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Plasminogen Activator Inhibitor-1 Deposition in the Extracellular Matrix of Cultured Human Mesangial Cells

J. Hagège,* M. N. Peraldi,* E. Rondeau,* C. Adida,* F. Delarue,* R. Medcalf,† W. D. Schleuning,‡ and J. D. Sraer*

From INSERM U64, Hôpital Tenon, Paris, France; Centre Hospitalier Universitaire Vaudois,† Lausanne, Switzerland; and the Department of Pharmaceutical Research,‡ the Institute of Biochemistry, Schering AG, Berlin, Germany

Human mesangial cells secrete tissue-type plasminogen activator (t-PA) and plasminogen activator inhibitor 1 (PAI-1), the latter being secreted in large excess* in vitro. *We demonstrate that PAI-1 is a major component of the extracellular matrix of cultured human mesangial cells, where its deposition is dependent on cell density. By immunogold silver staining, epipolarization microscopy and dispersive X-ray spectrometry, we have shown that matrix-associated PAI-1 is synthesized by spreading human mesangial cells, as indicated by the time-dependent accumulation of PAI-1 and the inhibitory effect of cycloheximide. Furthermore, by* in situ *hybridization, PAI-1 mRNA was detected in cultured mesangial cells. t-PA is present inside the cells, or at the cell surface, but is never associated with the extracellular matrix. Exogenous t-PA can remove matrix-associated PAI-1 without affecting cell adhesion. A similar effect was obtained by addition of urokinase-type plasminogen activator (u-PA) but not with fibrinolysis unrelated enzymes. In conclusion, PAI-1 is synthesized by human cultured mesangial cells and is deposited in the extracellular matrix by nonconfluent cells, whereas less PAI-1 is seen between confluent cells. This can explain the absence of detectable PAI-1 in normal human kidney biopsies. t-PA released by mesangial cells can bind and detach matrix PAI-1. (Am J Pathol 1992, 141:117–128)

Plasminogen activators are proteases that convert the plasma zymogen plasminogen into plasmin. Plasmin can degrade fibrin clots and extracellular matrix proteins during tissue remodeling.[1] Two types of plasminogen activators are synthetized by numerous cell types: tissue type plasminogen activator (t-PA) and urokinase-type plasminogen activator (u-PA). The active forms of these enzymes are inhibited by specific serine proteinase inhibitors, plasminogen activator inhibitor-1 (PAI-1) and 2 (PAI-2).[2] Plasminogen activators and their inhibitors play a major role in fibrinolysis, but also in the turnover of the extracellular matrix (ECM) and in the course of tumor growth and metastasis.[3–5] PAI-1 is found in the ECM of various cultured cells.[3,6–8] The induction of PAI-1 and its deposition in the extracellular space, where it could inhibit pericellular proteolysis, might be regulated in concert with synthesis of matrix components to favor accretion of the ECM and to influence cellular migration.[9] In the ECM of cultured cells, PAI-1 can bind to vitronectin.[10,11] The association of active PAI-1 with vitronectin increases PAI-1 stability. Furthermore, in endothelial cells, addition of t-PA to vitronectin-PAI-1 complexes results in the release of t-PA/PAI-1 complexes.[12]

The kidney glomerulus contains three different cell types. Endothelial cells and mesangial cells are closely associated at the inner surface of the glomerular basement membrane, whereas visceral epithelial cells cover its outer shell.[13] This complex structure is the site of glomerular filtration.[14] Mesangial cells have properties of both smooth muscle cells and macrophages. Accumulation of ECM in the mesangium plays a major role in the irreversible progression of glomerular sclerosis and chronic renal failure, as observed in the course of glomerulonephritis and diabetic nephropathy.[15,16] Mesangial cells have been reported to produce several ECM components, such as laminin, thrombospondin, fibronectin, collagen type I, III, and IV, and various proteoglycans.[17,18] We have previously demonstrated that human cultured glomerular mesangial cells synthesize and secrete t-PA and PAI-1, the latter being secreted in excess in the extracellular medium.[19]

Using immunocytochemical methods and *in situ* hybridization, we demonstrated that PAI-1 produced by cul-

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Address reprint requests to Dr. J. D. Sraer, INSERM U64, Hôpital Tenon, 4 rue de la Chine, 75020 Paris, France.

tured human mesangial cells is present in the ECM, with other matrix components and that PAI-1 can be released in the culture medium by plasminogen activators.

Material and Methods

Mesangial Cell Culture

Glomeruli were isolated as previously described.[19] Briefly, thin (2–4 mm) strips of decapsulated kidney cortex were minced in culture medium. Small fragments of tissue were pushed through 90-mesh stainless-steel screens with a glass pestle. The resulting mixture containing glomeruli was passed over a graded series of screens, and single, unencapsulated glomeruli were finally retained on a fine mesh screen, washed, and sedimented in basal medium. These steps resulted in a preparation of glomeruli virtually free of nonglomerular contaminants. For isolation of mesangial cells, isolated glomeruli were digested by collagenase (Sigma, St Louis, MO) type IV at the concentration of 750 U/ml for 30 minutes. The glomerular suspension was sieved over a stainless-steel mesh screen, and isolated epithelial cells were recovered in the filtrate after repeated washings. The remaining glomerular fragments were recovered and explanted in 10 mm Petri's dishes in complete Waymouth's medium supplemented with 20% fetal calf serum (FCS). The glomeruli were incubated at 37°C in a humidified 5% CO_2 incubator. By this method, smooth muscle-like mesangial cells appeared from the glomeruli approximately 8 to 14 days after attachment.[19] They were characterized by their morphologic stellate shape with irregular cytoplasmic projections, and biochemical characteristics as previously described.[20] They contain myosin filaments and smooth muscle cell a-actin,[21] and produce human renin.[22] No factor VIII-von Willebrand antigen was detected, excluding endothelial cell contamination. All experiments were performed between the third and fifth passages. When indicated, cells were grown on collagen-precoated filters (Costar 0,4 μ, Cambridge, MA).

Immunogold Silver Staining (IGSS) Technique with Brightfield and Epipolarization Microscopy

Mesangial cells, grown in plastic dishes in RPMI medium supplemented with 10% FCS, 100 UI/ml penicillin and 50 μg/ml streptomycin, were then plated on glass multiple slides and fixed and processed after 24 hours of subculture in RPMI medium containing 10% FCS. When indicated cells were plated in the same medium also containing 1 μg/ml cycloheximide (Sigma, St Louis, USA) or in RPMI medium, deprived of FCS, to exclude cross reaction with serum derived PAI-1.

After fixation in 2% glutaraldehyde in 0.1 M sodium phosphate buffer (PBS) or 1% paraformaldehyde for 10 min, glass slides were washed in PBS. The following antibodies have been used: monoclonal anti-human PAI-1 (PAI-1-201 Monozyme Aps Denmark), monoclonal anti-human t-PA (Biopool, Stockholm, Sweden), monoclonal anti-human vitronectin (Calbiochem, San Diego, CA), polyclonal anti-human fibronectin (Institut Pasteur, Lyon, France), and polyclonal anti-human collagen IV (Institut Pasteur, Lyon, France). The cells were incubated with one of these antibodies for 30 min at room temperature. After washing in PBS the slides were incubated with biotinylated anti-mouse IgG or anti-rabbit IgG (Vector, Burlingame, CA), rinsed in PBS and treated with 0,5% streptavidin, gold labeled (5 nm, Sigma, St Louis, MO) for 60 min. The slides were washed in distilled water before amplification with silver enhancement reagent (Biocell, Cardiff, UK) for 10 min, and counterstained with Giemsa.

Control experiments were done by addition of the second biotinylated antibody alone, with omission of the first specific antibody. Crossreactivity of anti-human protein antibodies with calf serum proteins was excluded with glass slides coated with RPMI containing 10% FCS in the absence of mesangial cells. These slides were then processed with the different antibodies as described.

For some experiments, cells were pretreated with either recombinant t-PA (Boehringer, Ingenheim, RFA), recombinant u-PA (Boehringer, Ingenheim, RFA), heparinase (Choay, Paris, France), collagenase (Sigma, St Louis, MO), or saponin (Sigma, St Louis, MO). Cells grown on filters were fixed with 2% glutaraldehyde in PBS. PAI-1 is revealed as described by IGSS, and filters were embedded in epoxy resin. Transversal semithin sections (1 μm) were made and counterstained by Toluidine blue. Photographs were taken with combined brightfield and epipolarization microscopy (Leitz-Orthoplan, Wild Leitz, Heezbrugg, Switzerland).

Scanning Electron Microscopy

For the scanning electron microscopic (SEM) examination, the cells were cultured on multiwell glass slides, fixed in 2.5% glutaraldehyde in phosphate-buffered saline (PBS), and rinsed in PBS. PAI-1 was revealed by IGSS. The glass slides were dried by the critical point method (Polaron E 3000 apparatus, Polaron, Watford, UK), coated by carbon streams and then observed by SEM Jeol at 7 to 15 kV. To fully characterize silver atoms in SEM micrographs, a Si(Li) detector for energy dispersive x-ray spectrometry (PHILIPS-EDAX, Eindhoven, Hol-

land) was used and blocked on the $Ag_{L\alpha}$ line. A map of silver atom distribution was thus obtained and compared with the SEM secondary electron image.

In Situ *Hybridization*

Adherent mesangial cells on multiwell glass slides were processed as previously described.[23] They were fixed in 2.5% glutaraldehyde for 10 minutes. Permeation was further obtained by treatment with HCl 0.2 mol/l for 10 minutes at room temperature followed by treatment with proteinase K 10 μg/ml. In control experiments, cells were treated with RNAse 10 μg/ml for 30 minutes at room temperature.

Labeling of the 1000-bp *Pst*1 insert of human PAI-1 cDNA (gift from D. J. Loskutoff, La Jolla, CA) with ^{35}S-dCTP (1000 Ci/mmol, Amersham, Buckinghamshire, UK) was performed using the multiprime labeling system (Amersham) according to the instruction of the manufacturer. After elution on a Sephadex G50 column (Pharmacia, Uppsala, Sweden), labeled cDNA was precipitated in ethanol, resuspended in 10 μg of water, denaturated at 100°C for 2 minutes, and mixed in the hybridization solution as previously described.[23] After dehydration in graded ethanol, cells were dried and coated for autoradiography with Kodak NTB2 (Eastman Kodak, Rochester, USA) nuclear track emulsion, exposed for the appropriate amount of time at 4°C, developed with the use of a standard method, and counterstained with May Grunwald Giemsa.

Results

Localization of PAI-1 and Other ECM Components

As shown in Figure 1a by IGSS, PAI-1 was found in the pericellular environment of spreading nonconfluent mesangial cells, in close contact with the plasma membrane. In confluent cells, less PAI-1 could be detected. Some PAI-1 was also detected at focal circular sites on the cell surface (Figure 1b). When cells were treated by EGTA for 5–10 minutes, some of them detached, and other cells exhibited signs of retraction but remained attached to the slide. In that case, PAI-1 was located mainly at the periphery of the cell but significant amounts were also detected under the cells (Figure 1c). Semithin sections of mesangial cells cultured on filters confirmed that PAI-1 was mainly associated with the cell substratum, but this staining consisted of a heterogeneous layer on the substratum (Figure 1d).

A similar pattern of PAI-1 deposition was shown in mesangial cells treated by IGSS and observed by SEM (Figure 2a, b). IGSS is used commonly in optical microscopy but not in SEM. To support this method for SEM and to further characterize the precise specific localization of PAI-1, we used dispersive x-ray spectrometry. Silver atom mapping corresponds to IGSS localization of PAI-1 as shown in Figure 2c, where the same cells as in Figure 2a are shown. Figure 2d shows the Lα line of silver, at 2.98 KeV, together with the two main Lβ lines and the channels used to produce the mapping image.

In contrast to PAI-1, t-PA was found inside the cells or at the cell surface, but was not observed in the ECM (Figure 3). Saponin-permeabilization of the cells was required to detect significant amounts of t-PA by IGSS, whereas the amount of extracellular PAI-1 was similar whether or not cells were treated by saponin (not shown).

The pattern of distribution of PAI-1 (Figure 4a) was different from that of other matrix proteins: fibronectin had a closer relation with cell membranes and gave a fibrillar pattern (Figure 4b); vitronectin exhibited a diffuse pattern in the ECM; and granular clusters were close to the cell membranes (Figure 4c). Collagen type IV or laminin was also observed in the cell substratum (not shown).

Figure 5 corresponds to the SEM image of PAI-1 (Figure 5a, b) and of vitronectin (Figure 5c, d), as compared with control cells (Figure 5e, f). Vitronectin had a homogeneous distribution in the extracellular space, whereas PAI-1 appeared dense close to the cells. Both PAI-1 and vitronectin were also found at local circular sites of the cell surface, suggesting a possible colocalization.

Evidence for PAI-1 Synthesis by Human Mesangial Cells

The kinetic analysis of ECM composition after cell seeding showed that PAI-1 appeared rapidly, since it was evident after 2 hours. Increasing amounts of matrix-associated PAI-1 were observed at the periphery of spreading cells between 4 and 48 hours of culture (Figure 6). However, in confluent areas, less PAI-1 was detected, suggesting a further removal after deposition.

Other matrix proteins were detected either at the same time (fibronectin, vitronectin) or later (collagen type IV) (not shown).

As shown in Figure 7, when cells were seeded in culture medium containing 1 μg/ml cycloheximide, an inhibitor of protein synthesis, the extracellular deposition of PAI-1 was completely suppressed after 24-hour incubation (Figure 7a). Furthermore, when cells were grown in serum-free medium, PAI-1 deposition was still observed (Figure 7b). Finally, by *in situ* hybridization using ^{35}S-labeled cDNA probe, PAI-1 mRNA was detected in the cytoplasm of cultured mesangial cells (Figure 7c, d), but not in RNAse-treated cells (Figure 7e). When *in situ* hybridization was performed at different degrees of conflu-

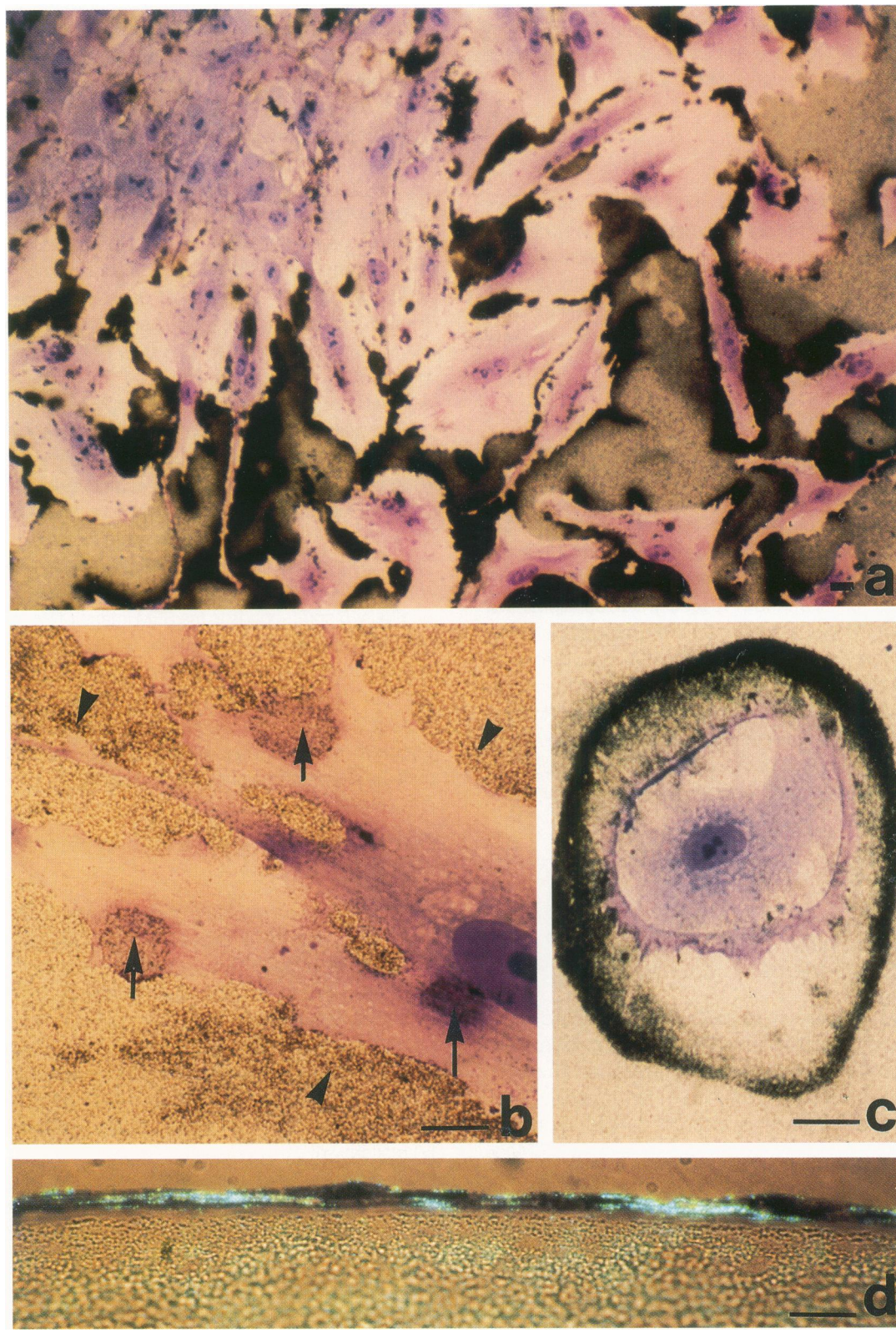

Figure 1. *IGSS of PAI-1 in human mesangial cells. After 24 hours of culture, human mesangial cells are stained with anti-PAI-1 monoclonal antibody. After IGSS, the preparation is counterstained with Giemsa.* **a**: *PAI-1 is mainly distributed in the pericellular matrix and is more abundant in nonconfluent than in confluent cells (brightfield illumination).* **b**: *Pericellular (arrowheads) and membrane-associated (arrows) PAI-1 is observed at higher magnification (brightfield illumination).* **c**: *After partial detachment by EGTA, PAI-1 appears more abundant in the pericellular matrix than in the subcellular matrix (brightfield illumination).* **d**: *Semithin sections of mesangial cells grown on filters confirm that most detectable PAI-1 is associated with the extracellular matrix and to a lesser extent at the surface of the cells (combined brightfield and epipolarization microscopy). Bars 10 μm.*

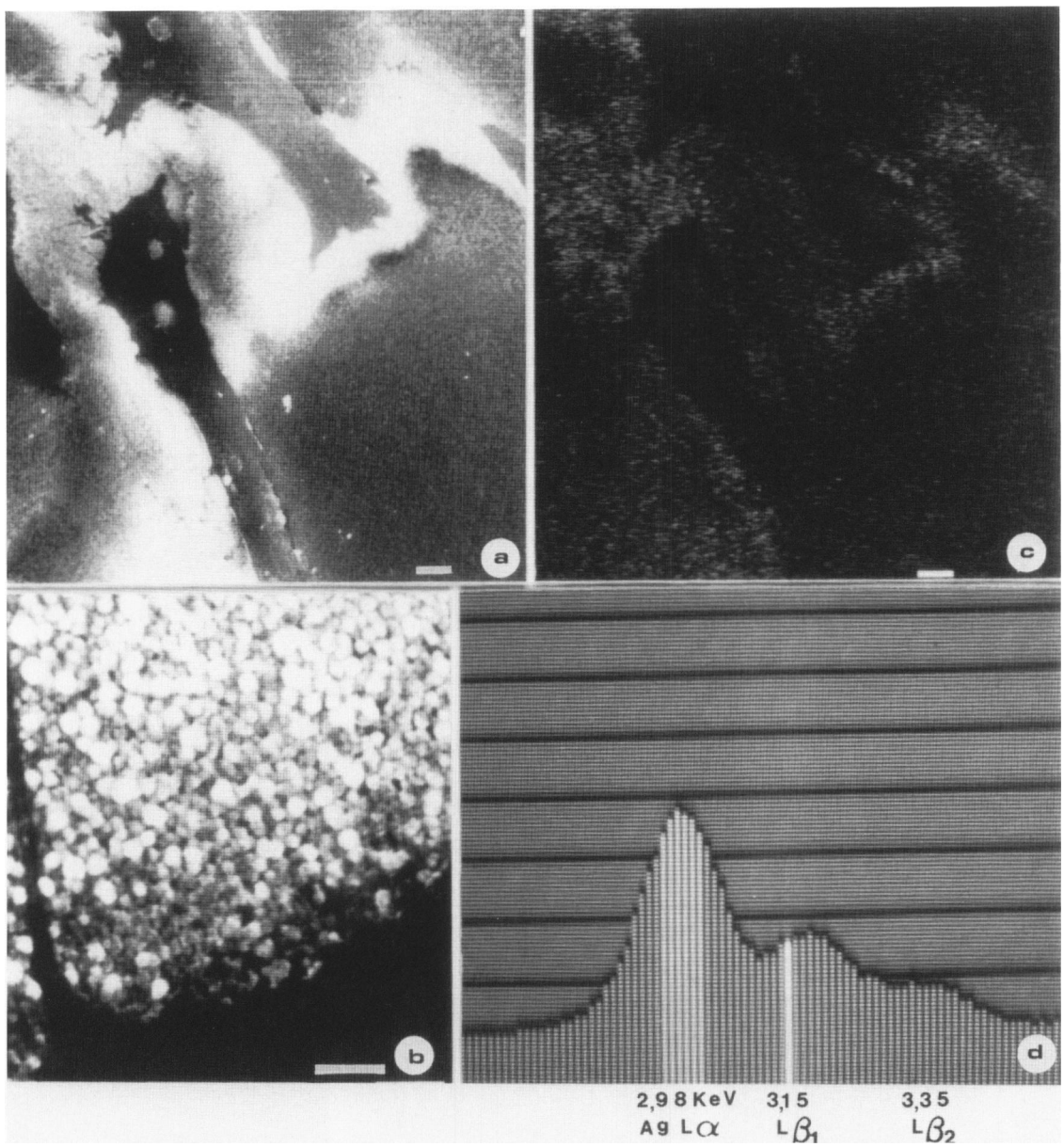

Figure 2. *IGSS of PAI-1 observed by scanning electron microscopy (SEM).* **a:** *The SEM secondary electron image shows a similar pattern of PAI-1 distribution as compared with the optical microscopy. Bar 10 μm.* **b:** *The presence of pericellular PAI-1 is seen as granular particles c.a. 0.05 to 0.3 μm. Bar 1 μm.* **c:** *The corresponding silver image of the same cells as in* **(a)** *clearly shows that PAI-1 antigen is essentially pericellular. Bar 10 μm.* **d:** *The L_α line of silver, at 2.98 KeV together with the two main L_β lines and the channels were used to produce the mapping image (in white).*

ency, we could not detect any difference in PAI-1 mRNA (data not shown).

Effects of Proteases on Matrix-associated PAI-1 Deposition

The amount of PAI-1 associated to mesangial cell matrix was dependent on cell confluency. It was probably regulated by the balance between PAI-1 deposition and removal during matrix remodeling by spreading cells.

As shown in Table 1 and Figure 8, addition of exogenous t-PA induced a dramatic decrease of matrix-associated PAI-1. A similar effect was obtained with another plasminogen activator, u-PA, and with a matrix-degrading enzyme collagenase. However, the latter also induced cell detachment (Table 1), which may indicate a nonspecific removal of pericellular matrix components. Conversely, heparinase, which hydrolyses specifically heparan-sulfate proteoglycans, and saponin, which dissolves membranes, did not affect matrix-associated PAI-1.

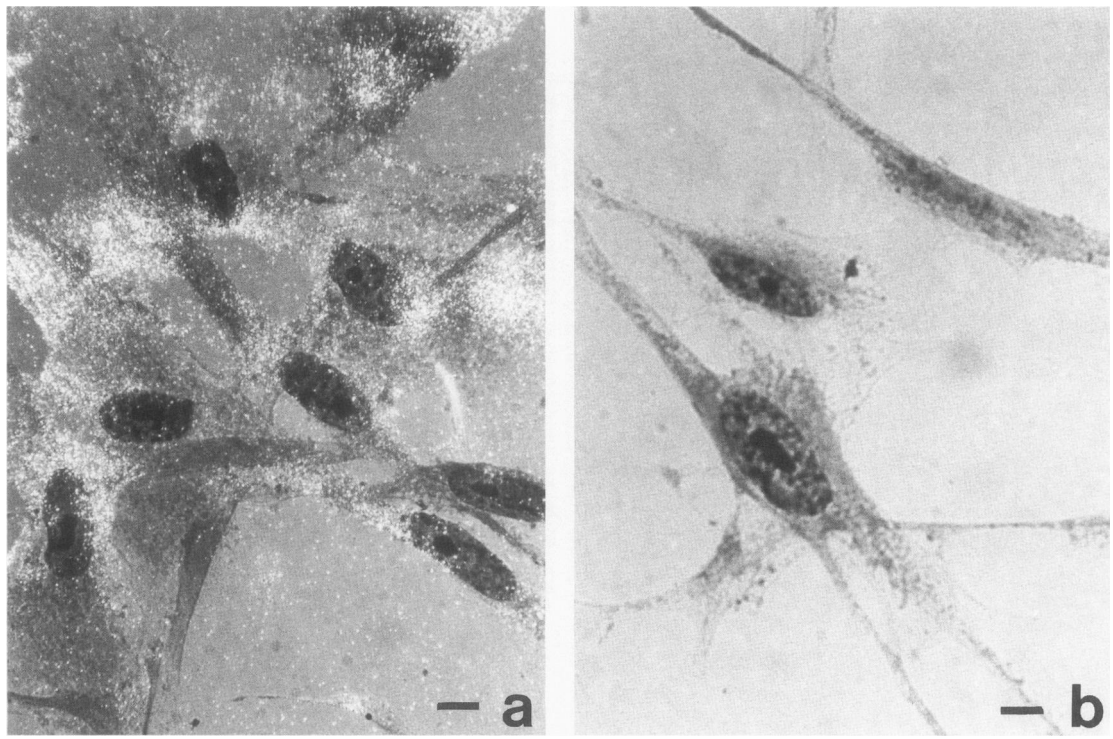

Figure 3. *IGSS of t-PA in cultured human mesangial cells.* **a**: *t-PA is present inside the cytoplasm of saponin-permeabilized cells.* **b**: *Control cells treated with a nonspecific serum (combined brightfield and epipolarization microscopy). Bars 10 μm.*

To further investigate the specific removal of matrix PAI-1 by t-PA, we compared the effects of exogenous t-PA before and after matrix fixation by paraformaldehyde. When t-PA (1 μg/ml) was added before fixation, less PAI-1 was detectable in the pericellular matrix (Figure 8b) compared with control preparations (Figure 8a), suggesting that PAI-1 was removed from the matrix. In that case, no t-PA was observed at the periphery of the cells (not shown). Conversely, when exogenous t-PA was added to fixed preparations, the amount of PAI-1 de-

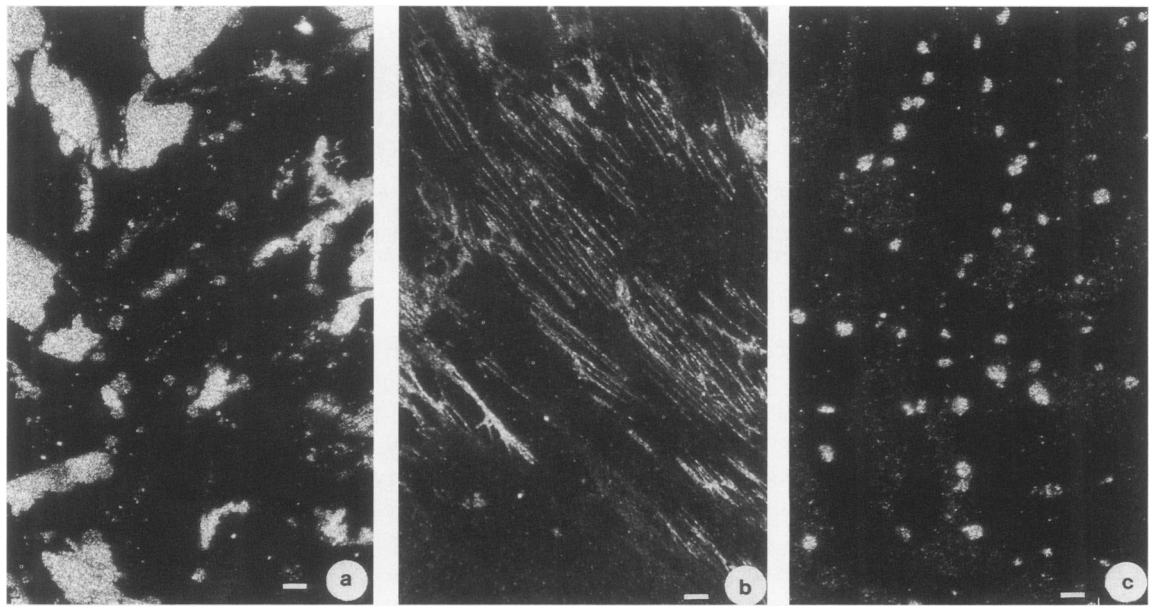

Figure 4. *Pattern of distribution of PAI-1 as compared with other matrix-associated proteins seen by IGSS.* **a**: *PAI-1 appears in white areas, whereas cells and their substratum are dark.* **b**: *Fibronectin has an extracellular fibrilar distribution.* **c**: *Vitronectin appears less abundant than PAI-1 in the extracellular environment, and is also associated to the cell membranes (epipolarization illumination). Bars 10 μm.*

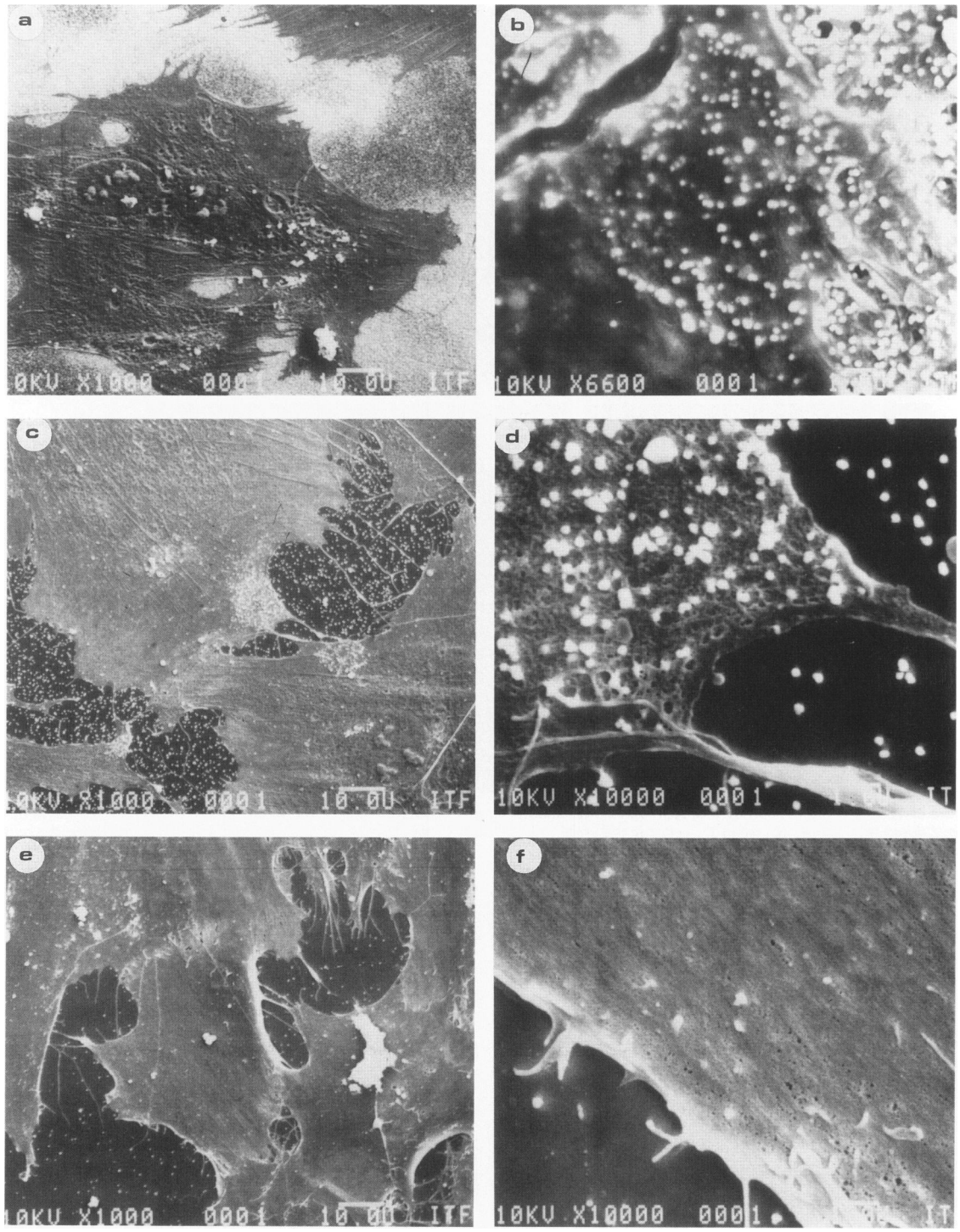

Figure 5. *IGSS of PAI-1* (a, b), *vitronectin* (c, d) *and control cells* (e, f) *observed by SEM. Extracellular PAI-1 appears bright and nonuniformly distributed around the cell* (a). *Extracellular vitronectin gives a sparse and uniform labeling* (c). *At higher magnification, cell-associated PAI-1* (b) *and cell-associated vitronectin* (d) *both show a similar granular membranous pattern. The IGSS background is weak, as shown in control cells* (e, f).

tected in the matrix was not decreased (Figure 8c). Furthermore, in these conditions, t-PA could bind to ECM and be detected by IGSS (Figure 8d). This suggested that, after fixation, PAI-1 could not be detached from the cell substratum but could still form complexes with t-PA.

Discussion

We demonstrated that PAI-1 is a component of the ECM of cultured human mesangial cells. Its deposition appears rapidly after seeding and seems to be regulated

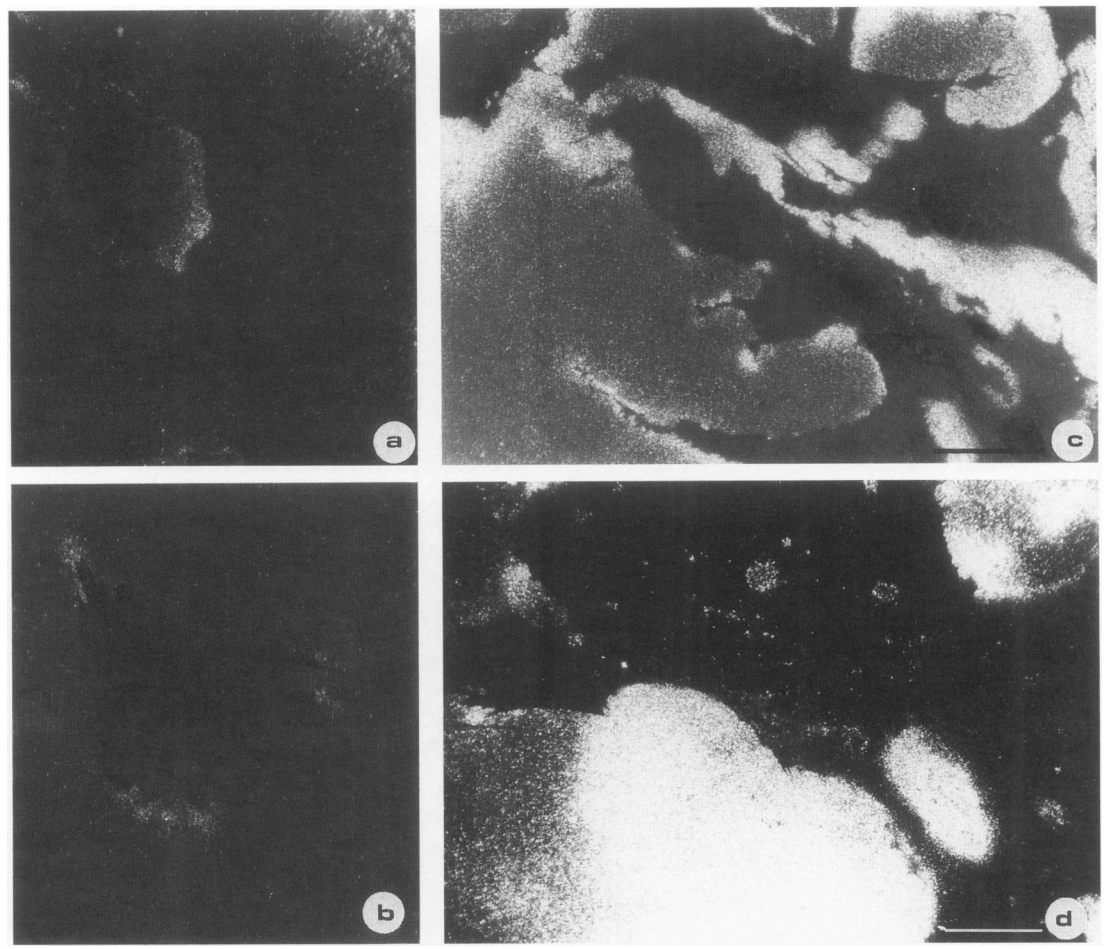

Figure 6. *Time-dependent deposition of PAI-1 in the extracellular matrix of mesangial cells. Increasing amounts of PAI-1 are found by IGSS after 2 hours* (a), *4 hours* (b) *when the cells are adhering and spreading, and after 24* (c) *and 48 hours* (d) *of culture, when the cells are growing (epipolarization illumination). Bars 10* μm.

by the rate of its synthesis and presumably by the rate of its removal from the ECM. This is in agreement with the recent reports that show that PAI-1 is a component of the extracellular matrix produced by various cell lines in culture.[24–28] However, this is the first demonstration that matrix-associated PAI-1 is synthesized by spreading human mesangial cells as shown by the time-dependent accumulation of PAI-1, the inhibitory effect of cycloheximide, and the direct detection of PAI-1 mRNA in cultured cells.

PAI-1 was localized by IGSS. The sensitivity of the IGSS method was increased by the use of epipolarization illumination and by SEM observation, which gives an overview of the whole cells and their environment. PAI-1 was mainly found in the pericellular environment. It has been reported that PAI-1 can interact with another cell matrix protein, vitronectin, to form complexes that retain both the cell-attachment–promoting activity of vitronectin and the capacity to inhibit u-PA and t-PA.[10,11] In our study, we found that extracellular vitronectin, which is present in FCS but could also be produced by mesangial cells, had a more diffuse and homogeneous distribution than PAI-1. The different patterns between matrix PAI-1 and vitronectin can be explained by the fact that most vitronectin is brought by FCS, as already published[29] and so is homogeneously distributed between cells. However, although we did not perform double-staining experiments, matrix-associated PAI-1 is probably bound to pericellular vitronectin. This is further suggested by the focal circular localization of PAI-1 and of vitronectin that we observed at the surface of mesangial cells. These focal cellular localizations could correspond to either sites of attachments to the cell membrane or to sites of secretion of these two proteins by mesangial cells. These focal circular sites could also correspond to integrin receptors for vitronectin since it is known that human mesangial cells have cellular receptors for fibronectin, laminin and collagen[30] and that rat mesangial cells express the vitronectin receptor.[31]

The localization of t-PA is more restricted. Immunoassay of t-PA in cell extracts showed low levels of t-PA,[32] and by IGSS most of the t-PA appeared to be inside the cell or at the cell surface, as described in endothelial

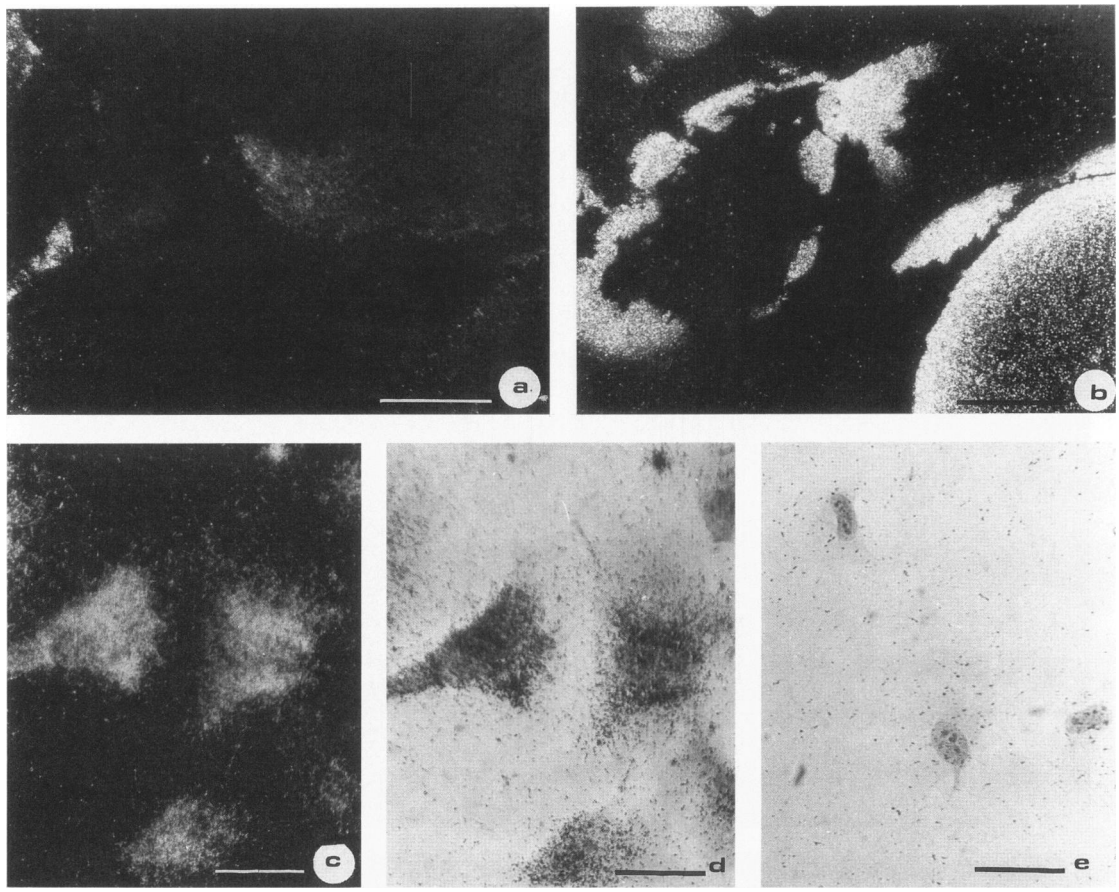

Figure 7. *Synthesis of PAI-1 by human mesangial cells grown on FCS-free RPMI. PAI-1 staining is clearly decreased after 24 hours of treatment in the presence of cycloheximide* (a) *as compared with control cells* (b) *(IGSS and epipolarization microscopy). PAI-1 mRNA is detected in the cytoplasm of mesangial cells by* in situ *hybridization using* ^{35}S *cDNA probe, as described in Methods. The same field is photographed using darkfield* (c) *or brightfield illumination* (d), *and is compared with the RNAse-treated control cells* (e). *Bars 10* μm.

cells.[33,34] t-PA has never been described in ECM,[27,35] whereas u-PA has been localized at focal contact sites of many cell types in culture,[24,36,37] including rat mesangial cells.[38]

The role of PAI-1 in the ECM is not well understood. It is suggested that, at focal contact sites, cell attachment is mediated by the interaction between extracellular vitronectin and cell vitronectin receptors that are concentrated at these sites.[6] PAI-1 associated to vitronectin could protect cell attachment from plasminogen activator-mediated proteolysis. On the other hand, it has been shown that u-PA can release PAI-1 from the vitronectin-PAI-1 complex,[39] suggesting that cell attachment at focal contact sites is reversible and can be modulated by local release of plasminogen activator. We have previously shown that human mesangial cells do not produce u-PA. The role of t-PA in these cells does not seem to be related to intravascular fibrinolysis. Although it has not been proven that t-PA is localized at focal attachment sites on mesangial cells, as described for u-PA,[39] t-PA may play a role in cell migration when limited pericellular proteolysis of the ECM is required since we demonstrated that exogenous t-PA was able to remove PAI-1 that was associated to the mesangial matrix without impairing cell

Table 1. *Effects of Enzymes on Matrix-associated PAI-1*

	Control	Heparinase (0.1 U/ml)	u-PA (500 U/ml)	t-PA (600 U/ml)	Collagenase (300 U/ml)	Saponin (0.05%)
Amount of PAI-1 (IGSS)	+	+	0	0	0	+
Cell detachment	0	0	0	0	+	+

After 24 hours culture in the presence of 10% FCS, cells were treated with the different compounds during 30 minutes at room temperature. Cells were then fixed and PAI-1 was stained by IGSS. PAI-1 deposition in the cell substratum was quantified as + (like in the control condition) or 0 (severely decreased or absent). The table precises if cells were detached (+) or still attached to the slides (0). Each experiment has been made in duplicate.

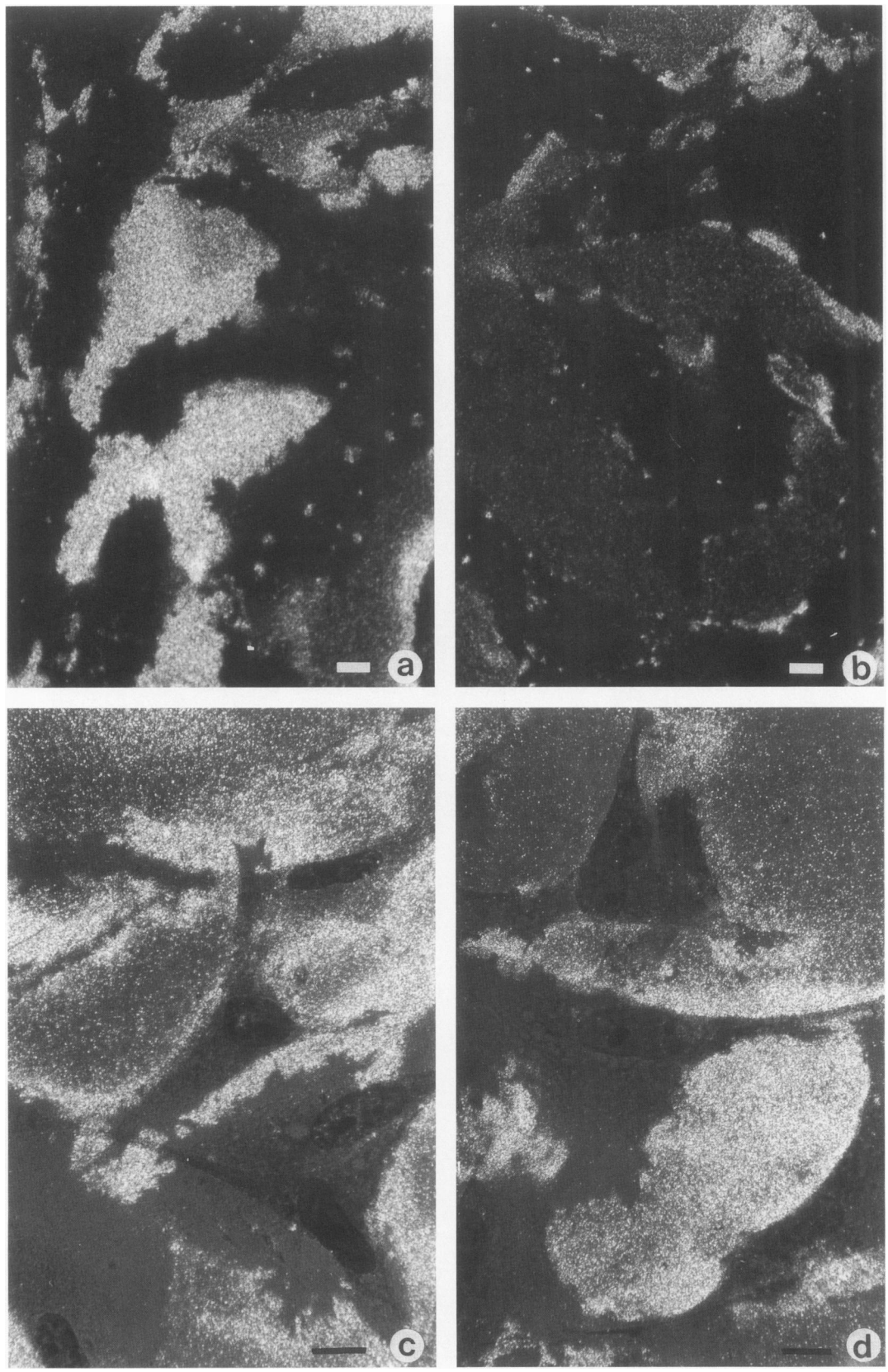

Figure 8. *Effects of treatment of cultured human mesangial cells by recombinant t-PA. When cells are pretreated by t-PA before fixation, less amounts of matrix-associated PAI-1 are detectable* (b) *when compared with untreated cells* (a). *When cells are treated by t-PA before fixation, similar amounts of PAI-1 are seen* (c) *as compared with untreated cells* (a), *but matrix-associated t-PA is detectable* (d) *in a pattern identical to matrix PAI-1 (IGSS and epipolarization microscopy). Bars 10 μm.*

adhesion. A similar effect was observed with exogenous u-PA but not with fibrinolysis-unrelated proteases, such as heparinase. Nonspecific release of PAI-1 was probably induced by collagenase, which also induced cell detachment. However, the proteolytic activity was required because saponin, which also induced cell detachment, did not affect matrix-associated PAI-1.

We have previously shown that mesangial cells release large amounts of PAI-1 compared with t-PA.[19] In conclusion, t-PA released by mesangial cells can bind to and detach matrix PAI-1 as reported for vascular smooth muscle cells[9] or can be rapidly inactivated by PAI-1 in the cultured medium.

The amount of PAI-1 in the ECM appears to be dependent on cell density. Large areas of positive PAI-1 staining were found by IGSS around nonconfluent spreading cells, but smaller areas were observed between subconfluent cells, suggesting that PAI-1 could be removed by t-PA from the matrix when cell density increases. This phenomenon could explain the absence of detectable PAI-1 by immunofluorescence in the mesangial matrix of normal human kidneys.[40] However, this may be due to a decreased sensitivity of the method since, by zymography, t-PA-PAI-1 complexes can be detected in extracts of human glomeruli.[41] Further studies are required to determine if PAI-1 accumulates in pathologic conditions with mesangial cell proliferation and mesangial matrix expansion.

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