Distinct Localizations of Urokinase-type Plasminogen Activator and Its Type 1 Inhibitor under Cultured Human Fibroblasts and Sarcoma Cells

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Abstract. We studied the immunocytochemical localization of urokinase-type plasminogen activator (u-PA) and the type 1 plasminogen activator inhibitor (PAI-1) in human fibroblasts and sarcoma cells, using both polyclonal and monoclonal antibodies. The u-PA was found to be located at discrete cell–substratum contact sites, and also at areas of cell–cell contacts, whereas PAI-1 was distributed as a homogenous carpet excluding strialike areas on the substrate under the cells. To confirm the extracellular localization of u-PA and PAI-1, we stained the cells live at 0°C before fixation. A double-labeling experiment showed different distribution of u-PA and PAI-1 under the cells, and especially their peripheral parts. The staining pattern of u-PA and PAI-1 resisted treatment with 0.2% saponin followed by mechanical removal of cells, a method previously reported to isolate focal contact membranes of fibroblasts. We further demonstrated the deposition of u-PA to the contact areas of cells obtained by saponin treatment by zymography, and that of PAI-1 by metabolic labeling, reverse zymography, immunoblotting, and immunoprecipitation. Fibronectin was also present in the preparations. The deposition of both PAI-1 and fibronectin by the sarcoma cells was enhanced, after treating the cells with 10^{-6} M dexamethasone. The confinement of u-PA to discrete contact sites and the more uniform distribution of PAI-1 on the cell substratum may explain how cells producing large amounts of enzyme inhibitors can produce PA-mediated focal proteolysis.

PLASMINOGEN activators (PAs)[1] are serine proteinases, which by limited proteolysis convert the abundant extracellular proenzyme plasminogen into the active proteinase plasmin. Two different types of PAs have been recognized in mammalian tissues: the urokinase type (u-PA) and the tissue type (t-PA), with approximate M_r 50,000 and 70,000, respectively. Evidence has accumulated indicating that u-PA plays a role in tissue degradation both in the normal organism and in cancer, whereas t-PA is supposed to be a key enzyme in thrombolysis (for reviews see references 12, 13, 16, 46, 58, 59).

Several of the plasma proteinase inhibitors interact with PAs. Specific protein inhibitors of PA have also been identified in a number of tissues, body fluids, and cell cultures (10, 14, 18, 19, 22, 26, 30, 35, 42, 44, 56, 66, 70, 73, 78, 80, 81, 83), though the mutual relationship of these inhibitors is not yet fully clarified. An M_r 54,000 plasminogen activator inhibitor (PAI) was recently purified from the human fibrosarcoma cell line HT-1080 (4), and immunologic evidence suggested that it is of the same type as the inhibitors from the rat hepatoma cell line HTC (66), bovine endothelial cells (18, 42, 44, 56, 70), and human blood platelets (19). This inhibitor has been designated plasminogen activator type 1 (PAI-1).[2] Other types of inhibitors acting on plasminogen activators include the protease nexin I (33, 64, 65) and that present in human placenta (PAI-2) (26, 30, 83).

Plasmin has a relatively broad specificity, being able to degrade known extracellular matrix glycoproteins and to activate latent collagenase (52, 54, 60). The affinity of plasminogen and plasminogen activators for laminin (61), fibronectin (62), and fibrin (45) further suggests the importance of these proteinases in the turnover of matrix components. Clearly, proteolysis taking place at cellular contact sites is of considerable importance for the interaction between membrane structures and the surrounding matrix components. Proteolytic targets of the pericellular matrices of cells in culture have been reported to include fibronectin (74) and an M_r 66,000 matrix-associated protein (31), which seems to be a plasminogen-independent cellular target for u-PA. In

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1. *Abbreviations used in this paper:* HES, human embryonic skin; PA, plasminogen activator; PAI, PAI-1, or PAI-2, plasminogen activator inhibitor, or specifically, PAI type 1 or type 2; t-PA, tissue-type plasminogen activator; u-PA, urokinase-type plasminogen activator; TNP, trinitrophenyl.

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addition, a metallo-endoproteinase seems to be involved in the focal degradation of fibronectin substrate by Rous sarcoma virus-transformed chicken and rat cells (11).

We now report that u-PA is located at cell–substratum and cell–cell contact sites of cultured human fibroblasts and sarcoma cells, whereas a specific M_r 54,000 PAI is homogeneously distributed on the substrate under the cells.

Materials and Methods

Cell Cultures

The human fibrosarcoma cell line HT-1080 was obtained from the American Type Culture Collection (CCL 121, Rockville, MD). The human embryonic skin fibroblast cultures (2-HES) were established locally by conventional methods and were used at the 10–20 passage level. The cells were grown either in plastic petri dishes (53 cm²) or in plastic Linbro wells (2 cm²; Flow Laboratories, Inc., McLean, VA) in Eagle's minimal essential medium (MEM) supplemented with 10% fetal calf serum (Gibco, Grand Island, NY), 100 IU/ml penicillin, and 50 µg/ml streptomycin. Cells were plated onto glass coverslips 24 h before extraction (see below).

Contact Area Preparations

Two procedures for removal of cells were used to prepare samples for immunofluorescence microscopic observations. Method I consisted of washing the cell layers at 0°C three times with PBS (10 mM NaPO₄, 145 mM NaCl, pH 7.4). The cultures were then placed in PBS supplemented with 0.2% saponin (white; BDH Chemicals Ltd., Poole, England) and shaken for 20 min at 0°C on a rotary shaker at 100 rpm. Then the coverslips were intensively pipetted in PBS and the material remaining bound to coverslips was fixed for 20 min in 3% (wt/vol) paraformaldehyde, prepared in buffered saline. In method II, instead of saponin, the cultures were incubated overnight in cold PBS, thereby letting the cells detach from the coverslips more slowly. In the morning the cultures were pipetted and washed in PBS and fixed as in method I.

Methods I and II were applied when the proteins remaining associated with the coverslip were to be analyzed. Cells were seeded onto Linbro wells, each well containing one coverslip. After a 24-h incubation at 37°C the medium was changed into serum-free MEM lacking methionine, and the cultures were further incubated for 2 h. The cultures were then metabolically labeled for 2 h with 50 µCi/ml [³⁵S]methionine (Amersham International, Buckinghamshire, England). The extraction procedure was carried out as detailed above (methods I and II). After excess buffer had been drained and removed by aspiration, the material remaining attached to the coverslips was dissolved at 0°C in Laemmli's sample buffer (1.25 M Tris-HCl, pH 6.8, containing 4% SDS, 20% glycerol, 0.005% bromphenol blue, and in reduced samples 10% 2-mercaptoethanol). The reduced samples were heated at 100°C for 2 min. Methods I and II gave quantitatively similar results.

Dexamethasone Treatment

Dexamethasone (Sigma Chemical Co., St. Louis, MO) was diluted from ethanol-based stock solution. The final dexamethasone concentration in the culture medium was 10^{-6} M.

Immunofluorescence

Polyclonal purified rabbit IgG and monoclonal (clone 12) mouse antibodies against u-PA, and mouse monoclonal (clone 1) and affinity-purified polyclonal rabbit IgG antibodies against PAI-1 from the HT-1080 cell line were prepared as described elsewhere (4, 34, 49, 50). Monoclonal antibody against the trinitrophenyl group, anti–TNP-IgG, was from the hybridoma clone designated Hy 2.15 (63). Rabbit antisera to fibronectin (25) and laminin (1) were produced and their specificity characterized as described. Mu-

rine monoclonal antibody fpg-9 to fibronectin was raised and its specificity in solid-phase radioimmunoassay, enzyme immunoassay, and immunoblotting established in collaboration with Drs. S. Barlati, V. Miggiano, and T. Vartio, as described elsewhere for monoclonal antifibronectin antibodies of the same series (76, 77). Rabbit antibodies to vinculin were from a commercial source (Transformation Research Inc., Framingham, MA). Fluorescein isothiocyanate (FITC)-conjugated sheep anti–rabbit immunoglobulin was from Wellcome (Beckenham, England) and tetramethylrhodamine isothiocyanate (TRITC)-conjugated goat anti–mouse immunoglobulins were from Nordic Immunological Laboratories (Tilburg, The Netherlands).

For immunofluorescent staining, coverslip cultures of cells were washed three times with cold PBS and fixed with either −20°C methanol or 3% (wt/vol) paraformaldehyde for 20 min. The intracellular antigens were exposed by detergent treatment (0.1% Triton X-100). The procedures for preparation of cellular contact areas have been detailed above. After additional washes with PBS the coverslips were incubated with the primary antibodies at a concentration of 50 µg/ml (antibodies to u-PA and the M_r 54,000 inhibitor) or diluted 1:100 (rabbit antifibronectin, rabbit antilaminin, and antivinculin sera) or 1:30 (monoclonal fpg-9 antifibronectin ascites) for 30 min. This was followed by a short rinse in PBS and incubation with fluorochrome (FITC/TRITC)-conjugated anti–rabbit/mouse immunoglobulins diluted 1:40 for 30 min. Staining of live cells was carried out at 0°C and consisted of incubation with the primary antibody prior to fixation with −20°C methanol and incubation with the fluorochrome-conjugated secondary antibody. For double-immunofluorescence studies of u-PA or PAI-1, the cells were incubated with antibodies in the following sequence: rabbit anti–u-PA IgG, sheep anti–rabbit immunoglobulins (FITC), mouse anti–PAI-1 clone 1, goat anti–mouse immunoglobulins (TRITC). Immunocytochemical controls included incubation with polyclonal antibodies preabsorbed with either highly purified u-PA (34) or PAI-1, and with preimmune rabbit IgG or mouse monoclonal anti–TNP-IgG, and omission of one of the steps in the staining procedure. After washing with PBS the coverslips were mounted in a solution consisting of glycerol and PBS in a one-to-one ratio. The specimens were viewed with a Leitz Dialux 20 EB microscope (E. Leitz, Wetzlar, Federal Republic of Germany) with epi-illumination. The light source was an HBO 200 high pressure mercury lamp and filter blocks I2 (excitation 450–490 nm, cutoff 515 nm) and N (excitation 530–560 nm, cutoff 580 nm) for FITC and TRITC, respectively, were used.

Immunoelectron Microscopy

The Linbro wells with cells were briefly rinsed three times with Dulbecco's PBS (DPBS) containing 0.2% BSA at 0°C, and then incubated with the primary antibody for 30 min, the entire experiment being made at 0°C. After rinsing three times with DPBS + 0.2% BSA, the cells were fixed with 1% glutaraldehyde (Sigma Chemical Co.) in 0.1 M phosphate buffer, pH 7.2, for 20 min, followed by treatment with 5 mg/ml NaBH₄ in the same phosphate buffer for 30 min. The cells were washed then three times with DPBS + 0.2% BSA, and then incubated with Perox Affini pure goat anti–rabbit IgG (H&L, Jackson Immuno Research Laboratories Inc., Avondale, PA) diluted 1:30 in DPBS + 0.2% BSA at 0°C for 30 min, and washed thereafter five more times. The peroxidase-catalyzed staining reaction was performed with the following mixture: 0.05% diaminobenzidine (Sigma Chemical Co.) and 0.001% H₂O₂ in 0.05 M Tris-HCl buffer, pH 7.6. After a 10-min staining, the cells were briefly rinsed three times with PBS, and postfixed with 1.5% OsO₄ in 0.1 M cacodylate buffer, pH 7.2, for 1 h at room temperature, and dehydrated with ethanol. The wells were mounted in Epon 812. After polymerization for 2 d, the Epon blocks were sectioned vertically against the cell layer. Thin sections (about 80 nm) were observed in a Jeol 100 CX TEMSCAN electron microscope at 40.0 kV at the Department of Electron Microscopy, University of Helsinki, Finland.

Polyacrylamide Gel Electrophoresis (PAGE) and Immunoblotting

Preparation and electrophoresis of samples in 8% and 5–16% gradient SDS polyacrylamide slab gels were as described by Laemmli (36). Radiolabeled material was detected by fluorography. Immunoblotting of proteins transferred electrophoretically to nitrocellulose sheets (type HAWP filter; Milli-

Figure 1. Immunofluorescence localization of u-PA and fibronectin in cultured human HT-1080 sarcoma cells and 2-HES fibroblasts. (*A, C,* and *F*) HT-1080, methanol fixation; (*E*) 2-HES, methanol; (*B, D,* and *G*) HT-1080, cells removed using the saponin procedure. The cells in *G* were cultured in the presence of 10^{-6}M dexamethasone. Primary antibodies: polyclonal anti–u-PA (*A–C* and *E*), monoclonal clone 12 anti–u-PA (*D*), polyclonal anti–u-PA absorbed with purified u-PA (*F*) and monoclonal fpg-9 anti-fibronectin (*G*). Indirect immunofluorescence and anti–rabbit IgG FITC (*A–C, E,* and *F*), and anti–mouse IgG TRITC (*D* and *G*) were used. Bars, 10 µm.
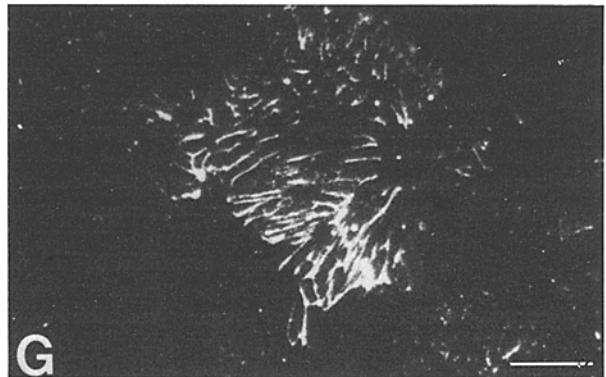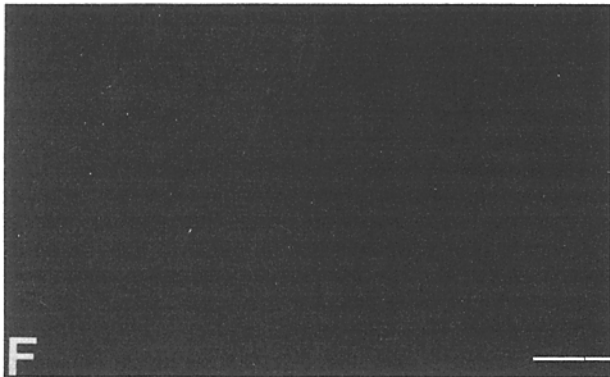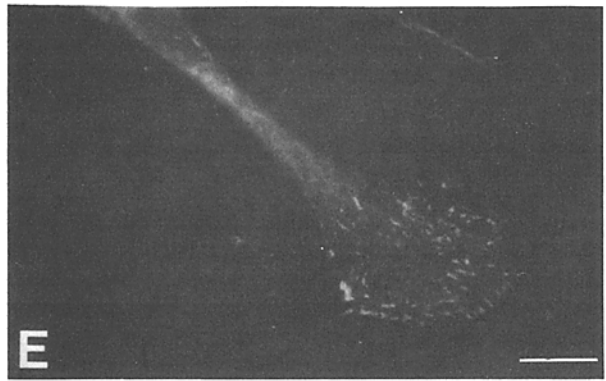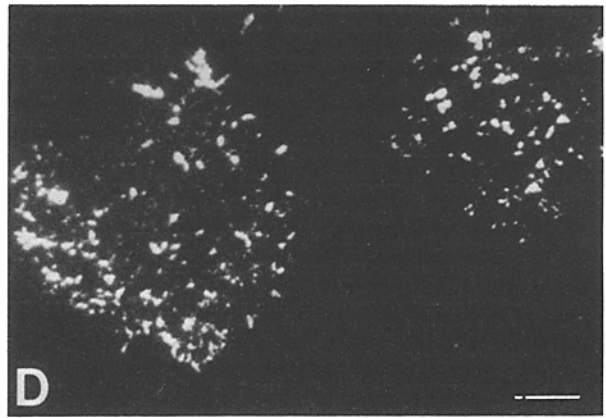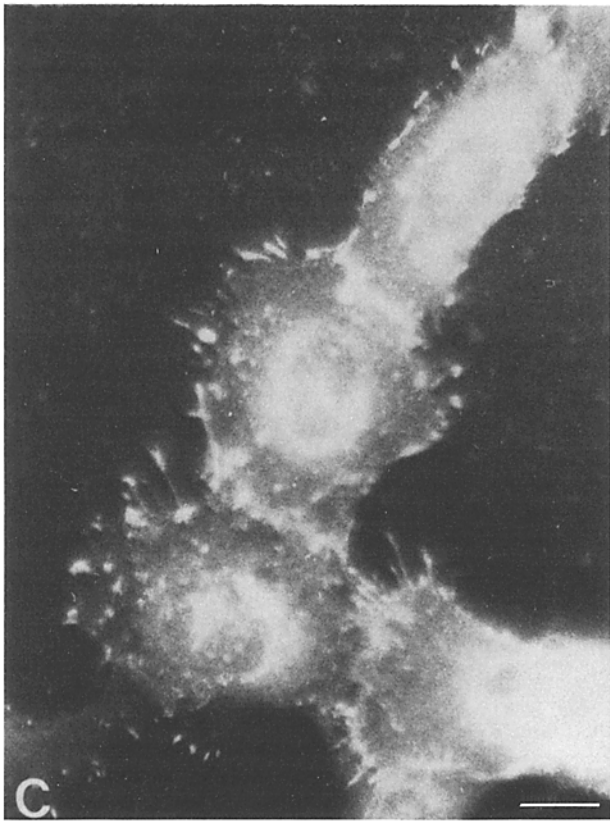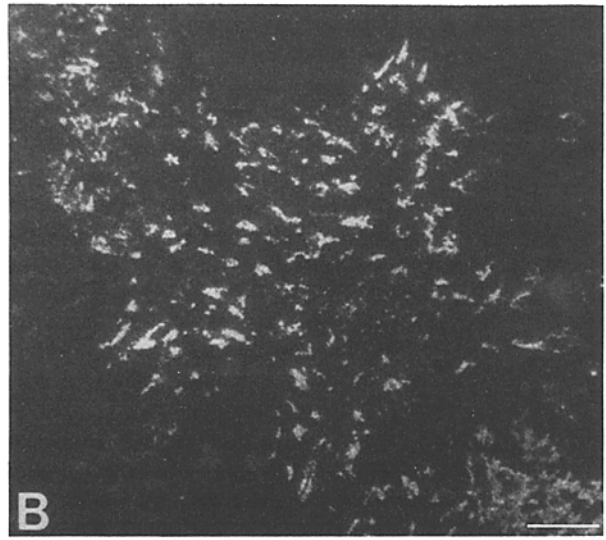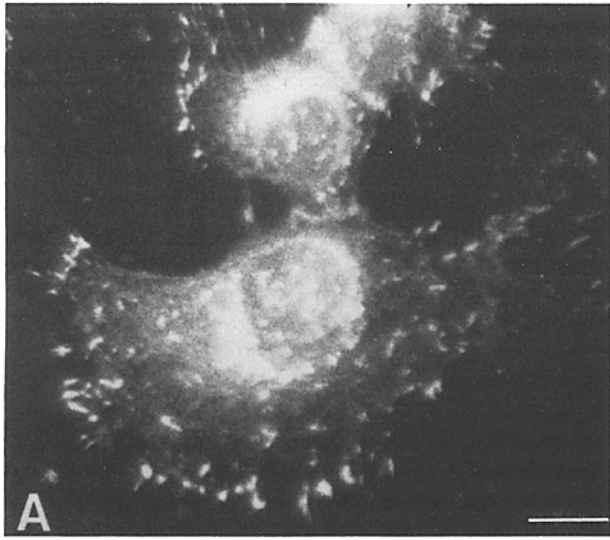
pore Corp., Bedford, MA) was performed using immunoperoxidase stain-
ing according to Towbin et al. (72) as modified (75). Commercially available
low molecular weight markers (Pharmacia Uppsala, Sweden) and ^{14}C-
labeled markers (Amersham International) were used.

Zymography

The contact area preparations were analyzed for PA activity as described
previously (23). Briefly, after electrophoresis in an 8% polyacrylamide slab
gel under nonreducing conditions and without heating the specimen, SDS
was removed from the gels with extensive washing on a gyratory shaker for
6 h in PBS with 2.5% Triton X-100, and an indicator agarose gel containing
casein and plasminogen was placed in contact with the polyacrylamide gel.
An agarose gel lacking plasminogen was used as a control. To determine
the type of the PA activity, contact area preparations were dissolved in 0.5%
Triton-X-100 and run through a column of Sepharose-coupled monoclonal
anti-u-PA or anti-t-PA before running the specimens in SDS PAGE. PAIs
in polyacrylamide gels were detected by washing the gel first in the same
solution as above, but containing 50 IU of u-PA in 100 ml of washing solu-
tion (Calbiochem Behring Corp., La Jolla, CA). When layered over an
agarose gel containing casein and plasminogen, a general lysis of the casein
took place except where inhibitors were present in the polyacrylamide gels.
These positions were revealed by opaque lysis-resistant zones: reverse
zymography (20).

u-PA and PAI-1 Preparations

u-PA was from a commercial preparation (M_r 54,000, 60,000 IU/mg, Cal-
biochem). PAI-1 was purified from conditioned culture medium of dexa-
methasone-treated HT-1080 cells as described in detail elsewhere (4). Iodi-
nation of u-PA and PAI-1 was carried out with the Iodo-Gen method (Pierce
Chemical Co., Rockford, IL).

Immunoprecipitation

For the immunoprecipitation experiment, metabolically labeled cells were
extracted using method I (see above), the contact area material being dis-
solved in 500 µl of 0.1% (wt/vol) SDS, 0.5% (vol/vol) Triton X-114, 50 mM
Tris-HCl buffer, pH 8.0, 150 mM NaCl. 70 µl of 10% (wt/vol) solution of
washed Protein A-Sepharose CL-4D beads (Pharmacia) in 50 mM Tris-HCl
buffer, pH 8.0, 150 mM NaCl was added, and after 15 min beads were sep-
arated by centrifugation at 9,000 g for 1 min. 5 µl of the monoclonal anti-
body against PAI-1 was added to the supernatant, and the mixture was in-
cubated overnight at 4°C. The samples were further incubated with Protein
A-Sepharose beads as above for 60 min at 37°C in an end-over mixer. The
precipitate was washed four times with the dissolving buffer and once with
50 mM Tris-HCl, pH 8.0, 150 mM NaCl, before resolvement by SDS PAGE
on 8% acrylamide gels. The dried gels were exposed on to Kodak XAR-5
film for autoradiography.

Conversion of Added^{125}I-u-PA to a High Molecular Mass Form in Contact Area Preparations

^{125}I-u-PA prepared as described above and diluted in PBS (10^6 cpm/ml),
was added to freshly prepared contact area material of 2-HES fibroblasts
obtained by saponin treatment as described. After incubation for 60 min at
4°C the preparations were washed with PBS, and the bound proteins were
dissolved in Laemmli's sample buffer under nonreducing conditions, and
analyzed in SDS PAGE followed by autoradiography.

Results

Immunocytochemical Localization of u-PA and PAI-1

HT-1080 and 2-HES cells were grown on glass coverslips
overnight. After rinsing with ice-cold PBS, the cells were
fixed or removed either by treating them with 0.2% saponin
or by letting them shed overnight in PBS at 4°C as described
in Materials and Methods. The fixed cells and the material
remaining on the coverslips were examined by immunofluo-
rescence microscopy.

Using both a polyclonal and a monoclonal antibody we
found u-PA to be located at cell substratum contact sites of
HT-1080 fibrosarcoma cells. Staining was also detectable in
areas of cell–cell contacts (Fig. 1, A and C). The label as-
sociated with the substratum resisted the saponin extraction
procedure, making the footprint contact pattern of immuno-
fluorescence even more evident (Fig. 1, B and D). When live
HT-1080 cells were stained, we detected patches of staining
at cell–cell contacts and a weak diffuse pattern of immuno-
fluorescence over the dorsal surfaces of cells (see Fig. 4 A),
in addition to that in the contact sites on the substratum (see
Fig. 4 B). These results demonstrate the extracellular loca-
tion of the u-PA antigen. Intracellular anti-u-PA staining was
also seen, showing highest intensity in the perinuclear region
(Fig. 1, A and C). This was particularly the case, when cells
were fixed with paraformaldehyde followed by various per-
meabilization procedures (not shown). At the electron mi-
croscopic level we could observe patches of polyclonal anti-
u-PA staining extracellularly in association with the plasma
membrane. Occasionally, a patch of stain formed a contact
between two neighboring HT-1080 cells (Fig. 2 A). Human
fibroblasts showed much lower staining intensity for u-PA
than sarcoma cells. Only rarely were we able to detect a con-
tact site staining pattern similar to that visualized in Fig. 1 E.

Staining with a monoclonal antibody to PAI-1 gave a simi-
lar pattern in fibrosarcoma cells and in fibroblasts. The im-
munofluorescence consisted of a homogeneously layer on the
substratum. There was also intracellular perinuclear staining
with very intensive labeling, presumably indicating PAI-1
synthesis (Fig. 3, A and B). After saponin extraction of cells,
the immunofluorescence consisted of a homogenous carpet
in the cell peripheral area interrupted by striae of negative
staining (Fig. 3 C and D), which were also very faintly pres-
ent in cells fixed with −20°C methanol (see Fig. 5 A).
Affinity-purified polyclonal rabbit anti-PAI-1 IgG gave simi-
lar staining patterns as the monoclonal antibody. In cells
stained live at 0°C with anti–PAI-1 antibodies, the immuno-
fluorescence was restricted to the peripheral parts of the cells
whereas the central areas appeared negative (Fig. 4 C). We
interpret this to be the result of antigen in accessibility on the
substrate under live cells in that the antigen was seen in the
saponin-treated preparation on the substratum (Fig. 3, C and
D). Double-stained HT-1080 cells, featured in Fig. 5, A and
B, show that u-PA and PAI-1 have distinct staining patterns
especially on the peripheral parts of the cells.

Fibronectin was detected with a polyclonal and the fpg-9
monoclonal antibody in both cell lines. The saponin contact
preparations of fibroblasts displayed a typical matrix pattern,
whereas in HT-1080 preparations the corresponding pattern
was dominated by dots and striae. Fig. 1 G shows the fpg-9
staining pattern in a contact area preparation of dexametha-
sone treated HT-1080 cells. Neither HT-1080 nor fibroblast
contact preparations displayed vinculin or laminin, as inves-
tigated by immunofluorescence using polyclonal antibodies.

Specificity controls including the use of up to 50 µg/ml
preimmune IgG corresponding to the anti–u-PA or anti–PAI-
1 and monoclonal anti–TNP-IgG were negative, as were ex-
periments in which one of the steps in the staining protocol
was omitted (Fig. 2 B). Absorption of the antibodies with
either highly purified u-PA (Fig. 1 F) or PAI-1 (not shown)
completely abolished the staining described above.

[^{35}S]Methionine Labeling and Immunoprecipitation

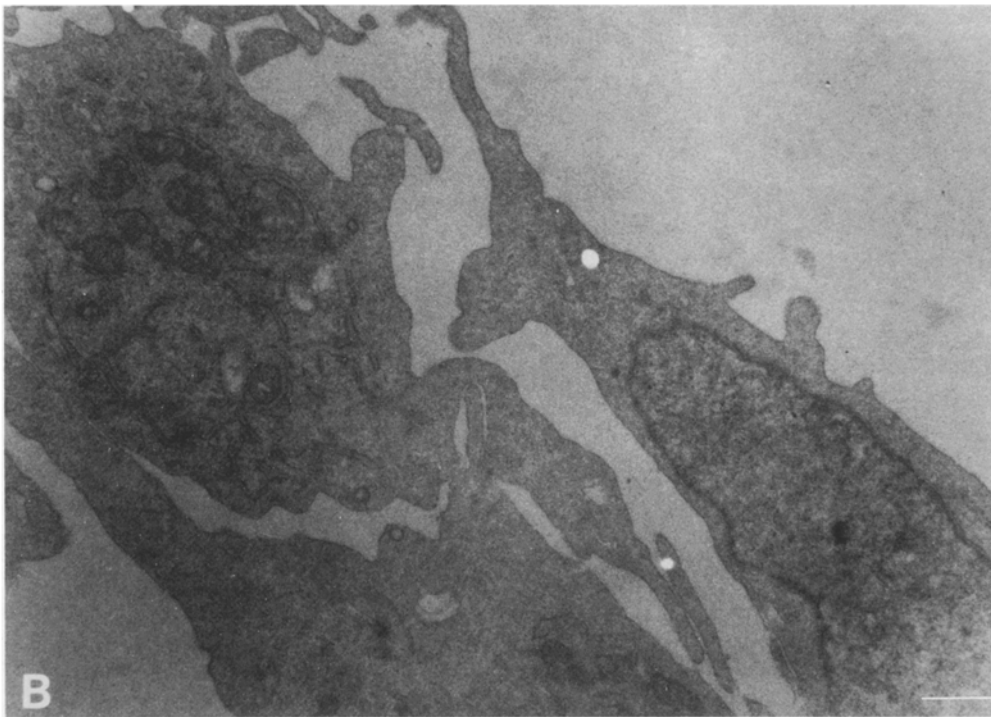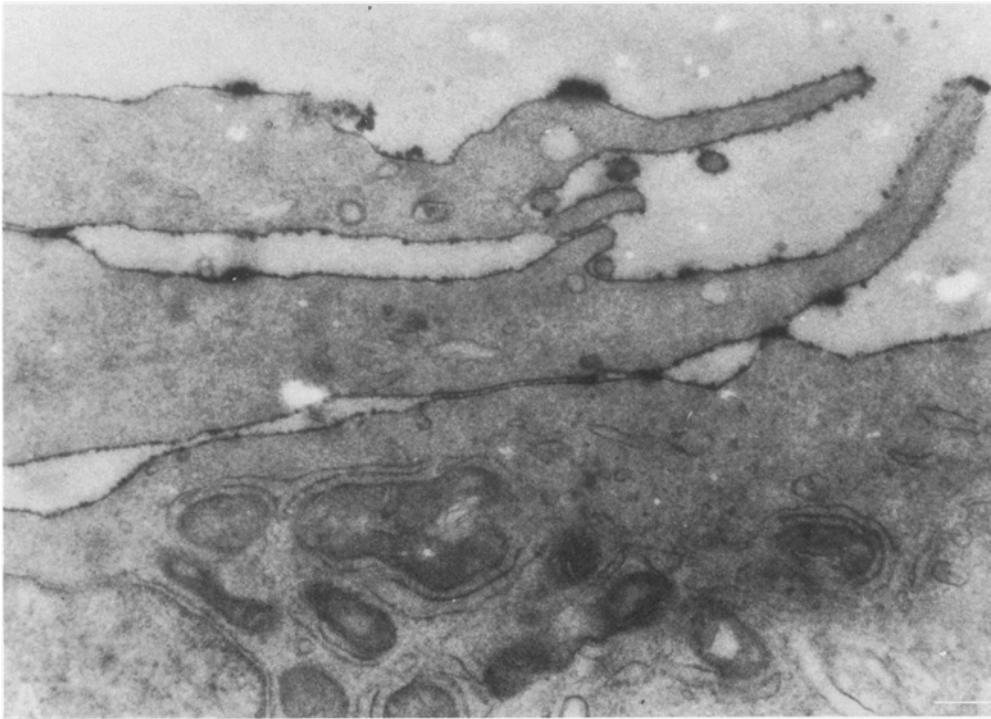To characterize the macromolecular components of the con-

Figure 2. Immunoelectron microscopic localization of u-PA in cultured HT-1080 sarcoma cells. The cells were incubated with the primary antibody (polyclonal anti–u-PA, *A*; none, *B*) at 0°C before fixation with glutaraldehyde and subsequent incubation with NaBH₄. Peroxidase-conjugated anti–rabbit IgG was used as the second antibody. The entire experiment was carried out at 0°C. Note the extracellular patches of the antigen on the cell surface and between cells. Bars, 0.2 μm.

tact area preparations obtained by method I, we labeled the cells metabolically with [^{35}S]methionine in the presence or absence of 10^{-6} M dexamethasone in the culture medium. The material attached to the glass coverslip was analyzed in SDS-PAGE followed by autoradiography. One major band run at M_r 54,000 and coelectrophoresed with purified radiolabeled PAI-1 (Fig. 6, lane *5*) in HT-1080 as well as fibroblast contact preparations. The monoclonal anti–PAI-1 antibody also immunoprecipitated a similar band from the preparation (see Fig. 10). However, the precipitation of PAI-1 did not re-

sult in a quantitative depletion of the M_r 54,000 band from the immune supernatant. Thus, it cannot be excluded that there are at least two M_r 54,000 proteins comigrating in the metabolically labeled contact area preparation. Enriched in the contact area preparations were also bands at M_r 60,000, 75,000, and 250,000. Dexamethasone in the culture medium increased the intensity of the M_r 54,000 and 250,000 bands on the HT-1080 contact preparations (Fig. 6, lanes *1–2* and *8–9*), as expected of the PAI-1 (2, 4, 14, 66) and fibronectin subunits (53). The M_r 54,000 band also appeared increased
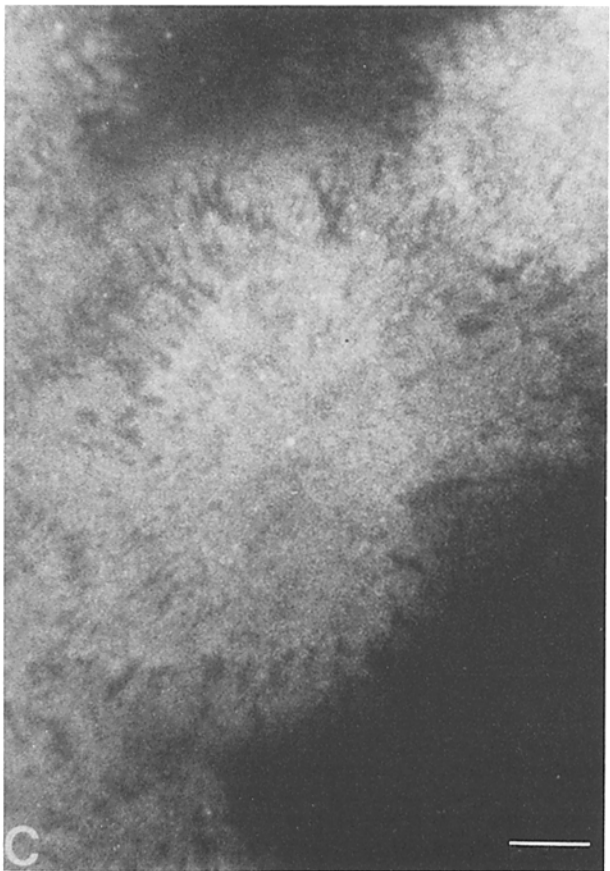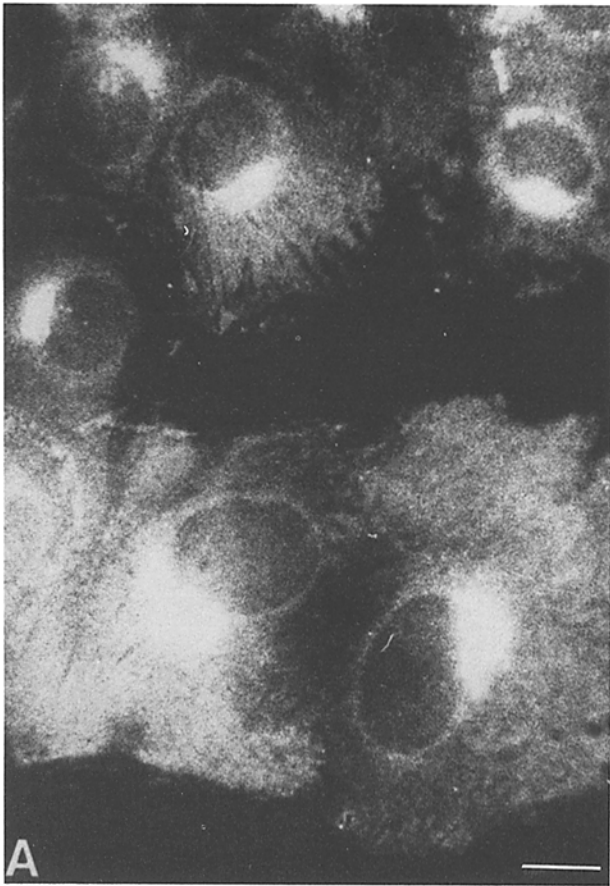
Figure 3. Immunofluorescence localization of PAI-1 in (*A* and *C*) cultured HT-1080 sarcoma cells and (*B* and *D*) 2-HES fibroblasts. Fixation was with methanol in *A* and *B*. Cells were removed using method I in *C* and *D*. Indirect immunofluorescence, monoclonal clone 1 anti–PAI-1 antibodies and TRITC-conjugated anti–mouse IgG were used. Note the lack of staining in strialike areas in the otherwise uniform staining under the cells in *A* and especially *C* and *D*. Bars, 10 µm.
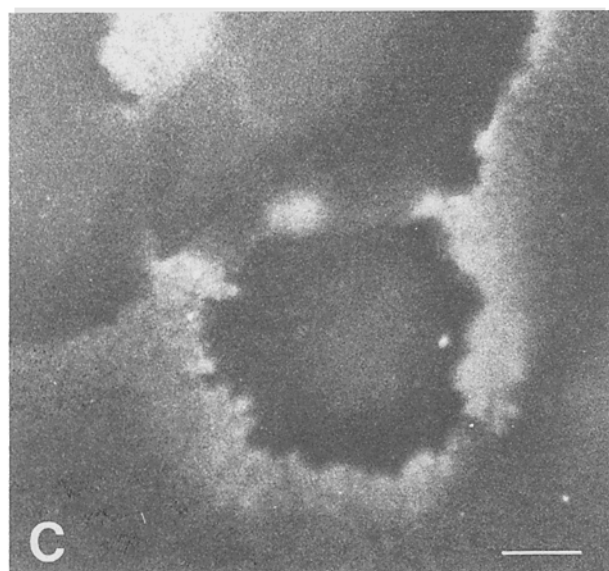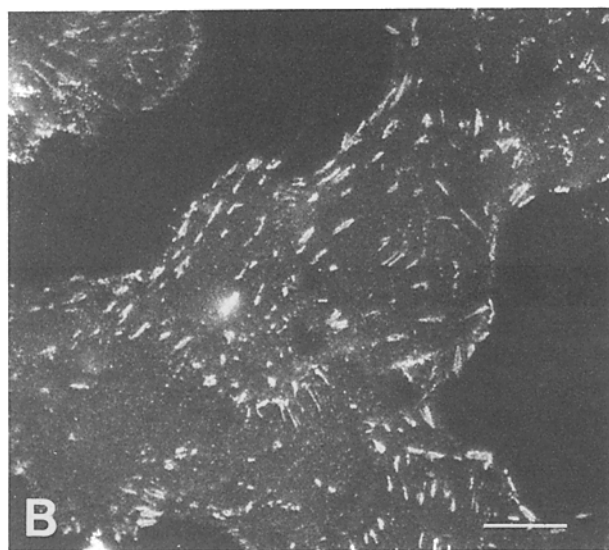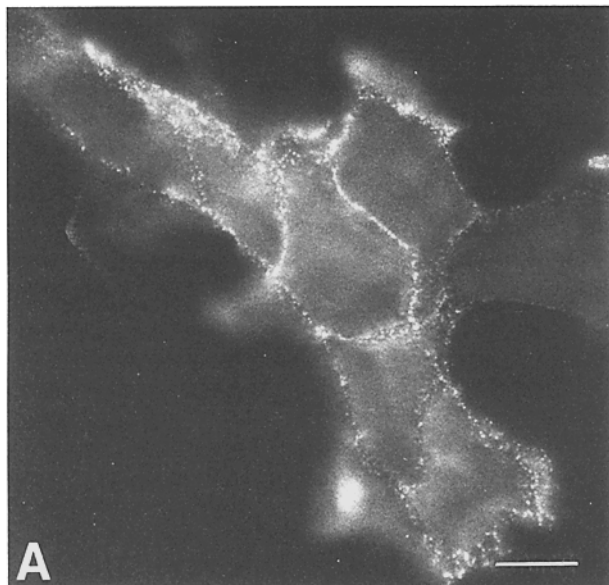
in the contact area preparations from dexamethasone-treated fibroblasts with SDS-PAGE under reducing conditions, but not with SDS PAGE under nonreducing conditions. This apparent discrepancy is due to the fact that less total protein was undeliberately applied to the gel lane corresponding to fibroblasts without dexamethasone electrophoresed under reducing conditions.

Zymographic and Reverse Zymographic Analysis of the Contact Area Preparations

Molecular forms of the plasminogen activators present in the contact area preparations were investigated by SDS-PAGE under nonreduced conditions followed by casein-agarose zymography (Fig. 7). In the contact area preparations of HT-1080 cells (lanes *1* and *2*), PA activity migrated as a well-defined band at M_r 50,000, as expected of u-PA. Additionally, higher molecular mass forms of PAs were detected as a smear of activity between M_r 70,000 and 100,000, with a distinctive band at the latter end. Dexamethasone slightly diminished the PA activity in the preparations. All the PA activity in the preparations is u-PA, in that preparations run through a column of Sepharose coupled monoclonal anti-u-PA antibodies lost all their PA activity, while treatment in a similar anti-t-PA column had no effect on the PA activity pattern of the preparations (data not shown). This indicates that PAI-1 and u-PA form a complex in the preparation which dissociates partly during electrophoresis, in agreement with previous findings (4). The reason that the lysis zone is not continuous between positions corresponding to M_r 50,000 and 100,000 can be ascribed to the fact that excess inhibitor migrates slightly behind uncomplexed u-PA, preventing lysis in that position. Virtually no PA activity was detected in fibroblast contact preparations using this method (lanes *3* and *4*); only after several days' exposure did a weak lysis appear at M_r 50,000. Control gels lacking plasminogen showed no caseinolytic activity.

Reverse casein agarose zymography was carried out to test for PA inhibiting activities (Fig. 8). Analysis of the contact area preparations of HT-1080 cells as well as fibroblasts revealed a major inhibitor zone at M_r 54,000. Dexamethasone markedly increased the intensity of the inhibitor zone of the HT-1080 contact area preparations (lanes *1* and *2*), but this effect was virtually non-existent in the fibroblast preparations (lanes *3* and *4*).

Immunoblotting

Contact area preparations of dexamethasone-treated HT-1080 cells were subjected to immunoblotting analysis under reducing conditions. The monoclonal PAI-1 antibody detected a single band at M_r 54,000 (Fig. 9, lane *1*). Lane 2 is a specificity control track immunoblotted with anti–TNP-IgG.

Figure 4. Immunofluorescence localization of (*A* and *B*) u-PA and (*C*) PAI-1 in HT-1080 cells. The cells were stained with the primary antibodies, (*A* and *B*) polyclonal rabbit anti–u-PA IgG, (*C*) mouse monoclonal anti–PAI-1, before fixation with methanol and incubation with the secondary antibodies. The entire experiment was performed at 0°C. In *A* the focus is above the growth substratum at the cell–cell contacts and in *B* and *C* the microscope was focussed on the substratum. Bars, 10 μm.
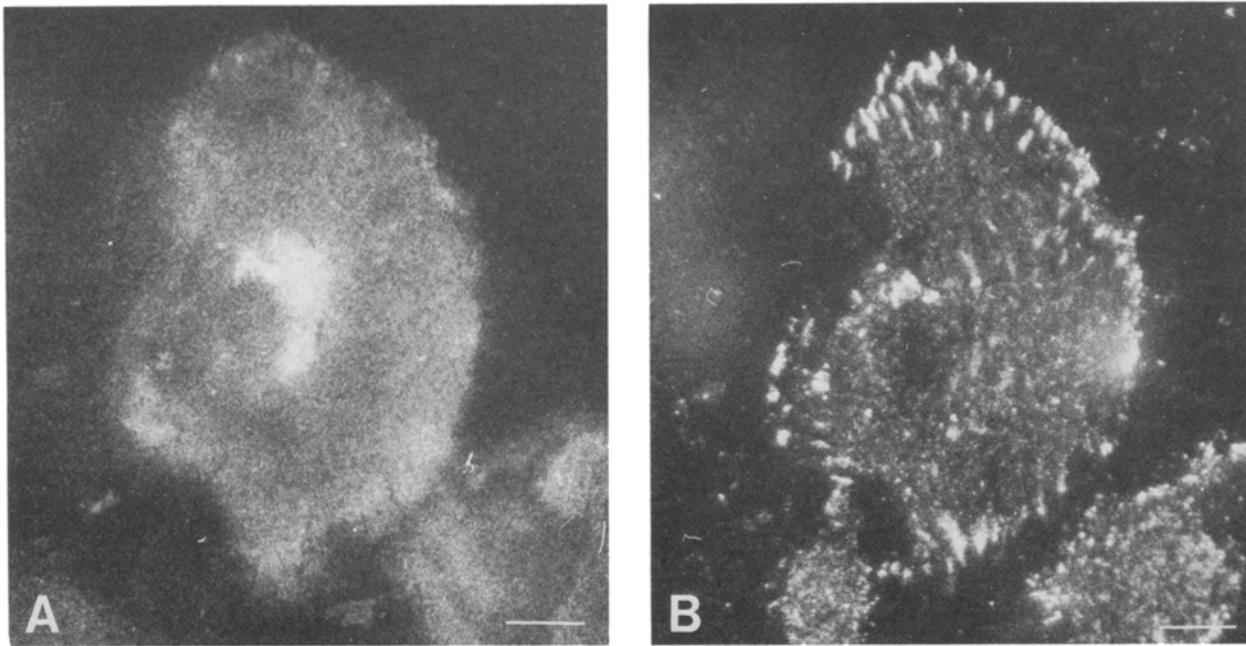
Figure 5. Double-immunofluorescence staining of methanol-fixed HT-1080 cells for (*A*) PAI-1 and (*B*) u-PA. The primary antibodies were (*A*) mouse monoclonal anti–PAI-1 and (*B*) polyclonal rabbit anti–u-PA IgG. The procedure is described in Materials and Methods. Bars, 10 μm.

Complexing of ^{125}I-u-PA in Contact Area Preparations of Fibroblasts

When ^{125}I-u-PA (M_r 50,000, Fig. 11, lane *a*) was added to preparations obtained from cultures of 2-HES fibroblasts using method I, and incubated for 60 min in the cold, a large proportion of the bound label was converted to a high molecular mass form: M_r 90,000 (Fig. 11, lane *b*). A similar

high molecular mass complex was obtained after incubation of ^{125}I-u-PA with excess amounts of PAI-1 purified from the HT-1080 cell line (lane *c*).

Discussion

In the present study we have described the distribution of u-PA and an M_r 54,000 PAI in cultured HT-1080 human fibrosarcoma cells and in human fibroblasts using both polyclonal and specific monoclonal antibodies. In our experiments, u-PA was found to be located at discrete cellular contact sites under the fibrosarcoma cells, whereas its inhibitor was distributed as a homogeneously thick carpet on the sub-
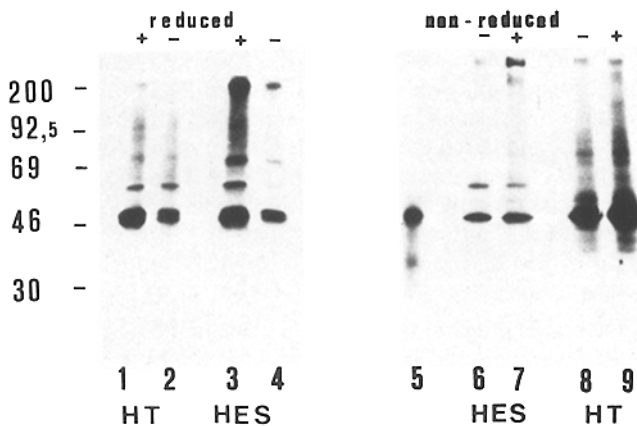

Figure 6. Macromolecular components of the contact area preparations. Metabolically labeled cells were extracted using the saponin procedure (method I), and the material remaining attached to the glass coverslips was dissolved in Laemmli's sample buffer, 500 μl per coverslip. 70 μl aliquots of the samples were analyzed by 5–16% polyacrylamide gradient SDS PAGE under reducing (lanes *1–4*) and nonreducing (lanes *5–9*) conditions, followed by autoradiography. The contact area preparations of dexamethasone-treated (lanes *1* and *9*) and untreated (lanes *2* and *8*) HT-1080 cells, and dexamethasone-treated (lanes *3* and *7*) and untreated (lanes *4* and *6*) 2-HES cells. Lane *5*: purified radiolabeled PAI-1. The molecular mass of the marker proteins (reduced) is shown in kilodaltons.

Figure 7. Casein-agarose zymography after 8% SDS PAGE of the contact area preparations. 70-μl samples of the contact area preparations of dexamethasone-treated (lane *1*) and untreated (lane *2*) HT-1080 cells, and dexamethasone-treated (lane *3*) and untreated (lane *4*) 2-HES cells were analyzed. Lane *5*: 250 mIU u-PA. Development at 37°C for 24 h. The positions of the M_r markers are indicated in kilodaltons on the right.
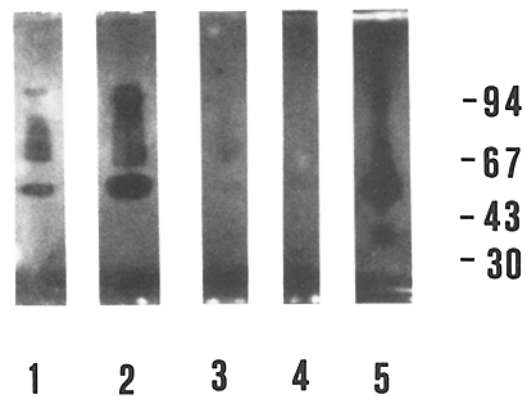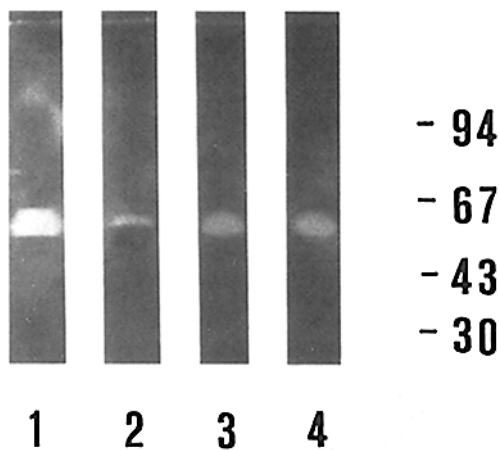
Figure 8. Reverse zymography after 8% SDS PAGE of the contact area preparations. 70-μl samples of the contact area preparations of dexamethasone-treated (lane *1*) and untreated (lane *2*) HT-1080 cells, and dexamethasone-treated (lane *3*) and untreated (lane *4*) 2-HES cells were analyzed. Development at 37°C for 24 h. The positions of the M_r markers are indicated to the right in kilodaltons.

stratum, but lacking in strialike areas. When live cells were stained, PAI-1 could not be detected under the central parts of the cells. Thus, we cannot exclude the possibilty that the negative strialike areas in saponin-treated cell preparations would be due to inaccessibility of the PAI-1 antigen to the antibodies used. At the electron microscopic level we detected patches of anti–u-PA staining associated with the plasma membrane. This result, obtained by staining live cells in the cold with the primary antibody, indicated that u-PA on the cell surface is externally located. The deposition of fibronectin was also detected using a monoclonal antibody. Normal human fibroblasts showed low intensity of anti–u-PA immunoreactivity, while producing PA inhibitor quite abundantly.

Additional lines of evidence were obtained, suggesting that u-PA and its inhibitor are indeed deposited to the contact areas of cells. First, the contact areas could be isolated with 0.2% saponin assisted mechanically with a stream of buffer. When such cell-free substrate-attached material, prepared from [³⁵S]methionine-labeled cells, was studied by fluorography, we found that an M_r 54,000 band was the major one in the preparations. This band includes PAI-1, as judged by immunoprecipitation experiments, although the presence of other proteins in the same position cannot be excluded. Still, its amount in the preparations was responsive to dexamethasone treatment of the HT-1080 cell cultures in agreement with the previous findings (2, 4, 14, 53, 66). PAI-1 in the contact area preparations could be immunoblotted with a specific monoclonal antibody. Apparently, u-PA is a relatively minor component in the contact area preparations; we were unable to detect u-PA in these preparations using immunoblotting. The PA activity of the contact area preparations was, however, confirmed with zymography which showed a major band at M_r 50,000 as well as a minor band at M_r 100,000. Reverse zymography showed a single M_r 54,000 inhibitor in the preparations. Furthermore, when ¹²⁵I-u-PA was incubated with the preparations, much of it was converted into a high molecular mass form (M_r 90,000). This observation is consistent with the presence of PAI-1 in con-
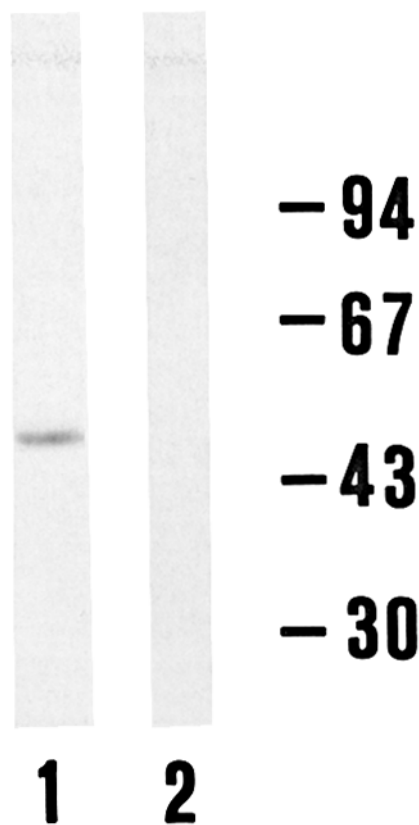

Figure 9. Immunoblotting of PA inhibitor under reduced conditions in a contact area preparation of dexamethasone-treated HT-1080 contact area preparation, using 50 μg/ml monoclonal anti–PAI, clone 1 (lane *1*) or monoclonal anti–TNP-IgG (lane *2*). The positions of the M_r markers are indicated in kilodaltons.

tact area preparations, but further experiments are needed to determine whether the complexing of ¹²⁵I-u-PA is specifically with PAI-1.

The accessibility of u-PA for antibodies at the contact sites differed under various fixation and permeabilization conditions, methanol fixation at −20°C giving the clearest results. Even then, the monoclonal antibodies gave much less staining than the polyclonal anti–u-PA antibodies we used. After removal of cells to obtain the contact area preparations both types of antibodies gave a similar pattern of staining, suggesting that the determinant of the monoclonal antibody is restrictedly exposed.

The subcellular localization of u-PA has been previously explored by cell fractionation studies and by the immunocytochemical approach. Despite conflicting results, there appears to be a consensus among the various cell fractionation reports that PA activity is often localized in membrane-rich fractions. In Rous sarcoma virus-transformed chicken embryonal fibroblasts, u-PA has been localized to fractions containing the Golgi apparatus and the plasma membrane (57), whereas in growing and virus-transformed 3T3 cells exclusively to the plasma membrane-enriched fraction (28, 29). However, in a study of HT-1080 cells PA of non-determined type was found associated solely with a cellular fraction representing either the Golgi apparatus or secretory granules rather than plasma membrane (39). Interestingly, either u-PA or PA of nondetermined type from several cell lines includ-
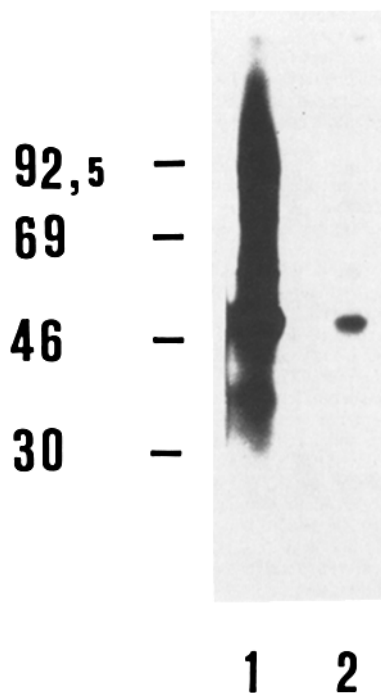
92,5 —
69 —
46 —
30 —

1   2

Figure 10. Autoradiography of 8% SDS PAGE of an immunoprecipitate of PAI-1 from the contact area preparation of dexamethasone-treated HT-1080 cells. The cells were labeled for 2 h in medium containing 50 μCi/ml [³⁵S]methionine. After extraction of cells, the labeled substrate-attached proteins were immunoprecipitated using monoclonal anti–PAI-1 (clone 1). Lane *1*: HT-1080 preparation; lane 2: the immunoprecipitate.

ing HT-1080, has been found resistant to various solubilization procedures that would be expected to solubilize non-membrane integrated proteins (28, 39, 41, 43, 57, 69). As for the light microscopic studies the subcellular distribution of u-PA differs greatly in different cell types (38, 67). Only one study at the electron microscopic level PA (apparently u-PA) immunoreactivity has been reported to be associated with the cell membranes of porcine kidney cells (55). Furthermore, in two recent studies of human monocyte-like U937 cells and peripheral blood monocytes (71, 79), u-PA was shown to bind with its noncatalytic aminoterminal domain to specific plasma membrane receptors, whereas the catalytic, carboxy-terminal domain remains exposed on the cell surface. Additionally, Fibbi et al. (21) have demonstrated the binding of a urokinase-ferritin conjugate to cell surface sites in A431 human epidermoid carinoma cells and proposed that these represented single and clustered receptor. Our data fit well with the findings above. If indeed u-PA is bound to a receptor also in HT-1080 cells, the anti–u-PA immunoreactivity at the contact sites and on the plasma membrane (Fig. 2 *A*) could be based on local clustering of such receptors.

Several different procedures have been used to isolate material designated either as cellular contact sites, cell substratum-attached material, foot pads, or focal contact membranes (5, 7, 9, 15, 37, 40, 47). These procedures include use of chelating agents, low concentrations of nonionic or zwitterionic detergents, or mechanical extraction. All these procedures to isolate the material from the growth substratum should be considered operational only, because the material obtained is expected to contain both extracellular matrix
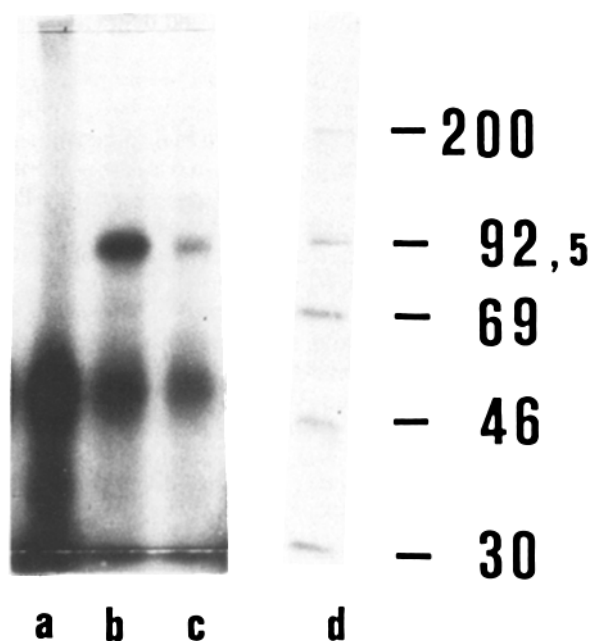

—200
— 92,5
— 69
— 46
— 30

a   b   c   d

Figure 11. Complexing of radioiodinated urokinase (¹²⁵I-u-PA, lane *a*) during incubation for 60 min at 4°C with contact area preparations of 2-HES cells (lane *b*) and with excess amounts (about 100-fold with respect to u-PA) of purified PA inhibitor (lane *c*). The samples were analyzed in 8% SDS PAGE followed by autoradiography. The positions of the M_r markers are indicated in kilodaltons (lane *d*).

components and material binding from the culture medium to the glass or plastic surface, besides the actual cellular contact site components. Saponin, which we used as a cholesterol-removing detergent, has also been used to isolate acethylcholine receptor clusters from cultured rat myotubes (8). In addition, using saponin and mechanical extraction Neyfakh et al. (47) found M_r 51,000 and 47,000 proteins in isolated focal contact membranes of mouse and chick fibroblasts, respectively. Interestingly, Bayley and Rees (7) detected a major new protein component with an M_r 50,000–55,000 in fibroblast focal adhesion preparations that remains attached to a glass substratum after cells are mechanically removed. Our results indicated that a contact area preparation obtained by saponin treatment contains PAI-1. However, the inhibitor was not confined to discrete focal contacts, nor was it found to have a matrix-type distribution, instead it was found to be deposited under the ventral surface of the cells excluding strialike areas.

Extracellular PA activity may be regulated at the level of cellular production and release of the activators themselves, and by modulators of the enzyme activity of the activators (for reviews, see references 16 and 59). Furthermore, the fact that PAs act as a part of a cascade reaction implies a more complicated set of control mecahnisms for the final proteolytic effect. Recent demonstration of proenyzmes with little or no activity (3, 17, 27, 32, 51, 67, 68, 82) suggests that mechanisms, although at present unknown, exist that control the conversion of zymogens to active enzymes in the extracellular space. Moreover, u-PA is inhibited by the abundant protease inhibitors of the human plasma, by the protease nexin, and by the recently reported more specific inhibitors (see our introductory text for references). Binding by pro-

tease nexin may initiate cellullar uptake and degradation of u-PA.

Our results demonstrating u-PA and PAI-1 in the contact area preparations add a novel dimension to the complex regulatory scenario of pericellular plasminogen activation. The protein concentration being too low in our preparations, we were not able to decide by immunoblotting, whether u-PA at the sarcoma cell contact sites is present as the one-chain inactive proenzyme. The results obtained by zymography, demonstrating a PAI-1/u-PA complex that is not formed by pro-u-PA (4), do not settle that question because pro-u-PA may have been activated during the zymography procedure. The difference in distribution between u-PA, being enriched into discrete contact sites, and PAI-1, being distributed as an extracellular carpet on the growth substratum, could conceivably permit focal u-PA-mediated proteolysis to occur.

The large amounts of the PAI on the growth substratum was a rather unexpected finding. This could be an indication of the need to secure the inhibition of PA in the pericellular area, to maintain cell surface integrity. Proteins bound in a similar way on to glass substrata include serum spreading factor (vitronectin), and the M_r 80,000 protein of serum-containing media (48). Vitronectin is an adhesive glycoprotein that promotes spreading of a variety of cell types on culture dishes (6, 24). The deposition of PAI-1 suggests that it may have functions other than enzyme inhibition. We are currently exploring this possibility.

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