PLASMINOGEN ACTIVATOR–SPECIFIC INHIBITORS
PRODUCED BY HUMAN MONOCYTES/MACROPHAGES

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Extracellular proteolysis is associated with cell migration and tissue remodelling (1). In particular, plasminogen activators (PAs) 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 pH 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

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1 Abbreviations used in this paper: PA, plasminogen activator; PAI, plasminogen activator inhibitor; PN, protease nexin; tPA, tissue-type PA; uPA, urokinase-type PA.
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-M₉ PAI from U 937 cells was the kind gift of Dr. E. K. O. Kruithof. ¹²⁵I-labeled plasma kallikrein and factor XII (10⁶ cpm/μg) were kindly provided by Dr. A. de Agostini. M, 55,000 uPA (1.5 x 10⁵ U/mg) was from the Green Cross Corporation (Osaka, Japan); M, 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-8159), 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-M₉ 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[¹²⁵I]L-[¹³⁵S]methionine, and En³Hance 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-M₉ PAI to trypsin, U 937 cells were collected, washed twice in serum-free RPMI 1640, and resuspended at 10⁶ 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.

**125I Labeling of u-PA, Thrombin, Trypsin, and Elastase.** The proteins were labeled with Iodogen as previously described (25): the specific activities of the 125I-enzymes were as follows: M, 55,000 uPA, 1–2 × 10⁷ cpm/μg; M, 33,000 uPA, 0.8–1.7 × 10⁷ cpm/μg; thrombin, 3 × 10⁶ cpm/μg; elastase, 7 × 10⁶ cpm/μg. Plasminogen (6–7 × 10⁶ cpm/μg) was converted to 125I-plasmin by incubation at 30°C for 18 h in presence of 100 U/ml uPA and 50% glycerol; the extent of conversion of 125I-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 125I-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 125I-enzyme.

Like the other inhibitors of the serpin family (32), U 937-produced PAI complex uPA essentially irreversibly. Therefore, the sensitivity of uPA to the PAIs cannot be measured by Kᵣ. 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 (kₐₑₛ) have been used to compare the specificity of α₁-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 kₐₑₛ 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 Mᵢ 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 × 10⁶ cells/ml in a modified culture medium containing 80 μCi/ml [³⁵S]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 ³⁵S-labeled conditioned media or cell extracts were incubated for 1 h at 4°C with M, 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 En³Hance 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, 33,000 125I-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 $M$, 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-a-D-mannopyranoside in PBS + 1 mg/ml BSA. Aliquots of the fractions collected were subsequently incubated with $M$, 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 $M$, 33,000 $^{125}$I-uPA and analyzed by SDS-PAGE and autoradiography.

Results

Synthesis and Secretion of PAIs by U937 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 $M$, 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 $M$, 72,000 complex with $^{125}$I-uPA was observed after 6 h of incubation in presence of PMA. A ligand forming a $M$, 94,000 complex with $^{125}$I-uPA was detected in the medium after 9 h (Fig. 1A); upon longer incubation (24 h), the conditioned medium of PMA-treated U 937 cells contained mainly the ligand forming the higher-$M$, complex (Fig. 1A). Both ligands were found to be inhibitors of uPA: as previously shown for the $M$, 72,000 complex (25), zymographic analysis revealed that the specific catalytic activity of the $M$, 94,000 complex was markedly lower than that of free uPA (data not shown). These ligands will thus be referred to as low-$M$, and high-$M$, PAIs, respectively. The induced secretion of the high-$M$, 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.
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FIGURE 1. Production of PAIs by U 937 cells in response to PMA. (A) Time course. U 937 cells were resuspended at 2 × 10⁶ 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 125I-αPA (2 ng), and analyzed by SDS-PAGE and autoradiography. M₉ were calculated from the positions of markers in the stained gel. (B) Effects of actinomycin D and cycloheximide. U 937 cells (2 × 10⁶ 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.

| Culture       | Conditioned medium | Cell extract |
|---------------|--------------------|--------------|
| Control       | 0.03               | 1.29         |
| PMA-treated   | 1.31               | 1.48         |

* The Mr 72,000 and 94,000 radiolabeled complexes formed upon incubation with 125I-αPA 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 125I-αPA 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-Mr PAI, and that the other samples contained only the low-Mr 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. 1B). The secretion of the high-Mr PAI was dependent upon ongoing RNA and protein synthesis, in contrast to the release of the low-Mr 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 [35S]methionine. The biosynthetically labeled conditioned media and cell extracts of control and PMA-treated cultures were incubated with M, 33,000 unlabeled urokinase, and the uPA/35S-labeled-PAI
Figure 2. Immunoprecipitation of [35S]methionine-labeled PAIs produced by U 937 cells: effects of PMA. U 937 cells were biosynthetically labeled with [35S]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, 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, 33,000 [125I]-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 autoradiography.

Complexes were immunoprecipitated by specific anti-uPA IgG. SDS-PAGE and autoradiography of the immunoprecipitates (Fig. 2) revealed that, in absence of PMA (Fig. 2A), an M, 72,000, radioactive complex was specifically immunoprecipitated from the U 937 cell extract by anti-uPA IgG (Fig. 2A, lane 3). We did not detect newly synthesized low-M, PAI in the corresponding conditioned medium (lane 7). In PMA-treated U 937 cells (Fig. 2B), the amount of newly synthesized low-M, PAI was increased in the cell extract (lane 3); additional bands were also specifically immunoprecipitated: they probably represent degradation products of the M, 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, PAI and its secretion into the corresponding culture medium (Fig. 2B, lane 7).

Thus, both low- and high-M, PAIs are synthesized by U 937 cells. Cell extracts contain the low-M, PAI; we could not detect any cell-associated high-M, PAI,
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FIGURE 3. The cell-associated low- Mr, 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 125I-uPA and processed for SDS-PAGE and autoradiography.

FIGURE 4. Kinetics of complex formation of 125I-uPA with the low- and high- Mr, U 937-produced PAIs. 125I-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- Mr, 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- Mr, PAI and secreted mainly the high- Mr, form in response to PMA (data not shown).

To determine whether the low- Mr, cell-associated PAI was exposed at the cell surface, we incubated U 937 cells in presence of trypsin (Fig. 3). The low- Mr, 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- Mr, PAI is intracellular.

Kinetics of Complex Formation between 125I-uPA and U 937-produced PAIs. The time course of complex formation between 125I-uPA and the low- Mr, PAI was comparable to that with the high- Mr, PAI (Fig. 4): half of the added 125I-uPA was complexed within the first 2–3 min incubation in U 937-conditioned media;
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 $k_{ass}$ of $8 \times 10^5$/M·s for the reaction of both low- and high-Mr 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 $^{125}$I-uPA 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. 5A) was found in fractions corresponding to an $M_r$ of 40,000.

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**FIGURE 5.** Determination of the $M_r$s 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.
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FIGURE 6. Glycosylation of the U 937-produced PAIs. (A) Effect of tunicamycin. U 937 cells (2 x 10⁶ cells/ml) were incubated for 10 h at 37°C in RPMI + 1 ng/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, 33,000 ¹²⁵I-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, 33,000 ¹²⁵I-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-Mₐ PAI secreted by PMA-treated U 937 cells (Fig. 5B) had a more heterogeneous Mₛ, in agreement with the broadness of the high-Mₛ complex band observed after SDS-PAGE and autoradiography; an average Mₛ of 55,000 was determined for this PMA-induced high-Mₛ PAI. Thus, the additive Mₛs 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 Mₛ 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-Mₛ 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. 6A). The production of the high-Mₛ PAI by PMA-treated cells (lane 4) was completely prevented by tunicamycin (lane 5); in contrast, the low-Mₛ PAI present in the culture medium of untreated U 937 cells (Fig. 6A, lane 2) or in cell extracts of untreated (lane 6) or PMA-treated cells (lane 8) appeared unaffected by treatment with tunicamycin (Fig. 6A, lanes 3, 7, and 9, respectively).

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

IEF of conditioned medium from PMA-treated U 937 cells gave different values of pl for the two PAIs (Table II). Upon neuraminidase treatment of the conditioned medium before IEF, the pl of the high-Mₛ PAI was increased to a value close to that of the low-Mₛ PAI; the pl 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, PAIs: Effect of Neuraminidase

| PAI type | pI | Neuraminidase-treated |
|----------|----|-----------------------|
| Low M,   | 5.2| 5.2                   |
| High M,  | 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.

Taken together, these results demonstrate that the secreted, high-Mr, 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, 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, PAI is inactivated in presence of propanol (25); this was also found to be the case for the high-M, PAI (Fig. 7A). The inactivation of the PAIs was detectable upon treatment with 10% propanol.

![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 125I-uPA. Alternatively, an aliquot of the same conditioned medium was first incubated with 125I-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 125I-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, 33,000 125I-uPA (lane 2); lane 1: untreated conditioned medium. (D) 125I-uPA/PAI complexes are dissociated by ammonium hydroxide. M, 33,000 125I-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 57°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).
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FIGURE 8. Specificity of the U 937-produced PAIs. (A) The PAIs react with both uPA and tPA. M, 53,000 125I-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, 55,000 125I-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.

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, 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. 8A): preincubation of the U
FIGURE 9. Comparison between the U937-produced elastase ligand and the PAIs. (A) The elastase ligand present in U937 cells does not react with uPA. U937 cells (2 x 10^7 cells/ml) were solubilized in PBS + 0.5% NP-40. 125I-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 U937 cells are antigenically different. 125I-elastase (lanes 1–3) or M, 33,000 125I-uPA (lanes 4–6) were added to the U937 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 125I-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 U937 cells.

Alternatively, we tested for formation of complexes with radioiodinated enzymes. 125I-labeled plasmin, thrombin, plasma kallikrein, and coagulation factor XII, failed to form SDS-resistant complexes when added to the U937-conditioned medium, although they did react with their respective ligands in the same experimental conditions (data not shown).

Incubation of 125I-elastase (Fig. 9A) with NP-40-solubilized U937 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. 9A, lane 3). Similar results were obtained with 125I-trypsin (data not shown). In addition, these ligands were not modulated by culturing the cells in presence of PMA. Furthermore, the complexes formed with 125I-elastase were not immunoprecipitated by an antibody raised against the PAI-2 present in placenta (Fig. 9B, lane 2), whereas 125I-uPA/PAI complexes were immunoprecipitated by this antibody (lane 5; see also Fig. 10). Thus, the ligands for trypsin and elastase present in U937 cultures are clearly different from the PAIs we have characterized. They probably correspond to α1-proteinase inhibitor, which is expressed in human monocytes/macrophages and in U937 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<sub>m</sub> U937-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
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FIGURE 111. Immunological characterization of the U937-produced PAIs: cross-reactivity with PAI-2 from human placenta. M, 33,000 $^{125}$I-uPA (1.5 μg/ml) (lane 1) was added to conditioned medium from PMA-treated U937 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-Mr, U937 PAI and the human plasma or serum antiproteases (Fig. 8B). When 55,000 M, $^{125}$I-uPA was incubated solely with serum (Fig. 8B, lane 1) or plasma (lane 2), several complexes were observed; these have been previously identified as complexes of the enzyme with $\alpha_2$-macroglobulin and antithrombin III (16). In contrast, if U937-conditioned medium (lane 3) was mixed with serum (lane 4) or plasma (lane 5), added $^{125}$I-uPA was almost completely complexed to the U937 PAI, as evidenced by the presence of a Mr 94,000 complex. These results indicate that the U937-produced PAIs could be preferential ligands for uPA under physiological conditions in vivo.

U937-produced PAIs Are Antigenically Related to Each Other and to PAI-2 Isolated from Human Placenta. The properties of the U937-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 U937 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-Mr, PAIs were specifically immunoprecipitated by antibodies directed to the placental PAI-2 (lane 8); in addition, a minor complex of intermediate Mr, that we had failed to resolve before immunoprecipitation because it was masked by the high-Mr complex, was immunoprecipitated by antibodies directed to the endothelial PAI-1 (Fig. 10, lane 9).

Thus, the low- and high-Mr, 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, PAI. Mr. 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.

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, 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. 11B). We occasionally observed a splitting of the low-M, PAI/uPA complex into a closely spaced doublet after

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SDS-PAGE of boiled samples; this may have resulted from a proteolytic cleavage of the low-\(M_c\) 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-\(M_c\) PAI secreted by peripheral blood monocytes/macrophages was antigenically related to PAI-2 (Fig. 11B, lane 8). Similar results were obtained with conditioned medium containing the high-\(M_c\) 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 \(M_c\)s provided a first evidence for the existence of distinct PAIs. Further studies revealed the following characteristics for these two PAIs: An \(M_c\) 40,000, pl 5.2, presumably nonglycosylated PAI was found in cell extracts under all conditions tested. A small fraction of the total amount of this low-\(M_c\) 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 \(M_c\), ranging from 50,000 to 65,000, was found exclusively in the conditioned medium. This PAI had a pl 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). 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

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of synthesis and secretion of PAIs in response to PMA. Using a similar approach, we have studied PAI production by cultured mouse macrophages. 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 Mr 40,000 form, presumably nonglycosylated, with a pI of 5.2, that is constitutively synthesized by these cells and that remains predominantly intracellular; a PMA-induced form of heterogeneous Mr (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 synthesized by U 937 cells. They are inactivated upon treatment with propanol, heat, and acid; the covalent and equimolar complexes formed between these PAIs and 125I-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.

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