THE ROLE OF PLASMINOGEN ACTIVATORS IN NEOPLASIA AND INFLAMMATORY LESIONS OF THE HUMAN INTESTINE

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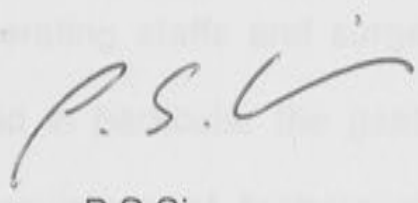
To my father and mother

CONTENTS

STATEMENT OF ORIGINALITY

The work embodied in this thesis is original and was carried out by myself and contains no material previously published or written by another person, except when due reference is made in the text of the thesis.



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SUMMARY

Despite numerous reports of the physiological importance of the role of proteolysis in vivo, it has been difficult to find correlations between the events observed and the presence of particular proteinases. The plasminogen-plasmin system, however, is important in the degradation of the extracellular matrix glycoproteins and the plasminogen activators are implicated as mediators of a proteolytic cascade which mammalian cells may employ to degrade the protein macromolecules of their immediate environment.

Four monoclonal antibodies raised against purified human plasminogen were characterized for their effects on the activation of plasminogen and on three enzymic properties of plasmin viz:- (a) thioesterolysis, (b) fibrinolysis, (c) conversion of high molecular weight human urokinase to its low molecular weight form.

None of the monoclonal antibodies inhibited plasminogen activation by urokinase. The monoclonal antibodies characterized in this study fell into three groups. The first group represented by anti-plg 1 inhibited (a), (b) and (c), while anti-plg 2, inhibited activities (a), (b) and (c) to varying degrees and in addition formed complexes with plasmin which were highly stable to sodium dodecyl sulphate. Anti-plg 3 and anti-plg 4 inhibited activity (c), but not (a) or (b).

Selective use of these monoclonal antibodies demonstrated unequivocally that plasmin mediates the activation of the proenzyme form of urokinase type plasminogen activator. Besides their use in affinity chromatography, therefore, these antibodies are valuable for defining the role of plasmin in the mechanisms of extracellular matrix degradation as shown in their use in Chapters 4-6.

Total plasminogen activator content (i.e. proenzyme plus active enzyme) was

found to be increased significantly in both colorectal carcinomas (n=20) and the premalignant adenomatous polyps (n=27) when compared to autologous normal mucosa. Activator content was also found to be increased in adenomatous polyps and autologous normal mucosa removed from familial polyposis coli patients.

The urokinase-type plasminogen activator was shown by a new monoclonal antibody technique to be present mainly as the proenzyme form.

For colon cancers, there was a significant correlation between their grade according to the Dukes' classification and the amount of proenzyme present ($P < 0.05$). Although a similar trend was evident, no significant correlation was, however, observed for total plasminogen activator content and the Dukes' grading.

For adenomatous polyps, no significant correlation was observed between enzyme content and the size or degree of dysplasia. However, plasminogen activator expression was found to be increased in the upper top or apical third of the polyps relative to the basal third or stalk.

These results suggest that the expression of increased levels of human plasminogen activator of 52,000 daltons proenzyme in dysplastic colon epithelial cells correlates with the extent of invasion. The polyp studies suggest that expression of human plasminogen activator of 52,000 daltons is a pre-requisite for invasion.

The study of the plasminogen activator content of colonic mucosa therefore offers a useful biochemical correlate of epithelial cell transformation.

The quantitative esterolytic assay incorporating monoclonal antibodies inhibitory to plasmin (chapter 3) was also used to elucidate the role of human plasminogen activator of 52,000 daltons in inflammatory bowel disease. The levels of proenzyme and active forms of human plasminogen activator of 52,000 daltons were compared in mucosa from patients with active inflammatory bowel disease (n=13), from inflammatory bowel disease patients in remission (n=7) and from uninvolved mucosa

(n=8). Preincubation of tissue homogenates with monoclonal anti-plg 1 and anti-plg 3 antibodies prevented activation of the proenzyme by plasmin.

The level (absorbance 412 nm) of total human plasminogen activator of 52,000 daltons enzyme activity (active + proenzyme) was markedly increased in active inflammatory bowel disease homogenates (1.10 $\pm$ 0.39), compared to those found in remission (0.48 $\pm$ 0.17) and in uninvolved mucosa (0.45 $\pm$ 0.20; $P < 0.01$). Assays of proenzyme levels showed a similar highly significant increase in active mucosa (0.80 $\pm$ 0.27) compared to remission (0.25 $\pm$ 0.15) and uninvolved mucosa (0.23 $\pm$ 0.15; $P < 0.01$). In actively diseased mucosa, 70% of the total human plasminogen activator of 52,000 daltons activity was present in the proenzyme form requiring extracellular cleavage by plasmin. These results suggest that human plasminogen activator of 52,000 daltons may be involved in the pathogenesis of tissue injury in inflammatory bowel disease by establishing the presence of a recognised pathway of inflammatory injury which is selectively and substantially enhanced in actively diseased tissue.

Subcellular fractionation of human intestinal macrophages was performed to define the localisation of the plasminogen activators and other serine hydrolase. Human intestinal macrophages, isolated from lamina propria and purified by centrifugal elutriation were disintegrated by nitrogen cavitation. The membranes were separated by equilibrium buoyant density using isopycnic centrifugation on a sucrose gradient. The subcellular membranes were localized using marker enzymes characteristic for plasma and intracellular membranes.

Mn^{2+}-stimulated leucine 2-napthylamidase was identified as a plasma membrane enzyme.

Analysis of membrane fractions identified a membrane bound esterase not detected in blood monocytes and exhibiting the same density and release characteristics of lysosomal hydrolase fraction. Whilst a portion of the esterase activity may reside in

the smooth endoplasmic reticulum, no evidence for plasma membrane esterase was btained. Unlike monocytes, the esterase activity was partially resistant to 40 mM sodium fluoride. The different properties of the human intestinal macrophages alpha-naphthyl esterase to that of the blood monocytes may have important significance in the light of recent evidence that alpha-naphthyl esterases are involved in the spontaneous cytotoxicity of monocytes toward tumour cells (see discussion on chapter 6).

Arylsulphatase C, an endoplasmic reticulum marker was detected in much denser fractions than that reported in monocytes.

Plasminogen activator of the urokinase-type was detected on the Golgi and plasma membranes fractions in human intestinal macrophages. The plasminogen activator was found to exist predominantly as proenzyme requiring plasmin for proteolytic activation. The findings that the plasminogen activator is membrane associated and exists mainly as proenzyme in the plasma and Golgi membrane fractions might have important biological implications and suggest a site of enzyme activity which may optimise their effectiveness.

The analysis of human intestinal macrophages plasma and intracellular membranes at high purity represents a valuable approach to elucidating the changes that characterize the differentiation of intestinal macrophages.

ABBREVIATIONS

PA	- plasminogen activator
u-PA	- urokinase type plasminogen activator
t-PA	- tissue type plasminogen activator
HPA66, HPA52, HPA36	- human plasminogen activator of 66,000; 52,000 and 36,000 daltons respectively
mPU	- milliploug unit
Ploug Unit	- unit of urokinase activity equivalent to approx. 1.4 CTA units
CTA	- Committee on Thrombolytic Agents
Plg	- plasminogen
Mr	- molecular weight
IBD	- inflammatory bowel disease
HIMØ	- human intestinal macrophages
DFP	- diisopropylfluorophosphate
SDS	- sodium dodecyl sulphate
SDS-PAGE	- polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulphate
EDTA	- ethylenediaminetetraacetic acid
NaOH	- sodium hydroxide
NaCl	- sodium chloride
DMEM	- dulbecco modified Eagle medium

HAT	-	hypoxanthine, aminoptherin, thymidine
FCS	-	foetal calf serum
Pen	-	penicillin
Gent	-	gentamycin
Strep	-	streptomycin
NPGB	-	p-nitrophenyl p'-guanidino-benzoate
EACA	-	ε-caproic-n-acid
BSA	-	bovine serum albumin
PBS	-	phosphate buffered saline

CHAPTER 1

GENERAL INTRODUCTION

PLASMINOGEN ACTIVATORS

SINCE the first observations by the pioneers of tissue culture on the liquefaction of clotted blood and the enhancement of fibrinolysis in vivo and in vitro in the 18th and 19th centuries, supporting experimental evidence accumulated until it promptoed Fisher (1925) to postulate about the proteolysis caused by cultured Rous sarcomas:-

> "We could imagine that the destructive process of the sarcoma cells in vivo would be as follows. First the stroma binding the cells together is liquefied. Consequently, reorganisation cannot take place as long as the lytic agent, the sarcoma cells, is present and because the fixed cells are deprived of their fibrin stroma".

Twenty years after his postulate, Fisher reported in 1946 that proteolysis caused by cultured cancer cells appeared to be due to their activation of an inactive proenzyme present in serum. It was left to Goldhaber et al., (1947), to suggest that the cancer cells released an enzyme which activated profibrinolysin to fibrinolysin. This activating enzyme is now widely known as plasminogen activator (PA), a serine protease which catalyses the conversion of inactive plasminogen to the active plasmin.

1.1. BIOCHEMISTRY OF PLASMINOGEN ACTIVATORS

In order to appreciate the possible implications of the role of plasminogen activators in tumorigenesis, cancer invasion and metastasis, an understanding of the biochemistry of these enzymes is necessary.

Christensen (1945) and Kaplan (1944; 1946) both demonstrated the existence of an inactive proenzyme, designated profibrinolysin or plasminogen which could be activated to the active protease fibrinolysin or plasmin. This was soon to be followed by demonstration of the presence of substances able to activate plasminogen in tissues and tissue extracts (Astrup & Permin 1947; Astrup & Stage 1952; Albreschten 1957 a,b), urine and blood (Williams 1951; Astrup & Sterndorff 1952; Sobel et al., 1952; Lewis & Ferguson 1951; Mullertz 1953; Sherry et al., 1959).

Human plasminogen, an inactive proenzyme, is present in abundant amounts in the extracellular fluid. Its native form is a single chain glycoprotein with a Mr of about 92,000 (Collen, 1980), containing about 2% carbohydrate and with NH_2-terminal glutamic acid (termed Glu-plasminogen). Glu-plasminogen is readily converted proteolytically to modified forms with NH_2-terminal lysine, valine or methionine (Wallen & Wiman, 1970; 1972) and the former therefore being termed Lys-plasminogen with Mr about 8000 lower than its native form. These conversions occur by hydrolysis of the Arg 67 - Met 68, Lys 76 - Lys 77 or Lys 77 - Val 78 peptide bonds. The complete plasminogen molecule consists of either 790 or 791 amino acids (Sottrup - Jensen et al., 1978a; Wiman 1978), containing 24 disulfide bridges as well as five homologous triple loop structures or "Kringles" (Sottrup-Jensen et al., 1978b).

In vitro activation of Glu-plasminogen to plasmin by plasminogen activator in a purified system occurs about 20 times more slowly than activation of Lys-plasminogen (Claeys & Vermylen 1974; Wallen & Wiman 1975; Thorsen et al., 1974; Lijnen & Collen 1982) but in either case, Lys-plasmin is formed. However, activation of the fibrinolytic system in vivo occurs by direct cleavage of the Arg 560 - Val 561

bond in Glu-plasminogen yielding Glu-plasmin and not via formation of the Lys-plasminogen intermediates (Holvoet et al., 1985).

Plasminogen is purified mainly by affinity chromatography with lysine-Sepharose introduced by Deutsch and Mertz (1970). Plasminogen is an inactive proenzyme, while its active form **PLASMIN** has a wide range of general proteolytic ability with a trypsin-like specificity. Plasmin consists of two polypeptide chains held together by disulfide bonds. The light chain, with a Mr of about 25,000, contains the active site and has considerable amino acid sequence homology with other serine proteases such as trypsin, chymotrypsin and pancreatic elastase.

Two different pathways of plasminogen activation by vertebrate plasminogen activators are shown schematically in Figure 1.1.

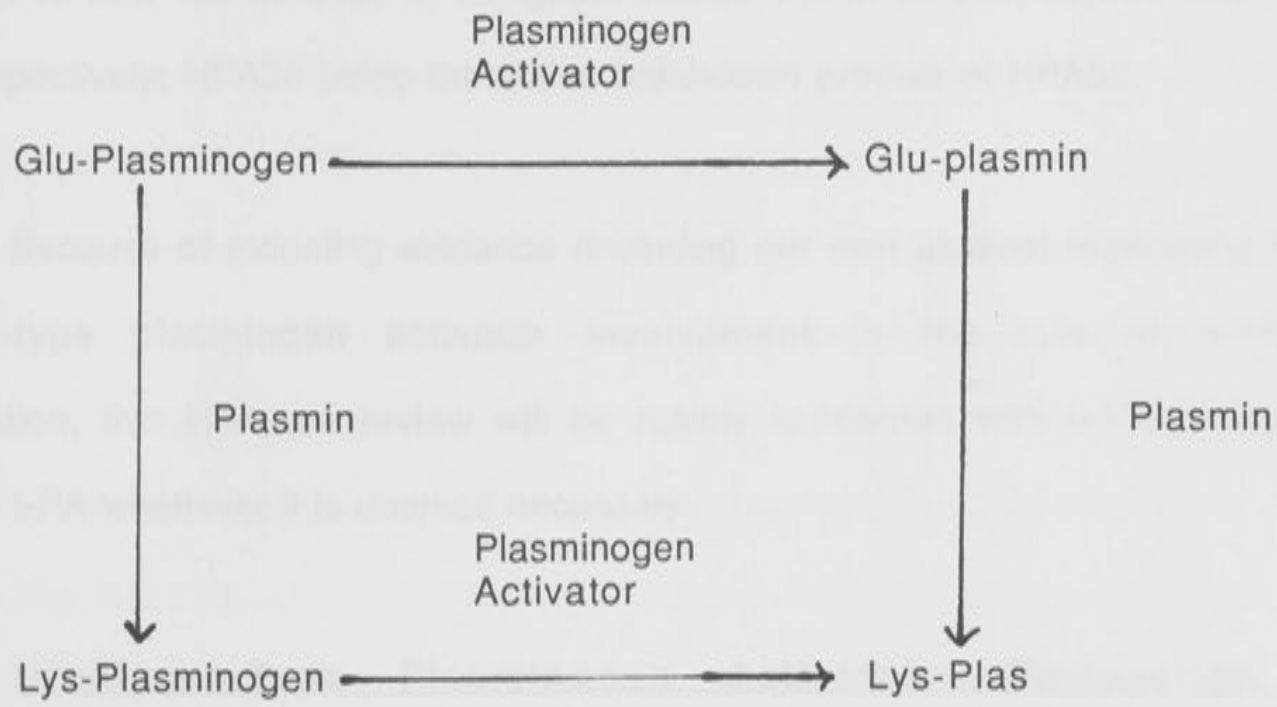


Figure 1.1. Activation of Plasminogen by Plasminogen Activator.

1.2 TYPES OF ACTIVATORS OF PLASMINOGEN

The existence of 2 different types of plasminogen activators, designated urokinase-type (u-PA) and tissue-type (t-PA) has been well documented. They differ in Mr, immunological reactivity, genotype and are the products of different genes (Aoki & Kaulla 1971; Unkeless et al., 1974b; Christman et al., 1975; Dano & Reich 1978; Granelli-Piperno & Reich 1978; Astedt 1979; Vetterlein et al., 1979; Dano et al., 1980b; Rijken et al., 1980; Roblin & Young 1980; Wilson et al., 1980; Rijken & Collen 1981; Gunzler et al., 1982b; Kaltoft et al., 1982; Schaller et al., 1982; Steffens et al., 1982; Edlund et al., 1983; Nielsen et al., 1983; Pennica et al., 1983). The human urokinase-type plasminogen activator has a Mr of about 50,000 - 52,000 (HPA52) while the tissue type has a Mr of about 70,000 (HPA66). Tissue-type plasminogen activator has also been termed extrinsic plasminogen activator (Collen 1980) although this term is less commonly used. In this and the following chapters, the term u-PA will be used to designate urokinase-type PA of any species while HPA66, HPA52 and HPA36 will be used to designate human PA of 66,000, 52,000 and 36,000 daltons respectively; HPA36 being the active breakdown product of HPA52.

Because of mounting evidence (including our own studies) implicating mainly urokinase-type plasmingen activator involvement in the role of malignant transformation, this literature review will be mainly concerned with u-PA and a brief mention of t-PA whenever it is deemed necessary.

1.2.1 Urokinase-Type Plasminogen Activator:- Purification and Occurence of u-PA

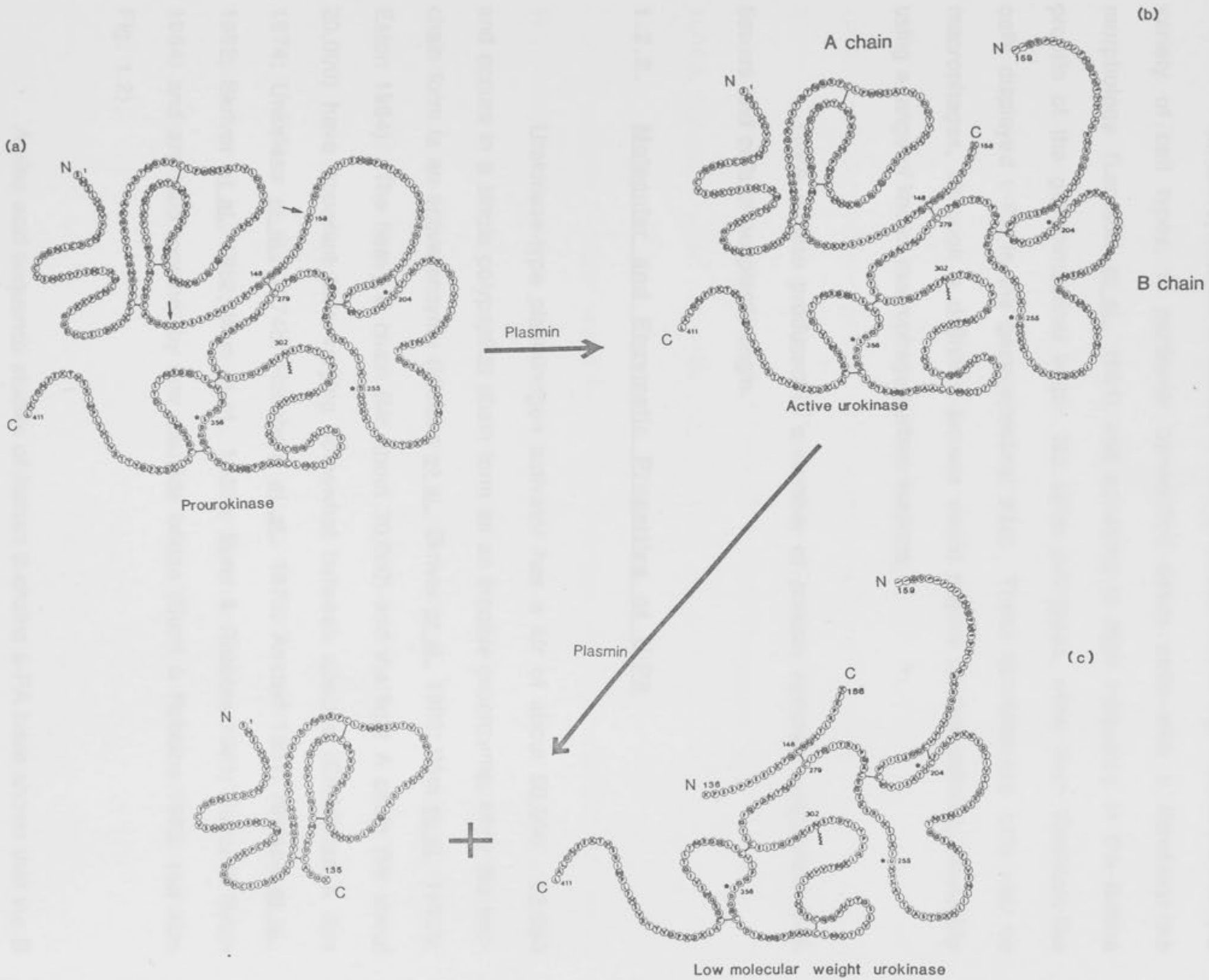
The presence of a high concentration of u-PA in human urine has for many years enabled its use as a commercial source for purification of the enzyme, although

some has been produced by cultures of kidney cells. More recently, recombinant urokinase has been produced after cloning of the human gene (Heyneker et al., 1983b; Verde et al., 1984), and this method will inevitably become the major commercial procedure.

Purification of u-PA to homogeneity from urine by a tedious sequence of precipitations and conventional chromatography steps was first described by Lesuk et al., (1965) and White et al., (1966). Since then, purification involving the use of affinity chromatography, especially the use of immobilized monoclonal antibodies, has been widely used (Kaltoft et al., 1982; Nielsen et al., 1982; Herion & Bollen 1983; Vetterlein & Calton 1983;). The purification of u-PA from human blood plasma and serum, human seminal plasma and from hyperplastic and malignant prostate tissue has also been reported. Because only very low concentration of u-PA are found in conditioned culture fluid of several types of cell lines, even after treatment of the cells with agents enhancing u-PA production e.g. phorbol esters (Goldfarb & Quigley 1980), urine is still the main source for the purification of u-PA. However, recently, Grondahl-Hansen et al., (1985), using a one-step procedure with monoclonal antibody immobilized on Sepharose, have been able to purify human u-PA to apparent homogeneity from the conditioned culture fluid of a human glioblastoma cell line.

As mentioned above, u-PA, or a plasminogen activator with similar properties, has been found in a number of biological fluids or tissue extracts (Kucinski et al., 1968; Granelli-Piperno & Reich 1978; Dano et al., 1980b; Astedt et al., 1977; Rijken et al., 1981; Astedt 1978; Shakespeare & Wolf 1979; Tissot et al., 1982; Wijngaards et al., 1982; Wun et al., 1982a; Casslen et al., 1981; Oshiba & Ariga 1983; Markus et al., 1980, 1983; Camiolo et al., 1984; Nakamura et al., 1984; Corasanti et al., 1980).

Figure 1.2 Schematic arrangement for the covalent structure of (a) prourokinase (b) urokinase and (c) low molecular weight urokinase. Plasmin cleavage sites are indicated by arrows. The A-chain of the human u-PA contains a "kringle" structure in its C-terminal part. The N-terminal of the B-chain contains the sequence Ile-Ile-Gly-Gly. The A-chain has Arg or Phe as its C-terminal amino acid. Nucleotide sequence around the cleavage site is Arg-Phe-Lys-Ile-Gly-Gly. Activation of prourokinase is effected by cleavage of a single bond resulting in the formation of A and B chains linked by a disulphide bridge. The Ser, His and Asp residues forming the active site in u-PA are marked with stars. Plasmin also cleaves urokinase A-chain at residues Lys 135-Lys 136 removing the kringle and N-terminal region, leaving only 21-23 residues linked to the B chain (Adapted from Heyneker et al., 1983a).


(a)
N
1
158
148
279
204
302
255
C
411
356
Prourokinase
Plasmin
(b)
A chain
N
1
N
159
C
158
148
279
204
302
B chain
C
411
255
356
Active urokinase
Plasmin
(c)
N
159
C
158
148
279
N
136
204
302
255
C
411
356
N
1
135
C
Low molecular weight urokinase

Immunocytochemical studies of normal mouse tissues has localized u-PA to a variety of cell types, in particular connective tissue cells with a fibroblast-like morphology (Larsson et al., 1984) and occurring in high frequency in the lamina propria of the gastrointestinal tract. No other cell types, other than fibroblast-like cells displayed u-PA in the gastrointestinal tract. These fibroblast-like cells may be macrophages, although a definitive answer would require double staining, possibility using a range of known macrophage surface markers.

U-PA is also produced in a number of primary cultures established from tissues and cells of neoplastic origin.

1.2.2. Molecular and Enzymatic Properties of U-PA

Urokinase-type plasminogen activator has a Mr of about 50,000 - 52,000 and occurs in a single polypeptide chain form as an inactive proenzyme, while the two-chain form is an active enzyme (Nielsen et al., Skriver et al., 1982; Wun et al., 1982b; Eaton 1984). The heavy B chain (Mr about 30,000) and the light A chain (Mr about 20,000) have apparent Mr's varying somewhat between species (Christman & Acs 1974; Unkeless et al., 1974b; Holmberg et al., 1976; Aasted 1981; Nielsen et al., 1982; Skriver et al., 1982; Wun et al., 1982b; Sumi & Robbins 1983; Sudol & Reich 1984) and are held together by one disulfide bridge (Sumi & Robbins 1983; see also Fig. 1.2).

Amino acid sequence studies of human 2 chains u-PA have shown that the B chain contains a heavy chain identical to that of the two chain 50,000 Mr forms and a light chain that is identical to the C-terminal 21 residues of the light chain of the native

50,000 Mr form. These findings demonstrate that the B chain (Mr about 30,000) is formed from the native 50,000 Mr form by proteolytic conversion (Gunzler et al., 1982a). This conversion probably occurs during storage and purification due to proteases present in urine and other biological fluids (Soberano et al., 1976b). The studies described in Chapter 3 showed that this conversion is mediated through plasmin and not by autocatalysis.

U-PA is a glycoprotein (McLellan et al., 1980; Steffens et al., 1982) with an isoelectric point of between 8.4 and 9.7. However, the exact value varies according to the species from which the enzyme originated and between different reports on u-PA from the same species (Christman & Acs 1974; Soberano et al., 1976a; Dano et al., 1980a; Nobuhara et al. 1981; Miwa et al., 1982).

Although plasminogen is the only well-documented protein substrate for u-PA, studies by Quigley et al., (1980) and Keski-Oja & Vaheri (1982) have suggested the existence of cellular non-plasminogen substrates. U-PA also hydrolyzes a variety of arginine and enzyme esters and amides and other low Mr substrates (Kjeldgaard & Ploug 1957; Ascenzi et al., 1982; Lottenberg et al., 1981; Friberger 1982).

The high degree of substrate specificity shown by u-PA is probably related to the presence of a specific binding pocket (Schoellman et al., 1982). U-PA also exhibits to some degree species specificity with respect to the plasminogen substrate (Reich, 1975).

Inhibition of the esterolytic activity (Landmann & Markwardt 1970) and plasminogen activating activity (Christman & Acs 1974; Unkeless et al., 1974b; Dano & Reich 1975; Astedt & Holmberg 1976) of u-PA from various species by the active-site

reagent for serine proteases diisopropylfluorophosphate (DFP) has indicated that these activators are serine proteases (Walsh and Wilcox, 1970).

The heavy B chain (Mr about 30,000 - 253 residues) carries the active site residues as demonstrated by affinity labelling with peptidyl choloromethyl ketone inhibitors (Ong et al. 1976) or [^{3}H] DFP (Christman & Acs 1974; Unkeless et al., 1974b; Nielsen et al., 1982; Skriver et al., 1982; Wun et al., 1982b), followed by SDS-polyacrylamide gel electrophoresis under reducing conditions.

The strong homology of the active site residues of u-PA with trypsin and the plasmin B-chain, is also illustrated by the fact that several compounds inhibiting u-PA also inhibit plasmin (Dano & Reich 1975, 1979). But as expected from the highly different substrate specificities of u-PA and plasmin, some inhibitors have different affinities for the two (Walton 1967; Dano & Reich 1975, 1979; Summaria et al., 1975).

1.2.3 Proenzyme of u-PA

There are several examples of serine proteases which are released from cells as inactive proenzymes which are converted to the corresponding active enzymes by limited proteolysis (for a review, see Neurath &Walsh 1976).

Studies by Bernik et al. (1974) and Nolan et al., (1977) suggested the presence of a latent form of u-PA in the conditioned medium from cultured cells, but definitive identification of an inactive u-PA proenzyme could not be achieved due to the lack of sufficient purified protein. Since then several authors (Sumi et al., 1982, Husain et al., 1983, Skriver et al., 1982, Eaton et al., 1984, Wun et al., 1982b,

Nielsen et al., 1982) have demonstrated a one-chain inactive polypeptide form of u-PA both in human and murine systems. This question is answered definitively by experiments using monclonal antibodies inhibitory to plasmin as reported in Chapter 3. The identification of proenzyme form of HPA52 has led to several proposed regulatory implications (see Chapters 4-6).

From the work described in Chapter 3 and that of others (Nielsen et al., 1982, Wun et al., 1982b) it is likely that the presumed mechanism of activation of pro-uPA is as depicted in Figure 1.3 below.

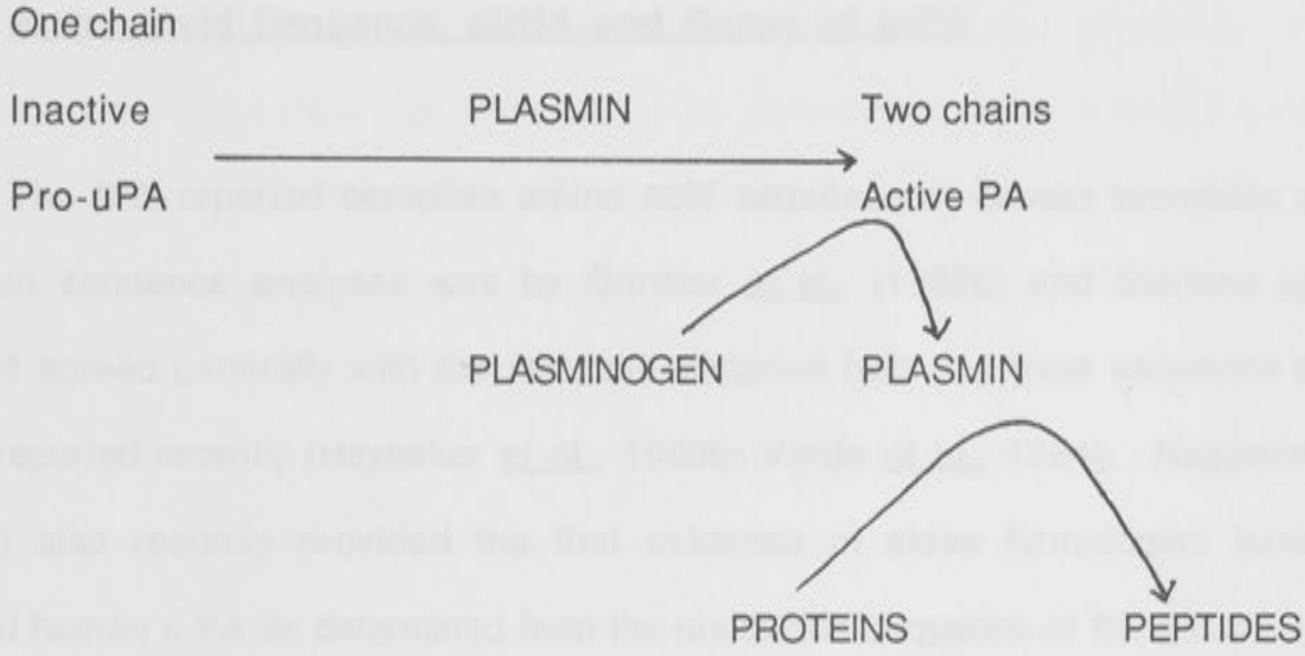


Fig. 1.3 Cascade reaction leading to u-PA initiated extracellular proteolysis. Pro-urokinase (Pro-u-PA) is an inactive single chain precursor molecule which is converted to an active two-chain polypeptide (Mr 20,000 and 30,000) linked by a single disulfide bridge, by cleavage of the Lys 158-Ile 159 peptide bond. The active u-PA activates plasminogen to plasmin by cleavage of the Arg 560 - Val 561 peptide bond.

The above reports, together with the findings that plasminogen activators of the urokinase type are present predominantly in proenzyme form in the colonic adenomatous polyps and carcinomas, in inflammatory bowel disease (see Chapters 4 and 5), extracellularly in a murine tumour (Skriver et al. 1984) and in various murine tissues and urine (Kielberg et al., 1985), strongly suggest that u-PA is generally released to the extracellular milieu in its inactive form, requiring plasmin mediated extracellular proteolysis to its active form. This inactive form is the predominant form of u-PA in intracellular stores and comprises a sizeable fraction of the u-PA in extracellular fluids in the intact organism.

1.2.4 Amino Acid Sequence, cDNA and Genes of u-PA

The first reported complete amino acid sequence of human two-chain u-PA from protein sequence analyses was by Gunzler et al., (1982b) and Steffens et al., (1982), and agreed generally with the structure deduced from the base sequence of u-PA cDNA reported recently (Heyneker et al., 1983b; Verde et al., 1984). Nagamine et al., (1984) also recently provided the first evidence of close homologies between porcine and human u-PA as determined from the nucleotide sequence of the porcine u-PA gene.

The sequencing of the cDNA for both u-PA and its comparison with that of t-PA has definitively established that each activator is the product of different genes as previously speculated.

The single structural gene for urokinase has been reported to be present on human chromosome 6, and nucleotide sequencing of genomic libraries indicated the presence of up to 13 introns (Kucherlapati et al., 1978, Holmes et al., 1985). However, Tripputi et al., (1985) using somatic cell hybrids and detection of genomic u-

PA DNA on Southern blots and in situ with a cDNA probe localized the human u-PA gene to the long arm of chromosome 10.

1.2.5 Tissue Type Plasminogen Activator

Tissue-type plasminogen activator (t-PA) can now be isolated quite readily from various sources and indeed milligram quantities had been purified from the tissue culture media of Bowes human malignant melanoma (Rijken & Collen 1981, Wallen et al., 1983).

Although the Mr to be expected from the nucleotide sequence of the corresponding cDNA of t-PA is 59,000, the Mr as determined by SDS-polyacrylamide gel electrophoresis is 66,000 daltons. Glycosylation of the t-PA molecule could account for this apparent discrepancy (McLellan et al., 1980).

Like u-PA, t-PA exists in two forms, a one-polypeptide chain form and a two-polypeptide chain form, the two chains in the latter, which is an active enzyme, being held together by disulfide bridges (Binder et al., 1979; Rijken & Collen 1981; Rijken et al., 1979; Aasted 1980; Wallen et al., 1981, 1982, 1983; Ranby et al., 1982; Nielsen et al., 1983). However, unlike u-PA, conflicting results as to the enzyme activity of the one-chain polypeptide form have been reported (Rijken & Collen 1981; Wallen et al., 1981, 1982, 1983; Ranby 1982; Rijken et al., 1982; Andreasen et al., 1984; Ichinose et al., 1984). Using monoclonal antibodies against human t-PA and a linear regression analysis using ^{3}[H]-DFP labelling, Andreasen et al., (1984) reported that the single chain t-PA represents a fibrinolytically inactive proenzyme; while Rijken et al., (1982) using a ^{125}I-labelled plasminogen conversion assay reported that the 2 forms of human t-PA had nearly identical activities. Others reported

that the one chain from of both human and porcine t-PA appeared to have considerable activity (Rijken & Collen, 1981; Wallen et al., 1981, 1982, 1983; Randy 1982).

Irrespective of whether it is accepted that the one chain form of t-PA is partly or completely inactive proenzyme, it could be converted by plasmin to a two chain form by the cleavage of an Arg 274-Ile bond, resulting in A and B chains linked by a single disulphide bond (Edlund et al., 1983; Pennica et al., 1983; Jornvall et al., 1983; Wallen et al., 1983). The B chain with a Mr of between 28,000 - 33,000 comprises the c-terminal region of the enzyme and is homologous with trypsin, plasmin and u-PA B chains and carries the active centre residues. An increase in amidolytic activity as a result of this bond cleavage (which does not affect the PA activity) has been used as an assay to measure the proportions of one and two-chains forms. The A-chain of human t-PA has a Mr of 37,000 - 40,000 with carbohydrate content differences or slight proteolytic modification at the N-terminal residues probably accounting for the small degree of heterogeneity (Wallen et al., 1983; Pennica et al., 1983). Whereas the A-chain of human u-PA contains one triple-loop kringle structure, the A-chain of human t-PA contains two triple loop kringle structures similar to those found in urokinase, plasminogen and prothrombin. Closer to the N-terminus, t-PA, like u-PA, contains a cysteine-rich domain like that found in blood coagulation factors IX and X and epidermal growth factor (Banyai et al., 1983; Pennica et al., 1983). However, controversy still persists as to whether the fibrin and lysine binding properties of t-PA reside in one or both kingles since these same properties are also exhibited by plasminogen, where they reside in the kringle structures (Banyai et al., 1983). Unlike u-PA, the one and two chain forms of t-PA and plasminogen both bind to Sepharose-bound L-lysine, arginine and to fibrin. Comparison of the four lysine-binding kringles (Reich 1978b) of plasminogen with the t-PA kringles, however failed to explain the lack of lysine or

fibrin-binding properties exhibited by urokinase kringle (Gunzler et al., 1982a; Pennica et al., 1983, Collen 1980).

As for u-PA, t-PA is a highly specific enzyme and plasminogen is the only known protein substrate. However, t-PA differs from u-PA in having very strong affinity for fibrin (Thorsen et al., 1972; Rijken & Collen 1981), an affinity that has even been used for purification of the enzyme by Wallen et al., (1982). Fibrin has also been found to strongly stimulate plasminogen activation by t-PA (Camiolo et al., 1971; Wallen 1978; Hoylaerts et al., 1982; Ranby 1982; Radcliffe 1983; Suenson et al., 1984).

Using Bowes melanoma cells, sufficient specific mRNA was synthesized to allow complete gene cloning of t-PA (Opdenakker et al., 1982; Edlund et al., 1983; Ny et al., 1984; Pennica et al., 1983). Like u-PA, t-PA is found in a number of places and is particularly prominent in the endothelial regions of tissues (Cano et al., 1985).

1.3 ASSAYS AND DETECTION METHODS FOR PLASMINOGEN ACTIVATORS

Because of the findings that u-PA is generally released from producer cells as an inactive proenzyme form requiring conversion to its active counterpart by limited proteolysis involving plasmin (see Chapter 3), methodological approaches in the enzymatic detection and quantitation of u-PA will therefore be highly influenced by the trace amounts of plasmin that are usually present in the plasminogen preparation used in the assay. Detection and quantitation of plasminogen activator activity could be further complicated by the presence of inhibitors of PA and plasmin since even minute amounts of plasmin inhibitors could strongly inhibit the assay when u-PA is present in its proenzyme form.

The increased interest during the past decade in PA involvement in fibrinolysis, tumorigenesis, inflammatory responses and the expression of hormonal regulation has led to a rising interest in the development of sensitive and precise methods for the specific assay of PA. The optimal assay method for PA obviously depends on the purpose for which it is to be used.

ENZYMATIC ASSAYS AND DETECTION METHODS

1.3.1 Plasminogen as Substrate

In these assay methods where plasminogen is used as the substrate, the PA activity is measured either directly by the amount of plasmin molecules formed or indirectly through the enzymatic activity of the plasmin generated, taking advantage of the amplification involved.

By the use of specific inhibitory antibodies against t-PA or u-PA, it is possible to distinguish between t-PA and u-PA since antibodies against the respective PA inhibit activation of plasminogen catalysed by that activator but not that catalysed by the other type (Kucinski et al.. 1968; Aoki 1974; Astedt & Holmberg, 1976; Vetterlein et al., 1979; Mackie et al., 1981; Corasanti et al. 1980; Rijken & Collen, 1981; Dano et al., 1980b; Kaltoft et al., 1982; Berger & Tuttle, 1983; Nielsen et al. 1983; Ossowski & Reich 1983).

Table 1.1 shows the common types of assays used to detect and quantitate PA activity using plasminogen as substrate:-

TABLE 1.1

ASSAY TYPE & GENERAL COMMENTS	REFERENCES
(a) FIBRIN PLATE	
Quantitation possible.	Jespersen & Astrup 1983
Most widely used assay.	Astrup & Mullertz 1952
Very sensitive to t-PA.	Lassen 1953
Detects about 10^{-16} mol u-PA.	Albrechtsen 1957b
Could be time consuming.	Brakman 1967
Quantitation depended on area of a lysis zone in comparison with standard PA preparation.	Haverkate & Brakman 1975
(b) ^{125}I-LABELLED FIBRIN PLATE	
Detects about 10^{-7} mol u-PA.	Larsson et al., 1984
Based on radioactivity release being proportional to concentration of PA.	Unkeless et al., 1973, 1974b
Can be used to demonstrate inactive pro-u-PA.	Barrett et al., 1977
Can be modified to detect both pro-u-PA and u-PA in biological samples containing inhibitors of pro-u-PA.	Skriver et al., 1982
Can detect t-PA as well.	
Assay quantitate total PA (i.e.	

active plus proenzyme).

Not suited for kinetic studies.

Several modifications based

on these method e.g. [^{3}H]

labelled fibrin.

(c) **CASEINOLYTIC**

Casein used as a substrate — Markus et al., 1980

for plasmin. — Kline 1971

Based on absorbance. — Kline & Reddy 1977

Sensitivity 2 x 10^{-15}

mol for u-PA.

Quantitation possible but

measures only total PA.

Has low sensitivity for t-PA.

(d) **SYNTHETIC PLASMIN SUBSTRATES**

Also based on absorbance. — Lottenberg et al., 1981

Quantitation possible. — Friberger 1982

Measured total PA but can be — Coleman & Green 1981

modified using monoclonal

antibodies to quantitate either

pro-u-PA and active u-PA. (See

Chapter 4 & 5).

Specific for u-PA in the absence

of added fibrin.

Synthetic substrate commonly

used are S-2251 (H-D-Val-Leu
-Lys-p-nitroanilide) and
thiobenzyl benzyloxycarbonyl
-L-lysinate (ZLS).
Can be used for kinetic and
binding studies.
Sensitivity 2 x 10^{-17} mol u-PA.

(e) **DIRECT PLASMINOGEN CONVERSION**

PA activity estimated directly by
the number of native plasminogen
molecules converted to
2-polypeptide chains plasmin
under reducing conditions.
Measured total PA only and can
used to demonstrate pro-t-PA.
Measurement of u-PA also
possible.
Useful for kinetic sudies and
studies of inhibition of plasminogen
activation.

Reddy & Markus 1972
Dano & Reich 1975, 1979
Eaton & Baker 1983
Lucas et al., 1983
Suenson et al., 1984
Mussoni et al., 1984

(f) **FIBRIN OVERLAY**

Distinction amount different types
of PA can be obtained by use of
specific inhibitory antibodies.
Quantitation not possible.

Rijken et al., 1980
Ljunger et al., 1983
Soreq & Miskin 1981
Smith et al., 1985

Assay influenced by inhibitors of PA and plasmin.
Can be used to detect proenzyme.
Several modifications based on this assay. Inferior to immuno-assay when used to detect PA in tissues.

(g) **ZYMOGRAPHIC DETECTION OF PA**

Can differentiate types of PA.
Assay based on diffusion of PA into gel containing plasminogen and fibrin leading to lysis zone.
However, assay semi-quantitative.
Effect of protease inhibitors minimal.
10^{-14} mol of u-PA & t-PA detectable.
Modification of this method with ^{125}-labelled casein which detects about 10^{-18} mol u-PA.
Can be modified to detect and demonstrate PA inhibitors and proenzyme forms of PA particularly u-PA.

Granelli-Piperno & Reich 1978
Dano et al., 1980b
Miskin & Soreq 1981b
Erickson et al., 1984

1.3.2 Enzymatic Assays with Synthetic PA Substrates

Although plasminogen is the only well-documented protein substrate for plasminogen activators, the ability of the enzymes to hydrolyze amide and ester derivatives of arginine and lysine in certain small peptides is being used in some assay methods.

The conversion of N-acetyl-L-lysine methyl ester into N-acetyl-L-lysine and methanol is one example. The methanol generated is then oxidized to formaldehyde which when mixed with chromotropic acid can be quantitated spectrophotometrically (White & Barlow 1970). In amidolytic assays, tripeptides derivatized with p-nitroaniline at the C-terminal amino acid (Friedman et al., 1977; Claeson et al., 1978; Lottenberg et al., 1981; Friberger 1982) are commonly used. Hydrolysis of the p-nitroanilide bond with the release of p-nitroaniline can then be measured spectrophotometrically.

In particular, such assays have been used to measure the conversion of proactivators to the active enzymes (Wun et al., 1982b; Andreasen et al., 1984) but their use in biological samples is limited by their non-specificity in cleavage by proteases other than PA.

1.3.3 Active-Site Titration

Active-site titration assays can only be used for the analysis of purified preparations of PA since the active-site titrants are not specific for PA. Rijken and Collen (1981) used incorporation of [^{3}H] DFP, an irreversible inhibitor of the active site of serine proteases as a measure of the numbers of active sites in the preparation of PA. Similarly, Wallen et al., (1981) and Andreasen et al., (1984) used [^{3}H] DFP

incorporation, together with SDS-polyacrylamide gel electrophoresis to quantitate the relative amouns of one and two chain forms of human t-PA in preparations containing both; while Someno et al., (1982) used a combination of the inhibitor and chromatography to quantitate the relative amouns of low and high Mr u-PA.

1.3.4 Immunological Assay and Detection Methods

The specificity of antigen-antibody interaction, coupled with the recent production of monoclonal antibodies (McAbs) against both types of human PA and plasmin (Kaltoft et al., 1982; Herion et al., 1981, 1983; Nielsen et al., 1983, Vetterlein & Calton, 1983; Salerno et al., 1984, and Chapter 3) is likely to enhance the specificity of assays employing antibodies, provided that inhibitors of PA do not compete with antibodies for binding.

Immunological assays may be superior since enzymatic assays for PA can often be prejudiced by the presence of interfering inhibitors of proteolytic enzymes in many biological fluids. These may interfere through direct inhibition of PA or through inhibition of the plasmin formed indirectly in coupled plasminogen activation-plasmin assays. Furthermore inhibitors of plasmin complicate the measurement of proenzymes due to interference with the plamin catalysed activation of proenzymes.

Commonly used immunological assays using both polyclonal and monoclonal antisera against PA are:-

(i) Radioimmunoassays (Astedt et al., 1975, 1981; Vetterlein et al., 1980, Urden & Blomback 1984, Wun et al., 1982a; Huber et al., 1984) with a detection limit of 10^{-15} mol for both human u-PA and t-PA.

(ii) Two-Site Immunoradiometric Assay (Holmberg et al., 1982; Rijken et al., 1983) Sensitivity 2 x 10^{-15} mol for both u-PA and t-PA.

(iii) Enzyme-Linked Immunosorbent Assay (ELISA) - Sensitivity 2 x 10^{-15} mol for both human u-PA and t-PA. (Bergsdorg et al., 1983, Rijken et al., 1984, Matsuo et al., 1983, Herion et al. 1983.)

(iv) Immunocytochemical Detection Although not really an "assay", nevertheless, the recent availability of strong and specific antibodies (polyclonal and monclonal) have seen their use in the localisation of murine u-PA and human u-PA and t-PA in tissues by immunofluorescence and peroxidase-antiperoxidase staining (Dano et al., 1982, Markus et al., 1983, Kristensen et al., 1984, 1985; Larsson et al., 1984; Nakamura et al., 1984; Salerno et al., 1984.)

(v) Immunoblotting This technique employed both SDS-PAGE and the transfer of separated proteins onto nitrocellulose paper followed by detection with antibodies for specific proteins. This has been used as a screening method for McAb production against t-PA (Nielsen et al., 1983), as a chain specificity assay of McAb against u-PA (Solerno et al., 1984) and for distinguishing between one-chain pro-u-PA and two chain active u-PA in impure biological products (Kielberg et al., 1985; Skriver et al., 1984).

1.4 OCCURRENCE AND FUNCTION OF u-PA IN NON-NEOPLASTIC CONDITIONS

The correlation of in vivo and in vitro studies of inflammatory processes and normal invasive or remodelling events, whether regulated temporally or/and hormonally have suggested the functional significance and occurrence of PA in non-neoplastic conditions and likely involvement of u-PA in extra-cellular proteolysis. In all the biological processes, the event and the associated extracellular proteolysis and tissue matrix degradation have been found to be coordinately controlled.

1.4.1 TISSUE INVASION AND DEGRADATION IN THE NORMAL ORGANISM

1.4.1.1 Ovulation

The ovum contained in the ovary follicle is enmeshed in several layers of cells and basement membrane. The presence of plasminogen in the follicular fluid and the temporal correlation between PA production and disruption in ovulation provided unambiguous evidence that PA is involved in the tissue degradation of the follicle wall (Strickland 1978; Beers et al., 1975; Strickland & Beers 1976; Beers 1975).

Granulosa cells when stimulated with gonadotrophins in vivo and in vitro at physiological levels that induce ovulation have been shown to produce significant levels of PA. It is postulated that the plasmin generated by the activation of plasminogen present in the follicular fluid by gonadotropin-induced PA disrupts the follicular wall and digests, with the help of other proteases like collagenase, the extracellular matrix leading to the eventual release of the ovum (Rohrlich & Rifkin 1979).

1.4.1.2 Implantation

Implantation of the fertilized egg into the uterine wall involves the invasion of the trophoblast through the uterine epithelium and the underlying basement membrane and into the stromal tissue of the endometrium (Kirby 1965, Schlafke & Enders 1975). The work of Strickland et al., (1976) with the in vitro pre-implantation of mouse blastocysts was later confirmed by Sherman (1980) and Kubo et al., (1981), who also found that the trophoblast cells expressed cell-associated PA as well as secreting the enzyme.

This activity was detectable only from days 6-10, during which time the mouse trophoblast was invasive in vivo, and was unrelated to the emergence of the embryo from the zona pellucida.

1.4.1.3 Mammary Gland Involution

Involution of the mammary glands after cessation of lactation is a classic example of tissue remodelling. The findings of Ossowski et al., (1979) indicated a positive temporal correlation between the onset of involution and the concomitant increase in the level of PA secretion by glandular epithelium. Various hormones such as hydrocortisone, aldosterone, prolactin and/or oxytocin which prevented the postlactational involution in vivo, also prevented PA increase in the mammary gland tissue. Similarly, factors such as epidermal growth factor or insulin which promoted glandular degeneration in organ cultures of mammary gland fragments induced high PA secretion especially after prolactin potentiation. U-PA but not t-PA was detected in the extracts of involuting mammary gland. Recent immunocytochemical studies by Larsson et al., (1984) confirmed previous indirect evidence that the u-PA was produced by the epithelial cells and also the lack of immuno reactive staining in the non-involuting glands.

1.4.1.4 Inflammation

The possible role(s) of PA and plasmin in inflammation has been of longstanding interest. Macrophages are derived from precursors in the bone marrow and circulate in the blood as monocytes and ultimately take up specific locations in tissues as macrophages. In sites of inflammation, these macrophages can become 'activated' to kill micro-organisms and tumour cells. However, in chronic inflammatory lesions, these cytocidal activities can be directed against the host to mediate tissue injury. Both in vivo and in vitro studies of the response of macrophages and polymorphonuclear leukocytes to inflammatory and inhibitory stimuli suggest the some effects of these agents influence the ability of the phagocytic cells to regulate the synthesis and secretion of plasminogen activators, collagenase and elastase. The findings reported by Unkeless et al., (1974a) and Gordon et al., (1974) that unstimulated murine macrophages produced PA of barely detectable levels, while cultured thioglycollate-stimulated murine macrophages produced and secreted PA of 10 times the resting amount, had renewed interest in the role of proteolytic proteases in inflammation. Since then, a number of agents have been found to either stimulate or inhibit the production/secretion of PA in macrophages. Exposure of macrophages to lymphokines (Klimetzek & Sorg 1977; Nogueira et al., 1977; Vassalli & Reich 1977; Gordon 1978; Gordon & Cohn 1978; Gordon et al., 1978; Greinder et al., 1979), asbestos (Hamilton et al., 1976), interferon (Hovi et al., 1981), colony-stimulating factor derived from cultured murine cells (Lin & Gordon 1979), Concanavalin A, and phorbol myristate acetate, a potent irritant, inflammatory agent and tumour promoter (Vassalli et al., 1977; Neumann & Sorg 1983) have all shown ability to stimulate the production of PA several-fold. PA synthesis and secretion by stimulated macrophages were inhibited and suppressed by physiological levels of anti-inflammatory steroids,

glucocorticoids, pharmacological agents like colchicine, vincristine and compounds affecting cyclic nucleotide metabolism and cholera toxins (Hamilton et al., 1976; Vassalli et al., 1976, 1977, 1980; Neumann & Sorg 1983). Similar findings have been reported for polymorphonuclear leukocytes, with respect to PA stimulation by concanavalin A and phorbol myristate acetate and inhibition by glucocorticoids (Granelli-Piperno et al., 1977).

All these observations pointed to the physiological importance of PA and led to the proposal that PA plays a role in inflammation and, in particular, in the migration of inflammatory cells to the sites of inflammation as well as subsequent plasmin formation by macrophages in the recruitment phase of the inflammatory response in vivo (Reich 1978a).

Since inflammation often involves degradation of the injured tissue, the u-PA released by macrophages and polymorphonuclear leukocytes may contribute to this process, with the help of other proteases, by extracellular degradation of proteins in a manner parallel to that proposed for cancer cells (see Section 1.5.2 in this Chapter). Hence, it should be noted that the secretion of elastase, collagenase and other serine proteases by stimulated macrophages and not by unstimulated macrophages have also been well documented (Unkeless et al., 1974a; Werb & Gordon 1975a,b; Werb et al., 1980; Wahl et al., 1974, 1975).

1.4.2 PLASMINOGEN ACTIVATOR IN NON-NEOPLASTIC PATHOLOGICAL CONDITIONS

The possible implications of proteolytic enzymes, in particular PA and plasmin have been briefly outline in Section 1.4.1 of this Chapter. Other non-neoplastic

pathological conditions in which PA has been implicated include the following, some of which involve inflammation and/or tissue degradation:-

Allergic vasculitis (Toki et al., 1982), Xeroderma pigmentosum (Miskin & Ben Ishai, 1981), rheumatoid arthritis (Berger, 1977; Meats et al., 1980 Hamilton & Slywka 1981; Hamilton et al., 1982), pemphigus (Hashimoto et al., 1982; 1984), experimental murine leprosy (Izaki et al., 1983), chronic inflammatory bowel disease (Doe & Dorsman, 1982; see Chapter 5), corneal ulceration (Berman et al., 1980), diabetes mellitus (Almer et al., 1975), demyelinating diseases (Cammer et al., 1978; 1981), chronic sinusitis (Kosugi et al., 1982) and protein-losing gastroenteropathy (Kondo et al., 1976).

1.5 OCCURRENCE AND FUNCTION OF PLASMINOGEN ACTIVATOR IN NEOPLASIA

Neoplastic cells display a diverse range of biochemical and physiological changes in comparison to their normal counterparts. Invasiveness and metastatic potentials are two features commonly associated with neoplastic cells. Destruction of the surrounding tissues and extracellular matrix that are normally 'cemented' together, by lytic enzymes, has been proposed to account for the invasiveness of tumour cells. One such lytic enzyme proposed is PA. It should, however, be stressed that in the following discussion, the attribution of definitive roles to PA and plasmin in various aspects of neoplasia/malignancy in NO WAY implies that these functions are specific to cancer-related events. Hydrolysis of the elaborated extracellular matrix that normally would serve as a barrier to the movement of tumour cells, would presumably allow migration of these cells and hence invasion. This view has been supported with ultrastructural evidence obtained from in vivo studies (Franks 1973) and immunocytochemical studies

(Markus et al., 1984; Camiolo et al., 1984; Skriver et al., 1984) where intensive staining and hence localisation of PA (u-PA) have been observed in areas with invasive growth and degradation of normal tussue. By contrast, no demonstrable staining has been observed in the larger parts of tumours where no invasion and degradation of normal tissue were occuring.

Early demonstration of PA in intact tumours using fibrin overlay methods most probably measured t-PA since fibrin stimulates t-PA activity and furthermore, no distinction was made between u-PA and t-PA. Recently, the production of high affinity specific antibodies has enabled the visualisation of the occurrence and distribution of u-PA in human colon adenocarcinomas and melanoma cells (Markus et al., 1983; 1984) and in transplantable murine Lewis lung tumours (Skriver et al., 1984). In the study by Skriver et al., (1984), these authors also noted that some immunoreactivity was localized in the cytoplasm of the tumour cells, often with a perinuclear localization. However, most of the immunoreactivity appeared to be localised extracellularly or near to the cell membrane.

To date, the occurrence of u-PA in benign tumours and of t-PA in malignant and benign tumours in immunocytochemical studies has yet to be demonstrated.

Extractable u-PA enzymatic activity has also been reported in human lung, colon and breast cancer (Corasanti et al., 1980; Markus et al., 1980; Evers et al., 1982), aspirates from patients with endometrial carcinomas (Niklasson et al., 1981), virus-induced mammary tumours and chemically induced rat mammary tumours (Mira-y-Lopez et al., 1983). PA of non-determined type has also been demonstrated for other types of tumours other than those mentioned above.

So far we have mentioned the occurrence of PA in intact tumours and tumour extract. PA has also been reported to occur in explants and cell cultures of neoplastic origin. PA of the urokinase type has been found in cultured cells from an ovarian carcinoma (Astedt & Holmberg 1976), in conditioned medium of tissue cultures derived from endometrial carcinomas (Astedt et al., 1978; Svanberg & Astedt 1979), mouse and rat mammary tumours (Mira-y-Lopez et al., 1983) and a number of cells lines of neoplastic origin (Hisazumi et al., 1977; Wu et al., 1977; Dano et al., 1980a, 1982; Naito et al., 1980, 1982; Rossman & Troll, 1980; Wilson et al., 1980, 1982, 1983; Harvey et al., 1982; Shyamala & Dickerman 1982; Strickland et al., 1983; Azzarone et al., 1983; see also Chapter 3).

1.5.1 Plasminogen Activator and Transformed Cells

Cellular transformation that occurs spontaneously in culture (Sanford et al., 1954), or by infection with oncogenic viruses (Termin & Rubin, 1958) or carcinogenic chemicals (Chen & Heidelberger, 1969) is believed to be closely related to malignancy in vitro.

PA has been shown to be elevated in primary turmour cells, cells transformed by both oncogenic viruses and chemical carcinogens and in a number of turmorigenic cell lines (Unkeless et al., 1974a, 1973; Ossowski et al., 1973; Rifkin et al., 1974; Goldberg 1974; Pollack et al., 1975). While there are a number of exceptions (Gallimore et al., 1977; Jones et al., 1975; Rifkin & Pollack 1977; Rosenthal et al., 1978; Wolf & Goldberg 1976), there is strong evidence in many cases that PA production often accompanies or precedes malignant transformation. This relationship has been most thoroughly studied in Rous sarcoma viruses (RSV), where malignant transformation of chicken embryo fibroblasts by the virus and with

temperature sensitive mutants, has clearly indicated a direct correlation between transformation and enhanced PA production (Unkeless et al., 1973; Rifkin et al., 1975; Quigley 1979). Studies with inhibitors of macromolecular synthesis indicated that induction of PA synthesis by RSV required both RNA and protein synthesis (Unkeless et al., 1973; Rifkin et al., 1975; Rifkin 1980).

Elevation of PA to very significant levels has also been reported in primary cultures of cells transformed by oncogenic DNA viruses e.g. simian virus (Ossowski et al., 1973; Christman & Acs 1974; Quigley et al., 1974), primary cultures of tumours cells, cells transformed by carcinogens, chemically induced tumours and in numerous cell lines, including tumour cell lines of human origin (Christman et al., 1977; Reich 1978a; Quigley 1979; Goldfard, 1982). By contrast, cytocidal DNA or RNA viruses and non-transforming avian leukosis viruses have no PA enhancing effect (Quigley 1979).

1.5.2 Functions of Plasminogen Activators in Neoplasia

The invasion and/or destruction of surrounding normal tissues that often characterised invasive growth of malignant tumours has always implicated the production and release of proteases responsible for the local and rapid hydrolysis of the extracellular matrix proteins (see Section 1.4). The availability of a large and widely distributed substrate for PA in the form of the zymogen plasminogen, and the finding that virus-induced transformation of embryonic chick fibroblasts was closely correlated with the synthesis of u-PA made it highly conceivable that the plasmin generated by the proteolytic cleavage of plasminogen by tumour-associated PA, together with other proteolytic enzymes, could play a major role in the breakdown of the complex extracellular matrices. As tissue degradation could play an important role in the invasive property of tumours and hence may be part of the process of metastasis (Fidler

et al., 1978), the above speculation could be extended to postulate a role for PA in invasion and metastasis.

Indeed, there is a large body of experimental evidence to support the above speculation/hypothesis:-

(a) Purified laminin, a major glycoprotein of the basement membrane has been shown to be susceptible to degradation by plasmin (Liotta et al., 1981a). Bogenmann & Jones (1983) have demonstrated a role for plasminogen in the degradation by tumour cells of an extracellular matrix elaborated in vitro.

(b) The ability of tumour promoters to induce plasminogen activators in cell culture (Wigler & Weinstein 1976).

(c) The findings that solid tumours contained significantly higher levels of PA (urokinase type) than their normal autologous tissues (See Section 1.5, see also Chapter 4; Corasanti et al., 1980; Evers et al., 1982; Markus et al., 1980).

(d) Transformation of cells by oncogenic DNA and RNA viruses induces a rapid synthesis of PA (See Section 1.5.1). Furthermore, only viruses that are oncogenic for the particular cell line induce PA; others, while they infect the cells, do not affect PA synthesis.

(e) The demonstration of the hormonal regulation of u-PA production resulting in controlled tissue degradation as part of physiological tissue injury events such as ovulation or breast involution (See Section 1.4).

(f) The immunocytochemical findings of intensive staining (u-PA) in areas of invasive growth and tissue degradation in experimental murine tumours. By contrast, no detectable staining was observed in large parts of the tumour where no invasion and degradation of the normal tissues was taking place (Skriver et al., 1984).

(g) The demonstration that the use of specific antibodies against human u-PA activity inhibited lung metastasis but not local growth of a human u-PA producing tumour, HEp-3, transplanted onto the chorioallantoic membrane of chicken embryo.

All the experimental evidence listed above supports a "prima facie" case that PA, in particular u-PA, through the generation of plasmin can play a causal and permissive role in cell invasiveness and metastasis. However, it should be pointed out that the role of other proteases, especially in combination with plasmin in tissue degradation and hence metastasis by invasive malignant cells should also be considered.

Plasmin, produced by the proteolytic activation of plasminogen by plasminogen activator, has been shown to have the ability to degrade laminin and fibronectin, both of which are glycoproteins components associated with the basement membrane (Goldfard 1982; Liotta et al., 1981a,b). In addition, plasmin also activates latent forms of type IV and type V collagenase that play a role in degradation of the collagenous components of the basement membrane (Goldfard 1982; Liotta et al., 1981a). Although native collagens (types I-III) in the extracellular matrix are resistant to plasmin degradation, PA could still play a role in the degradation of these types of collagen via a plasmin-mediated activation of latent collagenases (Stricklin et al., 1977; Werb et al., 1977; Dano et al., 1978; Paranjpe et al., 1980; O'Grady et al.,

1981). The collagenase can then initiate the catalytic cleavage of the collagen molecules, leaving them vulnerable to degradation by plasmin and other proteases (Burleigh 1977).

1.6 ROLE OF THE PLASMIN SYSTEM IN THE INVASIVE AND METASTATIC PROPERTIES OF NEOPLASIA

The association of PA and malignant cells and the role of PA, particularly u-PA in a variety of invasive and degradative biological processes has already been mentioned (See Section 1.5).

The PA activity associated with malignant transformation is dependent upon the interaction of PA, produced by transformed cells and the serum zymogen, plasminogen. Plasmin, the product of the proteolytic activation of plasminogen can be put to use for attacking purposes by cancer cells.

The implication of the plasmin/PA system in the invasive and metastatic properties of neoplasia although circumstantial, is supported by many observations. It is generally considered that dissolution of the fibrin in tumours by the plasmin-PA system enhances the local spreading of tumours. Identifiable fibrin (or fibrinogen) has been found in transplanted rat tumours (Day et al., 1959) and human tumours (Hiramoto et al., 1960; Bale et al., 1960) where it was localised within the tumour mostly in macroscopically identifiable clots as well as in necrotic areas (Markus 1983). Dvorak et al., (1979) suggested that a fibrin 'wrapping' found around tumour cell suspensions injected into syngeneic guinea pigs provided the angiogenesis factors required for blood vessel growth into the tumour. Yet, the contradictory reports of several studies (Wood 1958; Agostino & Cliffton 1963; Boeryd 1965; Brown 1973;

Grossi et al., 1960; Kodama & Tanaka 1978a) have also indicated that the formation of microthrombus was necessary for the successful lodgement of tumours and hence metastatic foci. Kodama & Tanaka (1978b) also reported that degradation of fibrin layers surrounding tumours by plasmin was necessary for migration of tumour cells into neighbouring areas and it has also been shown in numerous studies that primary tumours contained a significantly higher levels of u-PA activity compared to adjacent normal tissues. Such release of PA by cancer cells in circulation would tend to degrade the peripheral microthrombus deemed necessary for their arrest and subsequent invasion through the capillary wall. Following this hypothesis, then, metastatic cells should possess lower fibrinolytic potential than primary tumour cells and such findings had been reported (Markus et al., 1983).

Other functions thought to be mediated through the PA-plasmin system in the invasive and metastatic properties of neoplasia included the activation of latent collagenase, which could then degrade the collagen in the basement membrane (O'Grady et al., 1981; Paranjpe et al., 1980), degradation of laminin, a glycoprotein component in basement membrane and the release of cells from the primary tumour as the result of breakdown of cohesion between tumour cells.

1.7 SUBCELLULAR DISTRIBUTION OF PA

Defining the subcellular localisation of PA is of fundamental importance to the understanding of the many roles of the enzymes, in particular in the control of its production and release which may be regulated by some extracellular signal. In general, PA exists in a cell-associated form in addition to a soluble, extracellular form that is released into cell culture medium and body fluids.

The localisation of PA in the cells has been done mainly using cell homogenates separated by differential and gradient centrifugation and assay of the separated fractions with a series of marker enzymes specific for different subcellular organelles and PA activity. Early studies showed that the PA was localized in two particulate subcellular fractions:-

the lysosomal-rich fraction and the microsome fraction (Ali & Lack 1965). However, more recent studies since than have all indicated that PA is associated with membrane-rich fractions, rather than the cytosol fraction. Such fractions included plasma membrane-like and, in few cases, lysosomes and Golgi elements of the cell (Unkeless et al., 1974b; Quigley 1976; Loskutoff & Edgington 1977; Dvorak et al., 1978; Solomon et al., 1980; Fulton & Hart, 1981; O'Donnel-Tormey & Quigley 1981; Zisapel et al., 1982; Laug et al., 1983a,b; Lemaire et al., 1983).

Immunocytochemical studies at electron microsopic level by Paul et al., (1979) also indicated a plasma membrane association for PA in a wide variety of cells, but a recent immunofluorescence study (Dano et al., 1982) suggested that PA might be found in intracellular granules contaminating membrane fractions (Golgi ??). In tissue sections of murine Lewis long tumour, Skriver et al., (1984) demonstrated unevenly distributed intracellular staining in the cytoplasm with the highest staining at the perinuclear region.

Thus, in summary, most studies suggest that PA is closely associated with plasma membrane or Golgi membrane-like cellular elements. All recent studies concur that PA is not found in the lysosomal fractions. However, it should be noted that at the present moment, it has not been possible to determine if PA is an intergral membrane protein, or a secretory protein inside intramembrane granules on their way from their

intracellular site of synthesis to the extracellular space. Alternatively, the cell fractionation results could be simply artifactual due to the receptor-mediated or hydrophobic interaction of UK with plasma membrane subsequent to cell rupture, and not representative of the intracellular distribution in living cells.

The presence of membrane receptors fro the human u-PA or its amino acid fragment (residues 1-135) has been recently demonstrated on human blood monocytes, cells of the monocyte line U937 and on normal and RSV mouse fibroblasts (Vassalli et al., 1985; Stoppelle et al., 1985; Rosso et al., 1985).

1.8 SIGNIFICANCE AND AIMS

The PA system provides the means for correlation between the 2 essential features of malignancy namely (i) invasion (ii) metastasis.

The question therefore arises as to whether the transformation of human benign into neoplastic cells involves the expression of the PA enzymes since virus-transformed murine cells of neoplastic origin had been shown to produce and secrete u-PA.

The colonic polyp-cancer sequence and the inherited disorder multiple polyposis coli thus offer suitable models which can be exploited to address this question.

Because of the implication of PA in the mediation of tissue injury, it was also important to determine whether this enzyme also initiates inflammatory tissue injury. Inflammatory cells are recuited to sites of inflammation by a variety of factors including lymphokines and the complement fragment C5a (Hugli & Muller-Eberhard, 1978). A crucial step in the migration of these inflammatory cells is believed to be mediated by plasmin, which is the end product of a cascade of reactions mediated by PA. The plasmin beside mediating the migration of inflammatory cells, also lyses fibrin to release

biologically-active fibrin degradation products and also activates complement and kinin generation and initiation of the coagualtion cascade which all contribute to tissue injury. The influx of the inflammatory cells also assist in tissue injury by releasing degradative and hydrolytic enzymes as well as generating free oxygen radicals.

The inflammatory disorders of colon offered the opportunity to answer this question.

Several studies (see chapter 1) using in vitro cell cultures have shown the release of the proenzyme form of PA into the medium and implicated plasmin as a mediator involved in the proteolytic activation of the proenzyme into its active form.

The specific aims of this project were therefore to :-

(1) Develop a quantitative assay for the pro and active forms of HPA52 by raising monoclonal antibodies inhibitory to human plasmin and hence study the form of HPA52 in the extracellular environment.

(2) To explore the colonic polyp-cancer sequence and the inherited condition of polyposis coli to determine whether the expression of HPA52 is a correlate of malignant transformation.

(3) To determine the role of HPA52 in the mucosal tissue injury that characterises lesion of inflammatory bowel disease.

(4) To study intestinal macrophages as a possible source of HPA52 and its subcellular localization.

CHAPTER 2

MATERIALS AND METHODS

Unless otherwise stated, all chemicals and reagents were obtained from the best commercially grade available either from Sigma Chemical Co; Aldrich or BDH Chemicals. All solvents and acids/akaline were obtained from BDH Chemicals. All tissue culture media were from either Flow or Gibco Laboratories. All other methylumbelliferyl substrates were from Koch-Light or Sigma Chemical Co.

$Na^{125}I$, [2-^{3}H]AMP, [7-^{14}C]-tyramine, [carbonyl-^{14}C]NAD and UDP[6-^{3}H] galactose were from Amersham. Protein A, CN-Br activated Sepharose 4B, Sephadex G25, Lysine-Sepharose, Percoll and Ficoll-hypaque were all from Pharmacia.

A Perkin-Almer 500 Spectrophotometer was used to measure fluorescence.

2.1 ENZYMATIC ASSAYS

2.1.1 5'-Nucleotidase

5'-Nucleotidase was assayed by the method of Avruch and Wallach (1971) as implemented by Edelson and Cohn (1976). 100ul of gradient fraction was added to 500ul of 54 mM Tris-HCl pH 9.0/12 mM $MgCl_2$ containing 0.15 umoles 5' adenosine monophosphate and 10^5 dpm adenosine-[2-^{3}H]-5' monophosphate (ammonium salt). After incubation for 2 hr at 37^{o} C, the reaction was stopped by the addition of 0.2 ml of 0.25 M zinc sulphate and mixed. Then 0.2 ml of 0.25 M barium hydroxide was added, vortexed and spun at 600 g for 10 min. An aliquot (400 ul) of the supernatant was removed and counted in 5 ml of toluene scintillation fluid. Control samples contained gradient fractions with 0.2 ml of 0.25 M zinc sulphate added at zero time.

2.1.2 Mn^{2+}-Stimulated Leucine 2-Naphthylamidase

This enzyme was assayed by addition of 10-100 ul gradient fraction to 100 ul buffer (0.1M Hepes pH 7.0, 0.1% Triton X-100) with a final concentration of 1 mM $MnCl_2$ and 0.2 mM L-leucine 2-naphthylamide (Sigma Chemical Co.). After incubation at 37^0 C for 1 hr, the reaction was terminated by the addition of 2 ml 50 mM NaOH/glycine pH 10.4 containing 5 mM EDTA. The fluorescent product, 2-naphthylamide, was detected at 410 nm using an excitation wavelength of 340 nm.

2.1.3 NAD^+ Nucleosidase

NAD^+ nucleosidase was assayed by a modification of the method of Nakazawa et al., (1968). The reaction mixture contained 10 ul gradient fraction plus 1.6 nmol [carbonyl-^{14}C] NAD (5 nCi) in 15 ul 0.2 M Tris-HCl pH 8.0 containing 60 mM $MgCl_2$. After 60-90 min at 37^o C, the tubes were placed on ice and 2 ul of the reaction mix was spotted onto Whatman no:1 paper (Bioscientific Supplies) together with 1 ul of a 100 mM nicotinamide solution. Chromatrography was carried out with a solvent of 1 M ammonium acetate pH 5.0/ethanol (3:7). The nicotinamide spots were detected under ultraviolet light and cut out for liquid scintillation counting of [^{14}C] nicotinamide.

2.1.4 Alpha-naphthyl Acetate Esterase

This enzyme was determined at pH 7.5 by the method of Laug et al., (1983b) where 50-100 ul gradient fraction were added to 400 ul buffer (67 mM sodium phsophate pH 7.5, 0.01% Triton X-100) containing 1 mM alpha-naphthyl acetate (Sigma Chemical Co.) in final concentration. After incubation at 37^o C for 45 min, the

reaction was stopped by the addition of 1 ml 0.1 M sodium acetate buffer pH 4.5/0.1% Triton X-100 containing 10 mM mercury chloride and 4 mg Fast Red ITR (Sigma Chemical Co.) in final concentration. After 15 min, the absorbance was read at 550 nm.

2.1.5 Catalase

This was detected by a fluorimetric assay of Fayle et al., (1985). Gradient fraction (50 ul) was added to 100 ul buffer (0.1 M Hepes pH 7.0, 0.1% Triton X-100) containing 150 ul hydrogen peroxide, and incubated at room temperature for 20 min. Oxidation of the fluorescent compound scopoletin (7-hydroxy-6-methoxy-coumarin) (Sigma Chemical Co.) by hydrogen peroxide in the presence of horseradish peroxidase (Boehringer) was used to detect remaining hydrogen peroxide. 0.8 ml Hepes buffer containing 15 uM scopoletin and 80 ug horseradish peroxidase was added, and the fluorescence was measured at 460 nm using an excitation wavelength of 350 nm.

2.1.6 Monoamine Oxidase

Deamination of [^{14}C]tyramine was used to detect this enzyme (Summers & Hume, personal communication). 100 ul of gradient fraction was added to 100 ul of 10 mM sodium phosphate pH 7.0, 0.15 M NaCl containing 0.2 mM tyramine (Sigma Chemical Co.) and 2 uCi[^{14}C]tyramine. After incubation for 1-2 hr at 37^{o} C, the reaction was stopped by the addition of 0.2 ml 2 M citric acid. 1 ml of benzene/ethyl acetate (1:1) was added, and the tubes mixed vigorously on a vortex mixer (Scientific Instruments). An aliquot (600 ul) of the upper organic phase (containing p-hydroxyl [^{14}C] phenylacetaldehyde) was counted in 5 ml of scintillation fluid (0.5% 2,5-diphenyloxazole in xylene/Triton X-114, 2:1). Control samples contained gradient fraction, with citric acid added at zero time.

2.1.7 Arylsulfatase C

The method of Canonico et al., (1978), using a buffer stock to give 0.15 M Hepes pH 7.5, 0.13 M Na_2SO_4 and 0.13 M NaCl, with the addition of 0.1% Triton X-100 was used to detect arylsulfatase C. 25 ul of 100 mM barium chloride and 125 ul of buffer stock was added to 100 ul gradient fraction and allowed to incubate for 10 min. The Ba^{2+} and SO^{2-} and Cl^{-} inhibited sulphatase A and B respectively. After incubation, 10 ul of 0.38 mM methyl-umbelliferyl sulphate in final concentration was added and incubation continued for a further 1 hr at 37° C. Reaction was terminated with 2 ml 50 mM glycine/NaOH buffer pH 10.4 and the fluorescence was detected at 460 nm using an excitation wavelength of 363 nm.

2.1.8 Galactosyltransferase

Galactosyltransferase was assayed according to Baxter and Durham (1979) but in a buffer of 0.1 M Hepes/NaOH pH 7.0 containing 0.03% Triton X-100, 50 mM $MnCl_2$, 20mM β-mercaptoethanol (Sigma Chemical Co.) and with ovomucoid (Sigma Chemical Co; 10 mg/ml buffer) rather than desialo, degalacto-fetuin as acceptor (Jasaitis et al., 1982). 50 ul of buffer containing ovomucoid was added to 35 ul of gradient fraction and incubated for 10 min at 37° C, after which 5 ul of 200 uM UDP-galactose (Sigma Chemical Co.) and 1.2uCi[^{3}H]-UDP galactose was added and incubation continued for a further 60 min at 37° C. The reaction was stopped with 20 ul of 0.8 M EDTA and 45 ul of the reaction mixture was spotted onto a Whatman no:2 filter paper discs, dried and subsequently washed 3 times with 10% trichloroacetic acid at room temperature. The washed and dried paper counts were then used for liquid scintillation counting of [^{14}C] galactose.

2.1.9 Lactate Dehydrogenase

Lactate dehydrogenase was assayed according to the method of Kornberg (1955), using 0.75 mM sodium pyruvate and 0.33 mM NADH (Boehringer). The enzymatic reaction was initiated by the addition of 100 ul sodium pyruvate to a mixture of gradient fraction (100 ul), NADH (100 ul) and 1.2 ml 0.1 M phosphate buffer pH 7.4. Absorbance was measured at 340 nm every 30 seconds for 3 min.

2.1.10 Lysosomal Enzymes

β-glucuronidase was assayed by the fluorimetric method of Mead et al., (1955) using 0.1 M acetate buffer pH 4.5 containing 0.1% Triton X-100.

The β-N-acetyl-D-galactosaminidase activity was measured according to Leaback and Walker (1961) using 4-methyl umbelliferyl-2-acetamido-2-deoxy-β-D galactopyranoside as substrate and 0.5 M citrate pH 4.5/0.1% Triton X-100 buffer. The respective substrate solution (250 ul/30uM) was added to 100 ul gradient fractions and incubated for 1 hr at 37^{o} C. The reaction was terminated by addition of 2 ml cold 0.1 M glycine/NaOH pH 10.4 buffer. The fluorescence was measured at 460 nm using an excitation wavelenght of 363 nm.

2.2. PROTEIN ESTIMATION

Protein concentration was assayed by a modified Lowry method (Peterson 1977). One ml of water was added to standard protein solution, control and samples (50 ul) and mixed, followed by addtion of 100 ul 0.15% deoxycholate (BDH) and allowed to stand for 10 min. Then 100 ul of 72% trichloroacetic acid was added, mixed and

centrifuged for 20 min at 1000 g. The supernatant was carefully decanted and 400 ul of reagent A (comprising equal volume of 10% SDS, 0.8 N NaOH, water and CTC solution [0.2% sodium tartrate, 0.1% copper sulphate, 10% sodium carbonate]). After 10 min, 200 ul of 1:5 dilution of Folin reagent (Ajax Chemicals) was added and the absorbance read at 750 nm after 30 min at room temperature.

2.3 HYBRIDOMA (MONOCLONAL ANTIBODIES) PRODUCTION

Hybridoma production was performed under sterile conditions in a laminar air flow cabinet throughout.

2.3.1 Media

All media were sterilised by filtration through Sterifil Aseptic System (Millipore).

DMEM - This was prepared using a packet of Dulbeccos Modified Eagle Medium (Gibco) with addition of 74 ml of 5% $NaHCO_3$, 984 ml of distilled water and adjusting the media to pH 7.2 with 1.5 ml of 5N HCl.

DMEM + 10% Heat inactivated FCS - One ml of 100mM sodium pyruvate, 125 ul gent (Commonwealth Serum Lab.), 50 ul pen (Glaxo), 50 ul strep (Glaxo), 10 ml heat-inactivated FCS and 1 ml of 200mM glutamine (Sigma Chemical Co.) were added per 100 ml of DMEM.

DMEM + 2 x Gent/Pen/Strep/Fungizone - This was made up in identical manner to that described for DMEM with addition of 250 ul gent, 50 ul pen, 50 ul strep and 1 ml fungizone (Flow).

DMEM-HAT + 10% Heat inactivated FCS - This was prepared as described for DMEM + 10% heat inactivated FCS with addition of 1 ml aminopterin (Lederle Lab. Division), 1 ml thymidine (Sigma Chemical Co.) and 1 ml hypoxanthine (Sigma Chemical Co.).

Aminopterin (methotrexate) 10^{-4} M - Two ml of distilled water were added to a 50 mg commercial vial of methotrexate sodium injection and then 178 ul of this solution was added to 100 ml distilled water. This was then used as described above.

Thymidine - A 1.5 mM thymidine was made up in PBS pH 7.0.

Hypoxanthine - A 1 mM solution of hypoxanthine was made up in distilled water and pH to 11 with 10 M NaOH.

2.3.2 Preparation

(1) An immunized mouse (John Curtin School Animal House; see Chapter 3 for immunization regimen) was boosted 3-4 days before fusion with 60 ug of plasminogen in saline.

(2) Myeloma cells (X63 Ag8.653) that has been growing in mid-log phase at 3-5 x 10^5 cells/ml and were at least 95% viable.

(3) All media were warmed to 37° C on the day of fusion and polyethylene glycol (Koch-light 1540) were placed on 56° C water bath to melt. A solution of 30 ± 2% polyethylene glycol was prepared by adding 1 ml of warmed DMEM containing no FCS to 0.6 g of polyethylene glycol previously melted at 56° C.

2.3.3 Thymocytes

(1) 4-6 thymus glands from 4-8 week old mice were required to give a final suspension of at least 10^6 thymocytes/ml DMEM-HAT.

(2) Thymus glands were removed sterilely into a petri dish (Sterilin) of approximately 10 ml DMEM with 2 x gent/pen-strep/fungizone that had been previously warmed up to 37°C.

(3) In the sterile hood and using sterile technique, the thymus glands were rinsed through 4 more petri dishes of approximately 10 ml DMEM/2 x gent/pen-strep/fungizone and finally minced with scissors before straining through wire mesh into a final dish of medium using the rounded end of a spatula.

(4) The cells were resuspended and transferred to 15 ml sterile centrifuge tube (Sterilin) and centrifuged for 8 min at 300 g in room temperature.

(5) After centrifugation, the thymocytes were resuspended in 5-10 ml HAT and finally added to 250 ml DMEM-HAT (37° C) in 500 ml bottle which was then stored at 37° C (5% CO_2) until needed.

2.3.4 Spleen Cells

(1) The spleen from the immunized mouse was removed sterilely and placed in a petri dish of 5 ml DMEM/2 x gent/pen-strep/fungizone.

(2) The spleen was then transferred to a dish of fresh medium. Any fatty connective tissues were removed from around the organ, taking care to avoid breaking the sac.

(3) The spleen was rinsed through 4-5 more dishes of the same medium.

(4) In a final dish of this medium, spleen cell suspension was prepared by cutting the spleen close to one end with sterile scissors and teasing the cells out of the sac with 2 x 19G needles (Terumo) bent to approximately 90°. The spleen sac was discarded and the extruded cells resuspended and transferred to a 15 ml centrifuge tube.

(5) The cell suspension was allowed to settle for 5 min at room temperature, during which time large clumps settled to the bottom. The supernatant was removed and centrifuged at 300 g, 8 min at room temperature. The supernatant was discarded and the cell pellet resuspended in 10 ml of fresh DMEM/gent/pen-strep. The total cell count was normally 0.5-2 x 10^8 cells.

2.3.5 Myeloma Cells

The myeloma cell line X63 Ag8.653 was used in the fusion and had been maintained in mid-log phase by adjusting the cell count to 2 x 10^5 cells/ml every 2 days.

On the day of fusion, the myeloma cells were resuspended and placed in 50 ml centrifuge tube (Corning). The number of cells per ml and viability were determined, after which the appropriate number of myeloma cells were taken (ratio of 5:1, spleen:myeloma) and spun down at 300 g for 8 min. The cell pellet was then immediately resuspended in 10 ml DMEM/gent/pen-strep containing no FCS.

2.3.6 Spleen and Myeloma (Fusion)

The spleen and myeloma cell suspension (viability greater than 95%) were spun together at 300 g for 8 min and the supernatant removed by aspiration. The cell pellet was resuspended in DMEM/gent/pen-strep and recentrifuged. This process was repeated twice, before aspiration of all liquid from the cell pellet after the last spun. The pellet was loosened by gentle tapping of tube and then placed at 37^{o} C water bath to warm briefly.

One ml of 35% polyethylene glycol was added over a 1 min period, pipetting in a few air bubbles during the last 10 sec slowly to mix. This was then followed immediately by the addition of 1 ml DMEM containing 10% heat-inactivated FCS over 1 min, again mixing evenly, using a few air bubbles to finish. At any stage, pipetting of the cell suspension up and down was avoided. Addition of 1 ml DMEM containing 10% heat-inactivated FCS was repeated , followed by a further 8 ml of the same medium over

the next two minutes. After the addition, the fusion mix was spun at 300 g for 8 min. The supernatant was carefully removed by aspiration and the cell pellet resuspended in 5-10 ml of DMEM-HAT medium containing thymocytes(see 2.3.3). This was then added to 250 ml DMEM-HAT containing thymocytes, mixed gently by swirling the bottle and immediately plated out in 96-well plates (12 plates, 4 drops/well from 10 ml pipette, approximately 22 ml/plate, Flow Laboratories).

2.3.7 Maintenance

The plates were checked routinely for contamination. The fastest growing cells/hybridomas were visible at 5-6 days following fusion. On Day 7 after fusion, the plates were fed with HAT containing thymocytes prepared as in 2.3.3. Feeding was done by aspirating out approximately 100 ul of supernatant from each well and adding 2 drops of HAT containing thymocytes. At 2 1/2 weeks after fusion, the hybridomas were assayed using ELISA as detailed in Section 2.8 of this Chapter. Sometimes, slow-growing clones (usually stable antibody producing hybridomas) were positive only after 5-6 weeks.

2.3.8 Subcloning by Limiting Dilution

One ml of the primary hybridoma culture, harvested in mid-log phase, is adjusted to the concentration of 1×10^5 cells/ml. Ten fold serial dilutions in HAT medium containing thymocytes were prepared to obtain 23 ml of each of the following cell concentrations: 1×10^2/ml and 1×10^1/ml. Each of these cell suspensions was distributed at 100 ul/well in a 96 well tray. In 1-2 weeks, cloned colonies were detectable; according to the Poisson distribution, clones found in trays with less than 37% of clone-containing wells per tray are likely to arise from a single cell. Among the

subclones positive for antibody production, the ones showing better characteristics of growth and rate of antibody secretion were chosen for further subcloning and large scale expansion.

2.3.9 Freezing and Growth of Hybridomas as Ascites Tumours

Hybridomas were frozen in liquid nitrogen (according to standard cell freezing procedures) in 1 ml aliquots at a density of 1×10^7 cells/ml, in 95% heated-inactivated FCS and 5% dimethylsulfoxide (Sigma Chemical Co.).

Mice (Balb/C strain) were injected intraperitoneally with 0.5 ml pristane (2,6,10,14-tetramethylpentadecane, [Sigma Chemical Co.]) and then rested for 10-14 days. Hybridomas cells (0.2 ml, containing 1×10^6 to 1×10^7 cells in saline) were injected intraperitoneally. Ascites fluid was tapped several times after the tumour became palpable.

2.4 COUPLING OF MONOCLONAL ANTIBODIES TO CN-BR ACTIVATED SEPHAROSE 4B

The required amount of freeze-dried cyanogen bromide activated Sepharose 4B powdered (1 g approximately 3.5 ml, and 1 ml bound 5-10 mg protein) was swollen for 15 min in 1 mM HC1. The swollen gel was then washed on a sintered glass filter (porosity G3) with 1 mM HC1 (200 ml/g dry gel) in several aliquots, followed by washing with coupling buffer (0.25 M $NaHCO_3$ pH 8.5/0.5 M NaCl). This was then immediately transferred to a solution of the purified monoclonal antibody which had been previously dialysed against coupling buffer. A gel ratio of 1:2 gave suitable coupling ratio. The protein solution with gel suspension was mixed by rotation at 4^{o} C overnight.

After mixing, the coupling buffer was removed and replaced with 0.2 M glycine buffer pH 8.0 and mixing continued overnight. Following mixing, the gel suspension was transferred to a column and washed with coupling buffer followed by acetate buffer (0.1 M pH 4.0 with 0.5 M NaCl). This step was repeated for a further 2 times and then finally washed with 0.1 M Tris-HCl pH 8.1 containing 0.1% Triton X-100. Anti-plg 1 monoclonal antibody (600 ug) had been coupled in this manner and has been used for immunoaffinity purification of plasminogen as described in Section 2.4 of this Chapter.

2.5 GLUCOSE-OXIDASE COUPLING TO PROTEIN A AND TO F(ab)'$_2$

The method was based on Ternynck and Avrameas (1976). Initiation of the benzoquinone activation of glucose-oxidase (Boehringer Grade 1) involved dissolving 20 mg of glucose-oxidase in phosphate buffered saline pH 7.4 and dialysing against phosphate buffered saline pH 7.4 overnight, after which the dialysed glucose-oxidase was placed in a small tube with 300 ul 1 M phosphate buffer pH 6.0 and 500 ul of p-benzoquinone (Ajax Chemicals) solution (30 mg/ml in ethanol, spun at 8000 g for 25 min, then supernatant used) and shaken at 37^{o} C for 1 hr wrapped in foil. After shaking, it was centrifuged at 8000 g for 20 min and the supernatant (containing benzoquinone-activated glucose oxidase) was placed over Sephadex G25 column in 60 ml syringe (Terumo) containing 35 ml of gel. The protein (brown band) was eluted after the void volume, while the benzoquinone was retarded.

The benzoquinone-activated glucose oxidase eluate was then mixed with 2 mg F(ab)'$_2$ [Cappel Lab.] (previously dialysed overnight against phosphate buffered saline pH 7.4) or 2 mg Protein A (Cappel Lab.) and 200 ul of 1 M $NaHCO_3$ pH 8.8 and reacted for 48 hr at 4^{o} C with shaking. After 48 hr, 1 ml of 1M lysine in phosphage buffered saline pH 7.4 was added to react with remaining unreacted benzoquinone groups and shaking continued for 4 hr at 4^{o} C. Finally, the solution was dialysed against several

changes of phosphate suffered saline pH 7.4 overnight and concentrated to 1-2 ml by drying the dialysis bag in an air stream at room temperature. The final volume was recorded and an equal volume of glycerol (Ajax Chemicals) was added and then stored in several aliquots at -20^{o} C, where it remained unfrozen.

2.6 FIBRIN-PLATE OVERLAY GEL

The fibrin-overlay gel was prepared on a acid-washed glass plate (20 x 20 cm) which was equilibrated at 37^{o} C for about 15 min prior to pouring of the agarose gel mixture. The agarose gel mixture was made up in the following order:- 135 ul of thrombin (0.675 NIH units, Sigma Chemical Co.) was added to 1 ml of plasminogen (400 ug), after which it was mixed with 5 ml of agarose (125 mg in phosphate buffered saline; Sea-Plaque) previously melted at 65^{o} C in a water bath and then equilibrated at 40^{o} C. Finally, a 5 ml aliquot of fibrinogen (30 mg dissolved in 2 M glycine/0.1% Triton X-100 buffer pH 8.3; Sigma Chemical Co.) was added to the agarose mixture and mixed thoroughly before the final mixture was poured onto the centre of the plate and quickly spread evenly between layers of tape 14 cm apart by using a pipette as a roller. The gel (0.3-0.4 mm thick) was covered to prevent drying and allow 30 min at 37^{o} C for the clotting reaction to take place and a further 30 min at 4^{o} C for the agarose to harden.

After electropheresis of the SDS-polyacrylamide gel as described in Section 2.13, the gel slabs were washed in 0.1% Triton X-100 and developed by contact lysis of fibrin in an agarose gel. The development time for the fibrin lysis was usually 5 hr at 37^{o} C. Commercial enzymes and the position of stained standard proteins (Pharmacia HMW Kit) in the acrylamide gel were used to estimate the molecular weight of enzyme lysis bands.

2.7 PLASMINOGEN PURIFICATION

Plasminogen was purified on 2 cycles of lysine Sepharose affinity chromatography based on Deutsh and Mertz (1970).

One litre of plasma containing protease inhibitors (3mM EDTA, 10000 KIU Trasylol [Bayer], 0.3mM p-nitrophenylguanidinobenzoate [NPGB, Sigma Chemical Co.]) was applied to 25 x 3 cm column of lysine-Sepharose which had been previously washed with 0.1 M K_2HPO_4 pH 7.8/3 mM EDTA buffer and then equilibrated with 0.34 M K_2HPO_4 pH 7.8/0.3 M EDTA/0.3 mM NPGB containing 10000 KIU of Trasylol. The column was run at 50 ml/hr. When the plasma had completely run through the column, it was then washed with 0.34 M K_2HPO_4 pH 7.8/3 mM EDTA/0.3 mM NPGB/10000 KIU Trasylol until the OD_{280} was less than 0.3. The column was then eluted with 0.1 M K_2HPO_4 pH 7.8/3 mM EDTA/30 uM NPGB/0.2 EACA buffer and fractions with OD_{280} greater than 0.8 were collected and pooled. The eluate was dialysed against 0.1 M K_2HPO_4 pH 7.8/3 mM EDTA/30 uM NPGB overnight before reapplying to the column which had been previously regenerated with the same buffer used for dialysis and equilibrated with 0.34 M K_2HPO_4 pH 7.8/3 mM EDTA/30 uM NPGB buffer. The column was then eluted with the same elution buffer that had been used previously and fractions with OD_{280} greater than 0.5 were pooled together before dialysing against 50 mM glycine buffer pH 7.8 with several changes overnight. The dialysed plasminogen was then diluted with 50 mM glycine buffer pH 7.8 to give an absorbance 0.7 at 280 nm which is approximately equivalent to 400 ug/ml. It was then stored at -20^{o} C until use.

2.8 ENZYME-LINKED IMMUNOSORBENT ASSAY (ELISA)

Enzyme-linked immunosorbent assay (ELISA) was used as a primary screen for antibodies binding to plasminogen. Affinity-purified human plasminogen (50ng) in 50 ul 0.01 M sodium phosphate buffer pH 7.4 containing 0.15 M NaCl was incubated for 3 hr in each well of a Titertek "activated" immunoassay plate (Flow Laboratories) at 20° C, followed by blocking of non-specific binding sites with 0.2% gelatin (Ajax Chemicals) in 10 mM sodium phosphate buffer pH 7.4 containing 0.15 M NaC1, for 3 hr at 20° C. Hybridoma culture supernatants were then added and the plates incubated at 4° C for 16 hr, followed by washing with phosphate buffered saline pH 7.4/0.05% Tween 20 (Sigma Chemical Co). Each well was then incubated with 30 ul (12 ug) of $F(ab')_2$ - sheep anti-mouse IgG (heavy & light chain; Cappel Lab.) covalently coupled to glucose oxidase. Alternatively, a three-step assay was employed, in which an incubation with 30 ul rabbit anti-mouse IgG+M+A (Cappel) for 3 hr at 20° C was followed by a further incubation for 1 hr at 20° C with 30 ul (12 ug) Protein A (Pharmacia) covalently coupled to glucose oxidase.

In both cases, the wells were washed before application of 100 ul of reaction mixture (0.4 mg/ml o-phenylene diamine [Sigma Chemicals Co.], 20 mg/ml glucose, and 7.5 ug/ml horseradish peroxidase [Boehringer Grade 1] in 0.1 M citrate - 0.2 M phosphate buffer pH 5) in the dark. Colour development was stopped by the addition of 35 ul of 5N H_2SO_4.

2.9 CHARACTERIZATION OF ANTIBODIES (OUCHTERLONY)

The heavy and light chain types of the monoclonal antibodies were determined by Ouchterlony analysis on agarose gel of 50 times-concentrated antibody culture supernatant against commercial antisera specific for mouse IgM, IgA, IgG, subclasses of IgG and κ and λ light chains (Bionectics). Three ml of a 1% purified agar (Oxoid)

solution with 1% NaCl was layered onto a clean glass microscope slide. Approximately 15 ul of the concentrated antibody culture supernatant was added to adjacent, small 2 mm holes punched into the agar layer. The slide was incubated overnight in a humidified atmosphere at room temperature to allow formation of precipitin lines which were only observed when the immunoglobulin present in the concentrated culture supernant was of the correct specificity to allow complexing with the antiserum.

2.10 ISOLATION OF PURE IgG BY PROTEIN A-SEPHAROSE CHROMATOGRAPHY

Staphyloccal protein A, covalently linked to Sepharose CL-4B (Protein A-agarose), [Pharmacia] was washed and equilibrated with 0.1 M Tris-HCl pH 8.1 buffer. A column of bed volume 5 ml was prepared and used at 4^{o} C.

Ascites fluid or ammonium-sulphate concentrate of tissue culture supernatant was adjusted to pH 8.0 and applied to the column. The column was then washed with 0.1 M Tris-HCl buffer pH 8.1 until all unbound proteins were eluted. IgG was eluted with 0.1 M sodium acetate buffer pH 4.0 containing 0.15 M NaC1 at a flow rate of 12 ml/hr. Fractions of 1 ml were collected into tubes and neutralised with 600 ul of 1 M Tris-HC1 pH 9.0. Mouse IgG of all four subclasses was prepared using this technique. The IgG fractions were then pooled and dialysed overnight against 50 mM glycine pH 7.8 and used in all the experiments as described.

2.11 BINDING STUDIES

Plasminogen was dissolved to a concentration of 15 ug/ml in 10 mM sodium phosphate buffer, pH 8.0, containing 0.15 M NaCl and 100 ul (1.5 ug) of the dissolved

plasminogen was incubated for 4 hr at 20° C in each well of a Titertek "activated" immunoassay plate (Flow Laboratories). Non specific binding sites were blocked with 250 ul of a 1% BSA solution, in the same buffer, for 3 hr at 20° C. Various concentrations of the ^{125}I-labelled antibody, dissolved in 200 ul of 10 mM sodium phosphate buffer pH 8.0, containing 0.15 M NaCl were added to the coated wells and allowed to incubate at 37° C for 2 hr, followed by several washings with a 1% (w/v) solution of BSA. After the final wash, the wells were individually placed in test tubes and counted in a Packard Auto 500 gamma counter. These counts were then subtracted from controls in which the same concentration of ^{125}I-labelled antibody was incubated in the wells of a plate fully coated with bovine serum albumin. In the wells precoated with BSA, no more than 1% of the counts were found in any of the sample wells.

2.12 COLORIMETRIC ASSAY (YELLOW ASSAY)

Human plasmin was generated by incubating human urokinase (20 ul, containing 4 mPU; Sigma Chemical Co.), human plasminogen (20 ul containing 2 ug) and assay buffer (20 ul) for 45 min at 37° C. The assay buffer consisted of 50 mM glycine pH 7.8 containing 0.1% Triton X-100 and 0.1% gelatin. The reaction was stopped by the addition of 2.0 M KC1 (20 ul) so that continued activation of plasminogen to plasmin was inhibited by the high salt concentration. Either glycine buffer (for controls) or purified monoclonal antibodies (20 ul, 12 ug) that had been extensively dialysed against 50 mM glycine buffer pH 7.8 were then added and incubated for 1 hr at 20° C. The residual plasmin activity was then measured by the thioesterase colorimetric method of Coleman and Green (1981). Plasmin assay reagent (1 ml) containing 0.2 M phosphate (pH 7.5), 0.2 M KC1, 220 uM 5,5'-dithiobis [2-nitrobenzoic acid], 200 uM thiobenzyl benzyloxycarbonyl-L-lysinate (Peninsula Laboratories) and 0.1% Triton X100 was added and the mixture incubated for 1 hr at 37° C. The thioesterolytic

reaction was stopped by the addition of Trasylol (20 ul containing 15 ug) and the absorbance was read at 412 nm.

To test if preincubation of plasminogen with monoclonal antibodies had any effect on its activation by urokinase, dialysed monoclonal antibody (20 ul containing 12 ug) or assay buffer (in controls) was incubated with plaminogen (20 ul containing 2 ug) for 1 hr at 20^{o} C. Urokinase was then added and the mixture incubated at 37^{o} C for a further 45 min, after which 2.0 M KC1 (20 ul) was added and the assay continued as above.

Modification of the colorimetric assay for use in assays for pro and total HPA 52 was performed by incubating dialysed anti-plg 3 antibody (20 ul containing 12 ug IgG; for measuring active HPA52) or assay buffer (for total content of HPA52) with affinity purified (Deutsch & Mertz 1970) human plaminogen (20 ul, 2 ug) for 1.5 hrs at 20^{o} C. Diluted samples (20 ul) of the tissue homogenates which had been prepared in the presence or absence of anti-plg 3 antibody were then added and the mixture incubated for a further 45 minutes at 37^{o} C. The assay buffer consisted of 50 mM glycine pH 7.8 containing 0.1% Triton X-100 and 0.1% gelatin. The plasmin produced as a result of the activation of the plasminogen by the plasminogen activator was assayed as described above. The difference in the level of absorbance between the total HPA52 and the active HPA52 is equal to the amount of HPA52 proenzyme present.

Results for plasminogen activator activity were expressed after correction for the presence of plasminogen-independent proteases in the tissue extracts by subtraction of absorbances obtained in an indentical system to which neither plasminogen nor antibody was added.

2.13 POLYACRYLAMIDE GEL ELECTROPHORESIS IN THE PRESENCE OF SDS

The SDS-PAGE was performed in slab gels according to Laemmli (1970). The casting frames (two) were set up according to the manufacturer's notes (LKB instrument).

The lower separating gel was 3 mm thick, 15 cm wide and 13 cm tall and contained 11% acrylamide. The gel was prepared by mixing 14.8 ml 1.5 M Tris-HC1 pH 8.8, 0.4% SDS (Sigma Chemical Co.), 22 ml 30% acrylamide stock (Kodak), 2.5 g sucrose, 21 ml distilled water, 24 ul N,N,N',N'-tetramethylethylenediamine (Temed) [Ajax Chemical] and 100 ul of 10% ammunium persulphate [Ajax Chemical]. Twenty eight ml were poured into each frame and overlaid gently with water and left to polymerise for at least 1 hr. After the separating gel has set, the water overlay was poured off and overlaid with stacking gel with the appropriate comb inserted between the frames. The upper stacking gel consisted of 4% acrylamide and was prepared by mixing 5 ml stacking gel buffer (0.5 M Tris-HC1 pH 6.8/0.4% SDS), 2.6 ml 30% acrylamide stock and 12.5 ml distilled water. The stacking gel was left to polymerise for 1 hr before the comb was carefully removed and the sample wells washed out with distilled water. The samples were then carefully layered into the wells of the stacking gel, ensuring that mixing with the electrophoresis buffer was kept to an absolute minimum. The gels were then electrophoresed for 18 hr at 70V and at constant current using power-pack according to manufacturer's instruction (LKB).

2.14 AUTORADIOGRAPHY AND SDS-PAGE

SDS-PAGE was performed in slab gels according to Laemmli (1970) as described above (Section 2.13).

Samples containing ^{125}I-plasminogen, urokinase and Trasylol and the various additions were incubated at 37° C for various times as discussed in the results Section of Chapter 3. After incubation, the samples were boiled for 3 min in sample buffer (0.06 M Tris-HC1 pH 6.8 containing 20% v/v glycerol, 6% w/v SDS and 0.012% bromophenol blue) containing 5% beta-mercaptoethanol and subjected to SDS-PAGE for 18 hr at 70V, constant current and at room temperature. After electrophoresis, the gels were fixed and stained in 50% trichloroacetic acid, 0.1% coomassie brilliant blue for 30 min and destained in 10% glacial acetic acid and dried with a slab gel drier (Hoeffer Scientific Instruments). The dried gels were placed on Kodak X-omat films at -70° C to localise the radiolabelled protein.

2.15 AMINO ACID SEQUENCING

The terminal amino-acid sequence of lysine-Sepharose and antibody-affinity purified plasminogen was determined on 600 picomoles protein to ten residues on a Beckman 890 M amino-acid sequencer using a 0.1 M Quadrol program. The phenylthiohydantoin (PTH)-amino acids were identified by high-performance liquid chromatography (HPLC) on a Hewlett-Packard 1084B instument with an Altex 165 variable wave-length detector using a reverse phase C-18 column and sodium acetate-acetonitrile elution (Bledsoe & Pisano 1981).

2.16 FIBRIN AGAROSE RADIAL DIFFUSION GELS

A 1.25% agarose (Sea Plaque, FMC Corp. Rockland, Maine) gel matrix, 1.2 mm thick, containing fibrinogen (Sigma type X) and thrombin (Sigma Grade 1) was used for the detection of fibrinolysis. Wells of 3 mm diameter were cut for the application of

5 ul of plasmin/monoclonal antibody mixture which had been preincubated for 3 hr at 20° C. Radial diffusion was carried out in a humidified box for 20 hr at 37° C and then the gels were washed thoroughly in saline and stained with amido black.

2.17 PREPARATION OF COLO 394 CELL CULTURES

The human colonic epithelial cell line COLO 394 was a gift from Dr. R. Whitehead, Ludwig Cancer Research Institute, Melbourne (Frampton et al., 1982). These cells were grown in tissue culture medium (RPMI-1640; Flow Laboratories) with 10% heat-inactivated fetal calf serum containing 50 ug/ml gentamycin, 60 ug/ml penicillin and 20 ug/ml streptomycin and passaged twice a week and were maintained for several months. The secretion of the PA by these cells remained stable and constant throughout the period of culture as assessed every month using SDS-PAGE and fibrin plate overlay gel method as described in section 2.6 of this chapter.

For studies on proenzyme activation, 2 x 10^6 cells/well were plated in 6-place multi-well plates (Linbro). After adherence overnight, the cells were washed with serum-free RPMI-1640 and cultured in this for 24 hr before additions were made and incubations continued for a further 24 hr. Additions to the 3 experimental cultures were: (1) plasminogen (20 ug), (2) Buffer control (50 mM glycine pH 7.8), (3) plaminogen (20 ug), and anti-plg 1 (138 mg) mixture which had been previously incubated for 2 hr at 20° C.

2.18 EFFECT OF DFP ON PRO-ACTIVATOR AND ACTIVATOR

COLO 394 cell culture supernatants (30 ul) containing additions as described in legend of Fig. 3.6 were incubated with diisopropylfluorophosphate (10 ul, 0.25 mM)

(Sigma Chemical Co.), an irreversible inhibitor of serine proteases, for 5 hr at 20° C and the incubation stopped with SDS sample buffer to give a final volume of 80 ul. Untreated and treated samples were then applied to an 11% SDS electrophoresis gel under non-reducing conditions and detection of the proteases was performed using a fibrin overlay gel as described above (Section 2.6).

In the case of colorectal carcinomas, adenomatous polyps and inflammatory lesions, mucosal specimens were homogenised with (a) anti-plg 1 (1 ug/mg wet tissue), (b) buffer alone or (c) Trasylol (4 ug/mg wet tissue) and diluted 1:2 prior to assay. The diluted homogenates (20 ul) were then incubated with 20 ul (2 ug) of affinity-purified plasminogen for 45 minutes at 37° C which had been previously preincubated with 20 ul of either (a) anti-plg 1 (20 ug), (b) Trasylol (16 ug) or (c) buffer alone for 2 hr at 20° C.

Homogenates (30 ul) containing these additions were incubated with DFP or propylene glycol (Sigma Chemical Co.) as control (15 ul, 3.7 mM) for 5 hr at 20° C and the incubation stopped with SDS sample buffer to give a final volume of 85 ul. Untreated and treated samples were then applied to an 11% SDS electrophoresis gel under non-reducing conditions and detection of the proteases was performed using the fibrin-overlay gel described above (Section 2.6).

2.19 IODINATION

Iodination was performed according to the method of Morrison et al., (1971). The Enzymo-beads (BioRad) were rehydrated with 0.5 ml distilled water at least 1 hr before use. Into a disposable eppendorf tube (Bioscientific Supplies) were added 100 ul

of 0.2 M phosphate buffer pH 7.2, 20 ul protein solution (500 ug), 100 ul Enzymo-bead reagent, 50 ul 1% beta-D-glucose (BDH) and 30 ul 2 mCi $Na^{125}I$.

The reagents were mixed and iodination allowed to proceed at room temperature for 15 min. The appropriate fractions containing radioactivity were collected after desalting on a Sephadex G-25 fine column with phosphate buffered saline pH 7.4/0.1% gelatin.

2.20 ISOLATION OF HUMAN INTESTINAL MACROPHAGES FROM LAMINA PROPRIA

A single cell suspension of human intestinal lamina propria cells was prepared according to Golder & Doe (1983). Briefly, histologically normal mucosa resected from patients undergoing surgical resection for colonic carcinoma was immediately transported to the laboratory in ice-cold RPMI 1640 (Gibco, Grand Island Biological Co; Grand Island, N.Y.) supplemented with 100 IU/ml penicillin, 100 ug/ml streptomycin and 100 ug/ml neomycin. The muscularis mucosa was dissected free from the mucosa which was washed several times in calcium and magnesium-free Hank's balanced salt solution (CMF-HBSS) and then cut into small pieces. Sequential treatments of the tissues in CMF-HBSS supplemented with 1 mM dithiothreitol (Sigma Chemical Co; St. Louis, M.O.) and then 0.75 mM EDTA were continued until all epithelial cells were removed. After incubation overnight in 2 U/ml purified collagenase (CLSPA, Worthington Biochemical Corp; Freehold, N.J.), 5 U/ml DNase II (Calbiochem-Behring Corp.) and 5% human AB serum (Woden Valley Hospital Blood Bank), lamina propria cells were separated from the fragments of undigested tissues by filtration through surgical gauze. The supernatant was then centrifuged (300 g) for 15 min at room temperature. Viability of the cells from the disaggregation for all speciments was always greater than 95%.

2.21 PURIFICATION OF HUMAN INTESTINAL MACROPHAGES

Lamina propria cells were resuspended in a minimal volume of CMF-HBSS and introduced into the sample chamber for centrifugal elutriation which was carried out in a Beckman J2-21 centrifuge using a JE-6B rotor fitted with the standard elutriation chamber under sterile conditions. The elutriation buffer was 10 mM sodium phosphate pH 7 containing 0.15 M NaC1, 1 mM EDTA and 0.2% crude human serum albumin (Commonwealth Serum Laboratories, Melbourne, Australia). The temperature was maintained at 20^{o} C and the rotor speed at 2500 $\pm$ 10 rpm throughout the run. The elutriation separation chamber and the separation reservoir were pretreated with 1% Aquasil (Pierce Chemical Co; Rockford, IL, USA) and rinsed well prior to introduction of the lamina propria cells.

Cells were introduced into the elutriator when the elutriation pump was running at a flow rate of 9.0 ml/min and 65 ml were then collected. The flow rate was adjusted to 12.5 ml/min and a further 200 ml containing mainly erythrocytes and a small percentage of lymphocytes was collected. The pump flow rate was then increased to 15.0 ml/min and a fraction of 500 to 600 ml containing the majority of the lymphocytes was collected. The pump and then the rotor were stopped and the fraction collected from the elutriation chamber contained mainly neutrophils, some eosinophils, a small proportion of large lymphocytes, and the majority of the human intestinal macrophage population. Macrophages were further purified by underlaying with an equal volume of Percoll (Pharmacia, 1.064 g/ml, adjusted with CMF-HBSS) before centrifugation at 750 g for 40 min at 4^{o} C. Purified human intestinal macrophages were collected from the interface and washed in CMF-HBSS at 0-4^{o} C by successive centrifugations at 750 g for 10 min and 300 g for 10 min. The cells were then

resuspended in 10 ml of homogenisation buffer (10mM Hepes pH 7.4, 0.34 M sucrose, 1 mM EDTA, 0.1 mM $MgC1_2$).

2.22 SURFACE LABELLING OF PLASMA MEMBRANE USING WHEAT GERM AGGLUTININ

For cell surface-labelling with ^{125}I-labelled wheat germ agglutinin (^{125}I-WGA), the purified human intestinal macrophages were resuspended in cell resuspension buffer (Jesaitis et al., 1982) containing 0.1% human serum albumin and incubated with the ^{125}I-WGA for 5 min at 0° C. After centrifugation at 300 g for 10 min, the cells were washed successively with 30 ml cell resuspension buffer and 30 ml homogenisation buffer. The cells were then resuspended in 10 ml homogenisation buffer for nitrogen cavitation.

For routine plasma membrane preparation, 20 uM p-nitrophenylguanidinobenzoate and 100 KIU/ml Trasylol (bovine pancreatic trypsin inhibitor, Bayer) were included to reduce surface antigen degradation by serine proteinases. These inhibitors were omitted for gradients involving assays of plasminogen activators.

2.23 NITROGEN CAVITATION AND SUBCELLULAR FRACTIONATION BY ISOPYCNIC CENTRIFUGATION

The nitrogen cavitation and subcellular fractionation methods of Fayle et al., (1985) employed for human blood monocytes were applied to intestinal macrophages. Surface-labelled intestinal macrophages with ^{125}I-WGA in a 50 ml centrifuge tube and a stir bar were placed in a pre-cooled Parr cell disruption bomb (model 4635, Parr

Instruments, Moline, IL, USA) and then pressurized with nitrogen to 2750 KPa (400 lb in $^{-2}$) and stirred at 4^{o} C for 15 min before disintegration. The homogenate was then centrifuged at 1000 g for 10 min 4^{o} C and the supernatant collected. The pellets of 1000 g, containing cell debris, broken nuclei and most of the DNA, were discarded.

Subcellular fractionation by isopycnic centrifugation was performed on sucrose density gradients containing 10 mM Hepes pH 7.0 and formed with 3 ml 60% (w/w) sucrose overlaid with 22 ml or 25 ml of a linear gradient of 55-20% sucrose. After overnight equilibration of gradients at 4^{o} C, 10 ml of the cell homogenate was layered onto the gradient and centrifuged in a Beckman SW27 rotor at 25,000 rpm (108,000 g) for a minimum of 3 hr, by which time the gradient was at equilibrium. Fractions were then collected in equal volume by pumping at 1 ml/min from a thin tube lowered onto the bottom of the gradient. In experiments to test the effects of digitonin (Merck), sucrose density gradients were formed as before but with 1 ml 60% (w/w) sucrose overlaid with 8 ml of a linear gradient of 55-20% sucrose. Digitonin was added to one half of the membrane preparation at 0.51 mg/10^{8} human intestinal macrophages, in a volume of 3 ml homogenisation medium before subcellular fractionation.

2.24 IMMUNOFLUORESCENCE STAINING OF HUMAN INTESTINAL MACROPHAGES

Immunofluorescence was carried out in 96 well trays according to Loken & Stall (1982). A total of 1-2 x 10^{6} cells in medium (200 ul) were added to round bottom titertek 96 well plates at 0-4^{o} C. This was spun at 250 g for 5 min at 4^{o} C and the supernatant removed by tilting the plate and using suction system with needle attachment. 50 ul (monoclonal antibodies, 1% BSA, 0.1% Na N_3) in PBS were added and the cells resuspended by aggitation of bottom of wells. This was then left to incubate for

30 min on ice. After incubation, 100 ul of 2.5% BSA/0.1% NaN_3 in PBS were added and the wells spun at 250 g for 10 min at 4° C. The supernatant was removed and 50 ul of FITC second antibody (Cappel Laboratories) added and the cells resuspended as before. This was again left to incubate for 30 min on ice after which 100 ul of 1% BSA/0.1% NaN_3 were added and the wells spun as before. The supernatant was removed and the cells resuspended in 50 ul containing 1% BSA/0.1% NaN_3/0.2 ug per ml propridium iodide (Sigma Chemical Co.). The cells were counted under fluorescent microscope (8-10 ul per coverslip).

2.25 PREPARATION OF POOLED PA ENRICHED MEMBRANES FRACTIONS

The pooled peak PA enriched membrane fractions were prepared from a standard sucrose gradient fractions after assaying for PA activity using the colorimetric assay. Pooled sucrose gradients fractions enriched for PA activity were diluted with 4.4% NaC1 in 10 mM Hepes and centrifuged at 220,000 g for 18 hr at 4° C. The precipitate which contained the PA enriched membranes were resuspended in 200 ul of 50 mM glycine pH 7.8 containing 0.5% Triton X-100 and then used straight away for the experiments to determine the types and nature of the plasminogen activators in the pooled PA enriched membrane fraction using anti-plg 1 monoclonal antibody.

2.26 STATISTICS

Results have been expressed as mean ± SD. Statistical analysis of the data was performed using the non-parametric analysis of Wilcoxon and Mann-Whitney.

CHAPTER 3

MONOCLONAL ANTIBODIES INHIBITORY TO HUMAN PLASMIN: DEFINITIVE DEMONSTRATION OF A ROLE FOR PLASMIN IN ACTIVATING THE PROENZYME OF UROKINASE-TYPE PLASMINOGEN ACTIVATOR.

INTRODUCTION

Plasmin is a non-specific serine protease of the trypsin type which has an important role in a variety of both physiological and pathological processes. While its best studied action is in thrombolysis (fibrinolysis), it has also been proposed to facilitate a range of other proteolytic events involved in embryonic development, tissue remodelling and tumour invasion (Astrup 1978; Reich 1978a; Collen 1980).

Plasmin is generated under the action of plasminogen activating enzymes from the zymogen plasminogen which is present in abundant amounts in many extracellular fluids. Two distinct types of plasminogen activator have been characterised and shown to be the products of different genes (Aoki & Von Kaulla 1971; Unkeless et al., 1974b; Christman et al.,1975). They differ in immunological reactivity and have Mr of 52,000 daltons (U-PA) and 66,000 daltons (t-PA). Several reports (Skriver et al., 1982; Nielsen et al., 1983; Wun et al., 1982b; Andreasen et al., 1984) have indicated that the plasminogen activating enzymes are themselves produced as inactive proenzymes, requiring proteolytic conversion for the expression of plasminogen activator activity. A role for plasmin as the mediator of this conversion was indicated by experiments which employed purified preparations of plasmin and the potent serine protease inhibitor, trasylol. However, the participation of minor contaminant proteases

could not be absolutely ruled out by either the purity of the plasmin or the specificity of trasylol.

Although monoclonal antibodies have been developed against human plasmin (Ploplis et al., 1982), their use as selective inhibitors to study the actions of plasmin has not been explored. Nor has their potential for the affinity purification of plasminogen been exploited.

In this chapter, the production and characterisation of four monoclonal antibodies against human plasminogen are reported. The identification of plasmin as the enzyme molecule responsible for conversion of inactive pro-urokinase to active urokinase is unequivocally established, and the use of immunoaffinity chromatography for plasminogen purification is demonstrated.

METHODS AND MATERIALS

Monoclonal Antibody Production

Human blood was obtained from the Venesection Clinic, Woden Valley Hospital. Human plasminogen was purified by two cycles of affinity chromatography on lysine-Sepharose (Deutsch & Mertz 1970) as described in Chapter 2 Section 2.7. Eleven week-old Balb/c mice (Animal House, John Curtin School of Medical Research, ANU) were hyperimmunized with 5 weekly intraperitoneal injections of 15 ug plasminogen in Freund's adjuvant (Difco Laboratories). Fusion was carried out 4 days after the last injection.

Production of hybridomas was carried out between immune splenic lymphocytes and X63-Ag 8.653 murine myeloma cells (Kearney et al., 1979) according to the procedure as described in Chapter 2, Section 2.3. Enzyme-linked immunosorbent assay (ELISA) was used as a primary screen for antibodies binding to plasminogen as described in Chapter 2, Section 2.8.

Immediately after fusion, the cells were dispensed into twelve 96-well tissue culture plates (Nunc) in Dulbecco's modified minimal essential medium (DMEM) (Flow Laboratories) supplemented with hypoxanthine, aminopterin and thymidine (HAT) (Littlefield 1964), 10% heat inactivated fetal calf serum (FCS) (Flow Laboratories), 2 mM glutamine and antibiotics. Thymocytes (at least 2 x 10^6/ml) from 4-8 week old Balb/c mice were added at each feeding. Of 1,152 wells, 953 wells displayed vigorous hybridoma growth after 10 days. The hybridomas were grown for a total of 14 days in DMEM-HAT medium with a feeding at day 7 and adapted to DMEM with 10% FCS by gradually phasing out aminopterin and then hypoxanthine and thymidine. Antibodies against human plasminogen were detected in 41 culture supernatants. Cultures secreting antibodies were cloned by limiting dilution as described in Chapter 2, Section

2.3.8. However, only four of the monoclonal antibodies were selected since these four hybridomas were stable and continued to secrete antibodies after freezing and thawing. Furthermore, all these hybridomas still continued to show high secretion of antibodies even after being frozen in liquid nitrogen for a period of up to 6 months.

Characterisation and Purification of antibodies

Monoclonal antibodies were characterised using the Ouchterlony analysis on agarose gel as described in Chapter 2, Section 2.9. Monoclonal antibodies (IgG) were purified by precipitation from ascites fluid or hybridoma culture supernatant by 50% saturation with ammonium sulphate. After dialysis of the resuspended pellets against 0.1M Tris-HCl pH 8.1, antibodies were purified by protein A-Sepharose (Pharmacia) chromatography as described in Chapter 2, Section 2.10.

Antibodies of irrelevant specificities, and of the same class and light chains as of the anti-plg monoclonals were used for control experiments. WVH-1 is a monoclonal IgG antibody raised against human intestinal macrophages which reacts with an uncharacterised surface antigen present on monocytes and neutrophils (D. Fayle, unpublished). GAP-8.3 is a pan-leukocyte monoclonal IgG antibody (Berger et al., 1981, American Type Culture Collection, Rockville, Maryland, USA).

Binding (dissociation) constants for the I^{125}-labelled monoclonal anti-plasminogen antibodies to human plasminogen were assessed by a solid-phase assay previously described by Frankel & Gerhard (1979) and Ploplis et al., (1982) as described in Chapter 2, Section 2.11.

Affinity Chromatography

Anti-plasminogen monoclonal antibody was coupled to cyanogen bromide-activated Sepharose 4B as described in Chapter 2, Section 2.4. In the experiment shown

in Figure 3.2, a 1 ml column containing 600 ug of IgG, was run at a flow rate of 12 ml/hr.

Colorimetric Assay was performed according to the method of Coleman & Green (1981) as in Chapter 2, Section 2.12.

SDS-PAGE and Fibrin Overlay Detection of Proteases was carried out by the method of Granelli-Piperno & Reich (1978) as described in Chapter 2, Section 2.6.

Fibrin Agarose Radial Diffusion Gels

This was performed as described in Chapter 2, Section 2.16.

Preparation of COLO 394 cell cultures was carried as described in Chapter 2, Section 2.17.

Effect of DFP on Pro-activator and Activator was assessed as described in Chapter 2, Section 2.18.

Iodination of purified plasminogen and monoclonal anti-plasminogen antibodies was performed using Enzymo-Beads (BioRad) as desribed in Chapter 2, Section 2.19. The specific activities of the plasminogen and monoclonal antibodies were 1×10^5 cpm/ug and $0.5 - 1 \times 10^5$ cpm/ug respectively.

Autoradiography

The direct, plasmin-independent assay of Mussoni et al., (1984) employing SDS-PAGE and 2-mercaptoethanol was used to monitor urokinase-mediated conversion of single chain, ^{125}I-plasminogen to two chain plasmin as described in Chapter 2,

Section 2.14. The method was also used to determine the content of native plasminogen prepared by both the lysine and antibody affinity methods.

Protein was determined by a modified Lowry method (Peterson 1977) as described in Chapter 2, Section 2.2.

RESULTS

Affinity purification of human plasminogen from crude plasma

The monoclonal antibody anti-plg 1 (600 ug) was coupled to cyanogen bromide-activated Sepharose 4B and used to purify human plasminogen from fresh whole plasma by affinity chromatography. After extensive washing of the column, the applied plasminogen could be eluted by 0.1M glycine-HCl pH 2.5/0.1% Triton X 100 containing 0.5 M NaCl. The eluate contained pure plasminogen as evaluated by SDS-PAGE. Under reducing conditions, two very closely spaced Coomassie blue stainable bands were seen (Figure 3.2). Although only one band was visible as shown in the photograph in Figure 3.2, two very closely bands were nevertheless seen in the gel, one with much higher intensity than the other. Unfortunately, the photographic techniques used only managed to pick up the major band of higher intensity. The Mr was estimated from the electrophoretic mobility relative to stained standard marker proteins and was not significantly different from the agreed Mr of human plasminogen of about 94K (Sottrup-Jensen et al., 1978a). The immunoaffinity method gave a 26% higher relative yield than the lysine-Sepharose method. The immunoaffinity purified plasminogen preparation was comparable with that obtained from lysine-Sepharose affinity chromatography when assayed by the colorimetric method using urokinase.

The cleavage of I^{125}-plasminogen was used to assess the content of native plasminogen purified by the two methods. Quantitative data on ^{125}I-plasminogen cleavage by urokinase was obtained by removing the portions of the SDS-PAGE gel containing radio-labelled plasminogen and plasmin (the position of the radio-labelled polypeptides was determined by placing the X-ray film directly onto the gel) and

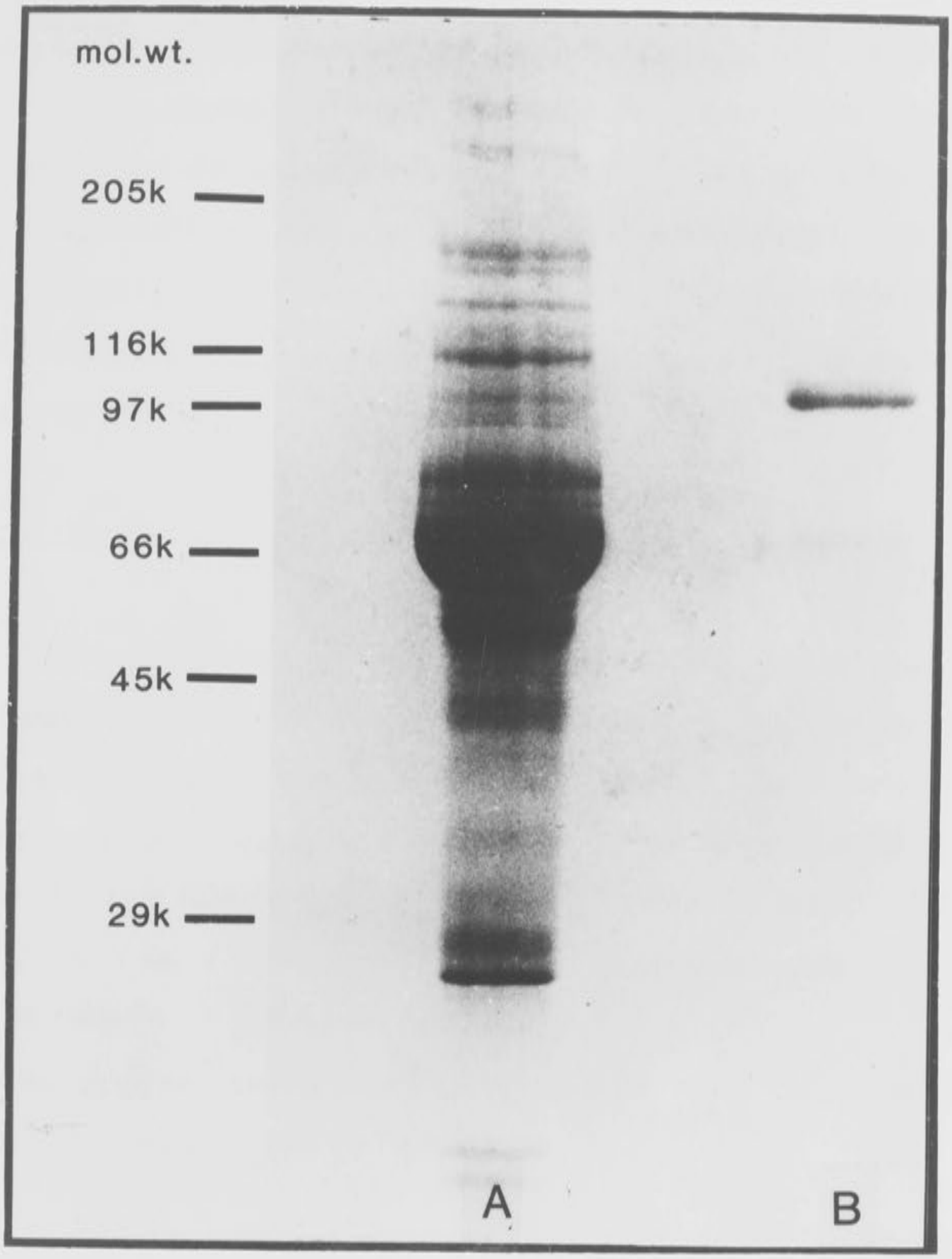


Figure 3.2 Affinity purification of plasminogen: Anti-plg 1 was coupled to Sepharose as outlined in Materials and Methods. The 1ml column was washed with Tris-HCl pH 8.1/0.1% Triton-X and the plasminogen eluted with 0.1M glycine pH 2.5 buffer. Starting material (lane A), Eluant fraction (lane B).

counting them in a Packard Auto Gamma 500 counter. Using 150 mPU of urokinase and 2 ug of the radio-labelled plasminogen prepared by either method, (in the presence of 80 ug/ml Trasylol) with an incubation period of 24 hr at 37° C, 96% of the antibody-purified plasminogen was converted to plasmin compared with 93% of the lysine-purified plasminogen. The predominant form of plasminogen from both methods was Glu-plasminogen as determined by the terminal amino acid sequence and was present at greater than 95%.

Affinity Studies

Binding (dissociation) constants for the ^{125}I-labelled monoclonal antibodies to human plasminogen were estimated as described under Materials and Methods of Chapter 2, Section 2.11. Figure 3.1 shows representative plots of 3 experiments in duplicate samples of the binding analysis for anti-plg 1 and anti-plg 2. The dissociation constants (K_D) for each were determined from the slopes of the lines and are summarized in Table 3.1. Anti-plg 1 has a slightly higher affinity than the other 3 antibodies which were similar to each other in their K_D values.

No competitive binding studies using the ELISA assay were performed with plasmin, DFP-plasmin, trypsin or chymotrypsin.

Effect of monoclonal anti-plasminogen antibodies on the susceptibility of plasminogen to activation by urokinase

The two-step coupled colorimetric assay of urokinase (Coleman & Green 1981) was used to determine whether the binding of monoclonal antibodies to human plasminogen altered its rate of activation by urokinase, or affected the subsequent assay of thioesterolytic activity by the plasmin formed. These two effects could be

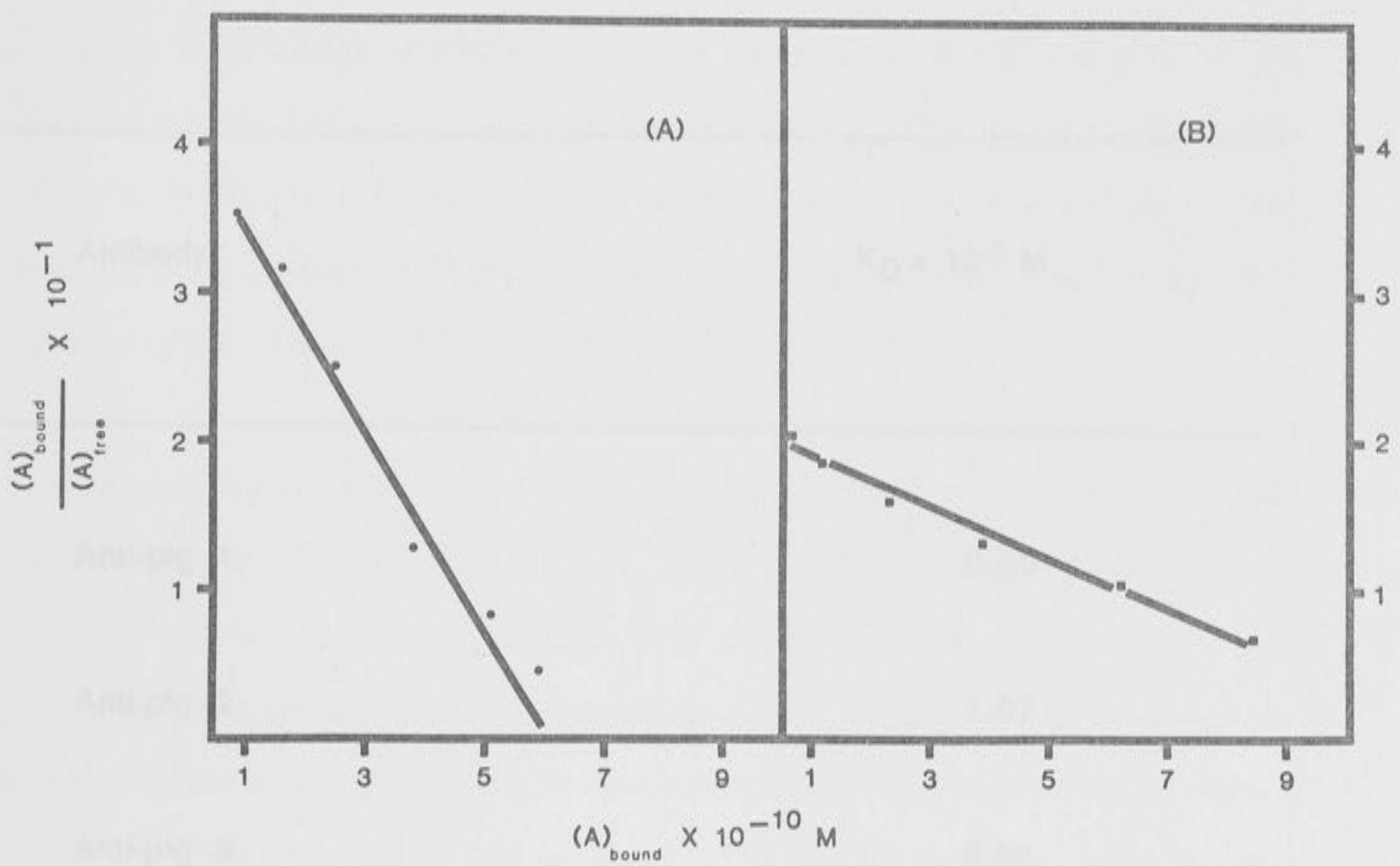


Figure 3.1 Modified Scatchard analysis of the binding of ^{125}I-labelled anti-plg 1 (A) and anti-plg 2 (B) to human plasminogen. In each case, the ratio of the concentration of $(A)_{Bound}$ to $(A)_{Free}$ antibody is plotted against $(A)_{Bound}$.

TABLE 3.1

Dissociation constants (K_D) for the 4 anti-plasminogen monoclonal antibodies to human plasminogen at 37° C.

Antibody	$K_D \times 10^{-8}$ M
Anti-plg 1	0.35
Anti-plg 2	1.07
Anti-plg 3	0.86
Anti-plg 4	0.73

distinguished by virtue of the two-step nature of the assay where, in the first step, plasminogen was activated to plasmin, and this reaction was then inhibited by the addition of 2 M KCl so that the plasmin activity already produced could be assayed by lysis of thioester in the second step.

When the protein-A purified monoclonal antibodies were preincubated with human plasminogen before the first (activation) step, both the monoclonal anti-plg 1 and anti-plg 2 antibodies produced a marked decrease in the colour yield of the subsequent plasmin assay step when compared with the antibody-free control (Table 3.2, Column 1). Monoclonal anti-plg 1 was somewhat more potent than anti-plg 2. Two other monoclonal anti-plasminogen antibnodies, anti-plg 3 and anti-plg 4, had no effect or gave a slight increase in colour yield. (Table 3.2, Column1).

Because the inhibitory effect of anti-plg 1 and anti-plg 2 could have resulted either from interference with the urokinase activation of plasminogen, or from direct inhibition of plasmin, the same antibodies were added to the incubation mixture after plasminogen activation, but before the plasmin assay step. Both anti-plg 1 and anti-plg 2 again produced a similar decrease in colour yield (Table 3.2, Column 2). Anti-plg 3 and anti-plg 4, however, either had no effect, or increased the colour yield to some extent, when compared with the antibody-free control (Table 3.2, Column 2). It was therefore apparent that while none of the monoclonal antibodies tested affected the susceptibility of human plasminogen to activation by urokinase, anti-plg 1 and anti-plg 2 strongly inhibited the thioesterase activity of pre-formed human plasmin. Direct colorimetric assay of the thioesterase activity of commercial porcine plasmin and bovine trypsin showed that none of the antibodies affected the activity of these enzymes (data not shown).

The observation that the monoclonal antibodies did not inhibit plasminogen activation was confirmed by examining the cleavage of ^{125}I-plasminogen by

TABLE 3.2

Inhibition of Colorimetric assay by monoclonal anti-plasminogen antibodies

	PRE-INCUBATION		POST-INCUBATION	
	Absorbance	% Inhibition	Absorbance	% Inhibition
Antibody				
Anti-Plg 1	0.115	101	0.143	95
Anti-Plg 2	0.390	53	0.357	57
Anti-Plg 3	0.868	-29	0.644	6
Anti-Plg 4	0.790	-16	0.730	-9
No Ab	0.695	-	0.679	-
No urokinase	0.118	-	0.112	-

Dialysed antibodies (20 ul containing 12 ug) were preincubated with plasminogen (20 ul containing 2 ug) for 1 hr at 20° C. In the case of post-incubation, the pre-formed plasmin was incubated with antibody in 0.4 M KCl for 1 hr at 20° C. Plasmin assay reagent was then added and the colorimetric assay continued.

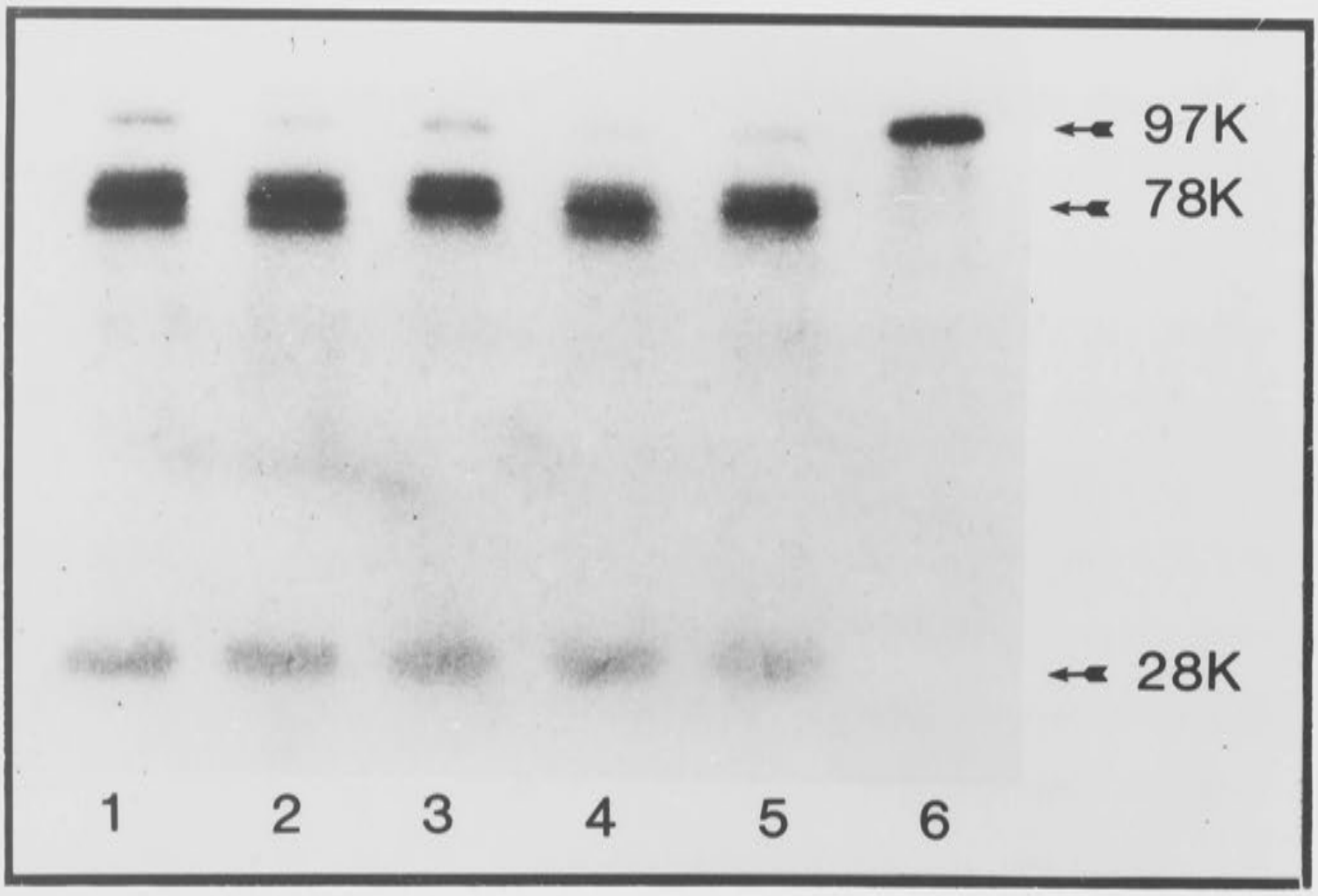


Figure 3.3 Autoradiogram showing the effect of monoclonal anti-plg antibodies on plasminogen cleavage: ^{125}I-plasminogen (2ug, lane 6) was incubated for 5 hrs with 150 mPU urokinase in the absence (lane 1) or presence of preincubation of plasminogen with the monoclonal anti-plg antibodies (16 ug/20ul). anti-plg 3 (lane 2), anti-plg 2 (lane 3), anti-plg 4 (lane 4) and anti-plg 1 (lane 5).

autoradiography after electrophoresis. In this assay, inhibition of plasminogen activation would have been indicated by the ability of the monoclonal antibodies to block urokinase-mediated cleavage of single chain plasminogen to the characteristic plasmin "heavy" and "light" chains (Dano & Reich 1979; Summaria et al., 1967). The results obtained (Figure 3.3) showed that none of the antibodies blocked the cleavage of plasminogen.

Effect of monoclonal anti-plasminogen antibodies on fibrinolytic activity of plasmin

The ability of the protein-A purified monoclonal antibodies to inhibit the fibrinolytic activity of plasmin was investigated using a fibrin radial diffusion method (Golder & Stephens 1983). Small amounts of human plasmin were generated by treatment of plasminogen with urokinase and the resulting preparation was incubated with each antibody for 3 hr at 20^{o} C. The plasmin-antibody mixtures were then applied to microwells cut in an agarose gel containing fibrin. Zones of fibrin lysis were formed as the diffusing plasmin degraded the insoluble fibrin to soluble fibrinopeptides.

As shown in Figure 3.4, anti-plg 1 (well 1) and anti-plg 2 (well 4) inhibited the fibrinolytic activity of human plasmin, with the former showing almost complete inhibition when compared with the irrelevant antibody WVH-1 (well 2) and the antibody-free (well 3) controls. Neither anti-plg 3 nor anti-plg 4 (wells 5 and 6 respectively) had any effect on the ability of plasmin to degrade fibrin.

Effect of monoclonal anti-plasminogen antibodies on plasmin-mediated conversion of HPA52 to HPA36

Active urokinase is a protein of 52,000 daltons which is readily cleaved by plasmin to a 36,000 dalton product that retains plasminogen activating activity (Barlow et al., 1981). Because anti-plg 1 and anti-plg 2 inhibited both the thioesterolytic and fibrinolytic activities of plasmin, the possibility arose that these antibodies interfered with the plasmin-catalysed cleavage of HPA52 was also investigated.

Each monoclonal antibody was preincubated with plasminogen for 2 hr at 20° C, then urokinase was added to generate small amounts of plasmin, which in turn cleaved some of the urokinase. The reaction was terminated with SDS and the proteins were separated by SDS-PAGE. Contact lysis of an overlay gel, containing fibrin and plasminogen was used to determine the position in the SDS-PAGE gel of plasminogen activators and plasmin from the incubated samples.

As shown in Figure 3.5, the urokinase preparations used contained both the high (HPA52) and low (HPA36) Mr forms of the enzyme (lane 1). When urokinase was incubated with plasminogen (in the absence of antibody), the plasmin produced by plasminogen activation cleaved almost all of the HPA52 form into the HPA36 form (lane 2). The plasmin responsible for this cleavage (Barlow et al., 1981) was evident by the lysis zone A at 85,000 daltons (lane 2-6, except lane 4 in the case of anti-plg 2 see below). All four anti-plasminogen antibodies (lanes 3 to 6) substantially inhibited tha plasmin-catalysed cleavage of the HPA52, indicating that this proteolytic activity of plasmin was inhibited by plasmin bound antibodies. This was not due simply due to substrate competition for plasmin between the protein of urokinase and the added IgG, since equivalent amounts of the irrelevant antibody had no effect (data not shown).

The stability of plasmin-antibody complexes

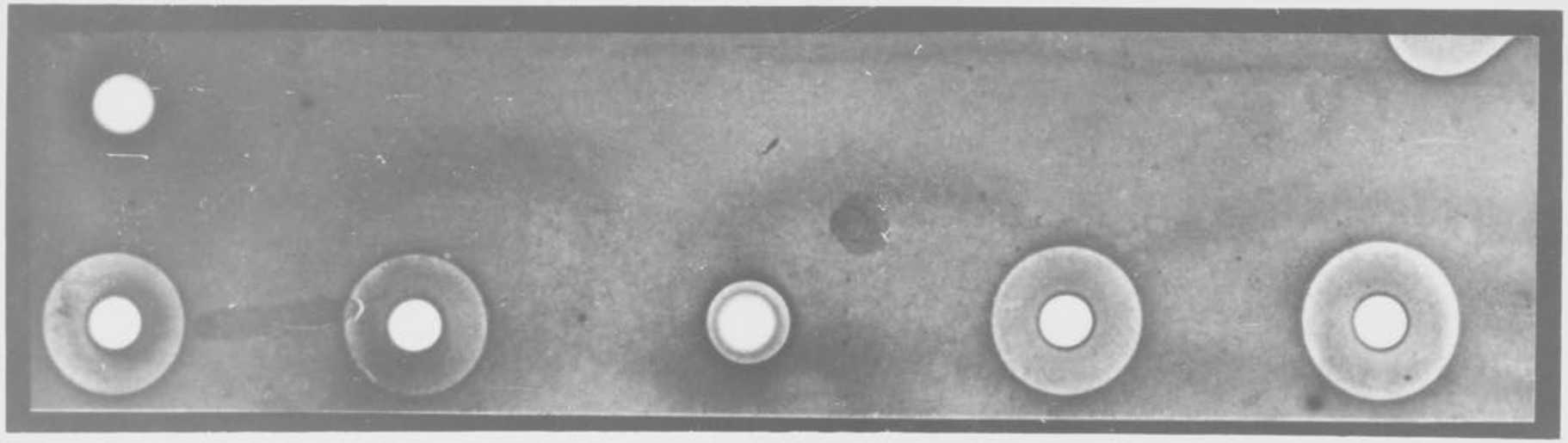


Figure 3.4 Effect of monoclonal antibodies on fibrinolysis: Human plasmin (10ul) was preincubated with 10ul of the respective antibody (7ug) for 3 hrs at 20°C and the mixture (5ul) applied to wells cut in a plasminogen-supplemented fibrin/agarose gel. The respective antibodies used were:- anti-plg 1 (well 1), WVH-1 (well 2), plasmin only (well 3), anti-plg 2 (well 4), anti-plg 3 (well 5) and anti-plg 4 (well 6). The gel was incubated for 20 hrs at 37°C.

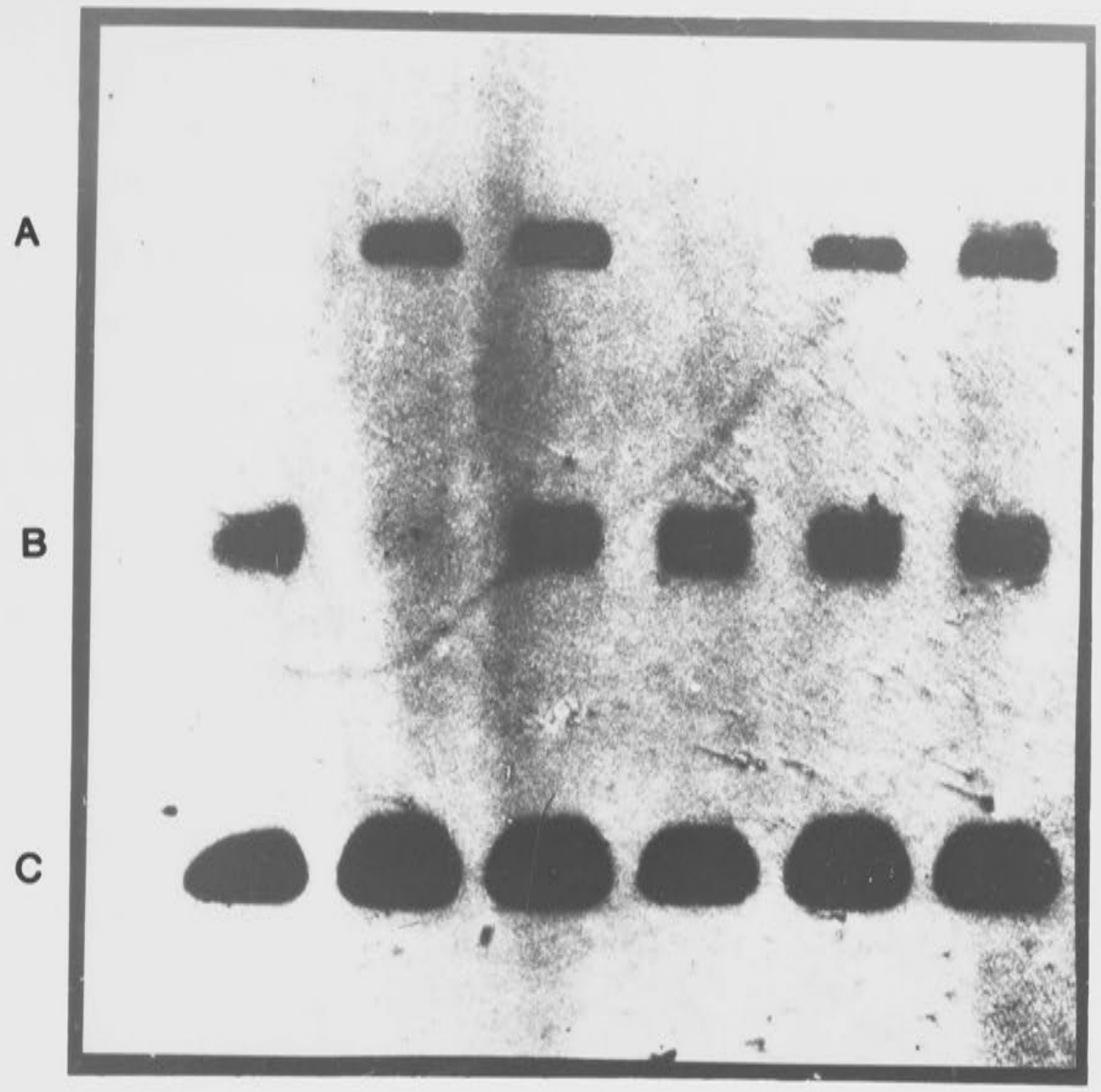


Figure 3.5 Effect of monoclonal antibodies on plasmin mediated cleavage of urokinase: SDS-PAGE after preincubation with antibodies. Plasminogen (10ul, containing 2ug) was preincubated with anti-plg antibodies (2lug) or with the assay buffer (control) for 2 hrs at 20°C and then further incubated with 30ul (6mPU) of urokinase at 37°C for 45 min before application to SDS-PAGE. The bands were developed in a fibrin overlay gel by incubation for 20 hrs at 37°C. Urokinase (lane 1), Urokinase incubated with plasminogen treated with:- control (lane 2), anti-plg 3 (lane 3), anti-plg 2 (lane 4), anti-plg 4 (lane 5) and anti-plg 1 (lane 6). Zones of lysis are: (A) Mr 85000 (plasmin), (B) Mr 52000 (urokinase-type plasminogen activator) and (C) Mr 36000 (degraded plasminogen activator).

In the experiment shown in Figure 3.5, (lane 4), it was also apparent that anti-plg 2 prevented the appearance of the band at 85K arising from plasmin formed in the incubation before electrophoresis, indicating the formation of a proteolytically-inactive antibody-plasmin complex which was stable to SDS treatment. The absence of the band was not due to inhibition of plasminogen activation by anti-plg 2, since anti-plg 2 did not inhibit plasminogen activation (see Fig. 3.3). The other anti-plg monoclonals formed complexes which were dissociated during SDS-PAGE, and active plasmin bands were produced at 85K.

Effect of monoclonal anti-plg 1 and DFP on the generation of COLO 394 plasminogen activator from the proenzyme

Plasminogen activators from several sources have now been shown to be produced as proenzymes (see Chapter 1). These proenzymes can be activated by plasminogen preparations containing traces of plasmin, but definitive evidence for this role of plasmin has not been reported.

In order to determine unequivocally whether plasmin was responsible for the conversion of the COLO 394 proenzyme to its active form, the ability of the anti-plg monoclonal antibodies to selectively inhibit plasmin activity was exploited for investigating this possible role of plasmin.

When the human colonic epithelial cell line COLO 394 was grown with serum-free RPMI 1640 medium, an appreciable amount of plasminogen activator activity was produced and secreted into culture supernatant. This activity consisted principally of HPA52 (Figure 3.6. lane 1) and was present as a proenzyme, since it gave the same lysis band after treatment with DFP, an irreversible inhibitor of serine proteases

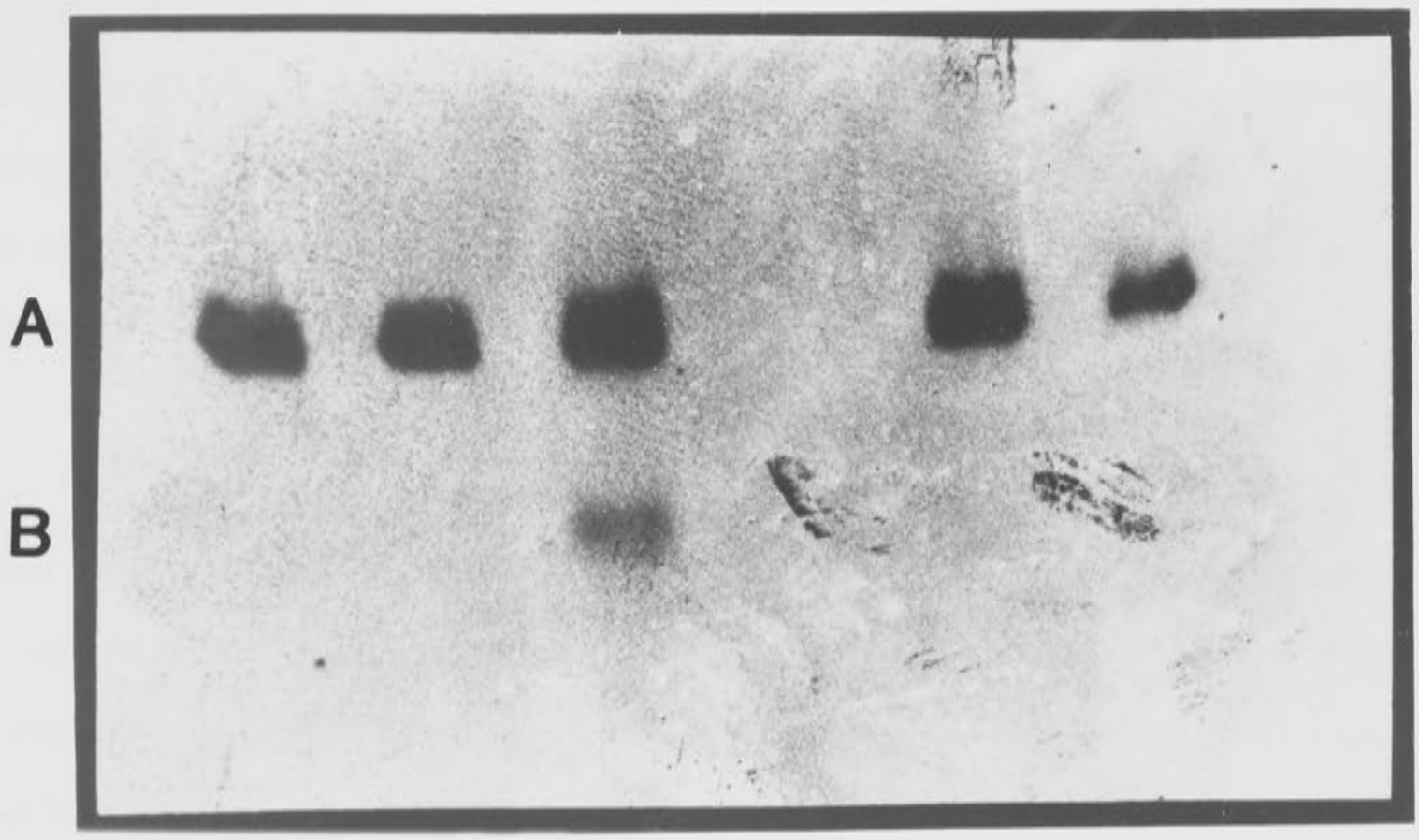


Figure 3.6 SDS-PAGE analysis of the role of plasmin in the conversion of proenzyme of COLO 394 plasminogen activator to active enzyme in cell culture incubated with :- assay buffer only (lane 1), assay buffer and treated with DFP (lane 2), plasminogen (lane 3), plasminogen and treated with DFP (lane 4), plasminogen pretreated with anti-plg 1 (lane 5), plasminogen pretreated with anti-plg 1 and treated with DFP (lane 6). Zones of lysis are:- (A) Mr 52000 (urokinase-type PA), (B) Mr 36000 (degraded urokinase-type PA).

(Figure 3.6, lane 2). The potentially active site in the proenzyme is inaccessible to DFP, in contrast to the active site in the active enzyme (Skriver et al., 1982). However, when untreated plasminogen was added to the COLO 394 cell culture growing in serum free RPMI-1640 and incubated for 24 hr, some of the high Mr activator (Zone A, lane 3) was cleaved to an active form of lower Mr (HPA36; Zone B, Lane 3). When this supernatant was treated with DFP both bands were absent (Figure 3.6. lane 4). This result indicated that the proenzyme had been activated, since the active site had become accessible to DFP.

To determine if the proenzyme activation observed could be attributed to traces of plasmin in the plasminogen, the preparation of plasminogen was preincubated with anti-plg 1 for 2 hr at 20^{o} C and then added to the COLO 394 cell culture and incubated for 24 hr. Only one band of HPA52 was seen (Lane 5) and there was no cleavage of the activator to the lower Mr form of HPA36 (cf Lane 3, where no anti-plg 1 was present). When this same culture superntant was treated with DFP, this HPA52 band was still present showing that the proenzyme was still present and that the antibody had therefore inhibited conversion of proenzyme to active enzyme. Since anti-plg 1 specifically inhibited plasmin, these results demonstrated that the plasmin present as a trace amount in the plasminogen preparation was responsible for the conversion of the pro-activator to its active form.

The above observation was confirmed by the coupled clorimetric assay of Coleman & Green using anti-plg 3 and anti-plg 4, which unlike anti-plg 1 and anti-plg 2, did not inhibit plasmin thioesterase activity, or the fibrinolytic activity of plasmin. Anti-plg 3 and 4 did, however, inhibit the plasmin-catalysed cleavage of active HPA52 (Figure 3.5). Plasminogen was therefore preincubated with anti-plg 3 or anti-plg 4 for 2 hr at 20^{o} C before the addition of COLO 394 proenzyme and assay buffer to the incubation.

Table 3.3 shows that in the presence of anti-plg 3 or anti-plg 4, the colour yield was markedly reduced compared to the control in which plasminogen was preincubated alone. When an irrelevant monoclonal antibody (GAP-8.3) was used, this reduction in colour yield was not seen. These results therefore confirmed that plasmin was responsible for the conversion of the proenzyme of COLO 394 plasminogen activator to its active form.

Beside secreting proenzyme into the culture supernatant, the COLO 394 cells themselves also have pro-PA present (unpublished observations).

TABLE 3.3

Inhibition of Conversion of COLO 394 Proenzyme to active plasminogen activator

Antibody	Absorbance
Anti-plg 3	0.375
Anti-plg 4	0.309
No Antibody	1.318
No Proenzyme	0.095
GAP-8.3	1.034

Dialysed anti-plasminogen monoclonal antibodies (20 ul containing 12 ug) were preincubated with plasminogen (20 ul, containing 2 ug) for 2 hr at 20° C, followed by incubation with proenzyme (20 ul) at 37° C for 45 min.

DISCUSSION

This chapter reports the properties of four monoclonal antibodies, all of IgG_1/K, raised against human plasminogen, which proved useful as selective inhibitors of plasmin. The use of ELISA as a primary screening method allowed the selection of all monoclonal antibodies that bound to plasminogen, while the effects on plasminogen activation and the enzymic properties of plasmin produced by these antibodies were demonstrated by three other methods. Definitive evidence that plasmin activates the proenzyme form of HPA52 secreted by a cancer cell line is described.

The dissociation constants obtained for the antibodies were of the same order of magnitude. However, the overall binding constants were obtained using a solid state binding assay and are thus only approximations. They may not be representative of those that could be obtained under equilibrium conditions. These antibodies were of slightly higher affinity than those of monoclonal antibodies 10F1 and 10VI, reported by Ploplis and co-workers (1982), which bind to human Glu-plasminogen and certain isolated fragments.

After coupling to Sepharose, one of these antibodies, anti-plg 1, was found to have an appropriate affinity and specificity for the purification of plasminogen to homogeneity from whole plasma by immunoaffinity chromatography. Anti-plg 1 did not bind significantly to any other proteins present in substantial amounts in plasma. The purification of plasminogen by one-step immunoaffinity chromatography provided an alternative to the current method of 2 cycles of affinity chromatography on lysine-Sepharose (Deutsch & Mertz 1970). The immunoaffinity method is rapid, minimizing the possibility of plasminogen degradation, and therefore conveniently provides highly-

purified plasminogen. All the other three anti-plg monoclonal antibodies were also able to purify plasminogen although not as effectively as anti-plg 1. The immunoaffinity purification of plasminogen by these monoclonal antibodies is a good indication of its specificity although a definitive demonstration would ideally also involve nitrocellulose immunoblotting with either serum or plasma.

Manipulation of the colorimetric assay of plasmin was used to distinguish antibody effects on plasminogen activation from effects on plasmin activity. From these experiments, it was clear that none of the antibodies interfered with the activation of plasminogen by the plasminogen activator, urokinase. This observation was confirmed by examining the effects of antibodies on the susceptibility of ^{125}I-plasminogen to cleavage, using autoradiography after electrophoresis. The autoradiogram clearly revealed that none of the antibodies blocked the urokinase-mediated cleavage of single chain plasminogen to the characteristic plasmin "heavy" and "light" chains.

By contrast, anti-plg 1 and anti-plg 2 bound to both human plasminogen and plasmin and strongly inhibited the fibrinolytic and esterolytic activities of plasmin, but bovine trypsin and porcine plasmin activities were unaffected.

In the experiments to investigate the cross reactivity of the anti-plg monoclonal antibodies, bovine and porcine plasmin as well as bovine chymotrypsin and trypsin were used. Even though only two species were tested, all these serine proteases beared structural similarity to other members of the group, including plasmin and elastase as well (Gunzler et al., 1982).

The anti-plasminogen antibodies were also evaluated by the SDS-PAGE fibrin overlay gel system which made it possible to examine not only their effect on the

fibrinolytic activity of plasmin but also on the proteolytic cleavage of urokinase that is mediated by plasmin. It was demonstrated that all the antibodies against plasminogen inhibited somewhat the plasmin cleavage of urokinase. However only anti-plg 2 was effective in inactivating plasmin by the formation of a complex stable in SDS.

The inhibition of human plasmin is likely to result from steric interference with the binding of substrate. It was therefore a little surprising that anti-plg 1 and anti-plg 2 were able to inhibit the action of plasmin on such a small substrate as the lysine thioester used in the colorimetric assay. Although no evidence is offered at this stage, it is tempting to speculate that the antibodies may have bound to the non-protease part of plasminogen, which contains the five closely homologous triple-loop structures, the so called kringles (Sottrup-Jensen et al., 1978b). These play important roles in the interaction of plasminogen with the ω-aminocarboxylic acids, fibrin and alpha$_2$-anti plasmin (Wiman & Wallen 1977; Wiman et al., 1979; Markus et al., 1979). Only kringle 5 may be excluded as the antibody combining site, since cleavage of the Arg 560-Val 561 bond by urokinase (Summaria et al., 1967,1975; Robbins et al., 1967) still occurs in the presence of anti-plg antibodies. Furthermore, a recent report (Marti et al., 1985) shows that the sequence of porcine miniplasminogen consisting of kringle 5 and the light chain portion of plasmin does not differ substantially from that of the known sequence of human miniplasminogen. It is therefore unlikely that the inhibition of plasmin produced by the monoclonal antibodies was due to binding at the catalytic site, since the amino acid sequence at the active site does not differ substantially between human and porcine plasmin (Marti et al., 1985) and in the experiments reported in this chapter, the antibodies failed to inhibit porcine plasmin. In this respect, it would be interesting to test whether the anti-plg monoclonal antibodies would bind to DFP-plasmin, since DFP is a irreversible inhibitor of the active site of serine proteases.

In a recent report by Ploplis et al., (1982) on the anti-plasminogen monoclonal antibnodies 10F1 and 10V1, it is worth noting that 10F1 was specific for an epitope on the kringle 4 domain of plasminogen or its fragments while 10V1 recognised an epitope in the kringle 1-3 domain of the plasminogen molecule. It had also been shown recently that the antibody 10F1 which interacted with a particular ε-amino caproic acid (EACA) binding site on the kringle 4 region of human Glu_1-plasminogen also enhanced the activation of Glu_1-plasminogen by urokinase, but not Lys 77-plasminogen (Cummings & Castellino 1985). In this respect, it is worth mentioning that anti-plg 3 appeared to enhance plasminogen activation (see Table 3.2, Column 1), although this enhancement was not significant. This enhancement was not attributed to either plasminogen or antibody being rate limiting or different preparations of plasminogen, since a dose-response curve (data not shown) was performed with various dilutions of the monoclonal antibodies and the concentration of the monoclonal antibody used in the experiments reported in this chapter was maximal. No changes was observed when different preparations of plasminogen and of the same concentration were used. A precedent for activation of an enzyme by a monoclonal antibody has been found before in the case of β-Galactosidase, where inhibitory, neutral and activating antibodies were demonstrated (Frackelton & Rotman 1980).

Previous work has identified a requirement for protease activity in enabling expression of plasminogen activator activity by cell lines in vitro (see Chapter 1). Plasmin has been indicated as one probable mediator of the conversion of inactive proenzyme to active plasminogen activator, and it has a specificity consistent with the proposed cleavage of the amino acid sequence of single-chain proenzyme to that of active two-chain enzyme. Thus, the peptide bond at lysine 158-isoleucine 159, as well as the Arg 156-phenylalanine 157 are likely plasmin cleavage sites (Gunzler et al., 1982a,b). The N-terminal amino acid of the B chain of active enzyme is isoleucine, and

the C-terminal amino acid of the A chain is arginine (Frackelton & Rotman 1980; Gunzler et al., 1982a,b). Since the amino acid sequence of urokinase is known (Gunzler et al., 1982a,b), the conversion of HPA52 into HPA36 can be explained on molecular basis. The Lys-Lys bond at positions 135-136 in the urokinase A-chain is prone to attack by plasmin (Gunzler et al., 1982b; Cederholm-Williams 1984; Sumi & Robbins 1983), resulting in the removal of the kringle and N-terminal region. Cleavage by plasmin at this position therefore results in the formation of a high (HPA52) and a low (HPA36) Mr form. This low Mr form of urokinase (HPA36) is enzymically similar to the large form with respect to retaining plasminogen activating activity (Barlow et al., 1981).

By selective use of these antibodies, it was possible to elucidate the role of plasmin in plasminogen activator expression in cell cultures of COLO 394. These cells secreted an inactive form of a urokinase type activator, which was converted to its active form when plasminogen containing traces of plasmin was present. The monoclonal antibodies employed here to investigate this conversion had absolute specificity compared with similar studies carried out previously using Trasylol.

The anti-plg antibodies completely inhibited the conversion of the proenzyme of plasminogen activator secreted by COLO 394 cells to active enzyme, thereby establishing a definitive role for plasmin in activating the proenzyme of plasminogen activator in this in vitro system.

Plasmin could be an in vivo physiological activator of urokinase proenzyme secreted by both malignant and normal cells because of the abundance of plasminogen in the extracellular fluid and because plasmin can enter the interstitial fluid (Fahey & McLanghlin 1963). Furthermore, only trace quantities of this protease are sufficient to activate urokinase proenzyme. It also appears that one chain urokinase PA (i.e.

proenzyme) is the most abundant/common form of PA produced by malignant human cells as our studies demonstrated (chapter 4).

Other proteases were not tested as activators of the proenzyme of COLO 394 cells but plasmin has been shown to be 20 fold more potent in the activation of pro-urokinase PA in HF cell than is trypsin or thrombin (Eaton et al.,1984). Wun et al., (1982) noted that plasmin was a more effective activator of HEP2 cell urokinase proenzyme PA than was thrombin, plasma kallikrein or factor XIIa.

In the study reported in this chapter, it was shown that plasmin could be a physiological activator of urokinase proenzyme secreted by both malignant and normal cells because plasmin can enter the interstial fluid (Fahey & MeLaughlin, 1963). Studies by others have shown that pro-urokinase from normal human foreskin fibroblasts or HEP 2 cells could be activated by thrombin, plasma kallikrein or factor XIIa, although much less efficiently. Werb & Aggeler (1978) have also shown that tissue kallikrein, activated factor X and a tissue kallikrein-like enzyme from conditioned medium of cultured melanoma cells could convert the single chain t-PA to the two-chain form. Others, as yet undiscovered, exogenous proteases could also activated pro-urokinase PA. The early increase of t-PA activity after injection with live but not lethally irradiated, tumorigenic cells into a mouse model by Colombi et al., (1986) is compatible with the hypothesis that tumorigenic cells might release factors able to activate the proenzyme.

In addition to their use in affinity purification, the availability of these monoclonal antibodies will facilitate the definition of the role of plasmin in the mechanisms of extracellular matrix degradation mediated by inflammatory cells as well as by tumour cells and in other functional processes thought to be mediated by plasmin.

CHAPTER 4

PROENZYME CONTENT OF UROKINASE-TYPE PLASMINOGEN ACTIVATOR IN COLORECTAL CARCINOMAS AND ADENOMATOUS POLYPS: QUANTITATIVE EXPRESSION AND RELATIONSHIP TO DEGREE OF INVASION

INTRODUCTION

Human plasminogen activating enzymes (HPA) exist in at least two forms distinguish from each other by Mr, immunological reactivity (for reviews see Christman et al., 1977; Astrup 1978; Collen 1980; Dano et al., 1985) and genomic composition (Steffens et al., 1982; Edlund et al., 1983; Pennica et al., 1983; Ny et al., 1984). Where as the human PA of Mr 66,000 (HPA66) is mainly found in vascular endothelium and has specialised fibrinolytic activity (Matsuo et al., 1981; Mattsson et al., 1981), the enzyme of 52,000 (HPA52), also known as urokinase, has been identified at sites of physiological and pathological tissue injury (see Chapter 1). Increased levels of HPA52 have been found in extracts of human cancer tissues (Markus et al., 1980; Camiolo et al., 1981; Evers et al., 1982; Corasanti et al., 1980; Elliot et al., 1984; Nagy et al., 1977) and there is evidence that secretion of HPA52 provides an important mechanism by which cancer cells invade and digest the intercellular matrix to permit invasion of normal tissue (see Chapter 1).

Recently, HPA52 has been identified in homogenates of colon cancer tissue (Corasanti et al., 1980; Elliott et al., 1984) and in the supernatant of neoplastic cell lines (Nielsen et al., 1982). Several studies suggest, however, that HPA52 is secreted as an inactive proenzyme which, as shown definitively in Chapter 3, requires proteolytic cleavage by plasmin to express activity. Because previous assays of

HPA52 in tissue homogenates used methods which do not permit the independent assay of active and proenzyme forms, this Chapter reports the development of a method which enables us to quantify the proportion of enzyme present in each form in order to provide a more detailed insight into the relationship between HPA52 expression, its molecular form and the development of colon cancer.

Clinico-pathological observations suggest that the majority of colon cancers arise from pre-existing adenomatous polyps by evolution through identifiable stages of epithelial dysplasia from mild to severe to carcinoma in situ (Muto et al., 1975). The aim of this study was to determine whether the expression of HPA52 is a correlate of malignant transformation in the colonic epithelium by comparing active and proenzyme levels of HPA52 in colon cancers and adenomatous polyps exhibiting different stages of dysplasia with those in metaplastic polyps, which do not predispose to malignant change (Kaye et al., 1973), and histologically normal tissues from the same patients and from control subjects.

MATERIALS AND METHODS

Tissue Samples and Homogenisation

Fresh polyps together with autologous normal mucosa were obtained immediately after removal from patients undergoing colonoscopic polypectomies while normal mucosa, polyps (when present) and carcinoma were also obtained from surgical resection for colon carcinoma. The tissues were dissected free from the muscularis mucosa and washed with Hank's balanced salt solution before they were divided into 2 parallel tissues. Two samples of each tissue specimen were obtained. One was fixed in buffered formalin for histology and Dukes' grading for carcinomas and the other piece was stored frozen at -20° C in homogenisation buffer (50 mM glycine buffer pH 7.8 containing 0.5% Triton X-100). Pedunculated polyps were cut into sections comprising the apical, middle, lower thirds and the stalk and stored frozen in homogenisation buffer at -20° C until assayed.

Twenty seven adenomatous polyps comprising 14 tubular, 9 tubulovillous and 4 villous and 7 metaplastic polyps were processed by conventional histological techniques and their epithelial dysplasia and types were classified according to the criteria described by Konishi and Morson (1982). Polyp size was measured in freshly isolated specimen as the largest diameter. Twenty colon cancers were studied and graded by Dukes' classification (Morson & Dawson 1979).

Thawed materials were later gently blotted, weighed and hand homogenised with a glass homogeniser in the presence or absence of 600 ng of the monoclonal antibody anti-plg 3, using 10 ul of homogenisation buffer per mg (wet weight) of

tissues. After centrifugation for 4 minutes at 8000 g using a Zentrifuge 3200 (Eppendorf), the supernatants were removed for assay and diluted 1:10 with homogenisation buffer prior to assay.

Monoclonal Antibodies

The monoclonal antibodies inhibitory to human plasmin, anti-plg 1 and anti-plg 3 as described in Chapter 3, were used to distinguish the proenzyme from the active enzyme activity of HPA52 in tissues.

Colorimetric-Antibody Assay for both the plasminogen activator proenzyme and total enzyme content of the tissue homogenates were quantified by modification of the colorimetric assay method of Coleman & Green (1981) as described in Chapter 2, section 2.12 using monoclonal anti-plasminogen antibodies as previously described (see Chapter 3).

Effect of DFP on Pro-activator and Activator using SDS-PAGE and Fibrin Overlay Zymogram

The method of Granelli-Piperno & Reich (1978) was used to detect the effect of the various additions and DFP on the plasminogen activator proenzyme and active enzymes of the tissue homogenates, using the fibrin overlay development process after SDS-PAGE as described previously in Chapter 2, Section 2.6.

RESULTS

Zymograms of plasminogen activators in homogenates and the effect of monoclonal anti-plg 1 and DFP.

To determine the nature of plasminogen activators expressed in colonic polyps and colon cancers, the SDS-PAGE fibrin overlay gel system was used to identify the different types of the enzyme and to determine whether they are represented in tumour tissues as proenzymes or in their active forms. As shown previously (Elliott et al., 1984), mucosal homogenates from both adenomatous polyps and colon carcinomas express the ubiquitous 66,000 Mr enzyme of endothelial origin (HPA66) and the urokinase-type plasminogen activator of Mr 52,000 (HPA52). The latter also has an active degradation product of Mr 36,000 (HPA36) (lane 3, figures 4.1 and 4.2) which is formed when HPA52 is incubated in the presence of plasminogen containing traces of plasmin.

Cleavage of the proenzyme of urokinase-type plasminogen activator to its active form was mediated by plasmin (see Chapter 3), and this finding was exploited in the experiments outlined here by using the ability of monoclonal antibodies inhibitory to plasmin and the plasmin inhibitor, Trasylol to inhibit the conversion of the proenzyme to its active form.

As the plasminogen used in the assay contains trace amounts of plasmin, it was preincubated with the monoclonal antibody inhibitory to plasmin, anti-plg 1, for 2 hrs at 37°C before incubation with the tissue homogenates. In other experiments, the plasmin inhibitor, Trasylol was added to both the plasminogen and the homogenates to inhibit in vitro activation of plasminogen by plasmin present in the tissue homogenates.

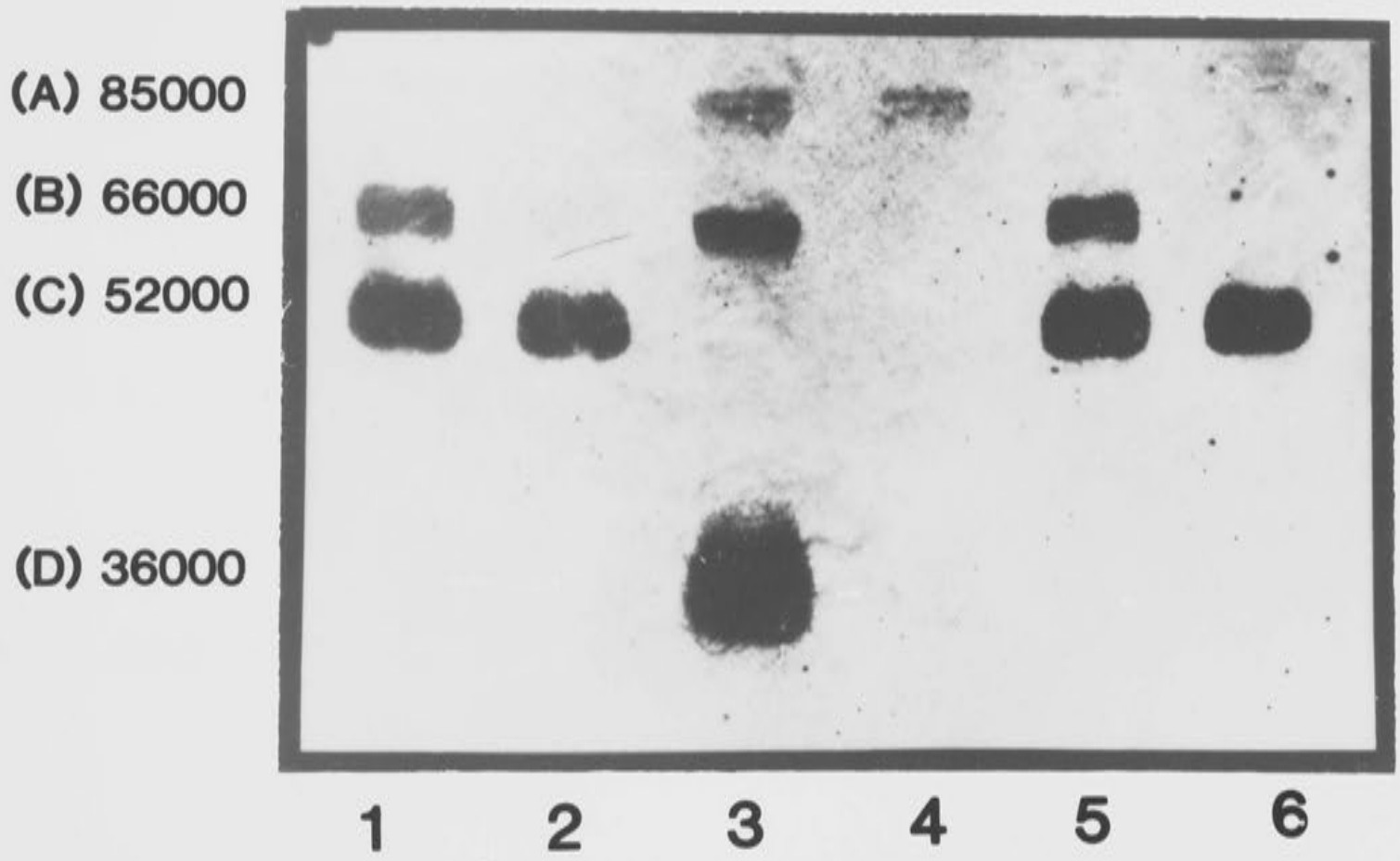


Figure 4.1 SDS-PAGE zymogram analysis of the effect of DFP and anti-plg 1 on the conversion of the plasminogen activators in homogenates of colorectal carcinomas treated with plasminogen pretreated with:- anti-plg 1 (lane 1), anti-plg 1 and treated with DFP (lane 2), assay buffer only (lane 3), assay buffer and treated with DFP (lane 4), Trasylol (lane 5), Trasylol and treated with DFP (lane 6). Zones of lysis are : (A) Mr 85000 (plasmin), (B) Mr 66000 (tissue type PA), (C) Mr 52000 (urokinase-type PA), (D) Mr 36000 (degraded urokinase type PA)

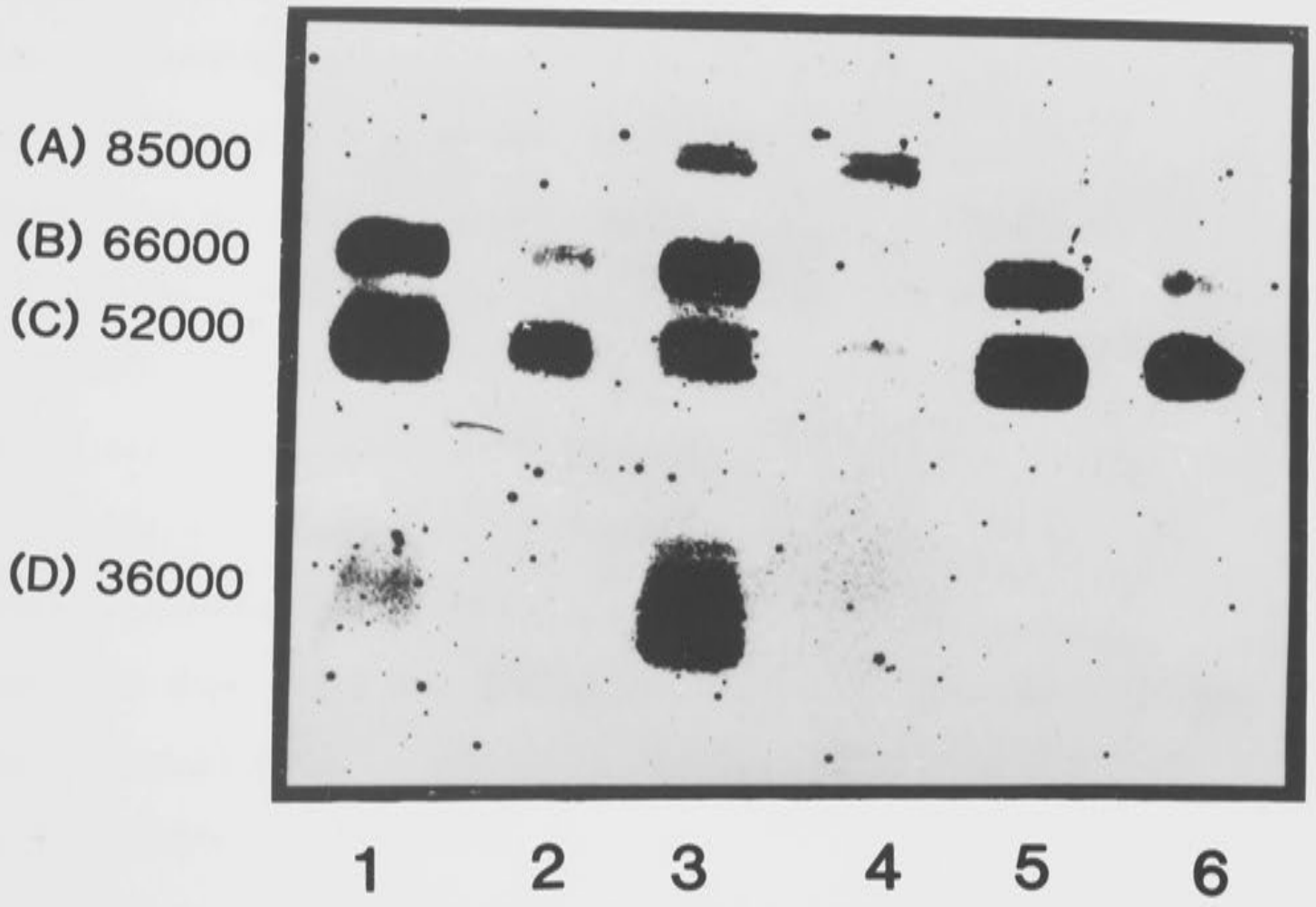


Figure 4.2 SDS-PAGE zymogram analysis of the effect of DFP and anti-plg 1 on the conversion of the plasminogen activators in homogenates of adenomatous polyps treated with plasminogen pretreated with:- anti-plg 1 (lane 1), anti-plg 1 and treated with DFP (lane 2), assay buffer only (lane 3), assay buffer and treated with DFP (lane 4), Trasylol (lane 5), Trasylol and treated with DFP (lane 6). Zones of lysis as in Figure 4.1.

Inhibition of plasmin in the plasminogen used in the assay and in the tissue homogenates prevented the degradation of HPA52 to its active breakdown product of Mr 36,000 (HPA36) as shown in lanes 1 (anti-plg 1) and 5 (Trasylol). To discern the extent to which the plasminogen activators were present in proenzyme form, the tissue incubates were treated with the irreversible inhibitor of serine proteases, diisopropylfluorophosphate (DFP). As shown in lane 2 (Figure 4.1 & 4.2), HPA52 was expressed strongly after DFP treatment in both polyps and colorectal carcinoma, indicating that this enzyme is present predominantly in the proenzyme form, in which the active site is inaccessible to DFP. By contrast, only a small proportion of the HPA66 exists as proenzyme in adenomatous polyps (Zone B, Fig. 4.2, lanes 2 & 6) whereas only the active form of the enzyme is detectable in colorectal carcinoma (Zone B, Fig. 4.1, lanes 2 & 6). In the case of tissue homogenates incubated with untreated plasminogen and DFP, however, all the plasminogen activator bands disappeared (Figs. 4.1 & 4.2, lane 4) indicating that all the plasminogen activator proenzyme present had been activated and consequently the active site was then accessible to DFP.

The plasmin band seen in the gels (Zone A, lanes 3 & 4) was due to the activation of plasminogen by traces of plasmin contained in the plasminogen preparation resulting in activation of the proenzyme to its active form, which in turn generated increasing amounts of plasmin from plasminogen at the location of the band on the SDS fibrin agarose gel. However, when the plasminogen preparation was incubated with either Trasylol or anti-plg 1 antibody, the plasmin band could not be seen since anti-plg 1 antibody and Trasylol bound to the plasmin present and hence prevented the conversion of the proenzyme to its active form, and the subsequent formation of more plasmin.

<u>Plasminogen activator HPA52) content</u>

To determine the expression of plasminogen activator HPA52 in its active and proenzyme forms, and its relationship to the development of malignancy in more detail, quantitative assays were developed. The total content of HPA52 (i.e. proenzyme plus active enzyme) in homogenates of tissues could be measured by the colorimetric assay because the plasminogen substrate used contained trace amounts of plasmin sufficient to activate all of the proenzyme present (see Chapter 3 and also Figures 4.1and 4.2, lane 3). Combination of the colorimetric assay with the selective use of monoclonal antibodies inhibitory to plasmin, allowed both the proenzyme and active enzyme content of tissues to be measured.

The importance of this novel method rests on the ability of the monoclonal antibody not only to inhibit plasmin in the plasminogen substrate but also to rapidly inhibit any plasmin generated by pre-existing active enzyme during the assay. In addition, the antibody used did not inhibit plasminogen activation. Thus when anti-plg 3 antibody was preincubated with plasminogen before the addition of tissue homogenates, the trace amounts of contaminating plasmin present in the preparation were inhibited, thereby preventing the conversion of proenzyme to its active form.

The colorimetric assay, in the absence of added fibrin, measured exclusively HPA52 since negligible HPA66 activity was detected when cell-free culture supernatant of the human melanoma cell line MM-170 (Whitehead & Little 1973) was used as a source of HPA66 (data not shown). Direct hydrolysis of the lysine thioester plasmin substrate by plasminogen-independent neutral proteases in the tissue homogenates did not contribute significantly to color development. Under conditions identical to those used for tissue homogenates, 2 mPloug units (equivalent to about 2.8m Committee on Thrombolytic Agents [CTA] units) of commercial urokinase produced an absorbance of 1.0 at 412 nm. As shown in Figure 4.3, the assay of known

activities of commercial HPA52 (urokinase) was unaffected by the presence of possible inhibitors in the tissue homogenates.

Although the results are expressed as absorbance, nevertheless, this represents the activity in 200 ug of tissue. The homogenisation technique is reproducible since several separate homogenisations of specimens from the same normal mucosa all showed the same enzyme activity. Unfortunately, activity in the sedimented fractions was never assayed but little activity would be expected since the homogenisation technique used is efficient and reproducible.

(i) **Colorectal Carcinomas.** The total and proenzyme plasminogen activator contents of the colon cancer homogenates, graded according to Dukes' classification, and their corresponding normal tissue homogenates are shown in Figures 4.4 and 4.5.

When plasminogen activator activity was assayed in the homogenates of colon cancer tissue, the activity of all cancer grades was 1.22 ± 0.41 for total enzyme and 0.86 ± 0.38 for the proenzyme. By contrast, the corresponding homogenates from histologically normal adjacent mucosa showed markedly lower activity of total enzyme (0.55 ± 0.18) and proenzyme content (0.26 ± 0.11). Thus the mean level of proenzyme was more than three times higher than in normal tissue homogenates. In addition, the proportion of HPA52 proenzyme present in cancer tissue was substantially higher (70 ± 3%) than that found in histologically normal mucosa from cancer bearing colons (47 ± 2%).

In every case analysed, the total activator content of cancer homogenates was very much higher than that of the normal tissue homogenates from the same patient,

even though there were considerable individual variations in plasminogen activator activity. The ratio of absorbances for pairs of cancer to autologous normal mucosal homogenates was 2.09 ± 1.3 (unpaired 2.22) for the total enzyme and increased to 3.94 ± 3.79 (unpaired 3.48) for the proenzyme.

The relationship of HPA52 in colon cancers graded according to their stage of dissemination at surgery using the Dukes' classification was also examined. For total HPA52 activity, each of the Dukes' grades showed significant increases when compared to the activity present in adjacent normal mucosa but no statistically significant difference was detected between the grades (Fig. 4.4). Significant differences were observed however, in the proenzyme contents of the Dukes' grades A, B and C + D (Fig. 4.5).

(ii) **Colonic Polyps** Both total HPA52 activities and the amount of proenzyme were assayed in metaplastic polyps and in adenomatous polyps which were classified as tubular, villous and tubulovillous. As shown in Figures 4.6 and 4.7, the total (0.43 ± 0.15) and proenzyme (0.31 ± 0.1) content of metaplastic polyps were similar to those observed in histologically normal tissue (0.48 ± 0.26 and 0.25 ± 0.17 respectively). By contrast, all three histological types of adenomatous polyp, displayed significantly higher values for both the total and the proenzyme activity. In addition, 68-72 % of the plasminogen activator content in the adenomatous polyps existed as preonzyme.

Neither the size of polyps (range 3 to 45 mm) nor the degree of dyplasia correlated with either the total or the proenzyme content of the polyps (data not shown). However, when the polyps were assayed in sections correponding to the upper, middle and lower thirds and stalk, the highest activities were found in the upper third of the polyps and the lowest in the basal third or stalk as shown in Table 4.1. A

similar gradient was also observed for proenzyme content. In 6 cases where metachronous polyps were present in colons bearing cancers, both the total and proenzyme content were found to be immediate between those for the cancer and those for normal mucosa (data not shown). In the 2 cases of familial polyposis coli patients studied, both the histologically normal mucosa and the polyps expressed high HPA52 activtiy (see arrows Figures 4.6 & 4.7).

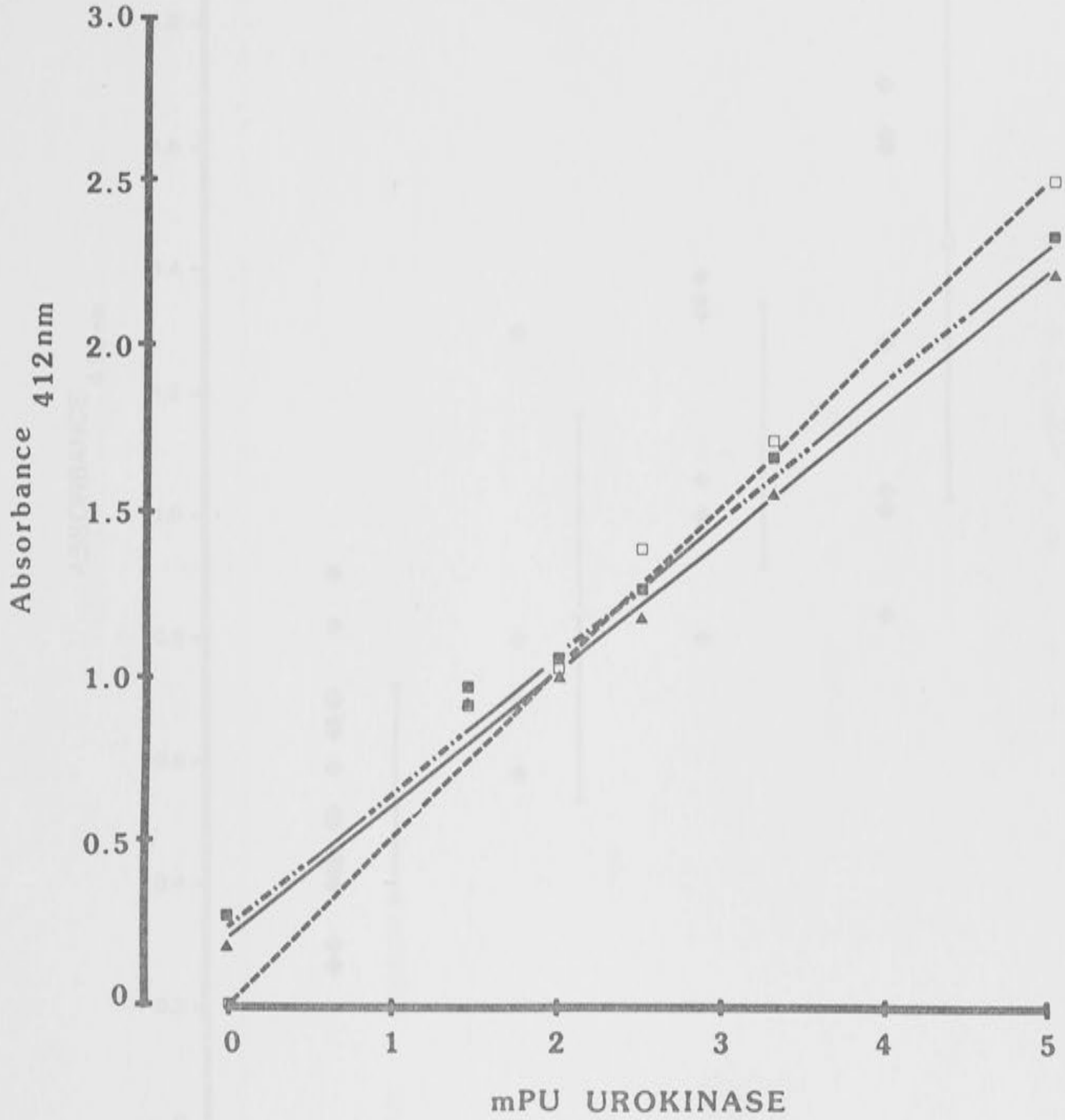


Figure 4.3 Standard curve for the expression of the colorimetric assay in the presence of known concentrations of commercial human urokinase where the urokinase is diluted by (■-·-·-■) anti-plg 3 + normal mucosa homogenate, (▲——▲) normal mucosa homogenate only, (□·····□) assay buffer only.

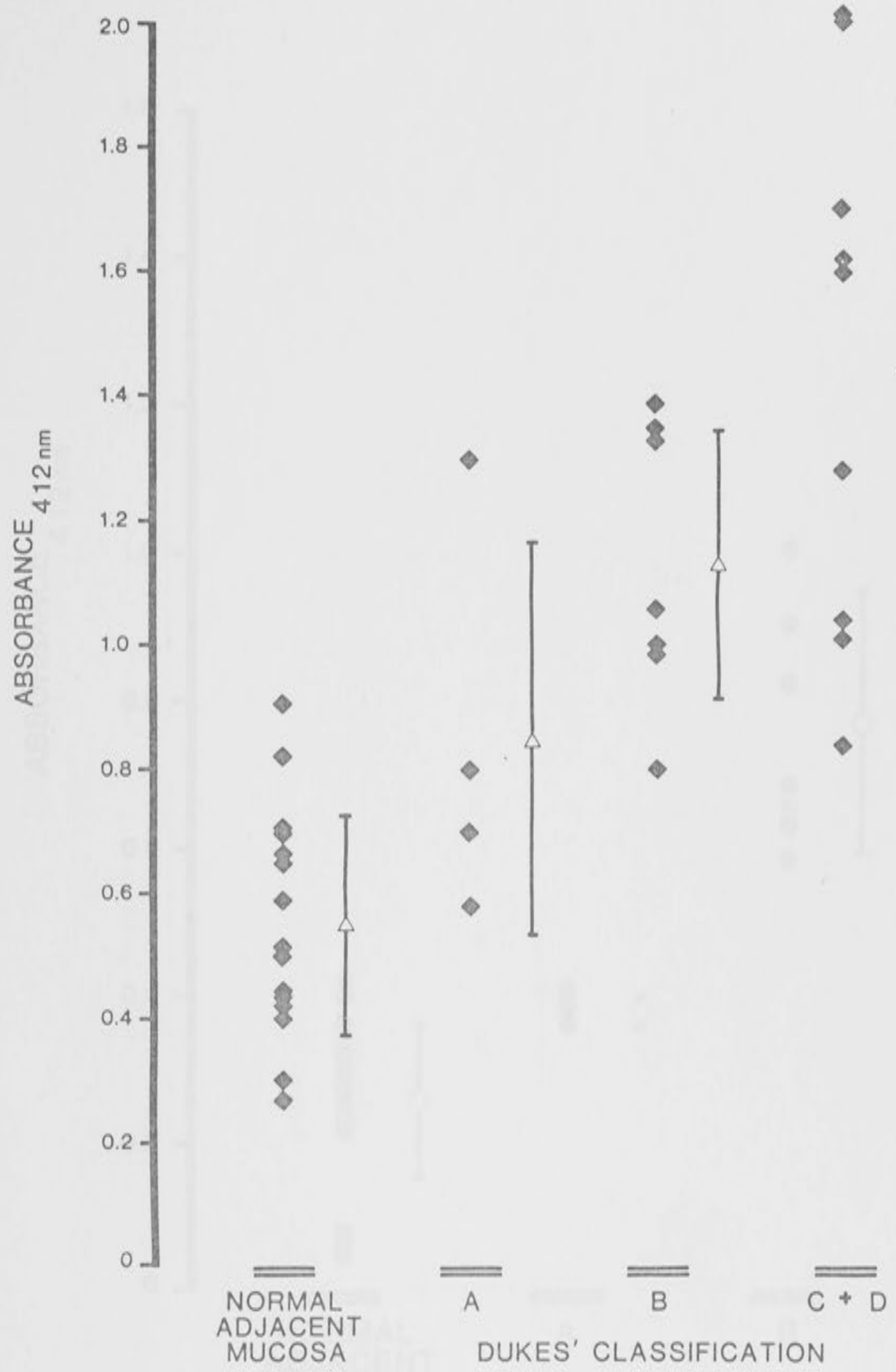


Figure 4.4 The relationship of Dukes' grading of colorectal carcinoma to the total activity of HPA52 in tissue homogenates of colorectal carcinomas and normal adjacent mucosa. Activity is expressed as absorbance at 412 nM. Under the same assay conditions, an absorbance of 1.0 at 412 nM is equivalent to 2mPU of commercial urokinase. (see Figure 4.3). Non-parametric test: Mucosa vs Dukes' B & C + D ($P < 0.01$). Dukes' A vs Dukes' C + D ($P < 0.05$)

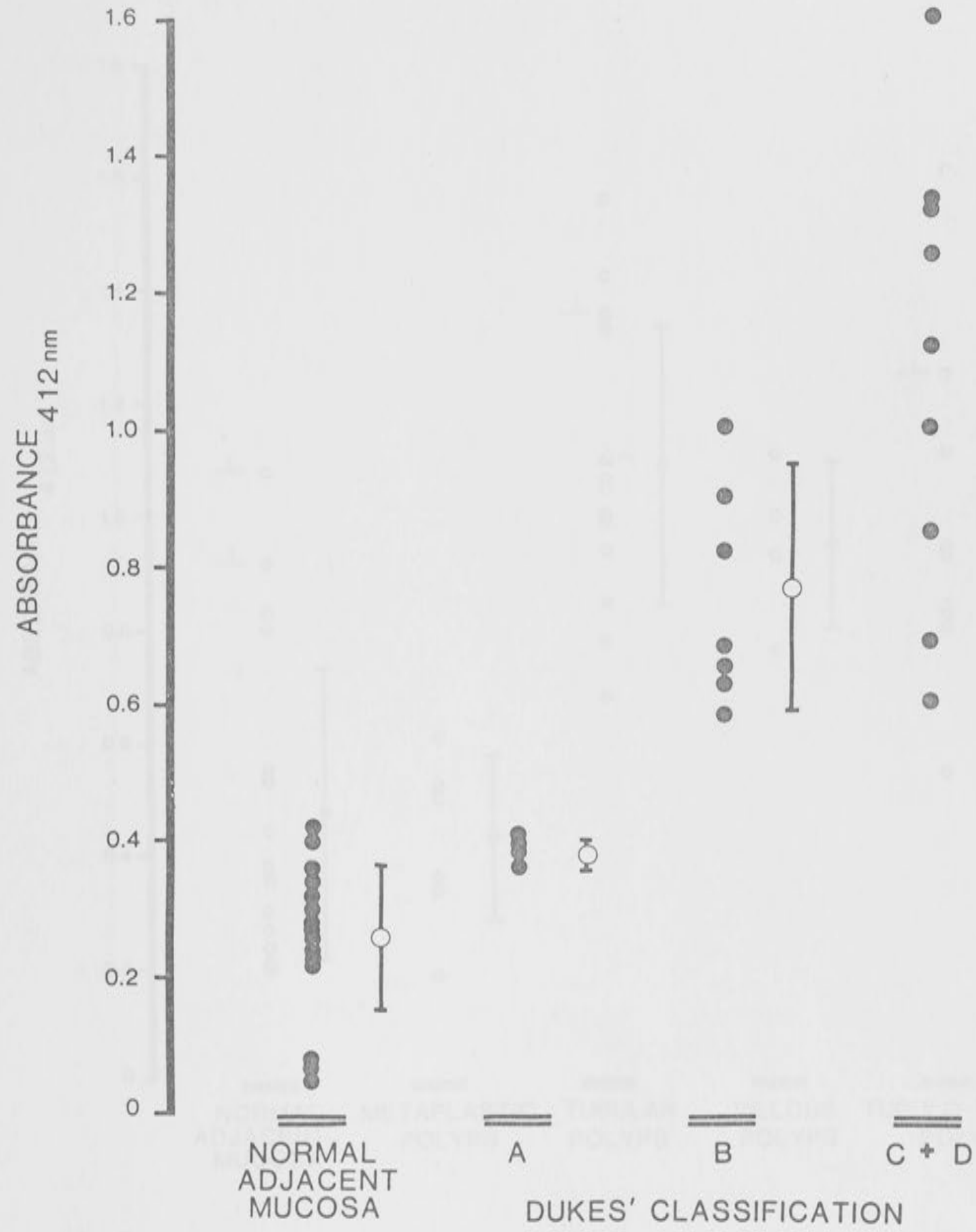


Figure 4.5 The relationship of Dukes' grading of colorectal carcinoma to the proenzyme content of HPA52. Activity is expressed as in Figure 4.4. Non-parametric test: Mucosa vs Dukes' B & C + D ($P < 0.01$). Mucosa vs Dukes' A ($P < 0.05$). Dukes' A vs Dukes' B, C + D ($P < 0.01$). Dukes' B vs Dukes' C + D ($P < 0.05$).

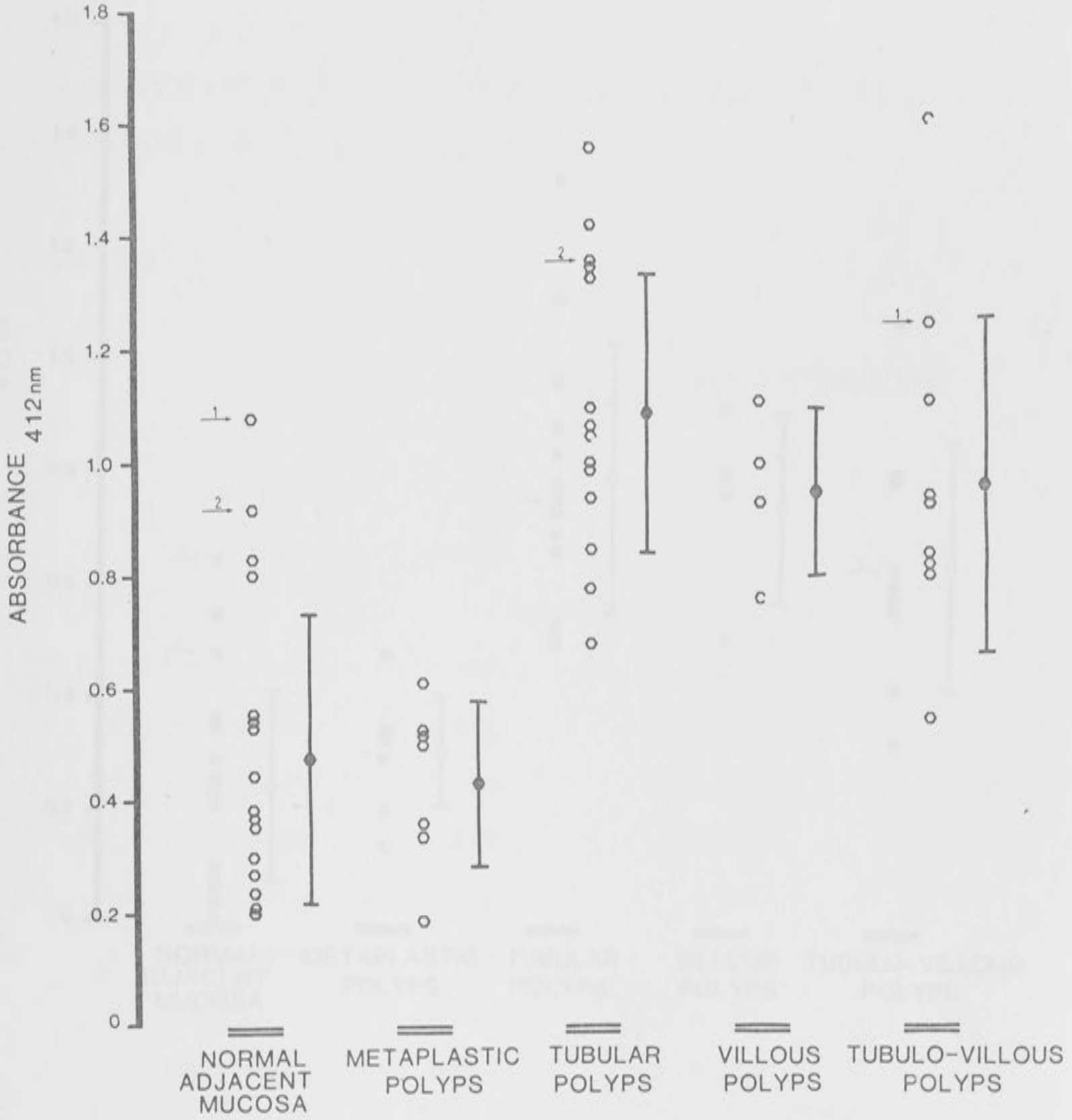


Figure 4.6 Total HPA52 activity expression in colonic adenomatous polyps, metaplastic polyps and normal adjacent mucosa. ←— 1,2 denotes polyposis coli patients. Mucosa vs tubular and tubulo-villous ($P < 0.01$). Mucosa vs villous ($0.05 > P > 0.01$). Metaplastic vs tubular, tubulovillous, and villous ($P < 0.01$ in all cases).

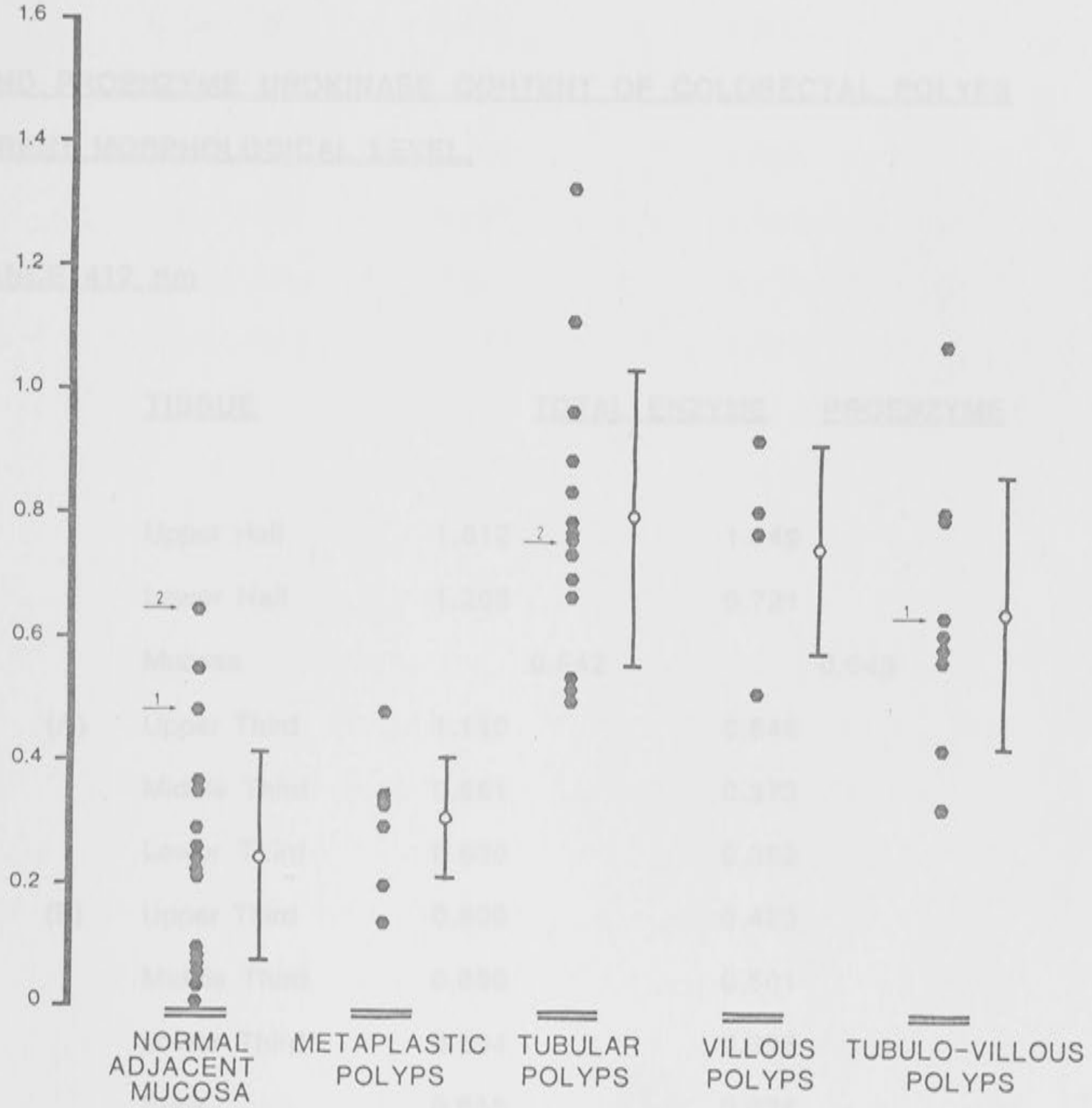


Figure 4.7 Proenzyme content of HPA52 in colonic adenomatous polyps, metaplastic polyps and normal adjacent mucosa. ←— 1,2 denotes polyposis coli patients. Mucosa vs tubular, villous and tubulovillous ($P < 0.01$ in all cases). Metaplastic vs tubular, villous and tubulovillous ($P < 0.01$ in all cases).

TABLE 4.1

TOTAL AND PROENZYME UROKINASE CONTENT OF COLORECTAL POLYPS AT DIFFERENT MORPHOLOGICAL LEVEL.

ABSORBANCE 412 nm

CASE		TISSUE	TOTAL ENZYME	PROENZYME
L.M.R.		Upper Half	1.612	1.049
		Lower Half	1.203	0.721
		Mucosa	0.542	0.043
H.E.	(A)	Upper Third	1.110	0.648
		Middle Third	0.661	0.373
		Lower Third	0.630	0.362
	(B)	Upper Third	0.800	0.423
		Middle Third	0.880	0.501
		Lower Third	0.694	0.398
		Stalk	0.615	0.226
W.M.		Upper Half	0.543	0.327
		Lower Half	0.523	0.302
		Stalk	0.267	0.114
J.B.		Upper Half	0.683	0.503
		Lower Half	0.631	0.475
		Stalk	0.479	0.341
J.J.		Upper Half	1.560	1.336
		Lower Half	0.929	0.801

	Stalk	0.200	0.071
L.M.	Upper Half	1.177	0.802
	Lower Half	0.916	0.599
	Stalk	0.780	0.563
J.M.	Upper Third	0.993	0.537
	Middle Third	0.830	0.562
	Lower Third	0.137	0.020

DISCUSSION

Using a sensitive colorimetric assay under conditions which were highly favorable to HPA52 proenzyme activation and therefore measured total enzyme content (i.e. proenzyme and active enzyme), this study demonstrates marked increases in the level of HPA52 activity in both adenomatous polyps and colon cancer, compared to paired specimens of histologically normal mucosa. Similar findings have been reported by Corasanti and co-workers (1980) who used an azocaseinolytic assay but whether the activity measured in tissue homogenates represents active enzyme, proenzyme and active eznyme, or active enzyme with substantial contribution from the proenzyme is unclear. Although apparently conflicting results have been reported by several groups, who found higher levels of plasminogen activator activity in normal mucosa and in the non malignant elements of cancers than in cancer tissues, these workers used a fibrinolytic assay which measures both HPA52 and the ubiquitous tissue plasminogen activators (Franklin et al., 1978; Szozepanski et al., 1982; Tissot et al., 1984). In addition, the fibrinolytic assay is more sensitive to the latter type of plasminogen activator which is also found at much higher levels in normal mucosa and submucosa than in cancers (Kohga et al., 1985). The results of greatly increased HPA52 activity reported in this Chapter are therefore at variance with those obtained by the fibrinolytic assay because the latter assay essentially measures tissue plasminogen activators and does not adequately reflect HPA52 enzyme activity.

The selective inhibition of plasmin using a monoclonal antibody inhibitory to human plasmin, has enabled the proenzyme and active enzyme content of HPA52 in tissue extracts to be determined for the first time. The results reported in this chapter demonstrate that a significant proportion of the plasminogen activator in adenomatous polyps and in colorectal carcinomas exists in the proenzyme form.

Moreover, all the normal tissues studied also contain low levels of HPA52 proenzyme. In the absence of anti-plg 3 which blocks plasmin, there was a considerable increase in the activity of the colorimetric assay, confirming that the predominant form of HPA52 in adenomatous polyps and colorectal carcinomas was the proenzyme. In the absence of added fibrin in the colorimetric assay, the type of plasminogen activator activity could only be attributed to the HPA52 and not to the ubiquitous HPA66 enzyme produced by endothelial cells. The recent immunohistochemical studies of Kohga et al., (1985) using antibodies specific for urokinase supports this conclusion.

The finding described in this chapter that the majority of the plasminogen activator was present as pro-HPA52 in both the colorectal carcinomas and adenomatous polyps was further substantiated by the results of DFP treatment followed by SDS-polyacrylamide electrophoresis and fibrin overlay gel zymography. Recent immunoblotting studies by Skriver et al., (1984) in the mouse Lewis lung carcinoma model have also shown that the majority of MPA48, the mouse analogue of HPA52, is present as the proenzyme. Taken together, the data suggest that the majority of plasminogen activator of the urokinase-type exists in tissues as the proenzyme. In support of this conclusion, the proenzyme forms of virus-transformed murine cells in culture (Skriver et al., 1982), and of human cells of neoplastic origin (Nielsen et al., 1982) have been well documented and have been shown to be the major enzyme form of plasminogen activators produced, and secreted, during culture.

Andreasen et al. (1985) using fractions of t-PA from supernatants of human melanoma cell lines (Bowes) purified by a single step monoclonal antibody showed the incorporation of the active site reactant DFP into pro-t-PA but not into pro-urokinase PA. In the data shown in this chapter and by Stephens (personal communication), DFP was incorporated in similar manner to that seen in the pro-urokinase PA. Whether this represents different properties in the t-PA in the

colorectal tissues compared to the Bowes cell lines is unclear. Electricwala & Atkinson (1985) describe the purification of two epithelial plasminogen activators from guinea pig keratocytes and human breast epithelial cells and showed that while these activators are immunologically identical to, and functionally behave like, human t-PA, their properties showed differences in isoelectric point, molecular mass and N-terminal amino acid sequence from melanoma PA. Furthermore, the melanoma Bowes cell line is not necessarily representative of the corresponding cell types in the intact organism with respect to production and release of plasminogen activators, because selection of cells i.e. phenotypic changes may take place when cultures are established, and because they may be differences in the presence of regulatory factors between the microenvironments of cells in the intact organism and in culture.

It is possible that t-PA as well as urokinase PA, could also have a role in tumour invasion and metastasis. Colombi et al., (1986) reported the sudden increased stimulation of t-PA type activity following injection of tumorigenic cells and the gradual increase of urokinase PA in plasma of Balb/C mice given injections of non metastasizing cell lines. The t-PA may function to maintain the vascular tree free of fibrin allowing the extravasation of tumorigenic cells to form metastases.

The finding of similar proenzyme and total PA content in benign tumour (metaplastic polyps) with that of histologically normal mucosa is not surprising. The production of plasminogen activators per se does not induce malignant behaviours in cells, but possible defects in the control of enzyme activity (or the activation of the secreted proenzyme) at the pericellular level may account for several of the growth properties of individual cells in the heterogenous population of tumour cells (Dexter & Calabresi 1982). The decisive difference between cancer cells and normal cells with regard to the mechanisms of tissue degradation is related to its regulation. Loss of actin organisation after treatment with tumour promoters in cultured, histologically

'late-stage' cells from human pre-neoplastic colon adenoma was connected with increased production of PA by these cells, whereas the actin structure of 'early-stage' preneoplastic adenoma cells not producing PA was unaffected by the promoters used (Friedman et al.,1984). This might indicate that the observed loss of actin organisation linked with production of plasminogen activators marks the transition from non-invasive early-stage tumours to invasively growing late-stage tumours. Plasmin is thought to play a crucial role in this transition. This observation could now be confirmed with the use of the monoclonal antibodies inhibitory to human plasmin (chapter 3).

In the study of the transplantable invasive and metastasizing murine Lewis lung tumour (Skriver et al.,1984), it was consistently found that the tumour contained both urokinase PA immunoreactivity and extractable urokinase PA enzyme activity. The most intensive staining was observed in areas with invasive growth and degradation of normal tissue, while in large parts of the tumours, where no invasion and degradation of normal tissue occurred, there was no demonstrable urokinase PA immunoreactivity. No immunocytochemical studies of the occurrence of urokinase PA in benign tumours and of t-PA in malignant and benign tumours have been reported.

Proteolytic activation of proenzyme to active enzyme has been definitively demonstrated to be mediated by plasmin (see Chapter 3) since the monoclonal antibodies used have absolute specificity compared to Trasylol. The extent of this conversion in vivo, is clearly important for the extracellular expression of plasminogen activator.

The role of plasmin in invasiveness by cancer cells has also been suggested by immunofluorescence studies using anti-plasminogen antibodies which stained the contours of tumour foci and tumour cells (Burtin et al., 1985). Plasmin may therefore

be required to activate the proenzyme of HPA52 in vivo thereby equipping tumour cells with a mechanism for their migration through restraining tissue structures both to invade and metastasize. The expression of HPA52 activity and the consequent initiation and amplification of a proteolytic cascade in the intercellular matrix in vivo is therefore likely to be influenced by the availability of proteases, such as plasmin, in the extracellular fluids.

Studies of HPA52 proenzyme levels in colon cancers show significantly increased levels for the Dukes' B and C + D stages when compared to the Dukes' A stage and normal mucosal levels. Although the numbers are small, there appears to be a gradient of proenzyme activity corresponding to the extent of invasion of the tumour. In addition, the proportion of proenzyme to active enzyme was substantially higher in the advanced stages B and C + D (69 & 76%) compared to Dukes' A (45%) and normal tissue (47%). A similar correlation showing that the higher the HPA52 content in cancer tissue, the worse is the prognosis has also been reported recently for breast cancer (Yang et al., 1982). Recent reviews have summarized systematic studies that also showed that the mean PA content of human tumours of the lung, colon, prostate and breast was higher than in the presumed normal tissues of origin (Corasanti et al.,1980; Markus et al.,1980; Evers et al.,1982; Camiolo et al.,1984). In the case of the breast tumours, using an assay carried out in the absence of fibrin, Evers et al. (1982) concluded that urokinase PA was the main form of PA in human breast carcinomas. O'Grady et al., (1985) showed that t-PA is the dominant form in benign breast tumours with urokinase PA only contributing approximately 10% of the total PA activity. However, all the malignant groups (primary carcinomas, axillary node metastasis and recurrences) contained significantly higher urokinase PA activity compared with the benign samples.

Further studies however are required to determine the significance of these findings to the invasive process of malignancy. In this context, immunohistochemistry of the murine Lewis lung carcinoma suggests that the mouse analogue of HPA52 is expressed principally at areas of invasive cancer growth and associated normal tissue degradation (Skriver et al., 984) whereas in the human carcinoma HEp3 model, antibodies which inhibit HPA52 activity prevent metastasis but not the primary tumour growth (Ossowski et al., 1983). Thus HPA52 may subserve different functions at different phases of cancer developmwent.

Significantly increased levels of HPA52 enzyme activity were found in all three types of adenomatous polyps when compared to metaplastic polyps and normal colonic mucosa. The majority of HPA52 was present as proenzyme as was found for the invasive stages of colon carcinoma. These findings support the histopathological data as to the premalignant nature of the adenomatous polyps and offer a biochemical correlate of epithelial cell transformation. However, these findings suggest a mechanism by which the hallmark of the malignant process, invasion of normal tissue, may occur. Although the numbers are small, no correlation was found between the degree of epithelial dysplasia and the level of HPA52 expression. The variations in the degree of dysplasia within single polyps however, made the study of the relationship between dysplasia and HPA52 levels very difficult. Largers numbers of more uniformly dysplastic polyps are required to establish or refute such a relationship.

When the site of HPA52 expression in polyps was studied, there was significantly greater proenzyme activity in the upper or apical third of the polyp than in the basal third or stalk. This may suggest that the site of biochemical transformation to increased HPA52 levels occurs first in the most exposed part of the polyp and correlates with recent histopathological studies showing that the earliest site of malignant transformation in adenomatous polyps usually occurs in the apical

third (Haggitt et al., 1985). Both histologically normal colonic mucosa, and adenomatous polyp tissue from two patients with familial polyposis coli, displayed high levels of HPA52. These finding are at variance with those of Corasanti et al., (1980) who reported low levels of plasminogen activator activity in both non-polypoid and cancer tissue from polyposis coli patients. This discrepancy may be due to the relative insensitivity of the azocaseinolytic assay used in that study.

Other enzymes that have been found to be elevated in colorectal cancer have included collagenases (for a review see Woolley 1982) and β-hexosaminidase (Plucinsky et al.,1986). In the report by Plucinsky et al. (1986), the authors found that the mean β-hexosaminidase activity and specific activity in crude supernatants of malignant colon tissue were significantly increased compared to those in the adjacent, uninvolved normal tissue. This increased enzymatic activity could be involved in tumour invasion of adjacent tissues and the metastatic spread of colorectal cancer, since β-hexosaminidase is a degradative enzyme that can hydrolyze glycoconjugate components of membranes and extracellular matrices.

CHAPTER 5

CORRELATION AND QUANTITATION OF PROENZYME OF UROKINASE TYPE PLASMINOGEN ACTIVATORS IN INFLAMMATORY BOWEL DISEASE

INTRODUCTION

Despite intensive research, the aetiopathogenesis of ulcerative colitis and Crohn's disease remains unknown. In the absence of a defineable aetiological agents(s) [e.g. bacterium, virus, toxin], interest has centred on elucidating the mechanisms of tissue injury in the inflammatory lesion of the mucosa so as to provide opportunities for more rational therapeutic intervention.

As mentioned in Chapter 1, plasminogen activators have been implicated in the mediation of tissue damage.

The aim of the experiments reported here was to determine whether the HPA52 enzyme is selectively expressed at the site of the inflammatory injury in ulcerative colitis and Crohn's disease.

MATERIALS AND METHODS

Patients

Biopsies of intestinal mucosa from 23 patients obtained at colonoscopy or immediately after intestinal resection were studied. The diagnosis of Crohn's disease, ulcerative colitis and infectious colitis were established by accepted clinical, microbiological, radiological, colonoscopic and histological criteria. The Crohn's group comprised 9 patients, 1 male and 8 females age range 14 to 70 years. Of the 4 in the ulcerative colitis group, there were 2 males and 2 females age range 33 to 63 years.

Biopsies were obtained from one patient suffering from Campylobacter jejuni colitis and from one patient suffering from an acute dysentery where no pathogen was isolated. In a further three cases, colonoscopies were performed on patients suffering from intermittent abdominal pains and diarrhoea but no definitive diagnosis was apparent at colonoscopy or from the histology of the colonic biopsies.

Multiple biopsies were taken from each patient and where possible involved and apparently uninvolved areas of mucosa were sampled from the same patient. Disease activity was assessed by colonoscopic appearances and by the histology of mucosal biopsies. The patients were then classified as active or quiescent.

Uninflamed mucosa was obtained from colonoscopies and intestinal resection performed on two groups of patients. The first comprised patients suffering from diarrhoea attributed to the irritable bowel syndrome or diverticular disease in whom the intestinal mucosa appeared uninflamed, stool microscopy showed no evidence of pus cells or red blood cells and the histologically appearances of the biopsy specimens were normal. The second control group consisted of tissues from uninvolved colonic or ileal mucosa obtained from polyp or cancer-bearing colons.

Mucosal tissues, dissected free of muscularis were stored frozen at -20°C in homogenisation buffer (50 mM glycine buffer pH 7.8 containing 0.5% Triton X100) until assayed. Thawed tissues (wet tissues) were later weighed and homogenised as previously described in Chapter 4 of Materials and Methods.

Colorimetric-antibody assay of plasminogen activators

The modified colorimetric assay of Coleman & Green as described in Chapter 2, section 2.12 with the selective use of monoclonal antibodies inhibitory to plasmin was used to quantify the proenzyme and total plasminogen activator content of the tissue homogenates. The results for the plasminogen activator activities were expressed after correction for the direct hydrolysis of the lysine thioester plasmin substrate used in the

assay by plasminogen-independent proteases in the tissue extracts by subtraction of absorbances obtained in an identical system to which no plasminogen and antibody had been added. This usually accounted for less than 5% of the total activity.

SDS-PAGE Fibrin Overlay Gel

The method of Granelli-Piperno & Reich as described in Chapter 2, section 2.6 was used to assess the effect of DFP on proactivator and activator as described in detail in Chapter 2, section 2.18.

RESULTS

SDS-PAGE zymogram analysis of the effect of monoclonal anti-plg 1 and DFP on the plasminogen activators in the tissue homogenates.

SDS-polyacrylamide gel electrophoresis (SDS-PAGE) with a fibrin overlay gel containing plasminogen was used to determine the types of PA present in the mucosal tissue homogenates from IBD and to ascertain the presence of inactive proenzyme PA.

As shown in chapter 3, inactive proenzyme of HPA52 can be converted to its active form by limited proteolysis involving the action of plasmin. Hence, the absolute specificity and ability of the monclonal antibodies, anti-plg 1, to selectively inhibit the plasmin mediated conversion of prourokinase to its active form was exploited. Proenzyme activation was monitored by DFP treatment followed by SDS-PAGE/fibrin overlay.

A preparation of plasminogen containing traces of plasmin was preincubated with or without anti-plg 1 antibody for 2 hr at 20° C before the incubate was added to the tissue homogenates and incubated at 37° C for a further 45 mins, after which the mixtures were incubated in the presence or absence of DFP for 6 hr at 20° C.

Figure 5.1, lane 5 shows the typical enzyme pattern produced by the IBD homogenate. A major band of Mr 52,000 daltons (HPA52) was present. Trasylol, a rapid and potent inhibitor of plasmin was added to plasminogen containing traces of plasmin and the tissue homogenates in these incubations to prevent in vitro activation as a result of the presence of plasmin in both the tissues and the plasminogen preparation. When this incubate was treated with DFP, an irreversible inhibitor of serine proteases, the HPA52 band was unaffected (lane 6). This result indicates that the enzyme of HPA52 existed predominantly in the tissue as its inactive proenzyme form, whose active site is inaccessible to DFP.

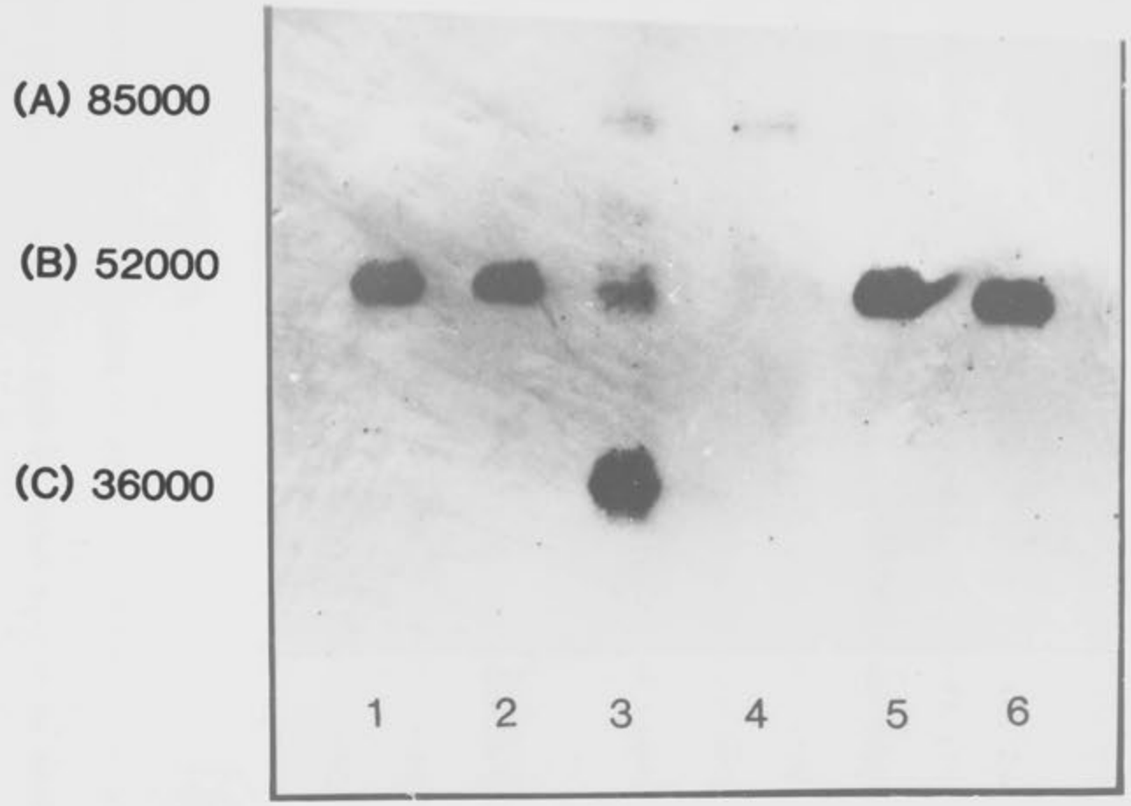


Figure 5.1 SDS-PAGE zymogram analysis of the effect of DFP and anti-plg 1 on the conversion of the plasminogen activators in homogenates of inflammatory bowel disease (Crohn's disease) treated with plasminogen pretreated with:- anti-plg 1 (lane 1), anti-plg 1 and treated with DFP (lane 2), assay buffer only (lane 3), assay buffer and treated with DFP (lane 4), Trasylol (lane 5), Trasylol and treated with DFP (lane 6). Zones of lysis are:- (A) Mr 85000 (plasmin), (B) Mr 52000 (urokinase-type PA), (C) Mr 36000 (degraded urokinase type PA).

By contrast, when plasminogen preincubated in the absence of anti-plg 1 antibody or Trasylol was added to likewise untreated tissue homogenate, a substantial proportion of the HPA52 (Zone B) was converted to an active form with a lower Mr of 36,000 daltons (HPA36; Zone C, lane 3 cf. lanes 1 and 5 when either anti-plg 1 antibody or Trasylol was present). As shown in lane 4, when the same homogenate was treated with DFP, both the HPA52 and HPA36 bands disappeared as expected, since the proenzyme form had been activated to its active form and the active site had now become accessible to DFP.

When anti-plg 1 antibody was used to block the plasmin mediated conversion of the proenzyme form to its active counterpart, however, a single major band of HPA52 in similar pattern to that displayed in Lane 5 was seen (lane 1). Again, when the same homogenate was treated with DFP, the same lysis band of the same Mr was still present indicating that the enzyme existed predominantly in tissue as an inactive proenzyme (lane 2).

The plasmin band with a Mr 85,000 daltons seen in lanes 3 & 4 (Zone A) was the result of the activation of plasminogen by HPA52 formed from the action of trace amounts of plasmin in the plasminogen preparation acting on the inactive proenzyme form of HPA52. The plasmin at the gel surface diffused into the agarose/fibrin containing overlay gel causing a lysis band. However, when the plasminogen preparation was incubated with either Trasylol or anti-plg 1 antibody, no plasmin band could be seen since both anti-plg 1 antibody and Trasylol bound to and inhibit the trace plasmin present, and therefore also prevented the conversion of the proenzyme form to its active form.

<u>Plasminogen Activator Content of IBD Tissue Homogenates.</u>

By the selective use of the monoclonal antibodies inhibitory to plasmin (see chapter 3), it was possible to use the Coleman & Green assay to quantify the proenzyme and total enzyme content of IBD tissues as described in chapter 2, section 2.12. The novelty of this assay method depended upon the ability of the antibodies to inhibit quickly any plasmin formed in the assay and on the absence of inhibition of plasminogen activation by the antibodies.

The monoclonal antibody inhibitory to the plasmin present in the preincubation with plasminogen prior to the addition of diluted tissue homogenates, inhibited these trace amounts of plasmin and thereby prevented the conversion of the HPA52 proenzyme to its active form.

The level of HPA52 activity was assessed for diseased, quiescent and unaffected mucosal tissues from the IBD intestine. The level was determined for both proenzyme and total enzyme (i.e. proenzyme plus active enzyme) as shown in Figures 5.2 and 5.3.

A significantly higher level of HPA52 was found in diseased mucosa from both ulcerative colitis and Crohn's disease patients when compared to paired normal biopsies (Table 5.1) or normal resected specimens from the same patients or to mucosa tissues from quiescent patients ($P < 0.01$). Quiescent tissue samples gave a mean activity of 0.480 ± 0.170 for total enzyme content and a mean activity of 0.250 ± 0.15 for proenzyme content (Figures 5.2 and 5.3), which was in the range determined for normal mucosa from carcinoma-bearing colons. The mean total and proenzyme content for the uninvolved mucosa (0.45 ± 0.20) and (0.230 ± 0.15) respectively was almost identical with that of the quiescent mucosa (0.480 ± 0.170) and (0.250 ± 0.15).

When the PA activity was measured in the disease/involved mucosa, the mean activity was 1.10 ± 0.39 for the total enzyme content and a corresponding mean value of 0.80 ± 0.27 for the proenzyme content.

In both the ulcerative colitis and Crohn's disease, the presence of active disease corresponded to increased levels of PA activity. The area of the bowel where the tissue

displayed the most severe disease, as assessed histologically, was also the area with the highest level of active PA. The majority (average 70%) of the HPA existed as proenzyme consistent with the earlier findings in the colorectal carcinomas and polyps (Chapter 4).

Table 5.2 shows the results for the two disease controls groups studied; infectious colitis and a group of undiagnosed chronic diarrhoea patients who were suspected of having Crohn's disease but in whom no definite diagnosis could be made. Although the numbers are small, the results indicate that the increase in both the total and proenzyme content of HPA52 found in inflamed mucosal tissues is not a finding specific to ulcerative colitis or Crohn's disease.

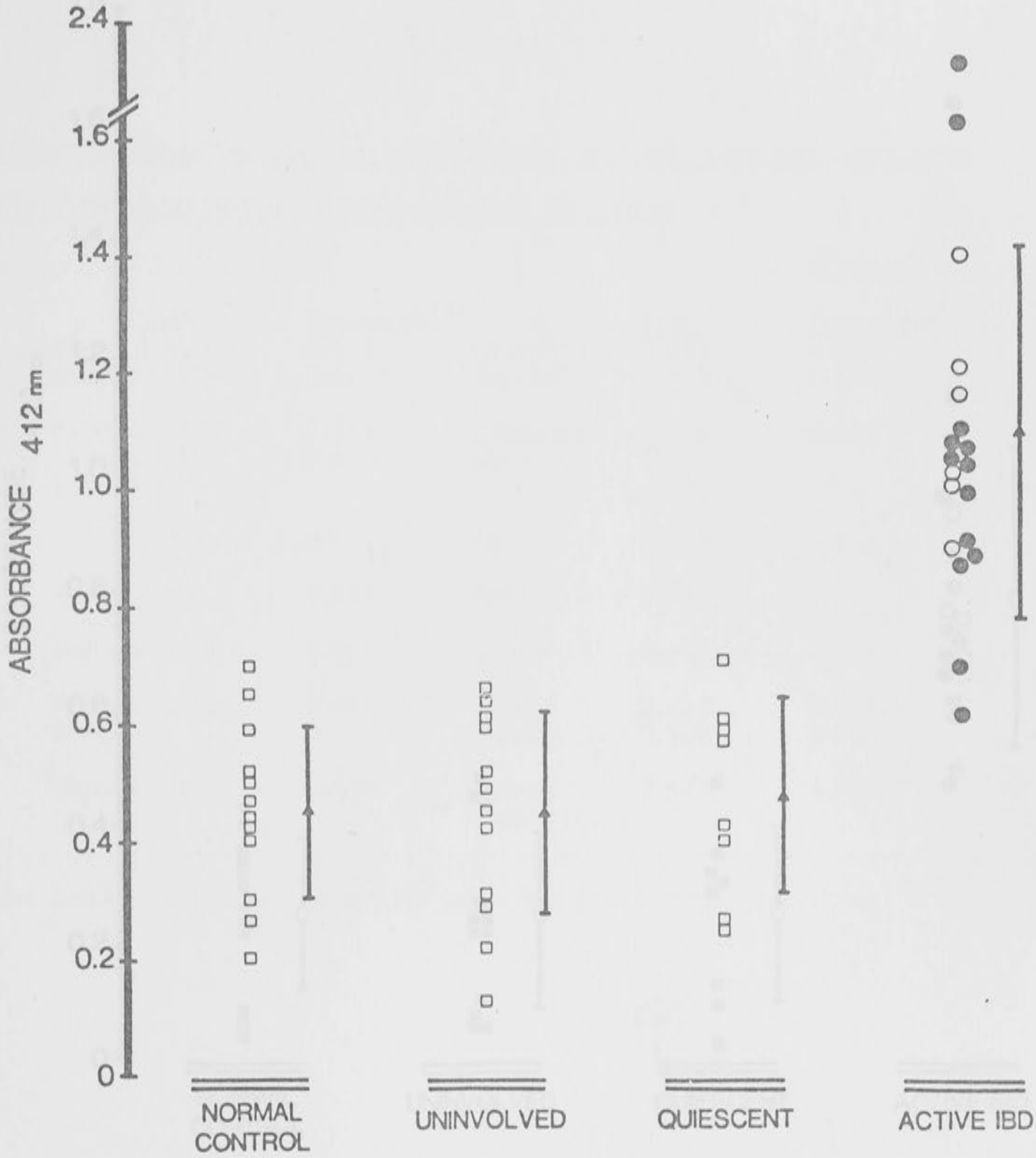


Figure 5.2 The relationship of inflammatory bowel disease to the total activity of HPA52 in tissue homogentaes of active IBD, Quiescent, uninvolved adjacent mucosa and normal mucosa control. Activity is expressed as in Figure 4.4. In the active IBD, symbol ● denotes Crohn's disease while ○ denotes ulcerative colitis. Uninvolved vs active IBD ($P < 0.01$). Quiescent vs active IBD ($P < 0.01$).

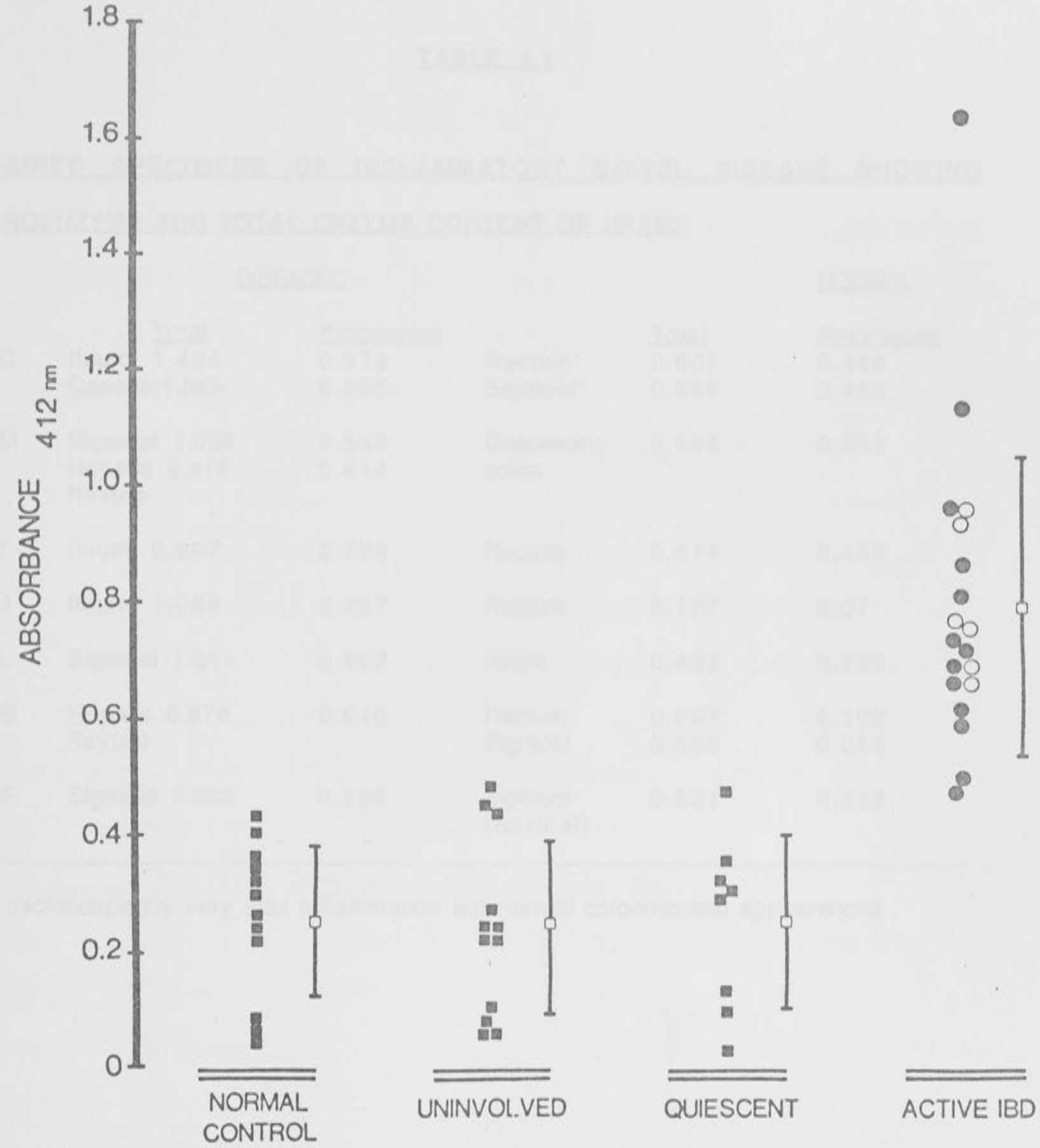


Figure 5.3 The relationship of inflammatory bowel disease to the proenzyme content of HPA52 in tissue homogenates of active IBD, Quiescent, uninvolved adjacent mucosa and normal mucosa control. Activity is expressed as in Figure 4.4. In the active IBD, symbol ● denotes Crohn's disease while ○ denotes Ulcerative colitis. Uninvolved vs active IBD ($P < 0.01$). Quiescent vs active IBD ($P < 0.01$).

TABLE 5.1

PAIRED SPECIMENS OF INFLAMMATORY BOWEL DISEASE SHOWING PROENZYME AND TOTAL ENZYME CONTENT OF HPA52

	DISEASED			NORMAL	
	Total	Proenzyme		Total	Proenzyme
SC	Ileum 1.404	0.973	Rectum*	0.601	0.449
	Caecum1.029	0.688	Sigmoid*	0.655	0.466
JM	Sigmoid 1.096	0.598	Descending colon	0.666	0.252
	Hepatic flexure 0.914	0.614			
JT	Ileum 0.907	0.729	Rectum	0.614	0.485
LJ	Ileum 1.058	0.857	Rectum	0.127	0.07
LL	Sigmoid 1.041	0.802	Ileum	0.453	0.229
DB	Hepatic flexure 0.878	0.646	Rectum	0.297	0.102
			Sigmoid	0.309	0.058
RF	Sigmoid 0.993	0.695	Sigmoid (normal)	0.521	0.228

* microscopically very mild inflammation but normal colonoscopic appearances

TABLE 5.2

MUCOSAL SPECIMENS OF INFLAMMATORY DISEASE CONTROLS AND UNDIAGNOSED CHRONIC DIARRHOEA SHOWING TOTAL AND PROENZYME CONTENT OF HPA52

Inflammatory Disease Controls

		Total	Proenzyme
JL*	Ileum	1.037	0.689
	Transverse colon	1.141	0.668
	Sigmoid colon	1.230	0.776
	Rectum	0.926	0.599
WG**	Rectum	0.430	0.005

Undiagnosed Chronic Diarrhoea associated with abdominal pain

		Total	Proenzyme
CM	Ileum	0.468	0.350
	Caecum	1.216	1.064
SM	Ileum	0.608	0.565
	Caecum	0.898	0.411
	Transverse colon	0.904	0.609
HR	Ileum	0.482	0.296

* - Infective organism unknown ** - Campylobacter colitis

DISCUSSION

The roles of the plasminogen activators in various biological processess have been documented in many studies. Indeed, a number of recent findings, especially in the area of cancer, have contributed substantially to a better understanding of the biochemistry of the complex proteolytic cascade of reactions leading to the formation of plasmin and the participation of PA in a diverse range of tissue degradation processes.

The association between PA and inflammation has always been guided by the hypothesis that the release of PA from inflammatory cells migrating to the site of inflammation, caused the proteolysis of components of the extracellular matrix and hence played a decisive role in the degradation of normal tissues leading to injury.

This Chapter reports for the first time, quantitative studies of the expression of HPA52 and its different forms (i.e. active enzyme and/or their inactive proenzyme form) that were expressed in chronic IBD.

The SDS-PAGE fibrin overlay gel was used to determine the types of PA and to ascertain the presence of proenzyme forms, while the sensitive colorimetric assay was used to quantify the amounts of HPA52 in the tissue homogenates.

The use of the sensitive colorimetric assay with the selective addition of a monoclonal antibody inhibitory to human plasmin (see Chapter 3) demonstrated that there was a significant increase in the level of HPA52 in the chronic inflammatory tissues in comparison with uninvolved tissues or tissues from quiescent patients ($P<0.01$).

The majority of the HPA52 in chronic IBD was presented to the extracellular milieu in its inactive form. Although it is impossible to define the cellular origin of the urokinase PA found in IBD using the enzyme assays, nevertheless, it is intriguing to speculate that the source of the urokinase PA might be from the activated macrophages or

from the epithelial cells. A definitive answer would certainly have to involve the use of immunocytochemistry. Both Crohn's disease and ulcerative colitis are chronic inflammatory disorders of the intestine of unknown aetiology. Macrophages are prominent in the inflammatory cell infiltrate in both conditions, but in Crohn's disease, a hallmark of the condition is the granuloma, where macrophages are particularly conspicuous. Evidence (Tanner et al.,1984) has been presented to indicate that in IBD, circulating monocytes are in an activated state and recuitment of these monocytes to the sites of tissue injury is evident both on histological grounds and on kinetic data. Therefore, the release of PA could just be one of the secretory products of the activated macrophages. Alternatively, the studies on pemphigus indicate that cells obtained from the normal dermis can be induced to secrete plasminogen activators when treated with the autoantibody that is found in the circulation of pemphigus patients.

To date, no studies have been performed on isolated human colonic epithelial cells to determine its ability to secrete HPA52 when either stimulated or induced. A report by Electricwala & Atkinson (1985) described the purification of two epithelial plasminogen activators from guinea pig keratocytes and human breast epithelial cells. These authors showed that while these activators are immunologically identical to, and functionally behave like, human t-PA, their properties showed differences in isoelectric point, molecular mass and N-terminal amino acid sequence from melanoma PA. These finding are not representative of all epithelial cell PA since the COLO 394 cell reported in Chapter 3 showed the secretion of HPA52.

On the other hand, the secretion of PA has been shown to correlate well with macrophage activation (Unkeless et al., 1974a) suggesting that the induction of the synthesis and secretion of PA may well depend on a number of sequential metabolic changes.

No quantitative data are available on the proportions of macrophages present in IBD tissue and in colon carcinoma tissue and no attempt was made to isolate macrophages from these tissues in order to measure pro PA and PA since it was not possible to obtain sufficient tissues to undertake the task.

The expression of the HPA52, whether in term of proenzyme or total enzyme, appears to be uninfluenced by the therapeutic administration of steroids in the therapy of these patients.

Previous studies of enzyme markers of inflammatory cells in the IBD using rectal biopsies showed that patients with ulcerative colitis have decreased activity of the lysosomal marker enzymes, N-acetyl-β-glucosaminidase, and acid phosphatase in both active and quiescent colitis. β-glucuronidase was only significantly reduced in acute colitis (O'Morain et al.,1984). Danovitch et al. (1972) have found decreased concentrations of arylsulphatases and β-glucuronidase but surprisingly, increased concentrations of acid phosphatases in biopsy tissue homogenates from patients with ulcerative colitis. This anomolous result probably was attributed to the fact that only 4 patients were examined. In another study by O'Morain et al. (1983), results showed that homogenates of rectal biopsies from patients with rectal Crohn's disease and active ulcerative colitis have increased levels of vitamin B_{12} binding protein, lysozyme and myeloperoxidase, specific markers for neutrophils. In contrast, in Crohn's disease when the rectum is spared, the activity of the neutrophils markers was similar to control values.

Monocytes isolated from subjects with IBD have higher N-acetyl-β-glucosaminidase and β-glucuronidase enzyme activities than corresponding controls (Ganguly et al.1978; Mee & Jewel 1980; Doe et al.,1980) and secretion of plasminogen

activators by monocytes is also markedly enhanced in patients with Crohn's disease and ulcerative colitis and this correlates with disease activity (Doe & Dorsman,1982).

The results reported here established an association between the HPA52 and the inflamed mucosa of ulcerative colitis and Crohn's disease, since biopsies from patients in remission or from uninvolved mucosa failed to show an increase in the level of the PA activity. These studies established that HPA52 may represent an important pathway in the pathogenesis of mucosal tissue injury and could therefore offer fresh insights into the pathogenesis of ulcerative colitis and Crohn's disease. The PA could promote injury through the generation of plasmin in the extracellular milieu resulting in generalised proteolysis, collagenolysis, complement activation, kinin generation and the initiation of the coagulation cascade.

Hence, future therapy could involve the use of substances modulating HPA52 production or plasmin generation and might therefore constitute a valuable contribution to current therapy.

CHAPTER 6

SUBCELLULAR FRACTIONATION OF HUMAN INTESTINAL MACROPHAGES: EVIDENCE FOR A LYSOSOMAL NON-SPECIFIC ESTERASE NOT FOUND IN MONOCYTES

INTRODUCTION

Intestinal macrophages are a prominent cell type in the normal intestinal mucosa (Golder & Doe1983). They derive from blood monocytes and display many of the diverse functional capabilities associated with macrophages in both immune and inflammatory networks (Reiko & Werb 1984).

The state of differentiation of macrophages is reflected in the composition and properties of the plasma membrane (Edelson 1981; Zuckerman & Douglas 1979). Particularly, the role of ectoenzymes in the human intestinal macrophages has received little attention. Before the commencement of the work reported in this chapter, both plasminogen activator (see Chapter 1, section 1.7) and non-specific esterase had been reported to be expressed as ectoenzymes on related cells. The work of Bozdech & Bainton (1981) concluded from cytochemical studies that human monocyte alpha-naphthyl butyrate esterases was a plasma membrane ectoenzyme and that there was negligible intracellular activity. By contrast, the granule activity seen cytochemically in the light microscope was interpreted as lysosomal and designated "non-specific acid esterase" (Parwaresch et al.,1981). A non-lysosomal cytoplasmic granular distribution of 2-naphthyl thiol acetate activity was seen in human monocytes under the electron microscope (Kim et al.,1982)

Hence, subcellular fractionation of human intestinal macrophage (HIMØ) was performed as reported in this chapter to define the localisation of the plasminogen activators and non-specific esterases. The isolation and characterisation of plasma membranes from purified preparations of intestinal macrophages using subcellular fractionation techniques also allowed the analysis of the localisation and properties of biochemical differentiation markers and comparison with the results previously reported for blood monocytes (Fayle et al., 1985). These may help to elucidate the changes that characterize the differentiation state of intestinal macrophages and their adaptation to the mucosal environment.

MATERIALS AND METHODS

Isolation and purification of human intestinal macrophages, surface labelling of plasma membrane using wheat germ agglutinin, nitrogen cavitation and subcellular fractionation were performed as described in Chapter 2, section 2.20-2.23.

Analytical Methods

An Atago type 500 refractometer was used to measure sucrose density. The binding of ^{125}I-labelled WGA was detected by counting each fraction in a Packard Auto-Gamma 500 counter.

Methods of assay for the following enzymes are described in detail in Chapter 2, section 2.1. β-hexosaminidase (β-N-acetyl-D-galactosaminidase EC 3.2.1.53) was measured according to Leaback and Walker (1961) using 4-methylumbelliferyl-2-acetamido-2-deoxy-β-D-galactopyranoside as substrate while β-Glucuronidase (EC 3.2.1.31) was assayed by the fluorimetric method of Mead et al., (1955). Arylsulfatase C (EC 3.1.6.1) was detected by the method of Canonico et al., (1978). Catalase (EC 1.11.1.7) was detected by oxidation of the fluorescent compound scopoletin (7-hydroxy-6-methoxy-coumarin) by H_2O_2 in the presence of horseradish peroxidase. Galactosyltransferase or UDP galactose: β-D-N-acetylglucosaminyl-glycoprotein galactosyltransferase (EC 2.4.1.38) was assayed according to Baxter and Durham (1979) using ovomucoid rather than desialo, degalacto-fetuin as acceptor (Fayle et al., 1985). Lactate dehydrogenase (EC 1.1.1.27) was assayed by the method of Kornberg (1954), using 0.75 mM sodium pyruvate and 0.33 mM NADH. Leucine-2-naphthylamidase (EC 3.4.11.2) was detected using 0.2 mM L-leucine 2-naphthylamide and deamination of [^{14}C] tyramine was used to detect monoamine oxidase (EC 1.4.3.4).

Alpha-naphthyl acetate esterase (EC 3.1.1) was determined at pH 7.5 by the method of Laug et al., (1983b) for gradient fractions and according to Yam et al., (1971) for cytocentrifuge preparations. 5'-Nucleotidase (EC 3.1.3.5) was assayed by the method of Edelson and Cohn (1976) and NAD^+ nucleotidase (EC 3.2.2.5) was assayed by a modification of the method of Nakazawa et al., (1968).

Plasminogen activator (urokinase EC 3.4.21.31) was assayed by the colorimetric assay method of Coleman & Green (1981) as described in Chapter 2, section 2.12.

Immunofluorescence was carried out in 96 well trays according to Loken & Stall (1982) using $F(ab')_2$ goat anti-mouse IgG (heavy + light chain; Cappel Laboratories, Cochranville, P.A, USA) as second antibody as described in Chapter 2, section 2.24.

SDS-PAGE Fibrin Overlay zymogram

The SDS-PAGE fibrin overlay gel method of Granelli-Piperno & Reich (1978) as described in Chapter 2, section 2.6 was used to determine the types of PA present in the pooled PA enriched membranes fractions and to ascertain the presence of inactive proenzyme PA using anti-plg monoclonal antibody. The samples applied to an 11% SDS electrophoresis gel under non-reducing conditions consisted of either plasminogen alone in assay buffer or plasminogen (10 ul, 1 ug) preincubated with monoclonal anti-plg 1 antibody for 2 hr at 20^o C and further incubated with 30 ul of pooled PA enriched membranes fractions at 37^o C for 45 min before stopping with SDS sample buffer to give a final volume of 100 ul. After electrophoresis, the gels slabs were washed in Triton X-100 and developed by contact lysis of fibrin in an agarose fibrin overlay gel.

Monoclonal antibody 25F9 & SC11 were the gifts of Prof. C. Sorg (Dept. Experimental Dermatology, Universitats-Hautklinik, D-4400 Munster, FRG) and Dr. R. I. Fox (Research Institute of Scripps Clinic, La Jolla, CA, USA) respectively. GAP-

8.3, L-243, 5E9, 63D3 & 4F2 were obtained from the American Type Culture Collection (Rockville, MD,USA). MO2 was purchased from Coulter Electronics, anti-CR3 & OKIa were from Ortho Diagnostics systems. Anti-cytokeratin 18 was from Boehringer Mannheim.

RESULTS

Isolation and Purification of Highly Enriched Human Intestinal Macrophages by Centrifugal Elutriation

In order to isolate human intestinal macrophage plasma membranes, a highly purified population of human intestinal macrophages was prepared by centrifugal elutriation followed by centrifugation over Percoll. This procedure resulted in an 8.5 fold enrichment and a mean purity of 85 $\pm$ 3%. Of the total disaggregated lamina propria cells, between 10-15 % were macrophages. Similar recoveries of intestinal macrophage at lower purity were recently reported by Beeken et al., (1984). Morphology, May-Gruenwald Giemsa staining, non-specific esterase staining, adherence and surface antigenic phenotype were used to verify the purity and suitability of the intestinal macrophages for plasma membrane preparation.

Contaminating cell types after Percoll treatment included large lymphocytes, residual granulocytes and occasionally, aggregated erythrocytes. Epithelial cell contamination, assessed by immunofluorescence using a monoclonal antibody against cytokeratin 18 (Debus et al., 1982), a constituent of colonic epithelial cells, was negligible.

Not all of the cells characterised morphologically as macrophages contained characteristic non-specific esterase staining; esterase positivity was found in about 75% of the cells in the final preparation. A similar proportion of cells displayed HLA-DR antigens (detected using the OKIa monoclonal antibody). Approximately 26% expressed the mature macrophage marker 25F9 (Hume et al., 1986), and 36%

expressed the MO2 antigen (Todd et al., 1981) at low levels. Only 5% of the macrophage preparation expressed the CR3 receptor found on monocytes, immature macrophages and residual granulocytes. Over 90% of the cells expressed the common leukocyte antigens detected by GAP-8.3 & SC 11, 80% expressed the transferrin receptor (5E9), and 84% & 41% carried the monocyte antigens detected by 4F2 (Haynes et al., 1981) and 63D3 (Ugolini et al., 1981) respectively.

Identification of plasma membrane after subcellular fractionation

Both ectoenzyme activities and trace radiolabelling were investigated for the identification of plasma membrane on the sucrose gradients. Nitrogen cavitation was used to disrupt the macrophage preparation which had been trace-labelled with the ^{125}I-labelled wheat germ agglutinin (WGA) and washed free of the unbound lectin.

The membrane fractions recovered from sucrose gradients after centrifugation to equilibrium showed that the distribution of bound WGA (Fig 6.1A) paralleled that of the ectoenzyme activities 5'-nucleotidase (Fig 6.1B) and Mn^{2+}-stimulated leucine 2-naphthylamidase (Fig 6.1D). The mean peak concentration of the plasma membrane determined from 16 separate preparations was at a density of 1.126 $\pm$ 0.005 g.cm^{-3}, in close agreement with that of the plasma membrane markers obtained for blood monocytes (Fayle et al., 1985). However, unlike human blood monocytes, human intestinal macrophage expressed very low levels of NAD^+ nucleotidase (data not shown). The plasma membrane 5'-nucleotidase activity was abolished by 0.1 mM $ZnSO_4$ but was unaffected by 10 mM sodium tartrate (data not shown). The proportions of each marker found in the density range 1.10-1.17 g.cm^{-3} were: 51% for 5'-nucleotidase. 39% for WGA and 38% for leucine 2-naphthylamidase.

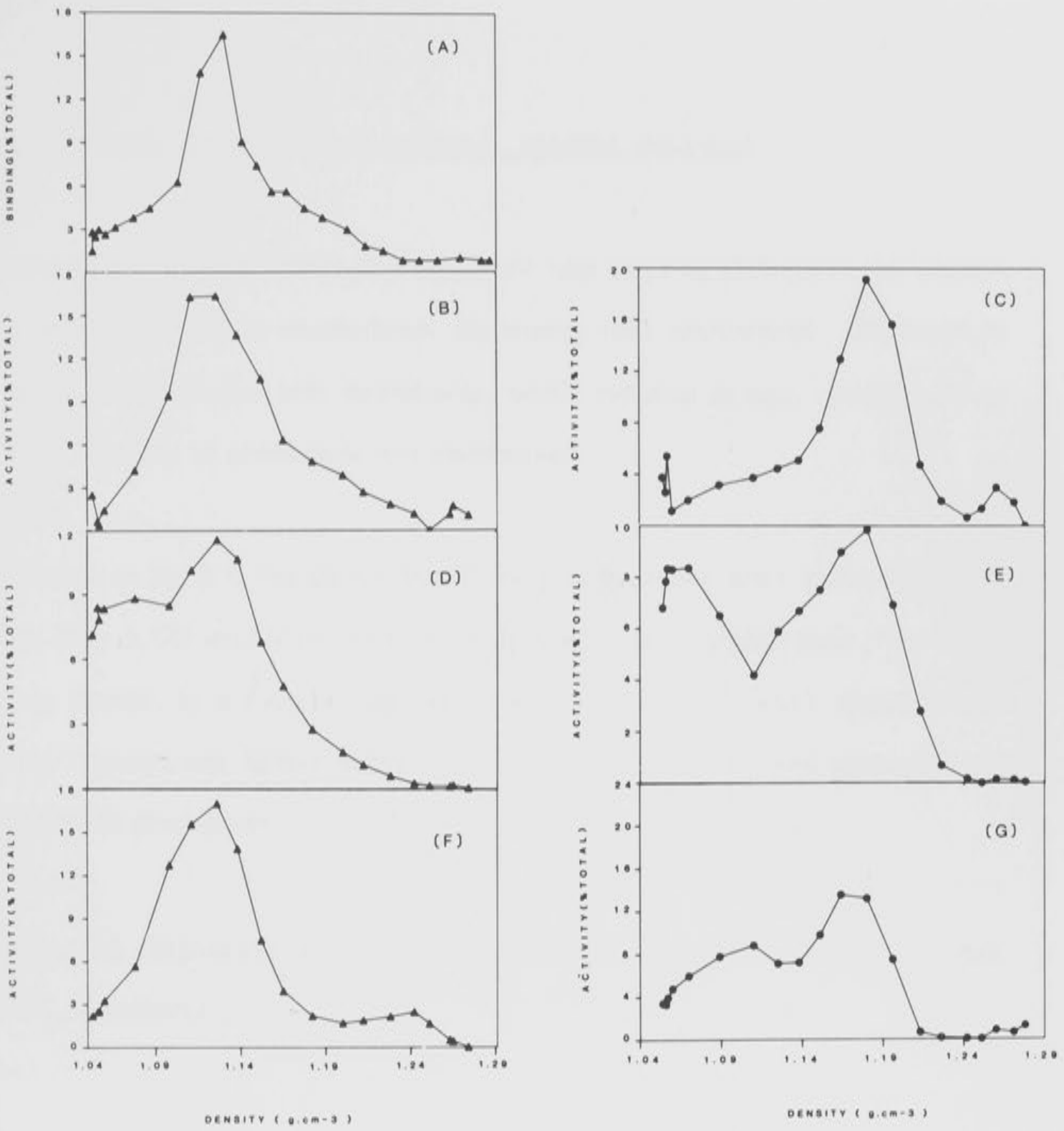


Figure 6.1 Plasma membrane marker distribution on sucrose gradients.

The 1000 x g supernatants from disrupted human intestinal macrophage were centrifuged to equilibrium on sucrose gradients either without (A,B,D,F ▲——▲) or with (C,E,G ●——●) pretreatment with digitonin. Results are plotted as the activity or binding in the fraction, as a percentage of the total recovered on the gradient, versus the density of the fraction. (A) ^{125}I-WGA (B,C) 5'-nucleotidase (D,E) leucine 2-naphthylamidase (F,G) plasminogen activator. Recoveries (sum of the activity of fractions with respect to the activity of the 1000 x g supernatants) are: 90 ± 10% for 5'-nucleotidase; 85 ± 5% for leucine 2-naphthylamidase; 107 ± 11% for plasminogen activator without digitonin treatment. In digitonin treatment, recoveries are: 87 ± 6% for 5'-nucleotidase; 75 ± 3% for leucine 2-naphthylamidase; 85 ± 4% for plasminogen activator.

Effect of digitonin on plasma membrane marker distribution

Digitonin, the cholesterol-binding detergent was used to distinguish the plasma membrane from intracellular membranes containing less cholesterol. Digitonin is believed to form a complex with cholesterol, which remains in situ, resulting in an increase in the density of cholesterol-rich membranes.

As shown in Fig 6.1, the distribution of the plasma membranes ectoenzymes 5'-nucleotidase (Fig. 6.1C) and Mn^{2+}-stimulated leucine 2-naphthylamiodase (Fig. 6.1E) was similarly altered, to a fraction with adensity of 1.18 $g.cm^{-3}$ when digitonin was added to the homogenate before subcellular fractionation, in amounts approximately equimolar with the cholesterol.

Distribution of Intracellular membranes and Cytosol markers after subcellular fractionation

Cytosol:

The distribution of the cytoplasmic enzyme lactate dehydrogenase on the gradient was largely that of a non-sedimentable enzyme (Fig 6.2A). However, about 8% of the activity penetrated into the heavier density region of the gradient (greater than 1.20 $g.cm^{-3}$).

Golgi apparatus:

The marker for the Golgi apparatus, UDP galactosyltransferase (Strous et al., 1983) was found predominantly in a density peak of 1.111 $\pm$ 0.005 $g.cm^{-3}$ (Fig. 6.2C)

almost superimposed on the plasma membrane. An estimated 71% of the activity was found in the density 1.10-1.20 $g.cm^{-3}$. Triton X-100 was used in this assay for the full expression of this activity. Digitonin treatment resulted in only a slight increase in the average density of this peak (Fig. 6.2D) to 1.125 $g.cm^{-3}$, indicating the presence of a low cholesterol concentration in the Golgi membranes.

Endoplasmic recticulum

Arylsulfatase C, a marker enzyme for endoplasmic recticulum in monocytes (Fayle et al., 1985), was distributed predominantly in a peak of density 1.21 $g.cm^{-3}$ (Fig. 6.2B), a density somewhat greater than that found for human blood monocytes (Fayle et al., 1985). Triton X-100 was included in this assay to allow for the full detection of this activity. An estimated 57% of the activity was found in the density range 1.171-1.231 $g.cm^{-3}$.

Mitochondria

The mitochondrial outer membrane enzyme, monoamine oxidase was found in a peak of a distinctly heavier fraction than the plasma membrane (1.16 $g.cm^{-3}$) but overlapping it (Fig. 6.2E). Digitonin treatment resulted in a slight increase in the density of this peak (Fig. 6.2F).

Peroxisomes

Most of the catalase activity was distributed throughout the free fraction and the lowest densities of the gradient (Fig. 6.2G). A small proportion of the catalase activity was found in a peak of density 1.18-1.22 $g.cm^{-3}$ (mean 1.196 ± 0.006 $g.cm^{-3}$).

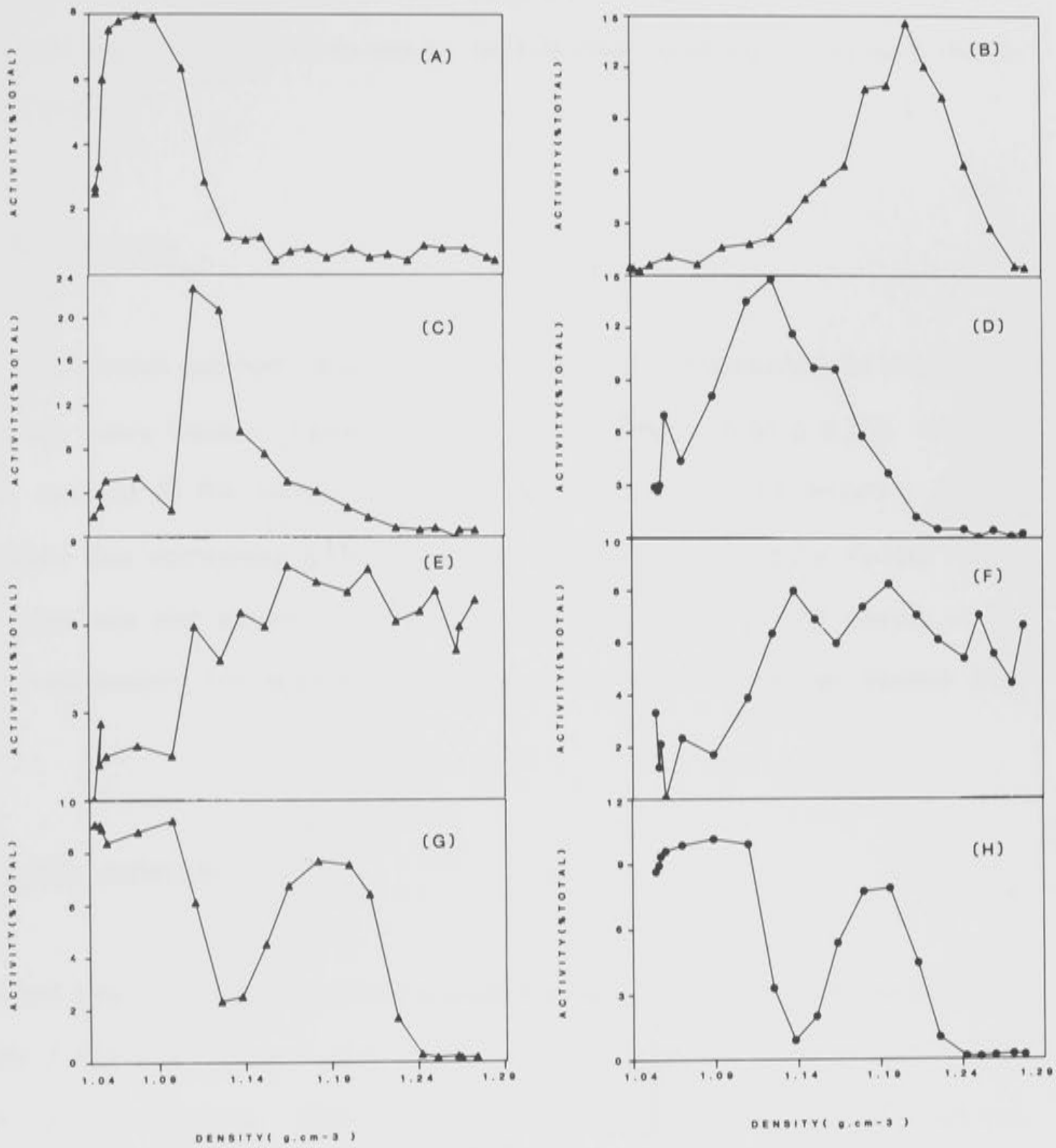


Figure 6.2 Distribution of markers of cytosol and intracellular membranes on sucrose gradients. The 1000 x g supernatants from disrupted human intestinal macrophage were pretreated with either with (D,F,H ●—●) or without (A,B,C,E,G ▲—▲) digitonin before centrifugation to equilibrium on sucrose gradients. Results are plotted as described in Figure 6.1. Markers, with their subcellular location in brackets were (A) lactate dehydrogenase (cytosol); (B) arylsulfatase C (endoplasmic reticulum); (C,D) galactosyltransferase (Golgi); (E,F) monoamine oxidase (mitochondria); (G,H) catalase (peroxisomes). Recoveries are: 98 ± 7% for (A); 129 ± 10% for (B); 89 ± 8% for (C); 85 ± 9% for (D); 98 ± 10%; (E) 94 ± 4% for (F); 120 ± 10% for (G); 115 ± 15% for (H).

Digitonin did not alter the profile of this distribution (Fig. 6.2H). Higher digitonin concentrations would be expected to disrupt peroxisomes, releasing the catalase (Amar-Costesec 1974).

Lysosomal vesicles

The lysosomal acid hydrolases β-glucuronidase and b-hexosaminidase were found in a relatively heavy fraction of peak density 1.21 $g.cm^{-3}$ (Fig. 6.3A & 6.3B). Nitrogen cavitation resulted in the release of substantial amounts of free enzyme, and β-glucuronidase was sometimes found in addition in a fraction of similar density to the plasma membrane (not shown). Digitonin treatment did not alter the density of the lysosomal membranes, but released most of the enzymes into the free fraction (Fig. 6.3C).

Non-Specific esterase

About 12% of the alpha-naphthyl acetate esterase activity was found in a fraction of density 1.165-1.242 $g.cm^{-3}$ (Fig. 6.3D). Over 67% of the esterase activity was found in the free fraction. Unlike the monocyte enzymes, the human intestinal macrophage esterases were partially resistant to 40 mM NaF (Fig. 6.3D). Again, digitonin did not alter the distribution but did solubilize most of the alpha-naphthyl acetate activity in the fraction of density 1.165-1.242 $g.cm^{-3}$ (Fig. 6.3E).

The residual esterase activity present at a density of 1.21 $g.cm^{-3}$ exhibited the same characteristics as the lysosomal enzymes, both in terms of density and release into the free fraction in the presence or absence of digitonin (compare Figs. 6.3A-E).

Figure 6.3 Distribution of lysosomal hydrolases and non-specific esterases on sucrose gradients.

The 1000 x g supernatants from the disrupted human intestinal macrophage were centrifuged to equilibrium on sucrose gradients either without (A,B,D, ▲——▲) or with (C,E ●—●) pretreatment with digitonin. Results are plotted as in Figures 6.1 & 6.2. (A) β-Glucuronidase; (B) N-acetyl-galactosaminidase; (C) β-Glucuronidase; (D,E) Non-specific esterase with (.....) or without (▲——▲) 40 mM sodium fluoride. Recoveries are: 92 ± 2% for (A); 87 ± 5% for (B); 98 ± 5% for (C); 101 ± 7% for (D) and 95 ±9% for (E).

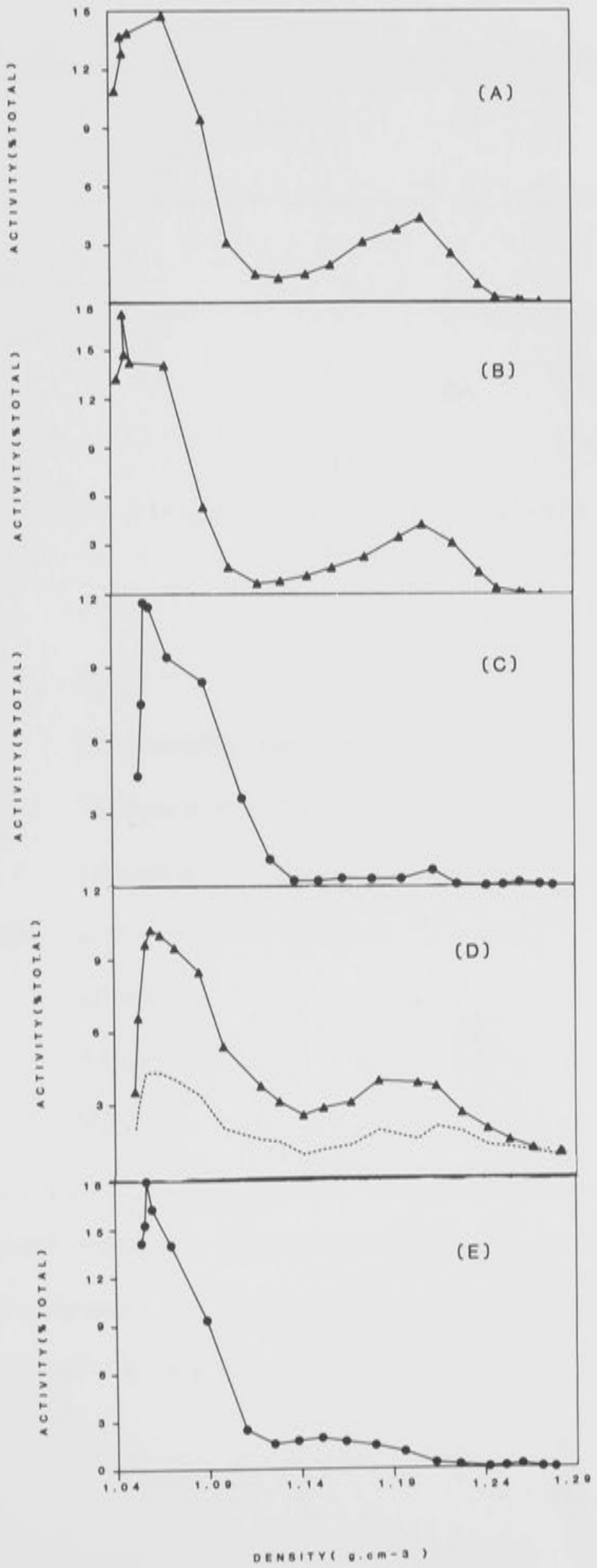

(A)
(B)
(C)
(D)
(E)
ACTIVITY(%TOTAL)
DENSITY(g.cm-3)
1.04
1.09
1.14
1.19
1.24
1.29

TABLE 6.1

Cytosol and Intracellular membrane content of plasma membrane fraction

Marker Enzyme	Location	Markers in density range (1.10-1.17 g,cm^{-3})**
		%
Galactosyltransferase	Golgi	48
Monoamine Oxidase	Mitochondria outer membrane	18
Arylsulfatase	Endoplasmic reticulum	7
Non-Specific esterase	Lysosome (membrane-associated)	9
N-Acetylgalactosaminidase	Lysosome	4
β-Glucuronidase	Lysosome	6
Catalase	Peroxisome	17
Lactate dehydrogenase	Cytosol	10

** The percentage of each marker in the plasma membrane fraction (density range 1.10-1.17 g.cm^{-3}) was estimated by the integral of the curve in this density range X 100, divided by the integral of the complete curve.

Intracellular Membrane Content of plasma membrane preparation.

In order to ascertain the purity of the plasma membrane preparation, the content of appropriate markers in the plasma membrane fraction (1.10-1.170 $g.cm^{-3}$) was used to indicate the non-plasma membrane content of this preparation (Table 6.1). Mitochondrial (monoamine oxidase) and Golgi (galactosyltransferase) membranes were the most prominent contaminants of the plasma membrane preparation. Other contaminants were minor. The selection of the peak plasma membrane fractions increased the purity in all cases but decreased the yield.

Plasminogen Activators

A cell-associated plasminogen activator activity with a peak density of 1.126 ± 0.003 $g.cm^{-3}$ was consistently found (Figure 6.1F). In most fractionation experiments, this peak density corresponded to the density of the plasma membrane markers. About 40% of the plasminogen activator activity was found in the density range 1.10-1.15 $g.cm^{-3}$. Digitonin treatment resolved the plasminogen activator activity into two fractions (Fig. 6.1G). Typically, 44% of the activity normally found in the density range 1.10-1.15 $g.cm^{-3}$ co-distributed with the plasma membrane markers (Fig. 6.1G; compare Fig.6.1C & E), whilst 28% co-distributed with the Golgi marker giving a peak density of 1.110 $g.cm^{-3}$ (Figure 6.1G; compare Fig. 6.2D).

Zymogram of plasminogen activators in pooled sucrose gradient fractions and the effect of monoclonal anti-plg 1 antibody.

To determine the nature of PA expressed in the membrane-fractions, the SDS-PAGE fibrin overlay gel system was used to determine whether the PA are represented as

proenzymes or in their active forms. The principle used in these experiments was defined in Chapter 4.

Untreated pooled sucrose gradients fraction produced only one major band of lysis on the fibrin-agrose zymogram after SDS-PAGE (Figure 6.4 lane 5). This band was produced by a plasminogen-dependent enzyme migrating with the same mobility as human urokinase (HPA52). A lysis band was formed in this system, apparently due to activation of the HPA52 in situ by plasmin present as a trace contaminant in the plasminogen used for the overlay gel. When the same plasminogen preparation was incubated with the pooled sucrose gradient fractions, activation of HPA52 occurred, with the concomitant formation of an active breakdown product of Mr 36,000 (Figure 6.4, lane 2, Zone D, cf control stopped at zero time with SDS, lane 1). The Mr 48,000 form (lane 2, Zone C) is a partial breakdown product of HPA52.

When the plasminogen used in the assay which contains trace amounts of plasmin was preincubated with the monoclonal antibody inhibitory to plasmin, anti-plg 1, for 2 hr at 37^{o}C begore incubation with the pooled sucrose gradients fractions, the degradation of HPA52 to its active breakdown product of Mr 36,000 (HPA36) was prevented (lane 4). When SDS-PAGE sample buffer was added to the preincubation mixtures at zero time before addition of the pooled sucrose gradient fractions, neither a plasmin band, nor evidence for the degradation of HPA52 was apparent, because of the inhibition of plasminogen activation by SDS sample buffer (Figure 6.4 lanes 1 & 3).

This result hence indicated that the PA in the HIMØ is predominantly present in the form of a proenzyme. Unfortunately, insufficient PA enzyme content in either the Golgi or plasma membrane fraction could be obtained to produce reliable and consistent gel patterns.

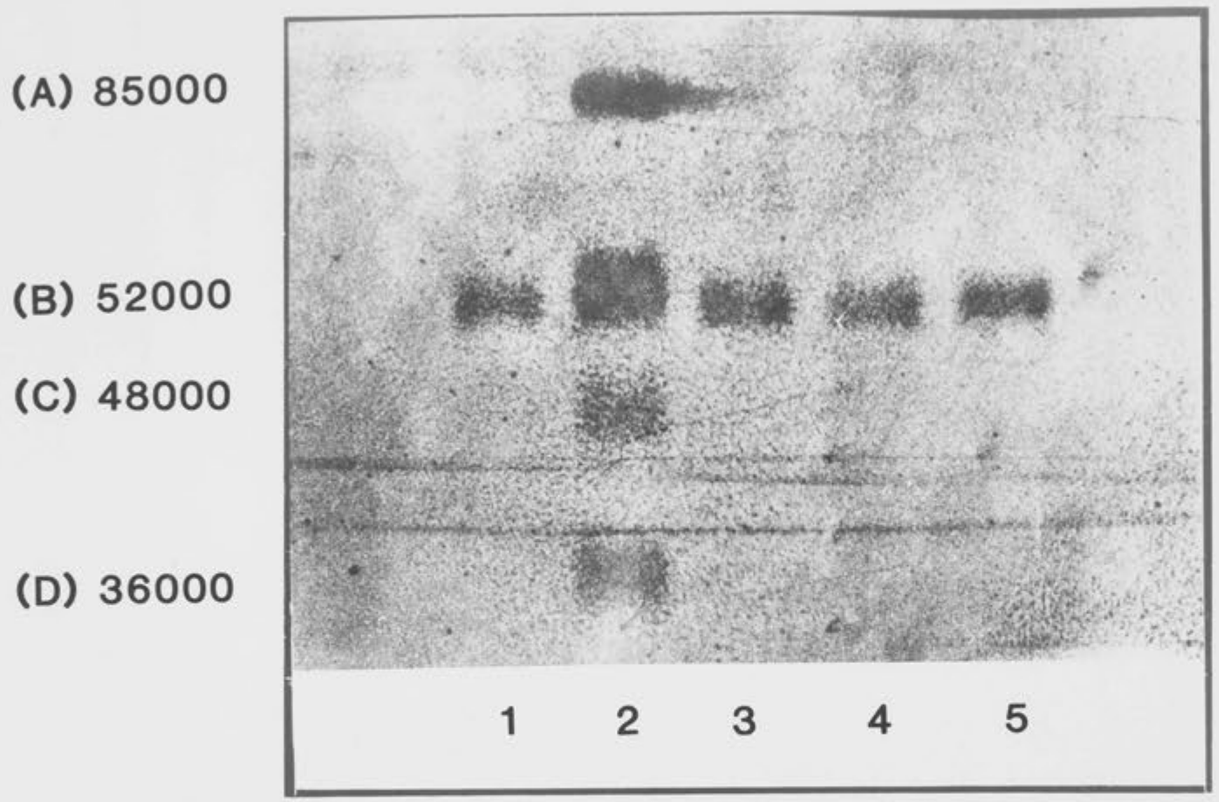


Figure 6.4 SDS-PAGE fibrin overlay gel showing the types and nature of PA in pooled sucrose gradient fractions (lane 5) pretreated with:- Lanes (1) plasminogen with SDS sample buffer added at zero time (2) plasminogen (3) anti-plg 1 and plasminogen previously incubated for 2 hrs at 20^{o}C and with SDS sample buffer added at zero time (4) anti-plg 1 and plasminogen mixture previously incubated for 2 hrs at 20^{o}C

DISCUSSION

The subcellular fractionation presented in this chapter provides a basic framework for the analysis of possible changes in the subcellular distribution or properties of many enzymes implicated in the differentiation of monocytes/macrophages. In order to define the localisation of PA and non-specific esterase, a characterisation of the extracellular and intracellular compartments is necessary.

The human intestinal macrophage PA, of the urokinase-type and predominantly in the form of proenzyme, was found to localise in the Golgi and plasma membrane fractions. Non-specific esterase was found in the lysosomal fraction and results also indicated that the membrane bound esterase of monocytes, which may be in the smooth endoplasmic reticulum, is also present in HIMØ. No evidence was found for a plasma membrane fraction.

Subcellular Fractionation:

The combination of centrifugal elutriation and subcellular fractionation by isopycnic centrifugation has proved suitable for the preparation of human intestinal macrophage plasma membrane and the subsequent analysis of the distribution of enzyme markers of intracellular membranes.

Mn^{2+}-stimulated leucine 2-naphthylamidase, a plasma membrane ectoenzyme, was also demonstrated for the first time on these cells.

The use of centrifugal elutriation to produce a well-defined cell preparation abolishes the need for an adherence step, and thus minimizes the functional and surface antigenic alterations to the cells. Traditionally, macrophages have been isolated by their propensity to adhere to glass or plastic surfaces but this approach has two drawbacks.

Firstly, it is not easy to release adherent cells without causing injury during scraping, exposure to local anaesthetics, chelators or cold. Secondly, macrophage function and biochemical characteristics may be altered by adherence. The elutriation procedure reported here gives an 8.5 fold enrichment of macrophages from the starting population of disaggregated lamina propria cells at high purity (85% ± 3%). These results compare favorably with those reported previously (Beeken et al., 1984) although the yields obtained in this studies are much higher.

Plasma membrane Marker Enzymes Distribution:

Plasma membrane vesicles prepared from isolated intestinal macrophages exhibited a characteristic density of 1.126 ± 0.0005 $g.cm^{-3}$, as judged by the presence in this fraction of three markers characteristic of the plasma membranes of monocytes and other cells; namely 5'-nucleotidase, Mn^{2+}-stimulated leucine-2 naphthylamidase activity and wheat germ agglutinin binding sites (Fayle et al.,1985).

5'-nucleotidase has been widely used as a marker of macrophage differentiation (Bianco & Edelson 1978), and was readily detected in the present work reported in this chapter. The absence of the inhibition of the enzyme activity by glycerol 2-phosphate (used to inhibit alkaline phosphatase) or sodium tartrate (used to inhibit acid phosphatase), together with the inhibition of AMPase activity by zinc sulphate, indicated that the activity was due to 5'-nucleotidase. The 5'-nucleotidase enzyme has been identified as an ectoenzyme of the plasma membrane in many species. Although this enzyme had been reported to be absent in freshly isolated monocytes (Johnson et al.,1977), work from our laboratory had found that this enzyme was readily detectable (Fayle et al., 1985).

Mn^{2+}-stimulated leucine 2-naphthylamidase, which we had previously found to be present in monocytes, was present in HIMØ and displayed a distribution profile paralleling that of 5'-nucleotidase. This report established for the first time, the presence of leucine 2-naphthylamidase enzyme in HIMØ. This enzyme has been extensively studied in mouse peritoneal macrophages (Wachsmuth & Wust 1982) and has also been demonstrated in the rabbit alveolar macrophages (Andrew et al., 1980) and recently on human moncytes and macrophages as an ectoenzyme (Fayle et al.,1985). A similar enzyme is a prominent component ileal brush border (Kenny & Maroux 1982) and had also been detected in the mucosa and the isolated microvillous fraction of the proximal colon of newborn pigs (Sepulveda & Smith 1979) and also in the human jejunal biopsy homogenates (Peters et al., 1975; Peters 1976).

In addition, the labelling of the cell surface glycoproteins and glycolipids by **wheat germ agglutinin** displayed a similar buoyant density profile to that of 5'-nucleotidase and Mn^{2+}-stimulated leucine 2-naphthylamidase plasma membrane markers. Digitonin was also used to distinguish the plasma membrane from the intracellular membranes. The plasma membrane markers of 5'-nucleotidase, Mn^{2+}-stimulated leucine 2-naphthylamidase and wheat germ agglutinin without the addition of the cholesterol binding detergent were found in a light membrane fraction of density range 1.10-1.15 $g.cm^{-3}$, with a peak density at 1.12 $g.cm^{-3}$. However, when digitonin was added to the homogenate before subcellular fractionation, in amounts approximately equimolar with the cholesterol content, the density profile was altered and the plasma membrane markers were then found in a sharp peak at a much heavier density of 1.18 $g.cm^{-3}$. These findings indicate that as with other cell types, the plasma membrane of the HIMØ is richer in cholesterol than other intracellular membranes. By contrast, digitonin treatment had little effect on the other subcellular markers investigated.

The above criteria established the unequivocal identification and isolation of HIMØ plasma membrane.

Intracellular Marker Enzymes:

In order to characterize the plasma membrane preparation fully, the content of contaminating intracellular membranes and soluble proteins were assessed using enzyme markers characteristic of the Golgi apparatus, endoplasmic reticulum, mitochondria, peroxisomes, lysosomal vesicles and cytoplasm.

Galactosyltransferases comprise a family of enzymes of the Golgi apparatus, each of which exhibits a characteristic acceptor specificity using UDP galactose as the substrate (Gerber et al., 1979; Kaplan & Hechtman 1984). The results reported here indicate that although this activity was found predominantly in a peak of density similar to that of the plasma membrane, less cholesterol was present in the Golgi membranes since digitonin treatment caused only a marginal shift of the buoyant density profile.

Galactosyl transferase, although found in the plasma membrane of several cell types (Hopper et al.,1986) is nevertheless specific for the Golgi in our cell type. The hardly noticeable shift in the density gradient following digitonin treatment unlike p.m. indicated that galactosyl transferase is useful as a marker in this instance and consequently the possible golgi localisation of PA is therefore convincing since the PA activity is separated from all other enzymic markers. The difference in density gradients of PA relative to catalase, 2 lysosomal enzymes and esterase, were sufficiently marked to support the conclusion that PA is not associated with peroxisomes, lysosomes or the endoplasmic reticulum.

Arylsulfatase C is an integral component of endoplasmic reticulum membranes, unlike the lysosomal arylsulfatases A and B (Milsom et al., 1972; Roy 1976; Moriyasu & Ito 1982). HIMØ arylsulfatase C, as expected, was found over a wide density range. The HIMØ endoplasmic reticulum membranes were considerably denser overall (mean density 1.21 $g.cm^{-3}$) than their counterparts in monocytes [1.17 $g.cm^{-3}$, Fayle et al., (1985)] and the levels of arylsulfatase C activity detected were lower than those found in human blood monocytes on a cellular basis.

The outer mitochondrial membrane enzyme **monoamine oxidase** (Schnaitman et al., 1967) was used to identify the mitochondrial component. The peak density of the mitochondrial marker (1.16 $g.cm^{-3}$) agreed with the published values for other cell types, which generally range over 1.16-1.18 $g.cm^{-3}$. However, the major contaminant of the plasma membrane fraction was mitochondrial particles.

Monoamine oxidase has been claimed to be an unsatisfactory marker for mitochondria as it is found in plasma membrane and enoplasmic reticulum fractions in certain cell types. In our study with HIMØ, monoamine oxidase is a satisfactory marker for mitochondria as it is not found in the p.m and the e.r fractions of this cells. Evidence to support this observation came from Table 6.1 where the monoamine contamination in the p.m marker density range 1.10-1.17 $g.cm^{-3}$ was only 18% and furthermore, the use of digitonin caused only a marginal shift in the mitochondria fraction unlike the p.m. fraction. Although succinate dehydrogenase would have been a much more satisfactory marker for mitochondria, unfortunately we have not been able to assay this marker in a reproducible manner.

Catalase, a component of peroxisomes displayed a membrane-associated peak at 1.18 $g.cm^{-3}$, with the remainder in the very low density or free fractions. The lack of effect of digitonin indicated that the peroxisomal membranes were cholesterol-poor. A

similar peak density and pattern of distribution for catalase has also been reported for peripheral blood monocytes (Fayle et al., 1985) and mouse peritoneal macrophages (Darte & Beaufay 1983). The current results do not exclude the possibility that some of the catalase found in the free fraction may be contributed by cytoplasmic catalase (Roels et al., 1977).

The HIMØ **lysosomal acid hydrolases** gave the highest buoyant density of all the intracellular membranes studied. Two representative lysosomal acid hydrolases, β-glucuronidase and N-acetylgalactosaminidase, gave a peak density at 1.21 $g.cm^{-3}$. An additional peak of similar density to the plasma membrane was sometimes found for β-glucuronidase, which otherwise had a similar distribution to that of N-acetylgalactosaminidase. As noted previously (Fayle et al., 1985), some β-glucuronidase may well be present on the endoplasmic reticulum.

HIMØ PA and Alpha-Naphthyl Acetate Esterase

The profile of **HIMØ alpha-naphthyl acetate esterase** activity after digitonin treatment closely resembled that previously seen in monocytes (Fayle et al., 1985). These results suggest that the membrane-bound esterase of monocytes, which may be in the smooth endoplasmic reticulum (Fayle et al., 1985), is also present in HIMØ. Residual activity at a density of 1.21 $g.cm^{-3}$ however, reflected the presence of an additional component of HIMØ esterase activity, that was more readily detectable without digitonin treatment (Figure 6.3D). This peak of esterase activity exhibited the same characteristics as the lysosomal enzymes, both in terms of density and release into the free fraction in the presence or absence of digitonin. The studies reported in this chapter indicate that HIMØ possess an esterase, probably lysosomal, which is not present in monocytes. The appearance of this enzyme may also be linked to the

appearance in HIMØ of esterase activity partially resistant to 40 mM NaF. Monocyte esterase activity was abolished by such treatment (Fayle et al., 1985). Changes in the esterase isoenzyme spectrum with maturation of human monocytes and monocyte-like cell lines have been well-documented (Parwaresch et al.,1981). In human leukocytes, the non-specific esterase isoenzyme profile correlates with the cell lineage and differentiation status. Despite some discrepancies, there is universal agreement that alpha-napthyl acetate esterase activity in cells of the monocyte lineage resides predominantly in 4-5 isoenzymes of pI 5.5-6.3 (Parwaresch et al.,1981; Scott et al., 1984; Yourno & Mastropaolo 1981). Recently it was discovered that monocytes differentiating in culture undergo pronounced alterations to this pattern. Thus the band at pI 6.1 rapidly decreases in relative activity, whilst a new isoenzyme appears at pI 6.3 within a day. The new band increases in intensity by day 3, when a second new band (pI 6.45) appears. The resulting pattern is identical to that of human resident peritoneal macrophages (Parwaresch et al.,1981). Patterns almost identical to the monocyte and macrophage profiles (designated Mon-2 and Mon-3 respectively) were recently reported in different cases of acute myeloid leukaemia (Scott et al., 1984). Taken together, these results suggest that the alpha-naphthyl acetate isoenzyme profile is highly-correlated with differentiation status in monocytes. Indeed, Oertel et al., (1985) using the alpha-naphthyl acetate esterase inhibitor, bis [4-nitrophenyl]-phosphate showed that human monocytes alpha-napthyl acetate esterases were involved in the spontaneous cytotoxicity of monocytes toward tumour cells. Whether the alpha-naphthyl acetate esterase in HIMØ may act against colorectal tumour cells requires further study.

The **PA distribution** closely paralleled the distribution of the plasma membrane enzyme, 5' nucleotidase, WGA and Mn^{2+}-stimulated leucine aminopeptidase before digitonin treatment. After digitonin treatment, the PA distribution resolved into

2 peaks, one paralleled the Golgi fraction and the other with the plasma membrane fraction. The implication that part of PA was associated with Golgi membrane fractions suggests that the PA has the potential to be incorporated into the plasma membrane and subsequently gain excess to the extracellular environment. Despite the finding that the PA is membrane associated and exists predominantly as proenzyme in the plasma and Golgi membrane fractions, macrophages isolated from histologically normal mucosa have little urokinase PA in comparison to activated monocytes and tumour cells and therefore argue against PA from macrophages isolated from histologically normal mucosa having a significant function in regulating extracellular proteolysis. Whether the HIMØ could produce its own plasminogen, hence amplifying the effect of the small amount of PA in the generation of a proteolytic cascade, remains to be studied.

It has often been suggested that treatment of intact cells rather than homogenates with digitonin including the use of ecto-enzyme inhibitors like DFP could have helped clarify the plasma membrane localisation of the plasminogen activators. The use of DFP or whole cells with digitonin or ectoenzyme inhibitors to show whether PA can be liberated in soluble form does not necessarily argue against a localisation within carrier vesicles if it is not liberated in soluble forms. In fact, PA could be bound at the internal surface of the membrane of the secretory vesicles and become exposed on the cell surface after fusion of the vesicles with the plasma membrane. It could then display its enzymatic action either on the cell surface or after shedding into the pericellular space. Since digitonin resolved PA into 2 peaks: (a) Golgi and (b) plasma membrane, this suggested that the PA in the golgi could become incorporated into the plasma membrane and subsequently gain acess to the extracellular environment. As our result indicated that the PA is released as proenzyme urokinase type PA and that plasmin activated the proenzyme of urokinase type PA into active enzyme (chapter 3), then the definitive localisation of the PA in the cell could involve the use of the monoclonal antibodies inhibitory to plasmin. An experimental approach would be to gently activate whole HIMØ

cells in the presence or absence of plasmin and in the presence or absence of plasmin and the monoclonal antibody inhibitory to plasmin together using conditions that do not impair cell viability. If PA is localised at the plasma membrane, then we would expect the activation of the proenzyme to active enzyme and subsequently we could then use the "activated" PA in the plasma membrane of HIMØ to activate the plasminogen in the yellow colorimetric assay.

The newly found membrane receptor for urokinase PA on human monocytes maintained in culture (Vassalli et al.,1985; Stoppelli et al.,1984) is intriguing. Tumour cells (and probably macrophages or epithelial cells in IBD) start to release inactive pro-urokinase PA during their differentiation/transformation. How and where the activation takes place, still remains unsolved but this process clearly involves plasmin. Plasminogen dependent fibrinolysis on the surface-attached parts of the human monocyte membrane indicates that these contact areas somehow trigger the activation of pro-urokinase PA. The presence of a receptor for two chain urokinase PA on the monocyte-macrophage plasma membrane (Vassalli et al.,1985; Stoppelli et al.,1984) may protect the enzyme from inhibitors at the contact areas of the cells and help to focus the proteolytic effect.

CHAPTER 7

GENERAL DISCUSSION

The main findings of the work presented in this thesis were discussed in the concluding sections of each chapter and were outlined in the Summary (page viii). This chapter discusses these findings in the context of the importance and biological role of proteolytic enzymes as summarised up recently by Neurath (1984).

"Proteolytic enzymes are not only a physiological necessity but also a potential hazard, since, if uncontrolled, they can destroy the protein components of cells and tissue."

7.1 Colorectal carcinomas

The hypothesis of PA playing a role in tissue degradation in cancer and the documentation that most cell types in the normal organism do not produce detectable amounts of PA, imply that an abberration in the regulation of PA activity exists in tumour cells.

It has been suggested that the production of PA by invasive tumour cells and the generation of plasmin are key events in the hydrolysis of all matrix components, since glycoprotein removal from the matrix is required for the maximal degradations of collagens and elastin. The availability of the monoclonal antibodies inhibitory to human plasmin (chapter 3) will now be able to be used to elucidate this mechanism in the same manner as in the study performed by Ossowski and Reich (1983) where antibodies to urokinase were shown to inhibit experimental metastasis.

It is generally believed that the total PA activity in a tissue is the net result of several factors; including the rate of biosynthesis, release of the enzyme from the producer cells, the rate of proenzyme activation and the presence of stimulatory factors and/or inhibitors in the extracellular milieu.

A putative regulatory mechanism at the level of activation of the proenzyme to plasminogen activator is only likely to be of any significance if the proenzyme in intact tissues is not always converted to its active counterparts immediately after secretion e.g. by trace amounts of plasmin that might be present in the extracellular space. The findings in chapters 4 and 5 indicated that this is the case. Furthermore, the finding that the majority of the plasminogen activators of the urokinase type existed as proenzyme in these tissues deserved comments.

The lack of inhibition between proteinase inhibitors and pro-urokinase PA is a consistent finding(Eaton et al.,1984; Vassalli et al., 1984; Andreasen et al., 1986). The demonstration of the lack of reaction of the inhibitors with pro-uokinase PA may have important regulatory implications. Even if inhibitor and pro-urokinase PA coexist extracellularly, no reaction will occur until pro-urokinase is converted to active urokinase PA. But conversion will be followed by the rapid inhibition of the enzyme activity of active urokinase PA. In the intact organism, this may serve to confine PA activity to times and places with high rates of conversion of pro-urokinase PA to the active enzyme. This role of the inhibitor may be particularly important in the biology of tumours. Although it is at present unknown whether tumours in vivo have high production of PA inhibitors like the HT 1080 cell line (Andreasen et al., 1986) , it may be speculated that production of PA inhibitor by the cancer cells, together with urokinase PA, may help to confine plasminogen activation mediated proteolysis to areas of contact between the tumour and the normal tissue and thereby protect the tumour against the tissue destruction that it imposes upon the surrounding normal tissue.

Whether tumour cells possess a receptor for two chain urokinase PA like the monocyte-macrophage cells (Stoppelli et al.,1984; Vassalli et al.,1985) which might protect the pro-urokinase PA once activated from inhibitors as well as focusing proteolytic effect, is also unknown.

The cascade reactions leading to plasminogen activation provide effective proteolytic activity, which can be regulated at the level of single cells and used for strictly controlled proteolysis and degradation of extracellular matrix. The mere secretion of PA has no actual correlation with disturbances of cell growth. It may be a prerequisite for certain steps of neoplastic growth, such as metastasis and invasion by tumour cells. The differences between physiological proteolysis and proteolysis related to neoplasia may be in the control of either the pericellular activation of proenzymes or removal of the active proteinases from the microenvironment of cells, for example through alpha 2 macroglobulin. Removal of proteinases from the extracellular and intravascular fluid is the best characterised function of alpha 2 macroglobulin. The differences in alpha 2 macroglobulin levels in normal and malignant tissues are consistent with the widely held notion that proteolytic activity accompanies transformation. Defects in the regulation of pericellular proteolysis might be involved in the disruption of cell-matrix connections and in the altered phenotype of transformed cells.

Comparison of the studies of the occurrence of plasminogen activators in tumours with those of their occurrence in the normal organism (chapter 4) indicates that plasminogen activators of the urokinase type may prove to be a useful but not specific tumour marker especially when expressed in proenzyme forms.

Further studies are needed to explore this possibility, including histochemical studies of the occurrence of urokinase PA in benign tumours and in pre-malignant

conditions. The considerable amount of urokinase PA immunoreactivity detected extracellularly or attached to the cell membrane in malignant tumours (see Dano et al.,1985) and the low content of urokinase PA immunoreactivity in some organs (e.g. brain and liver) also points to a possible use of radio labelled antibodies against urokinase PA for detection of tumours and metastases by radio imaging.

It was not possible to define the cellular origin of the urokinase type PA using the colorimetric enzyme assay, although the variability in the amount of PA detected in tumours could be accounted for by tumour cell heterogeneity. The major cause of this heterogeneity is probably a genetic instability of cancer cells, which may give rise to new variant subclones during tumour development (Nowell 1976). A strong candidate for the source of urokinase type PA, however, could be transformed epithelial cells within tumours, as other studies have shown the production of these enzymes by epithelial cells derived from both colorectal carcinomas (Carretero et al.,1985; Friedman et al.,1984) and adenomatous polyps (Friedman et al., 1984) in culture. Immunocytochemical studies have also shown urokinase to be associated with neoplastic epithelial cells in colonic cancer (Kohga et al., 1985; Burtin et al., 1985) and it has been postulated that urokinase may be released by tumour epithelial cells into the stroma, where it has also been detected (Kohga et al., 1985). However, immunocytochemical studies of normal mouse tissues have localised urokinase PA to a variety of cell types, in particular connective tissue cells with a fibroblast-like morphology (Larsson et al., 1984) and these occurr in high frequency in the lamina propria of the gastrointestinal tract. No other cell types, other than fibroblast-like cells, displayed urokinase PA in the gastrointestinal tract. Double staining using both the urokinase PA antibodies and the monoclonal antibodies inhibitory to human plasmin (chapter 3) might help to define the cellular origin more precisely since we would expect a localisation of the plasmin in areas where there is a high concentration of PA.

The contribution of PA by infiltrating white cells represents a further potential source of protease activity in tumours especially when activated or transformed since it was demonstrated that lymphocytes produce negligible amounts of PA unless they were transformed - for example, by human T cell lymphotropic virus type III - when appreciable amounts of PA of urokinase type were apparent (Hinuma et al., 1985).

However, the studies reported in chapter 5 that the urokinase PA content of colonic mucosa from IBD was very much higher than from normal mucosa imply that the production of this enzyme is not a specific feature of transformed cells, even though a contradictory study from ours had been reported (Verspaget et al., 1986).

A hypothetical mechanism for the activation of plasminogen in vivo is schematically represented in Figure 7.1.

Initially, the pro-urokinase may directly activate plasminogen to plasmin. The generated plasmin then converts cellular or tissue inactive pro-urokinase into active two chain urokinase PA which in turn activates plasminogen to plasmin. In a plasma milieu, activation is prevented by competitive inhibition of a yet uncharacterised inhibitor of the activation of plasminogen. However, the addition of fibrin prevents the inactivation of plasminogen activation by the competitive inhibitor. This concept is supported by the work of Lijnen et al., (1986). Using recombinant pro-urokinase (Rec-pro-UK), obtained by the expression of the human pro-urokinase gene in Escherichia coli, Lijnen et al., (1986) in an in vitro purified system showed that in mixtures of Rec-pro-UK and plasminogen, both active urokinase and plasmin are quickly generated. Addition of plasmin inhibitors (aprotinin or α_2-anti plasmin) abolishes the conversion of Rec-pro-UK to urokinase but not the activation of plasminogen to plasmin; suggesting that the Rec-pro-UK activates plasminogen directly. However, evidence that the conversion of Rec-pro-UK to urokinase is abolished but not the activation of plasminogen to plasmin cannot be conclusive owing to the possibility of

contaminant protease activity in the plasmin preparation and the plasmin inhibitors used in the experiment. A definitive answer would involve the use of the specific monoclonal antibody inhibitory to human plasmin generated in chapter 3.

Since plasmin and soluble active urokinase PA are rapidly inhibited by circulating and cell-secreted inhibitors, plasminogen and PA activation therefore occurred at the cellular contact area (receptor), thus being protected from the inhibitors.

The finding that autologous normal mucosa contained little HPA52 compared with colon cancer tissue (chapter 4) requires comment. The low level of PA activity may result from normal mucosa making HPA52 as well as an excess of HPA52 inhibitor. The net effect would be the absence of detectable activity as was the case reported for human umbilical vein endothelial cells (Dosne et al.1978). Tumour cells, on the other hand, may make HPA52 but no inhibitor. However, the findings of a higher proportion of active enzyme in the normal mucosa homogenates (53%) against 30% in tumours (see chapter 4) would argue against the increased production of HPA52 inhibitor in normal mucosa compared to the tumours. Furthermore, if the normal mucosa produced relatively more endogenous HPA52 inhibtor, then the inhibition of the colorimetric assay in normal mucosa would be seen. This was not the case (see chapter 4).

Rather, the increase in the level of HPA52 activity in tumour compared to adjacent normal mucosae is more likely to result from a change in transcription and translation of the HPA52 gene (Becker et al.,1981; Hamilton 1983; Nagamine et al.,1983). Experiments with inhibitors of mRNA have suggested that these agents may affect the rate of PA gene transcription (see review by Dano et al.,1985). With the probes of cloned cDNA for PA mRNA becoming available, it will be possible to study in detail the regulation of HPA52 in normal and neoplastic tissue.

Further studies are also required on factors capable of affecting the HPA52 proenzyme conversion. These include the availability of plasmin to activate HPA52 and the interaction of inhibitors released by other cells for example, minactivin (from activated monocytes; Golder & Stephens 1983) and protease-nexin (from human fibroblast; Baker et al.,1980). Such inhibitors could regulate PA activity directly or by influencing PA synthesis.

The possible role of macrophages in the regulation of proteolysis around tumours must also be taken into account. Subcellular fractionation of HIMØ was performed to define the localisation of the serine hydrolases, PA and non-specific esterase. The analysis of HIMØ plasma and intracellular membranes at high purity represents a valuable approach to elucidating the changes that characterize the differentiation of intestinal macrophages.

The finding that the serine hydrolase, alpha-naphthyl acetate esterase in the intestinal macrophages was partially resistant to 40mM NaF, in contrast to human blood monocytes, is of interest. The HIMØ alpha-naphthyl acetate esterase may be involved in the mediation of cytotoxicity against colorectal tumour cells in view of recent report by Oertel et al., (1985) who showed that human monocyte alpha-naphthyl acetate esterases were involved in the spontaneous cytotoxicity of monocytes toward tumour cells. Whilst the alpha-naphthyl acetate esterases are a major and characteristic enzyme in cells of the monocyte lineage, their role and physiological substrates are unknown.

The finding that the PA in the HIMØ is membrane-associated might have widespread implications (see chapter 6) especially in the interactions with other cell types which might induce changes in the production or metabolism of PA. However, the amount of PA released by HIMØ isolated from histologically normal mucosa is relatively small compared with activated monocytes and tumours and this finding argues against HIMØ plasma or Golgi membrane PA having a significant function in regulating any

possible extracellular proteolysis on its own. No attempt was made to study the pro PA/PA of activated macrophages from either inflammatory tissues or colon carcinomas since it is not possible to obtain sufficient tissues. Activated blood monocytes were studied by Fayle et al. who showed an elevated PA secretion in the proenzyme form (unpublished observations). The use of the macrophages isolated from normal mucosa at least serves as a comparison in the absence of a more suitable tissue. In the event that monoclonal antibody markers are developed to distinguish between activated macrophages from those resident in the tissues, a qualitative study of activated macrophages and their potential for secreting urokinase PA could be undertaken using immunohistochemical techniques.

The effects of interactions between different types of cell leading to modulation of production of PA has been documented in many studies (for a review, see Saksela 1985). Such interactions between various types of cell, which induce changes in the metabolism of plasminogen activators, are particularly interesting in connection with the possible correlation between PA and malignancy. Enhancement of local proteolytic activity brought about by contacts between certain cell types, may be involved in determining which cell in a tumour can metastasize and where the secondary tumours can arise. Such mechanisms also suggest a possible alternative to the PA production by the malignant cell itself. In this respect, whether HIMØ could be induced to secrete large amounts of PA perhaps in conjunction with other enzymes or cells or even to produce their own substrate for PA, plasminogen, and hence regulate extracellular proteolysis remains to be studied.

The studies reported in this thesis indicate that PA may be a useful early correlate of the development of malignancy in the colon. However, further studies are still required to delineate as yet unknown factors which might contribute to the regulation of PA production in cancer cells. In particular, the monoclonal antibodies

inhibitory to human plasmin could be used to complement further studies in the human carcinoma HEp3 model where it was demonstrated that antibodies which inhibited HPA52 activity also prevented metastasis but not the primary tumour growth (Ossowski et al.,1983). The use of the antibodies might shed more light into the role of plasmin in vivo in the regulation of invasion and metastasis.

In summary, the hypothesis of a causative role of PA in tissue degradation in neoplasia (and in normally scheduled events of tissue degradation) is based on the assumption that PLASMIN, in combination with other proteases, can degrade the extracellular matrix, including the basement membrane. The generation of the monoclonal antibodies to plasmin reported in chapter 3 will now enable the many proposed roles of plasmin to be delineated further.

7.2 Inflammatory Bowel Disease (Crohn's disease and Ulcerative colitis).

The aetiology of inflammatory bowel disease remains elusive. Among the many attempts to identify the pathogenesis of these diseases have included the characterisation of mediators of inflammation in tissues.

The results reported in chapter 5 established an assocaition between increased HPA52 activity and the inflamed mucosa of the ulcerative colitis and Crohn's disease.

The increased production of HPA52, most probably by epithelial cells, might involve anti-colon antibodies. These antibodies could bind to the epithelial cells and trigger the increased secretion and production of PA. Anti-colon antibodies have been found in sera of up to 90% of patients with IBD (for review see Kirsner & Shorter

1982). They react with the mucus of epithelial globlet cells of the small and large intestine. Immune complexes have also been detected in sera of patients with IBD, although little is known of the nature of their antigen composition (see review by Kirsner & Shorter 1982). In this respect, the immune complex, such as epithelial cells coated with autoantibody, could also act as the first or initiation signal by binding to the Fc receptors of the macrophages (and neutrophils) triggering a sequential metabolic changes (Gordon et al.,1974) in the human intestinal macrophages causing activation. The necessary secondary/second signal may be provided from lymphokine or endotoxin excreted by commensal enteric organisms entering the privileged environment of the lamina propria. Activated HIMØ may then release tissue damaging oxygen metabolites and other degradative enzymes like lysosomal enzymes (Mee & Jewell 1980; Doe et al., 1980) as well as stimulating a secondary tissue damaging immunological mechanisms. The release of oxygen-derived free radicals may also promote inflammation through the inactivation of serum inhibitors of protease activity resulting in increased tissue damage (Carp & Janoff 1979; Matheson et al.,1979). Proteases liberated at the sites of inflammation may then remain free to attack the tissue structures if high enough concentration of oxidants are present.

Although anti-colon antibodies in the intestinal mucosa have not been directly demonstrated, one must ask whether the intestinal plasma and B cells are capable of producing auto-reactive antibodies. Unfortunately, few plasma cells appear to survive the conventional isolation of lamina propria by collagenase digestion of mucosa stripped of its epithelium with EDTA. The percentage of B cells is also decreased (MacDermott et al.,1981).

A similar observation had been described in the bullous skin disorder pemphigus where autoantibodies are produced against an uncharacterised intercellular substance of skin and mucosa. The binding of these antibodies to human epidermal cells in an

experimental system stimulates synthesis and secretion of HPA52 by the cells (Hashimoto et al., 1984; Hashimoto et al.,1983).

Further evidence that the interaction of epithelial cells and autoantibodies might contribute to the increased production of PA came from a report described by Becker et al., (1981). These authors purified the IgG of rabbit antisera raised against two cell lines - LLC-PK_1 derived from pig kidney and from Kirsten sarcoma virus transformed BALB/3T3 cells and using in vitro cell cultures of respective target cells, they found a progressive rise in PA production after IgG addition. The induction of PA synthesis was reversible and required the continued presence of IgG for its maintenance. The increase in PA production depended on gene transcription and translation.

Beside the possibility of anti-colon antibodies, whether other cell types like macrophages, once activated could release a factor which subsequently modify PA production by the epithelial cells is as yet unknown. Macrophages are prominent in the inflammatory cell infiltrate in both conditions; in Crohn's disease, a hallmark of the condition is the granuloma, where macrophages are particularly conspicuous. There is no doubt that macrophages play a prominent role in a variety of chronic inflammatory conditions. Evidence has been presented to indicate that, especially in IBD, circulating monocytes are in an activated state.

The possible role of plasminogen activator inhibitors in the regulation of PA in IBD requires comment. Although inhibitors of PA have yet to be isolated from IBD tissues, nevertheless it is conceivable that extracellular PA activity in these inflamed tissues may be regulated at the level of biosynthesis, secretion from producer cells and the activation of inactive proenzyme forms and by the stimulation or inhibition of the enzyme activity of the activation, in the same manner as that proposed for tumour cells. The possible interactions of plasminogen activators with cell-derived components include binding of activated urokinase PA by protease nexins and miniactivin. These

proteinase inhibitors have been found mainly in fibroblastoid cells and activated monocytes respectively and may play a central role in the cellular metabolism of urokinase PA and some other serine proteinases (Knauer & Cunningham 1984).

A very recent study by Lawrence & Loskutoff (1986) showed that PA inhibitor purified from cultured bovine aortic endothelial cells could be inactivated by oxidants in similar manner to the human a_1-protease inhibitor. At this point in time, no direct studies have as yet been performed to determine whether the inhibitors of urokinase type PA could be inactivated by oxidants released by activated macrophages in IBD particularly since they are conspicuous in these diseases. The sensitivity of the PA inhibitor purified from cultured bovine aortic endothelial cells to oxidants suggests that oxidants liberated by activated inflammatory cells (Weiss & Regiani 1984) in IBD may also inactivate PA inhibitor and this unleash a whole cascade of tissue-destructive proteinases, including elastase and plasmin. The recent demonstration of dramatically elevated plasmin in rheumatoid synovial fluid (Inman & Harpel 1986) is consistent with this hypothesis. The plasmin, generated through the action of PA is probably required for mediation of diapedesis of inflammatory cells through the vessel wall and onto the site of inflammation in addition to its capacity to mediate tissue injury. The availability of monoclonal antibodies inhibitory to plasmin will now be able to test this hypothesis in IBD to indicate elevated levels of plasmin and together with monoclonal antibodies to urokinase type PA in a double staining immunochemistry, might also help to delineate the cellular origin of PA in this inflamed tissues.

Finally, it is unlikely that total PA content and pro-PA could serve as specific markers in the IBD since two disease control groups studied; infectious colitis and a group of undiagnosed chronic diarrhoea patients who were suspected of having crohn's disease but in whom no definite diagnosis could be made (chapter 5) also indicated increased levels of pro-PA and total PA. However, the enzyme might be a useful

biochemical marker of inflammation and a thorough appreciation and understanding of the role of proteinases in the biochemical mechanism of inflammation may assist in the early diagnosis of IBD and in the development of therapeutic agents for the prevention or treatment of this diseases.

In summary, the pathogenesis of IBD could involve anti-colon antibodies reacting with epithelial cells, causing an elevated level of PA leading to chronic tissue injury. PA release is able to promote injury through the generation of plasmin with subsequent fibrinolysis, complement activation, kinin generation and the initiation of the coagulation cascade as well as activating collagenase (Gordon et al.,1978).

The above comments on the possible pathogenesis of IBD provide a useful working model for further investigation of the pathogenesis of IBD, even though future findings may invalidate some of its aspects. In particular, the availability of a novel monoclonal antibody against colonic epithelial cells (Sim, Gibson & Doe, manuscript in preparation) and the monoclonal antibodies inhibitory to plasmin will help to either support or refute some aspects of the hypothesis in particular with regard to increased PA production by colonic epithelial cells and the roles of plasmin in these inflamed tissues.

In conclusion, Figure 7.2 shows a hypothetical model illustrating the mechanism of regulation of urokinase plasminogen activator and its subsequent mediation in extracellular matrix degradation. Plasminogen activator and collagenolytic metallo proteases both produced by metastasizing cells or transformed / inflammatory cells in its inactive form may act in concert to degrade the whole basement membrane after its activation. Plasminogen activator can generate plasmin from the extracellular

plasminogen (see chapter 1 & Figiure 7.2). The plasmin produced by this reaction can directly degrade non-collagenous extracellular matrix components such as laminin and fibronectin. In addition, plasmin can activate latent metallo proteases which would degrade extracellular matrix collagenous and proteoglycan components. Other degradative enzymes could also be involved, like for example β-hexosaminidase. This lysosomal glycosidase can hydrolyse glycoconjugate components of membranes and extracellular matrices. The actual cascade of extracellular matrix degradation in vivo probably occurs adjacent to the tumour or inflammatory cell surface where the local concentration of enzymes is enough to override natural protease inhibitors and this is also help by the presence of putative urokinase receptors on these cells. The presence of these receptors on the plasma membrane also enables "focal and localised" proteolysis to occur at the cell surface in such a way that it is protected from circulating inhibitors like α_2-antiplasmin or α_2-macroglobulin. Such inhibitors are ubiquitous throughout the extracellular matrix and are present in serum. At any point in time only a small proportion of the total tumour or inflammatory cell population may be expressing extracellular matrix degrading protease activity. Although the inter-relationship between the coagulation, fibrinolysis and kallikrein systems are not shown in the model, nevertheless they should also be considered, particularly their involvement in the migration of these tumour or transformed/inflammatory cells.

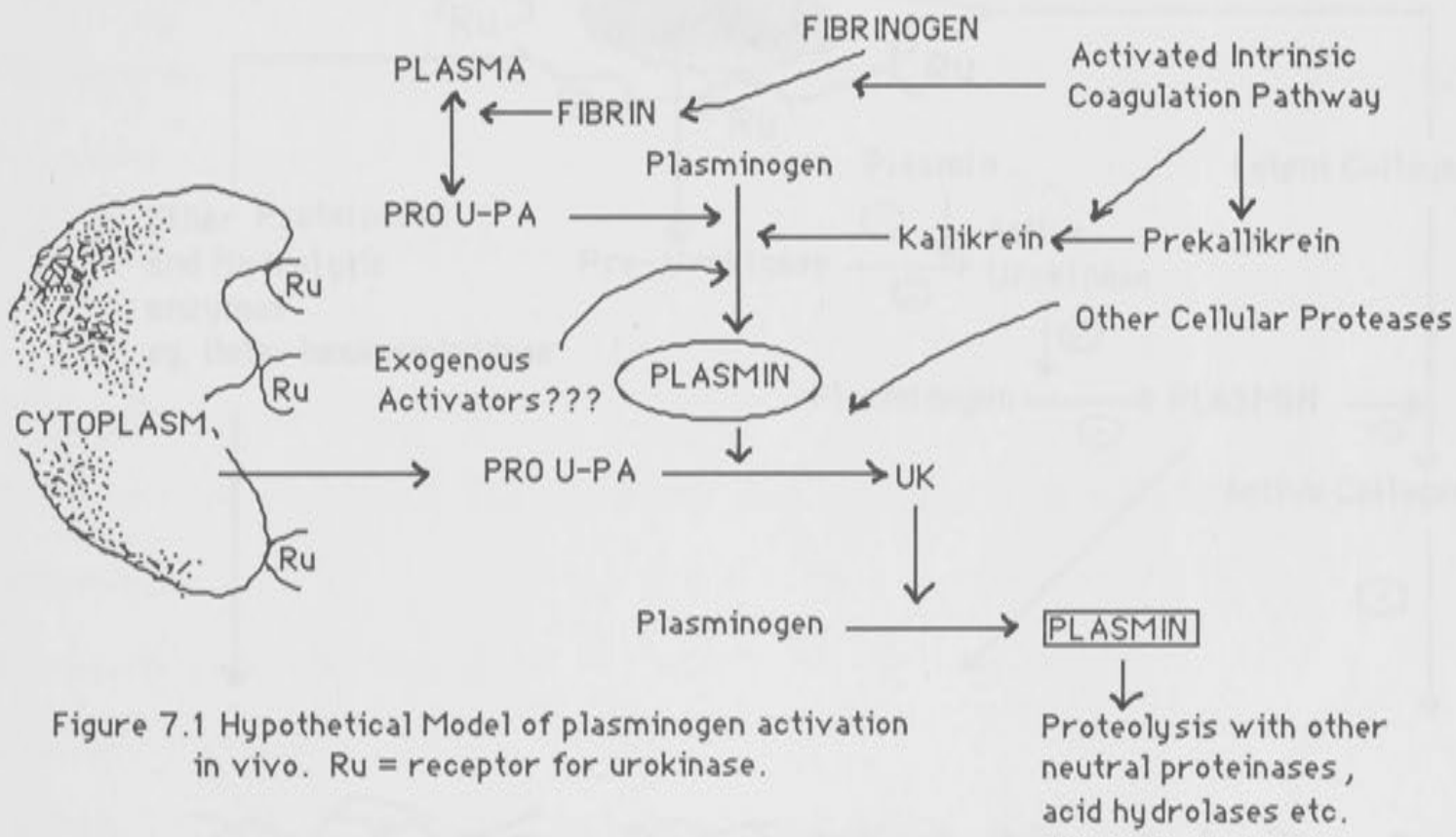


Figure 7.1 Hypothetical Model of plasminogen activation in vivo. Ru = receptor for urokinase.

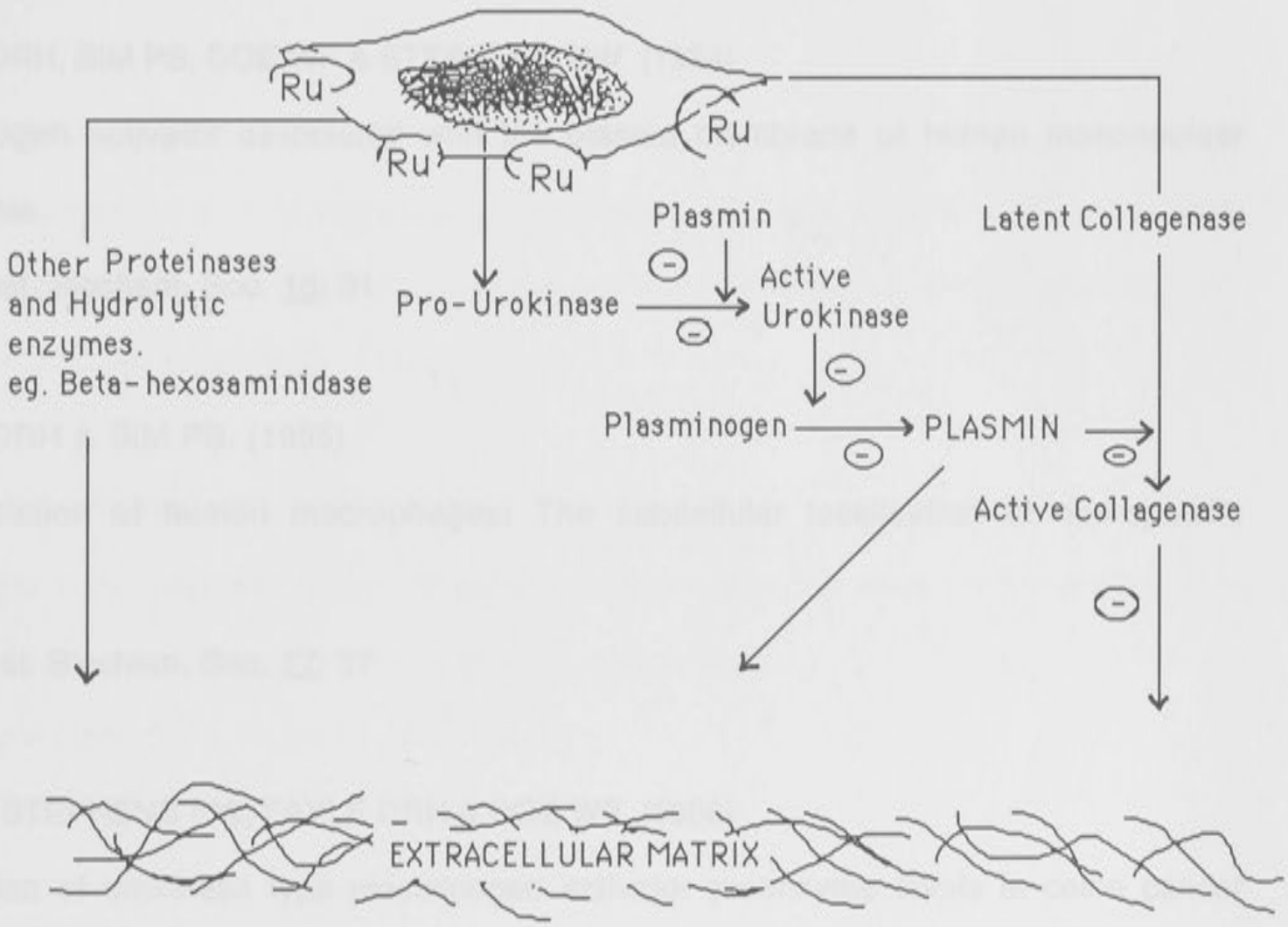


Figure 7.2 Hypothetical model showing the degradation of the extracellular matrix <u>in vivo</u> by an interplay of proteolytic enzymes released from inflammatory or transformed cells.
RU = putative urokinase receptor ⊖ = Possible inhibition steps by extracellular/host inhibitors

APPENDIX I

Much of the work reported in this thesis have either been published or submitted for publication.

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Cell Biochemistry and Function (submitted)

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