higher molecular weight bands that just migrated into the gel. Plasminogen treatment (Figure 15, +) resulted in a reduction of monomeric ^{125}I-PAI-2 compared to no treatment (–). Two lower molecular weight bands of 30 and 26kDa, similar to those observed in the ^{125}I-PAI-2/u-PA standard, were also observed only in the plasminogen treated samples. The presence of even lower molecular weight bands (14 and 12kDa) in the plasminogen treated samples provides evidence of further degradation of ^{125}I-PAI-2 which was not apparent in the ^{125}I-PAI-2/u-PA standard. It appeared that plasminogen treatment of HCT116 cells resulted in binding and subsequent degradation of ^{125}I-PAI-2 by the plasma membrane preparations.

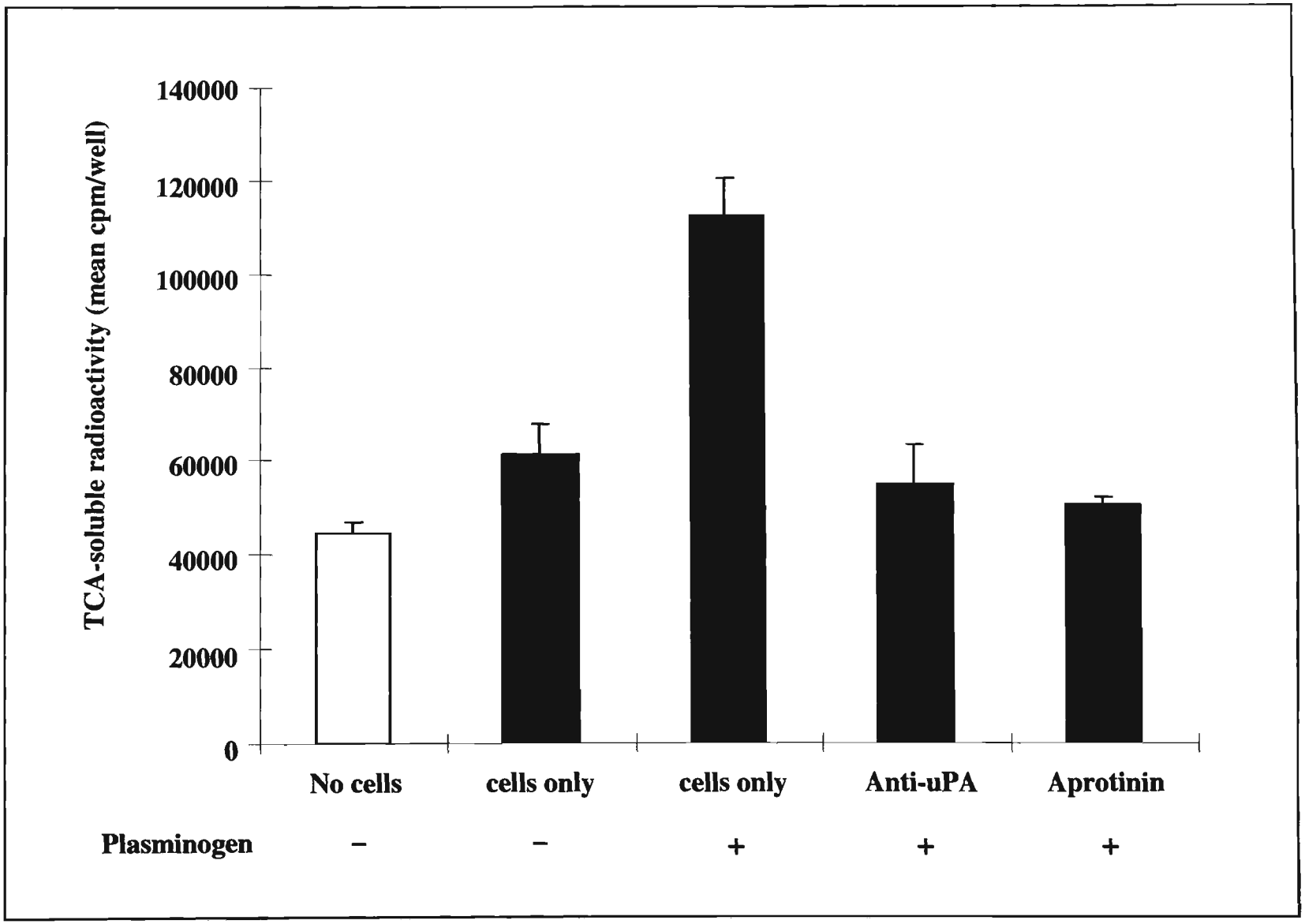
2.3.12 Processing of ^{125}I-PAI-2 at 37°C

At 37°C, HCT116 cells treated with plasminogen prior to addition of ^{125}I-PAI-2 resulted in approximately a 2-fold increase in TCA-soluble radioactivity compared to cells that had not been treated (Figure 16). In the presence of an u-PA inhibitor (the monoclonal antibody #394) and an inhibitor of plasmin activity, aprotinin (Ellis *et al.*, 1987; Cajot *et al.*, 1989), a 51% and 55% reduction in TCA-soluble radioactivity was observed, respectively, compared to cells in the absence of inhibitors. In the absence of cells, the ^{125}I-PAI-2 preparation contained approximately 72% of free ^{125}I compared to ^{125}I-PAI-2 incubated in the presence of cells not treated with plasminogen at 37°C.

2.4 DISCUSSION

The main aim of this chapter was to examine the ability of ^{125}I-labelled human recombinant PAI-2 to bind to the human colorectal cancer cell line HCT116. Since PAI-2 is known to bind to u-PA, initially flow cytometric studies were implemented to assess the expression of cell-surface u-PA, as well as u-PAR on HCT116 and LIM1215, which are less aggressive than HCT116 cells. Results from flow cytometric analysis of these two human colorectal cell lines showed that the highly metastatic cell line, HCT116, possessed more cell surface u-PA and u-PAR than the less metastatic LIM1215. This result was in concordance with studies of colorectal

Figure 16. Processing of ^{125}I-PAI-2 by HCT116 cells at 37°C. Cells were grown to confluency in monolayer in 24 well plates. The cells were either treated (+) or not treated (-) with plasminogen prior to addition of the inhibitors (anti-uPA and approtinin) and ^{125}I-PAI-2. The cells were then incubated at 37°C for 8h. After 8h TCA precipitation was carried out and the resultant TCA-soluble radioactivity was measured. The control with no cells present (□) was also placed at 37°C and TCA precipitation was also carried out after 8h. The results are expressed as the mean ± SD from three separate experiments, with duplicates of each sample in each experiment.


TCA-soluble radioactivity (mean cpm/well)
140000
120000
100000
80000
60000
40000
20000
0
No cells
cells only
cells only
Anti-uPA
Aprotinin
Plasminogen
–
–
+
+
+

carcinomas which found that one of the markers of neoplastic growth and metastatic spread in adenocarcinomas is the increase of both u-PA antigen and activity (Sier *et al.*, 1991). Scatchard analysis of ligand binding found that the more aggressive human colon carcinoma cells expressed 17-fold more u-PAR per cell compared to the less aggressive cells (Boyd *et al.*, 1988) facilitating the focalisation of plasminogen activation at the cell surface (Ellis and Danø, 1991). The HCT116 cells, which were shown to express cell suface u-PA, have also been shown to not express PAI-2, hence HCT116 was chosen as the cell line to be used for all subsequent experiments.

Incorporation of the ^{125}I label can cause considerable changes in the protein structure and hence its biological activity and behaviour. These were important aspects to consider when radiolabelling PAI-2. Characterisation of ^{125}I-PAI-2 resulted in formation of complexes with purified human u-PA in a 1:1 molar ratio in solution, as was found by Kiso *et al.*, (1988). The ability of ^{125}I-PAI-2 to form complexes with u-PA indicated that incorporation of the radionuclei did not alter the activity of PAI-2 with respect to it's interaction with u-PA. Other findings of this chapter indicates that; 1) ^{125}I-PAI-2 binds to HCT116 at 4°C; 2) active u-PA is responsible for the majority of binding of ^{125}I-PAI-2 on HCT116 cells; and 3) HCT116 can catabolise ^{125}I-PAI-2 at 37°C.

The conversion of plasminogen to plasmin by active u-PA not only serves for the degradation of ECM components (Vassalli *et al.*, 1991), but it may also serve to

produce more active u-PA, since plasmin has been shown to activate single-chain pro-u-PA into it's active two-chain u-PA form (Kasai *et al.,* 1985; Ellis *et al.,* 1987). Direct increases in expression of cell surface active u-PA on viable HCT116 and LIM1215 cells were observed by flow cytometry when cells were treated with plasminogen (section 2.3.1). In contrast, plasminogen treatment had no effect on cell surface u-PAR expression in these two cell lines. In a study by Pöllänen *et al.,* (1990), it was shown that pro-u-PA can be activated by the addition of plasminogen and that PAI-2 can inhibit the activity of cell-associated active u-PA. Using a fluorimetric peptidolytic assay, Baker, M.S. *et al.,* (1990) observed that treatment of LIM1215 and COLO205 cells with plasminogen increased specific u-PA activity, and with the addition of PAI-2 the u-PA activity was returned close to control levels. This study supported those observations, because using a modified version of the Coleman and Green (1981) assay the addition of PAI-2 inhibited the activity of u-PA in HCT116 cell lysates.

Jensen *et al.,* (1990) using an anti-tubuline drug capable of disrupting vesicle transport and inhibitors of lysosomal acid hydrolases, found that u-PA/PAI-1 complex and to a lesser extent u-PA, are internalized by vesicular traffic and transferred to lysosomes for degradation into amino acids at 37°C. Approximately 50% of cell bound u-PA/PAI-1 and u-PA/PAI-2 complexes were degraded to TCA-soluble products, while only 15% of cell bound u-PA and DFP-u-PA were degraded. Hence

all cell binding experiments in this chapter with ^{125}I-PAI-2 were carried out at 4°C to minimize internalization and subsequent degradation.

^{125}I-PAI-2 bound to HCT116 cells in a time- and concentration-dependent manner under the conditions stated in this chapter. Plasminogen treated cells bound more ^{125}I-PAI-2 than untreated cells, probably as a result of conversion of pro-u-PA to active u-PA via the generated activity of plasmin from plasminogen, as was seen by the increase in u-PA expression by HCT116 cells via flow cytometric analysis.

The number of PAI-2 binding sites per HCT116 cell pretreated with plasminogen was 20 times lower than reported for u-PA binding sites (ie. u-PAR sites per cell) on this and other cell lines. u-PA binding sites for HCT116 cells have been reported in the range of 106,075 ± 4,649 sites per cell (Boyd *et al.*, 1988) and up to 100,000 for HT29 cells (Reiter *et al.*, 1993). This may be due to one of two reasons; either all the u-PAR on HCT116 cells were not occupied with u-PA, or that treatment of cells with plasminogen under the conditions of this study did not result in activation of all pro-u-PA into active two-chain u-PA, as the extent of activation of u-PA by plasminogen pre-treatment was not assessed in this study. Nevertheless, in either case, the subsequent result would be limited number of active u-PA available for binding PAI-2.

It remains unclear as to which proteins/moieties are responsible for the binding of PAI-2 on cancer cells. In the presence of u-PA inhibitors the binding of ^{125}I-PAI-2 was significantly reduced demonstrating that HCT116 cells bind ^{125}I-PAI-2 via active u-PA. In the presence of inhibitors against t-PA and plasmin, no decrease was observed on ^{125}I-PAI-2 binding to cells, indicating that PAI-2 binding to HCT116 cells were also independent of cell surface t-PA or plasmin. Pöllänen *et al.*, (1990) found that recombinant PAI-2 inhibited plasmin generation from plasminogen added exogenously, by inhibiting u-PA activity on the cell-surface of human rhabdomyosarcoma cells. Furthermore, PAI-2 was found co-localized with endogenous u-PA and intracellular vinculin at the focal adhesions of the rhabdomyosarcoma cells (Pöllänen *et al.*, 1990).

Evidence of PAI-2/u-PA complexes occurring on the cell surface have been documented in the literature. Reinartz *et al.*, (1996) were able to elute PAI-2/u-PA complexes from the surface of HaCaT cells. They also observed PAI-2/u-PA complexes with the addition of exogenous HMW-u-PA to HaCaT cell lysates. In this study, treatment with plasminogen resulted in complex formation between ^{125}I-PAI-2 and u-PA (94kDa protein band) found on HCT116 cells. However, it cannot be ruled out that the 94kDa protein band thought to be ^{125}I-PAI-2/u-PA complexes may be a degradation product of polymeric forms of ^{125}I-PAI-2. There was also a 101kDa protein observed in the cell lysates, but it is unlikely to be PAI-2/u-PA complex

because it was found in both ^{125}I-PAI-2 standard and in cells not treated with plasminogen.

PAI-2 was also found to bind to HCT116 cells not pretreated with plasminogen suggesting that PAI-2 may be binding to something else other than active u-PA. Crosslinking experiments were implemented to determine other PAI-2 binding proteins/moeities. No definitive results were obtained from these experiments, so no other PAI-2 binding proteins/moieties on plasma membranes from HCT116 cells could be identified. Jensen *et al.,* (1996) found members of phospholipid binding proteins, annexins I, II, IV and V, bound ^{125}I-PAI-2 without abolishing it's inhibitor activity. Binding to annexins was via the region corresponding to exon 3 of the gene sequence which is thought to be important for PAI-2 functions. It is speculated that the fraction of ^{125}I-PAI-2 binding to HCT116 cells that is not due to u-PA may be via annexin proteins.

Annexin I, also an actin binding protein, is thought to play a role in signal transduction to the cytoskeleton. Elevated protein levels of annexin I was observed in rat adenocarcinoma cell lines, with higher levels of proteins found in the more metastatic cells compared to weakly metastatic cells (Pencil and Toth, 1998). Annexin I expression has also been observed in human mammary adenocarcinomas (Pencil and Toth, 1998), human breast cancers (Ahn *et al.,* 1997) and human hepatocellular carcinoma (Masaki *et al.,* 1996). Annexin II has been shown to bind

calcium as well as plasminogen and t-PA (Redlitz and Plow, 1995) and is a substrate for protein-tyrosine kinases. The expression of annexin II both at the mRNA and protein level was found to increase in pancreatic adenocarcinoma cell lines when compared to the normal pancreas (Vishwanatha *et al.,* 1993). This increase was also observed in primary pancreatic and metastatic tumours (Vishwanatha *et al.,* 1993). In small cell lung cancer cell line, H69AR, elevated levels of annexin II mRNA were found (Cole *et al.,* 1992). Evidence of annexin II (Wildrick *et al.,* 1992) and annexin IV (Satoh *et al.,* 1996) expression was observed in the human colon cancer cell line, HT-29. A form of annexin IV was found to exist spanning the membrane of HT-29 cells (Satoh *et al.,* 1996).

With evidence throughout the literature on the involvement of annexins in cancers and the location of these binding proteins at the cell surface, it is possible that annexins I, II and IV may be responsible for binding PAI-2 on HCT116 cells, where active u-PA is not involved. It is not known if HCT116 cells express any forms of annexins. Hence possible work using flow cytometric analysis of HCT116 cells could be performed to determine whether these cells express any types of annexins. If expression of annexin(s) was found then the use of antibodies directed against annexin I, II and IV in a competition assay could be employed to determine if any of the annexin(s) are responsible for binding PAI-2 on HCT116 cells.

The binding of ^{125}I-PAI-2 to active u-PA on the surface of HCT116 cells at 37°C appeared to have resulted in the internalization and subsequent degradation of the ^{125}I-PAI-2/u-PA complex. In another study Estreicher *et al.,* (1990) found that incubation of THP-1 cells with PAI-2 at 37°C after the cells were incubated with ^{125}I-u-PA resulted in loss of cell-associated radioactivity at a much faster rate compared to cells not incubated with PAI-2. This indicates that ^{125}I-u-PA/PAI-2 complexes were being cleared rapidly from the cell surface. In addition, the binding of preformed ^{125}I-u-PA/PAI-2 complexes by THP-1 cells at 37°C resulted in the disappearance of cell-associated radioactivity with time and the appearance of degradative intermediates (Estreicher *et al.,* 1990; Ragno *et al.,* 1995). Ragno *et al.,* (1995) found that the α_2-macroglobulin receptor, shown to be responsible for the internalization of PAI-1/u-PA complex, was not involved in the internalization of PAI-2/u-PA. Estreicher *et al.,* (1990) concluded that u-PAR acted as a clearance receptor for ^{125}I-u-PA/PAI-2 complexes from the cell surface of THP-1 cells. This was supported by the work of Jensen *et al.,* (1990) which observed that degradation of u-PA/PAI-1 and u-PA/PAI-2 complexes was approximately three times greater than degradation of uncomplexed u-PA at 37°C, and u-PAR was shown to participate in the clearance of u-PA/inhibitor complexes in the human trophoblastic choriocarcinoma cells. Other proteins have been found to be involved with the clearance of inhibitor/protease complexes. Some of these proteins are discussed in the next chapter.

The physiological role(s) of PAI-2 still requires elucidation. PAI-2 has been shown to be expressed broadly by a variety of cell and tissue types ranging from placental tissue (Hofmann *et al.*, 1994) to epidermal cells (Lyons-Giordano *et al.*, 1994) to monocytes/macrophages (Wohlwend *et al.*, 1987). PAI-2 has also been thought to have a role in cellular differentiation (Rehemtulla *et al.*, 1990) and apoptosis (Dickinson *et al.*, 1995). PAI-2 is the physiological inhibitor of u-PA whether in solution or receptor associated. Recombinant PAI-2 can now be synthesised readily making available large quantities of the protein for use. Results of this study and others have shown that recombinant PAI-2 can bind to cell surface u-PA on a variety of cancer cells and inhibit u-PA activity on these cells. Therefore future works in the following chapters will focus on determining the biodistribution and clearance of ^{125}I-PAI-2 in a nude mouse model with xenografts of HCT116 cells. Thus the aims will be to investigate the imaging and therapeutic potential of PAI-2 in treating u-PA expressing cancers.

3. DETERMINATION OF THE CLEARANCE AND BIODISTRIBUTION OF IODINATED PAI-2 (^{125}I-PAI-2) USING A SUBCUTANEOUS XENOGRAFT NUDE MOUSE MODEL OF THE HUMAN COLORECTAL CANCER CELL LINE HCT116

3.1 INTRODUCTION

There have been several studies examining the imaging of colorectal cancer. These primarily include the use of radiolabelled antibodies TAG and/or CEA. A monoclonal antibody to TAG labelled with rhenium (^{188}Re) was found to localise to the human colorectal cancer LoVo in nude mice, with a tumour-to-blood contrast of 2.04±0.44 (Hosono *et al.*, 1998). More uses for the antibodies in imaging have been studied in radioimmunoguided surgery (RIGS) (Buraggi *et al.*, 1987; de Nardi *et al.*, 1997; Bakalakos *et al.*, 1998; Bertoglio *et al.*, 1998a, 1998b; di Carlo *et al.*, 1998; Percivale *et al.*, 1998; Renda *et al.*, 1998). This has been found to be just as successful as current chemotherapeutic strategies, and may even improve the sensitivity of detection of metastasis.

Human cancers including breast (Reilly *et al.*, 1991; Sumiyoshi *et al.*, 1992; Del Vecchio *et al.*, 1993), colonic (Corasanti *et al.*, 1980; Miseljic *et al.*, 1995), gastric (Cho *et al.*, 1997), and urinary bladder (Hasui *et al.*, 1992) have been shown to have increased levels of u-PA. In the Lewis lung tumour model, the highest level of u-PA was found associated with areas of invasive growth (Skriver *et al.*, 1984). In breast

cancer, u-PA was established to be a superior prognostic marker (Janicke *et al.*, 1991; Reilly *et al.*, 1991). Refer to the literature review for further evidence implicating the involvement of u-PA with cancer invasion and metastasis. Since u-PA has been implicated as a major candidate of cancer spread it would appear that u-PA may be used as a target for imaging or treating of cancer metastasis. To date, one study has trialled using u-PA as a target for cancer imaging (Hang *et al.*, 1998), and research has been focused on treating cancer using u-PA as the target (Ossowski and Reich, 1983a,b; Hearing *et al.*, 1988; Ossowski *et al.*, 1991).

Using an antibody for imaging requires the development of an antibody that is only specific for u-PA and does not cross-react with other antigens. One problem associated with the use of antibodies are that the antibody itself is a relatively big molecule, therefore steric hinderance may prevent the antibody from binding to u-PA. This problem has been alleviated through the use of $F(ab')_2$/Fab' fragment mixtures, which have been found to be more sensitive at early detection of colorectal cancer lesions compared to intact immunoglobulin (Behr *et al.*, 1995a, 1995b, 1995c). In addition the applied antibodies are immunogenic and often stimulate the patient's immune system (Blanco *et al.*, 1997; Grossbard *et al.*, 1999; Kricka, 1999; Lacic *et al.*, 1999). This can be overcome by using immunosuppressants, but this leaves a patient open to other infectious diseases. However, the use of humanised murine monoclonal antibodies may provide a solution to both problems (Vaswani and Hamilton, 1998; Rebello *et al.*, 1999; Richards *et al.*, 1999).

A logical approach would be the use of specific u-PA inhibitors like PAI-1 or PAI-2. PAI-2 is believed to react mainly with u-PA and forms covalently complexes. Consequently, it was postulated that PAI-2 may be a useful imaging or therapeutic agent in cancers where u-PA is involved. The imaging of u-PA by the use of PAI-2, on the surface of *in vivo* cancer cells may provide a gauge of the invasiveness of the cancer cells. Finally, the inhibition of u-PA by PAI-2 may be able to prevent the growth of tumours or metastasis.

Results from the previous chapter demonstrated that ^{125}I-PAI-2 can bind specifically to active cell-associated u-PA and also inhibit u-PA activity of HCT116 cells. Therefore from these results, the three main aims of this chapter were to determine; (1) the biodistribution of ^{125}I-PAI-2 in a HCT116 colorectal tumour xenograft bearing nude mouse model, (2) the clearance of ^{125}I-PAI-2 in that model and (3) whether intravenously administered ^{125}I-PAI-2 localises to the colorectal tumour xenografts. These experiments will allow assessment of the potential use of PAI-2 as an imaging agent in human cancers where u-PA is involved. Two different species of PAI-2, human PAI-2 (hPAI-2) and mouse PAI-2 (mPAI-2), were used in experiments carried out in this chapter. This was to determine if mPAI-2 would be more effective at imaging the colorectal tumour xenografts compared to hPAI-2. The PAI-2 used in individual experiments are specified in each method and result section.

3.2 MATERIALS AND METHODS

3.2.1 Reagents

Recombinant hPAI-2, mPAI-2 and murine u-PA were from Biotech Australia, Roseville, Australia. Heparin was purchased from Calbiochem-Novabiochem Pty., Alexandria, NSW, Australia.

3.2.2 Cell culture

Details of all media reagents and cell culture conditions were given in the previous chapter (section 2.2.1 and 2.2.2). To avoid variation from different passages in the cells used, one culture of cells were grown to large quantities and frozen. Aliquots of cells from the same passage were employed in all *in vivo* experiments.

3.2.3 Animals

Four-week old athymic mice (Swiss outbred nude Nu/Nu mice) were obtained from the Animal Services Division, John Curtin School of Medical Research, ANU, Australia. The mice were age- and sex-matched in each experiment. Throughout all experiments, the mice were maintained in a laminar flow cabinet under specific-pathogen-free conditions and monitored regularly. The animal experiments carried

out in this thesis were approved by the animal care and ethics committee of the University of Wollongong. All mice were sacrificed with CO_2 (2-3L/min) in an anesthetic chamber at the end of all experiments.

3.2.4 Xenografts of human colon cancer in nude mice

Xenografts of human colon cancer were induced by subcutaneously injecting either 10^6 or $2x10^6$ HCT116 cells (in 100µl of RPMI) with 26.5G needles in the right flank of four -week-old athymic Swiss outbred Nu/Nu mice. The mice were monitored daily after injection to ensure no adverse effects on the well being of the mice as a result of the treatment. Mice were checked daily for the appearance of subcutaneous tumours. Visible tumours of approximately 1-2mm in diameter were reproducibly observed 7-10 days after injection of the cells. In cases where no tumour xenografts were observed after 7-10 days, the mice were re-injected with cells as above.

3.2.5 Iodination of human and murine PAI-2 proteins

Both hPAI-2 and mPAI-2 were radiolabelled with ^{125}I using the Iodo bead^R method as detailed in chapter 2 (section 2.2.4.1). The characterisation of ^{125}I-hPAI-2 was carried out as stated in chapter 2 (section 2.2.4.2). Characterisation of ^{125}I-mPAI-2 was carried out in a similar manner with the exception that murine PAI-2 was complexed

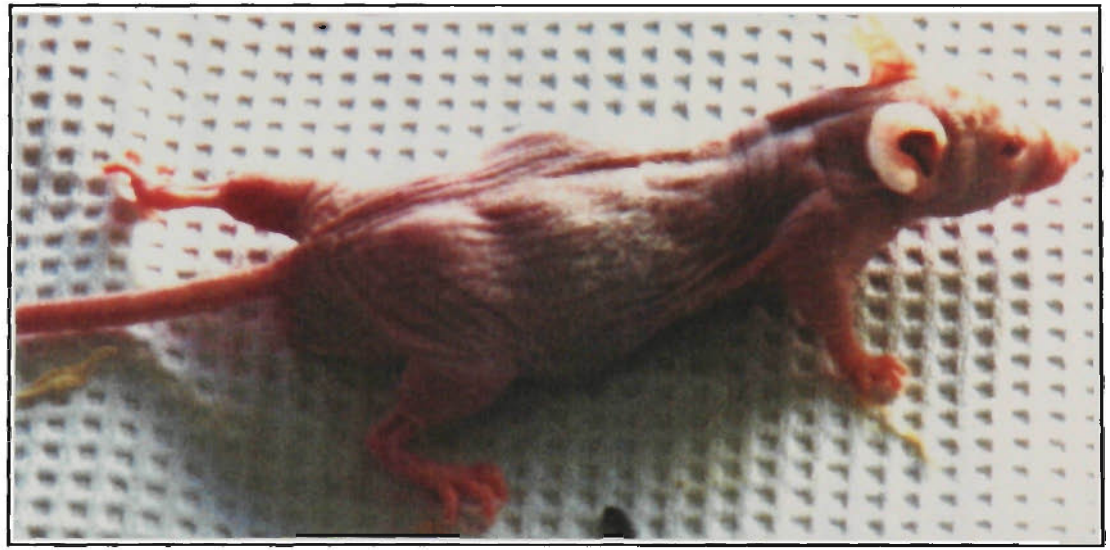
with murine u-PA and not with human u-PA. The murine u-PA was not inactivated with EGR-CMK prior to the addition of ^{125}I-mPAI-2.

3.2.6 Biodistribution and kinetic studies of ^{125}I-hPAI-2 in control and tumour-bearing nude mice

In pharmacokinetics and biodistribution studies, five to twelve-week-old control mice, without tumour xenografts (Figure 1, control mouse), were injected with ^{125}I-hPAI-2 diluted in 100µl of PBS through the lateral tail vein. In one experiment, the mice were killed from 0.5min to 24h after injection (Table I). In another experiment, the mice were killed 5min after intravenous injection of ^{125}I-hPAI-2. Blood was collected from the caval vein in the presence of heparin (50 USP Units/mouse). Following this, plasma was separated from cells by centrifugation at 14,000rpm for 10min at RT in a microfuge. The radioactivity was then measured in 30-50µl duplicate samples of plasma. Urine samples from these mice were collected by puncturing the bladders after the mice were sacrificed. The radioactivity was then measured in duplicate samples of urine.

In tumour-bearing mice, once tumour xenografts in all animals had reached approximately 1cm in diameter (Figure 1, tumour-bearing mouse), ^{125}I-hPAI-2 diluted in 100µl of PBS was injected into the lateral tail vein. Mice were sacrificed from 1 to 240min after injection of ^{125}I-hPAI-2. Blood was collected from each mouse and

A)



B)

Figure 1. Athymic Swiss outbred Nu/Nu mice. A) A control mouse without a tumour xenograft. B) A tumour-bearing mouse with a subcutaneous xenograft of the human colon cancer cell line, HCT116. The diameter of the tumour xenograft on the mouse in this picture is approximately 12mm.

treated as above to obtain plasma samples for measurement of radioactivity. The muscle, skin, liver, kidneys, spleen, heart, lungs and tumour xenografts were also collected for radioactivity measurements. Radioactivity from intact organs were counted, with the exception of the skin and muscle. Instead a small sample of skin and muscle was always taken from the right hind leg and measured for radioactivity. After this the tissues/organs were placed into pre-weighed 25ml tubes and the tissues/organs were weighed. Three separate repeat experiments were carried out. Refer to Table I for an outline of the experiments with the control and tumour-bearing mice.

Table I: Outline of the experiments analysing the kinetics and biodistribution of ^{125}I-hPAI-2 in control and tumour bearing mice.

		Amount of ^{125}I-PAI-2 per mouse	**Number of mice per time point**
Control mice	Exp. 1	100ng	3
	Exp. 2	3.6μg	4
Tumour-bearing mice	Exp. 1	1mg	2
	Exp. 2	200ng	2
	Exp. 3	200ng	3

3.2.7 Calculation of $T_{1/2\alpha}$ and $T_{1/2\beta}$ for control and tumour-bearing mice

The $T_{1/2}$ for the α- and β-phase of ^{125}I-hPAI-2 clearance were calculated using a two-compartment non-linear regression method (Markrides *et al.*, 1996) with the aid of the program Graphpad Prism. The equation used is given below. In the tumour-bearing studies, all data were expressed as the mean ± SD of all the half lives obtained from

the three separate experiments. The data from control mice were expressed as the mean ± SD from one experiment with three mice at each time point.

$$C = Be^{-\beta t} + Ae^{-\alpha t}$$

Where;

C = drug concentration in the central compartment.

B = intercept on the y-axis from back-extrapolation from the line of the second decay.

A = intercept on the y-axis from back-extrapolation from the line of the initial decay.

β = the slope of the extrapolated line of the second decay.

α = the slope of the extrapolated line of the initial decay.

$t_{1/2\alpha}$ = α phase half-life

$$t_{1/2\alpha} = 0.693/\alpha$$

$t_{1/2\beta}$ = β phase half-life

$$t_{1/2\beta} = 0.693/\beta$$

3.2.8 Characterisation of ^{125}I-hPAI-2 in mouse plasma after intravenous injection

The aims of these experiments were to determine the stability of ^{125}I-hPAI-2 in plasma and whether ^{125}I-hPAI-2 still retained activity in mouse plasma after intravenous injection. To assess the stability of ^{125}I-hPAI-2, plasma samples (1:4 dilutions) obtained from mice killed at various times after intravenous injection were added to 4x non-reducing SDS-PAGE sample buffer (1:4 dilutions) and left at RT for 5min prior to being loaded into the wells of 12% SDS-PAGE gels. SDS-PAGE was run as outlined in Chapter 2 (section 2.2.5.2), dried and exposed to Hyperfilm at -70°C for autoradiography. Standards of purified ^{125}I-hPAI-2 were present in all gels, along with molecular weight protein standards (either Mark 12 or Seeblue).

To examine the activity of ^{125}I-hPAI-2 in plasma, human u-PA (1-2μg) was added to plasma samples (diluted 1:2-1:4 in PBS) from mice that had been injected with ^{125}I-hPAI-2 and left for 40min at RT (total reaction was 15 or 30μl). The reactions were stopped by the addition of 4x non-reducing SDS-PAGE sample buffer (5 or 10μl). The samples were loaded into wells of 12% SDS-PAGE gels and the gels conducted as previously stated in Chapter 2 (section 2.2.5.2). Following this, the gel was dried for 3hr using a Bio-Rad gel dryer. Dried gels were then exposed to Hyperfilm and left at -70°C for autoradiography. Purified ^{125}I-hPAI-2 and pre-formed ^{125}I-hPAI-2/u-PA complexes (refer to chapter 2, section 2.2.5.2) were used as standards.

3.2.9 Biodistribution of ^{125}I-mPAI-2

Eight nude mice were intravenously injected with ^{125}I-mPAI-2 (100ng per mouse) and killed at various times after injection (refer to Table II). Blood was collected in 100µl of heparin (5,000IU/ml) and plasma samples obtained as described above (section 3.2.4). Two 50µl samples of plasma from each mouse were collected for radioactivity measurements. The liver, kidneys, spleen, lungs, heart, stomach, skin, muscle and tumour xenografts were also collected for radioactivity measurements. The data from this experiment were processed in the same manner as in section 3.2.5.

Table II: Experimental outline of the biodistribution studies of ^{125}I-mPAI-2.

Number of mice	Time of ^{125}I-mPAI-2 incubation	Presence of tumour xenograft
2	5min	No
2	5min	Yes
2	30min	Yes
2	60min	Yes

3.2.10 Effect of frequency and route of ^{125}I-hPAI-2 injection on ^{125}I-hPAI-2 biodistribution

To determine the influence of the frequency and the route of administration on the biodistribution of PAI-2 in the tumour xenograft of tumour-bearing mice, three injections of ^{125}I-hPAI-2 were given with a 2h interval between each injection (200ng/injection), given either intravenously (i.v., n=4 mice), intraperitoneally (i.p.,

n=4 mice) or subcutaneously (s.c., n=3 mice). The mice were killed 30min after the third and final injection. Mice in the control group (n=3 mice) received a single intravenous injection of ^{125}I-hPAI-2. Samples of liver, skin and tumour were collected and the relative radioactivity determined. The Student-Newman-Keuls multiple comparison test was used to identify statistically significant differences in the amount of radioactivity measured in the tumour, liver and skin between four groups (1. single i.v. injection; 2. multiple i.v. injections; 3. multiple s.c. injections and 4. multiple i.p. injections).

3.2.11 PAI-2 antigen levels

An ELISA was used to determine whether tumour tissues contained hPAI-2 protein. After being left for 2-3months at -20°C to allow for the decay of radioactivity, livers, kidneys and tumour xenografts (n=2 for all three) obtained from the above kinetics and biodistribution studies (section 3.2.4) were homogenised in 10mM Tris, 2mM $MgCl_2$, 0.5mM PMSF, pH 7.5 (8ml/g tissue) using a Dounce homogeniser. Then NaCl (final concentration, 0.15M) was added to the homogenates to restore tonicity. The homogenates were then centrifuged at 4°C for 5min at 400G to pellet the nucleus. To the supernatant, 5% (v/v) Triton X-100 was added (final concentration 0.5% (v/v)) to solubilise membranes for 16h at 4°C. After another centrifugation (1000g, 10min @ 4°C), the supernatant was collected for ELISA.

Polystyrene 96-well microtitre plates were pre-coated with 5μg/ml of goat polyclonal antibody directed against human PAI-2. Purified human recombinant PAI-2 was used for the standard curve which ranged from 0 to 25ng/ml. Homogenate samples (400μg of total protein) were added to the wells and either left overnight at 4°C or for 2h at RT. PAI-2 antigen captured in the wells was then detected with immunopurified polyclonal PAI-2 antibody (1:100 dilution or 4μg/ml) conjugated to horseradish peroxidase. The substrate OPD (150μl 0.2M Na_2HP0_4, pH5.0 with citric acid containing 400μg/ml OPD and 0.012% (v/v) H_20_2) was used for colour detection. Readings were made using a Bio-Rad plate reader at 495nm. The detection limit of the ELISA was 0.31ng/well.

3.2.12 Immunoprecipitation of PAI-2

Tumour-bearing mice were injected intravenously with ^{125}I-hPAI-2 (1μg/mouse). The mice were killed 30min after the injection. Samples of blood, organs and tissues were collected. The organs and tissues were homogenised as described in section 3.2 (5ml/g for the liver, 1.25ml/g for the tumour). The homogenates were then centrifuged at 1000g for 15min. PAI-2 antibody (American Diagnostica Inc., #3750) was added into the supernatants (final concentration, 1μg/ml) and incubated at 4°C for 8h. The mixture was then incubated with 50μl of reconstituted protein A

Sepharose beads (20% (w/v) in 50mM Tris, 3M NaCl, pH7.8) at 4°C for 16h. The protein A Sepharose beads were pelleted and washed (50mM Tris, 0.1M NaCl) at 1000g for 5min. Proteins bound to the beads were extracted with 50µl SDS-PAGE sample buffer. After centrifugation (1000g, 3min), 25µl of the supernatants was separated on a 10% non-reducing SDS-PAGE gel. The dried gel was exposed to a high performance autoradiography film and developed for autoradiography.

3.2.13 Urokinase activity assay

Tumour, liver and kidney tissues were obtained from mice from the kinetics and biodistribution experiments killed at various times. The tissues and organs were homogenised in a volume of 10ml/g as described above (section 3.2.7) with the exception that PMSF was not present. The protein concentration of homogenates was measured using the Bio-Rad DC protein assay kit as per manufacturer's instructions. u-PA activity of the tumour homogenates and kidney homogenates was determined as previously described in Chapter 2 section 2.2.4.2. The generation of plasmin through the activation of plasminogen by u-PA was quantified by the addition of the synthetic plasmin substrate ZLS resulting in a color product. u-PA activity was also measured in homogenates in the presence of PAI-2 (10µg/ml) and an antibody against human u-PA (#394, 10µg/ml). Data were expressed as the mean u-PA activity (IU/mg of total

protein) ± SD in tumour (n=5) and kidney (n=4) homogenate samples. Readings were made using a BioRad plate reader at 405nm.

3.2.14 Phosphoimaging experiments for ^{125}I-hPAI-2

Three 10-11 week old athymic mice with subcutaneous tumour xenografts of HCT116 cells were intravenously injected with ^{125}I-hPAI-2 (10^7 cpm). Mice were sacrificed either 5min or 60min post-intravenous injection. In all experiments the major organs (liver, kidneys, lungs, heart, spleen, stomach, intestines and tumour) were removed and placed adjacent to the skeletal remains of the mouse on a phosphor screen, in order to detect tissue associated radioactivity. The amount of radioactivity in each organ and tissue were visualized using the Molecular Dynamics Storm software. The amount of radioactivity from the organs and tissues were quantified using the program Imagequant and expressed as intensity/pixel for each organ and tissue. Refer to Table III for mass of mice and diameter of tumours.

Table III: Imaging studies experimental outline.

	Mouse mass (g)	Tumour diameter (mm)
Experiment 1	24.4	25
Experiment 2	25.4	28
	30.4	23

3.2.15 Radioactivity data analysis

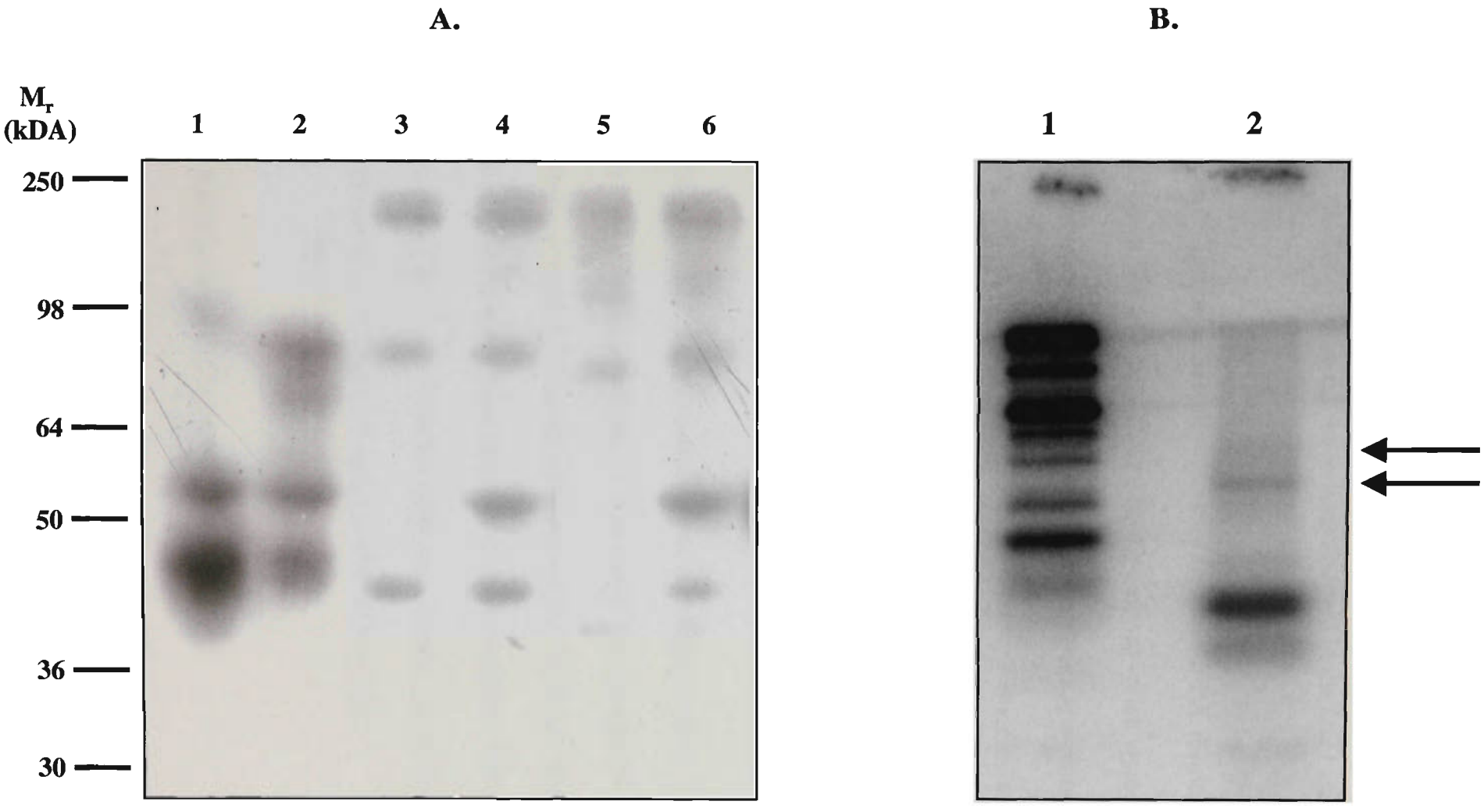
Radioactivity measurements obtained for all plasma and urine samples were expressed as cpm/ml. To standardise results, the cpm/ml data was divided by the total radioactivity injected per mouse and expressed as a percentage. This was called relative radioactivity[a]. Radioactivity measurements for all organs and tissues from all ^{125}I-PAI-2 experiments were expressed as cpm/g. The values of cpm/g for individual organs/tissues were then divided by the total radioactivity injected per mouse. This resulting value was then expressed as a percentage and termed relative radioactivity[b].

3.3 RESULTS

3.3.1 ^{125}I-labeled mouse PAI-2

Comparing the iodinations of mPAI-2 and hPAI-2, similar protein bands were observed for Fractions 4 and 5 of ^{125}I-mPAI-2 (Figure 2A, lanes 3 & 4) obtained from NAP-10 columns as was observed for ^{125}I-hPAI-2 (lane 1). Monomeric ^{125}I-hPAI-2 and ^{125}I-mPAI-2 had a molecular weight of ~46kDa. Higher molecular weight bands were also present in both samples. Reaction of ^{125}I-hPAI-2 with human u-PA resulted in new protein bands with molecular weights of 92kDa and 140kDa (lane 2). When ^{125}I-mPAI-2 was reacted with human u-PA, one of the fractions appeared to be

Figure 2. Characterisation of ^{125}I-mouse PAI-2 (mPAI-2). The two autoradiographs were preparations from two different iodinations. A) Comparison between ^{125}I-hPAI-2 and ^{125}I-mPAI-2. ^{125}I-hPAI-2 only (lane 1) and ^{125}I-hPAI-2 incubated with active human uPA (lane 2) were analysed along with fractions 4 (lane 3) and 5 (lane 4) from purification of ^{125}I-mPAI-2 by a NAP-10 column. ^{125}I-mPAI-2 from fractions 4 and 5 were incubated also with human uPA (lanes 5 & 6, respectively). The samples were separated on 12% SDS-PAGE under non-reducing conditions. Seeblue molecular weight protein standards were used. B) Interaction of ^{125}I-mPAI-2 with mouse uPA. ^{125}I-mPAI-2 (1μg) was reacted with mouse uPA (10μg) (lane 2). Lane 1 is ^{125}I-mPAI-2 by itself. The arrows denotes ^{125}I-PAI-2/uPA complexes. The proteins were separated on 12% SDS-PAGE under non-reducing conditions, followed by exposure to a phosphor-imaging screen. Both images were obtained using the Storm system with the computer program, Imagequant. Molecular weight protein standards were not present in autoradiography B.


A.
B.
M_r (kDA)
1
2
3
4
5
6
250
98
64
50
36
30
1
2

degraded by the human u-PA (lane 5), while no evidence of complex formation between the inhibitor and protease was observed in the other (lanes 6).

However, ^{125}I-mPAI-2 in the presence of mouse u-PA resulted in two new protein bands (Figure 2B, lane 2; indicated by the arrows) which were not present in the ^{125}I-mPAI-2 only sample (lane 1). This suggests complex formation between ^{125}I-mPAI-2 and mouse u-PA. In addition, there is a decrease in the molecular weight of the ^{125}I-mPAI-2 indicating a degradation following reaction with mouse u-PA. Therefore it appears, as was observed with hPAI-2, that the iodination process did not alter the ability of mPAI-2 binding to mouse u-PA.

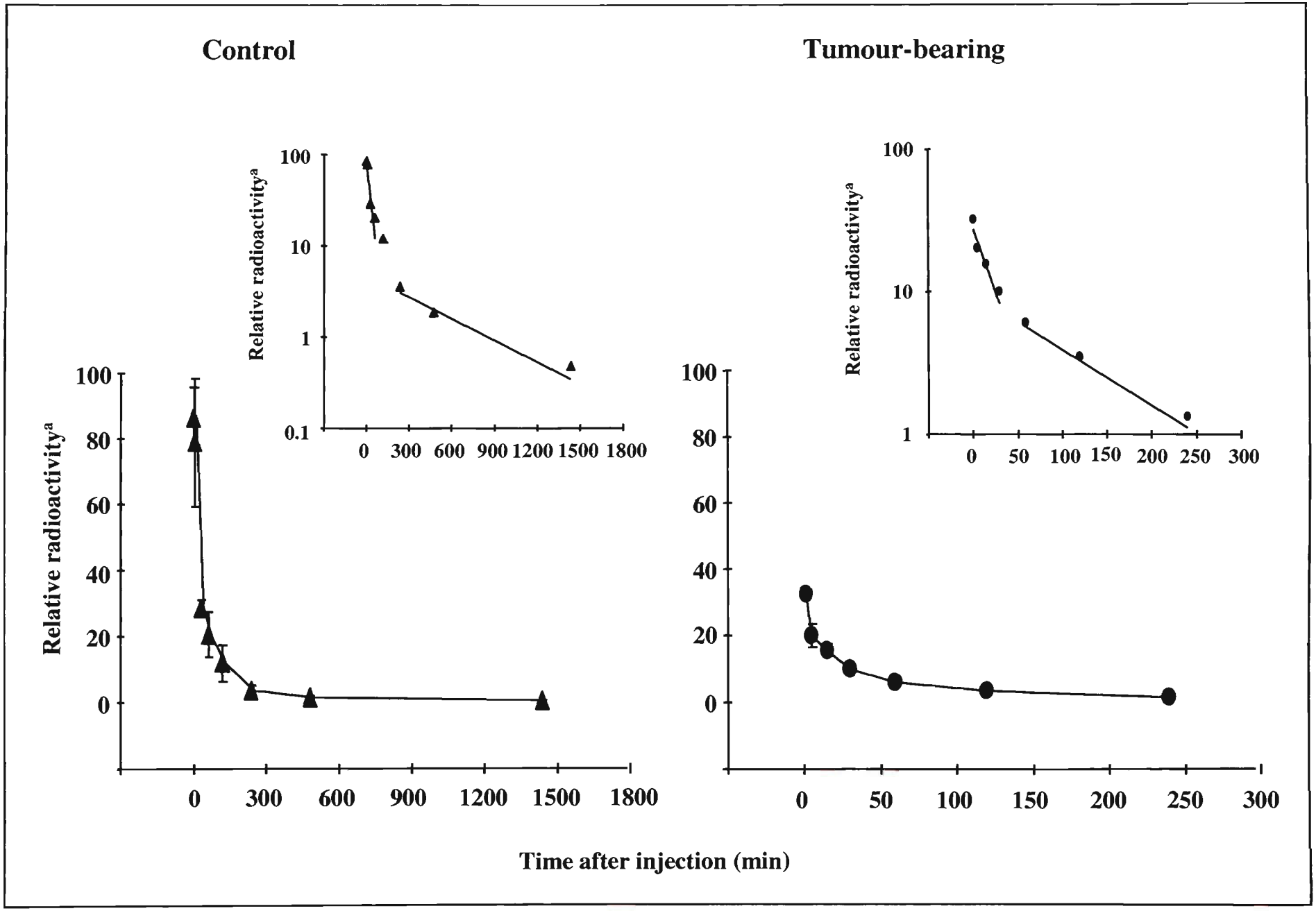
Multiple protein bands were observed in the preparation of ^{125}I-mPAI-2 (Figure 2B, lane 1). At Biotech Australia, it was noted that storage of PAI-2 preparations at concentration greater than 1mg/ml increased the incidence of polymerization, as well as rendering the protein more susceptible to degradation. The stock concentration of mPAI-2 was 12mg/ml. Therefore, this may explain the presence of multiple protein bands observed in ^{125}I-mPAI-2 separated by SDS-PAGE.

3.3.2 Kinetic studies of systemic ^{125}I-PAI-2

The clearance pattern of ^{125}I-hPAI-2 from the circulation of both control and tumour-bearing mice (Figure 3) was observed to be biphasic. The $T_{1/2}$ of the distribution

Figure 3. Biphasic clearance of ^{125}I-hPAI-2 from plasma of nude mice with or without xenografts of the human colon cancer cell line, HCT116. All mice were injected with ^{125}I-hPAI-2 (refer to Table 1) and sacrificed at indicated times. The clearance curve presented for the tumour-bearing mice (●) is from one representative experiment from a total of three experiments. The clearance curve presented for the control mice (▲) is from one experiment. Each point from each curve is the mean ± SD of relative radioactivity[a] (n=3 mice at each time point). The $T_{1/2\alpha}$ and $T_{1/2\beta}$ were calculated using a two-compartment non-linear regression model (Markrides *et al.*, 1996) with the computer program, Graphpad prism.

Relative radioactivity[a]: Taken as the radioactivity in plasma expressed on a per ml basis and divided by the total amount of radioactivity injected per mouse. This value was then expressed as a percentage.


Control
Tumour-bearing
Relative radioactivity[a]
Time after injection (min)
100
80
60
40
20
0
0 300 600 900 1200 1500 1800
0 50 100 150 200 250 300
10
1
0.1

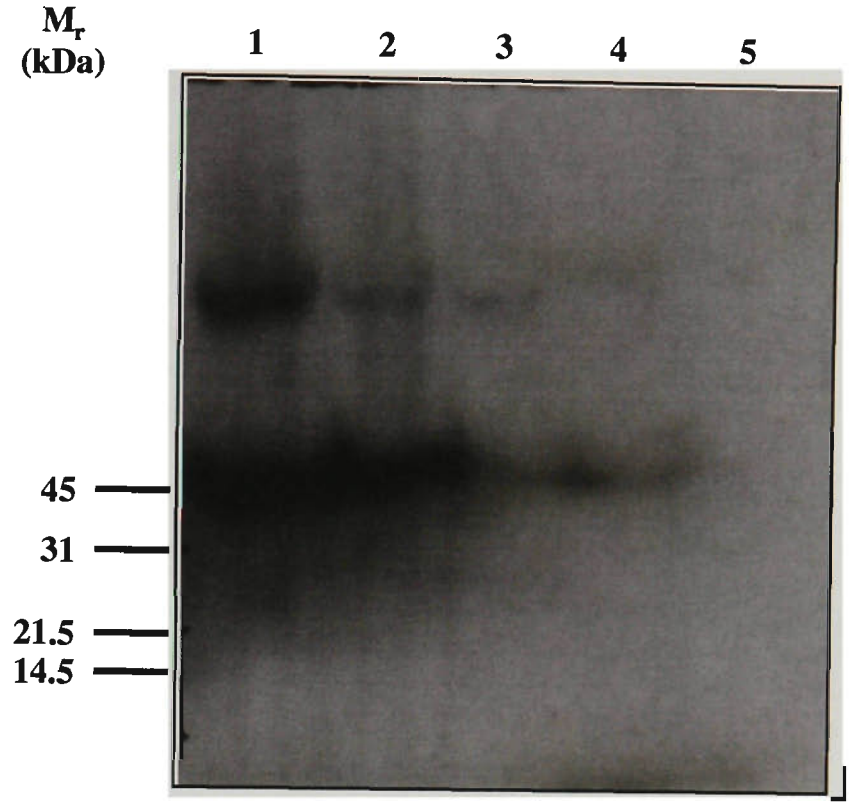
phase ($T_{1/2\alpha}$) in control and tumour-bearing mice were 12.5min and 1.4min, respectively. The $T_{1/2}$ of the elimination phase ($T_{1/2\beta}$) in the control and tumour-bearing mice were 342min and 29min, respectively. At 60min post-injection, 11.2 ± 3.1% and 6.9 ± 2% of total injected dose remained in the plasma of control and tumour-bearing mice, respectively. These data reflect a faster clearance of ^{125}I-hPAI-2 in tumour-bearing mice. Additionally, the influence of the size of injected dose of ^{125}I-hPAI-2 (100-500ng/mouse) on clearance kinetics in control mice was investigated by a post-doctoral fellow in the lab and found to have no significant effect on $T_{1/2}$ (data not shown).

The stability of ^{125}I-hPAI-2 in plasma was tested on samples taken at various times after injection into control (Figure 4A) and tumour-bearing mice (Figure 4B). In plasma samples from control mice, the characteristic monomer, dimer and larger bands were observed up to 2h post injection of ^{125}I-hPAI-2 (Figure 4A, lanes 1-4). These bands were also observed in plasma samples at 4h post injection (lane 5). However, the intensity of the bands at 4h were much lighter when compared to the intensity of the bands at other time points. At 8h (not shown) no protein bands were visible in plasma samples from control mice.

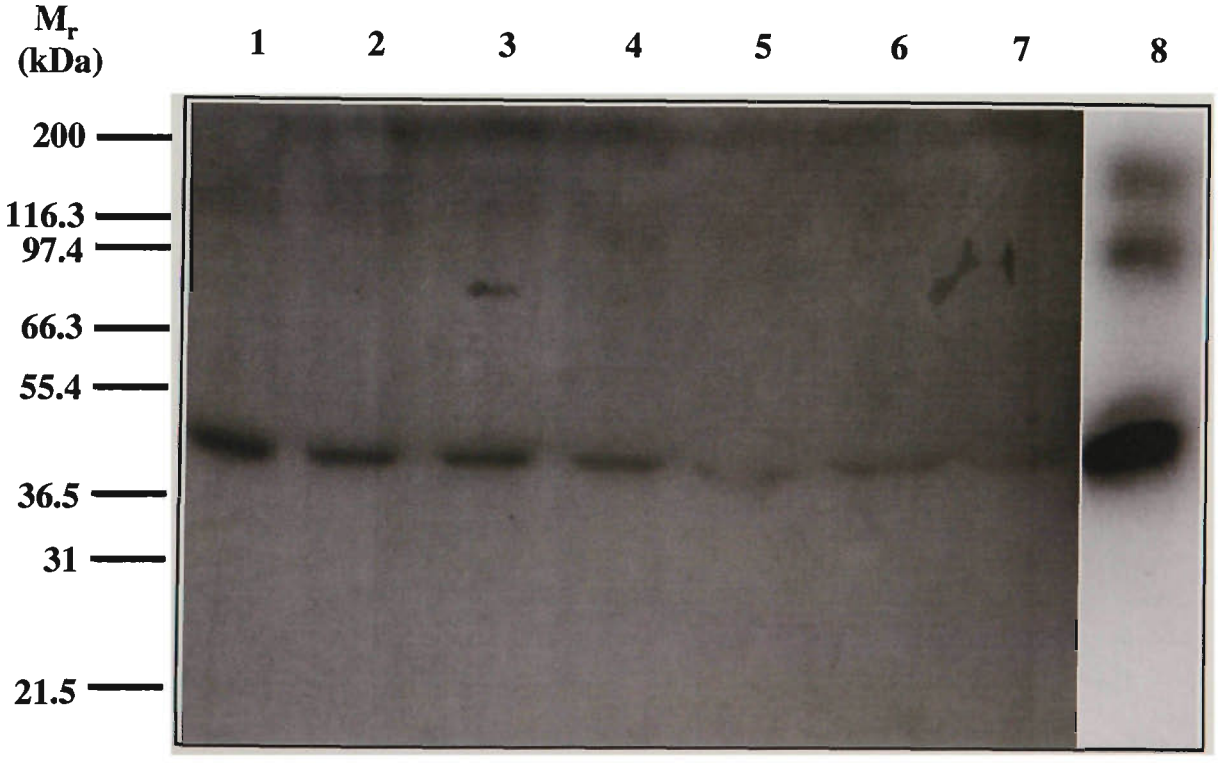
Plasma samples from tumour-bearing mice injected with ^{125}I-hPAI-2 also showed the characteristic monomer and larger molecular weight bands from 1min up to 4h post injection (Figure 4B, lanes 1-7) indicating that the radioactivity detected was

Figure 4. Analysis of plasma from control (A) and tumour-bearing mice (B) after intravenous injection with ^{125}I-hPAI-2. Mice were intravenously injected with ^{125}I-hPAI-2 (100ng/mouse for control; 200ng/mouse for tumour-bearing) and sacrificed at various times. A) Plasma samples at 5min (lane 1), 30min (lane 2), 60min (lane 3), 120min (lane 4) and 240min (lane 5) after the injection were separated on 4-20% gradient SDS-PAGE and exposed to film (16 days at -70°C) for autoradiography. Low molecular weight protein standards were used. B) Plasma samples obtained from tumour-bearing mice killed at 1min (lane 1), 5min (lane 2), 15min (lane 3), 30min (lane 4), 60min (lane 5), 120min (lane 6) and 240min (lane 7) post injection were separated on 12% SDS-PAGE. ^{125}I-hPAI-2 only standard was also present (lane 8). The lanes 1-7 were exposed to film for 4 days at -70°C, while the ^{125}I-hPAI-2 only standard (lane 8) was exposed to film overnight at RT, for autoradiography. Mark 12 molecular weight protein standards were used.

A)


M_r
(kDa)
1
2
3
4
5
45
31
21.5
14.5

B)

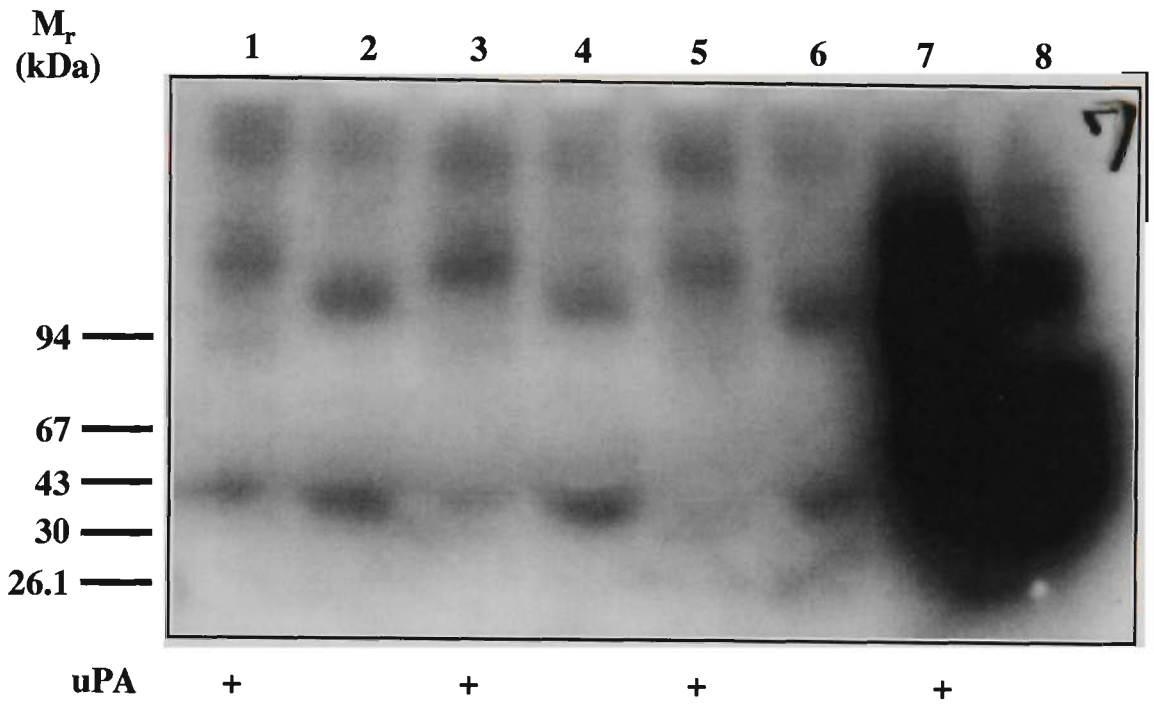

M_r
(kDa)
1
2
3
4
5
6
7
8
200
116.3
97.4
66.3
55.4
36.5
31
21.5

representative of intact PAI-2 protein. However, with increasing time following injection, the intensity of the monomeric band was seen to decrease. The molecular weight of monomeric ^{125}I-hPAI-2 in plasma samples were 43kDa, while the three higher molecular weight bands were 117kDa, 141kDa and 198kDa. The ^{125}I-hPAI-2 standard (Figure 4B, lane 8), present with plasma samples from the tumour-bearing mice, contained a monomeric band of 43kDa and two higher molecular weight bands of 96kDa and 141kDa. Free ^{125}I represented less than 10% of the total radioactivity found in the plasma samples as determined by TCA precipitation. These results illustrate that human PAI-2 does not form detectable SDS-stable complexes with any mouse plasma proteins (Figure 4A & B).

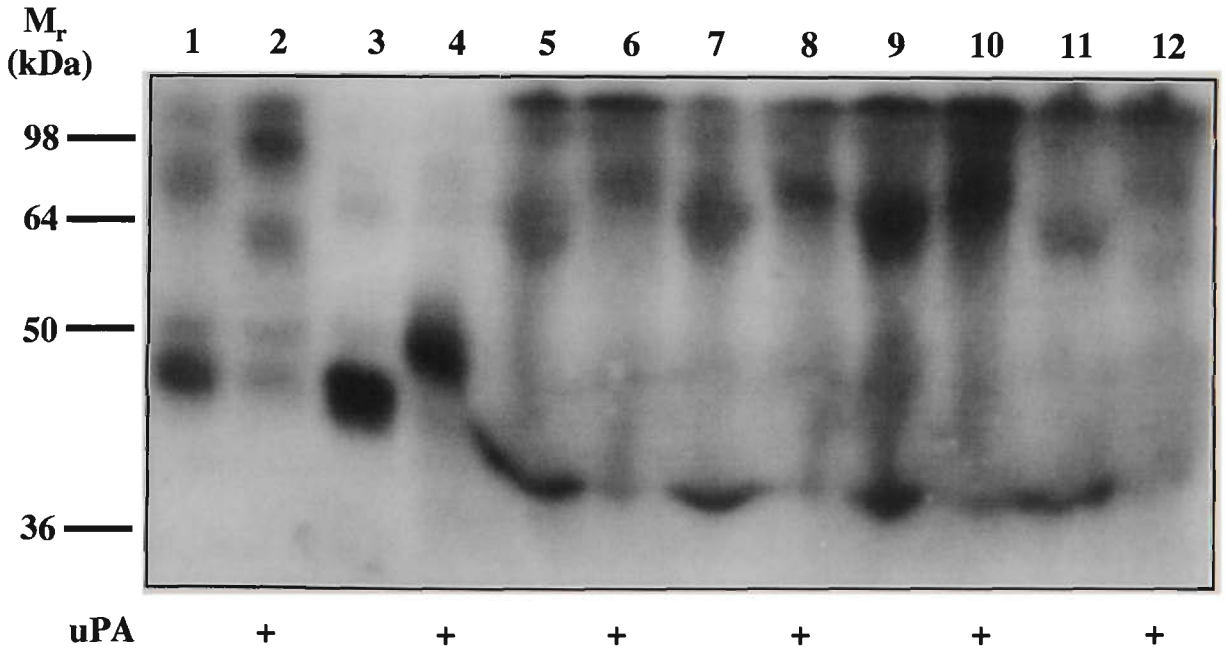
The activity of ^{125}I-hPAI-2 in plasma samples of tumour-bearing mice injected intravenously with ^{125}I-hPAI-2 was examined. Plasma samples from tumour-bearing mice injected with ^{125}I-hPAI-2 were reacted with human u-PA. Figure 5 shows two autoradiographs of plasma samples which had either been incubated with (denoted by +) or without u-PA. The samples from both autoradiographs were the same plasma samples, with the exception that the bottom autoradiograph contains plasma samples from one extra mouse. Also, plasma samples from the top autoradiograph were separated on a gel and exposed to film 20 days after the mice were killed, while samples from the bottom autoradiograph were separated and exposed to film 40 days after the mice were sacrificed.

Figure 5. Characterisation of intravenously injected ^{125}I-hPAI-2 in plasma from tumour-bearing mice. Plasma samples from tumour-bearing mice which had been intravenously injected with ^{125}I-hPAI-2 (2.5μg/mouse) were either reacted with (denoted by +) or not reacted with human uPA. The samples from both autoradiographs were the same plasma samples, with the exception that the bottom autoradiograph contains another plasma sample from a different mouse. A) Murine plasma samples are in lanes 1-6. ^{125}I-hPAI-2 alone (lane 8) and ^{125}I-hPAI-2/uPA complexes (lane 7) were overexposed (2 days exposure at -70°C), hence are not discernable in this figure but were discernable from the original autoradiography. Plasma samples were separated using a 12% SDS-PAGE gel and exposed to film 20 days after the mice were sacrificed. B) ^{125}I-hPAI-2 alone (lanes 1 & 3) and ^{125}I-hPAI-2/uPA complexes (lanes 2 & 4) were conducted under non-reducing (lanes 1 & 2) and reducing (lanes 3 & 4) conditions. Murine plasma samples from mouse 1 are in lanes 5-8 and from mouse 2 in lanes 9-12. Samples were separated using a 12% SDS-PAGE gel and exposed to film 40 days after the mice were sacrificed.

A.


M r (kDa)
1
2
3
4
5
6
7
8
94
67
43
30
26.1
uPA
+
+
+
+

B.


M r (kDa)
1
2
3
4
5
6
7
8
9
10
11
12
98
64
50
36
uPA
+
+
+
+
+
+

^{125}I-hPAI-2 (Fig. 5A, lane 7; Fig. 5B, lanes 1 & 3) and ^{125}I-hPAI-2/u-PA complex standards (Fig. 5A, lane 7; Fig. 5B, lanes 2 & 4) had similar banding patterns to the ^{125}I-hPAI-2 standards in both autoradiographs as observed previously, with the molecular weight of monomeric ^{125}I-hPAI-2 being 43kDa. High molecular weight bands were also observed in the ^{125}I-hPAI-2 standard from both autoradiographs. The reaction between ^{125}I-hPAI-2 and u-PA under non-reducing conditions resulted in protein bands with molecular weights of 65kDa and 84kDa which were not present in the ^{125}I-hPAI-2 standard.

Similar protein bands were observed in the plasma samples and ^{125}I-hPAI-2 standard in autoradiograph A. In autoradiograph B, the molecular weight of monomeric ^{125}I-hPAI-2 in plasma was 37kDa which was lower than the molecular weight of monomeric ^{125}I-hPAI-2 standard (43kDa). This was thought to be due to degradation of the plasma ^{125}I-hPAI-2 during storage, because the analysis of other plasma samples less than 10 days after the mice were killed showed the molecular weight of monomeric ^{125}I-hPAI-2 to be similar to the molecular weight of monomeric ^{125}I-hPAI-2 in the standard (Refer to Figure 4B). In plasma samples where u-PA was present, a decrease in intensity or sometimes the disappearance of monomeric ^{125}I-hPAI-2 was observed as compared to samples without u-PA (Figure 5A, lanes 1, 3 & 5; Figure 5B, lanes 6, 8, 10 & 12). This decrease in monomeric ^{125}I-hPAI-2 was accompanied by the appearance of a major band with molecular weight of 76kDa and a fainter band of 63kDa (as calculated from autoradiograph B). This suggests that

plasma ^{125}I-hPAI-2 in tumour-bearing mice has retained its inhibitory activity since it was still able to form SDS-stable complexes with exogenously added active human u-PA.

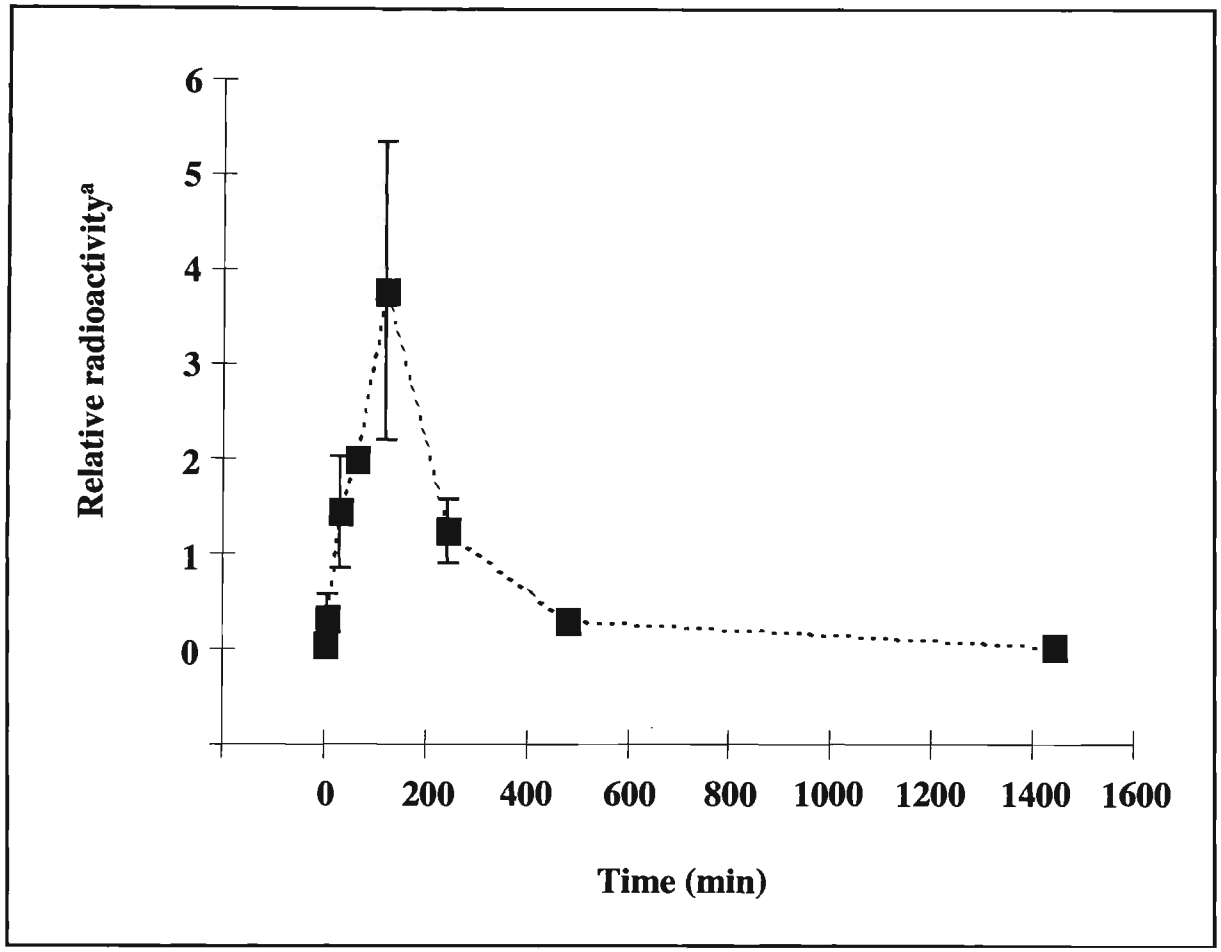
3.3.3 Radioactivity in urine of mice injected with ^{125}I-PAI-2

Radioactivity appeared in urine as early as 0.5min after intravenous injection of ^{125}I-PAI-2 (Figure 6A) in control mice. A steep increase was observed in relative radioactivity from 0.5min to 2h. At 2h after injection, the maximal amount of relative radioactivity was achieved and was 77-fold greater than at 0.5min. This was followed by a steep decline in relative radioactivity at 4h after injection, which was approximately 33% of the maximal amount at 2h. A slower rate of decrease was observed between 4 to 8h. At 24h after injection, the relative radioactivity was approximately 0.5% of the relative radioactivity observed at 2h. As determined by SDS-PAGE and autoradiography, all radioactivity found in urine after 2h was derived from degraded ^{125}I-PAI-2 unlike plasma samples where minimal degradation had occurred (Figure 6B).

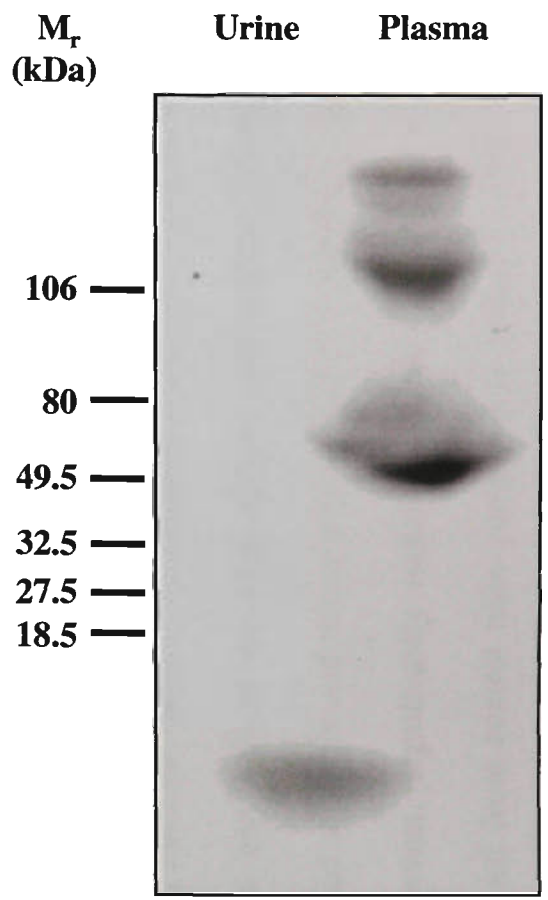
Figure 6. Clearance of ^{125}I-hPAI-2 in mouse urine. A) Control mice were intravenously injected with ^{125}I-hPAI-2 (100ng/mouse) and killed at various times. Urine samples were collected after the mice were killed by puncturing the bladders. The data is presented as mean relative radioactivity ± SD from three mice per time point. B) A mouse was injected with 500ng (or $5x10^6$cpm) of ^{125}I-hPAI-2. After 2h plasma and urine were collected and analysed by 4-20% gradient non-reducing SDS-PAGE. The gel was exposed for 2.5 days at -70°C prior to being developed. Low range pre-stained SDS-PAGE molecular weight protein standards were used.

Relative radioactivity[a]: Taken as the radioactivity in the urine expressed as cpm/ml divided by the total cpm injected per mouse.

A.


Relative radioactivity[a]
6
5
4
3
2
1
0
0
200
400
600
800
1000
1200
1400
1600
Time (min)

B.


M_r (kDa)
Urine
Plasma
106
80
49.5
32.5
27.5
18.5

3.3.4 Organ and tissue biodistribution of human ^{125}I-PAI-2

Radioactivity data showed that ^{125}I-hPAI-2 distributes amongst a range of organs and tissues after i.v. injection into both tumour-bearing and control mice. Radioactivity levels peaked at 5min in kidneys, liver, lung and spleen of control mice (Figure 7A & B) and decreased rapidly over the following 4h. In skin and muscles, radioactivity levels peaked at 30min followed by a comparatively slower decline (Figure 7C).

In tumour-bearing mice, radioactivity levels in the lung (Figure 8B), heart and muscle (Figure 8C) peaked at 1min after i.v. injection. The radioactivity levels in the kidneys peaked after 5min (Figure 8A), while the liver (Figure 8A), spleen (Figure 8B) and skin (Figure 8D) peaked at 15min after i.v. injection of ^{125}I-hPAI-2.

The amount of radioactivity in all organs and tissues at the first time point from control mice was greater compared to the amount of radioactivity in the organs and tissues from tumour-bearing mice. In control mice the majority of radioactivity was located in the kidneys (Figures 9A) 5min after ^{125}I-hPAI-2 injection, while in tumour-bearing mice the majority of radioactivity was located in the liver. This corresponded to 13% and 37% of the total injected radioactivity, respectively. This was also observed at 30min and 60min after ^{125}I-hPAI-2 injection (Figure 9B & C). At most time points, significantly higher levels of radioactivity remained in all tissues examined from control mice compared to tumour-bearing mice. This reflects the

Figure 7. The uptake and clearance of ^{125}I-hPAI-2 by organs and tissues from control mice. ^{125}I-hPAI-2 was injected into control mice and sacrificed at various times after injections. The organs and tissues were taken for radioactivity measurements. A. Liver and kidney. B. Lung and spleen. C. Skin and muscle. The data was expressed as mean relative radioactivity[b] ± SD from one experiment with three mice per time point.

Relative radioactivity[b] : Taken as the radioactivity in the organs/tissues on a per gram basis then divided by the total amount of radioactivity injected per mouse and expressed as a percentage.

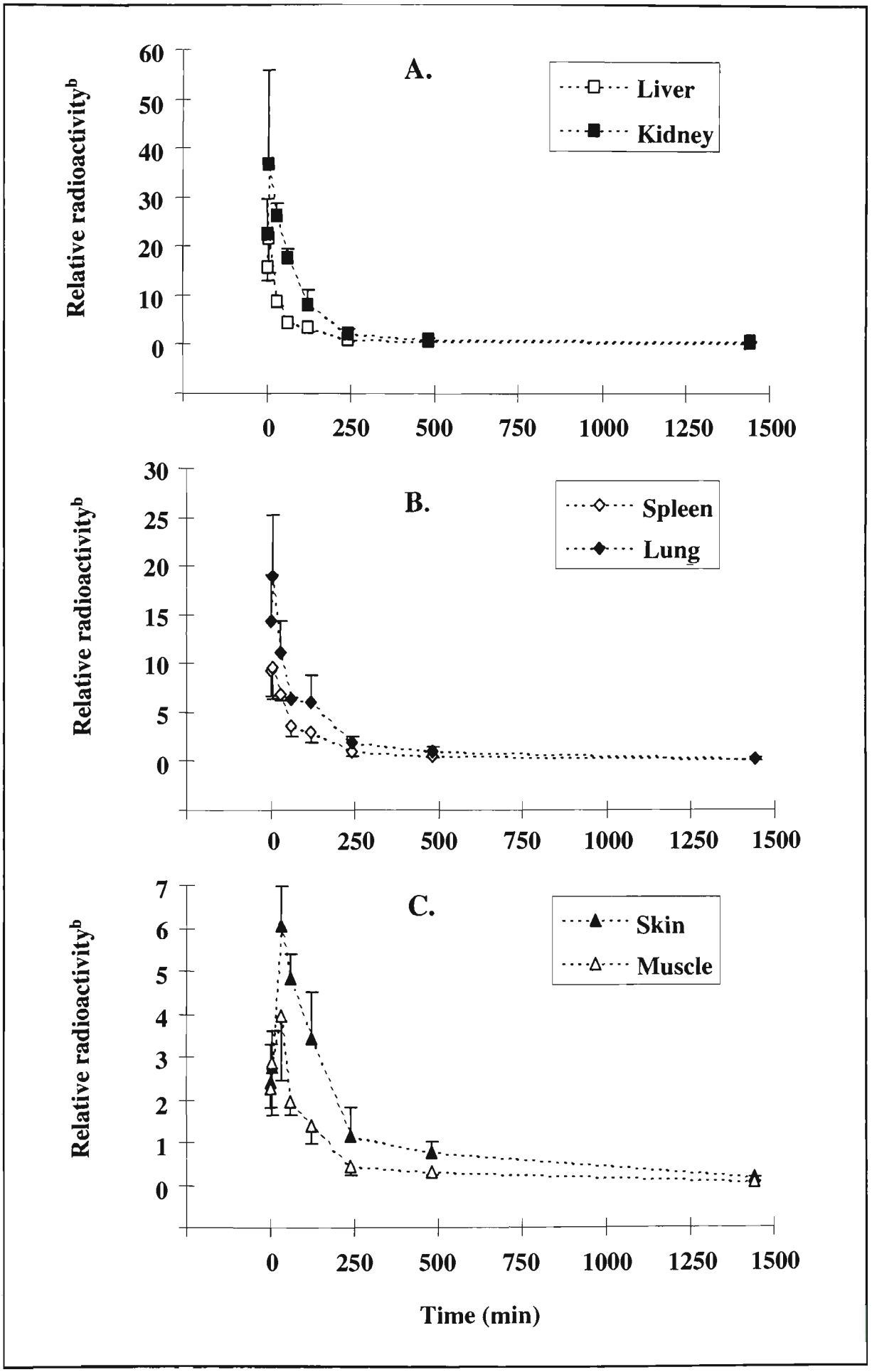

A.
Liver
Kidney
Relative radioactivity[b]
60
50
40
30
20
10
0
0
250
500
750
1000
1250
1500
B.
Spleen
Lung
Relative radioactivity[b]
30
25
20
15
10
5
0
0
250
500
750
1000
1250
1500
C.
Skin
Muscle
Relative radioactivity[b]
7
6
5
4
3
2
1
0
0
250
500
750
1000
1250
1500
Time (min)

Figure 8. The uptake and clearance of ^{125}I-hPAI-2 by organs and tissues from tumour-bearing mice. Data were obtained from one experiment with three mice at each time point. The mice were intravenously injected with ^{125}I-PAI-2 and sacrificed at the indicated time. The major organs including the liver, kidney, spleen (A), lung, heart (B), muscle and skin (C) were removed and the radioactivity determined. The tumour was also removed and the radioactivity determined (C). The data was expressed as mean relative radioactivity[b] ± SD from a representative experiment with three mice per time point.

Relative radioactivity[b]: Taken as the radioactivity in the organs/tissues on a per gram basis then divided by the total amount of radioactivity injected per mouse and expressed as a percentage.

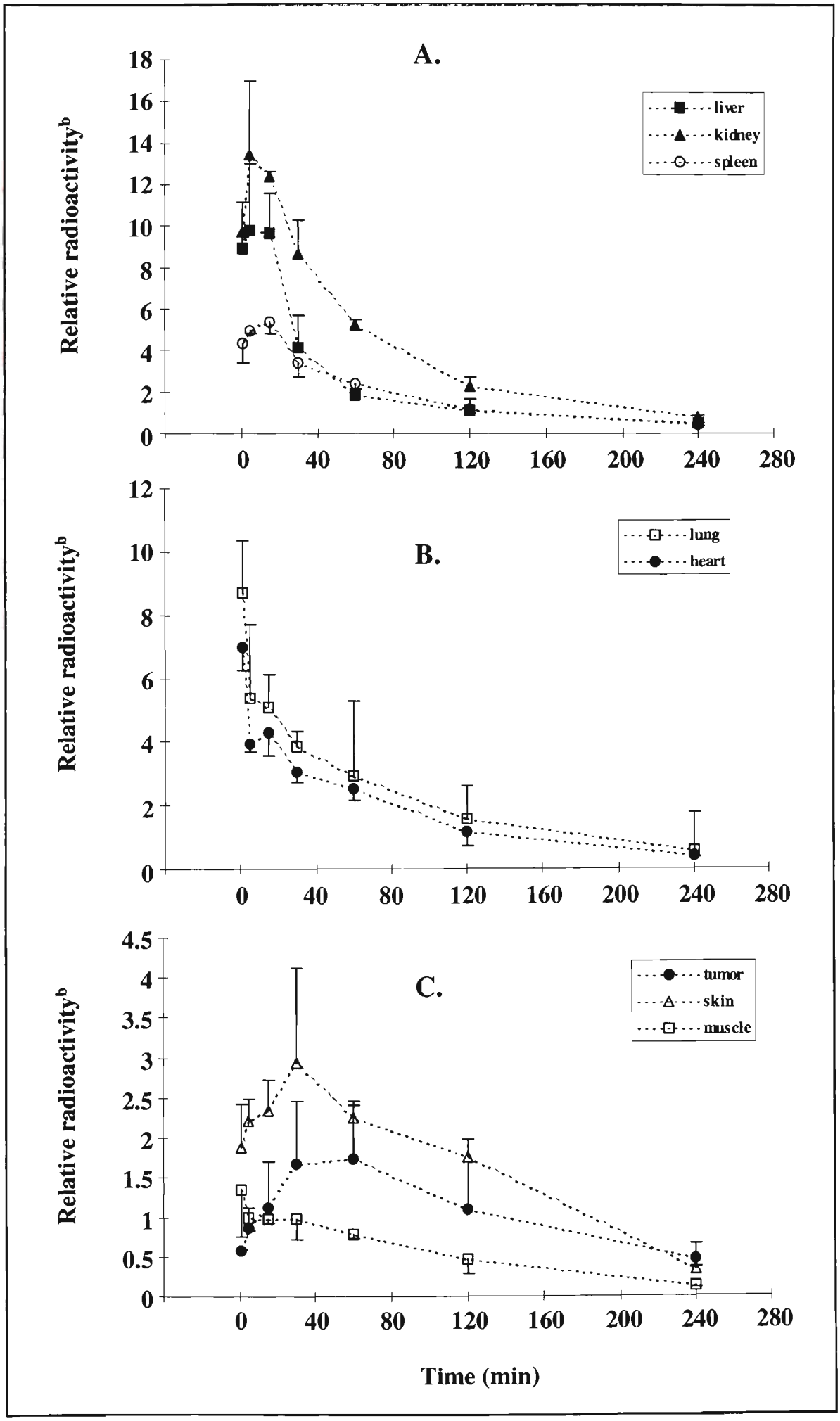
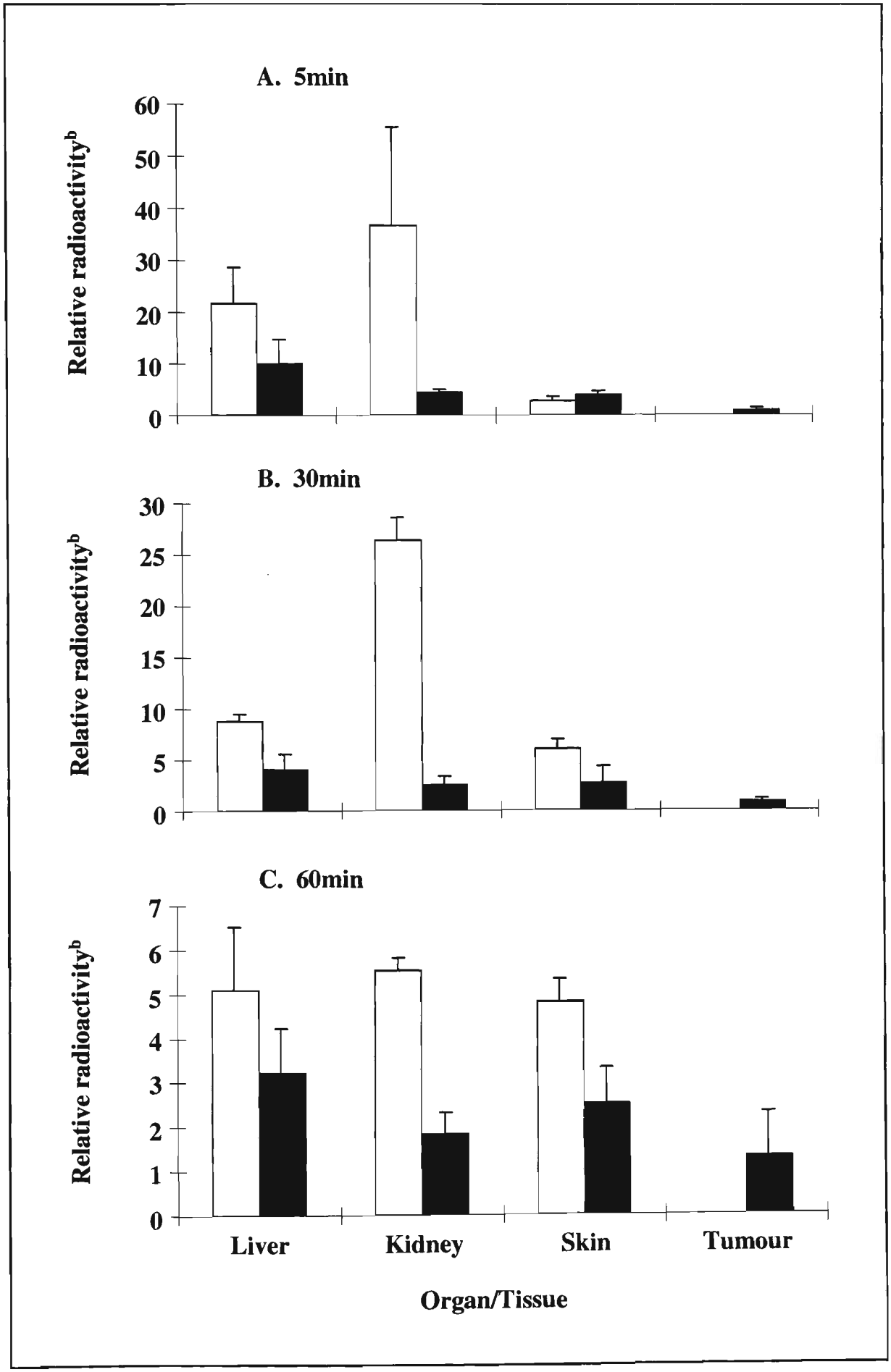

A.
Relative radioactivity[b]
liver
kidney
spleen
B.
lung
heart
C.
tumor
skin
muscle
Time (min)

Figure 9. Tissue distribution of ^{125}I-hPAI-2 at 5min (A), 30min (B) and 60min (C) post-intravenous injection in control and tumour-bearing mice. Control (open bars) and tumour-bearing (filled bars) mice were intravenously injected with ^{125}I-hPAI-2 and sacrificed at various times. The organs and tissues were removed and the radioactivity determined. Data is presented as the mean relative radioactivity[b] ± SD of three mice.

Relative radioactivity[b] : Taken as the radioactivity in the organs/tissues on a per gram basis then divided by the total amount of radioactivity injected per mouse and expressed as a percentage.


A. 5min
Relative radioactivity[b]
60
50
40
30
20
10
0
B. 30min
Relative radioactivity[b]
30
25
20
15
10
5
0
C. 60min
Relative radioactivity[b]
7
6
5
4
3
2
1
0
Liver
Kidney
Skin
Tumour
Organ/Tissue

longer elimination phase observed in control mice (control mice $T_{1/2\beta}$ = 342min, tumour-bearing mice $T_{1/2\beta}$ = 29min). However, between 5min to 4h the rate of decrease in radioactivity levels was approximately 50% faster in organs and tissues of control compared to tumour-bearing mice. Radioactivity levels peaked in the stomach by 2h and this was followed by a sharp reduction to background levels. In contrast, the thyroid steadily accumulated free iodine over 24h.

3.3.5 Tumour distribution of human ^{125}I-PAI-2

Radioactivity was found in tumour xenograft tissues remarkably quickly (1min post ^{125}I-hPAI-2 injection), reaching a plateau between 30min and 60min (Figure 8C). Assessment of tumour uptake indicated a peak of 1.5 $\pm$ 0.5% of the total radioactivity injected post ^{125}I-hPAI-2 injection at 60min (Figure 9C). Tumour tissues also retained radioactivity longer than the other major organs. At 4h radioactivity in the tumour xenografts had decreased from peak levels 90% slower compared to the kidney, liver and lung. Compared to the spleen and skin the peak radioactivity had decreased 70% slower. The amount of relative radioactivity in the tumour xenograft was found to be proportional to the tumour mass (Figure 10), when examined at 30min and 60min after intravenous injection of ^{125}I-hPAI-2.

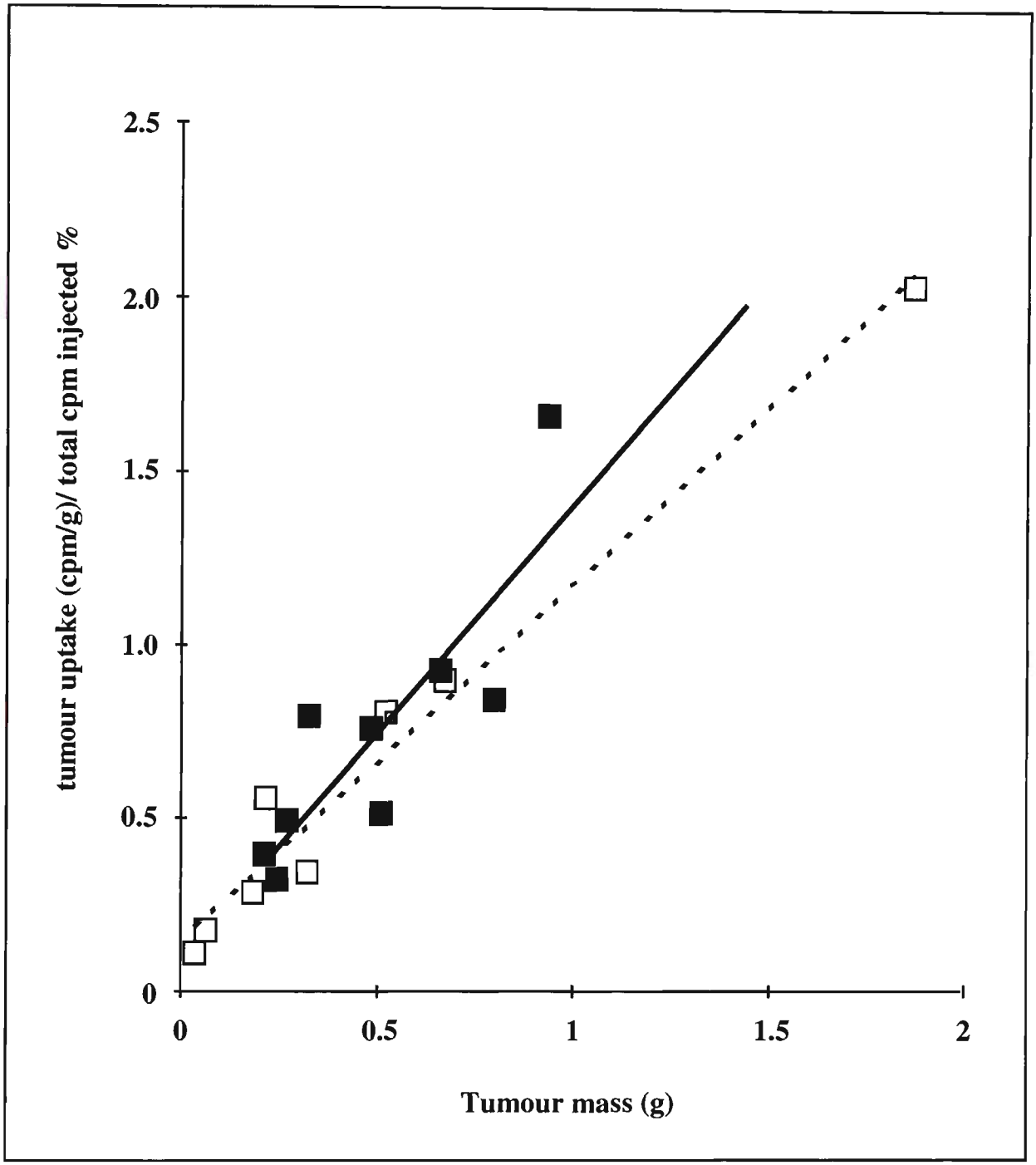


Figure 10. Relationship between tumour mass and the amount of radioactivity accumulated in the tumour. The data are presented as the amount of radioactivity in the tumour (cpm/g) divided by the total radioactivity injected per mouse and expressed as a percentage. The graph represents tumour mass and the corresponding radioactivity measured from four separate experiments (n=1 at each point) taken at 30min (open squares, R^2=0.98) and 60min (closed squares, R^2=0.72) after intravenous injection of human ^{125}I-PAI-2.

3.3.6 Tissue biodistribution of mouse ^{125}I-PAI-2

Similar biodistribution patterns were observed with ^{125}I-mPAI-2 as was seen with ^{125}I-hPAI-2, after intravenous injection. The majority of radioactivity was found in the plasma post-intravenous injection of ^{125}I-mPAI-2 (Figure 11A). An 18% and 33% reduction in radioactivity in plasma from tumour-bearing mice was observed at 30min and 60min, respectively, compared to the radioactivity in plasma 5min post injection. Similar results were observed with injected ^{125}I-hPAI-2 in mouse plasma (section 3.3.2).

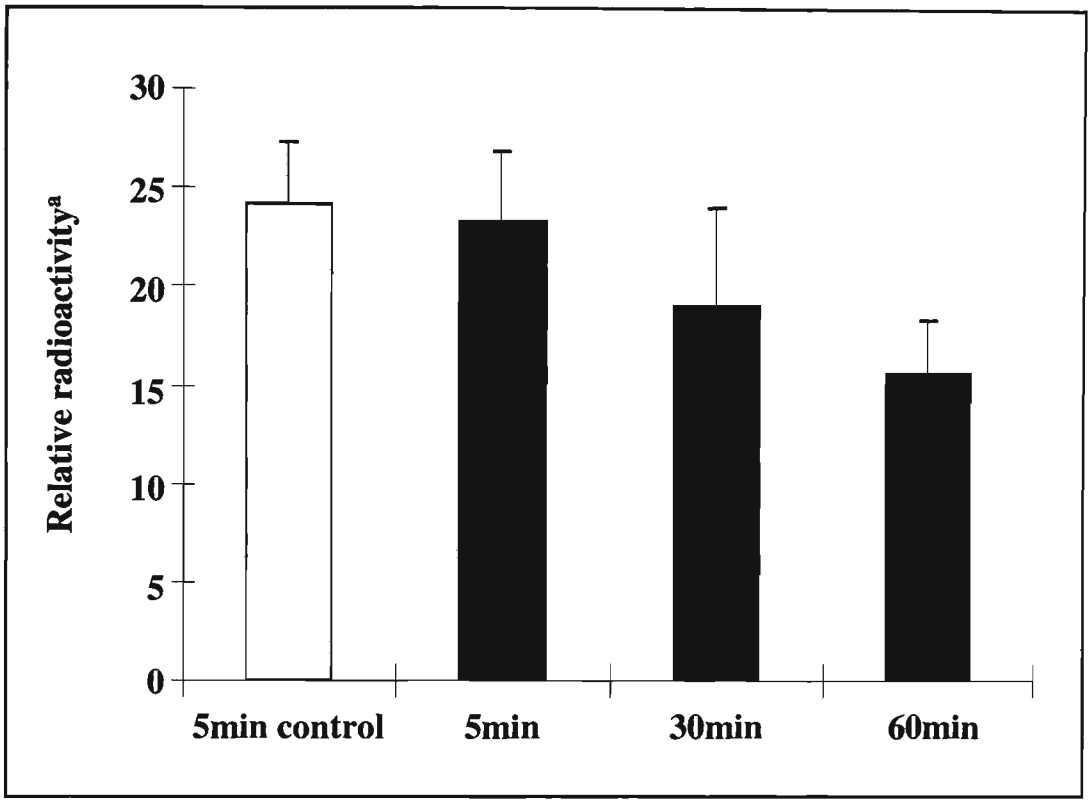
As shown by the autoradiography (Figure 11B), monomeric ^{125}I-mPAI-2 in plasma had a molecular weight of 38kDa, which was lower than the molecular weight of monomeric ^{125}I-mPAI-2 (44kDa) of the standard. This was thought to be due the storage of plasma for a lengthy period after the mice were sacrificed prior to analysis, as was seen with some of the results for ^{125}I-hPAI-2 (Figure 5). Higher molecular weight bands of 100kDa, 132kDa and 169kDa were also evident in plasma samples.

At 5min (Figure 12A) and 30min (Figure 12B) post injection of ^{125}I-mPAI-2, the kidney had the most radioactivity levels at 12% and 20% of total injected radioactivity, respectively. The lungs and stomach were the other two organs in which high amounts of radioactivity was measured. Radioactivity in most organs had significantly decrased by 60min (Figure 12C) in tumour-bearing mice, with the

Figure 11. ^{125}I-mPAI-2 in plasma of control and tumour-bearing mice. Control (open bar) and tumour-bearing (filled bars) mice were intravenously injected with ^{125}I-mPAI-2 (100ng/mouse) and sacrificed either 5, 30 or 60min after injection. Plasma samples from each mouse were taken for radioactivity measurements. A) The data is presented as mean relative radioactivity[a] ± SD (n=2 mice) at each time point. B) An autoradiography of ^{125}I-mPAI-2 in plasma of control (lanes 2 & 3) mice 5min post injection and tumour-bearing mice at 5min (lanes 4 & 5), 30min (lanes 6 & 7) and 60min (lanes 8 & 9) post injection. An ^{125}I-mPAI-2 standard was in lane 1. Samples were separated using 12% SDS-PAGE and dried.

Relative radioactivity[a]: Taken as the radioactivity in the plasma expressed as cpm/ml divided by the total cpm injected per mouse.

A.


Relative radioactivity[a]
30
25
20
15
10
5
0
5min control
5min
30min
60min

B.

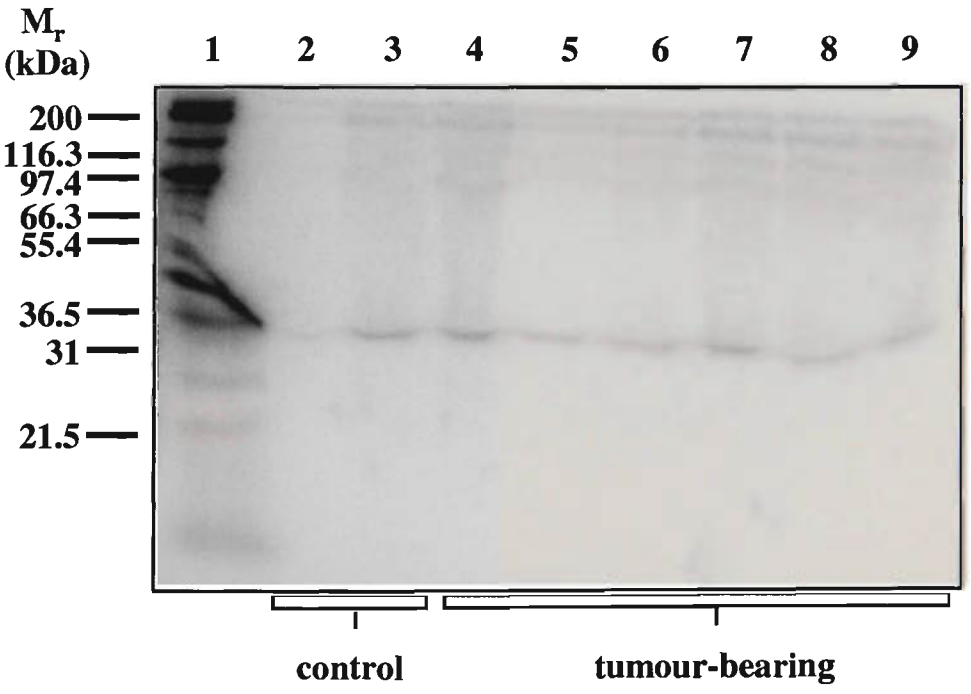
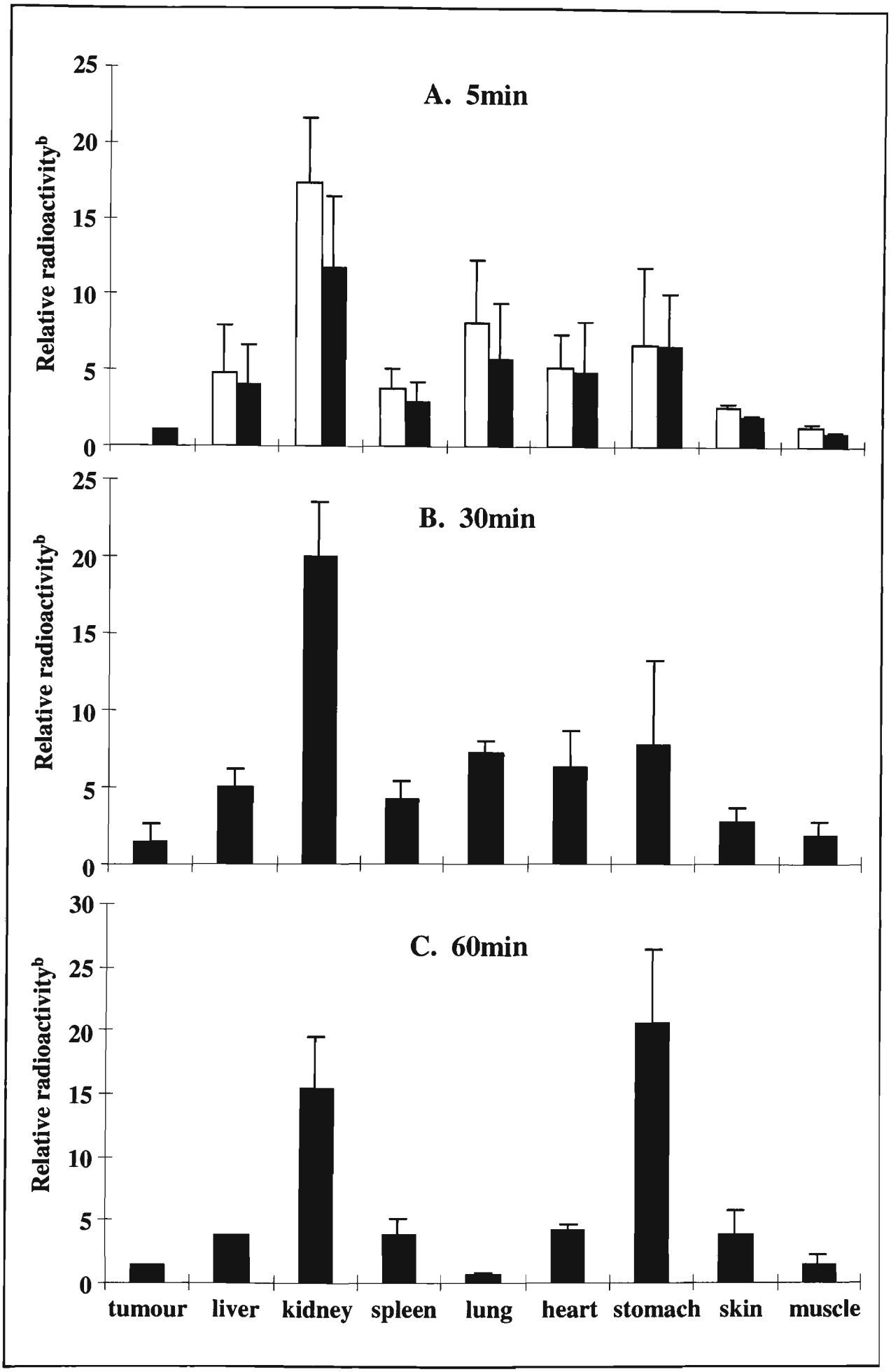

M_r (kDa)
1
2
3
4
5
6
7
8
9
200
116.3
97.4
66.3
55.4
36.5
31
21.5
control
tumour-bearing

Figure 12. Tissue distribution of ^{125}I-mPAI-2 at 5min (graph A), 30min (graph B) and 60min (graph C) post-intravenous injection in control and tumour-bearing mice. Both control (open bars) and tumour-bearing (filled bars) mice were intravenously injected with ^{125}I-mPAI-2 (100ng/mouse) and sacrificed at various times. The organs and tissues were collected for radioactivity measurement. Data is presented as the mean relative radioactivity[b] ± SD of two mice at each time point.

Relative radioactivity[b]: Taken as the radioactivity in the organs/tissues on a per gram basis then divided by the total amount of radioactivity injected per mouse and expressed as a percentage.


A. 5min
B. 30min
C. 60min
Relative radioactivity[b]
25
20
15
10
5
0
30
tumour
liver
kidney
spleen
lung
heart
stomach
skin
muscle

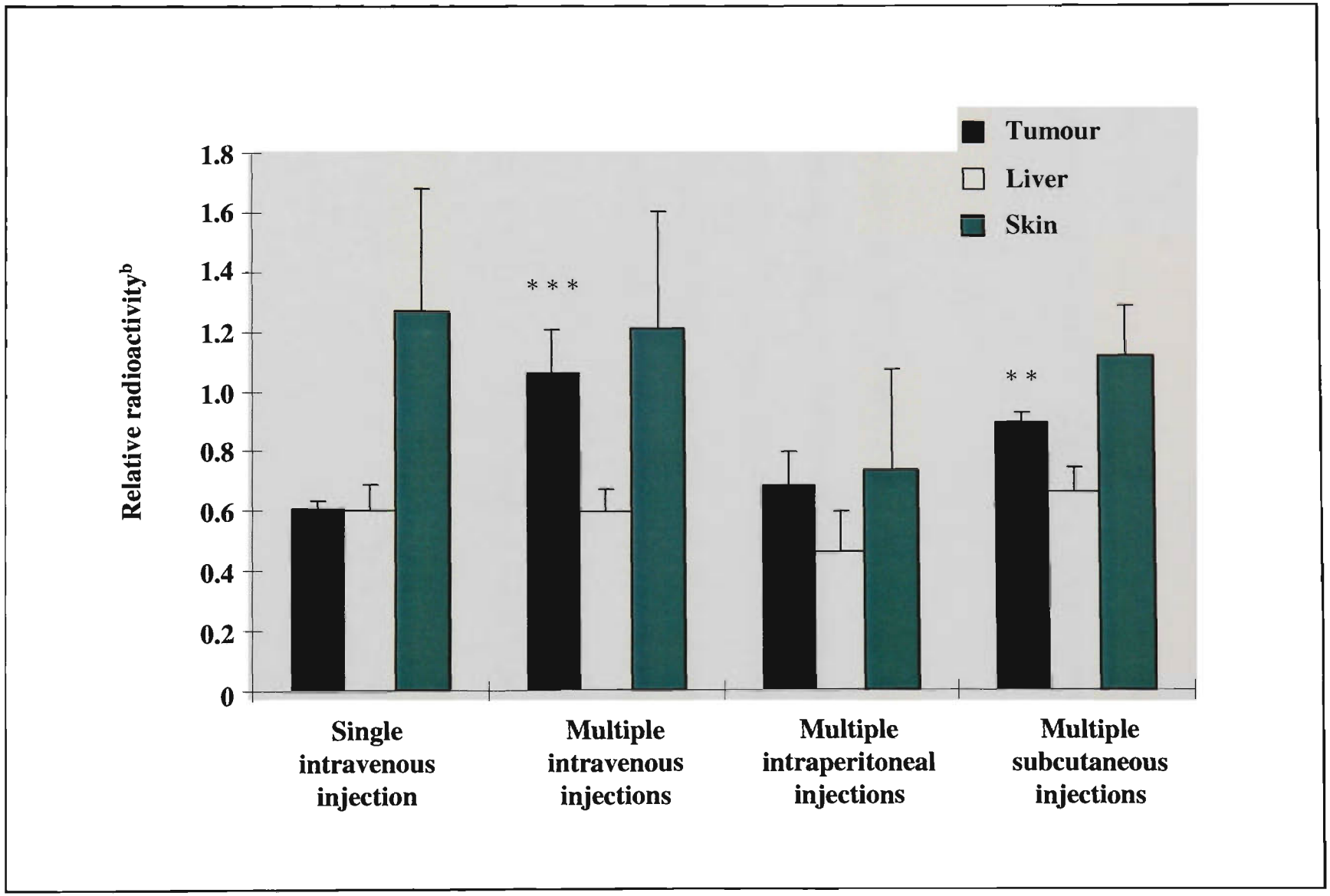
exception of the stomach and tumour tissue. As was observed with ^{125}I-hPAI-2, intravenous injection of ^{125}I-mPAI-2 resulted in increased radioactivity in the stomach and tumour between 5min and 60min post injection. A maximal amount of radioactivity of 1.4% was observed in tumour tissues at 30min (± 1.2%) and 60min (± 0.05%) post-intravenous injection of ^{125}I-mPAI-2. In contrast to ^{125}I-hPAI-2, no significant differences were observed in the radioactivity levels in all tissues between control and tumour-bearing mice at 5min post injection of ^{125}I-mPAI-2.

3.3.7 Multiple routes and multiple injections of ^{125}I-PAI-2

Multiple injection strategies and routes were examined in an attempt to increase the amount of PAI-2 associated radioactivity localising in tumour xenografts. Since radioactivity took longer to peak in tumour xenografts and had a longer clearance time compared to most organs, multiple intravenous injections of ^{125}I-hPAI-2 resulted in a significant increase ($p<0.001$) in accumulation of radioactivity in the tumour without any accompanying increase in other organs such as the liver and skin (Figure 13). A similar pattern was obtained when mice were given multiple s.c. injections of ^{125}I-hPAI-2, but the increase, though significant ($p<0.05$), were not as large as those seen via the i.v. route. Multiple i.p. injections did not result in significant increases in radioactivity compared to a single i.v. injection.

Figure 13. Increased accumulation of radioactivity in the tumors after multiple injections of ^{125}I-hPAI-2. Three mice were given a single intravenous injection of ^{125}I-PAI-2 and were sacrificed 30min after the injection. The other groups of mice were given three injections of ^{125}I-PAI-2 either intravenously (n=4), intraperitoneally (n=4) or subcutaneously (n=3). The injections were given at two hour intervals and the mice were sacrificed 30min after the final injection. The tumour, liver and skin were collected from each mouse and the radioactivity measured and expressed as cpm per gram. Using the student-Newman-Keuls multiple comparison test a significant difference between the single injection and multiple intravenous injections (* * * , $P<0.001$) and between single injection intravenous and multiple subcutaneous injections (* * , $P<0.05$) was observed in tumour tissues.

Relative radioactivity[b] : Taken as the radioactivity in the organs/tissues on a per gram basis then divided by the total amount of radioactivity injected per mouse and expressed as a percentage.


Tumour
Liver
Skin
Relative radioactivity[b]
1.8
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0
* * *
* *
Single intravenous injection
Multiple intravenous injections
Multiple intraperitoneal injections
Multiple subcutaneous injections

3.3.8 ^{125}I-hPAI-2 antigen content in tissues from both tumour-bearing and control mice

The amount of human recombinant PAI-2 protein localising to various tissues was quantified using a PAI-2 specific ELISA. Despite the fact that there was more than 5 times the radioactivity in the liver compared to tumour xenografts at 5min after injection of ^{125}I-hPAI-2, similar levels of PAI-2 antigen were detected in liver and tumour samples (Figure 14). In kidney samples, where radioactivity levels were also much higher than that of tumour samples 5min after intravenous injection of ^{125}I-hPAI-2, the levels of PAI-2 antigen were higher than those observed in other tissues. PAI-2 antigen levels decreased in the liver between 5 and 60min. In contrast, the opposite was observed in kidney and tumour samples (Figure 14) suggesting that PAI-2 accumulated in these tissues. This was especially the case with tumour tissues and reflects the finding that radioactivity peaked between 30 and 60min after injection and decreased thereafter at a slower rate than in both the liver and kidneys (Figure 8A & D).

Although immunoreactive PAI-2 was difficult to detect in any tissues by Western blotting, immunoprecipitation with an antibody directed against human PAI-2 detected a PAI-2 band of approximately 48kDa in tumour samples taken 30min after injection of ^{125}I-hPAI-2 (Figure 15). Bands corresponding to the molecular weights

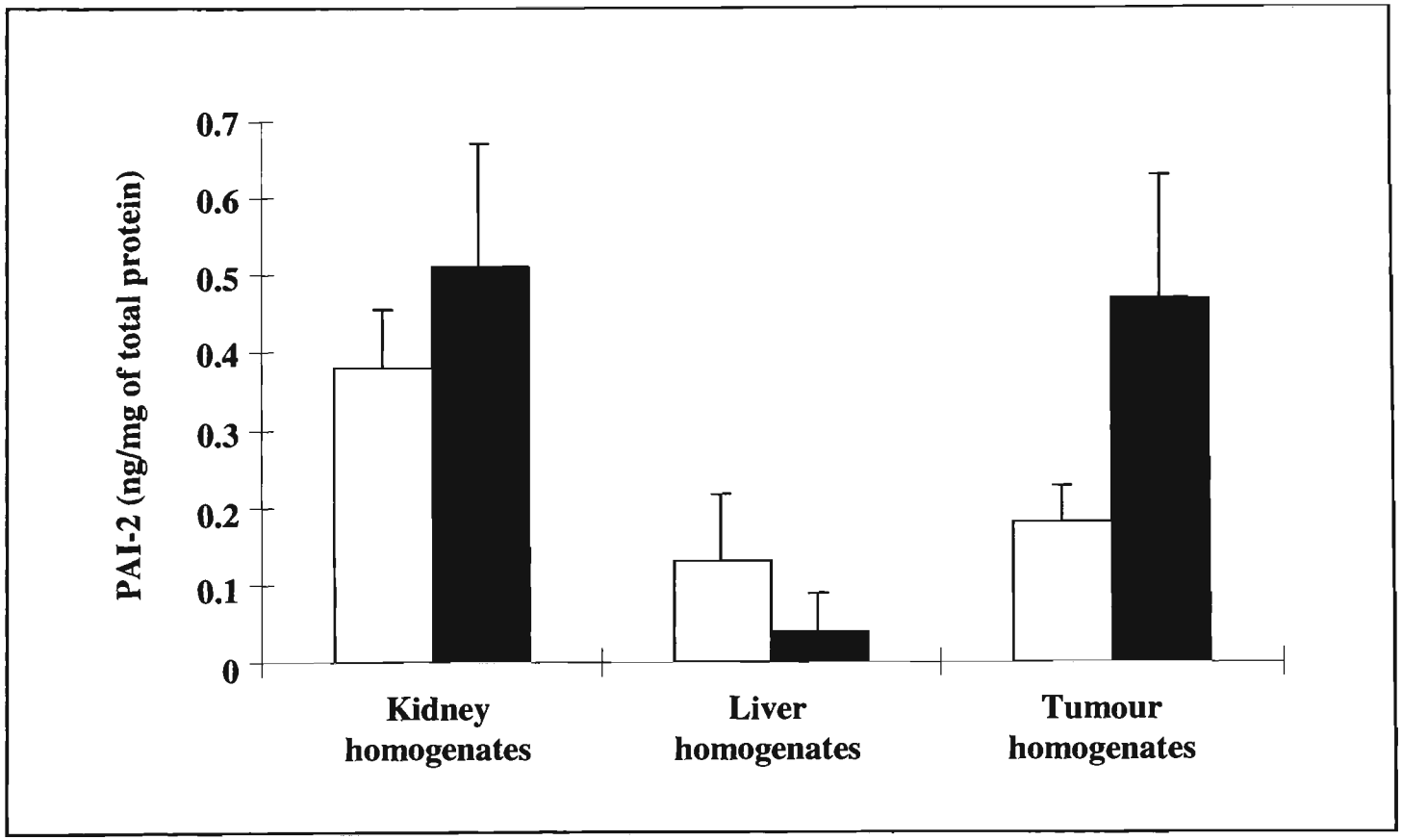


Figure 14. PAI-2 antigen in liver, kidney and tumour xenograft homogenates. Liver, kidney and tumour xenograft were obtained from mice killed at 5min (open bars) and 60min (filled bars) post-intravenous injection from one kinetic and biodistribution experiment. The organs and tumour tissue were homogenised and the resulting homogenates were assayed for the presence of PAI-2 antigen by ELISA. Results were from 2 mice at each time point. Units are expressed as mean ng of PAI-2/mg of total protein ± SD.

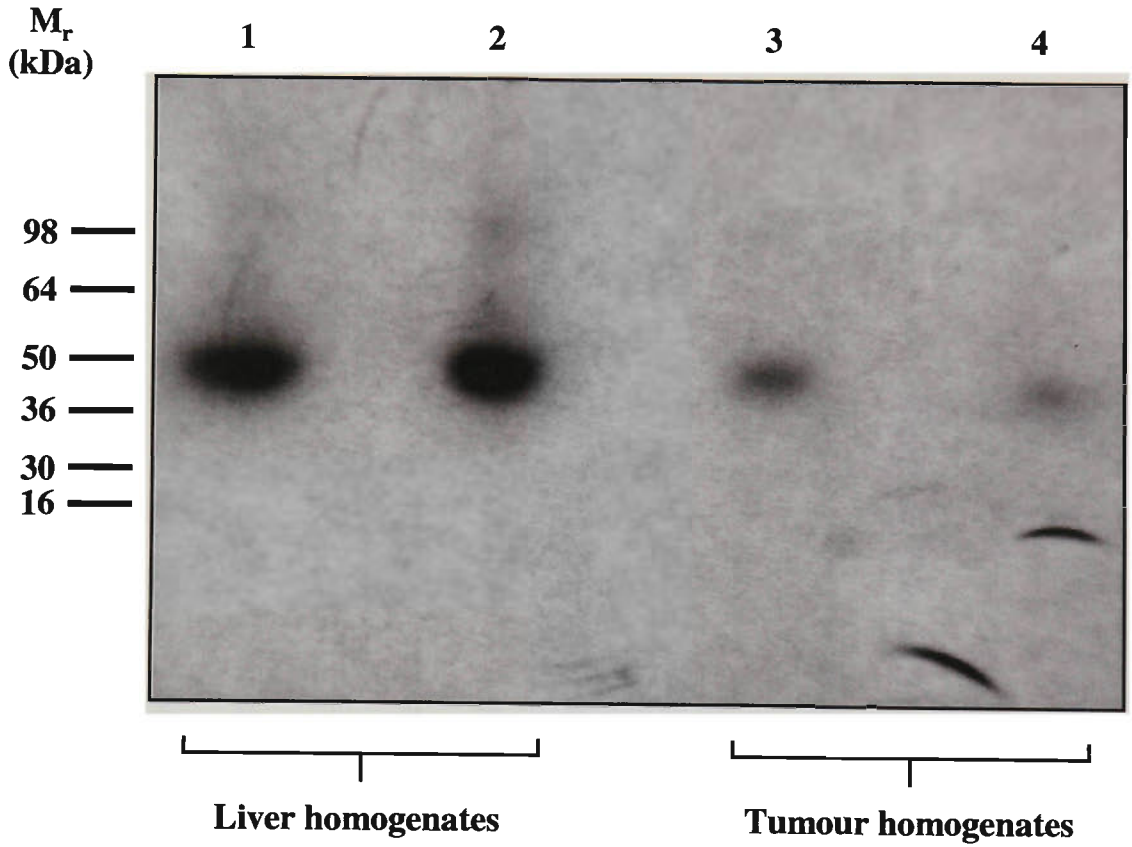


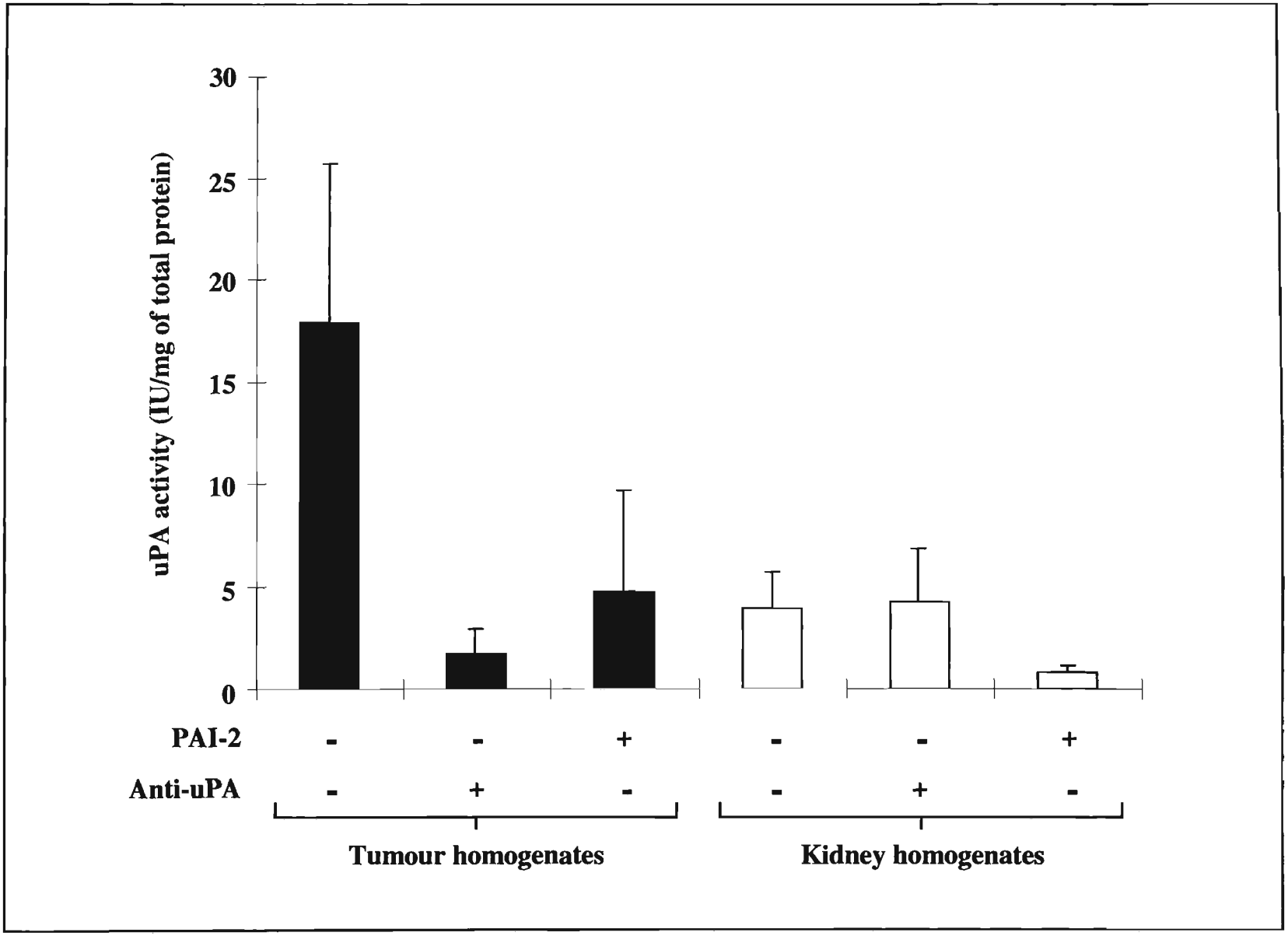
Figure 15. Immunoprecipitation of hPAI-2 from liver and tumour xenograft homogenates. Lanes 1 & 2 are immunoprecipitates of ^{125}I-hPAI-2 from liver homogenates. Lanes 3 & 4 are immunoprecipitates of ^{125}I- hPAI-2 from tunour homogenates. The samples were separated using a 10% non-reducing SDS-PAGE gel. The dried gel was exposed to film at -70°C for 8 days.

of u-PA:PAI-2 complexes (i.e. bands of ~82kDa and 98kDa) were never detected, suggesting rapid dissociation and/or degradation after complex formation.

3.3.9 Urokinase activity in tumour and kidney homogenates

Using a plasmin substrate, the specific u-PA activity (IU/mg of total protein) of tumour and kidney homogenates were measured indirectly by examining the generation of plasmin activity. The u-PA activity in tumour homogenates was 4.5 times greater than that of kidney homogenates (Figure 16). In the presence of added PAI-2 the u-PA activity in both tumour and kidney homogenates was approximately 26% and 20% of control (no inhibitors present), respectively. The monoclonal antibody against human u-PA resulted in a reduction of u-PA activity in tumour homogenates to approximately 9% of control activity. In contrast, no reduction in u-PA activity was observed with kidney homogenates in the presence of the u-PA inhibitory monoclonal antibody. This was expected because the monoclonal antibody is specifically directed against human u-PA and not mouse u-PA.

Figure 16. uPA activity measured in tumour xenograft and kidney homogenates. Tumour xenografts (n=5) and kidneys (n=4) were obtained from mice sacrificed after a biodistribution and kinetic experiment and homogenised. The Coleman and Green assay (1979) was used to measure uPA activity in either the presence (+) or absence (-) of an inhibitory monoclonal antibody against human uPA (Anti-uPA) or human recombinant PAI-2. The data is expressed as mean uPA activity (IU) per mg of total protein ± SD.


30
25
20
15
10
5
0
uPA activity (IU/mg of total protein)
PAI-2
Anti-uPA
- - + - - +
- + - - + -
Tumour homogenates
Kidney homogenates

3.3.10 Phosphoimaging of radioactivity in organs and tissues of tumour bearing mice

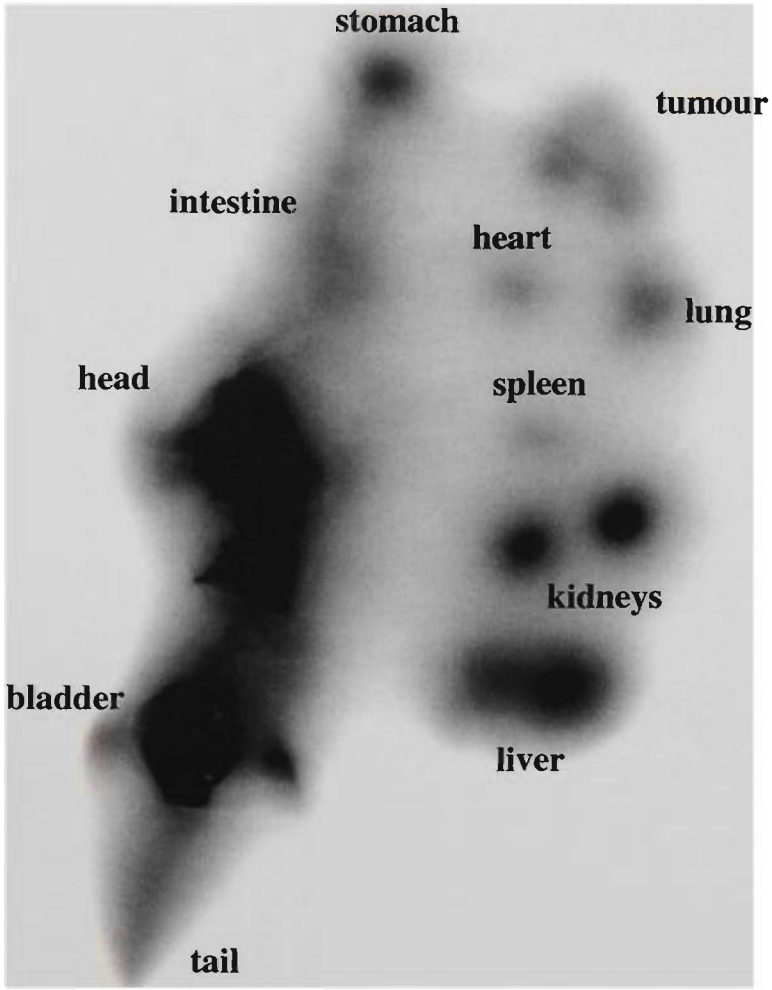
Phosphoimaging results confirmed results obtained from the kinetics experiments at 5min and 60min post-intravenous injection of ^{125}I-hPAI-2. Images of radioactivity in the various tissues and organs from mice killed 5min and 60min after injection are shown in Figure 17. Using the computer program, Imagequant, the intensity of radioactivity in each organ and tissue was obtained and depicted (intensity/pixel) in the bar graph 5min (Figure 18A) and 60min (Figure 18B) after injection. At 5min after intravenous injection of ^{125}I-hPAI-2, the majority of radioactivity was in the liver, kidneys and stomach (Figures 17A & 18A). At 60min, the intensity of radioactivity in the liver, kidneys, heart, lungs and spleen were reduced compared to 5min (Figures 17B & 18B). The opposite was observed for the stomach, intestines and tumour xenograft. At 60min, after injection of ^{125}I-hPAI-2, the intensity of radioactivity in these organs and tissues were greater than at 5min (Figures 17B & 18B).

3.4 DISCUSSION

This study is the first to describe the pharmacokinetics and biodistribution of human, recombinant ^{125}I-PAI-2 *in vivo* in both control and tumour-bearing animals. The use

Figure 17. Radioactivity in organs and tissues of tumour-bearing mice at 5min and 60min after i.v. injections of ^{125}I-hPAI-2. The mice were injected with ^{125}I-hPAI-2 and sacrificed either 5min (A) or 60min (B) after injection. The organs including, liver, kidneys, lungs, heart, spleen, stomach and intestines were removed along with the tumour xenograft, and all, including the remains of the mice, were exposed to a phosphor screen for 24h. Images were obtained from the Storm system using the software Imagequant.

A. 5min



B. 60min

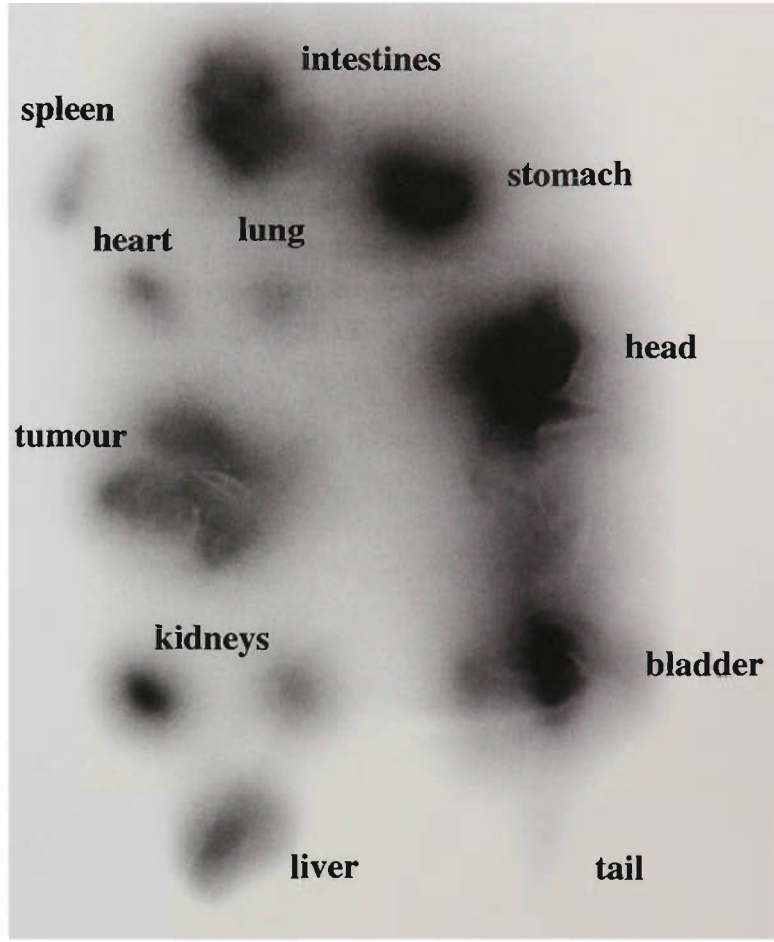
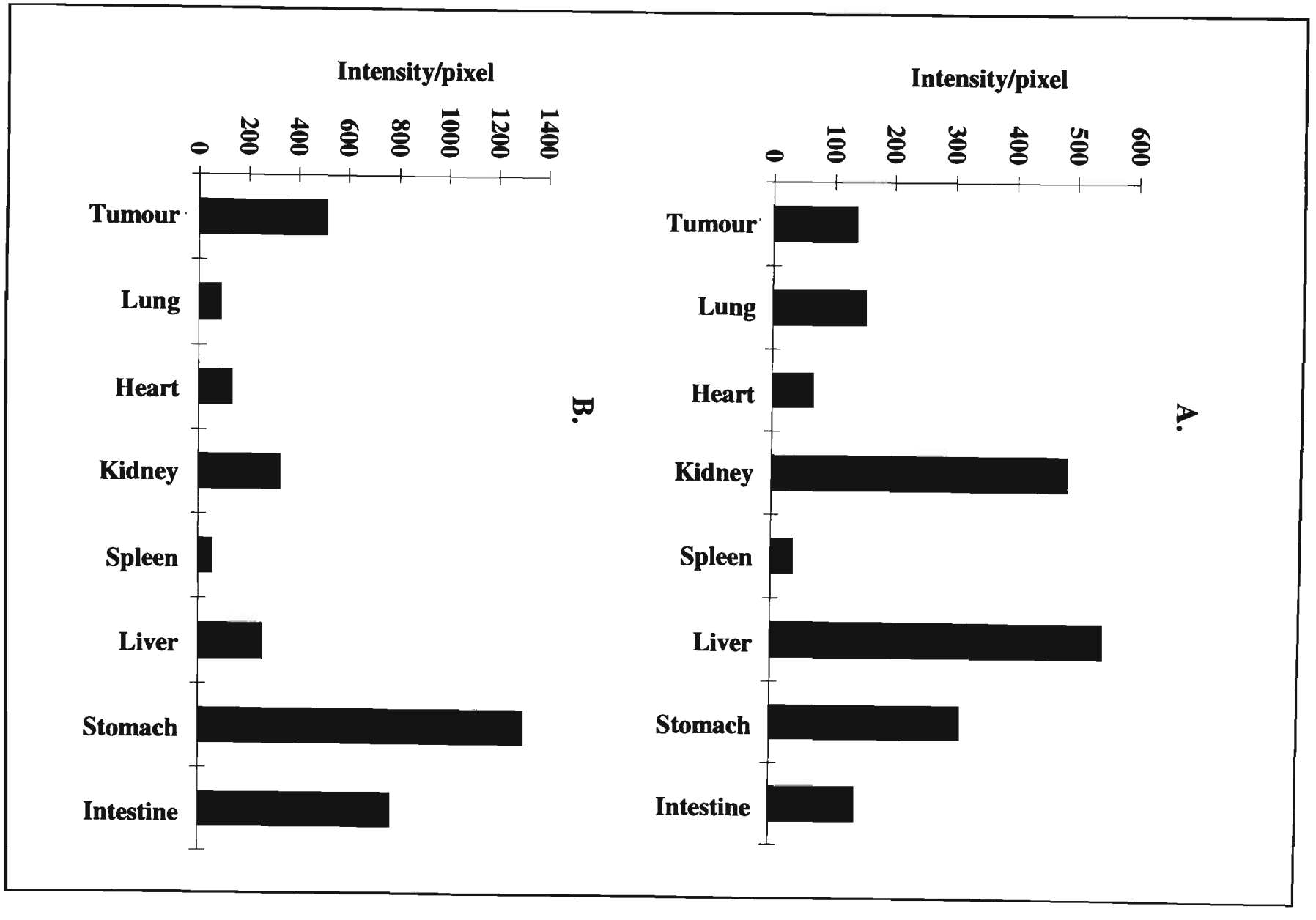


Figure 18. Graphical representation of the distribution of radioactivity (intensity/pixel) in organs and tissues of tumour-bearing mice, 5min (A) and 60min (B) after i.v injection of ^{125}I-hPAI-2. The organs and tumour xenografts removed and exposed to a phosphor screen. The software Imagequant was used to analyse images of radioactivity in the organs, tumour xenograft and remains of the mice.


Intensity/pixel
0
100
200
300
400
500
600
Tumour
Lung
Heart
Kidney
Spleen
Liver
Stomach
Intestine
A.
Intensity/pixel
0
200
400
600
800
1000
1200
1400
Tumour
Lung
Heart
Kidney
Spleen
Liver
Stomach
Intestine
B.

of nude mice bearing xenografts of human colon cancer HCT116 cells, provided a useful model to assess whether PAI-2 may serve as a useful imaging agent. HCT116 cells express high levels of u-PA, and elevated levels of u-PA in cancer have been widely documented throughout the literature (Bouchet *et al.,* 1994; Nagayama *et al.,* 1994; Mulcany *et al.,* 1994; Naitoh *et al.,* 1995; Gleeson *et al.,* 1992). For the imaging of cancer, radiolabelled antibodies have been used (Buraggi *et al.,* 1987; de Nardi *et al.,* 1997; Bakalakos *et al.,* 1998; Bertoglio *et al.,* 1998a, 1998b; di Carlo *et al.,* 1998; Hosono *et al.,* 1998; Percivale *et al.,* 1998; Renda *et al.,* 1998). Since PAI-2 binds covalently to u-PA, the binding of PAI-2 to the tumour mass via u-PA binding to its cell-surface receptor may serve to identify tumours and metastasis. In addition, PAI-2 has the potential to be used in the treatment of any human cancer that expresses active u-PA. Data from this chapter shows that; (1) there was a difference in clearance and organ uptake between control animals and those bearing tumour xenografts, and (2) that PAI-2 associated radioactivity accumulates in tumour xenografts, especially after repeat intravenous injections of ^{125}I-hPAI-2, without an accompanying increase in major organs.

^{125}I-hPAI-2 in murine plasma in both tumour-bearing and control animals was found as a non-degraded monomer, dimer and larger forms. Thus ^{125}I-hPAI-2 in plasma is stable and does not form SDS-stable complexes with circulating mouse proteins such as mouse t-PA or mouse u-PA. This may reflect differences in species specificity, since recombinant human PAI-2 is not as active an inhibitor of rat or mouse u-PA as

it is of human u-PA (Kruithof *et al.,* 1995; Andrews, 1996). In addition, the absence of detectable mouse t-PA/^{125}I-hPAI-2 complexes may indicate that single chain t-PA reacts too slowly with hPAI-2 to form detectable complexes with hPAI-2 (Thorsen *et al.,* 1988). The possibility that injected ^{125}I-hPAI-2 may have lost activity *in vivo* exists, and may be the reason as to why no inhibitor/protease complexes were detected. However, it was shown that ^{125}I-hPAI-2 in plasma of mice retained inhibitor activity because ^{125}I-hPAI-2/u-PA complexes were observed in the presence of exogenously added human u-PA.

Like the clearance of u-PA and t-PA *in vivo* (Fuchs *et al.,* 1985; Stump *et al.,* 1987), human PAI-2 is cleared from the circulation in a biphasic manner, suggesting the presence of a peripheral compartment for the distribution of injected ^{125}I-PAI-2. In both phases the clearance from plasma after ^{125}I-hPAI-2 injection was much faster in the tumour-bearing ($T_{1/2}\alpha$ = 1.4min and $T_{1/2}\beta$ = 29min) than in the control mice ($T_{1/2}\alpha$ = 12.5min and $T_{1/2}\beta$ = 342min). This suggests that the presence of the tumour xenograft has an effect on the rate of clearance of ^{125}I-hPAI-2 from mouse plasma either via effects on the homeostatic mechanisms within the mouse, or via binding and degradation through the elevated u-PA levels the tumour is known to produce. In a study by Colombi *et al.,* (1986) the development of a s.c. murine tumor in BALB/c mice was associated with the appearance of increasing levels of u-PA in the blood. In addition, there may be an increase in specific binding sites for PAI-2 due to the presence of tumour emboli found within blood vessels, or an increase in secretion of

u-PA by the tumour mass. Both could lead to an increase in ^{125}I-hPAI-2/u-PA complex formation and subsequent clearance of the complex.

In a study by Mast *et al.*, (1991) implementing plasma elimination studies and transverse urea gradient gel electrophoresis, it was observed that inhibitor-proteinase complexes were removed from the circulation more rapidly than native or modified inhibitor (in which the inhibitory activity was lost). The rapid clearance of inhibitor-proteinase complexes was thought to be due to the effects of conformational differences of native, modified and inhibitor-proteinase complex. Another potential mechanism may be that the presence of the tumour may lead to increasing levels of circulating antigens. Even though nude mice are thymic deficient, they are still able to mediate immune responses through the functions of B-cells, macrophages and natural killer cells (Rygaard *et al.*, 1982). Watanabe *et al* (1989) and Beatty *et al* (1990) found that larger tumours may give rise to higher levels of circulating antigen resulting in enhanced immune complex formation with the injected radiolabelled antibodies and subsequently leading to more rapid clearance from the blood.

The differences observed in levels, peak times and clearance of radioactivity between the organs and tissues may be due to differences in tissue blood flow through the major organs. In humans for instance, the kidneys, which represent 0.5% of the body weight, receive 23% of the cardiac output, whereas resting muscle, which accounts for approximately 40% of the body weight, receives only 16% of the cardiac output

(Rothe *et al.,* 1976; Ganong *et al.,* 1989). de Boer *et al.,* (1992) observed in human subjects and in rat liver, that liver blood flow was a major determinant in the clearance of exogenous and possibly endogenous recombinant t-PA.

Biodistribution studies of ^{125}I-mPAI-2 in tumour-bearing animals yielded results similar to that obtained with ^{125}I-hPAI-2 ie., the majority of ^{125}I-PAI-2-associated radioactivity was found in the major organs such as the kidneys, liver, lungs and heart. However, different tissue blood flow may be only one of the factors influencing the biodistribution of injected ^{125}I-PAI-2. Radioactivity can also be seen in sites where free iodine is commonly taken up, such as the thyroid and stomach. Another potential factor that may be influencing the biodistribution of injected ^{125}I-PAI-2, is the presence and distribution of binding moieties or receptor-mediated localisation of ^{125}I-PAI-2 in the organs and tissues.

While complexes of PAI-2 with u-PA were not detectable in tumour xenografts, monomeric forms of hPAI-2 were observed in liver and tumour samples. The presence of PAI-2 antigen supports the kinetic data indicating that while radioactivity peaked early in the liver and decreased with time, the opposite was true in the tumour xenografts. The kinetic and ELISA data, together with data from the multiple injection experiments, indicate a rapid uptake of radioactivity in the liver and kidneys following ^{125}I-hPAI-2 injection. However, after an initial rapid uptake, the liver processes PAI-2 antigen more rapidly than other organs or tumours. While the kidney

is also involved in rapid uptake and accumulating a higher proportion of radioactivity, the rate of turnover appears to be slower compared to the turnover in the liver. This results in slower elimination of PAI-2 antigen from the kidney via the urine where a degraded form of ^{125}I-PAI-2 was observed 2h after the injection. It may be that the liver is responsible for the catabolism of ^{125}I-hPAI-2 as it is for human t-PA and u-PA (Kuiper *et al.*, 1988; Kuiper *et al.*, 1992).

To date, little is known about the catabolism of PAI-2 *in vivo*. It is possible that the clearance of PAI-2 is mediated by a group of molecules referred to as serpin receptors which recognize and eliminate complexes of proteinases with their related serpins. Two types of serpin receptors have been identified on hepatic cells: serpin receptor 1 (SR1) and serpin receptor 2 (SR2) (Mast *et al.*, 1991). SR1 recognizes and eliminates complexes of proteinases with α_1-proteinase inhibitor (α_1PI), α_1-antichymotrypsin (α_1ACT), antithrombin III (ATIII) and heparin cofactor II. SR2 recognizes and eliminates complexes of proteinases with α_2-antiplasmin (α_2AP). Mast *et al.*, (1991) proposed that serpin receptors recognise the proteinase-complexed form (ie.: "relaxed" conformation) of serpins but not the native form (ie.: "stressed" conformation). In this study no evidence was found that human PAI-2 forms complexes in plasma with either mouse u-PA or t-PA.

Another group of molecules which may be involved in the clearance of PAI-2 are LRP and gp330. LRP is a multi-ligand receptor and has been found to be responsible

for the clearance of injected human u-PA or t-PA from the circulation in animals by mediating the cellular internalization and degradation of t-PA or u-PA in a PAI-1-dependent or a PAI-1-independent way (Mast *et al.,* 1991; Bu *et al.,* 1994). Some LRP expressing cells such as COS-1 (a simian fibroblast-like transformed cell line) and U937 are able to internalize and degrade t-PA and u-PA in a PAI-1-dependent manner (Strickland *et al.,* 1994; Orth *et al.,* 1992). Other LRP expressing cells such as the rat hepatoma cell line MH1C1 and the human hepatoma cell line Hep G2, are able to internalize and degrade t-PA and u-PA via a PAI-1-independent pathway (Orth *et al.,* 1992; Cubellis *et al.,* 1990; Bu *et al.,* 1992).

The cellular uptake of t-PA and u-PA, or the complexes can be blocked by anti-LRP antibodies or the receptor associated protein (RAP) (Strickland *et al.,* 1994; Orth *et al.,* 1992; Cubellis *et al.,* 1990). Warshawsky *et al.* (1993) reported that administration of RAP *in vivo* prolonged the plasma half-life of ^{125}I-t-PA from 1min to 5-6min indicating that a RAP-sensitive receptor, most likely to be LRP, is involved in the clearance of t-PA from circulation by mediating its internalization. LRP and all components of the plasminogen activation system are expressed in mice (Teesalu *et al.,* 1996) and murine LRP has been shown to bind and internalise several different human serpins (Kounnas *et al.,* 1996).

gp330 is also a member of the low density lipoprotein receptor family which shares many structural features with LRP and binds similar ligands including plasminogen

activator-inhibitor complexes (Bu *et al.*, 1994). Unlike LRP which is expressed by hepatocytes as well as other type of cells, gp330 is much more limited in its distribution and is found primarily on absorptive epithelium including epithelium in the renal proximal tubules. This implicates it's involvement in the uptake and degradation of plasminogen activator-inhibitor complexes at these sites (Zhang *et al.*, 1994). These findings, together with results of this chapter show that injected PAI-2 distributed mainly to the liver and kidneys and is finally eliminated through the urine in degraded forms, suggest that LRP and/or gp330 may be involved in the catabolism and clearance of PAI-2 *in vivo*.

Radio-iodinated PAI-2 was cleared from plasma in a biphasic process after intravenous injection in both control and tumour-bearing animals. However, the clearance of radioactivity from the circulation was more rapid in tumour-bearing animals. Since ^{125}I-hPAI-2 interacted specifically with HCT116 cell-associated active u-PA (results from chapter 2), it is likely that the ^{125}I-hPAI-2 injected into HCT116 tumour xenograft bearing mice interacted with the tumour cells through active, cell-surface receptor-bound u-PA. Approximately 1% of PAI-2-associated radioactivity localised to the tumour xenograft after a single intravenous injection. More importantly, multiple i.v. injections of ^{125}I-hPAI-2 resulted in a statistically significant increase of tumour-associated radioactivity to 1.6%. Compared to single dose, multiple s.c. injections and multiple i.p. injections also resulted in increased tumour-associated radioactivity. However, the overall accumulation of radioactivity in the

tumour by these routes was lower than that achieved by i.v. injection. This was not observed in other tissues or organs and there was no evidence of toxicity in any animals given multiple ^{125}I-hPAI-2 injections. Overall, these results suggest that human recombinant PAI-2 may be useful as a therapeutic agent of invasive human cancer.

However, for imaging purposes ^{125}I-hPAI-2 does not appear to be a good candidate as judged from results obtained under the experimental conditions carried out in this chapter. The tumour to normal tissue ratio of radioactivity was ≤1:1 for all organs with the exception of muscle. To be acceptable as an imaging agent, the ratio should be higher than 1:1. Imaging ratios of colorectal cancer have been reported from 2:1 to 3:1 (Hosono *et al.,* 1998; Renda *et al.,* 1998). The distribution of PAI-2 was not limited to the tumour xenograft but rather the majority of the radioactivity was found in the major organs, as discussed above. The clearance of radioactivity was much faster in the presence of tumour xenografts, therefore would not provide sufficient time for imaging purposes. An extended $T_{1/2}$ *in vivo* would allow more time for PAI-2 to reach the tumour xenograft, as well as reducing the dose of PAI-2 required to inhibit u-PA activity. Strategies for extending the half-life of proteins for imaging purposes documented in the literature include, deglycosylation of protein (Fulton *et al.,* 1988) or fusion of the protein to a stabilizing protein known to have a longer $T_{1/2}$ (Makrides *et al.,* 1996).

The choice of radio isotope is another important consideration when assessing the imaging potential of a protein. For imaging purposes, the radio isotope used must emit energy around 40-100KeV, with 100KeV being optimum for detection and image production by the gamma cameras. While ^{125}I, has a longer $T_{1/2}$, it is a very low energy emitter (25-30KeV). The isotope ^{131}I is preferred for imaging purposes, because it is a high energy emitter (Bomanji *et al.*, 1998; Murray *et al.*, 2000; Cailleux *et al.*, 2000). Other isotopes currently in use are technetium (^{99}Tc) (Bomanji *et al.*, 1998; Ortapamuk *et al.*, 1999; Wang, S. *et al.*, 1999; Auzeloux *et al.*, 2000; de Cicco *et al.*, 2000) and indium (^{111}In) (de Jong *et al.*, 1999; Fuster *et al.*, 1999; Manyak *et al.*, 1999; Saga *et al.*, 1999; Sawada *et al.*, 1999). Another potential isotope is ^{188}Re, which was found to be superior to ^{125}I for imaging colorectal cancer (Hosono *et al.*, 1998). There is also the possibility of incorporating the ^{125}I by using thiol-reactive diethylenetriaminepentaacetic acid-D-peptide adducts, which were found to be superior to the chloramine method (Stein *et al.*, 1999). Increasing the specificity of PAI-2 for tumour xenograft, as well as extending the $T_{1/2}$ of PAI-2 and using another radio-label may make PAI-2 more suitable for imaging purposes.

In conclusion, this study found that recombinant wild type PAI-2 is not suitable as an imaging agent in *Nu/Nu* mice with human colon cancer xenografts, due to the relatively low specificity for the tumour xenograft. However, *Nu/Nu* mice with human colon cancer xenografts may be a useful model in which to assess the long term therapeutic efficacy of PAI-2 in cancer growth and metastasis. Since PAI-2 does

not target a specific cancer (like most antibody strategies), and has been proposed to be a physiological inhibitor of u-PA, it could potentially be used against a wide range of malignant human cancers, wherever cellular u-PA over-expression is thought to play crucial roles in invasion, angiogenesis and metastasis.

4. ASSESSMENT OF RECOMBINANT HUMAN PAI-2 AS A THERAPY FOR HUMAN COLORECTAL CANCER

4.1 INTRODUCTION

Within a population of cancer cells there exist subpopulations of cells with variations in cell size, cell shape, and nuclear and nucleolar size. In addition their relationship to the cells surrounding them varies. Subpopulations of cancer cells show different tumourigenicity and metastatic potential in nude mice. It is the heterogeneity of cancer cells that poses difficulties in the treatment of cancer patients. Heterogeneity in solid tumours is a major factor in the inability to treat such tumours, as the cells within the tumour may vary widely in their response to therapy. Also, the primary tumour can give rise to metastasis that differ radically from the primary tumour in their biological behaviour, resistance to drugs and marker production.

There is accumulating documentation throughout the literature showing the involvement of u-PA and the plasminogen/plasmin cascade in tumour invasion and metastasis (Andreasen *et al.,* 1997). Cancer cells use this enzyme cascade to degrade components of the ECM. This in turn facilitates subsequent invasion of the surrounding environment and the development of metastasis at distant sites from the primary tumour growth. As mentioned previously, this evidence includes; inhibition of cancer by inhibition of u-PA (Evans & Lin, 1995); the conferral of the metastatic

phenotype by transfection with the u-PA gene (Achbarou *et al.*, 1994) and a correlation between shortened disease-free interval as well as survival in patients with different cancers and high levels of u-PA (Evers *et al.*, 1982; Duffy *et al.*, 1988; Duffy *et al.*, 1990; Jänicke *et al.*, 1991; Reilly *et al.*, 1991; Spyratos *et al.*, 1992; Foekens *et al.*, 1992; Grøndahl-Hansen *et al.*, 1993; Maeda *et al.*, 1996; Cantero *et al.*, 1997).

Several therapeutic strategies have examined the inhibition of components of the plasminogen/plasmin cascade such as the activity of u-PA. Some therapeutic strategies include inhibition of u-PA activity with antibodies. It has been found that u-PA antibodies could inhibit lung metastasis (Ossowski and Reich, 1983a,b), murine melanoma experimental metastasis (Hearing *et al.*, 1988) and local invasion by LB6 cells (Ossowski *et al.*, 1991). Other studies have used specific u-PA inhibitors. It was found that amiloride could inhibit the growth of H6 hepatoma and DMA/J mammary adenocarcinomas (Sparks *et al.*, 1983). Amiloride, *p*-aminobenzamidine and PAI-1 were found to be able to inhibit the growth of human prostate cancer, albeit that the amiloride was found to be toxic to the animals. A recent study used a SERPIN derived from myoepithelial cells from the mammary gland that shares 58% sequence simliarity with PAI-2 to treat breast cancer (Xiao *et al.*, 1999). Transfection of breast cancer cells with this SERPIN lead to a significant inhibition of primary tumor volumes, axillary lymph node metastasis, and lung metastasis. Other therapeutic strategies may include interference with the interaction between u-PA and u-PAR (Stephens *et al.*, 1988; Ellis *et al.*, 1989; Ellis *et al.*, 1990). With the success

of these studies it appears that PAI-2, the physiological inhibitor of u-PA, would therefore be a prime candidate for the suppression of the invasive and metastatic propensity of u-PA expressing cancers *in vivo*.

From results of the previous chapter it was observed that with multiple intravenous injections of ^{125}I-PAI-2 an increase in tumour-associated radioactivity was observed. This increase in radioactivity was not found in the liver or skin. This suggested that multiple injections allowed for the accumulation of PAI-2 in the tumour xenograft and not in other major oragns. From the kinetics and biodistribution studies in control and tumuor-bearing mice, exposure to PAI-2 up to 24h, as well as large amounts of the protein was not toxic to the mice. Thus, the main aim of this chapter was to assess the efficacy of recombinant human PAI-2 as a therapeutic agent of human cancer, with the use of a nude mouse model of subcutaneous xenografts of the human colon cancer cell line, HCT116. It was hypothesised that the inhibitory activity of PAI-2 towards u-PA would serve as a useful tool in abolishing u-PA mediated cancer cell growth and metastasis. The aims of the first part will examine the effects of PAI-2 on the growth of the tumour xenograft in nude mice. The second part will examine the effects of PAI-2 on the morphology of the tumour xenografts histologically.

4.2 MATERIALS AND METHODS

4.2.1 Materials

Recombinant human non-glycosylated PAI-2 (47kDa) was from Biotech Australia, Roseville, Australia. The PAI-2 preparation used in these set of experiments had already been used in animal trials carried out by Hoechst and was found to produce no harmful effect on the animals.

4.2.2 Cell culture

Details of all media reagents and cell culture conditions were given in the chapter 2, section 2.2.1 and 2.2.2.

4.2.3 Animals

Nude mice were used as described in the previous chapter, section 3.2.1.

4.2.4 Preparation of cells for injection

The cells used in all of the experiments in this chapter were originally passaged from one flask and stored as 1.5ml aliquots in liquid nitrogen until needed. Upon removal from liquid nitrogen the cells were quickly thawed out and placed into 75cm^2 flask. RPMI+10% (v/v) FBS was then added (total volume of 20-30ml) and the cells were left to grow in a humidified incubator at 37°C in 5% (v/v) CO_2. Changes of media were carried out when neccessary. Once confluent, cells were initially rinsed with RPMI (without any FCS) and then incubated in trypsin/EDTA for 3min at 37°C. The cells were then pelleted (centrifugation for 3min at 200g) and resuspended in RPMI. A cell count was carried out using the trypan blue exclusion method. The cells were kept under sterile conditions throughout all procedures.

4.2.5 Xenografts of human colon cancer in nude mice

Xenografts of human colon cancer were induced by subcutaneously injecting with either 10^6 or $2x10^6$ HCT116 cells (in 100µl of RPMI) in the right flank of athymic Swiss outbred Nu/Nu mice with 26.5G needles. Visible tumours were reproducibly observed 7 days after the injection of cells. In cases where tumours were not observed after 7 days the mice were re-injected with cells.

4.2.6 Characterisation of PAI-2

Prior to the start of all therapy experiments, the PAI-2 used was characterised for activity. PAI-2 (1μg) was reacted with 1μg u-PA for 40min at RT (total volume of 15μl). PAI-2 was also left to react with u-PA that had been inactivated with 0.5mM EGR-CMK for 20min at room temperature (total volume of 15μl). The reaction was stopped with the addition of either 5μl 4x reducing or non-reducing sample buffer and the samples were separated on a 12% SDS-PAGE. After electrophoresis the gel was stained in Coomassie Blue R250 and dried as described in chapter 2, section 2.2.5.2.

4.2.7 Therapy experiment 1

The mice were injected with $2x10^6$ HCT116 cells and monitored regularly for any adverse effects and for tumour development. Once a tumor mass was visible in all mice PAI-2 injections were started. The mice were injected twice daily, once in the morning and once in the evening around the same time each day. The mice were injected with 50μg PAI-2 in 50μl PBS, pH 7.4 which was given peri-tumourally. The mice were divided into three experimental groups (Table I). One group of mice received PAI-2 injections for 35 days, another group received PAI-2 injections for 28 days and the last group, which was a control group, received no injections.

The first injection was always given in the afternoon, with only one injection of PAI-2 for the first day. On the final day of the experimental period the mice were sacrificed using CO_2 (2-3L/min) in an anesthetic chamber, 30min after the final injection of 50μg PAI-2 (given in the morning). The mice were weighed prior to being sacrificed. Tumour mass and diameter were measured after removal from the mouse. A visual examination for lung or lymph node metastasis were carried out on all animals. Lung metastasis could be seen on the surface of the lung while lymph node metastasis was defined as visible enlargement of the lymph nodes.

Table I. Experimental outline of therapy experiment 1.

Experimental procedure	Number of mice in the group
PAI-2 injections for 35 days	2 females & 3 males
PAI-2 injections for 28 days	3 females & 2 males
No injection given	3 males

4.2.8 Therapy experiment 2

As in the above experiment $2x10^6$ HCT116 cells were injected per mouse and once the tumours were observed the PAI-2 injections started. The experiment consisted of two main groups (Table II). The same amount of PAI-2 (50μg PAI-2/injection with

two injections/day) was injected into the mice as in the above experiment. This time the mice in the control group were injected with 50µl of autoclaved and 0.22µm filtered PBS, pH 7.4. Male mice were either injected with PAI-2 or PBS for a total of 19 days, while the female mice were injected for a total of 10 days. The mice were weighed and sacrificed as described above. Tumour mass and diameter were measured.

Table II. Experimental outline of therapy experiment 2.

Experimental procedure	Number of mice in the group
PAI-2 injections	5 females & 8 males
PBS injections	5 females & 8 males

4.2.9 Therapy experiment 3

The mice were injected with 10^6 HCT116 cells. Six days after injection of the cells 14 out of 30 mice had developed tumours (ranging from 1-3mm in diameter). These mice were pooled together and divided into two boxes, while the mice that had not developed tumours were re-injected with another 10^6 HCT116 cells. Two days after re-injection of the cells, another 9 mice had developed tumours. A total of 17 mice with subcutaneous xenografts of HCT116 tumours were used in this experiment

(Table III) with tumour xenografts ranging from 1 to 10mm in diameter. As before one group was injected with PAI-2, while the control group received PBS injections.

Throughout the duration of the experiment the mice were weighed every second day and the diameter of tumour xenografts were measured every day with calipers. The mice were injected twice daily for a total of three weeks, each injection consisting of 50μg PAI-2 in a total volume of 50μl PBS. The PAI-2 was injected peritumourally. The injections were rotated 90° around the tumour xenograft each time (Figure 1). Control mice were injected with 50μl PBS, twice daily at similar sites around the tumour xenograft as with PAI-2 injections.

Table III. Experimental outline of therapy experiment 3.

Experimental procedure	Number of mice in the group
PAI-2 injections	5 females & 4 males
PBS injections	4 females & 4 males

4.2.9.1 PAI-2 ELISA

ELISAs were carried out on plasma samples, liver, kidney and tumour homogenates obtained from mice in therapy experiment 3. Plasma samples were obtained as stated in Chapter 3, section 3.2.6 at the start and end of the experiment. Organs were

Figure 1. A photograph of a nude mice, with a tumour xenograft of HCT116 cells. The arrows denotes the sites at which PAI-2 injections were given. The diameter of the tumour xenograft on this mouse was approximately 3mm.

homogenised and and along with plasma samples were analysed by ELISA as stated in Chapter 3, section 3.2.11. Total protein from each homogenate sample (400μg) or plasma samples (10μl) were analysed in duplicates in each ELISA. The ELISA was repeated three times for each sample. After subtraction of background, the results were expressed as ng of PAI-2/mg of total protein for each homogenate sample and ng/ml of plasma.

4.2.9.2 u-PA activity assays

4.2.9.2.1 Indirect colorimetric u-PA assay

The u-PA activity of homogenates from liver, kidney and tumour xenografts from therapy experiment 3 were measured using the Coleman and Green (1981) assay. The method for this assay has been described in Chapter 3, section 3.2.13. Values are presented as the mean ± SD of at least three repeated experiments for each tissue homogenate.

4.2.9.2.2 Zymography

The method used was similar to that developed by Heussen and Dowdle (1980) (Refer to Appendix). Samples from therapy experiment 3 were separated on 12% SDS-PAGE gels containing plasminogen and gelatin. The gels were cooled to 4°C with

ice-cold SDS-PAGE running buffer and conducted at 200V for 1h at 4°C. Next the gels were placed in ice-cold wash buffer (2.5% (v/v) Triton X-100) and left at RT for 30min with constant shaking. This wash step was then repeated. Following this the gels were placed in ice-cold lysis buffer (0.1M glycine-NaOH, pH 8.3) containing 10mM EDTA and left to develop at RT overnight with constant shaking. Development of lysis bands occured in the presence of EDTA to eliminate any metalloproteinase activity which may be present in the homogenates. The gels were then stained with 0.1% (w/v) amido black for 30min at RT. To remove excess stain the gels were then placed in 50% (v/v) methanol and 10% (v/v) acetic acid and left at RT for several hours until the desired staining intensity was achieved. Purified human u-PA (1μg/well) was used as a positive control. Seeblue molecular weight protein standards were used for molecular weight estimation.

4.2.9.3 Histology and Immunohistochemisty

Organs including liver, kidney, lung as well as tumour xenograft tissue were obtained from mice at the end of experiments. The organs were weighed then placed in 25-40ml of the fixative, 5% (w/v) formaldehyde. The organs were sent to the Southern Pathology Histology Department (Wollongong, NSW, Australia) for embedding and sectioning. Hematoxylin and Eosin (H&E) and Masson's Trichrome staining were carried out at North Wollongong hospital in the Department of Histology (North Wollongong, NSW, Australia). Refer to Appendix for staining protocols.

The Dako staining kit was used for the staining of human u-PA or PAI-2 antigen in the sections. Staining was carried out as per manufacturer's instructions. Briefly the sections were initially immersed in a pool of 3% (v/v) hydrogen peroxidase and left for 5min to neutralize the endogenous peroxidase activities of the sections. The sections were then washed three times with PBS. Sections were washed the same way between all of the following steps. To block any non-specific binding sites, sections were incubated with PBS+1% (w/v) BSA for 15min. Monoclonal antibodies to u-PAR, u-PA or PAI-2 were used next, at a concentration of 5-10μg/ml in PBS+1% (w/v) BSA. As negative controls, isotype control antibodies were used at the same concentration. Primary antibodies were incubated with the slides for 15min. Sections were next incubated with the linking reagent for 15min. This was followed by incubation in streptavidin peroxidase for 15min. Finally, antigen was detected with the substrate-chromagen solution. The slides were then gently rinsed with distilled water and mounted in DPX and coverslipped.

4.2.10 Calculations and statistical analysis of data

All basic calculations, means and standard deviation values were calculated using Microsoft Excel. The final weight of the mice was calculated by subtracting the weight of the tumour xenograft from the measured weight of the mouse at the end of

experiment. Tumour diameter gradient was obtained when fitting a linear regression to the graph of days vs tumour diameter. Weight gained was calculated by subtracting the final weight (the weight of the animals at the end of the experiment) by the initial weight (the weight of the animal at the start of the experiment). For experiment 3 the change in tumour diameter was calculated by subtracting the initial diameter of the tumour xenograft from the final diameter of the tumour xenograft. Tumour growth rate was calculated by dividing the change in tumour diameter by 21, the total number of days of injections. Calculations of the slope from the linear regressions were carried out using the computer program, GraphPad Prism. The student's t-test was carried out on pairs of data to determine if there were statistical differences between the different experimental groups, using a P-value < 0.05 as significant.

4.3 RESULTS

4.3.1 Active PAI-2

The PAI-2 preparation alone (Figure 2, lane 1) under non-reducing conditions gave rise to a major band with a M_r of 47kDa. There were also three minor bands with molecular weights of 31kDa, 52kDa and 124kDa. The 47kDa band represented monomeric PAI-2, while the 31kDa band was thought to be a degraded form of PAI-2 present in the preparation. The 51kDa band, which was present in all samples under

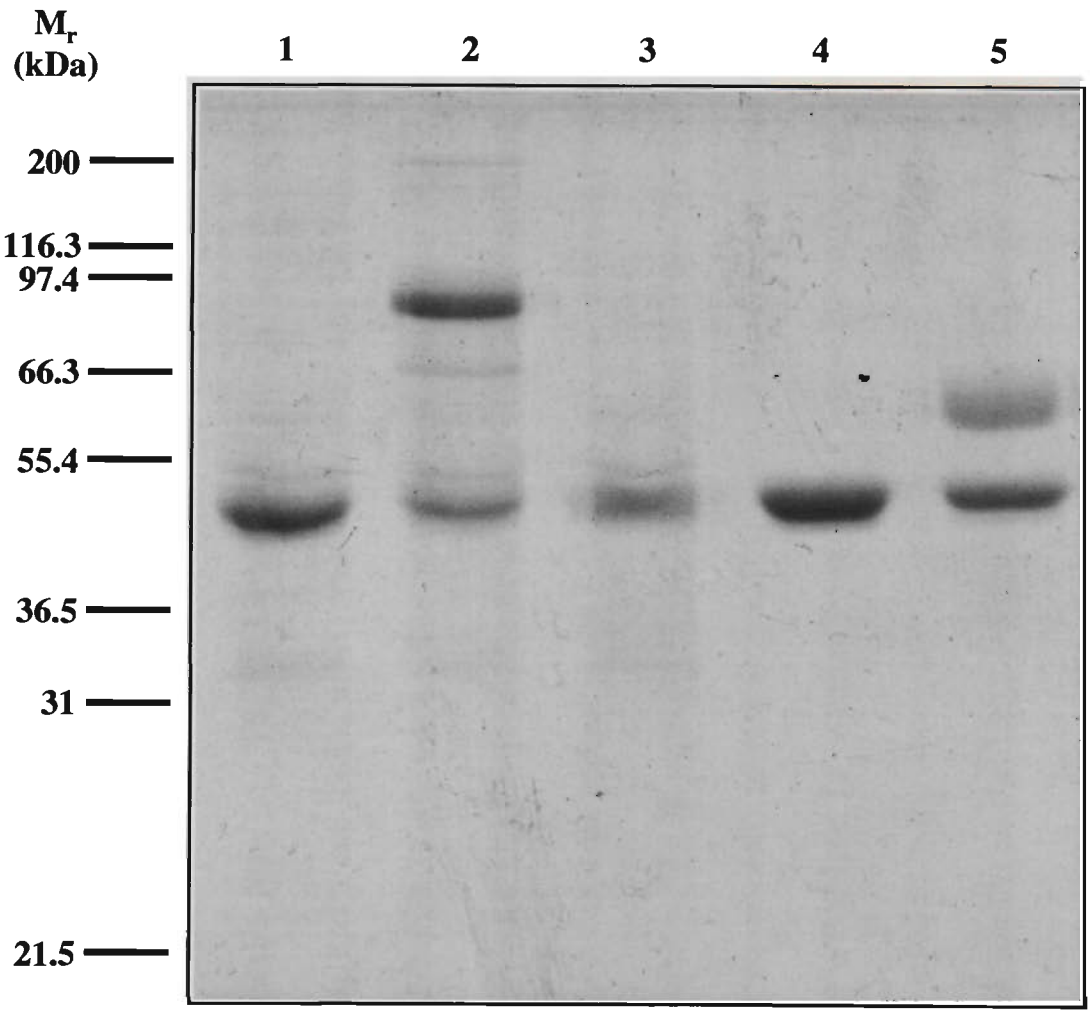


Figure 2. Characterisation of human recombinant PAI-2 for use in therapy experiments. PAI-2 alone (lanes 1&4), PAI-2/uPA complexes (lanes 2&5) and PAI-2 complexed with EGR-CMK-inactivated uPA (lane 3) were separated on a 12% SDS-PAGE under non-reducing (lanes 1-3) and reducing (lanes 4&5) conditions. The gel was stained with coomassie blue for visualization of bands. Mark 12 molecular weight markers were used as protein standards.

non-reducing conditions and was absent when samples were ran under reducing conditions was thought to be polymers formed either between monomeric PAI-2 and degraded PAI-2 fragments, or between the degraded PAI-2 fragments themselves, found in the preparation. The 124kDa band may be polymers of PAI-2.

The presence of u-PA resulted in the appearance of three new protein bands, a major band of 88kDa and two other bands of lesser intensity with molecular weights of 70kDa and 161kDa (lane 2). The 88kDa band was thought to be complex formed between 47kDa PAI-2 and 55kDa u-PA. The 70kDa band may have been complexes of 47kDa PAI-2 and 33kDa u-PA. The 161kDa band may have been complexes formed between u-PA and polymeric forms of PAI-2. The same three bands (31kDa, 47kDa & 51kDa) that were present in the PAI-2 only sample were also present in the sample containing u-PA. In support of complex formation between PAI-2 and u-PA, a reduction in the intensity of monomeric PAI-2 protein band was observed only in the presence of u-PA (lane 2). Incubation of PAI-2 with u-PA inactivated by EGR-CMK (lane 3) resulted in the same protein bands as the PAI-2 only standard (lane 1). Under reducing conditions the PAI-2 preparation gave rise to a single band with a M_r of 47kDa (lane 4). When the u-PA/PAI-2 complex was analysed under reducing conditions the 88kDa protein band was replaced by a 60kDa protein band (lane 5). The 60kDa band was thought to be a complex between 47kDa PAI-2 and 33kDa u-PA.

4.3.2 Experiment 1

A summary of the gross anatomical observations made when the mice were killed and dissected at the end of the experiment was detailed in Table IV. Two of the three mice from the control group showed local invasion or lung metastasis. Meanwhile, from the group of mice injected with PAI-2 for 35 days, two of the five mice had metastasis. One mouse had metastasis to the lungs and another mouse had metastasis to nearby lymph nodes (Figure 3). H&E sections of lung metastasis is shown in Figure 4. Ulceration of the skin on the tumour was evident in all three experimental groups.

The range of the weight of the mice at the end of the experiment for the two PAI-2 injected groups (35 days of injection, 22.3-27.6g; 28 days of injection, 21.4-27.4g) was relatively broad while the range for the control group (24.7-26.5g) was more narrow (Figure 5A). There were no significant differences in the mean weight between the mice given PAI-2 injections for 35 days (24.9 ± 2.5g), the mice given PAI-2 injections for 28 days (24.5 ± 2.3g), or the control group (25.8 ± 0.9g), which were not given injections of PAI-2 (Figure 5B).

The tumour xenograft diameter from the group injected for 28 days with PAI-2 had a broader spread (1-10mm) compare to the control group (5-8mm, Figure 6A). In the group injected for 35 days, 4 mice had tumour diameters comparable to the control group, whereas one mouse had a tumour of a larger size (13mm). The mice given

Table IV. The macroscopic observations of the effect PAI-2 treatment had on the tumour xenograft at the end of therapy experiment 1.

	Observations			
Experimental procedure	Lung metastasis	Invasion of surrounding tissues	Enlarged lymph nodes	Ulcerations
35 days of PAI-2 injection	1/5	0/5	1/5	2/5
28 days of PAI-2 injection	0/5	0/5	0/5	1/5
No injection (control)	1/3	1/3	0/5	1/3

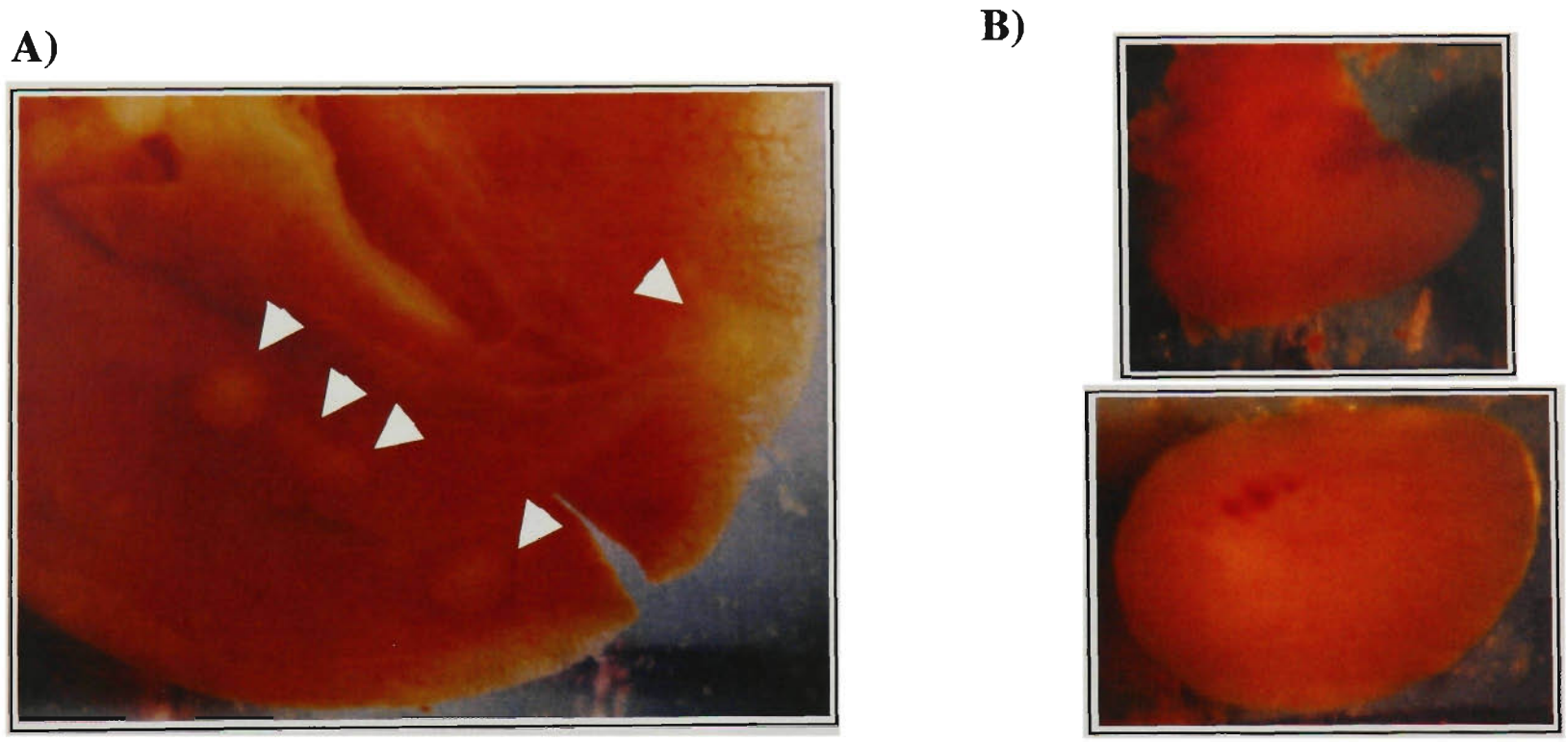
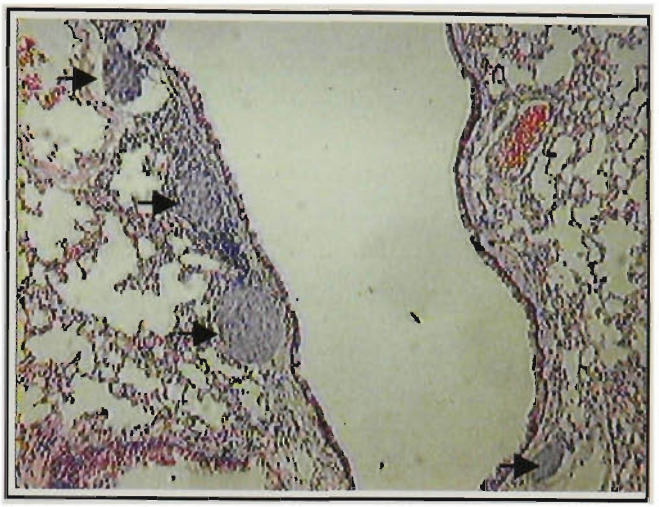


Figure 3. Photographs of a lung metastasis (A) and an enlarged lymph node (B). The arrows in A) indicate the lung metastasis. In B) the top photograph is a normal mouse lymph node with some adipose tissue on top and the bottom photograph is an enlarged lymph node. All photographs were taken under 40x magnification.

A)



B)

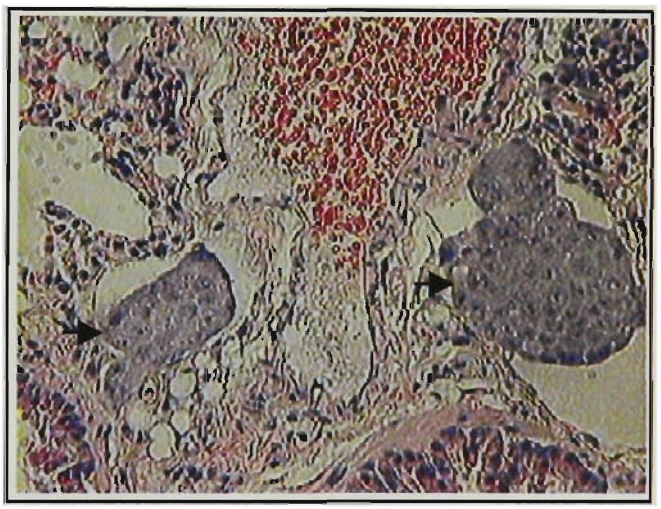


Figure 4. H&E staining of pulmonary metastasis in nude mice with subcutaneous tumour xenograft from therapy experiment 1. Section of lung with metastasis (denoted by the arrow) at 25x (A) and 40x (B) magnification.

Figure 5. The effect of PAI-2 injection on the weight of mice from therapy experiment 1. The mice were either injected with PAI-2 for 35 (n=5) or 28 (n=5) days. Control mice (n=3) were not given injections of PAI-2. A) Each filled square represents the weight (g) of one mouse. B) A bar graph representing the mean weight (g) ± SD from each experimental group.

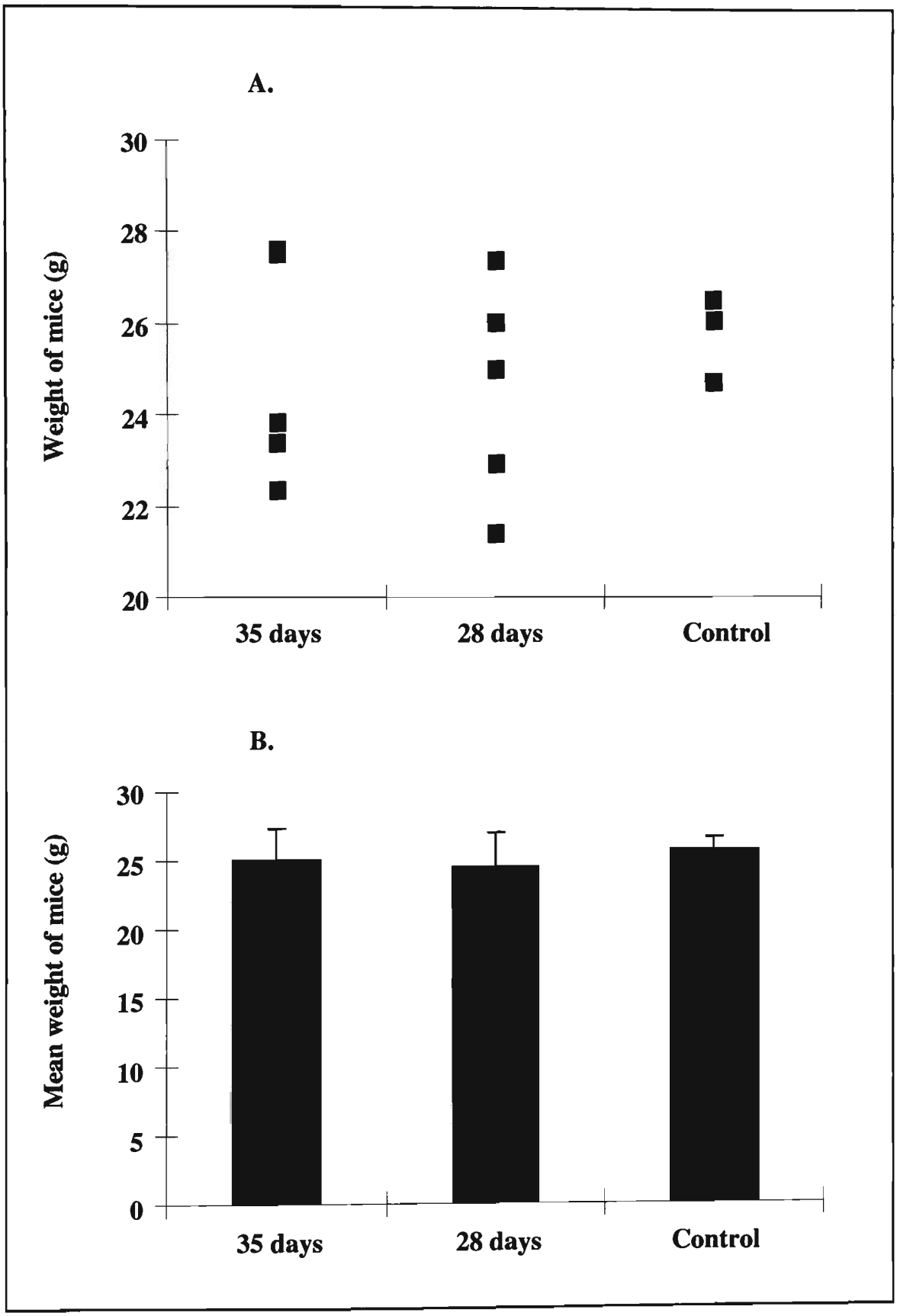
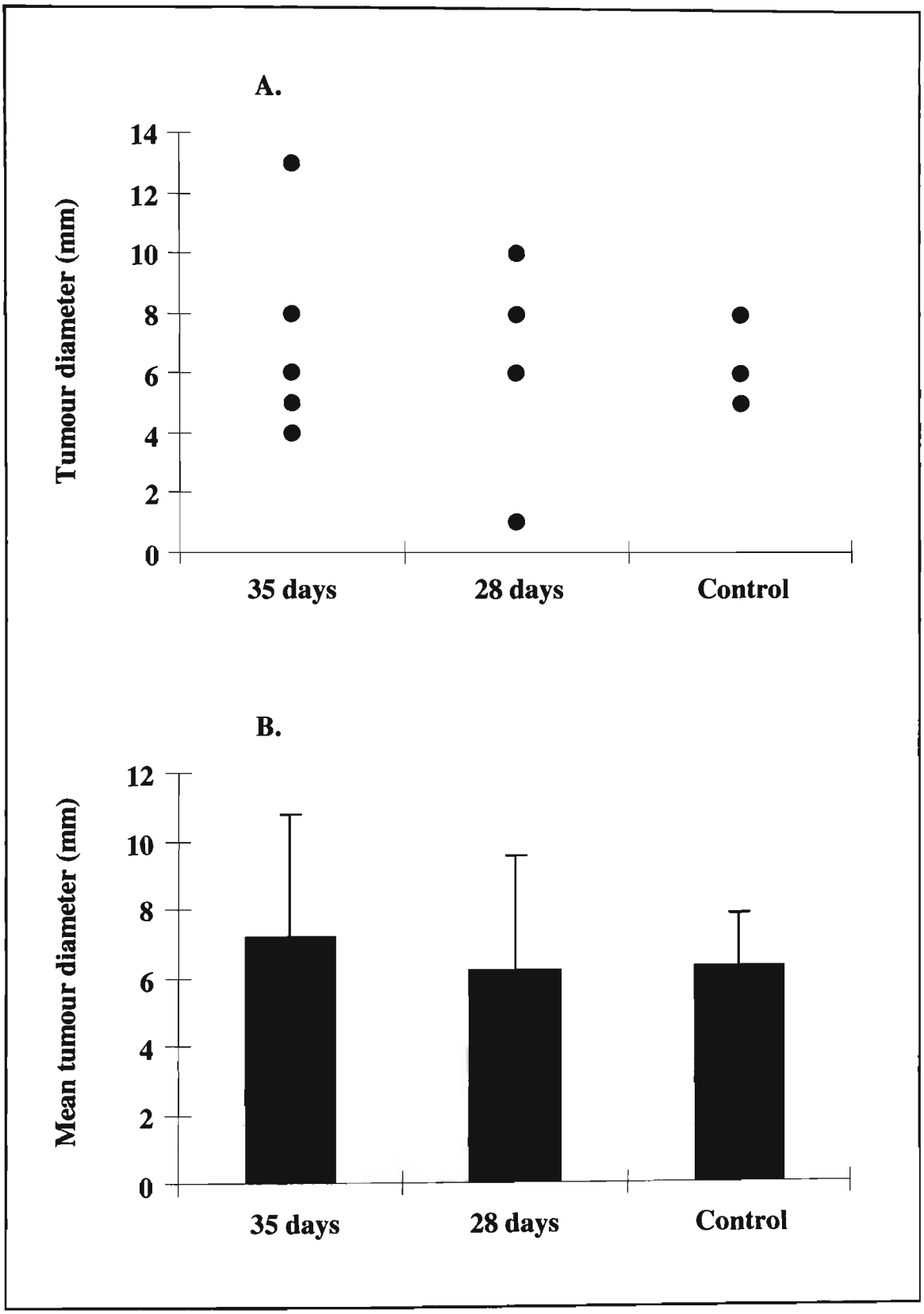

A.
Weight of mice (g)
30
28
26
24
22
20
35 days
28 days
Control
B.
Mean weight of mice (g)
30
25
20
15
10
5
0
35 days
28 days
Control

Figure 6. The effect of PAI-2 injection on the diameter of tumour xenografts from therapy experiment 1. The mice were either injected with PAI-2 for 35 (n=5) or 28 (n=5) days. The control group consisted of mice (n=3) which were not given injections of PAI-2. A) Each filled circle represents the diameter (mm) of a tumour xenograft obtained from one mouse. B) A bar graph representing the mean tumour diameter (mm) ± SD from each experimental group.


A.
Tumour diameter (mm)
14
12
10
8
6
4
2
0
35 days
28 days
Control
B.
Mean tumour diameter (mm)
12
10
8
6
4
2
0
35 days
28 days
Control

PAI-2 injections for 35 days showed the largest mean tumour xenograft diameter (7.2 ± 3.6mm). While, the group of mice given PAI-2 injections for 28 days (6.2 ± 3.3mm) and the control group (6.3 ± 1.5mm) had similar mean tumour xenograft diameter, although the standard deviation for the control group was much less than that of the PAI-2 injected group (Figure 6B).

The range of tumour masses for the two group injected with PAI-2 (35 days of injections, 0.04-1.46g; 28 days of injections, 0.007-0.3g) were similar to that of the control group (0.07-0.58g), with the exception of one mouse that received PAI-2 injections for 35 days (Figure 7A). This mouse weighed about the same as all the other mice in the experiment, however the tumour xenograft on this mouse weighed approximately 3 times more (1.46g) than the next largest tumour xenograft from this experiment. PAI-2 injections for 28 days resulted in the lower mean tumour mass (0.2 ± 0.1g) compared to the control group (0.3 ± 0.3g), while the group given PAI-2 injections for 35 days showed the largest mean tumour mass and the largest SD value (0.5 ± 0.6g, Figure 7B). However, there was no significant difference in mass between tumour xenografts from the mice injected with PAI-2 or those from control mice.

A linear relationship was found to exist between tumour diameter and tumour mass in all three groups (Figure 8). Tumour diameter was found to be proportional to tumour mass (ie. the larger the tumour the more it weighed). The gradient of the linear

Figure 7. The effect of PAI-2 injection on the mass of tumour xenografts from therapy experiment 1. The mice were either injected with PAI-2 for 35 (n=5) or 28 (n=5) days. The control group consisted of mice (n=3) which were not given injections of PAI-2. A) Each filled diamond represents the mass (g) of a tumour xenograft obtained from one mouse. B) A bar graph representing the mean tumour mass (g) ± SD from each experimental group.

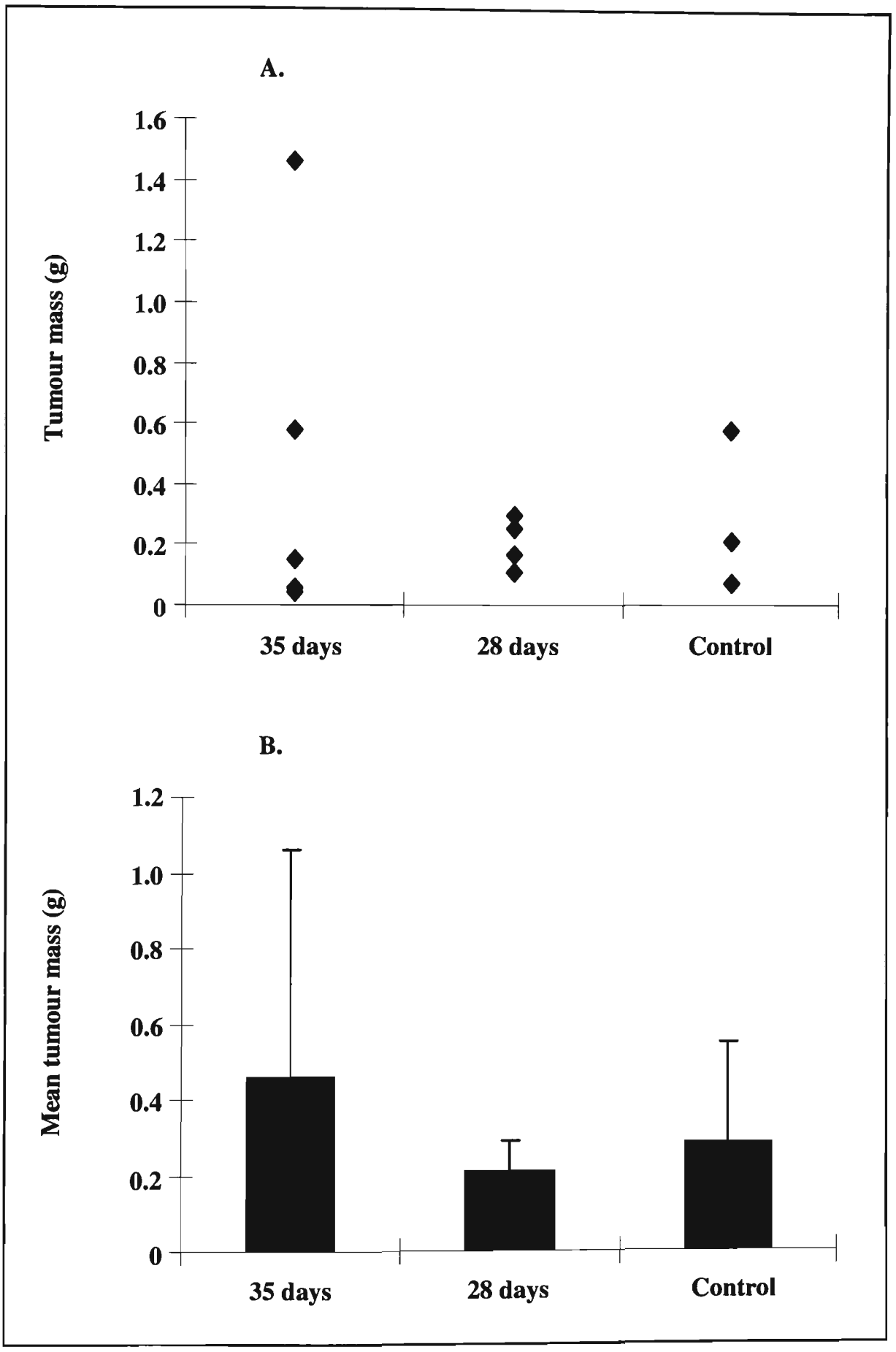
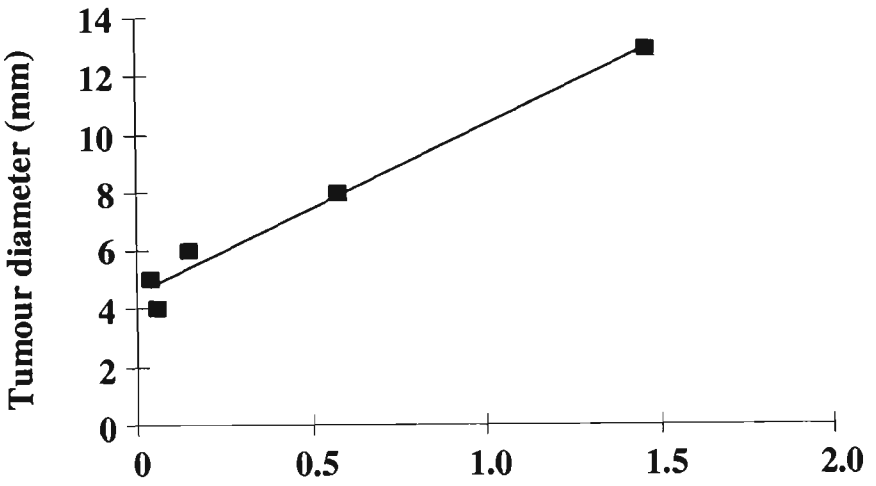
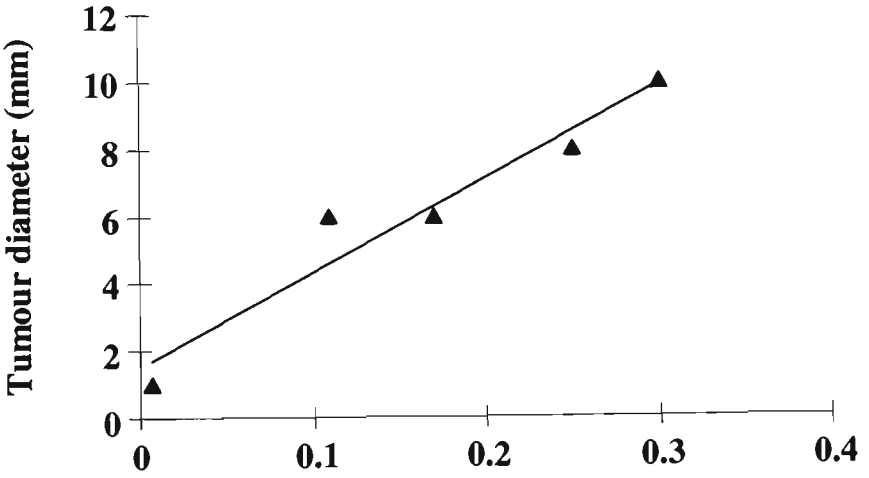

A.
Tumour mass (g)
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0
35 days
28 days
Control
B.
Mean tumour mass (g)
1.2
1.0
0.8
0.6
0.4
0.2
0
35 days
28 days
Control

Figure 8. The relationship between tumour mass and tumour diameter in therapy experiment 1. Linear regressions are shown fitted through data from: A) 35 days of PAI-2 injection ($r^2 = 0.98$, n=5); B) 28 days of PAI-2 injection ($r^2 = 0.94$, n=5) and C) No injection of PAI-2 ($r^2 = 0.99$, n=3).

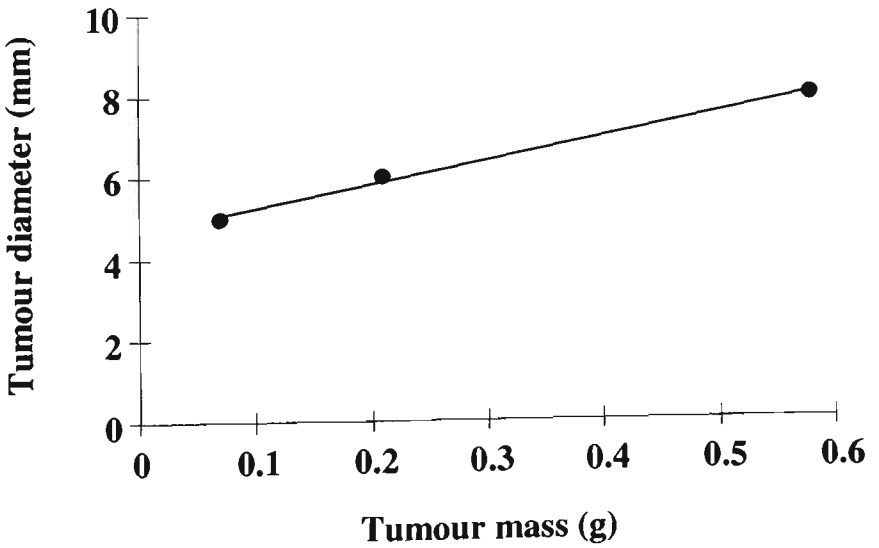
A. 35 days of PAI-2 injection



B. 28 days of PAI-2 injection



C. No injection



regressions for the group injected with PAI-2 for 35 days (5.8 ± 0.5, r^2 = 0.98; Figure 8A) was similar to the gradient obtained for the control group (5.8 ± 0.4, r^2 = 0.99; Figure 7C). In contrast, the gradient of the linear regression for mice injected with PAI-2 for 28 days was much steeper (28.0 ± 4.2, r^2 = 0.94; Figure 8B). Therefore, larger diameter tumour xenografts from the group injected for 28 days did not weigh as much as the larger tumour xenografts from the other two groups.

4.3.3 Experiment 2

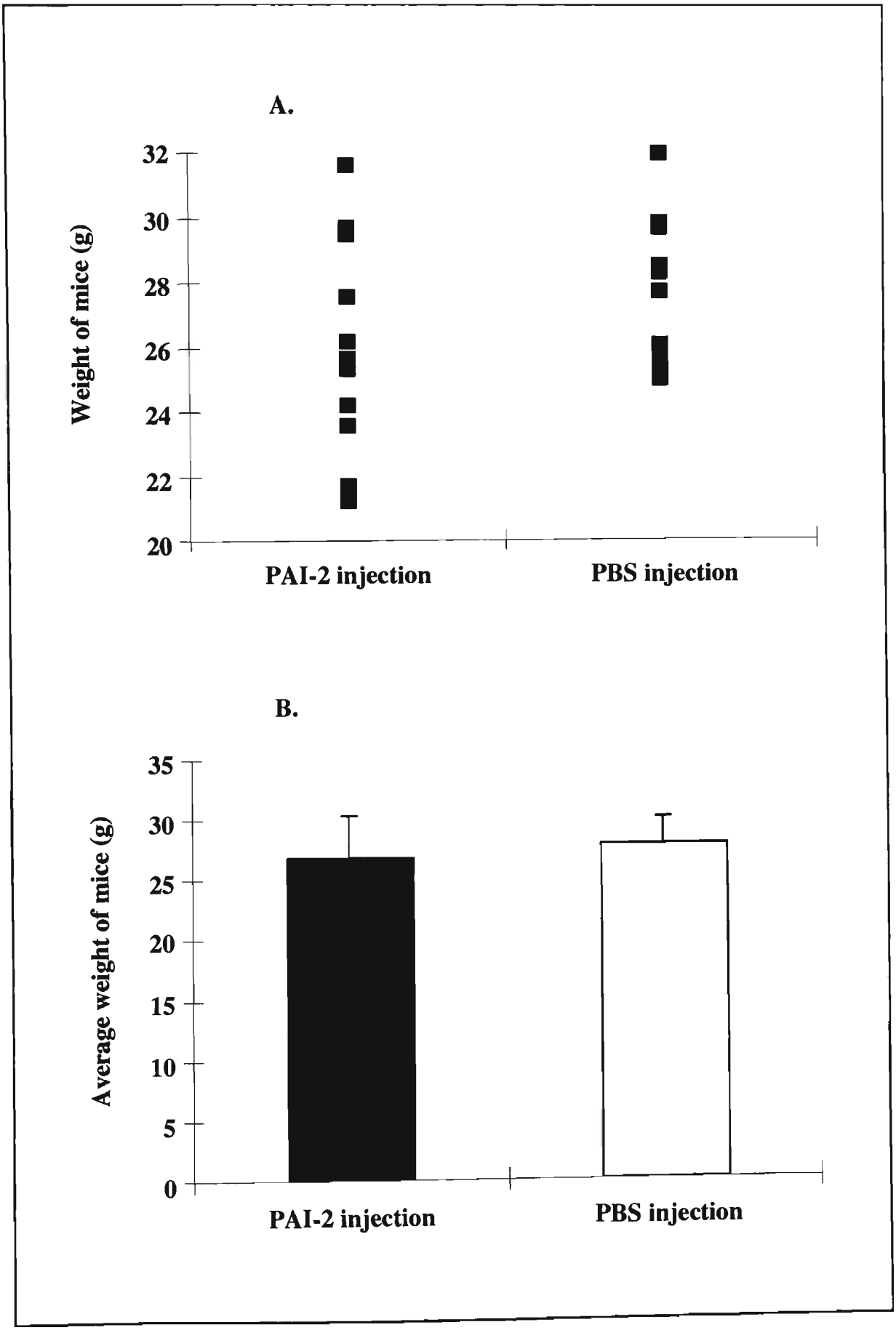
Tumour-bearing mice were given peritumoural injections of either PAI-2 or PBS (as controls) twice daily for a total of 20 days. The gross anatomical observations from the two groups showed that mice injected with PAI-2 had a higher percentage of local invasion (46%) compared to PBS injections (23%; Table V). Metastasis to the lungs (31%) were observed only in the group injected with PAI-2. In addition, there was no encapsulation of the tumours from mice injected with PAI-2, while the number of mice with ulcerations and enlarged lymph nodes were the same for the two groups.

As was observed in the previous experiment, the range of weights at the end of the experiment of mice injected with PAI-2 (21.2-31.6g) was slightly broader compared to the PBS group (25-32g; Figure 9A). However, no significant differences in the mean weight of the mice were observed between PAI-2 (25.5 ± 3.3g) or PBS (27.6 ± 2.3g) injections (Figure 9B).

Table V. The macroscopic observations of the effect of PAI-2 treatment on the tumour xenograft at the end of therapy experiment 2.

	Observations				
Experimental procedure	Encapsulated tumour mass	Lung metastasis	Invasion of surrounding tissues	Enlarged lymph nodes	Ulcerations
PAI-2 injected	0/13	4/13	6/13	1/13	2/13
PBS injected	3/13	0/13	3/13	1/13	2/13

Figure 9. The effect of PAI-2 treatment on the weight of the mice from therapy experiment 2. The mice were given peritumoural injections of either PAI-2 (n=13) or PBS (n=13) twice daily for 20 days. A) Each filled square represents the weight (g) of one mouse. B) A bar graph representing the mean weight (g) ± SD from each experimental group.


A.
Weight of mice (g)
32
30
28
26
24
22
20
PAI-2 injection
PBS injection
B.
Average weight of mice (g)
35
30
25
20
15
10
5
0
PAI-2 injection
PBS injection

The range of tumour diameters from mice injected with PAI-2 and PBS was 8-30mm and 2-20mm, respectively (Figure 10A). The mean diameter of the tumour xenografts from mice injected with PAI-2 (17 ± 7.3mm, P-value=0.034) was significantly larger compared to the diameter of the tumour xenografts from the PBS injections (11.2 ± 5.6mm; Figure 10B). The range of tumour mass from mice injected with PAI-2 (0.6-3g) was much broader compared to the range of tumour mass from mice injected with PBS (0.002-0.8g; Figure 11A). The mean mass of tumour xenografts from the PAI-2 (1.3 ± 0.8g) injected group was greater than four times the mean mass of tumour xenografts from the PBS group (0.3 ± 0.2g; Figure 11B).

In contrast to experiment 1, no correlation was found between tumour diameter vs tumour mass in this experiment (Figure 12). Although gradients were obtained from the linear regression of tumour diameter versus tumour mass for both groups, the correlation coefficient (r^2) for the linear regression from both the PAI-2 ($r^2 = 0.46$) and PBS ($r^2 = 0.36$) treated mice were below 0.5, indicating that the data points did not fit to a linear regression analysis.

4.3.4 Experiment 3

In the previous two therapy experiments, when mice were injected with $2x10^6$ HCT116 cells/mouse, tumour xenografts were observed in the majority of mice 7

Figure 10. The effect of PAI-2 injection on the diameter of tumour xenografts from therapy experiment 2. The mice were given peritumoural injections of either PAI-2 (n=13) or PBS (n=13) twice daily for 20 days. A) Each filled circle represents the diameter (mm) of a tumour xenograft obtained from one mouse. B) A bar graph representing the mean tumour diameter (mm) ± SD from each experimental group.

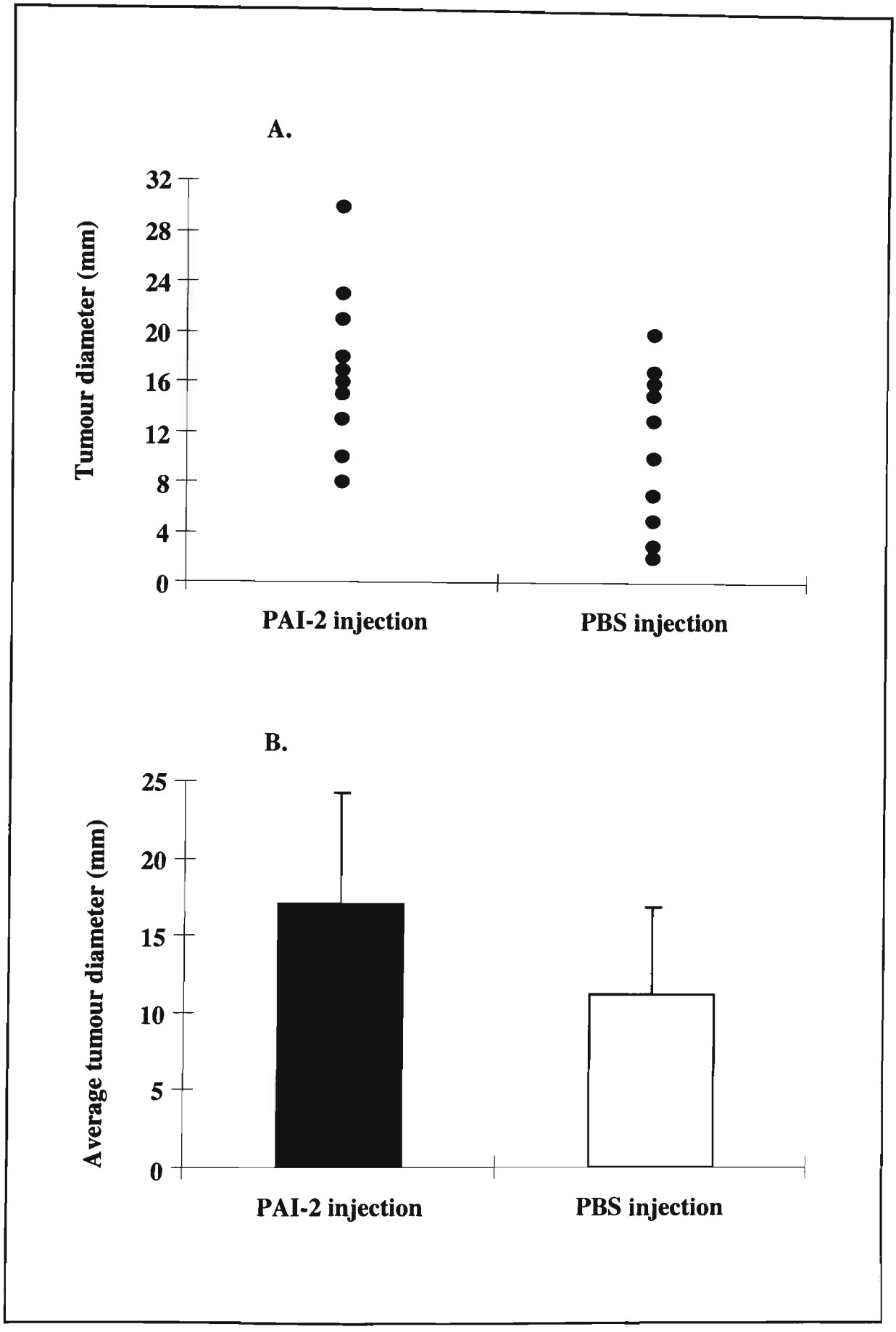
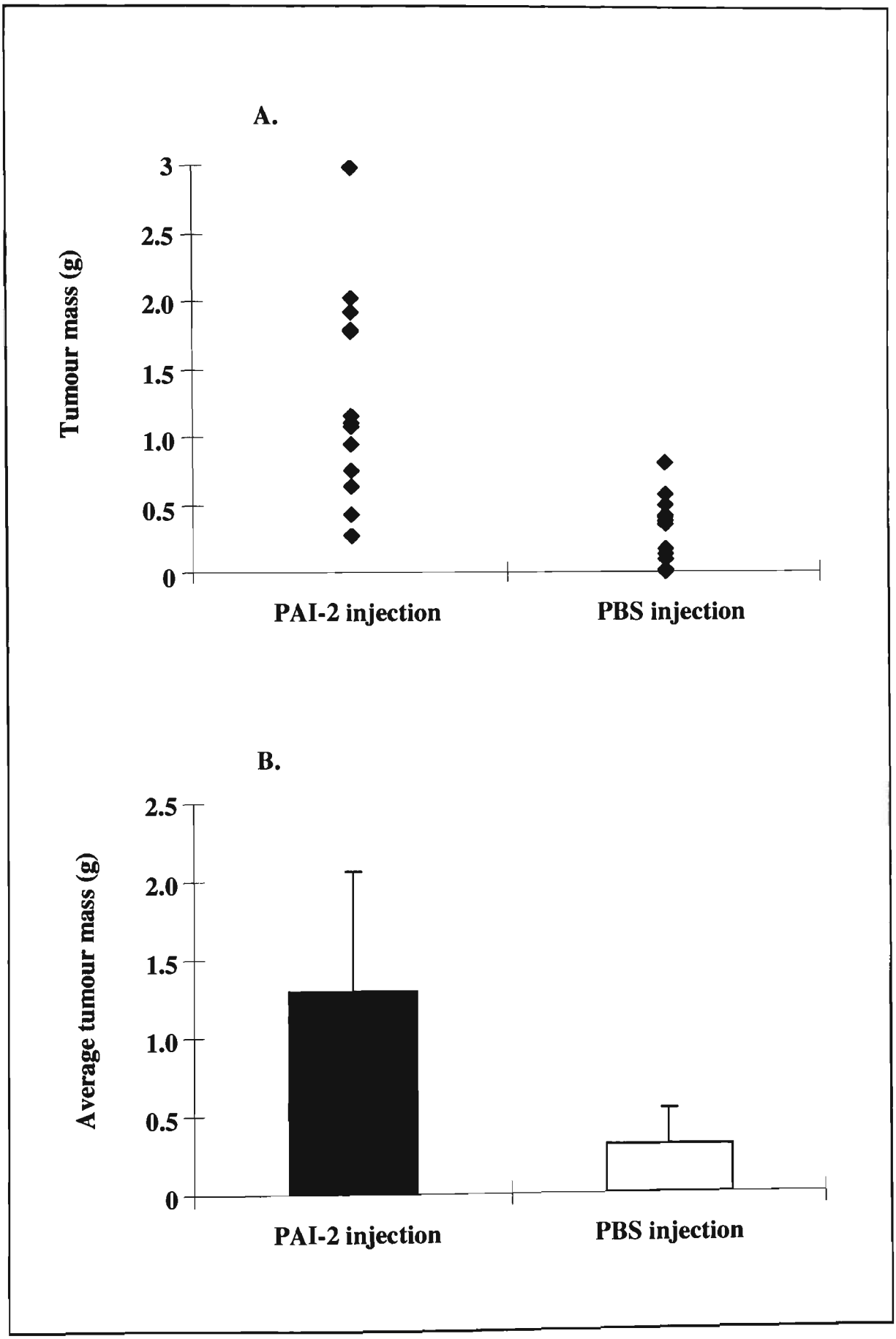

A.
Tumour diameter (mm)
32
28
24
20
16
12
8
4
0
PAI-2 injection
PBS injection
B.
Average tumour diameter (mm)
25
20
15
10
5
0
PAI-2 injection
PBS injection

Figure 11. The effect of PAI-2 injection on the mass of tumour xenografts from therapy experiment 2. The mice were given peritumoural injections of either PAI-2 (n=13) or PBS (n=13) twice daily for 20 days. A) Each filled diamond represents the mass (g) of a tumour xenograft obtained from one mouse. B) A bar graph representing the mean tumour mass (g) ± SD from each experimental group.


A.
Tumour mass (g)
3
2.5
2.0
1.5
1.0
0.5
0
PAI-2 injection
PBS injection
B.
Average tumour mass (g)
2.5
2.0
1.5
1.0
0.5
0
PAI-2 injection
PBS injection

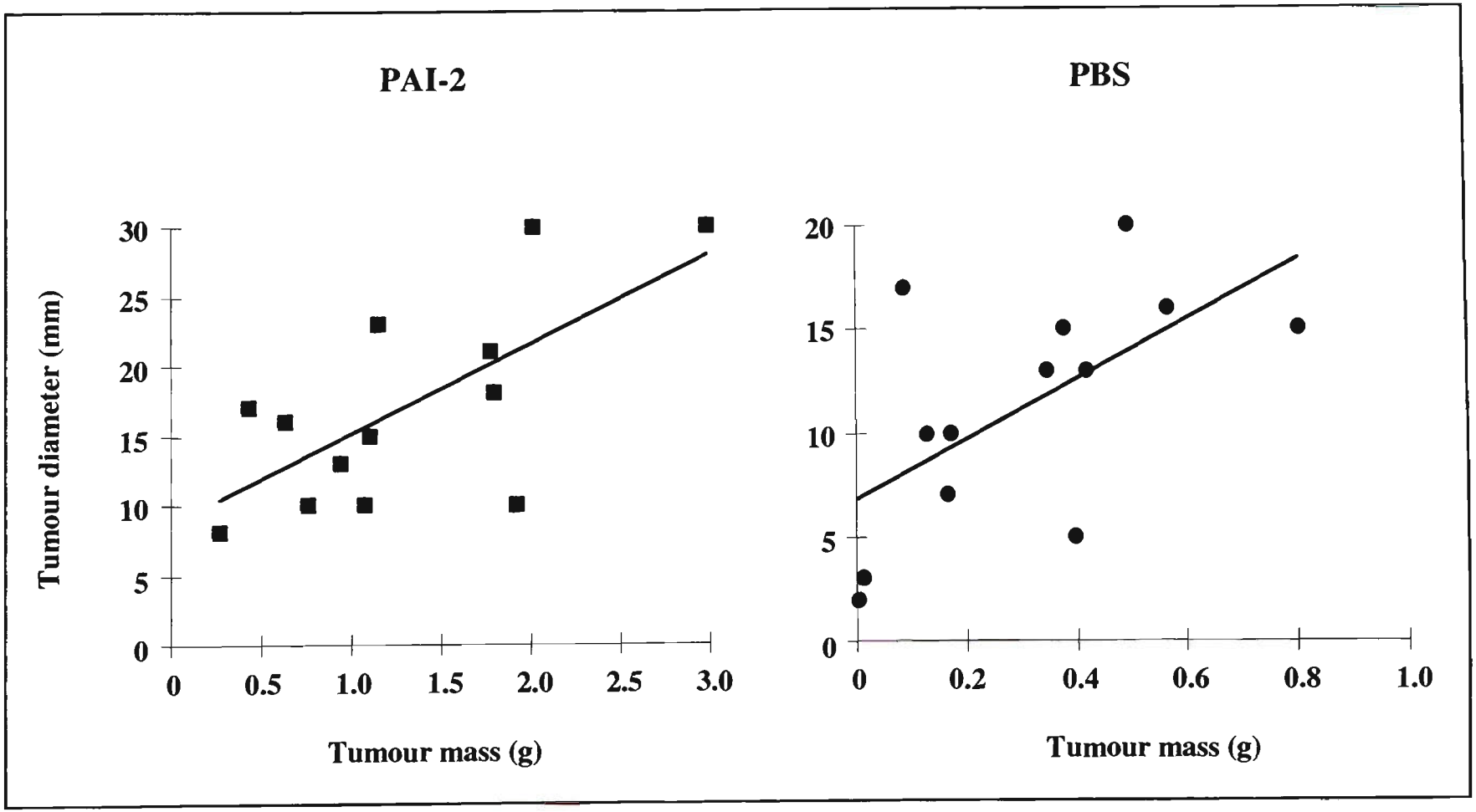


Figure 12. The relationship between tumour mass and tumour diameter in therapy experiment 2. A linear regression is shown fitted through all data points from the mice injected with PAI-2 ($r^2 = 0.46$) or PBS ($r^2 = 0.36$).

days after injections. In contrast, when mice were injected with 10^6 HCT116 cells/mouse in this therapy experiment, only 47% of mice development tumour xenografts 7 days after injections of the cells.

From the gross examination (Table VI), the mice injected with PAI-2 were found to have a higher incidence of enlarged lymph nodes (44%) compared to the mice injected with PBS (25%). However, the PBS injected group showed a higher incidence of lung metastasis (37%) compared to mice injected with PAI-2 which showed no incidence of lung metastasis. In addition, control mice had more visible vasculature (62%) on the tumour mass compared to the mice injected with PAI-2 (11%). The tumours from the control mice also showed a higher incidence of tumour necrosis (87%), compared to the mice injected with PAI-2 (33%). Incidences of ulceration of the skin above the tumour xenograft was higher in PBS (25%) injected group than the PAI-2 (11%) injected group.

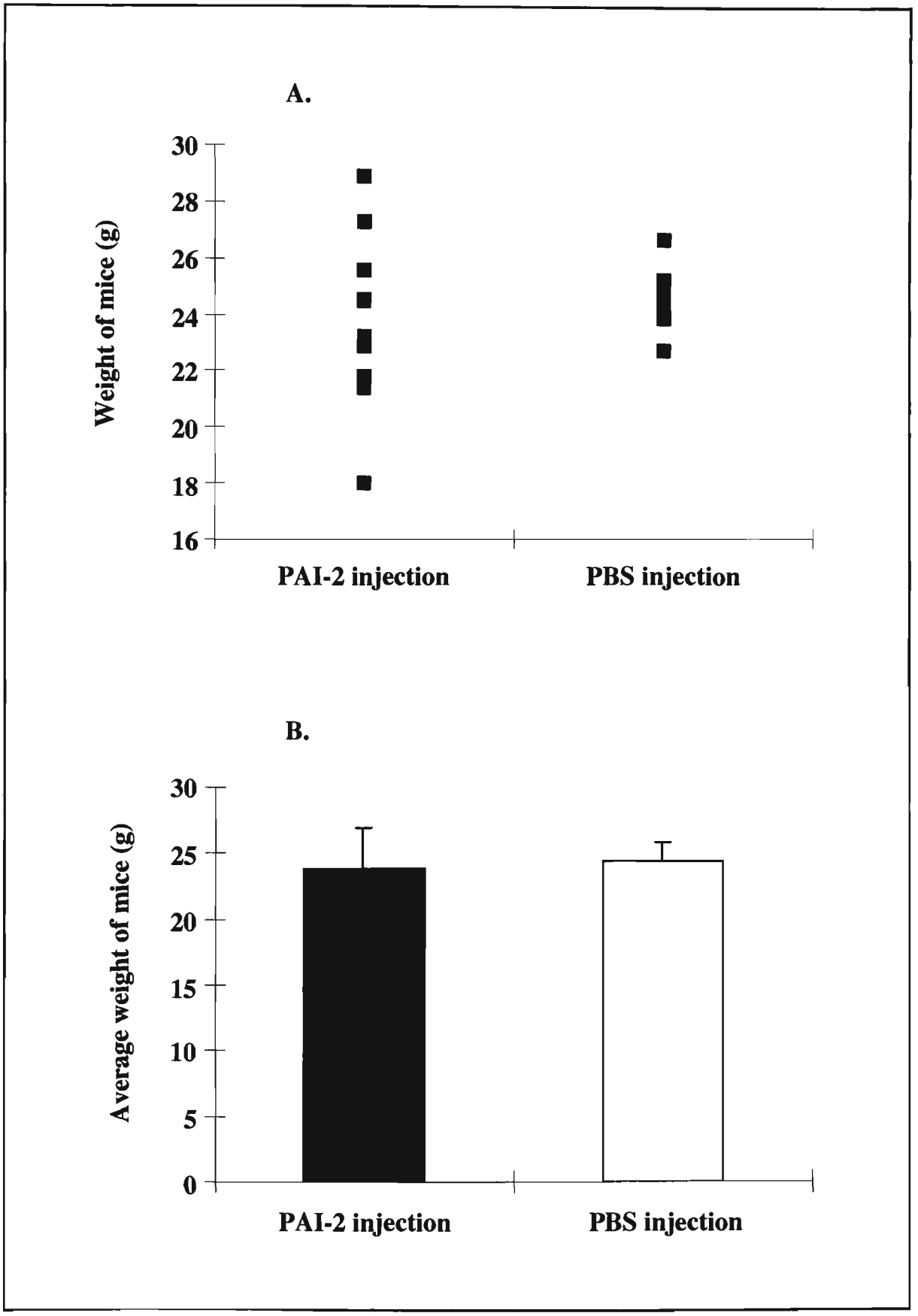
The range of the final weight of mice from the group injected with PAI-2 (21.3-28.9g) was broader compared to the final weight of mice from the PBS group (22.7-26.7g; Figure 13A). As in the two previous experiment, there was no significant difference in the mean weight of mice between PAI-2 (24.4 ± 2.5g) and PBS (24.4 ± 1.3g) injections (Figure 13B).

Table VI. The macroscopic observations of the effect of PAI-2 treatment on tumour xenografts at the end of therapy experiment 3.

	Observations				
Experimental procedure	Necrosis of tumour mass[a]	Lung metastasis	Ulceration of skin above tumour mass	Enlarged lymph nodes	Visible vasculature on tumour mass
PAI-2 injected	3/9	0/9	1/9	4/9	1/9
PBS injected	7/8	3/8	2/8	2/8	5/8

[a] It was observed that some of the tumour masses were not solid and the inner core of tumour mass contained a yellow viscous substance. Therefore, this was defined as necrosis of the tumour mass.

Figure 13. The effect of PAI-2 injection on the weight of the mice from therapy experiment 3. The mice were given peritumoural injections of either PAI-2 (n=9) or PBS (n=8) twice daily for 21 days. A) Each filled square represents the weight (g) of one mouse. B) A bar graph representing the mean weight (g) ± SD from each experimental group.


A.
Weight of mice (g)
30
28
26
24
22
20
18
16
PAI-2 injection
PBS injection
B.
Average weight of mice (g)
30
25
20
15
10
5
0
PAI-2 injection
PBS injection

The range of tumour diameter (Figure 14A) and mass (Figure 15A) from the mice injected with PAI-2 (0-20mm and 0-0.9g, respectively) were broader compared to those of the PBS (9-17mm and 0.2-1.2g, respectively) injected mice. There was no significant difference in the mean tumour xenograft diameter between the mice injected with PAI-2 (10 ± 7.6mm) or PBS (13.6 ± 2.9mm; Figure 14B). There was also no significant difference in the mean mass of the tumour xenografts between the mice injected with PAI-2 (0.4 ± 0.3g) or PBS (0.6 ± 0.4g; Figure 15B).

In two of the mice from the group given PAI-2 injections the tumour xenografts had disappeared before the end of the three weeks of injections. A tumour xenograft with a diameter of 1mm was observed in both mice up to 8 days after the start of the injections. However around day 9 the tumour xenografts in both of these mice were harder to detect. Two weeks after the initial injections it was determined that the two tumour xenografts on these two mice receiving PAI-2 injections were no longer visible. At the end of the three weeks of injections, no tumour xenografts were found under the dermis of these two mice.

Measurement of tumour diameter throughout the 21 days of injection allowed for analysis of tumour growth rate in the PAI-2 and PBS injected group. The change of tumour diameter (Figure 16A) was similar for both the PAI-2 (6.6 ± 3.7mm) and PBS (5.4 ± 2.1mm) group. There was no significant difference in the rate of tumour growth (Figure 16B) between the PAI-2 (0.31 ± 0.18mm/day) or PBS (5.4 ±

Figure 14. The effect of PAI-2 injection on the diameter of tumour xenografts from therapy experiment 3. The mice were given peritumoural injections of either PAI-2 (n=9) or PBS (n=8) twice daily for 21 days. A) Each filled circle represents the diameter (mm) of a tumour xenograft obtained from one mouse. B) A bar graph representing the mean tumour diameter (mm) ± SD from each experimental group.

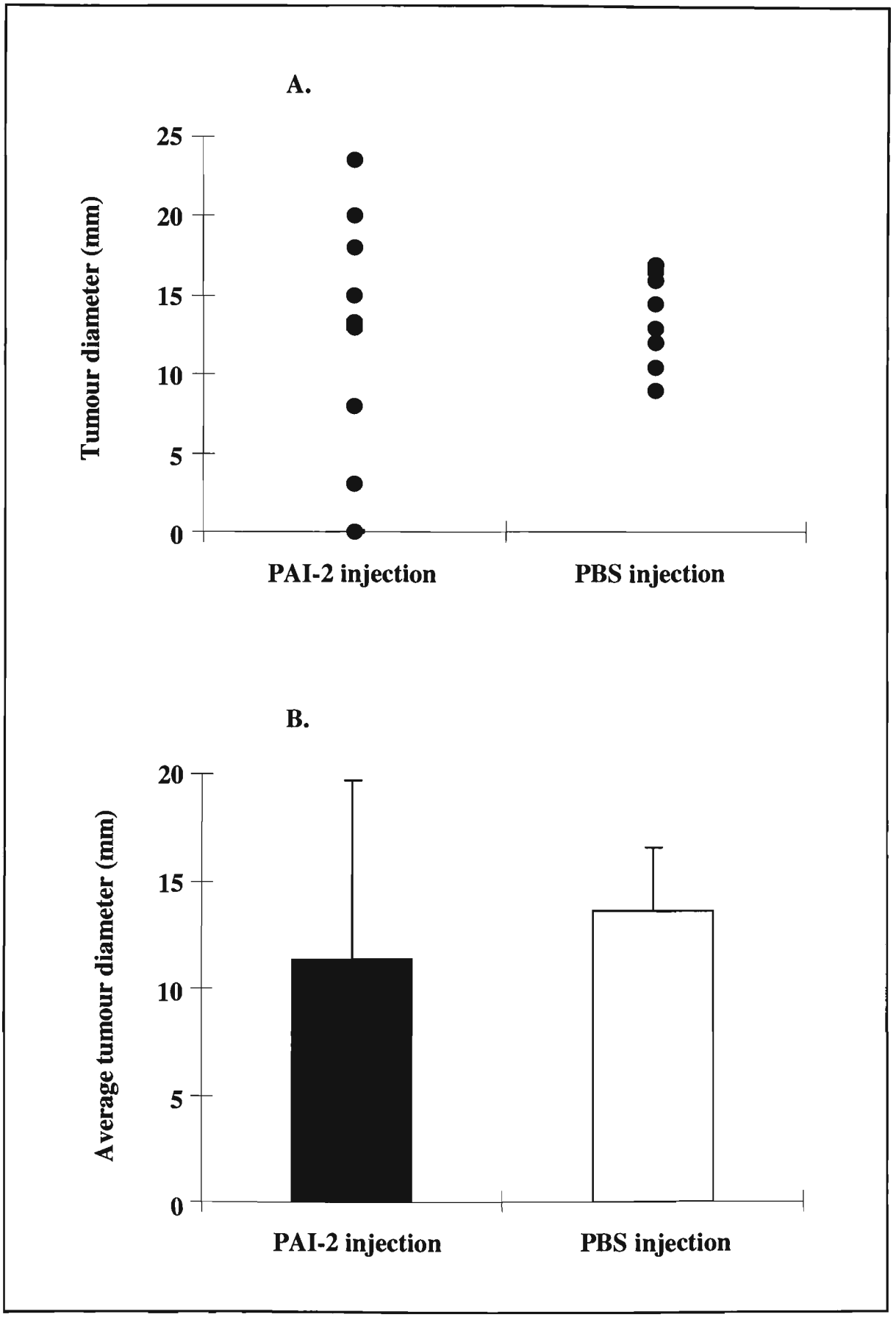
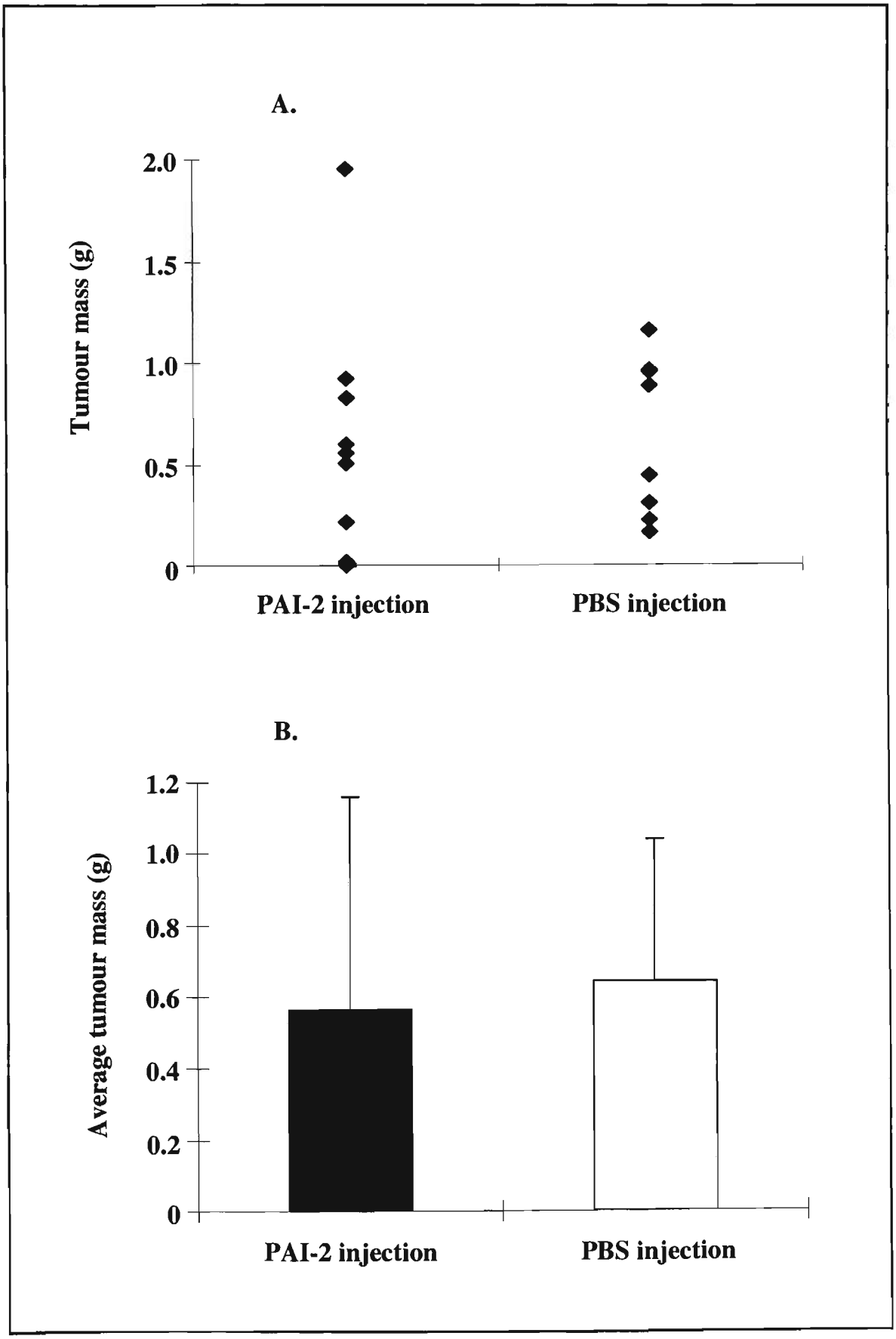

A.
Tumour diameter (mm)
25
20
15
10
5
0
PAI-2 injection
PBS injection
B.
Average tumour diameter (mm)
20
15
10
5
0
PAI-2 injection
PBS injection

Figure 15. The effect of PAI-2 injection on the mass of the tumour xenografts from therapy experiment 3. The mice were given peri tumoural injections of either PAI-2 (n=10) or PBS (n=8) twice daily for 21 days. A) Each filled diamond represents the mass (g) of a tumour xenograft obtained from one mouse. B) A bar graph representing the mean tumour mass (g) ± SD from each experimental group.


A.
Tumour mass (g)
2.0
1.5
1.0
0.5
0
PAI-2 injection
PBS injection
B.
Average tumour mass (g)
1.2
1.0
0.8
0.6
0.4
0.2
0
PAI-2 injection
PBS injection

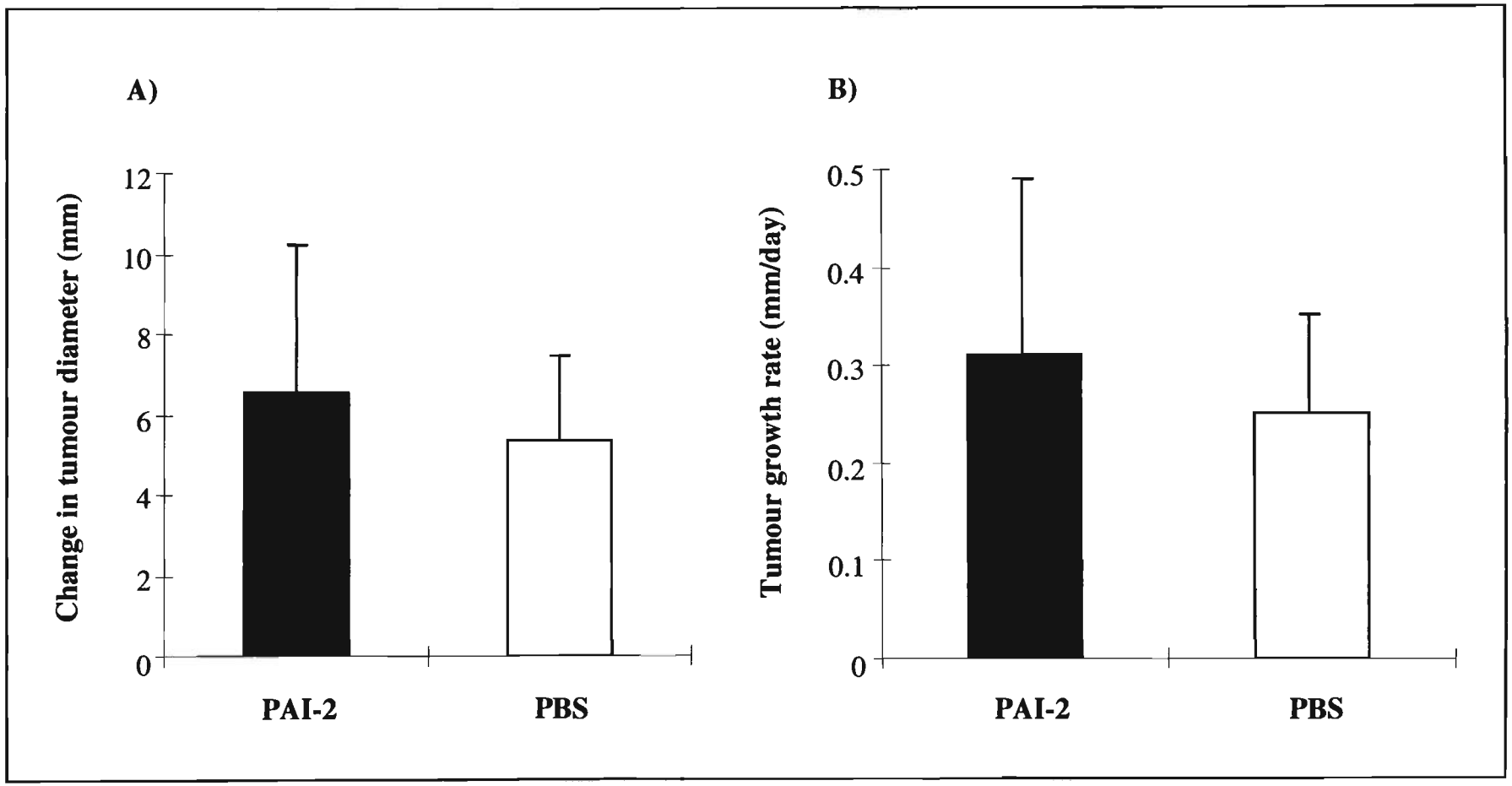


Figure 16. The effects of PAI-2 treatment on tumour diameter and growth rate. Data from both charts represents the mean and SD of all data from the PAI-2 (filled bars) and PBS (open bars) injected groups. A) The change in tumour diameter (mm) was calculated by subtracting the initial tumour diameter by the final diameter. B). Tumour growth rate (mm/day) was calculated by dividing the change in tumour diameter by 21, the total number of days of injections.

2.1mm/day) group. As was observed in the first experiment, a linear correlation was found between tumour diameter and tumour mass (Figure 17). The gradient of the linear regression of the PAI-2 (20.6 ± 1.7, r^2 = 0.95) group was much steeper compared to the gradient of the PBS group (6.7 ± 1.3, r^2 = 0.81).

4.3.4.1 PAI-2 ELISA

Plasma samples taken from the start of each therapy experiment were negative for PAI-2 antigen in the mice injected with PAI-2 or PBS, hence these data were not shown. PAI-2 ELISA results from the plasma samples taken at the end of therapy experiment 3 are shown in figure 18A. In the PBS injected group, no PAI-2 could be detected in the plasma, whereas in the group injected with PAI-2 there was PAI-2 (20.3 ± 7.7ng/ml) detectable in the plasma. No PAI-2 could be detected in the liver homogenates from the PBS injected mice, but there was some reactivity within homogenates of the kidneys (0.7 ± 0.1ng/mg) and tumour xenografts (0.2 ± 0.05ng/mg; Figure 18B). In contrast, the liver (0.7 ± 0.4ng/mg), kidney (3.2 ± 2.3ng/mg) and tumour xenograft (4.7 ± 1.7ng/mg) homogenates from mice receiving PAI-2 injections contained considerably more PAI-2 antigen.

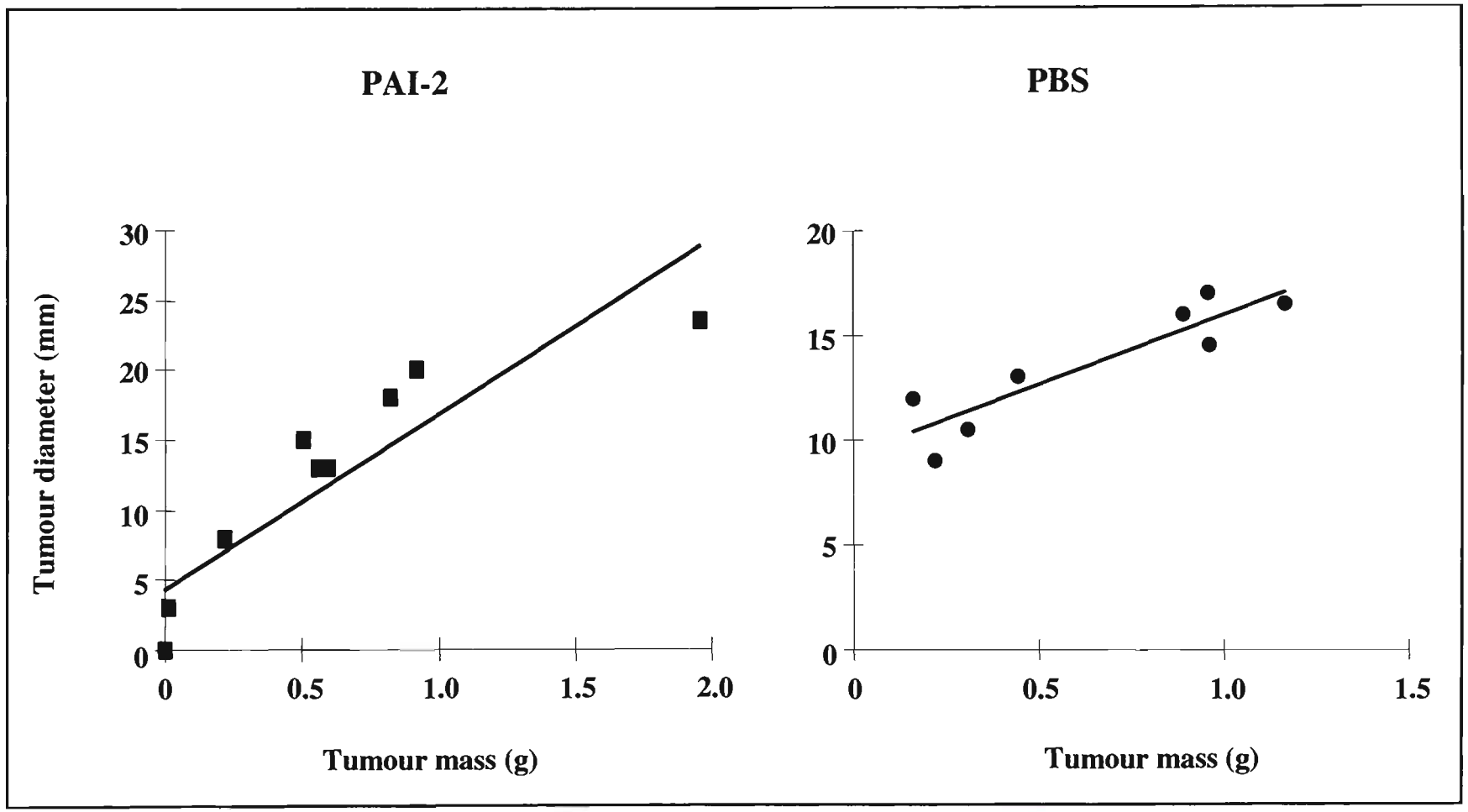
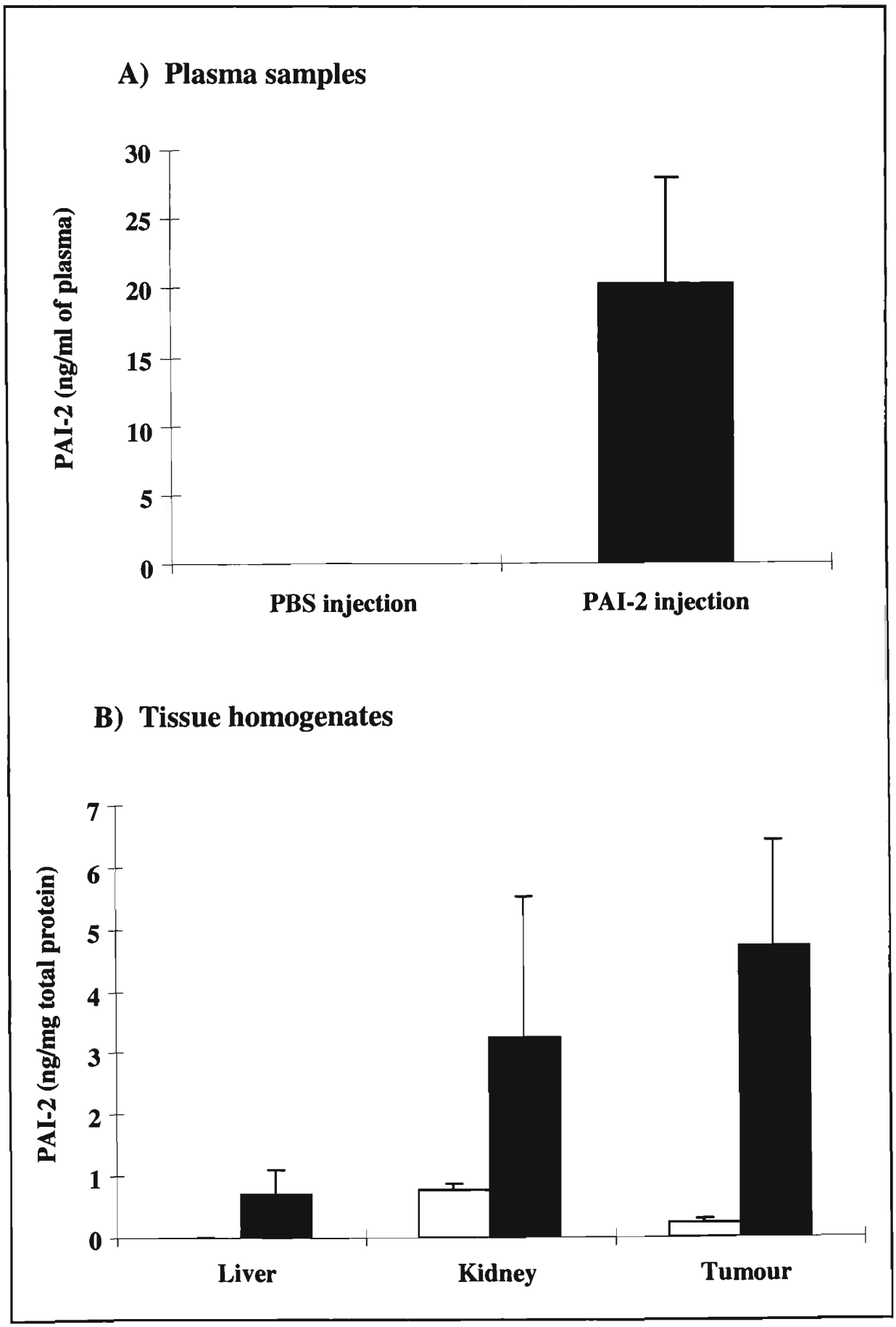


Figure 17. The relationship between tumour diameter and tumour mass in therapy experiment 3. Linear regressions are shown fitted through all data points from the mice injected with PAI-2 ($r^2 = 0.95$) and the mice injected with PBS ($r^2 = 0.81$).

Figure 18. PAI-2 antigen in plasma samples and tissue homogenates from the third therapy experiment. A) Blood was collected at the end of the experiment after the mice were killed and plasma samples obtained. PAI-2 antigen in plasma samples from mice injected with either PAI-2 (filled bars) or PBS (open bars) were measured via ELISA. B) Tumour xenografts, livers and kidneys were also obtained from the mice after they were killed. The organs and tumour xenografts were homogenised and PAI-2 antigen was also measured via ELISA in the homogenates. All the data are expressed as the mean ± SD of n=9 mice injected with PAI-2 and n=8 mice injected with PBS, with duplicates samples in each ELISA. Units are in ng PAI-2/ml of plasma or ng PAI-2/mg of total protein.


A) Plasma samples
PAI-2 (ng/ml of plasma)
30
25
20
15
10
5
0
PBS injection
PAI-2 injection
B) Tissue homogenates
PAI-2 (ng/mg total protein)
7
6
5
4
3
2
1
0
Liver
Kidney
Tumour

4.3.4.2 u-PA activity of tissue homogenates

4.3.4.2.1 Indirect colorimetric u-PA assay

u-PA activity of liver, kidney and tumour xenograft homogenates were measured by indirect measurement of plasmin generation (Figure 19). Homogenates of tumour xenograft (1916 ± 560mIU/mg protein) from the mice injected with PAI-2 contained the most u-PA activity, followed by kidney homogenates (1317 ± 582mIU/mg protein) and liver homogenates (77 ± 35mIU/mg protein). Similar results were obtained for tumour (1921 ± 560mIU/mg protein), kidney (1468 ± 293mIU/mg protein) and liver (69 ± 33mIU/mg protein) homogenates from the mice injected with PBS. No significant differences were observed in the level of u-PA activity in homogenates of the liver, kidney and tumour xenograft between mice injected with either PAI-2 or PBS.

4.3.4.2.2 Zymography

Liver and tumour homogenates obtained from mice injected with PAI-2 or PBS were analysed by zymography. Purified u-PA samples gave rise to a major band of lysis around the molecular weight of 55kDa (Figure 20A & B, lane 1). In homogenates of tumour xenograft from mice injected with either PAI-2 or PBS, there were three major bands with M_r of 33kDa, 55kDa and 98kDa (Figure 20A). There was also a weak band of lysis that just migrated into the resolving gel with a M_r of >250kDa.

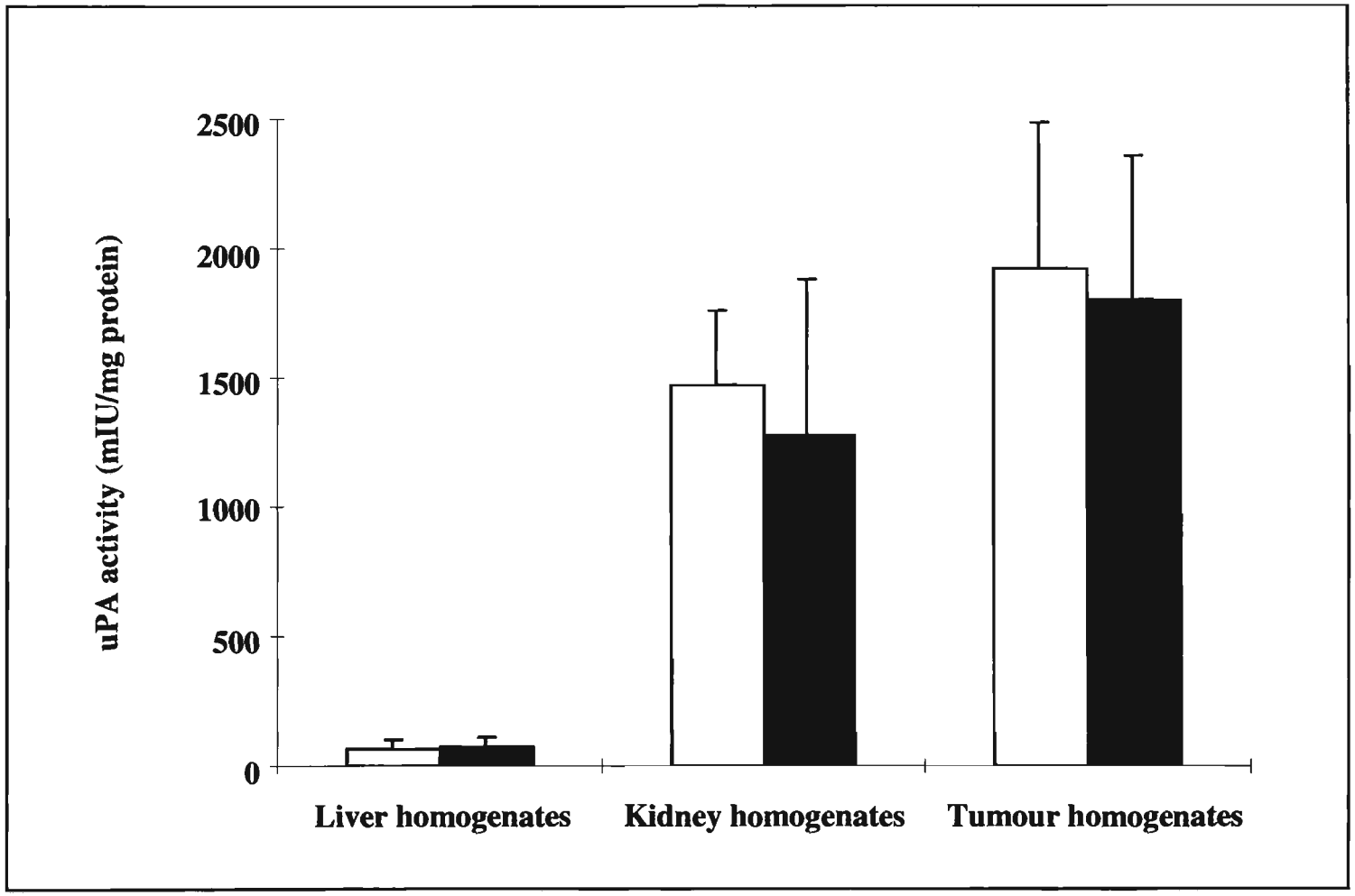
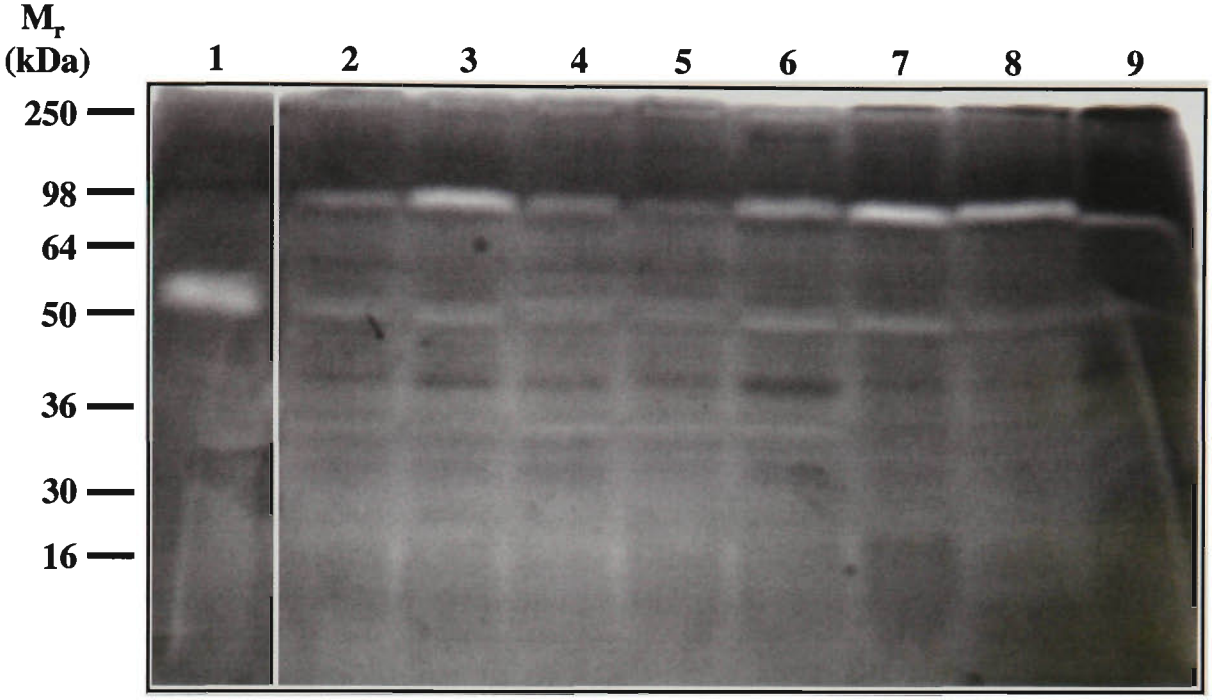


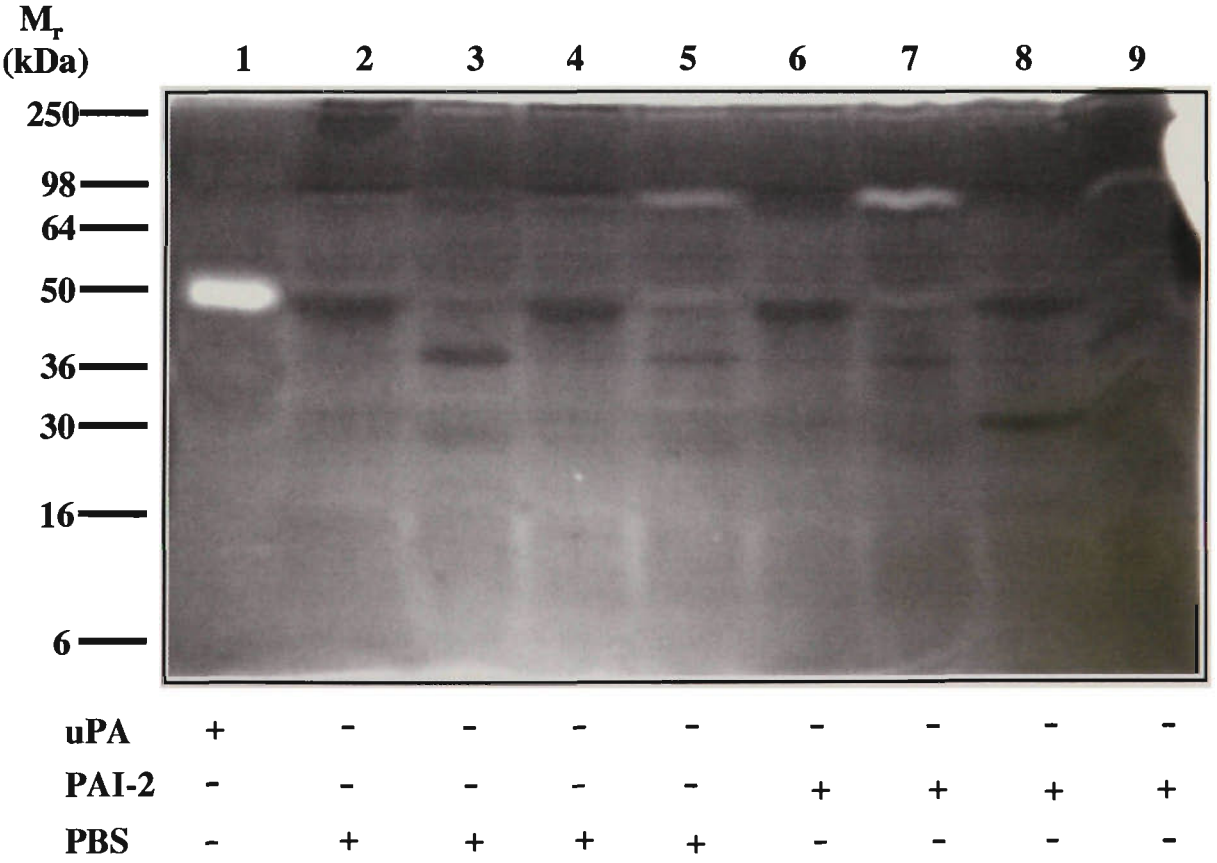
Figure 19. uPA activity of tissue homogenates from therapy experiment 3. uPA activity of homogenates of liver, kidney and tumour xenograft from mice given peritumoural injections of PBS (open bars) or PAI-2 (filled bars), were measured indirectly by measurement of plasmin generation. Units are in mIU/mg protein. Data is expressed as the mean±SD of n=9 for PAI-2 injections and n=8 for PBS injections.

Figure 20. Zymographs of homogenates of tumour xenograft and liver from therapy experiment 3. A) This zymography consists of homogenates from tumour xenografts only. Lanes 3, 6, 8 and 9 contains homogenates of tumour xenografts from mice injected with PAI-2. Lanes 2, 4, 5 and 7 contains homogenates of tumour xenografts from mice injected with PBS. Lane 1 contains purified uPA. B) This zymography contains homogenates of liver and tumour xenograft. Lane 1 is purified uPA. Samples in lanes 2-5 are from PBS injected mice and lanes 6-9 are from mice injected with PAI-2. Lanes 2, 4, 6 and 8 are liver homogenates and lanes 3, 5, 7 and 9 are homogenates of tumour xenografts.

A)


M_r (kDa)
1 2 3 4 5 6 7 8 9
250
98
64
50
36
30
16
uPA + - - - - - - - -
PAI-2 - - + - - + - + +
PBS - + - + + - + - -

B)


M_r (kDa)
1 2 3 4 5 6 7 8 9
250
98
64
50
36
30
16
6
uPA + - - - - - - - -
PAI-2 - - - - - + + + +
PBS - + + + + - - - -

However, the most predominant band of lysis was the 98kDa band. This 98kDa band is thought to be PAI-1:u-PA complexes, as there is no evidence of visible PAI-2:u-PA complexes by zymography. The55kDa and 33kDa bands were thought to be the high and low molecular weight forms of u-PA, respectively. The identity of the >250kDa is not known as there is no evidence in the literature showing u-PA:u-PAR complexes remain stable during SDS electrophoresis. There was no difference in the intensity of the bands of lysis with PAI-2 or PBS injections. Some of the liver homogenates from mice injected with PAI-2 or PBS contained very weak expression of the 33kDa, 55kDa and 98kDa bands (Figure 20B, lanes 2, 4, 6 and 8), while other samples did not. Overall, the homogenates of tumour xenografts (Figure 20B, lanes 3, 5, 7 and 9) expressed more u-PA than homogenates of liver from both the PAI-2 and PBS injected mice.

4.3.4.3 Histology and immunohistochemistry

With H&E the nuclei were stained purple and remaining structures were stained pink. An example of cultured HCT116 cells stained with H&E and tumour sections from mice injected with PAI-2 or PBS are presented in Figure 21. Staining of HCT116 cells revealed a heterogeneous population of cells (Figure 21A). The nucleus can be seen to occupy the majority of the cell. The staining of the tumour sections from mice injected with PBS or PAI-2 were similar in both groups (Figure 21B & C). Similar to the cultured HCT116 cells, cells from the tumour xenografts were also heterogeneous

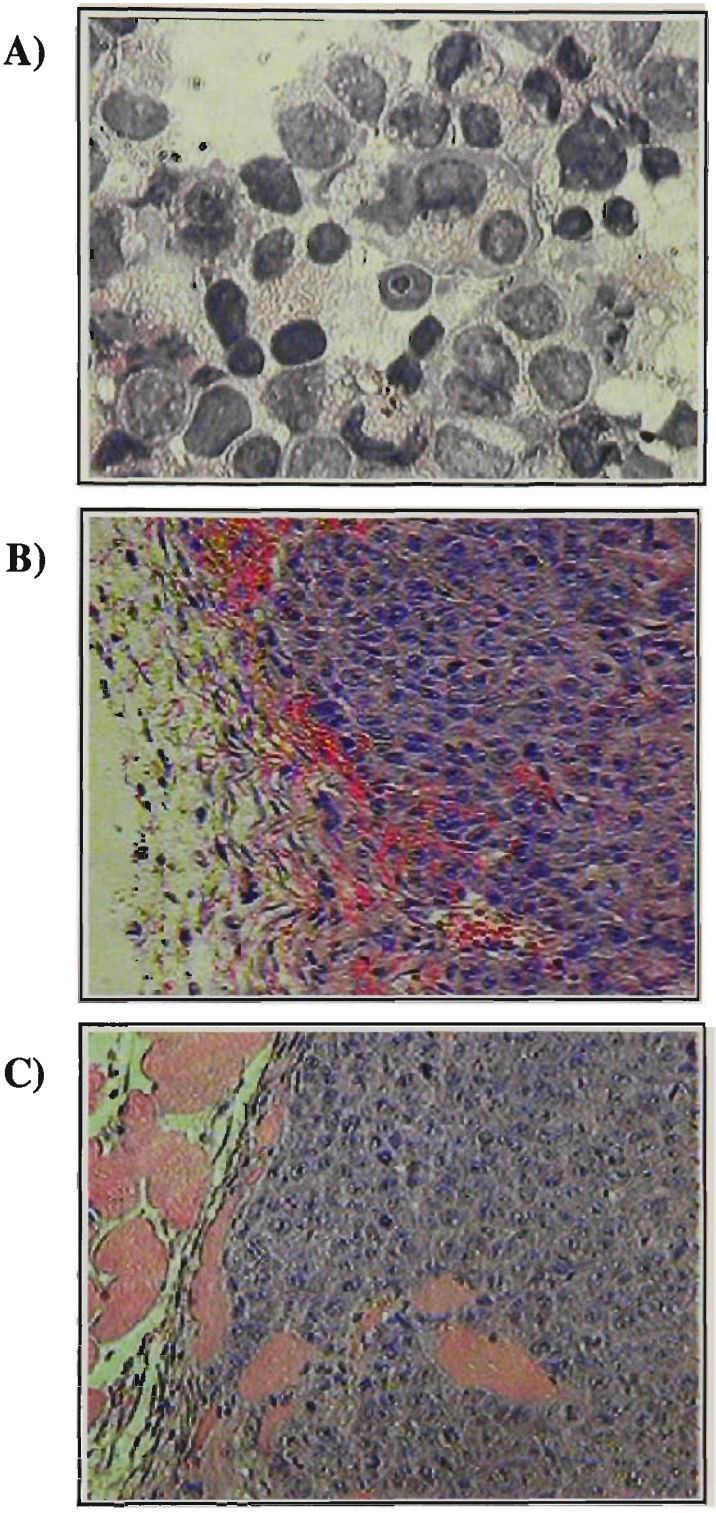


Figure 21. H&E staining of cultured HCT116 cells and tumours derived from HCT116 cells. A) HCT116 cells cultured on a glass coverslip (100x). B) Section of a tumour from PBS injected mouse (25x). C) Section of a tumour from a mouse injected with PAI-2 (25x). Local invasion of surrounding muscle is seen.

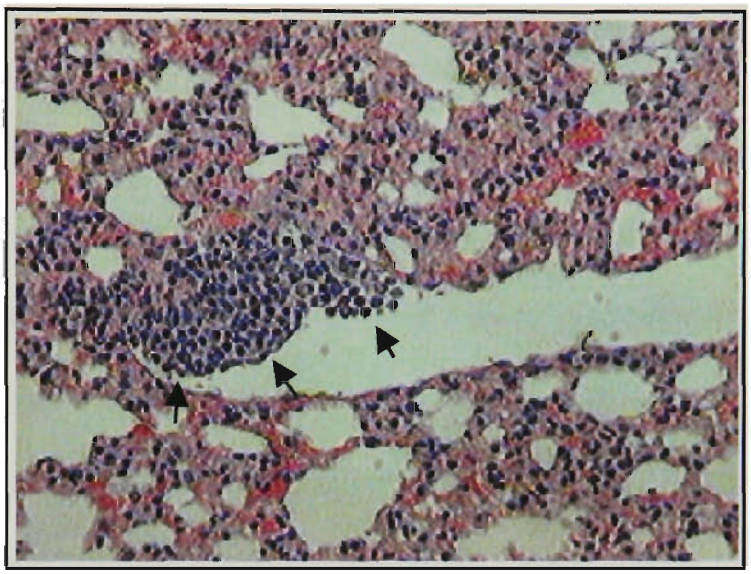
in size and shape. The sections of lung with metastasis from mice injected with PAI-2 (Figure 22B) or PBS (Figure 22A) were also very similar. There were no observable differences between the normal tissue or the metastasis from either group of mice.

The results from the immunohistochemistry were very unclear. The staining obtained from the test antigens (u-PA, u-PAR and PAI-2) were similar to that obtained for the isotype controls (IgG1 and IgG2a), which were used as negative controls (data not shown). This is thought to be due to cross reactivity between the secondary antigen supplied in the kit with the primary antibody hence no conclusive data was obtained. When a different staining protocol was implemented, which abolished the use of the second antibody, all the sections were only weakly stained. Hence this alternative method was not sensitive enough to detect the PAI-2 antigen in the sections, which was detected by ELISA.

4.4 DISCUSSION

An important factor to consider for a potential therapeutic agent is that there should be no adverse effects as a result of administration of the therapeutic agent. The well being of the mice with respect to their behaviour (ie. interaction with other mice in the cage as well as food and water intake) and ability to gain weight was not affected by

A)



B)

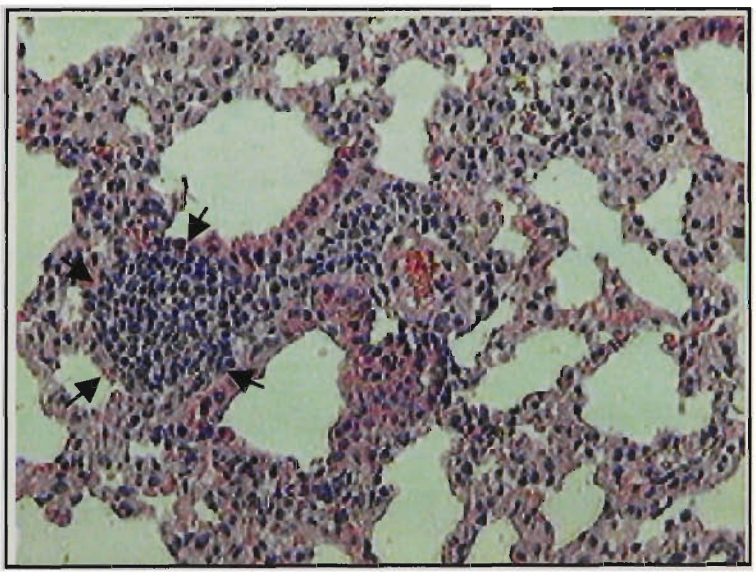


Figure 22. H&E staining of pulmonary metastasis in nude mice with subcutaneous tumour xenograft. A) Section of lung with metastasis (denoted by the arrow) from a mouse injected with PBS (25x). B) Section of lung with metastasis from a mouse injected with PAI-2 (25x).

injections of either PAI-2 or PBS. The mice which were either injected with PAI-2 or PBS showed similar final weights in all three therapy experiments.

Unfortunately, PAI-2 injections did not appear to inhibit the formation of metastasis. There was no inhibition of lymph node metastasis in any of the three therapy experiments. In fact, for therapy experiments 1 and 3 mice injected with PAI-2 had a higher incidence of lymph node metastasis. In the case with lung metastasis the results showed more promise. In therapy experiment 3, where PAI-2 injections were started when the tumour xenograft were approximately 1mm in diameter, there was a marked decrease in the percentage of lung metstasis compared to the PBS injections. This decrease in pulmonary metastasis when using PAI-2 has been observed before. A study by Evans and Lin (1995), showed that infusion of PAI-2 resulted in a significant decrease in pulmonary metastasis of rat mammary cancer cells in female Fisher 344 rats. The effects of a polysulfonated naphthylurea compound, suramin, found to increase PAI-2 production while decreasing u-PA production in the human renal cell carcinoma cell line, SN12C-PM6, correlated to its significant inihibition of pulmonary and hepatic metastasis of SN12C-PM6 in nude mice (Marutsuka *et al.*, 1995).

Results from therapy experiment 1 and experiment 2, suggested that tumour xenografts from mice injected with PAI-2 had a more aggressive behaviour, having a higher percentage of local invasion and pulmonary metastasis. A possible

explanation for the difference in results observed between experiment 3 versus experiments 1 and 2 may be that the tumour xenografts were much larger before PAI-2 injections were started. In the first two therapy experiments PAI-2 injections were started after the appearance of tumour xenografts in all mice. This meant that some of the tumour xenografts which appeared earlier would have been larger in diameter when the injections started. In studies carried out by various investigators where suppression of pulmonary metastasis was observed using inhibitors of u-PA, such as amiloride, B428, and antibodies against u-PA, the inhibitors were given either before, at the same time or less than one week after innoculations of the cancer cells (Kobayashi *et al.,* 1994b; Rabbani *et al.,* 1995; Evans *et al.,* 1998; Evans & Sloan-Stakleff, 2000b).

The PAI-2 injections did not appear to have any effect on the final mass or diameter of tumour xenografts. However, from therapy experiment 3, tumour xenografts from two of the mice given PAI-2 injections had disappeared by the end of the experiment. Similarly, a study by Rabbani *et al.,* (1995) found that an inhibitor of u-PA, B428, resulted in a reduction of primary tumour volume in male rats with subcutaneous tumours of the rat prostate cancer cell line Dunning R3227. Apart from those two mice, data from the rest of the experiment showed no significant difference in the final mass or diameter of tumour xenografts.

The results from all three therapy experiments suggested that PAI-2 may only be effective in the treatment of small tumour xenografts. That is, the smaller the tumour xenograft when PAI-2 injections were started, the more effective PAI-2 was at preventing tumour growth. PAI-2 injections had no effect on the growth of larger tumour xenografts. This observation could be due to three possibilities. The first may be that larger tumour mass may impose a larger surface area thus reducing the ability of PAI-2 to effectively reach individual tumour cells. The second possibility may be that the greater extent of vascularization of the tumour and thus the greater extent of blood vessels may dilute the amount of PAI-2 injected. Hence not enough PAI-2 can reach individual cancer cells. The third consideration is the amount of u-PA present in a larger tumour is greater than in smaller ones. The u-PA secreted by tumour cells may bind PAI-2, reducing the amount of PAI-2 available for binding to cancer cells. Ellis *et al.,* (1990) found that PAI-2 inhibited the activity of u-PA bound to U937 cells with an association rate that was lower than that obtained for u-PA in solution. The expression of u-PA on the surface of cancer cells may be in excess of the amount of PAI-2.

A difference was found between PAI-2 and PBS injections in the relationship between tumour diameter and tumour mass, in the first and last therapy experiments. For a given tumour diameter the mass of the tumour xenograft from the PAI-2 injected group did not weigh as much as the tumour xenografts from the PBS injected group. Along with this, was the observation that tumour xenografts from mice

injected with PAI-2 in the therapy experiment 3 had a higher percentage of necrosis in the inner core of the tumour mass. This may reflect that PAI-2 was inducing more necrosis, perhaps by blocking angiogenesis in the inner core of the tumour xenograft.

From the results, it is clear that injected PAI-2 can be detected in the plasma of mice, and does accumulate in the liver, kidneys and tumour xenografts. Therefore, PAI-2 can and does reach the tumour xenografts. However, PAI-2 did not appear to have any effect on u-PA activity in liver, kidney or tumour xenograft as observed from the u-PA activity assay and zymography data. These data suggests that there may be other PAI-2 binding sites other than u-PA within the tissues. One possible candidate may be the components of the surrounding extracellular matrix. In a study, by Ritchie *et al.,* (1999) it was observed that PAI-2 could be cross-linked to fibrin.

PAI-2 has been shown to have some potential as a therapeutic agent of human cancer. Using tumour xenografts derived from A431 cells it was observed that treatment with PAI-2 produced significant reduction in tumour mass compared to a control group injected with PBS (Shinkfield *et al.,* 1992). For that same study, *in vitro* results found recombinant PAI-2 to have a stimulatory effect on the growth of A431 cells. In another study the infusion of PAI-2 resulted in a significant decrease in pulmonary metastasis (Evans & Lin, 1995). Finally, the use of recombinant PAI-2 as a specific high-affinity therapeutic agent for u-PA would be ideal as PAI-2 can discriminate

between the active and inactive forms of u-PA since PAI-2 only binds to the active site of u-PA (Wun *et al.*, 1987).

The results obtained from studies carried out in this chapter are promising in two aspects. Extended exposure and a large concentration of PAI-2 appeared to have no detrimental effects on the well being of the mice under the experimental conditions of these studies. The disappearance of tumour mass with PAI-2 injections in two of the mice, as well as a reduction in pulmonary metastasis with PAI-2 treatment compared to PBS is encouraging. Thus, it appears in order to have an effect the cancer must be detected as early as possible so that the tumour is as small as possible. However, it must be stressed that the results obtained from these studies are only preliminary. More work is still required to adequately and accurately assess the potential use of recombinant human PAI-2 as a therapeutic agent for the treatment of human cancers.

Future experiments may implement larger amounts of PAI-2 administered per mouse per day, as results have shown u-PA activities from homogenates of tumour xenografts from either PAI-2 or PBS injections in these studies did not differ. In a study by Evans and Sloan-Stakleff (2000b) the maximal effect for suppression of pulmonary metastasis was at a concentration of 10mg/kg/day of amiloride in rats. The average weight of the mice use in therapy experiments 1 to 3 was around 26g, converting the weight to kg the amount of PAI-2 injected was approximately 4mg/kg/day. A different method of administration could be implemented, which may

enhance the inhibitory effects of PAI-2 on u-PA activity. Rather then peritumoural injection of the u-PA inhibitor, PAI-2 may be administered via osmotic pump continually throughout the duration of the experiment (Evans & Lin, 1995; Evans & Sloan-Stakleff, 2000b) or orally via drinking water (Evans *et al.,* 1998). PAI-2 may need to be administered with other known inhibitors of u-PA as well as inhibitors of matrix metalloproteinases to have a more potent effect to abolish cancer metastasis.

5. CONCLUSION

The results from this thesis further support the interaction of PAI-2 with cellular u-PA. The cell binding experiments clearly demonstrate that PAI-2 binds to HCT116 cells via active u-PA. It was difficult to observe u-PA/PAI-2 complexes in HCT116 cells, and was thought to be due to rapid turnover of the complexes. Very few studies exists that have observed u-PA/PAI-2 complexes produced endogenously, or from the addition of exogenous PAI-2. Reinartz *et al.*, (1998) observed u-PA/PAI-2 complexes on the surface of HaCaT cells by flow cytometry. A study by Ragno *et al.*, (1995) examined the processing of u-PA/PAI-2 complexes by the addition of exogenous u-PA/PAI-2 complex to THP-1 cells. At 4°C the complexes could still be observed, but at 37°C a 70kDa fragment was released, while a 22kDa fragment remained bound to the cell surface.

^{125}I-PAI-2 did not appear to be an effective direct imaging agent of tumour metastasis in this nude mouse model using the human colorectal cancer. However in the presence of a tumour xenograft the clearance of ^{125}I-PAI-2 from the circulation was found to be much quicker than that in control animals. Therefore, it is possible that ^{125}I-PAI-2 administered exogenously may be used to obtain information as to the presence of cancers in the subject as determined from the clearance rate. It may be

that patients with cancers will clear ^{125}I-PAI-2 faster from the circulation than those free of cancer.

Colorectal cancer has been imaged primarily by using tumour associated glycoprotein (TAG) antibody B72.3 and carcinoembryonic antigen (CEA) antibody FO23C5. A good correlation has been observed between detecting radiolabelled biotinylated B72.3 using a gamma detecting probe and immunohistochemistry (Mangili *et al.*, 1993). B72.3 labelled with ^{188}rhenium was found to localise to the human colorectal cancer LoVo in nude mice, with a tumour-to-blood contrast of 2.04±0.44 (Hosono *et al.*, 1998). The antibodies have primarily been radiolabelled with ^{125}I and used in radioimmunoguided surgery (RIGS) (Buraggi *et al.*, 1987; de Nardi *et al.*, 1997; Bakalakos *et al.*, 1998; Bertoglio *et al.*, 1998a, 1998b; di Carlo *et al.*, 1998; Percivale *et al.*, 1998; Renda *et al.*, 1998). Using these antibodies in combination with RIGS has the potential to improve the outlook for patients with colorectal cancer by increasing the sensitivity of metastasis detection. Unfortunately PAI-2 does not appear to be as effective as these antibodies, as an imaging agent.

The treatment of colorectal cancer has also focused on using TAG monoclonal antibody and/or CEA monoclonal antibody. Several studies have focused on using TAG monoclonal antibodies (Divgi *et al.*, 1995; Mulligan *et al.*, 1995; Meredith *et al.*, 1996; Triozzi *et al.*, 1997). The TAG monoclonal antibody CC49 (Divgi *et al.*, 1995) has been labelled with ^{131}I or the β-emitter Lu (Mulligan *et al.*, 1995) but did

not lead to any clinical anti-tumour effects. A combination of ^{131}I labelled CC49 and the CEA monoclonal antibody COL-1 were administered with α interferon to increase TAG and CEA antigen (Meredith *et al.*, 1996). The amount of radiation observed at the tumour were increased compared to historical controls, however the amount of radiation was still below that required for tumour regressions. Unlabelled CC49 has also been used in conjunction with IL-2 to treat metastatic colorectal cancer, without any observable clinical responses (Triozzi *et al.*, 1997).

There has been some research into CEA on colorectal cancer as a therapuetic target. In an animal model of colorectal carcinoma high and low CEA monoclonal antibodies radiolabelled with ^{131}I were used for treatment (Behr *et al.*, 1999a, 1999b). If mice were treated with the antibodies ten days after tumour inoculation the low affinity antibody had a 20% permanent cure rate, while the high affinity antibody had an 80% permanent cure rate (Behr *et al.*, 1999a). In human patients though, the high affinity monoclonal antibody showed promise, 18% of patients showed partial remission determined from an objective response (Behr *et al.*, 1999a, 1999b). Similar to the TAG antibody, PAI-2 does not appear to be a magic bullet for well established colorectal cancer, but shows some promise on small tumours. However, this does require further study and confirmation.

Several therapeutic strategies in animal models have examined the inhibition of components of the plasminogen/plasmin cascade such as the activity of u-PA. Some

therapeutic strategies include inhibition of u-PA activity with antibodies. It has been found that u-PA antibodies could inhibit lung metastasis (Ossowski and Reich, 1983a,b), murine melanoma experimental metastasis (Hearing *et al.,* 1988) and local invasion by LB6 cells (Ossowski *et al.,* 1991). Other studies have used specific u-PA inhibitors. It was found that amiloride could inhibit the growth of H6 hepatoma and DMA/J mammary adenocarcinomas (Sparks *et al.,* 1983). Amiloride, *p*-aminobenzamidine and PAI-1 were found to be able to inhibit the growth of human prostate cancer, albeit that the amiloride was found to be toxic to the animals (Jankun *et al.,* 1997). PAI-2 has been tested before and it was found to be able to reduce the mass of an epidermoid carcinoma tumour (Shinkfield *et al.,* 1992). Other therapeutic strategies may include interference with the interaction between u-PA and u-PAR (Stephens *et al.,* 1988; Ellis *et al.,* 1989; Ellis *et al.,* 1990). With the success of these studies it appears that PAI-2, the physiological inhibitor of u-PA, would therefore be a prime candidate for the suppression of the invasive and metastatic propensity of u-PA expressing cancers *in vivo.*

There are possible future experiments, both *in vitro* and *in vivo. In vitro* it would be interesting to examine the growth of HCT116 cells in the presence of PAI-2, by TUNEL and PCNA assay. The HCT116 cells should be checked for the expression of the annexins to see if they are the PAI-2 binding moiety detected. To take that a step further, the yeast two hybrid system could be used to identify other PAI-2 binding moities. To improve the tumour/background ratio in the biodistribution and kinetic

study, an isotope different to ^{125}I could be used. One such source may be the use of technicium 99 (Tcm99). For the therapy experiments, more treatment needs to be done on smaller tumours.

In conclusion, PAI-2 does bind to HCT116 cells *in vitro* via active u-PA. PAI-2 is not suitable as an imaging agent due to poor tumour-to-normal tissue discrimination compared to current agents used. That however may be improved by the trial of other isotopes, in conjunction with multiple doses. As a potential therapeutic agent, PAI-2 has some promise. PAI-2 treatment did lead to the cure of 2/9 small tumours. However, PAI-2 was ineffective against large tumours or in preventing metastasis to the lymph nodes. It was encouraging that in the final experiment PAI-2 appeared to inhibit pulmonary metastasis. PAI-2 shows potential, but more work is required to fully assess it's capabilities as an inhibitor of cancer metastasis where the involvement of u-PA is determined.

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APPENDICES

Buffers for the Coleman and Green assay

GTGE; 50mM Glycine pH 7.8
0.1% (v/v) Triton X-100
0.1% (v/v) gelatin
5mM 6-aminocaproic acid
The gelatin required boiling for dissolving.

PLA; 200mM K_2HPO_4
200mM KCl
0.1% (v/v) Triton X-100
220μM DTNB

ZLS; 500mg Z-lysine thiobenzylester / 5.75ml dimethylformamide
Store at 4°C in glass
Actual use: 50μl ZLS / 50ml PLA

The equation used for calculating $T_{1/2\alpha}$ and $T_{1/2\beta}$

$C = Be^{-\beta t} + Ae^{-\alpha t}$
C = drug concentration in the central compartment.
A = intercept on the y-axis from back-extrapolation from the line of the initial decay.
B = intercept on the y-axis from back-extrapolation from the line of the second decay.
α = the slope of the extrapolated line of the initial decay.
β = the slope of the extrapolated line of the second decay.
$t_{1/2\alpha}$ = α phase half-life
$t_{1/2\alpha} = 0.693/\alpha$
$t_{1/2\beta}$ = β phase half-life
$t_{1/2\beta} = 0.693/\beta$

Zymography gels

Resolving gel (bottom layer):

30% (w/v) Acrylamide & 1% (w/v) bis-acrylamide	4.12ml
1.5M Tris-HCl, pH 8.8/0.4% (v/v) SDS	2.81ml
Human plasminogen (stock concentration of 400μg/ml)	0.3ml
1% (w/v) Gelatin	1.125ml

10% (w/v) Ammonium persulphate	0.02ml
TEMED	0.01ml
H_2O	2.81ml

Stacking gel (top layer):

30% (w/v) Acrylamide & 1% (w/v) bis-acrylamide	0.4ml
0.5M Tris-HCl, pH 6.8/0.4% (v/v) SDS	0.5ml
10% (w/v) Ammonium persulphate	0.02ml
TEMED	0.02ml
H_2O	4.04ml

Amido black for staining of gels:

Amido black	0.5g
Methanol	150ml
Acetic acid	50ml
H_2O	300ml

H & E staining of tissue sections from therapy experiments

Place slides in an oven set at 40°C for 2min prior to dewaxing with histolene and alcohols.

2 x histolene	1min
3 x 100% alcohol	1min
wash with H_2O	1min
Haematoxylin	3min
wash with H_2O	1.5min
1% HCl in 70% alcohol	0.5min
wash with H_2O	1.5min
Scott's Tap water	1min
wash with H_2O	1min
eosin solution	1min
2 x 100% alcohol	1min
histolene	2min

Sections were mounted with DPT.

Haematoxylin:
Used Shandon instant haematoxylin purchased from Edward Keller.
Mix solution A with solution B as per manufacturer's instruction. Leave mixture overnight and filter with whatman's paper the following day.

Scott's Tap water:

Magnesium sulphate ($3H_2O$)	14.23g
Sodium bicarbonate	3.5g
distilled water	1000ml

Add a crystal of thymol.

Eosin stock:

1% Eosin Y in 80% alcohol

For use;

1 part of eosin
3 parts 80% alcohol
0.5ml glacial acetic acid per 100ml

Masson's Trichrome staining

Staining was carried out in the histology department at the North Wollongong hospital. Solutions were all premade and the following protocol as set up by the staff in the histology department was used to stain all sections.

2 x histolene	1min
3 x 100% alcohol	1min
wash with H_2O	1min
Mordant in Bouin's solution @ 56°C	60min
Wash in running water until colour disappears	
rinse in distilled H_2O	1min
Weigerts iron haematoxylin solution	10min
wash in running H_2O	10min
rinse in distilled H_2O	1min
Biebrich scarlet-acid fuchsin solution	2min
rinse in distilled H_2O	1min
Phosphomolybdic-phosphotungstic acid solution	15min
straight into aniline blue solution	15min
rinse in distilled H_2O	1min
glacial acetic acid	3min
2 x 100% alcohol	1min
histolene	2min

Sections were mounted with DPT.