PLASMINOGEN ACTIVATOR-SPECIFIC INHIBITORS PRODUCED BY HUMAN MONOCYTES/MACROPHAGES

By ANNELISE WOHLWEND,* DOMINIQUE BELIN,‡ AND JEAN-DOMINIQUE VASSALLI*

*From the *Institute of Histology and Embryology, and the ‡Department of Pathology, University of Geneva Medical School, 1211 Geneva 4, Switzerland*

Extracellular proteolysis is associated with cell migration and tissue remodelling (1). In particular, plasminogen activators (PAs)[1] are produced by monocytes/macrophages involved in inflammatory reactions (2, 3). In the context of the focal control of PA-mediated extracellular proteolysis, the cellular production of specific protease inhibitors and its possible hormonal control are thus of particular interest.

The existence of protease inhibitors with a high affinity for PAs has been demonstrated in several tissues and cultured cell types. An inhibitor of the urokinase-type PA (uPA), first identified in human placenta by Kawano et al. (4), has been purified (5) (T. C. Wun and E. Reich, manuscript in preparation). This PA-specific inhibitor (PAI), named PAI-2, forms SDS-resistant, presumably covalent, complexes with uPA and tissue-type PA (tPA), and such complexes lack enzymatic activity; PAI-2 has a very high affinity for PAs (6) and does not react with plasmin or thrombin. Human fibroblasts have also been shown to secrete a variety of protease ligands, named protease-nexins (PN) (7, 8). The protease-PN complexes bind to fibroblasts through their PN moiety, and are rapidly internalized and degraded (9). PN1, which has been purified (10, 11), can be clearly distinguished from PAI-2 by pI and by its reactivity towards other serine proteases, such as thrombin and plasmin. Another PAI, named PAI-1, produced by bovine endothelial cells (12, 13) and present in human platelets (14), has also been characterized. The unusual stability of this PAI to acid or SDS is in contrast to the lability of the other protease inhibitors discussed above.

Until recently, no specific PAI had been identified in plasma, and the generally accepted view was that the already known plasma protease inhibitors were solely responsible for the inactivation of PAs in plasma (15, 16). The existence of fast-acting PAI(s) in plasma has now been conclusively demonstrated (17–19); some of these are antigenically related to the PAI-2 (20), and others to the PAI-1 (21).

Mononuclear phagocytes also secrete fibrinolytic inhibitors (22, 23), and recent studies (24–28) have established some of the characteristics of PA-specific ligands secreted by monocytes/macrophages. In view of the importance of controlled

This work was supported by grant 3.075-0.84 from the Swiss National Science Foundation and by the Sir Jules Thorn Charitable Overseas Trust. Address correspondence to A. Wohlwend, Institute d'Histologie et d'Embryologie, CMU, 1, rue Michel-Servet, 1211 Genève 4, Switzerland.

[1] *Abbreviations used in this paper:* PA, plasminogen activator; PAI, plasminogen activator inhibitor; PN, protease nexin; tPA, tissue-type PA; uPA, urokinase-type PA.

 J. Exp. Med. © The Rockefeller University Press · 0022-1007/87/02/0320/20 $1.00
Volume 165 February 1987 320–339

extracellular proteolysis in inflammatory reactions, a detailed understanding of the characteristics of the PAI(s) produced by mononuclear phagocytes is of obvious interest. We have previously shown that cells from the human histiocytic lymphoma–derived U 937 line, as well as monocytes purified from human peripheral blood, simultaneously secrete the single-chain zymogen pro-uPA and an inhibitor of active two-chain uPA (25). We provide here an analysis of the biochemical properties of the monocyte and U 937-produced PAIs. In particular we show that monocytes/macrophages synthesize two functionally and antigenically related forms of PAI: a predominantly intracellular protein, corresponding to that described in our previous work (25), and a glycosylated, preferentially secreted form. The production of these PAIs is enhanced under conditions that promote the differentiation of mononuclear phagocytes (29) and modulate their functional activity (30).

Materials and Methods

Materials. Purified human tPA from HeLa cells, and rabbit antibody to human uPA were the kind gift of Dr. W. D. Schleuning. Affinity-purified rabbit IgG raised against PAI-2 from human placenta was kindly provided by Dr. T. C. Wun; rabbit antiserum to PAI-1 from bovine aortic endothelial cells was the generous gift of Dr. D. J. Loskutoff; rabbit antiserum to the purified low-M_r PAI from U 937 cells was the kind gift of Dr. E. K. O. Kruithof. ^{125}I-labeled plasma kallikrein and factor XIIf (10^6 cpm/μg) were kindly provided by Dr. A. de Agostini. M_r 55,000 uPA (1.5×10^5 U/mg) was from the Green Cross Corporation (Osaka, Japan); M_r 33,000 uPA was generously provided by Serono (Denens, Switzerland). Actinomycin D (A-4262), cycloheximide (C-6255), Clostridium perfringens neuraminidase (N-2133), NP-40 (N-6507), phorbol 12-myristate 13-acetate (PMA) (P-8139), porcine pancreatic trypsin (T-0134), thrombin from human plasma (T-6759), tunicamycin (T-7765), and soybean trypsin inhibitor (T-9003) were from Sigma Chemical Co. (St Louis, MO). Trasylol was obtained from Bayer (Zurich, Switzerland). Porcine pancreatic elastase was obtained from Elastin Products Co. (Pacific, MI). Bovine serum albumin (BSA) was obtained from Miles (Rehovot, Israel); stock solutions were acid-treated before use. Sephadex G-50 and G-200, Concanavalin A–Sepharose, carrier ampholytes (Pharmalytes; pH 3–10 and 4–6.5), Percoll, and low-M_r electrophoresis calibration kit were from Pharmacia Fine Chemicals (Uppsala, Sweden), Ultrogel ACA 54 from LKB Instruments Inc. (Bromma, Sweden), *Streptococcus aureus* (Pansorbin) from Calbiochem Behring (La Jolla, CA) and methyl-α-D-mannopyranoside from Fluka Chemical Co. (Buchs, Switzerland). Na[^{125}I],L-[^{35}S]methionine, and En^{3}Hance were obtained from Amersham Corp. (Amersham, Great Britain). RPMI 1640 and FCS were from Gibco Laboratories, (Grand Island, NY), Triton X-100 from Merck and Co., Inc. (Rahway, NJ), SDS from Bio-Rad Laboratories (Richmond, CA), Iodogen from Pierce Chemical Co. (Rockford, IL), and acrylamide, bisacrylamide, *N,N,N′,N′*-tetramethylethylenediamine and ammonium persulfate from Eastman Kodak Co. (Rochester, NY). All the other chemicals were of the best commercial grade available. Tissue culture plasticware was from Falcon Labware (Oxnard, CA). Plasminogen was purified from human plasma (31).

Cell Culture. Cultures of U 937 cells and human peripheral blood monocytes, and preparation of conditioned media were performed as previously described (25); incubations were performed in RPMI 1640 + 1 mg/ml BSA. Cells were collected by centrifugation (10 min at 300 *g*), washed three times with PBS + 1 mg/ml BSA, and lysed in 0.2% Triton X-100, 0.1 M Tris HCl, pH 8.1. Nuclei and cell debris were removed by centrifugation for 10 min at 750 *g*, and the cell extracts stored at −20°C.

Trypsin Treatment. To investigate the accessibility of the cell-associated low-M_r PAI to trypsin, U 937 cells were collected, washed twice in serum-free RPMI 1640, and resuspended at 10^6 cells/ml in RPMI 1640 + 1 mg/ml BSA. Alternatively, the cells were lysed at the same density in PBS + 0.2% Triton X-100, and the nuclei were discarded by

centrifugation for 10 min at 1,000 *g*. Intact and lysed cells were further processed in parallel. Trypsin was added to the samples at a final concentration of 5 μg/ml, and the cells were incubated for various times at 37°C. Proteolysis was blocked by cooling the samples on ice and by the concomitant addition of soybean trypsin inhibitor at a final concentration of 50 μg/ml. Intact trypsin-treated cells were subsequently lysed as described above.

^{125}I Labeling of u-PA, Thrombin, Trypsin, and Elastase. The proteins were labeled with Iodogen as previously described (25); the specific activities of the ^{125}I-enzymes were as follows: M_r 55,000 uPA, 1–2 × 10^7 cpm/μg; M_r 33,000 uPA, 0.8–1.7 × 10^7 cpm/μg; thrombin, 3 × 10^6 cpm/μg (two radioactive peptides were present in the preparation of ^{125}I-thrombin; one unidentified contaminant of M_r 20,000, and thrombin itself, at M_r 34,000; trypsin, 3.4 × 10^6 cpm/μg; elastase, 7 × 10^6 cpm/μg. Plasminogen (6–7 × 10^6 cpm/μg) was converted to ^{125}I-plasmin by incubation at 30°C for 18 h in presence of 100 U/ml uPA and 50% glycerol; the extent of conversion of ^{125}I-labeled one-chain plasminogen into two-chain plasmin was determined by SDS-PAGE under reducing conditions and autoradiography.

Formation of Enzyme/Inhibitor Complexes. Unless specifically indicated, 5 μl of ^{125}I-enzyme (0.5–2 ng) were added to 10 μl of the samples to be tested; after 1 h at 4°C, the reaction was stopped by adding an equal volume of double-strength electrophoresis sample buffer. For competition studies, the samples were preincubated for 30 min at 4°C with a 20–100-fold excess unlabeled enzyme before their incubation with ^{125}I-enzyme.

Like the other inhibitors of the serpin family (32), U 937-produced PAIs complex uPA essentially irreversibly. Therefore, the sensitivity of uPA to the PAIs cannot be measured by K_i. Formation of nondissociating protease/inhibitor complexes can be kinetically resolved into two steps: (*a*) initial formation of a reversible complex that dissociates at a finite rate, and (*b*) conversion of the reversible complex into a stable complex. At protease and inhibitor concentrations below the micromolar range, the reactions proceed according to simple second-order kinetics. The corresponding association constants (k_{assoc}) have been used to compare the specificity of α-1 proteinase inhibitor (33) and that of protease nexin (11) for different proteases. In the case of the U 937-produced PAIs, the plots of $1/V$ versus time (calculated from the data illustrated in Fig. 4) were linear up to 10 min; this was consistent with simple, second-order kinetics, and has allowed us to determine the k_{assoc} of uPA with the U 937-produced PAIs.

SDS-PAGE. SDS-PAGE and processing of the electrophoretic gels for autoradiography were performed as previously described (25). Molecular weight standards were run on the same gels, and their positions are indicated on the margin; the M_r values of the different species present were calculated from the positions of the markers.

Biosynthetic Labeling. U 937 cells were collected by centrifugation, washed three times in PBS + 1 mg/ml BSA, and resuspended at 2 × 10^6 cells/ml in a modified culture medium containing 80 μCi/ml [^{35}S]methionine, 90% methionine-free DMEM, 10% RPMI 1640, 1 mg/ml BSA, and, when indicated, 30 ng/ml PMA. After 24 h at 37°C, the conditioned media were collected by centrifugation. The cells were washed once with PBS, centrifuged, and resuspended in 0.6% Triton X-100, 0.1 M Tris HCl, pH 8.1, and 200 KIU/ml Trasylol; Triton X-100 and Trasylol were also added to the conditioned media to obtain the same final concentrations as in the cell extracts. Aliquots of the ^{35}S-labeled conditioned media or cell extracts were incubated for 1 h at 4°C with M_r 33,000 unlabeled uPA (1.5 μg/ml) or an equal volume of PBS. The samples were then incubated for 30 min at 20°C with *S. aureus*, centrifuged, and the pellets discarded. The supernatants were then immunoprecipitated with anti-uPA IgG (36 μg/ml) or nonimmune IgG (40 μg/ml) (see below); the supernatants and pellets of immunoprecipitation were subjected to SDS-PAGE. The gels were fixed, stained, treated with En^{3}Hance before drying, and exposed to Kodak SB-5 films at −80°C.

Immunoprecipitation. This was performed as previously described (25) with the following modifications: the samples were first incubated with M_r 33,000 ^{125}I-uPA; 15 μl aliquots were subsequently mixed with 2 μl of anti-uPA IgG (0.5 mg/ml), anti–placental PAI-2 IgG (0.6 mg/ml), antiserum to endothelial PAI-1 (1:5 dilution), or nonimmune IgG (1.2

mg/ml). After 2 h at 4°C, *S. aureus* (15 μl) were added, the samples were further incubated for 30 min at 20°C, and the suspensions were centrifuged (5 min; Beckman microfuge). The supernatants were mixed with an equal volume of double-strength electrophoresis sample buffer, containing 5% 2-ME. The pellets were washed three times with NET-TS (0.5 M NaCl, 1 mM EDTA, 50 mM Tris HCl, pH 8.1; 1% Triton X-100, 0.2% SDS), once with NET-TU (as NET-TS, expected that SDS is replaced by 1 M urea), and once with NT-S (0.5 M NaCl, 50 mM Tris HCl, pH 8.1; 0.1% SDS), and finally eluted with 30 μl of sample buffer + 5% 2-ME. Both supernatants and immunoprecipitates were heated 5 min at 100°C before SDS-PAGE, and the gel was processed for autoradiography.

Gel Filtration. Ultrogel ACA 54 was equilibrated in 0.2 M ammonium acetate, pH 7.0. Conditioned media (5–10 ml) were lyophilized, resuspended in 0.5–1 ml 0.2 M ammonium acetate, pH 7.0, applied to the column, and 2 ml fractions were collected. Aliquots of the fractions were subsequently incubated with M_r 33,000 ^{125}I-uPA and analyzed by SDS-PAGE and autoradiography.

Concanavalin A–Sepharose. Conditioned media (0.4–0.6 ml) were loaded on a Con A–Sepharose column (1 ml), previously equilibrated in PBS. The column was washed with PBS and eluted with 0.2 M methyl-α-D-mannopyranoside in PBS + 1 mg/ml BSA. Aliquots of the fractions collected were subsequently incubated with M_r 33,000 ^{125}I-uPA and analyzed by SDS-PAGE and autoradiography.

Analytical Isoelectric Focusing. Conditioned media (1 ml) were lyophilized, resuspended in 0.1 ml water, and applied to spun Sephadex G-50 columns (34) equilibrated in carrier ampholytes (pH 3–10 or pH 4–6.5). The samples were then subjected to IEF in Sephadex G-200 previously equilibrated in the corresponding ampholytes, as described in the instruction manual (Pharmacia Fine Chemicals). Alternatively, the samples were incubated for 1 h at 37°C in presence of 0.1 U/ml neuraminidase before IEF. The gels were subsequently fractionated and the fractions were reconstituted in 0.25 ml water. The pH of the fractions was measured and subsequently brought back to neutrality by addition of 0.1 ml 1 M Tris HCl, pH 7.4. The samples were further incubated with M_r 33,000 ^{125}I-uPA and analyzed by SDS-PAGE and autoradiography.

Results

Synthesis and Secretion of PAIs by U 937 Cells in Response to PMA. To investigate a possible modulation of production of the PAI we had previously identified (25), U 937 cells were cultured in presence of PMA. Conditioned media were collected at different times, and incubated with M_r 33,000 ^{125}I-uPA. The presence of PAIs was detected by the formation of radiolabeled enzyme/ligand complexes; these complexes were stable in SDS-PAGE, and could be visualized by autoradiography as radioactive bands of slower electrophoretic mobility than the free enzyme. An increase in release of a ligand forming an M_r 72,000 complex with ^{125}I-uPA was observed after 6 h of incubation in presence of PMA. A ligand forming a M_r 94,000 complex with M_r 33,000 ^{125}I-uPA was detected in the medium after 9 h (Fig. 1 *A*); upon longer incubation (24 h), the conditioned medium of PMA-treated U 937 cells contained mainly the ligand forming the higher-M_r complex (Fig. 1 *A*). Both ligands were found to be inhibitors of uPA: as previously shown for the M_r 72,000 complex (25), zymographic analysis revealed that the specific catalytic activity of the M_r 94,000 complex was markedly lower than that of free uPA (data not shown). These ligands will thus be referred to as low-M_r and high-M_r PAIs, respectively. The induced secretion of the high-M_r PAI was detectable down to a concentration of 1 ng/ml of PMA.

The amount of PAI present in conditioned media and cell extracts of control and PMA-treated U 937 cells was determined; the values from a representative experiment are presented in Table I.

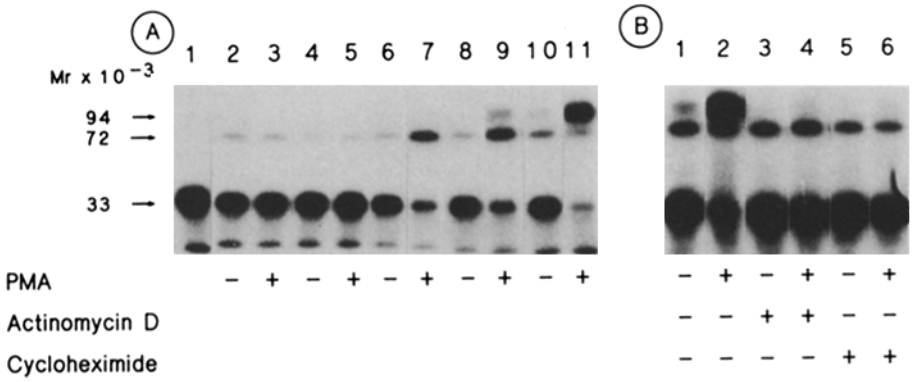

FIGURE 1. Production of PAIs by U 937 cells in response to PMA. (*A*) Time course. U 937 cells were resuspended at 2×10^6 cells/ml in RPMI + 1 mg/ml BSA alone (lanes *2*, *4*, *6*, *8*, and *10*) or containing 10 ng/ml PMA (lanes *3*, *5*, *7*, *9*, and *11*), and incubated for 1 h (lanes *2* and *3*), 3 h (lanes *4* and *5*), 6 h (lanes *6* and *7*), 9 h (lanes *8* and *9*), or 24 h (lanes *10* and *11*); control: culture medium without cells (lane *1*). Samples (10 μl) of control and conditioned media were incubated for 1 h at 4°C with ^{125}I-uPA (2 ng), and analyzed by SDS-PAGE and autoradiography. M_r were calculated from the positions of markers in the stained gel. (*B*) Effects of actinomycin D and cycloheximide. U 937 cells (2×10^6 cells/ml) were incubated for 16 h in RPMI + 1 mg/ml BSA with or without 10 ng/ml PMA, 5 μg/ml actinomycin D, and 5 μg/ml cycloheximide, as indicated. The conditioned media were then processed as described in *A*.

TABLE I
Quantitation of PAI in U 937 Cultures

Culture	[PAI]* (micrograms per 2×10^6 cells per 20 h)	
	Conditioned medium	Cell extract
Control	0.03	1.29
PMA-treated	1.31	1.48

* The M_r 72,000 and 94,000 radiolabeled complexes formed upon incubation with ^{125}I-uPA were quantitated by cutting the appropriate regions of the electrophoretic gels and counting their radioactivity. The amount of PAI was calculated on the basis of the specific activity of ^{125}I-uPA and of the stoichiometry of the complexes (Fig. 5); this calculation takes into account the fact that the conditioned medium of PMA-treated cells contained mainly the high-M_r PAI, and that the other samples contained only the low-M_r PAI.

To investigate the mechanism of PMA-mediated induction of PAI secretion, we incubated U 937 cells with PMA in presence of drugs that interfere with RNA or protein synthesis, i.e., actinomycin D and cycloheximide, respectively (Fig. 1*B*). The secretion of the high-M_r PAI was dependent upon ongoing RNA and protein synthesis, in contrast to the release of the low-M_r PAI, which appeared unaffected by these drugs.

To determine whether PMA enhanced the synthesis of these PAIs, U 937 cells were incubated in presence of [^{35}S]methionine. The biosynthetically labeled conditioned media and cell extracts of control and PMA-treated cultures were incubated with M_r 33,000 unlabeled urokinase, and the uPA/^{35}S-labeled-PAI

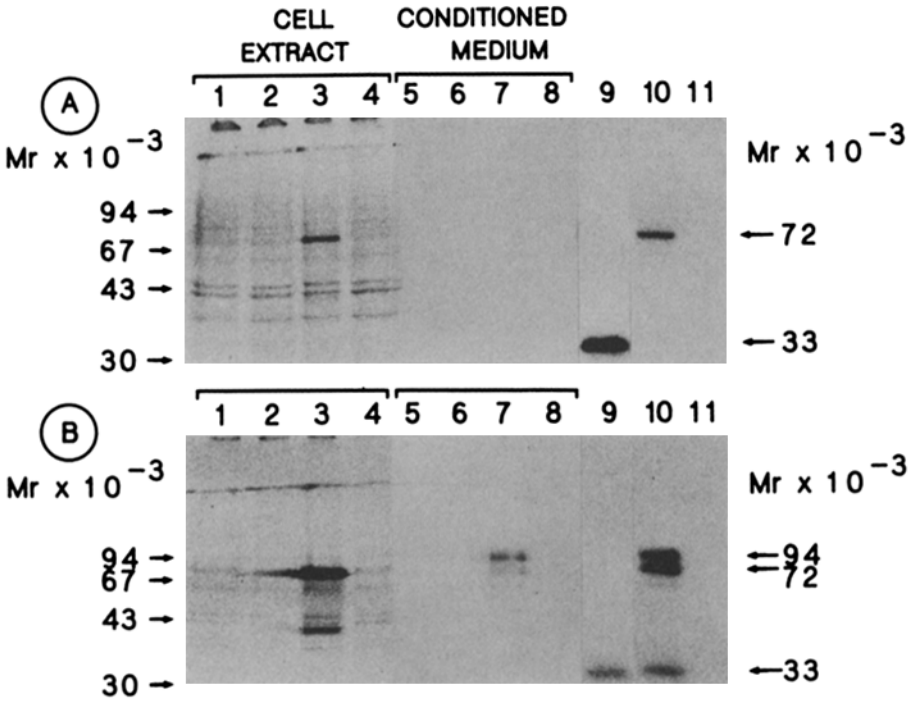

FIGURE 2. Immunoprecipitation of [^{35}S]methionine-labeled PAIs produced by U 937 cells: effects of PMA. U 937 cells were biosynthetically labeled with [^{35}S]methionine, in absence (*A*) or in presence (*B*) of 30 ng/ml PMA. Cell extracts (lanes *1–4*) and conditioned media (lanes *5–8*) were collected after 24 h of culture, and incubated with M_r 33,000 unlabeled uPA (1.5 μg/ml) (lanes *3*, *4*, *7*, and *8*) or an equal volume of PBS (lanes *1*, *2*, *5*, and *6*), before immunoprecipitation with anti-uPA IgG (lanes *1*, *3*, *5*, and *7*) or nonimmune IgG (lanes *2*, *4*, *6*, and *8*). For comparison, M_r 33,000 ^{125}I-uPA (lane *9*, *A* and *B*) was added to aliquots (10 μl) of cell extract from control cultures (*A*, lanes *10* and *11*) or conditioned medium from PMA-treated cultures (*B*, lanes *10* and *11*) before immunoprecipitation with anti-uPA IgG (lane *10*, *A* and *B*) or nonimmune IgG (lane *11*, *A* and *B*). The immunoprecipitates were processed for SDS-PAGE under reducing conditions, and the gels were analyzed by fluorography.

complexes were immunoprecipitated by specific anti-uPA IgG. SDS-PAGE and autoradiography of the immunoprecipitates (Fig. 2) revealed that, in absence of PMA (Fig. 2*A*), an M_r 72,000, radioactive complex was specifically immunoprecipitated from the U 937 cell extract by anti-uPA IgG (Fig. 2*A*, lane *3*). We did not detect newly synthesized low-M_r PAI in the corresponding conditioned medium (*lane 7*). In PMA-treated U 937 cells (Fig. 2*B*), the amount of newly synthesized low-M_r PAI was increased in the cell extract (lane *3*); additional bands were also specifically immunoprecipitated: they probably represent degradation products of the M_r 72,000 complex. Although U 937 cells have been shown to secrete the single-chain proenzyme form of uPA, the amount of enzyme produced by these cells is much lower than that of the PAIs (25) and was therefore not detected in control immunoprecipitates (Fig. 2, lanes *1* and *5*). Furthermore, PMA induced the synthesis of the high-M_r PAI and its secretion into the corresponding culture medium (Fig. 2*B*, lane 7).

Thus, both low- and high-M_r PAIs are synthesized by U 937 cells. Cell extracts contain the low-M_r PAI; we could not detect any cell-associated high-M_r PAI,

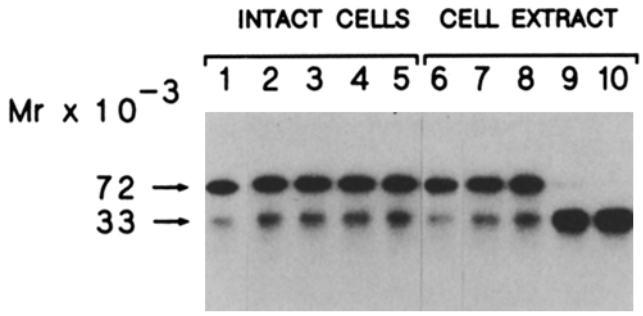

FIGURE 3. The cell-associated low-M_r PAI is not accessible to trypsin in intact cells. U 937 cells (10^6 cells/ml) were resuspended in RPMI + 1 mg/ml BSA (lanes *1–5*) or solubilized in PBS + 0.2% Triton X-100 (lanes *6–10*). The samples were then incubated at 37°C with 5 μg/ml trypsin. Proteolysis was blocked by the addition of soybean trypsin inhibitor (50 μg/ml) at 0°C after 1 min (lanes *3* and *8*), 30 min (lanes *4* and *9*), or 60 min (lanes *5* and *10*). *1* and *6*: nonincubated samples. *2* and *7*: samples incubated for 90 min at 37°C in the absence of trypsin. Intact cells were then lysed by addition of 0.2% Triton X-100; all samples were subsequently incubated with ^{125}I-uPA and processed for SDS-PAGE and autoradiography.

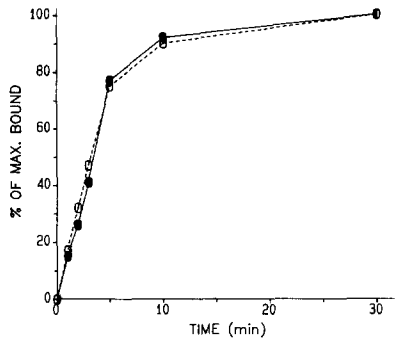

FIGURE 4. Kinetics of complex formation of ^{125}I-uPA with the low- and high-M_r U 937-produced PAIs. ^{125}I-uPA (1.8 nM) was added to conditioned media from control (○) or PMA-treated (●) U 937 cells, which had been diluted with fresh culture medium to contain 1–1.5 nM free low- or high-M_r PAIs. The reaction mixtures were incubated at 37°C; aliquots were taken at indicated times and processed for SDS-PAGE and autoradiography. The fixed and stained gel was sliced and radioactivity in the complexes counted in a gamma counter.

which thus appears to be preferentially secreted. This pattern was not due to the coexistence of different subpopulations in the U 937 cell line; indeed, all of 40 single cell–derived cloned cultures of U 937 cells contained the low-M_r PAI and secreted mainly the high-M_r form in response to PMA (data not shown).

To determine whether the low-M_r, cell-associated PAI was exposed at the cell surface, we incubated U 937 cells in presence of trypsin (Fig. 3). The low-M_r PAI present in intact, live cells was totally resistant to proteolysis (lanes *1–5*), whereas it was completely degraded when cells had been solubilized before their incubation with trypsin (Fig. 3, lanes *6–10*). This indicates that the low-M_r PAI is intracellular.

Kinetics of Complex Formation between ^{125}I-uPA and U 937-produced PAIs. The time course of complex formation between ^{125}I-uPA and the low-M_r PAI was comparable to that with the high-M_r PAI (Fig. 4): half of the added ^{125}I-uPA was complexed within the first 2–3 min incubation in U 937-conditioned media;

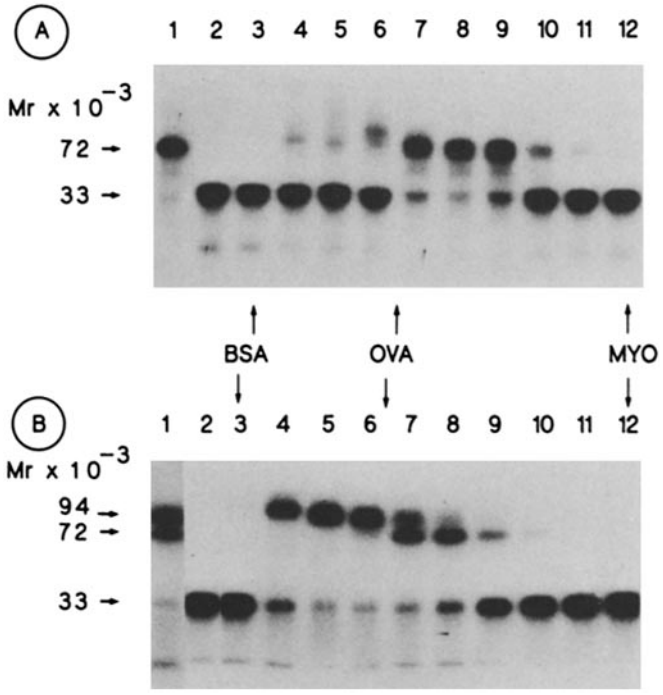

FIGURE 5. Determination of the M_rs of the unreacted PAIs by gel filtration. Conditioned media from control (*A*) or PMA-treated (*B*) U 937 cells were analyzed by gel filtration on Ultrogel ACA 54. Aliquots of the conditioned media before gel filtration (lane *1*, *A* and *B*) and of the fractions eluted from the column (lanes *2–12*) were incubated with M_r 33,000 ^{125}I-uPA, and the samples were analyzed by SDS-PAGE and autoradiography. The ACA 54 column was calibrated before each analysis: the position of elution of the markers is indicated: *BSA*, M_r 67,000; *OVA*, M_r 45,000; *MYO* (myoglobin), M_r 18,000.

these PAIs can thus be classified as fast-acting PAIs. Despite a slight decrease in the rate of complex formation at 4°C by comparison with that at 37°C (data not shown), most of the incubations in the present work were performed at 4°C to minimize nonspecific proteolysis, particularly when crude cell extracts were assayed.

On the basis of the data illustrated in Fig. 4, and using the same approach as Eaton et al. (35) (see Material and Methods), we calculated an association constant (k_{assoc}) of 8×10^5/M·s for the reaction of both low- and high-M_r U 937-produced PAIs, with M_r 33,000 or 55,000 human ^{125}I-uPA.

Biochemical Characterization of U 937-produced PAIs. The comparison of the M_r of free ^{125}IuPA with that of uPA/inhibitor complexes does not provide information on the M_r of the free, unreacted PAIs; indeed the M_r of the PAIs might be affected by their reaction with uPA, as already observed for other protease/inhibitor interactions (36). To determine the M_r of the PAIs before their interaction with uPA, we analyzed the conditioned medium of control and PMA-treated U 937 cells by gel filtration; the eluted fractions were incubated with ^{125}I-uPA, and the samples were analyzed by SDS-PAGE and autoradiography (Fig. 5). The low-M_r PAI present in conditioned medium of untreated U 937 cells (Fig. 5*A*) was found in fractions corresponding to an M_r of 40,000.

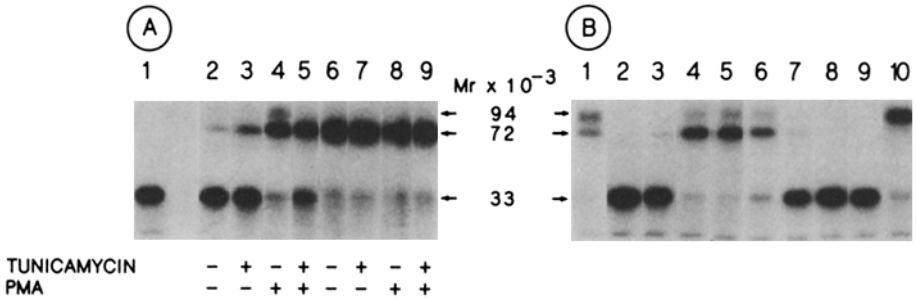

FIGURE 6. Glycosylation of the U 937-produced PAIs. (*A*) Effect of tunicamycin. U 937 cells (2×10^6 cells/ml) were incubated for 10 h at 37°C in RPMI + 1 mg/ml BSA in absence or in presence of 10 ng/ml PMA and/or 1 μg/ml tunicamycin, as indicated. The conditioned media (lanes *2–5*) and cell extracts (lanes *6–9*) were then incubated with M_r 33,000 ^{125}I-uPA (lane *1*), and analyzed by SDS-PAGE and autoradiography. (*B*) Affinity chromatography of the U 937 PAIs on Con A–Sepharose. Conditioned medium (0.6 ml) from PMA-treated U 937 cells was loaded on a Con A–Sepharose column; the column was loaded and washed extensively with PBS (lanes *2–7*) and eluted with 0.2 M methyl-α-D-mannopyranoside in PBS + 1 mg/ml BSA (lanes *8–10*). M_r 33,000 ^{125}I-uPA was added to aliquots of the conditioned medium before chromatography (lane *1*) or to the eluted fractions (lanes *2–10*). The samples were finally processed for SDS-PAGE and autoradiography.

The high-M_r PAI secreted by PMA-treated U 937 cells (Fig. 5*B*) had a more heterogeneous M_r, in agreement with the broadness of the high-M_r complex band observed after SDS-PAGE and autoradiography; an average M_r of 55,000 was determined for this PMA-induced high-M_r PAI. Thus, the additive M_rs of uPA and free PAIs are close to that of the complexes. This suggests that the enzyme/inhibitor complexes are equimolar, and that reaction with uPA does not result in the cleavage and release of a large polypeptide; the uncertainties inherent to the methods of M_r determination used in our studies do not exclude the possible release of a small peptide upon enzyme/inhibitor reaction.

As the observed size heterogeneity of the high-M_r PAI may have been the consequence of differential glycosylation, U 937 cells were treated with PMA in presence of tunicamycin, an antibiotic that prevents N-linked glycosylation (Fig. 6*A*). The production of the high-M_r PAI by PMA-treated cells (lane *4*) was completely prevented by tunicamycin (lane *5*); in contrast, the low-M_r PAI present in the culture medium of untreated U 937 cells (Fig. 6*A*, lane *2*) or in cell extracts of untreated (lane *6*) or PMA-treated cells (lane *8*) appeared unaffected by treatment with tunicamycin (Fig. 6*A*, lanes 3, 7, and *9*, respectively).

This first evidence for glycosylation of the high-M_r PAI was confirmed by the results of affinity chromatography of the conditioned medium from PMA-treated U 937 cells on ConA–Sepharose (Fig. 6*B*): the high-M_r PAI was selectively retained on the column, from which it could be eluted with the appropriate sugar (lanes *8–10*); the low-M_r PAI was recovered in the flow-through of the column (Fig. 6*B, lanes 2–7*).

IEF of conditioned medium from PMA-treated U 937 cells gave different values of pI for the two PAIs (Table II). Upon neuraminidase treatment of the conditioned medium before IEF, the pI of the high-M_r PAI was increased to a value close to that of the low-M_r PAI; the pI of the latter was not significantly affected by neuraminidase (Table II).

TABLE II
Values of pI Obtained for U 937–produced Low- and High-M_r PAIs: Effect of Neuraminidase

PAI type	pI	
	Untreated	Neuraminidase-treated
Low M_r	5.2	5.2
High M_r	4.7	5.0

The values of pI were calculated from the data obtained upon isoelectric focusing of the conditioned media from control or PMA-treated U 937 cells.

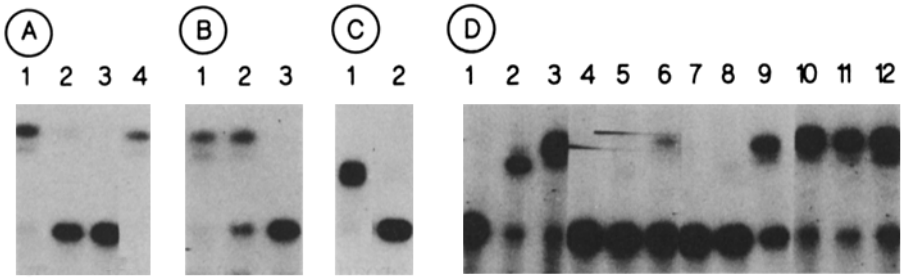

FIGURE 7. Chemical stability of the U 937-produced PAIs. (*A*) Both PAIs are inactivated by propanol. Aliquots of conditioned medium from PMA-treated cells were first supplemented with 0% (lane *1*), 10% (lane *2*), or 20% (lane *3*) 1-propanol, which was then immediately removed by lyophilization; the samples were reconstituted to their original volume with water and incubated with ^{125}I-uPA. Alternatively, an aliquot of the same conditioned medium was first incubated with ^{125}I-uPA before treatment with 40% 1-propanol, lyophilization, and reconstitution with water (lane *4*), as described above. The samples were further processed for SDS-PAGE and autoradiography. (*B*) The PAIs are heat sensitive. Aliquots of conditioned medium from PMA-treated U 937 cells were heated for 15 min at 50°C (lane *1*), 60°C (lane *2*), or 70°C (lane *3*). The samples were then incubated with ^{125}I-uPA and analyzed by SDS-PAGE and autoradiography. (*C*) Both PAIs are acid-labile. Conditioned medium from PMA-treated U 937 cells was adjusted to pH 3 and neutralized 2 h later before reaction with M_r 33,000 ^{125}I-uPA (lane *2*); lane *1*: untreated conditioned medium. (*D*) ^{125}I-uPA/PAI complexes are dissociated by ammonium hydroxide. M_r 33,000 ^{125}I-uPA (lanes *1*, *4*, *7*, and *10*) was incubated with conditioned medium from control (lanes *2*, *5*, *8*, and *11*) or PMA-treated (lanes *3*, *6*, *9*, and *12*) U 937 cells. Aliquots of these samples were either analyzed directly (lanes *1–3*) or made 1 M in ammonium hydroxide (lanes *4–12*) and lyophilized after 30 min of incubation at 37°C. The samples were reconstituted in water, and analyzed directly (lanes *4–6*) or after a second 1-h incubation at 4°C without (lanes *7–9*) or with untreated conditioned medium from PMA-treated cells (lanes *10–12*).

Taken together, these results demonstrate that the secreted, high-M_r, PMA-induced PAI is indeed glycosylated and carries terminal sialic acid residue(s); under the same experimental conditions, we have not found evidence for glycosylation of the low-M_r PAI.

Stability of U 937-produced PAIs. To further study the similarities and differences between the two PAIs and their possible relationship with other inhibitors, we tested their relative lability upon exposure to different conditions.

We had previously observed that the low-M_r PAI is inactivated in presence of propanol (25); this was also found to be the case for the high-M_r PAI (Fig. 7*A*). The inactivation of the PAIs was detectable upon treatment with 10% propanol

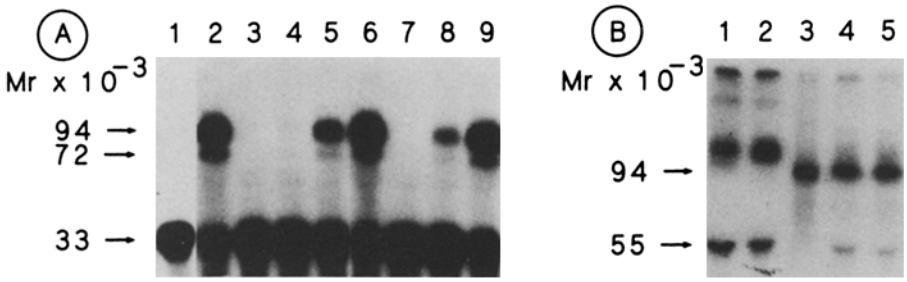

FIGURE 8. Specificity of the U 937-produced PAIs. (*A*) The PAIs react with both uPA and tPA. M_r 33,000 ^{125}I-uPA (5 ng) (lane *1*) was added to aliquots of conditioned medium from PMA-treated U 937 cells preincubated for 30 min at 4°C with 50 ng tPA (lane *3*), 17 ng tPA (lane *4*), 5 ng tPA (lane *5*), 1.7 ng tPA (lane *6*), 50 ng uPA (lane *7*), 17 ng uPA (lane *8*), 5 ng uPA (lane *9*), or an equal volume of PBS + 1 mg/ml BSA (lane *2*). The samples were further processed for SDS-PAGE and autoradiography. (*B*) The U 937-produced PAIs are preferential ligands for uPA. M_r 55,000 ^{125}I-uPA (2 ng) was added to 30 μl of 1:1 dilutions in fresh culture medium of human serum (lane *1*), human plasma (lane *2*), U 937-conditioned medium (lane *3*), or to 1:1 mixtures of U 937 conditioned medium and human serum (lane *4*) or human plasma (lane *5*). The samples were incubated for 1 h at 4°C and further processed for SDS-PAGE and autoradiography.

(lane *2*), whereas exposure to up to 40% propanol did not significantly affect the preformed complex (lane *4*). Both low- and high-M_r PAIs were similarly inactivated upon incubation at elevated temperature (Fig. 7*B*): the formation of complexes with ^{125}I-uPA was partially affected after treatment of conditioned medium at 60°C (Fig. 7*B*, lane *2*), and completely prevented at 70°C (lane *3*). Pretreatment of the PAIs at pH 3 (Fig. 7*C*) completely prevented their subsequent reaction with ^{125}I-uPA (lane *2*), whereas the preformed ^{125}I-uPA/PAI complexes were stable under these conditions (data not shown). Ammonium hydroxide (Fig. 7*D*) caused an almost complete dissociation of preformed enzyme/inhibitor complexes (lanes *5* and *6*). Dissociation of the complexes resulted in the recovery of active enzyme, but not of functional PAI. Indeed, reincubation of the treated sample (after removal of ammonium hydroxide by lyophilization) allowed the formation of some high-M_r complexes, presumably by reaction of dissociated uPA with remaining native PAI (Fig. 7*D*, lanes *8* and *9*); upon further addition of untreated U 937 conditioned medium (lanes *10–12*), all the ammonium hydroxide–dissociated radiolabeled enzyme was again recovered in high-M_r complexes.

These results are compatible with the model proposed for the reaction of proteases with inhibitors of the serpin family (32): upon enzyme/inhibitor reaction, there is an irreversible alteration (presumably a cleavage) of the inhibitor, and the formation of an ester bond between the enzyme and the modified inhibitor. This ester bond is cleaved in presence of ammonium hydroxide, with release of active enzyme and inactivated inhibitor.

Since both low- and high-M_r PAIs were similarly affected by the different experimental conditions, they may be structurally related and their mode of action may be identical.

Specificity of PAIs Produced by U 937 Cells. To investigate the interaction of the PAIs with other proteases, we used two different approaches. We analyzed their reaction with tPA by competition studies (Fig. 8*A*): preincubation of the U

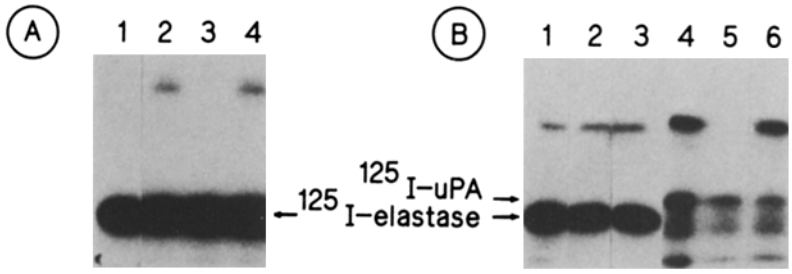

FIGURE 9. Comparison between the U 937-produced elastase ligand and the PAIs. (*A*) The elastase ligand present in U 937 cells does not react with uPA. U 937 cells (2×10^7 cells/ml) were solubilized in PBS + 0.5% NP-40. ^{125}I-elastase (2 ng) (lane *1*) was then added to aliquots (30 μl) of the cell extract preincubated for 20 min at 37°C with 20 ng unlabeled elastase (lane *3*), 20 ng unlabeled uPA (lane *4*) or an equal volume of PBS + 1 mg/ml BSA (lane *2*). The samples were further incubated for 20 min at 37°C and analyzed by SDS-PAGE and autoradiography. (*B*) The PAIs and the elastase ligand produced by U 937 cells are antigenically different. ^{125}I-elastase (lanes *1–3*) or M_r 33,000 ^{125}I-uPA (lanes *4–6*) were added to the U 937 cell extract (prepared as described in *A*); after 20 min at 37°C, aliquots of the samples were analyzed directly (lanes *1* and *4*) or immunoprecipitated with anti-placental PAI-2 IgG (lanes *2* and *5*) or nonimmune IgG (lanes *3* and *6*). Immunoprecipitation supernatants (lanes *2*, *3*, *5*, and *6*) were further processed for SDS-PAGE and autoradiography.

937 PAIs with excess unlabeled tPA (lanes *3–6*) or uPA (lanes *7–9*) completely prevented the formation of complexes with ^{125}I-uPA. In view of the very restricted specificity of tPA, which renders a nonspecific degradation of the PAIs by the added enzyme most unlikely, this result strongly suggests that tPA reacts with the PAIs produced by U 937 cells.

Alternatively, we tested for formation of complexes with radioiodinated enzymes. ^{125}I-labeled plasmin, thrombin, plasma kallikrein, and coagulation factor XIIf, failed to form SDS-resistant complexes when added to the U 937-conditioned medium, although they did react with their respective ligands in the same experimental conditions (data not shown).

Incubation of ^{125}I-elastase (Fig. 9*A*) with NP-40-solubilized U 937 cells resulted in the formation of an SDS-resistant enzyme/ligand complex (lane *2*); however, the formation of this complex was not prevented by preincubation with an excess of unlabeled uPA (lane *4*), whereas it was completely abolished upon preincubation with the same molar excess of unlabeled elastase (Fig. 9*A*, lane *3*). Similar results were obtained with ^{125}I-trypsin (data not shown). In addition, these ligands were not modulated by culturing the cells in presence of PMA. Furthermore, the complexes formed with ^{125}I-elastase were not immunoprecipitated by an antibody raised against the PAI-2 present in placenta (Fig. 9*B*, lane *2*), whereas ^{125}I-uPA/PAI complexes were immunoprecipitated by this antibody (lane *5*; see also Fig. 10). Thus, the ligands for trypsin and elastase present in U 937 cultures are clearly different from the PAIs we have characterized. They probably correspond to α_1-proteinase inhibitor, which is expressed in human monocytes/macrophages and in U 937 cells (37), and/or to the fast-acting elastase inhibitor recently described in these cells by Remold-O'Donnell (38).

In conclusion, the low- and high-M_r, U 937-produced PAIs are different from the major plasma protease inhibitors and from protease nexin, which also reacts with thrombin and plasmin. In view of their restricted specificity, they can be

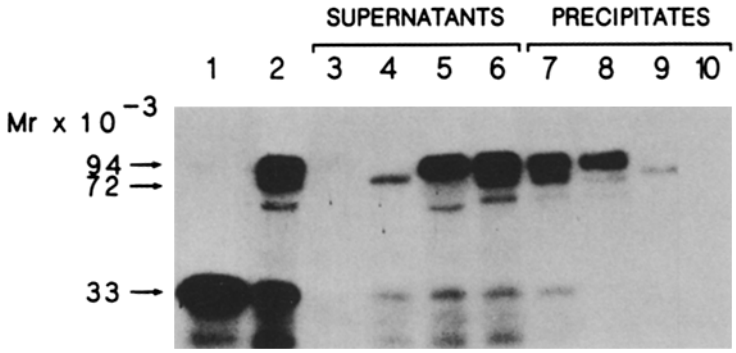

FIGURE 10. Immunological characterization of the U 937-produced PAIs: crossantigenicity with PAI-2 from human placenta. M_r 33,000 ^{125}I-uPA (1.5 μg/ml) (lane *1*) was added to conditioned medium from PMA-treated U 937 cells (lane *2*); after 1 h at 4°C, aliquots of the sample were immunoprecipitated with anti-uPA IgG (lanes *3* and *7*), anti–placental PAI-2 IgG (lanes *4* and *8*), antiserum to the endothelial PAI-1 (lanes *5* and *9*), or nonimmune IgG (lanes *6* and *10*). The supernatants (lanes *3–6*) and the immunoprecipitates (lanes *7–10*) were analyzed by SDS-PAGE and the gel was processed for autoradiography.

referred to as PA-specific inhibitors. Since both PAIs follow the same specificity pattern, they are, at least functionally, closely related.

To further characterize these PAIs, we compared the relative affinities for uPA of the low-M_r U 937 PAI and the human plasma or serum antiproteases (Fig. 8*B*). When 55,000 M_r ^{125}I-uPA was incubated solely with serum (Fig. 8*B*, lane *1*) or plasma (lane *2*), several complexes were observed; these have been previously identified as complexes of the enzyme with α_2-macroglobulin and antithrombin III (16). In contrast, if U 937-conditioned medium (lane *3*) was mixed with serum (lane *4*) or plasma (lane *5*), added ^{125}I-uPA was almost completely complexed to the U 937 PAI, as evidenced by the presence of a M_r 94,000 complex. These results indicate that the U 937-produced PAIs could be preferential ligands for uPA under physiological conditions in vivo.

U 937-produced PAIs Are Antigenically Related to Each Other and to PAI-2 Isolated from Human Placenta. The properties of the U 937-produced PAIs are reminiscent of those of the PAI-2 first identified in human placenta (4, 5, 39). In addition, we have indirect evidence that these PAIs are different from the PAI-1 produced by endothelial cells (12) and also present in human platelets (14) and in serum (21); indeed, this PAI is unusually stable to SDS, acid, and to heating at 100°C (12). To confirm this, we immunoprecipitated the complexes formed by addition of ^{125}I-uPA to conditioned medium of PMA-treated U 937 cells (Fig. 10), using antibodies directed against human uPA (lanes *3* and *7*), placental PAI-2 (lanes *4* and *8*), endothelial PAI-1 (lanes *5* and *9*), or nonimmune IgG (lanes *6* and *10*). The major complexes corresponding to the low- and high-M_r PAIs were specifically immunoprecipitated by antibodies directed to the placental PAI-2 (lane *8*); in addition, a minor complex of intermediate M_r, that we had failed to resolve before immunoprecipitation because it was masked by the high-M_r complex, was immunoprecipitated by antibodies directed to the endothelial PAI-1 (Fig. 10, lane *9*).

Thus, the low- and high-M_r PAIs that we have characterized are immunologi-

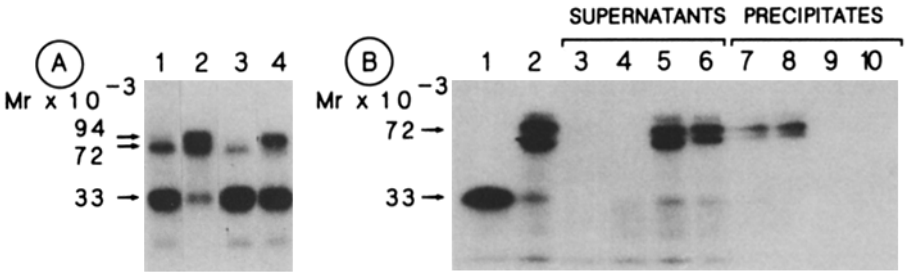

FIGURE 11. Production of PAIs by human peripheral blood monocytes/macrophages. (*A*) Human peripheral blood monocytes/macrophages also secrete the high-M_r PAI. M_r 33,000 ^{125}I-uPA (2 ng) was added to 10 μl of conditioned medium from U 937 cells (lanes *1* and *2*) or from human monocytes (lanes *3* and *4*) that had been previously cultured for 24 h in absence (lanes *1* and *3*) or in presence (lanes *2* and *4*) of 30 ng/ml PMA. After 1 h at 4°C, the samples were analyzed by SDS-PAGE and autoradiography. (*B*) These PAIs are antigenically related to the placental PAI-2. Monocyte-conditioned medium was analyzed exactly as described in Fig. 10. The supernatants (lanes *3–6*) and the pellets (lanes *7–10*) of immunoprecipitations with anti-uPA IgG (lanes *3* and *7*), anti–placental PAI-2 IgG (lanes *4* and *8*), antiserum to the endothelial PAI-1 (lanes *5* and *9*), or nonimmune IgG (lanes *6* and *10*) were subsequently processed for SDS-PAGE and autoradiography.

cally related to a functionally similar protein (PAI-2) purified from human placenta; this also indicates that they are related to each other, and hence that they may represent postsynthetic modifications of a same precursor. The presence of low levels of PAI-1 in conditioned medium of U 937 cultures indicates that a presumably homogeneous cell population can produce different PAIs.

Human Monocytes/Macrophages Isolated from Peripheral Blood Secrete the High-M_r Form of PAI. We had previously shown (25) that primary cultures of peripheral blood monocytes/macrophages released the low-M_r PAI. Subsequent analysis of other conditioned media revealed that primary cultures can release the high-M_r PAI, in response to PMA (Fig. 11*A*), and also under resting conditions (data not shown). The variable ratio of low- to high-M_r PAIs observed among different primary cultures presumably reflects: (*a*) the differential viability of the cells in different cultures; since the low-M_r PAI is abundant in cell extracts (see Table I), lysis of a small fraction of the cells will result in accumulation of this PAI in the conditioned medium; (*b*) the status of activation of the cells; indeed, in cultures of mouse peritoneal macrophages, the production of both low- and high-M_r PAIs varies as a function of the inflammatory state of the animal.[2]

PAIs Secreted by Human Monocytes/Macrophages Are Antigenically Related to Placenta-derived PAI-2. The antigenic relationship of the PAIs produced by human peripheral blood monocytes/macrophages with those from human placenta or endothelial cells was investigated by immunoprecipitation. The low-M_r complex formed upon incubation of monocytes/macrophages-conditioned medium with ^{125}I-uPA was analyzed using specific antibodies directed to the placental PAI-2 and the endothelial PAI-1 (Fig. 11*B*). We occasionally observed a splitting of the low-M_r PAI/uPA complex into a closely spaced doublet after

[2] Wohlwend, A., D. Belin, and J.-D. Vassalli. Plasminogen activator-specific inhibitors in mouse macrophages: in vivo and in vitro modulation of their synthesis and secretion. Manuscript submitted for publication.

SDS-PAGE of boiled samples; this may have resulted from a proteolytic cleavage of the low-M_r PAI upon its interaction with uPA, as suggested by Kruithof et al. (40) and Nielsen et al. (41). Like the PAIs produced by the U 937 cell line, the low-M_r PAI secreted by peripheral blood monocytes/macrophages was antigenically related to PAI-2 (Fig. 11*B*, lane *8*). Similar results were obtained with conditioned medium containing the high-M_r PAI (data not shown). In contrast to what was observed for the U 937 cell line, we did not detect a complex related to the PAI-1 (lane *9*) in cultures of human monocytes/macrophages.

Discussion

The results of the present work provide conclusive evidence for the synthesis and secretion of PAIs by cultured human monocytes/macrophages. In particular, we have shown that these cells produce two structurally distinct PAIs that differ in their cellular localization, although they are immunologically and functionally indistinguishable. Their lack of reactivity toward a number of other serine proteases besides uPA and tPA suggests that they may be PA-specific. In view of their high rate of association with uPA, they could be effective PAIs in vivo; this contention is supported by the observation that they are preferential ligands of uPA, when added to human plasma or serum, at a concentration several orders of magnitude lower than that of the major circulating antiproteases. Together, these results point to a possible role of these PAIs in limiting PA-catalyzed extracellular proteolysis, in particular in the context of inflammatory reactions involving mononuclear phagocytes.

The production of PAIs by human or murine monocytes/macrophages has been reported previously (22–28, 42). Our results confirm in particular those of Chapman and Stone (26) and of Kopitar et al. (28); indeed, the immunological crossreactivity and biochemical similarity of the PAIs from mononuclear phagocytes with the previously characterized placenta-derived PAI-2 have been verified. In addition, we demonstrate here the synthesis of these PAIs by the cultured cells, and its regulation by PMA, a hormonal-like modulator. Furthermore, our study has revealed a hitherto unrecognized feature of this antiprotease system: the existence of two distinct, but related, forms of PAI. This observation was greatly facilitated by the analytical method we used, i.e., the detection of stable complexes between radiolabeled enzyme and inhibitors by SDS-PAGE and autoradiography (7). The presence of complexes of different M_rs provided a first evidence for the existence of distinct PAIs. Further studies revealed the following characteristics for these two PAIs: An M_r 40,000, pI 5.2, presumably nonglycosylated PAI was found in cell extracts under all conditions tested. A small fraction of the total amount of this low-M_r PAI was also found in culture supernatants. Although synthesized by the cells during the culture, its release was not affected by inhibitors of macromolecular synthesis; this release may thus correspond to secretion of preformed stored PAI, or to leakage from damaged cells. The resistance to proteolysis of this PAI when intact cells were incubated with trypsin indicates that it is intracellular, and not cell surface–associated; subcellular fractionation will be required to determine its precise localization. A PAI of higher and more heterogeneous M_r, ranging from 50,000 to 65,000, was found exclusively in the conditioned medium. This PAI had a pI of 4.7, and was

glycosylated with terminal sialic acid residue(s). Its synthesis and secretion were markedly stimulated when cells were cultured in presence of PMA. No functional difference was found between these two PAIs; in particular, their respective lability, enzymatic specificity and immunological reactivity were identical. We conclude that they probably represent two forms of the same PAI, which are related to PAI-2.

The characteristics of the PAI purified from placental tissue suggest that it is comparable to the low-M_r, predominantly intracellular PAI from monocytes/macrophages. Thus, cell and tissue extracts may in general contain mostly the low-M_r form of the protein, whereas the high-M_r form would be found exclusively in extracellular compartments. This model is supported by the recent identification of an M_r 70,000 PAI, immunologically related to the PAI-2 protein, in human pregnancy plasma (20). Furthermore, we have observed that a number of cell lines unrelated to mononuclear phagocytes also contain and secrete PAIs that react with antibodies to PAI-2 (43); as shown here for monocytes, a low-M_r form was consistently found in cell lysates, whereas conditioned media contained predominantly a high-M_r form (A. Estreicher, manuscript in preparation).

The chromatographic and electrophoretic differences between these two forms of PAIs can probably be accounted for by differences in glycosylation. Indeed, after removal of sialic acid residues by neuraminidase, the size of the high-M_r PAI is only slightly above that of the low-M_r species (data not shown), and their pIs are also very close. Hence, the PMA-induced secretion of the high-M_r form may be due to post-translational modification (i.e., glycosylation) of newly-synthesized low-M_r PAI; it could also result from saturation of a postglycosylation modification process, which generates the preferentially intracellular low-M_r species by removing the carbohydrate side chains from the high-M_r form. Alternatively, PMA may enhance the synthesis of a distinct PAI, carrying appropriate determinants for glycosylation and secretion; this could result from transcription of a different gene for the high-M_r PAI, or from alternate splicing of a common precursor mRNA. These issues can only be resolved by determination of the structure of the two PAIs. A first step in this direction is the recent purification and partial sequencing of the low-M_r, U 937-produced PAI, which belongs to the antithrombin III family of antiproteases (40); antibodies raised against the purified protein also react with the high-M_r PAI.

It is well established that the plasminogen-dependent proteolytic activity of mononuclear phagocytes varies considerably as a function of the inflammatory status of the animal (2); similarly, PA production by cultured monocytes/macrophages, as measured by overall proteolytic activity, can be modulated by a variety of hormones and mediators (30, 44, 45). The PA activity of mature mononuclear phagocytes is entirely due to uPA, and all the observed modulations are associated with changes in the rate of transcription of the uPA gene and in the level of uPA mRNA (46).[3] However, it appears that the production of PAIs is also a phenotypic property of monocytes/macrophages that can be modulated: endotoxin (23) and muramyl dipeptide (24) have been shown to increase PAI levels in macrophage and monocyte cultures, and our results show the modulation

[3] Collart, M. A., D. Belin, J.-D. Vassalli, and P. Vassalli. c-*fos* expression in differentiated macrophages: early changes during modulation of functional activity. Manuscript submitted for publication.

of synthesis and secretion of PAIs in response to PMA. Using a similar approach, we have studied PAI production by cultured mouse macrophages.[2] We have identified and characterized two PAIs synthesized by these cells, which are biochemically, functionally and antigenically similar to the human proteins described here. Their production varies dramatically as a function of the inflammatory status of the animal, and can also be hormonally modulated in vitro; the balance between uPA and its inhibitors is likely to be central to the control of proteolysis in inflammation.

Summary

Human monocytes/macrophages produce plasminogen activator–specific inhibitors (PAIs) that form covalent complexes with urokinase-type plasminogen activator (uPA). We have characterized two functionally and antigenically related forms of PAIs produced by resting and phorbol myristate acetate (PMA)-treated U 937 cells: an M_r 40,000 form, presumably nonglycosylated, with a pI of 5.2, that is constitutively synthetized by these cells and that remains predominantly intracellular; a PMA-induced form of heterogeneous M_r (50,000–65,000) with a pI of 4.7, that is preferentially secreted; this PAI is glycosylated with terminal sialic acid residue(s).

Biosynthetic labeling experiments demonstrated that both PAIs are synthetized by U 937 cells. They are inactivated upon treatment with propanol, heat, and acid; the covalent and equimolar complexes formed between these PAIs and ^{125}I-uPA are dissociated by ammonium hydroxide, suggesting that the PAIs are linked to uPA via an ester bond. Human peripheral blood monocytes/macrophages also produce the two forms of PAI. These PAIs are clearly different from the main plasma protease inhibitors and they are both antigenically related to the PAI-2 characterized in human placenta.

We thank Glenn Marchi, Mireille Vaucher, and Ariane Widmer for excellent technical assistance, Nicolas Gerber for skillful photographic work, and Dr. S. Strickland for helpful discussions in the preparation of the manuscript.

Received for publication 26 September 1986.

References

1. Reich, E. 1978. Activation of plasminogen; a general mechanism for producing localized extracellular proteolysis. *In* Molecular Basis of Biological Degradative Processes (Berlin, R. D., M. Herrman, and I. H. Lepow, editors) Academic Press, New York, pp. 155–169.
2. Unkeless, J. C., S. Gordon, and E. Reich. 1974. Secretion of plasminogen activator by stimulated macrophages. *J. Exp. Med.* 139:834.
3. Gordon, S., J. C. Unkeless, and Z. A. Cohn. 1974. Induction of macrophage plasminogen activator by endotoxin stimulation and phagocytosis, evidence for a two-stage process. *J. Exp. Med.* 140:995.
4. Kawano, T., K. Morimoto, and Y. Uemura. 1970. Partial purification and properties of urokinase inhibitor from human placenta. *J. Biochem.* 67:333.
5. Astedt, B., I. Lecander, T. Brodin, A. Lundblad, and K. Low. 1985. Purification of

a specific placental plasminogen activator inhibitor by monoclonal antibody. *Thromb. Haemost.* 53:122.

6. Christensen, U., L. Holmberg, B. Bladh, and B. Astedt. 1982. Kinetics of the reaction between urokinase and an inhibitor of fibrinolysis from placental tissue. *Thromb. Haemost.* 48:24.
7. Baker, J. B., D. A. Low, R. L. Simmer, and D. D. Cunningham. 1980. Protease-nexin: a cellular component that links thrombin and plasminogen activator and mediates their binding to cells. *Cell.* 21:37.
8. Knauer, D. J., and D. D. Cunningham. 1984. Protease nexins: cell-secreted proteins which regulate extracellular serine proteases. *TIBS (Trends Biochem. Sci.).* 9:231.
9. Low, D. A., J. B. Baker, W. C. Koonce, and D. D. Cunningham. 1981. Released protease-nexin regulates cellular binding, internalization, and degradation of serine proteases. *Proc. Natl. Acad. Sci. USA.* 78:2340.
10. Scott, R. W., and J. B. Baker. 1983. Purification of human protease nexin. *J. Biol. Chem.* 258:10439.
11. Scott, R. W., B. L. Bergman, A. Bajpai, R. T. Hersh, H. Rodriguez, B. N. Jones, C. Barreda, S. Watts, and J. B. Baker. 1985. Protease nexin. Properties and a modified purification procedure. *J. Biol. Chem.* 260:7029.
12. Loskutoff, D. J., J. A. van Mourik, L. A. Erickson, and D. Lawrence. 1983. Detection of an unusually stable fibrinolytic inhibitor produced by bovine endothelial cells. *Proc. Natl. Acad. Sci. USA.* 80:2956.
13. van Mourik, J. A., D. A. Lawrence, and D. J. Loskutoff. 1984. Purification of an inhibitor of plasminogen activator (anti-activator) synthesized by endothelial cells. *J. Biol. Chem.* 259:14914.
14. Erickson, L. A., M. H. Ginsberg, and D. L. Loskutoff. 1984. Detection and partial characterization of an inhibitor of plasminogen activator in human platelets. *J. Clin. Invest.* 74:1465.
15. Rijken, D. C., I. Juhan-Vague, and D. Collen. 1983. Complexes between tissue-type plasminogen activator and proteinase inhibitors in human plasma, identified with an immunoradiometric assay. *J. Lab. Clin. Med.* 101:285.
16. Waller, E. K., W.-D. Schleuning, and E. Reich. 1983. Complex-formation and inhibition of urokinase by blood plasma proteins. *Biochem. J.* 215:123.
17. Kruithof, E. K. O., C. Tran-Thang, A. Ransijn, and F. Bâchmann. 1984. Demonstration of a fast-acting inhibitor of plasminogen activators in human plasma. *Blood.* 64:907.
18. Thorsen, S., and M. Philips. 1984. Isolation of tissue-type plasminogen activator-inhibitor complexes from human plasma. *Biochim. Biophys. Acta.* 802:111.
19. Wiman, B., J. Chieleweska, and M. Ranby. 1984. Inactivation of tissue plasminogen activator in plasma. *J. Biol. Chem.* 259:3644.
20. Lecander, I., and B. Astedt. 1986. Isolation of a new plasminogen activator inhibitor from pregnancy plasma. *Br. J. Haematol.* 62:221.
21. Erickson, L. A., C. M. Hekman, and D. J. Loskutoff. 1985. The primary plasminogen activator-inhibitors in endothelial cells, platelets, serum, and plasma are immunologically related. *Proc. Natl. Acad. Sci. USA.* 82:8710.
22. Klimetzek, V., and C. Sorg. 1979. The production of fibrinolysis inhibitors as a parameter of the activation state in murine macrophages. *Eur. J. Immunol.* 9:613.
23. Chapman, H. A., Jr., Z. Vavrin, and J. B. Hibbs, Jr. 1982. Macrophage fibrinolytic activity: identification of two pathways of plasmin formation by intact cells and of a plasminogen activator inhibitor. *Cell.* 28:653.
24. Golder, J. P., and R. W. Stephens. 1983. Minactivin: a human monocyte product

which specifically inactivates urokinase-type plasminogen activators. *Eur. J. Biochem.* 136:517.

25. Vassalli, J.-D., J. M. Dayer, A. Wohlwend, and D. Belin. 1984. Concomitant secretion of prourokinase and of a plasminogen activator-specific inhibitor by cultured human monocytes/macrophages. *J. Exp. Med.* 159:1653.
26. Chapman, H. A., Jr., and O. L. Stone. 1985. Characterization of macrophage-derived plasminogen activator inhibitor (similarities with placental urokinase inhibitor). *Biochem. J.* 230:109.
27. Saksela, O., T. Hovi, and A. Vaheri. 1985. Urokinase-type plasminogen activator and its inhibitor secreted by cultured human monocyte-macrophages. *J. Cell. Physiol.* 122:125.
28. Kopitar, M., B. Rozman, J. Babnik, V. Turk, D. E. Mullins, and T.-C. Wun. 1985. Human leucocyte urokinase inhibitor. Purification, characterization and comparative studies against different plasminogen activators. *Thromb. Haemost.* 54:750.
29. Nilsson, K., K. Forsbeck, M. Gidlung, C. Sundstrom, T. Totterman, J. Sallstrom, and P. Venge. 1981. Surface characteristics of the U 937 human histiocytic lymphoma cell line: specific changes during inducible morphologic and functional differentiation in vitro. *Haematol. Blood Transf.* 26:215.
30. Vassalli, J.-D., J. Hamilton, and E. Reich. 1977. Macrophage plasminogen activator: induction by concanavalin A and phorbol myristate acetate. *Cell.* 11:695.
31. Deutsch, D. G., and E. T. Mertz. 1970. Plasminogen purification from human plasma by affinity chromatography. *Science (Wash. DC).* 170:1095.
32. Carrel, R., and J. Travis. 1985. $Alpha_1$-antitrypsin and the serpins: variation and countervariation. *TIBS (Trends Biochem. Sci.).* 10:20.
33. Beatty, K., J. Bieth, and J. Travis. 1980. Kinetics of association of serine proteinases with native and oxidized $alpha_1$-proteinase inhibitor and $alpha_1$-antichymotrypsin. *J. Biol. Chem.* 255:3931.
34. Maniatis, T., E. F. Fritsch, and J. Sambrook. 1982. Chromatography through Sephadex G-50: spun-column procedure. *In* Molecular Cloning: A Laboratory Manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY. 466–467.
35. Eaton, D. L., R. W. Scott, and J. B. Baker. 1984. Purification of human fibroblast urokinase proenzyme and analysis of its regulation by proteases and protease nexin. *J. Biol. Chem.* 259:6241.
36. Travis, J., and G. S. Salvesen. 1983. Human plasma proteinase inhibitors. *Ann. Rev. Biochem.* 52:655.
37. Perlmutter, D. H., F. Sessions Cole, P. Kilbridge, T. H. Rossing, and H. R. Colten. 1985. Expression of the $alpha_1$-proteinase inhibitor gene in human monocytes and macrophages. *Proc. Natl. Acad. Sci. USA.* 82:795.
38. Remold-O'Donnell, E. 1985. A fast-acting elastase inhibitor in human monocytes. *J. Exp. Med.* 162:2142.
39. Holmberg, L., I. Lecander, B. Persson, and B. Astedt. 1978. An inhibitor from placenta specifically binds urokinase and inhibits plasminogen activator released from ovarian carcinoma in tissue culture. *Biochim. Biophys. Acta.* 544:128.
40. Kruithof E. K. O., J.-D. Vassalli, W.-D. Schleuning, R. J. Mattaliano, and F. Bachmann. 1986. Purification and characterization of a plasminogen activator inhibitor from the histiocytic lymphoma cell line U-937. *J. Biol. Chem.* 261:11207.
41. Nielsen, L. S., P. A. Andreasen, J. Grondhal-Hansen, L. Skriver, and K. Dano. 1986. Plasminogen activators catalyse conversion of inhibitor from fibrosarcoma cells to an inactive form with a lower apparent molecular mass. *FEBS (Fed. Eur. Biochem. Soc.) Let.* 196:269.
42. Lemaire, G., J. C. Drapier, and J. F. Petit. 1983. Importance, localization, and

functional properties of the cell-associated form of plasminogen activator in mouse peritoneal macrophages. *Biochim. Biophys. Acta.* 755:332.

43. Busso, N., D. Berlin, C. Failly-Crepin, and J.-D. Vassalli. 1986. Plasminogen activators and their inhibitors in a human mammary cell line (HBL-100). *J. Biol. Chem.* 261:9309.
44. Vassalli, J.-D., J. Hamilton, and E. Reich. 1976. Macrophage plasminogen activator: modulation of enzyme production by anti-inflammatory steroids, mitotic inhibitors, and cyclic nucleotides. *Cell.* 8:271.
45. Vassalli, J.-D., and E. Reich. 1977. Macrophage plasminogen activator: induction by products of activated lymphoid cells. *J. Exp. Med.* 145:429.
46. Collart, M. A., D. Belin, J.-D. Vassalli, S. de Kossodo, and P. Vassalli. 1986. Gamma interferon enhances macrophage transcription of the tumor necrosis factor/cachectin, interleukin-1 and urokinase genes, which are controlled by short lived repressors. *J. Exp. Med.* 164:2113.