Plasminogen activator inhibitor-type I is a major biosynthetic product of retinal microvascular endothelial cells and pericytes in culture

Ann E. CANFIELD,*|| Ana M. SCHOR,* David J. LOSKUTOFF,† Seth L. SCHOR‡ and Michael E. GRANT§

*Cancer Research Campaign Department of Medical Oncology, Christie Hospital and Holt Radium Institute, Manchester, M20 9BX, U.K., †Department of Immunology, Scripps Clinical Research Foundation, La Jolla, CA 92037, U.S.A., ‡Department of Cell and Structural Biology and §Department of Biochemistry and Molecular Biology, School of Biological Sciences, University of Manchester, Manchester M13 9PT, U.K.

Previous studies have shown that a glycoprotein of M_r 47000 (designated Gp47) is a major biosynthetic product of retinal endothelial cells *in vitro* (Canfield, Schor, West, Schor & Grant (1987) *Biochem. J.* **246**, 121–129). We now present data indicating that (a) an identical protein is secreted by bovine retinal pericytes, (b) this protein is plasminogen activator inhibitor-type I (PAI-1), as revealed by immunoprecipitation with specific antibodies and reverse fibrin zymography, and (c) retinal endothelial cells and pericytes synthesize different species of matrix macromolecules, that is: type IV collagen is the major collagen secreted by endothelial cells, whereas pericytes produce predominantly type I collagen; fibronectin and thrombospondin are synthesized by both cell types. Our studies also indicate that PAI-1 is produced, albeit at considerably lower levels, by large vessel vascular cells (aortic endothelial and smooth muscle cells) and human skin fibroblasts. PAI-1 produced by human skin fibroblasts appears to be a distinct molecular species compared to its bovine counterpart as assessed by its slower mobility on SDS/polyacrylamide-gel electrophoresis. The potential significance of elevated PAI-1 production by retinal endothelial cells and pericytes, as well as their distinctive patterns of matrix biosynthesis, is discussed in terms of the involvement of these cells in the maintenance and remodelling of microvessel basement membrane.

INTRODUCTION

Retinal microvessels are characterized by an unusually thick basement membrane, and contain two types of cells: endothelial cells lining the lumen of the vessel, and pericytes embedded within the basement membrane. All of these components are involved in neovascularization (for reviews see Schor & Schor, 1983; Folkman, 1984; Furcht, 1986; Folkman & Klagsbrun, 1987), which is a characteristic feature of the microangiopathy associated with diabetes mellitus (for reviews see Ashton, 1974; Brownlee and Cerami, 1981). We are particularly interested in the involvement of the two types of microvascular cells in the assembly and maintenance of the vascular basement membrane, and their relative roles in the remodelling of this structure. Accordingly, we have isolated endothelial cells and pericytes from bovine retinal vessels (Schor & Schor, 1986) and have studied the synthesis of matrix macromolecules by these cells (Canfield *et al.*, 1986, 1987a). Our studies have demonstrated that retinal microvascular endothelial cells retain their capacity to synthesize basement membrane proteins *in vitro*, although the substratum on which the cells are cultured modulates both the morphology (Schor & Schor, 1986) and biosynthetic phenotype (Canfield *et al.*, 1986) of these cells. In addition, we noted that a glycoprotein of M_r 47000 (Gp47) was a major component secreted into the medium by the endothelial cells. The biochemical properties of this protein were investigated further (Canfield *et al.*, 1987b), and it was demonstrated that Gp47 is

a non-collagenous glycoprotein with a pI of 4.6–5.5, which is not disulphide-bonded into higher M_r -multimers and which does not bind to either gelatin-Sepharose or heparin-Sepharose but is retained by concanavalin A-Sepharose. Approximately 10% of the molecular mass of this protein was attributed to asparagine-linked oligosaccharide. Furthermore, it was concluded that Gp47 was not related to other proteins of similar M_r , namely, SPARC/osteonectin, urokinase, tissue plasminogen activator or proteoglycan link proteins (Canfield *et al.*, 1987b).

In this present study we have analysed the proteins synthesized by endothelial cells and pericytes maintained under standard tissue culture conditions. We demonstrate that Gp47 is an inhibitor of plasminogen activator (PAI-1) and that it is also secreted in large amounts by retinal pericytes *in vitro*. We also show that other vascular cells (bovine aortic endothelial cells and smooth muscle cells) and non-vascular cells (human skin fibroblasts) secrete this protein but in significantly lower amounts than the microvascular cells. The significance of these findings is discussed in terms of the role of retinal endothelial cells and pericytes in basement membrane maintenance and remodelling.

EXPERIMENTAL

Eagle's minimal essential medium, donor calf serum, sodium pyruvate, glutamine, non-essential amino acids

Abbreviation used: PAI, plasminogen activator inhibitor.

|| To whom correspondence should be addressed.

and antibiotics were obtained from Gibco BRL Ltd., Paisley, Scotland, U.K. Ascorbic acid was obtained from BDH Chemicals Ltd., Poole, Dorset, U.K. *N*-Chlorosuccinimide, β -aminopropionitrile fumarate, phenylmethanesulphonyl fluoride, *N*-ethylmaleimide, 2-mercaptoethanol, Nonidet-P40, Tween-80 and agarose (type I) were purchased from Sigma Chemical Co., Poole, Dorset, U.K. Protein A-Sepharose (CL-4B) was obtained from Pharmacia Fine Chemicals, Milton Keynes, U.K. Thrombin and urokinase (reference standard) were purchased from Leo Laboratories, Aylesbury, Bucks., U.K. and fibrinogen (grade L) was obtained from KabiVitrum, Stockholm, Sweden. Gel bond film was purchased from ICN Biomedical, High Wycombe, Bucks., U.K.

L-[³⁵S]Methionine (1443 Ci/mmol) and [¹⁴C]methylated standard protein mixture (M_r 14 300–200 000) were purchased from Amersham International, Bucks., U.K. Rabbit anti-(human α_1 -antitrypsin) was purchased from Dako Ltd., High Wycombe, Bucks., U.K.

Radiolabelling of cell cultures and isolation of newly synthesized proteins

Bovine retinal microvascular endothelial cells (passage 3–6), retinal pericytes (passage 1–4), aortic endothelial cells (passage 10–12) and aortic smooth muscle cells (passage 3–13) were isolated and cultured on plastic Petri dishes or on gelatin-coated Petri dishes as previously described (Schor *et al.* 1983; Schor & Schor, 1986). Human skin fibroblasts (passage 3–13) were obtained from explant cultures as described by Ham (1980). Stock cultures were routinely maintained in Eagle's minimum essential medium supplemented with either 15% (v/v) donor calf serum (for fibroblast cultures) or 20% (v/v) donor calf serum (for vascular cell cultures), ascorbic acid (50 μ g/ml), 2 mM-glutamine, 1 mM-sodium pyruvate, non-essential amino acids, penicillin (100 units/ml) and streptomycin (0.1 mg/ml). Cultures were incubated at 37 °C in a moist atmosphere of CO₂/air (1:19). Cells were radiolabelled for 24 h at confluence with [³⁵S]methionine (20–50 μ Ci/ml) using the incubation conditions described previously (Canfield *et al.* 1986, 1987b). The medium containing the newly-synthesized proteins was centrifuged (16 000 g, 2 min) to precipitate cell debris; proteinase inhibitors (2 mM-phenylmethanesulphonyl fluoride and 10 mM-*N*-ethylmaleimide) were added to the supernatants and the samples were either analysed immediately or stored at –20 °C before analysis.

Immunoprecipitation

Samples of freshly collected [³⁵S]methionine-labelled medium proteins were tested for the presence of plasminogen activator inhibitor (PAI) by immunoprecipitation using specific antibodies raised against bovine endothelial cell PAI-1 (Van Mourik *et al.*, 1984) following the method described by Cooper *et al.* (1981). Normal rabbit serum was used as a control. Briefly, aliquots (100–200 μ l) of conditioned medium were mixed with 1 ml of 50 mM-Tris/HCl, pH 8.0, containing 0.4 M-NaCl, 5 mM-EDTA and 1% (v/v) Nonidet-P40, and 5 μ l of antiserum or normal rabbit serum was added. After incubation at room temperature for 1.5 h, 30 μ l of a 50% (v/v) suspension of protein A-Sepharose in the above buffer was added and the incubation was continued at 4 °C for a further 1.5 h. The complex of antigen, antibody

and protein A-Sepharose was collected by centrifugation, washed twice with the above buffer and twice with 10 mM-Tris/HCl, pH 6.8. Bound proteins were released by heating at 100 °C for 3 min in double-concentration Laemmli (1970) sample buffer containing 10% (v/v) 2-mercaptoethanol.

Peptide mapping

Peptide mapping techniques were employed to compare the proteins of M_r 47 000 synthesized by retinal endothelial cells and retinal pericytes. Peptide fragments were generated using *N*-chlorosuccinimide as previously described (Canfield *et al.*, 1987b).

Electrophoretic analyses

Discontinuous SDS/polyacrylamide-gel electrophoresis was carried out on the conditioned medium with or without reduction by 5% (v/v) 2-mercaptoethanol as described elsewhere (Canfield *et al.*, 1986). Newly-synthesized proteins were revealed by fluorography (Bonner & Laskey, 1974; Laskey & Mills, 1975). [¹⁴C]Methylated M_r standards were myosin (M_r 200 000), phosphorylase *b* (a doublet of M_r 100 000 and 92 500), bovine serum albumin (M_r 69 000), ovalbumin (M_r 46 000), carbonic anhydrase (M_r 30 000) and lysozyme (M_r 14 300).

Zymography

Samples of conditioned medium was tested for PAI activity by reverse fibrin zymography as originally described by Erickson *et al.* (1984) and modified by Booth *et al.* (1985). Samples were incubated with an equal volume of sample buffer [4% (w/v) SDS/8 M-urea/40 mM-iodoacetamide/0.2 M-Tris/HCl, pH 8.0] for 30 min at 37 °C and were then made 10% (v/v) with respect to glycerol. SDS/polyacrylamide-gel electrophoresis was conducted using 3% and 10% acrylamide stacking and separating gels respectively. The gels were washed for 90 min in 2 $\times$ 500 ml of 2.5% (v/v) aqueous Triton X-100, rinsed in distilled water and then applied to a detector gel. The detector gel contained fibrinogen (2 mg/ml), plasminogen (2.0–3.0 μ g/ml, present as a contaminant of the fibrinogen), thrombin (0.06 unit/ml), urokinase (0.04 i.u./ml), 17.5 mM-NaCl, 60 mM-Tris/HCl, pH 6.8 and 0.8% (w/v) agarose at the final concentrations indicated. After 16–20 h at 30 °C the polyacrylamide gel was removed and the detector gel photographed.

RESULTS

Comparison of the proteins synthesized by retinal endothelial cells and pericytes *in vitro*

Cultures of retinal microvascular cells were grown to confluence and incubated with [³⁵S]methionine as described in the Experimental section. The newly-synthesized proteins secreted into the medium were fractionated with (NH₄)₂SO₄ at 30% saturation (which specifically precipitates collagens and other high- M_r proteins) and at 30–80% saturation (which precipitates the remaining proteins which are predominantly of lower M_r) and then analysed by SDS/polyacrylamide-gel electrophoresis. Fig. 1 clearly demonstrates that the major protein present in the 30–80% (NH₄)₂SO₄ fraction from the medium conditioned by both cell types is of M_r approx. 47 000 (Fig. 1, tracks 2 and 4). This protein,

Fig. 1. Fluorograms of the [^{35}S]methionine-labelled proteins secreted into the medium by retinal endothelial cells and pericytes

Confluent cultures of endothelial cells and pericytes were incubated with [^{35}S]methionine (50 $\mu\text{Ci}/\text{ml}$) for 24 h at 37 $^{\circ}\text{C}$. The medium was then decanted and fractionated with $(\text{NH}_4)_2\text{SO}_4$ as described in the Experimental section. The newly synthesized proteins were separated by electrophoresis under reducing conditions and detected by fluorography. Proteins secreted by endothelial cells are shown in tracks 1 and 2, those secreted by pericytes are in tracks 3 and 4. Tracks 1 and 3 contain species precipitating at 0–30% $(\text{NH}_4)_2\text{SO}_4$ saturation; tracks 2 and 4 contain proteins precipitating at 30–80% $(\text{NH}_4)_2\text{SO}_4$ saturation. The migration positions of fibronectin (Fn), thrombospondin (Tsp), procollagen types I and IV and PAI-1 (Gp47) are indicated.

secreted by retinal endothelial cells has previously been designated Gp47 (Canfield *et al.*, 1987b) and subsequent studies (described below) demonstrate that it is identical to the species of M_r 47000 secreted by retinal pericytes. The major high- M_r proteins synthesized by retinal endothelial cells and pericytes [concentrating in the fraction precipitating at 30% saturation with $(\text{NH}_4)_2\text{SO}_4$] were found to be distinctive for each cell type (Fig. 1, compare tracks 1 and 3). The endothelial cells secrete predominantly type IV (basement membrane-specific) collagen into the culture medium (Fig. 1, track 1), whereas pericytes secrete mainly interstitial type I collagen (Fig. 1, track 3). Fibronectin and thrombospondin were secreted into the medium by both cell types. The identification of the newly-synthesized proteins secreted by endothelial cells had already been published (Canfield *et al.*, 1986). The same methods (i.e. mobilities on SDS/polyacrylamide-gel electrophoresis, immunoprecipitation with specific antibodies and susceptibilities to pepsin and bacterial collagenase) have been used to analyse the proteins synthesized by retinal pericytes, and a full account of this work will be published elsewhere. This present communication is mainly concerned with the identification of protein Gp47.

Identification of protein Gp47 as type I-plasminogen activator inhibitor (PAI-1)

Several lines of evidence demonstrate that protein Gp47 is an inhibitor of plasminogen activator. Direct confirmation was achieved by immunoprecipitation using

Fig. 2. Identification of protein Gp47 as plasminogen activator inhibitor-type I by immunoprecipitation and reverse fibrin zymography

(a) Confluent cultures of cells were incubated with [^{35}S]methionine for 20–24 h at 37 $^{\circ}\text{C}$. At the end of the incubation period samples of the medium were used for immunoprecipitation experiments as described in the text. Samples in tracks 1–8 were as follows: 1, standard [^3H]acetylated rat-tail tendon collagen; 2, total proteins secreted into the medium by pericytes; 3, proteins immunoprecipitated from pericyte medium with anti-(endothelial cell PAI-1) serum; 4, proteins precipitating from pericyte medium with normal rabbit serum; 5, [^{14}C]methylated proteins of known M_r ; 6, total proteins secreted into the medium by retinal endothelial cells; 7, proteins immunoprecipitated from endothelial cell medium with anti-(endothelial cell PAI-1) serum; 8, proteins precipitated from endothelial cell medium with normal rabbit serum. The migration positions of PAI-1 and [^{14}C]methylated proteins of known M_r are indicated. (b) Confluent cultures of pericytes were washed twice with serum-free minimal essential medium (MEM) for 2 h. The medium was removed and fresh serum-free MEM was added, and the incubation was continued for a further 24 h at 37 $^{\circ}\text{C}$. The medium was decanted, centrifuged to precipitate cell debris and diluted 1:2 with 0.1 M-Tris/HCl, pH 7.4, containing 0.1% (v/v) Tween-80. Aliquots (10 μl) were used for reverse fibrin zymography as detailed in the text. The resulting zymogram is shown.

specific antibodies and by reverse fibrin zymography (Fig. 2). Fig. 2(a) demonstrates the specific precipitation of Gp47 from the medium conditioned by retinal pericytes (compare tracks 2 and 3) and endothelial cells (compare tracks 6 and 7) using antibodies raised against bovine aortic endothelial cell PAI-1. In control experiments, antiserum raised against human α_1 -antitrypsin [a protein which has approx. 30% sequence similarity with PAI-1 (Loskutoff *et al.*, 1986)] failed to immunoprecipitate any proteins from the conditioned medium (results not shown). The presence of inhibitory activity in these samples was also demonstrated by the technique of reverse fibrin zymography. Thus, PAI activity shows as a lysis-resistant opaque band, which corresponds to protein Gp47, in a fibrin-agarose indicator gel (Fig. 2b). Final confirmation that the proteins of M_r 47000 synthesized by retinal endothelial cells and pericytes were

Fig. 3. Peptide maps of radiolabelled proteins obtained by using *N*-chlorosuccinimide

Confluent cultures of endothelial cells and pericytes were incubated with [^{35} S]methionine for 20 h at 37 °C. Newly synthesized proteins present in the medium were denatured under reducing conditions, separated on 8% polyacrylamide slab gels and detected by fluorography. Bands corresponding to proteins Gp47 were excised from the gels and incubated for 30 min at room temperature with 0.15 M *N*-chlorosuccinimide as described by Lischwe & Ochs (1982). The resulting peptides were separated by electrophoresis on 15% polyacrylamide slab gels and detected by fluorography. Samples in tracks 1 and 2 are as follows: 1, PAI-1 (Gp47) synthesized by retinal pericytes; 2, PAI-1 (Gp47) synthesized by retinal endothelial cells. The migration positions of [^{14} C]methylated proteins of known M_r are indicated.

identical was achieved by peptide mapping studies using *N*-chlorosuccinimide (Fig. 3).

Supporting evidence that protein Gp47 is PAI-1 was obtained by comparing some of the biochemical properties reported for PAI-1 (Van Mourik *et al.*, 1984) with those found for protein Gp47. Protein Gp47 is a glycoprotein (pI of 4.6–5.5) which does not bind to heparin (Canfield *et al.*, 1987b), which migrates in the same position when it is electrophoresed under reducing and non-reducing conditions (Fig. 4), and which is non-collagenous, as demonstrated by its resistance to digestion by highly purified bacterial collagenase (see Fig. 6). All of these results are consistent with the reported characteristics of PAI-1 (Van Mourik *et al.*, 1984; Collen, 1986).

Synthesis of PAI-1 by other cell types

A series of experiments were conducted in order to investigate whether PAI-1 was synthesized in equally high levels by other vascular and non-vascular cells in culture. Accordingly, confluent cultures of bovine retinal

Fig. 4. Fluorogram of [^{35} S]methionine-labelled proteins secreted into the medium by bovine retinal endothelial cells, aortic endothelial cells, aortic smooth muscle cells and human skin fibroblasts

Confluent cultures of cells were incubated with [^{35} S]methionine for 20 h at 37 °C. At the end of the incubation period the medium was decanted and the newly synthesized proteins were separated by electrophoresis on 8% polyacrylamide slab gels and detected by fluorography. Samples in tracks 1–10 are as follows; 1 and 7, total proteins secreted by retinal endothelial cells; 2 and 8, total proteins secreted by aortic endothelial cells; 3 and 9, total proteins secreted by aortic smooth muscle cells; 4 and 10, total proteins secreted by human skin fibroblasts; 5, standard [^{3} H]acetylated rat-tail tendon collagen; 6, [^{14} C]methylated proteins of known M_r . Samples were electrophoresed either in the presence (tracks 1–6) or absence (tracks 7–10) of 2-mercaptoethanol. The migration positions of [^{14} C]methylated proteins of known M_r are indicated. * Indicates a non-collagenous protein of M_r approx. 40 000 secreted by endothelial cells.

endothelial cells, bovine aortic endothelial cells, bovine aortic smooth muscle cells and human skin fibroblasts were incubated with [^{35} S]methionine, and the newly-synthesized proteins secreted into the medium were analysed by SDS/polyacrylamide-gel electrophoresis. Fig. 4 shows the electrophoretic profiles generated when samples of conditioned medium were analysed either with reduction (tracks 1–4) or without reduction (tracks 7–10). All four cell types were found to secrete a protein of approx. M_r 47 000 which was not disulphide-bonded into higher M_r multimers. However, the microvascular cells synthesize significantly more of this protein than do the other cell types (Fig. 4, compare tracks 1–4). Confirmation that the proteins of M_r 47 000 synthesized by aortic endothelial cells and human skin fibroblasts were PAI-1 was obtained by immunoprecipitation using antibodies raised against bovine aortic endothelial cell PAI-1 (Fig. 5a) and by reverse fibrin zymography (Fig. 5b). It is of particular interest that the protein precipitating with anti-PAI-1 from human-fibroblast conditioned medium has a significantly slower mobility than PAI-1 from bovine vascular cells. This difference in electrophoretic mobility was a consistent observation first noted in Fig.

Fig. 5. Identification of PAI-1 in the culture medium from aortic endothelial cells and human skin fibroblasts

(a) Cultures of aortic endothelial cells and human skin fibroblasts were incubated with [^{35} S]methionine for 20 h at 37 °C and samples (100 μ l) of medium were used for immunoprecipitation experiments as detailed in the text. The products were separated by electrophoresis under reducing conditions on a 6.5 % polyacrylamide slab gel and revealed by fluorography. Samples in tracks 1–6 are as follows: 1, total proteins secreted into the medium by skin fibroblasts; 2, proteins immunoprecipitating from fibroblast medium with anti-(endothelial cell PAI-1) serum; 3, proteins precipitating from fibroblast medium with normal rabbit serum; 4, total proteins secreted into the medium by aortic endothelial cells; 5, proteins immunoprecipitating from endothelial cell medium with anti-(endothelial cell PAI-1) serum; 6, proteins precipitating from endothelial cell medium with normal rabbit serum. The migration positions of [^{14} C]methylated proteins of known M_r are indicated. (b) Serum-free conditioned medium was collected from confluent cultures of aortic endothelial cells and skin fibroblasts as described in the legend to Fig. 2(b). Samples were diluted 1:2 with 0.1 M-Tris/HCl, pH 7.4, containing 0.1 % (v/v) Tween-80 and aliquots (10 μ l) were used for reverse fibrin zymography as described in the Experimental section. Samples in tracks 1 and 2 are from skin fibroblast and aortic endothelial cell cultures respectively. The migration positions of PAI-1 secreted by fibroblasts and endothelial cells are marked by arrowheads.

4, where the migration of PAI-1 secreted by fibroblasts suggested it was of greater M_r than PAI-1 from vascular cells.

Since all the various cell types used in this study synthesize PAI-1, it was important to assess whether these cells differ from each other regarding the synthesis of other proteins. These studies clearly demonstrate that each cell type synthesizes its own specific population of extracellular matrix proteins. Proteins secreted into the medium by retinal endothelial cells, aortic endothelial cells, aortic smooth muscle cells and human skin fibroblasts are shown in Figs. 4 and 6. The proteins secreted by retinal pericytes are shown in Fig. 1 (tracks 3 and 4).

Fig. 6. Effect of bacterial collagenase on the newly synthesized proteins secreted into the medium by retinal endothelial cells, aortic endothelial cells, aortic smooth muscle cells and human skin fibroblasts

Cell cultures were labelled with [^{35} S]methionine for 24 h. Proteins secreted into the medium were digested with highly-purified bacterial collagenase (30 units) for 3 h at 37 °C in 62 mM-Tris/HCl buffer, pH 7.4, containing 5 mM- CaCl_2 . The products were separated by electrophoresis under reducing conditions on an 8 % polyacrylamide slab gel and revealed by fluorography. Samples in tracks 1–9 are as follows: 1 and 2, samples from retinal endothelial cell cultures; 3 and 4, samples from aortic endothelial cell cultures; 5, [^{14} C]methylated proteins of known M_r ; 6 and 7, samples from smooth muscle cell cultures; 8 and 9, samples from human skin fibroblast cultures. Samples in tracks 1, 3, 6 and 8 were incubated for 3 h without enzyme; samples in tracks 2, 4, 7 and 9 were incubated for 3 h with bacterial collagenase. The migration positions of fibronectin (Fn), thrombospondin (Tsp), pro α_1 (I) and pro α_2 (I) chains of type I collagen and [^{14} C]methylated proteins of known M_r are indicated.

These proteins have been identified by their characteristic electrophoretic mobilities before and after reduction (Fig. 4), their susceptibilities to digestion by bacterial collagenase (Fig. 6) and to pepsin (not shown). Thus, smooth muscle cells, pericytes and skin fibroblasts synthesize and secrete more bacterial collagenase-sensitive proteins than the endothelial cell cultures (Fig. 6). These collagens have been identified by pepsin digestion as a mixture of collagen types I and III (results not shown). By comparison, the retinal endothelial cells synthesize type IV collagen, and the population of aortic endothelial cells used in this study secrete only minor amounts of collagenous proteins. All five cell types synthesize the high- M_r non-collagenous glycoprotein fibronectin (see Figs. 1 and 6). However, thrombospondin, which is a major glycoprotein secreted into the medium by the endothelial cells and pericytes, is synthesized in much lower amounts by smooth muscle cells and skin fibroblasts. Several other, at present uncharacterized, proteins may also be distinctive for each cell type. For example, the endothelial cells secrete a non-collagenous protein of M_r approx. 40 000 (* in Fig. 4), which is not

detected in either the smooth muscle cell, pericyte or fibroblast culture medium. Previous studies also indicate that this latter protein binds to heparin-Sepharose (Canfield *et al.*, 1987b). Furthermore, proteins of M_r approx. 90000, 32000 and 35000–39000 appear to be specific for the smooth muscle cell cultures (Fig. 4).

DISCUSSION

The structural rigidity of the wall of small blood vessels is in a large part attributable to the capillary basement membrane. This structure undergoes extensive remodelling during the process of neovascularization: there is an initial localized degradation of the basement membrane through which the endothelial cells then migrate, and this is followed by the synthesis of a new blood vessel wall. The factors which regulate this complex process remain to be fully elucidated, but are likely to include collagenases, stromelysin and plasmin, together with their respective activators and inhibitors (Schor & Schor, 1983; Murphy & Reynolds, 1985; Saksela, 1985; Furcht, 1986; Moscatelli & Rifkin, 1988; Reich *et al.*, 1988). In this present communication we demonstrate that retinal microvessel endothelial cells and pericytes synthesize and secrete substantial amounts of an inhibitor of plasminogen activator (PAI-1) when maintained in monolayer culture. This protein inhibits the conversion of plasminogen to plasmin by plasminogen activators (for recent reviews, see Blasi *et al.*, 1987; Sprengers & Kluft, 1987; Loskutoff *et al.*, 1988). The high levels of PAI-1 synthesis by the microvascular cells may play an important role in regulating the degradation of the vessel wall *in vivo* (see Reich *et al.*, 1988).

The biosynthesis of PAI-1 by retinal vascular cells was demonstrated by (a) the specific precipitation of a protein of M_r 47000 using antiserum raised against bovine aortic endothelial cell PAI-1 (Fig. 2a); (b) the presence of a lysis-resistant opaque band (corresponding to a protein of M_r 47000) in a fibrin-agarose detector gel (Fig. 2b); and (c) comparing some of the biochemical properties of this protein (Figs. 1, 4 and 6, and Canfield *et al.*, 1987b) with those previously reported for PAI-1 (Van Mourik *et al.*, 1984). Accordingly, we have demonstrated that this protein (M_r 47000) contains approx. 10% carbohydrate; thus this species can be radiolabelled with [^{3} H]fucose and [^{3} H]mannose, it binds to concanavalin A-Sepharose, and in experiments in which the cells were incubated with tunicamycin, the M_r was reduced to 42000 (Canfield *et al.*, 1987b). These results are consistent with the presence of three potential *N*-glycosylation sites in endothelial cell PAI-1, which has been predicted from sequence analyses (Ginsburg *et al.*, 1986; Ny *et al.*, 1986; Pannekoek *et al.*, 1986). We also demonstrate that bovine microvessel PAI-1 migrates in the same position when it is electrophoresed under reducing or non-reducing conditions (Fig. 4), in agreement with previous reports (Loskutoff *et al.*, 1983). This finding is also in accordance with the reported absence of cysteine residues in PAI-1 (Ny *et al.*, 1986; Pannekoek *et al.*, 1986).

Our studies also demonstrate that other cells, namely bovine aortic endothelial cells and human skin fibroblasts, synthesize and secrete PAI-1 *in vitro* (Figs. 4 and 5). Bovine aortic smooth muscle cells also secrete a protein of M_r approx. 47000 which we tentatively identify as PAI-1, for it also migrates in the same position when it is electrophoresed under reducing and non-reducing

conditions (Fig. 4). Furthermore, it is noteworthy that the PAI-1 produced by human skin fibroblasts had a markedly reduced electrophoretic mobility compared with the protein produced by bovine vascular cells, suggesting there may be some variation between PAIs synthesized by cells derived either from different tissues or species. The secretion of PAIs by bovine aortic endothelial cells (Van Mourik *et al.*, 1984; Erickson *et al.*, 1985; Hekman & Loskutoff, 1985; Lawrence & Loskutoff, 1986), human umbilical vein endothelial cells (Gelehrter & Sznycer-Laszuk, 1986; Ginsburg *et al.*, 1986; Kooistra *et al.*, 1986; Levin, 1986; Pannekoek *et al.*, 1986; Booth *et al.* 1987; Levin & Santell, 1987), bovine aortic smooth muscle cells (Laug, 1985) and human lung fibroblasts (Laiho *et al.*, 1986; Lund *et al.*, 1987; Pollanen *et al.*, 1987) has previously been reported. This is the first report, to our knowledge, showing that retinal pericytes can secrete PAI-1.

The maintenance and integrity of a normal blood vessel wall *in vivo* depends on the controlled turnover of connective tissue components. Any disturbance in the balance between synthesis and degradation of these proteins will have a dramatic effect on vascular function. The present paper shows that the different cells of the vasculature appear to synthesize specific matrix proteins (Figs. 4 and 6); in addition, they also secrete various metalloproteinases, together with activators and inhibitors of these enzymes (this communication; Laug, 1981, 1985; Gross *et al.*, 1982; Booyse *et al.*, 1984; Van Mourik *et al.*, 1984; Erickson *et al.*, 1985; Ginsburg *et al.*, 1986; Moscatelli, 1986; Moscatelli & Rifkin, 1988). However, retinal microvascular cells secrete markedly more PAI-1 than cells derived from the aorta or human skin fibroblasts. It can be speculated that the secretion of high levels of PAI-1 by both pericytes and endothelial cells may play a role during tumour extravasation and angiogenesis by preventing the degradation of the normal microvessel wall. It is interesting in this context that certain angiogenic factors like basic fibroblast growth factor and transforming growth factor β modulate the synthesis PAI-1 (Saksela *et al.*, 1987). The loss of pericytes during diabetic retinopathy may therefore decrease the total amount of PAI-1 present in the retinal microvessels and this may be a contributory factor leading to neovascularization.

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