Plasminogen Activator Inhibitor Type I Stabilizes Vitronectin-dependent Adhesions in HT-1080 Cells

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Abstract. Polyclonal antibodies against plasminogen activator inhibitor type-I (PAI-1) caused rapid retraction and rounding of substrate-attached HT-1080 cells. The kinetics and extent of antibody-mediated cell rounding were not dependent on either urokinase or plasmin activity. Cells adherent to vitronectin-coated substrates detached within 2 h of antibody addition. Cells adherent to fibronectin were unaffected by the antibodies. Immunoblotting of substrate-attached material indicated that HT-1080 cells deposited PAI-1 into vitronectin, but not fibronectin, dependent contacts. These data suggest that the antibody-mediated cell rounding resulted from a steric disruption of vitronectin-dependent adhesions, indicating that the binding site on vitronectin for PAI-1 is near, but does not overlap, the binding site for vitronectin receptor. The accumulation of PAI-1 into vitronectin-dependent adhesion sites correlated temporally with the preferential degradation of fibronectin from the substrate. HT-1080 cells adherent to either fibronectin or vitronectin were able to activate exogenous plasminogen to plasmin. Plasmin levels were increased 200% on cells adherent to fibronectin and 100% on cells adherent to vitronectin. In the presence of a neutralizing antibody against PAI-1, vitronectin adherent cells activated plasminogen to the same extent as fibronectin adherent cells. Plasmin levels of 200% above baseline were associated with retraction of cells from the substrate. The ability of vitronectin adherent cells to activate exogenous plasmin was completely blocked in the presence of neutralizing antibodies against urokinase. These data represent the first demonstration that vitronectin-associated PAI-1 regulates urokinase in focal contact areas.

NUMEROUS studies over the past several years have suggested a relationship between neoplastic growth and the fibrinolytic system (reviewed in Dano et al., 1985; Markus, 1988). In vitro, cell transformation is often associated with increased production of plasminogen activators, which convert the proenzyme plasminogen to plasmin. HT-1080 is a highly proteolytic cell line derived from a human fibrosarcoma (Rasheed et al., 1974). The rapid degradation of extracellular matrix by these cells can be partially blocked by inhibitors of urokinase-type plasminogen activators, suggesting a role for plasmin in the destruction of matrix (Bergman et al., 1986; Reich et al., 1988). HT-1080 cells secrete urokinase which has been localized to vinculin-rich areas of cell–substratum adhesion, suggesting that the enzyme is concentrated in focal contacts (Pollanen et al., 1987, 1988; Herbert and Baker, 1988). In culture, a number of cell types have been shown to form focal contacts on the adhesive glycoproteins, fibronectin, and vitronectin, present in serum (Singer et al., 1988). It is clear that in order for a proteolytically active cell to maintain adhesion, the urokinase and/or plasmin in contact areas must be tightly regulated.

Plasminogen activator inhibitor type-I (PAI-1)[1] is the most important specific inhibitor of plasminogen activators. In vivo, PAI-1 has been isolated from serum, platelets, and placenta (Kruithof, 1988). In vitro, PAI-1 is secreted by endothelial cells (Loskutoff and Edgington, 1977), fibroblasts (Laiho et al., 1986), and HT-1080 cells (Andreasen et al., 1986). PAI-1 secreted by endothelial cells retains activity through association with the extracellular matrix (Mimuro et al., 1987) but becomes inactive in solution (Levin, 1986). Unlike endothelial cells, HT-1080 cells do not assemble significant amounts of extracellular matrix (Oliver et al., 1983; McKeown-Longo and Etzler, 1987); however, they do deposit PAI-1 onto the substratum (Pollanen et al., 1987). Treating HT-1080 cells with dexamethasone, which increases PAI-1 levels in the substratum (Pollanen et al., 1987), also alters cell morphology. The cells appear well spread and more adherent to the substratum (Oliver et al., 1983; McKeown-Longo and Etzler, 1987). The studies presented here were undertaken to investigate the role of

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1. *Abbreviations used in this paper:* PAI-1, plasminogen activator inhibitor type-I; SAM, substrate-attached material.

PAI-1 in maintaining HT-1080 cell adherence. The results indicate that immediately after plating, the cells degrade fibronectin and deposit PAI-1 onto vitronectin. This PAI-1 protects vitronectin from plasmin degradation and the cells remain adherent to vitronectin–PAI-1 complexes.

Materials and Methods

Cell Cultures

Human fibrosarcoma cells, HT-1080, were a gift from Dr. Noellyn Oliver (Tufts University), and stock cultures were grown in plastic tissue culture dishes (55 cm²; Corning Glass Works, Corning, NY) in MEM (Gibco Laboratories, Grand Island, NY) supplemented with 10% FBS (HyClone Laboratories, Inc., Logan, UT), 100 IU/ml penicillin, and 100 μg/ml streptomycin (pen/strep; Flow Laboratories, Inc., McLean, VA). Experiments were performed in either 12-well Linbro tissue culture plates (4.5 cm²; Flow Laboratories, Inc.) or 24-well tissue culture plates (1.9 cm²; Corning Glass Works).

For those experiments requiring the absence of plasminogen, FBS (100 ml) was depleted of plasminogen by three passes over a Lysine-Sepharose 4B (Sigma Chemical Co., St. Louis, MO) column equilibrated with 0.3 M potassium phosphate (pH 7.4) containing 1 mM EDTA (Deutsch and Mertz, 1970). HT-1080 cells were plated in MEM with 10% FBS depleted of plasminogen and grown to confluence. To remove cell-bound plasmin, cells were washed in PBS containing 1 mM tranexamic acid. Human plasminogen was purified from serum by affinity chromatography on lysine-Sepharose as described above. Plasminogen was eluted from the column with 0.1 M potassium phosphate (pH 7.4) containing 0.2 M E-amino caproic acid. Plasminogen was dialyzed against PBS and sterile filtered before use. Plasminogen did not contain any active plasmin when tested in the Spectrozyme PL assay.

Purified human fibronectin and human vitronectin were the generous gift of Dr. Deane F. Mosher (Departments of Medicine and Physiological Chemistry, University of Wisconsin Medical School, Madison, WI). Purified proteins were used to coat 24-well tissue culture plates. Proteins were diluted in PBS and incubated in wells for 1 h. The wells were then washed three times with PBS. Wells received a second coat of PBS containing 1% BSA and were then washed three times with PBS. HT-1080 cells to be plated onto these wells were trypsinized from stock culture plates, washed, and resuspended at 5 × 10⁵ cells/ml in MEM supplemented with 1% BSA. 1 ml of the cell suspension was plated into each well.

Substratum-attached material (SAM) was isolated by a modification of the method of Rosen and Culp (1977). Briefly, cell layers were rinsed three times with 4°C MEM and incubated in 10 mM Hepes, 1 mM EGTA (pH 7.4) at 37°C for 30 min. The cells were removed with gentle agitation and the wells were washed three times with PBS. The SAM left on the bottom of each well was solubilized with reducing gel sample buffer.

The following reagents were used in inhibition studies: Aprotinin (#517; American Diagnostica, New York, NY); Diisopropyl-fluorophosphate (DFP), p-nitrophenyl-guanidino-benzoate (NPGB), and PMSF (Sigma Chemical Co.). The following inhibitory mAbs were also used: an anticatalytic antibody to human urokinase (anti-uPA, #394; American Diagnostica) and to human tissue plasminogen activator (anti-tPA, #374B; American Diagnostica); and an antibody to the active site of PAI-1 (#379; American Diagnostica). Specific amounts are provided in the figure legends.

Purification of PAI-1 and Isolation of Antibodies

HT-1080 cells were seeded into plastic roller bottles (850 cm²; Corning Glass Works) and allowed to grow to confluency. The medium was removed and the incubation continued for 24 h in medium containing 10⁻⁷ M dexamethasone (Sigma Chemical Co.). At this time, the medium was removed and MEM containing 10⁻⁷ M dexamethasone without serum was added and the cells incubated for another 24 h. This conditioned medium was collected and PMSF was added to a final concentration of 1 mM. Conditioned medium was filtered through a 0.22-μm nylon filter (Corning Glass Works) and concentrated ~40-fold by ultrafiltration on a YM30 membrane (mol/wt cut-off 30,000; Amicon Corp., Danvers, MA). The concentrate was dialyzed against 10 mM sodium phosphate (pH 7.4) and PAI-1 was isolated by chromatography on heparin-Sepharose CL-6B (Pharmacia, Uppsala, Sweden). Bound proteins were eluted with 1 M NaCl, dialyzed against PBS, and passed over gelatin-Sepharose 4B (Pharmacia) to remove fibronectin.

The pass through was collected, lyophilized, resuspended in reducing gel sample buffer, and further purified by preparative SDS gel electrophoresis. When analyzed by Western immunoblotting, purified PAI-1 contained no urokinase. Since this assay can detect 100 ng of urokinase, the PAI-1 preparation contained <1% contamination with urokinase (data not shown). This preparation of PAI-1 was used as an immunogen in rabbits for the production of antibodies. Specificity and titer of antiserum was determined by Western immunoblotting and ELISA.

Immunoglobulins (IgGs) were isolated from antiserum using protein A-Sepharose. Immune or nonimmune rabbit serum was passed over a protein A-Sepharose CL-4B (Pharmacia) column equilibrated with 100 mM sodium phosphate (pH 7.0). Bound IgG was eluted with 1 M acetic acid, 100 mM glycine (pH 3.0), and the eluate was immediately neutralized to pH 7.4 with 1 M Tris (pH 10.0). The eluate from the immune serum was designated anti-PAI-1 IgG and that from the nonimmune serum was designated normal IgG. Both eluates were then dialyzed against PBS and the concentration determined by the OD at 280 nm using the absorbance at 280 nm of a 1% solution of rabbit IgG (i.e., 13.5). The samples were filter sterilized and stored at −80°C for future use.

For the preparation of Fab fragments, anti-PAI-1 or normal IgG was dialyzed against 20 mM sodium phosphate, 10 mM EDTA (pH 7.0), and concentrated to ~20 mg/ml in dialysis tubing using dry polyethylene glycol (mol wt 15,000; Aldrich Chemical Co., Milwaukee, WI). IgG (10 mg/ml) was digested with 1.75 U of immobilized papain (cross-linked 6% beaded agarose, 7 U/ml; Pierce Chemical Co., Rockford, IL) in the presence of cysteine. The digestion solution was incubated overnight on a shaker platform at high speed at 37°C. The resulting solution was clarified by centrifugation. The supernatant was applied to a protein A-Sepharose column under the same conditions as described above. The pass through (containing the Fab fragments) was collected and dialyzed against PBS and the concentration determined by the OD at 280 nm using the absorbance at 280 nm of a 1% solution of rabbit Fab fragments, i.e., 15.0). The samples were filter sterilized and stored at −80°C for future use.

Iodination of Proteins

Fibronectin was iodinated as previously described (McKeown-Longo and Mosher, 1983). Vitronectin was iodinated in a similar fashion except the chloramine-T reaction was stopped with an equimolar concentration of sodium metabisulfite (Fisher Scientific, Fair Lawn, NJ). Free iodine was removed by chromatography on G-25 (Sigma Chemical Co.) in PBS. Iodinated proteins were stabilized with 1% BSA, 0.1 mM PMSF, and then dialyzed against PBS. Samples were frozen at −80°C for future use. Integrity of the labeled protein was assessed by gel electrophoresis and autoradiography. Specific activity of each protein was adjusted to 1 mCi/mg before use.

Plasmin Assay

Plasmin activity was assayed using the chromogenic substrate, Spectrozyme PL (#251; American Diagnostica). To measure cell-associated plasmin, cells in 24-well plates were washed three times with phenol red-free MEM. Spectrozyme PL, 0.25 mM in MEM (250 μl), was added to each well and incubated for 90 min at 37°C. Samples (150 μl) were removed from each well and added to 50 μl 50% acetic acid in 96-well ELISA plates (Corning Glass Works). The OD was measured at 410 nm using a Dynatech MR600 multiplate reader. Wells that did not contain cells were used as controls. To measure total plasmin activity, the conditioned medium was not removed from the well before the assay.

Results

Purification of PAI-1

Previous studies have shown that dexamethasone induces a 10-fold increase in the synthesis and secretion of PAI-1 by HT-1080 cells (Andreasen et al., 1987). This increase in PAI-1 synthesis is accompanied by accumulation of a fibronectin containing extracellular matrix and by changes in cell morphology (Oliver et al., 1983; McKeown-Longo and Etzler, 1987). The cells appear more well spread and better attached to the substrate. It is not clear from these earlier
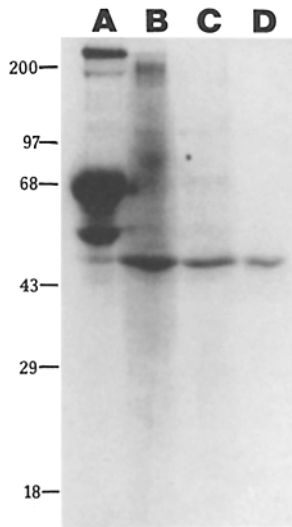
Figure 1. PAI-1 purification. (lane *A*) TCA precipitation of serum-free conditioned medium from HT-1080 cell monolayers treated with dexamethasone. (lane *B*) Heparin-Sepharose bound fraction of pooled conditioned medium. (lane *C*) Gelatin-Sepharose pass through of heparin-bound fraction. (lane *D*) Protein purified by preparative SDS-PAGE (*arrowhead*). This protein reacted with mAbs to PAI-1 in an immunoblot (data not shown). Position of molecular weight markers is shown on the left.

studies whether the morphological changes preceed or follow the accumulation of matrix. The purpose of the current study was to probe the role of PAI-1 in the maintenance of HT-1080 cell adhesion.

Human PAI-1 was purified from the conditioned medium of dexamethasone-treated HT-1080 cells. Fig. 1, lane *A*, shows that PAI-1 (designated by the *arrowhead*) can be detected in the conditioned medium of dexamethasone-induced HT-1080 cells. When the conditioned medium is passed over a heparin affinity column, the preparation is enriched for both PAI-1 and fibronectin (Fig. 1, lane *B*). Fibronectin is removed from the preparation by gelatin affinity chromatography (Fig. 1, lane *C*) and the PAI-1 is further purified by preparative SDS-PAGE (Fig. 1, lane *D*). This preparation was used as immunogen to produce polyclonal anti-PAI-1-antibodies in rabbits.

Anti-PAI-1 IgG was purified as described in Materials and Methods and the specificity of the antibodies was determined by Western immunoblots (Fig. 2). When tested against the proteins recovered from HT-1080 conditioned medium, the antibodies reacted with both a 48,000 and a 44,000 mol wt protein (Fig. 2, lanes *A* and *a*). These two proteins were also recognized using an mAb against human PAI-1 (data not shown), indicating that they represent intact PAI-1 and the inactive PAI-1 cleavage product (Nielsen et al., 1986). No reactivity of the antibodies could be detected against fibronectin, vitronectin, urokinase, or any other proteins present in the conditioned medium (Fig. 2).

Anti-PAI-1 Antibodies Cause HT-1080 Cells to Detach from the Substratum

To probe the role of PAI-1 in maintaining HT-1080 cell adhesion, polyclonal antibodies to PAI-1 were added to HT-1080 cell monolayers. HT-1080 cells grew as substrate-attached cells when cultured on tissue culture plastic; however, like most transformed cells, they did not exhibit contact inhibition. Once the cells covered the bottom of the dish, they continued to grow in clusters on the surface of the monolayer (Fig. 3 *A*). IgGs prepared from both normal rabbit serum and anti-PAI-1 antiserum were incubated with confluent cultures of HT-1080 cells and compared for their effects on cell mor-

phology. Addition of anti-PAI-1 IgG resulted in disruption of cell–substratum adhesion. Cells retracted from one another and partially detached from the substratum, resulting in a rounded appearance and exposure of underlying plastic (Fig. 3 *B*). Antibody concentrations as low as 12.5 μg/ml disrupted cell adhesion as effectively as 100 μg/ml (data not shown). This response could be reproduced with the Fab portion of the antibody (Fig. 3 *D*). Time course experiments showed that cell retraction was evident 3 h after the addition of anti-PAI-1 IgG (data not shown). By 5 h all cells were completely rounded but still attached to the substrate (Fig. 3). With longer incubation times (18–24 h), cells eventually detached from the substrate and floated in clusters in the medium (data not shown). This effect was specific for anti-PAI-1 IgG as polyclonal antibodies against urokinase did not disrupt adhesion (data not shown). The antibody was not toxic to the cells since detached cells excluded vital dyes and replated normally after removal of the antibody (data not shown). An mAb against the active site of PAI-1 was also not effective in mediating cell detachment in similar studies, however, the addition of a secondary antibody directed against the monoclonal did result in disruption of adhesion (data not shown). These data suggest that the antibody-
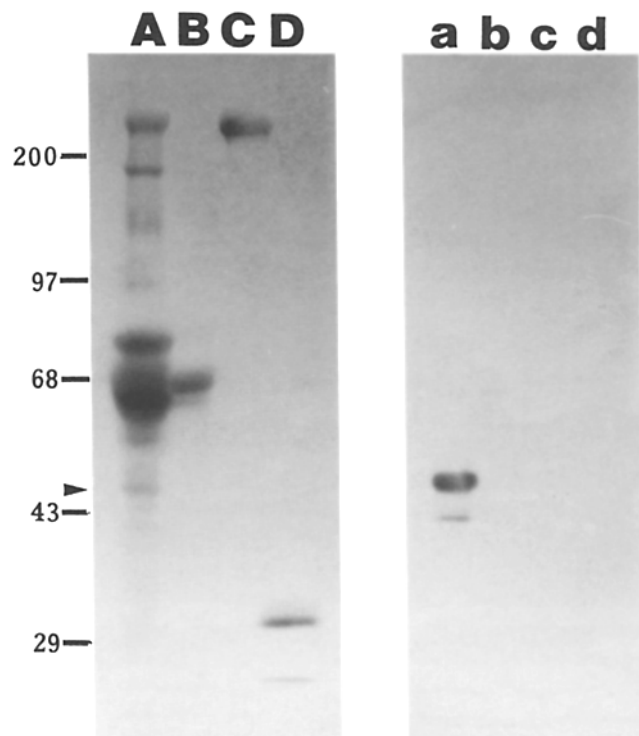
Figure 2. Immunoblot analysis of anti-PAI-1 antibody specificity. (lanes *A–D*) Amido Black stain of nitrocellulose containing electroblotted proteins. (lanes *a–d*) The nitrocellulose was incubated with the anti-PAI-1 antibodies (25 μg/ml) and developed using 4-chloro-1-naphthol and HRP-conjugated goat anti–rabbit IgG. (lanes *A* and *a*) TCA precipitation of HT-1080 cell–conditioned medium. (lanes *B* and *b*) Purified human vitronectin (10 μg). (lanes *C* and *c*) Purified human fibronectin (10 μg). (lanes *D* and *d*) Purified active human urokinase (10 μg). Intact PAI-1 is indicated by the arrowhead. Position of molecular weight markers is shown on the left.
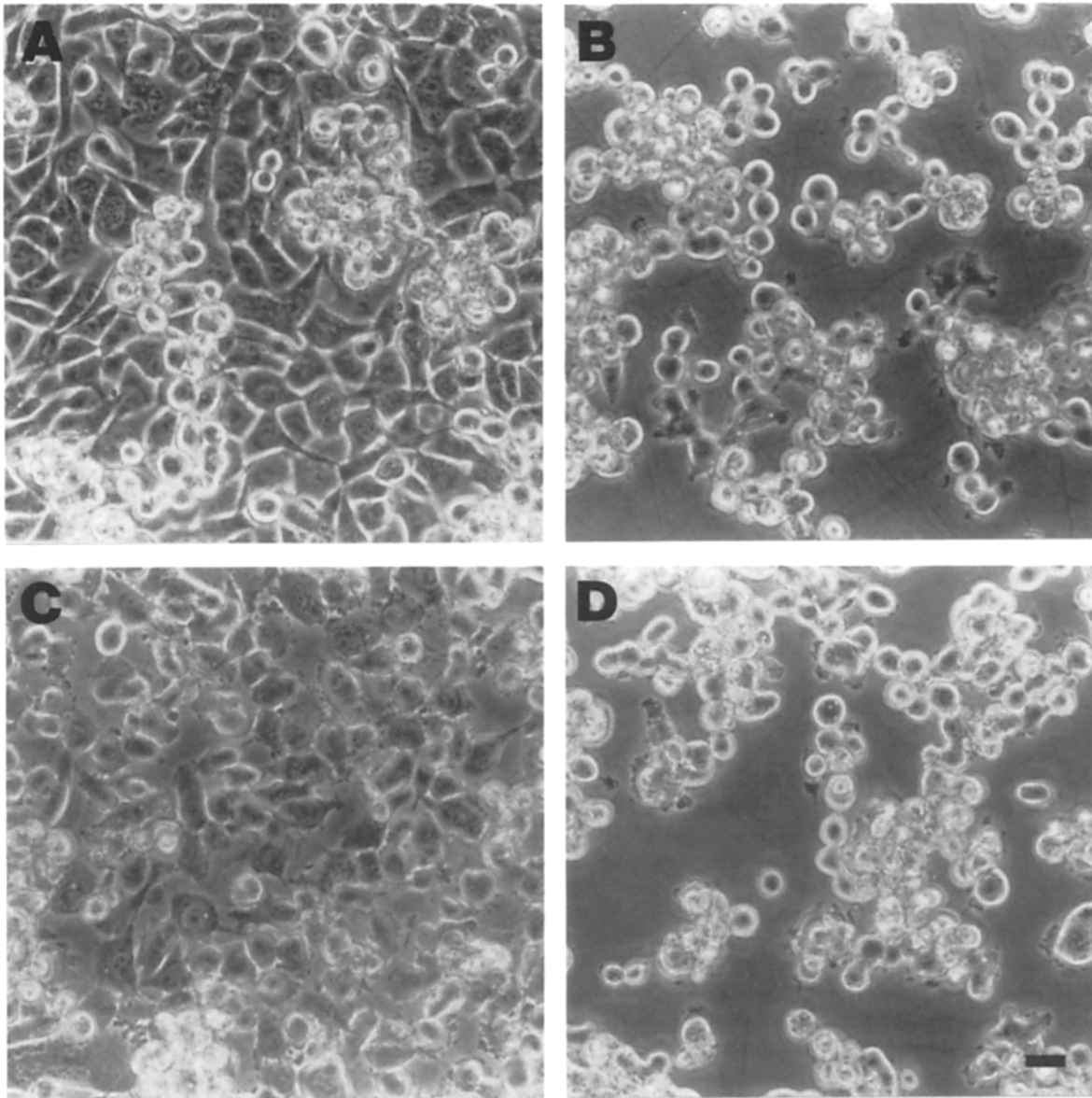
Figure 3. Effect of anti-PAI-1 on HT-1080 cells. Confluent cultures of cells were incubated with normal or anti-PAI-1 IgG or Fab fragments for 5 h. Antibodies were added to cells in either complete medium (*A* and *B*) or MEM (*C* and *D*). (*A*) Normal IgG 100 μg/ml; (*B*) anti-PAI-1 100 μg/ml; (*C*) normal Fab 50 μg/ml; (*D*) anti-PAI-1 Fab 50 μg/ml. Bar, 25 μm.

mediated rounding was not due to inhibition of PAI-1 activity.

Additional experiments were done to address whether anti-PAI-1 IgG could be interferring with PAI-1-dependent inhibition of cell-associated urokinase. Experiments were done to address directly the possible role of urokinase and plasmin in the cell rounding phenomenon. To eliminate urokinase-dependent plasminogen activation, an mAb against the active site of urokinase was used. Potential residual plasmin activity was inhibited with aprotinin. In combination, these modulators had no effect on the kinetics or degree of anti-PAI-1-mediated cell rounding (Fig. 4 *C*). In other experiments, cells were washed with tranexamic acid to remove bound plasmin (Stephens et al., 1989) and cultured for one passage in plasminogen-depleted serum. The serine protease inhibitor p-nitrophenyl-guanidino-benzoate (100 μM)

was added in combination with aprotinin (200 KIU/ml) to these cultures. No effect on the anti-PAI-1-mediated rounding was observed (data not shown). The addition of the serine protease inhibitors, PMSF (1 mM), or diisopropylfluorophosphate (250 μM) also had no effect on antibody-mediated cell rounding (data not shown). Results shown in Figs. 3 and 4 suggest that the rounding of the cells in response to anti-PAI-1 is not due to enhanced proteolysis but results from a steric disruption of the interaction of the cells with the substrate.

Anti-PAI-1 Disrupts HT-1080 Cell Adhesion to Vitronectin and Not to Fibronectin

HT-1080 cells do not assemble detectable amounts of extracellular matrix (Oliver et al., 1983; McKeown-Longo and
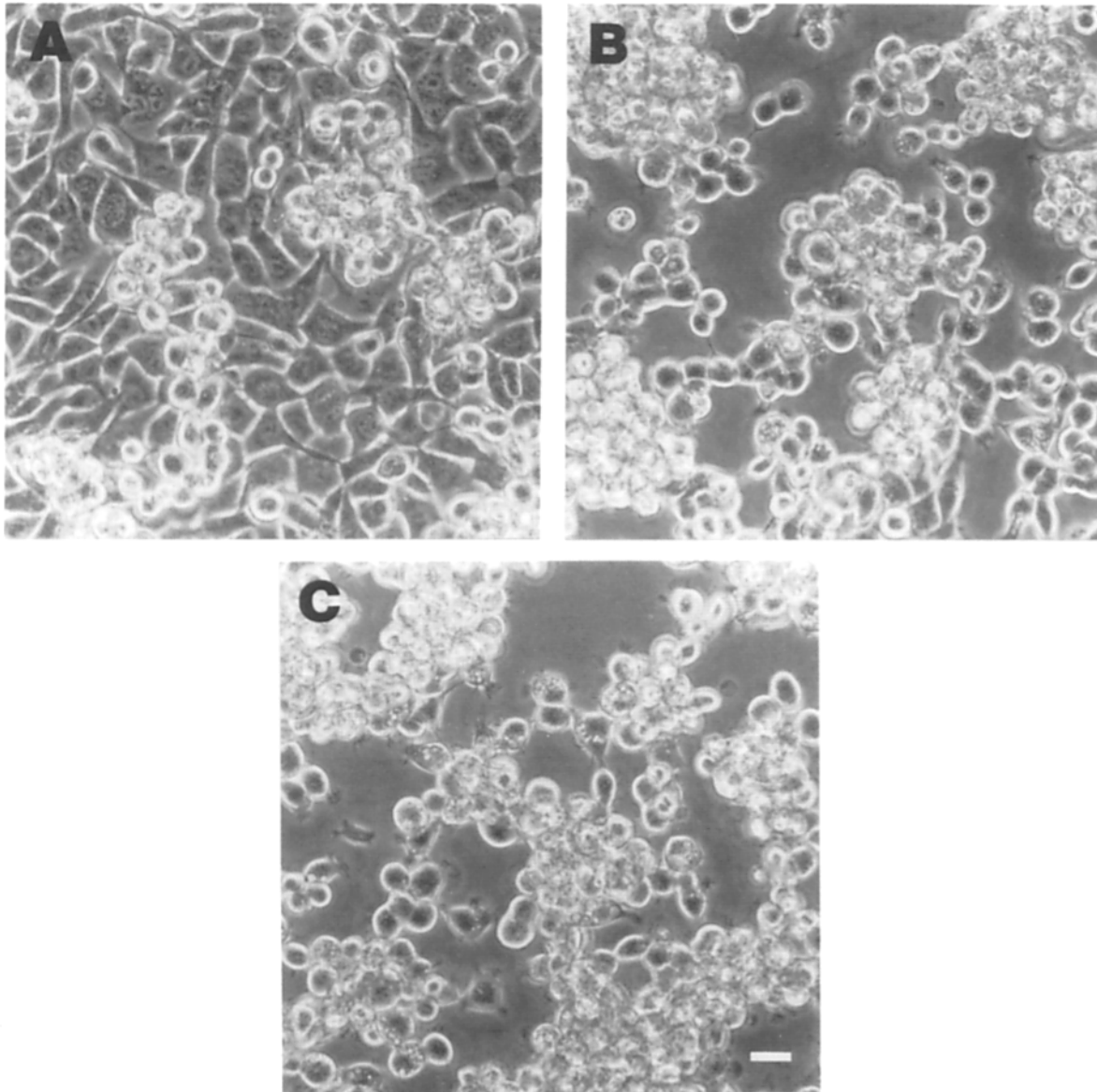
Figure 4. Effect of plasmin inhibition on anti-PAI-1–mediated detachment. Monolayers of HT-1080 cells were grown to confluence in complete medium. The cells shown in *C* received 100 μg/ml of anticatalytic antiurokinase antibody and aprotinin (200 KIU/ml) to inhibit urokinase and plasmin activity. Normal or anti-PAI-1 IgG (100 μg/ml) was added to the cultures and photographs were taken 5 h later. (*A*) Normal IgG; (*B* and *C*) anti-PAI-1 IgG. Bar, 25 μm.

Etzler, 1987). Therefore, in culture, the cells are dependent on the serum proteins fibronectin and/or vitronectin for adhesion (Hayman et al., 1982). Experiments were designed to test whether the anti-PAI-1 IgG disrupted adhesion of HT-1080 cells to either vitronectin or fibronectin. Cells were plated in wells coated with either vitronectin or fibronectin. After a 5-h incubation, cells on both substrates appeared equally adherent and well spread. At this time, anti-PAI-1 IgG was added to the wells. Cells plated in wells coated with vitronectin began to retract within 2 h. By 5 h the cells were completely rounded but still attached to the dish (Fig. 5 *B*). Cells plated on fibronectin were not affected by the antibody (Fig. 5 *C*). Cells plated on a mixture of vitronectin and fibronectin rounded as rapidly as cells plated on vitronectin alone (Fig. 5 *D*). These data suggest that HT-1080 cells

maintain adhesion by interacting with vitronectin–PAI-1 complexes.

HT-1080 Cells Deposit PAI-1 on Vitronectin but Not on Fibronectin-coated Substrates

To determine whether HT-1080 cells deposit endogenous PAI-1 preferentially on vitronectin- or fibronectin-containing adhesion sites, SAM from cells plated into wells coated with either vitronectin or fibronectin was analyzed by Western immunoblotting. Fig. 6 indicates that SAM prepared from vitronectin-coated wells was rich in PAI-1 (lane 2). PAI-1 was also detected in SAM prepared from wells coated with both fibronectin and vitronectin (lane 3). However, PAI-1 was not detected in SAM prepared from fibronectin-coated wells
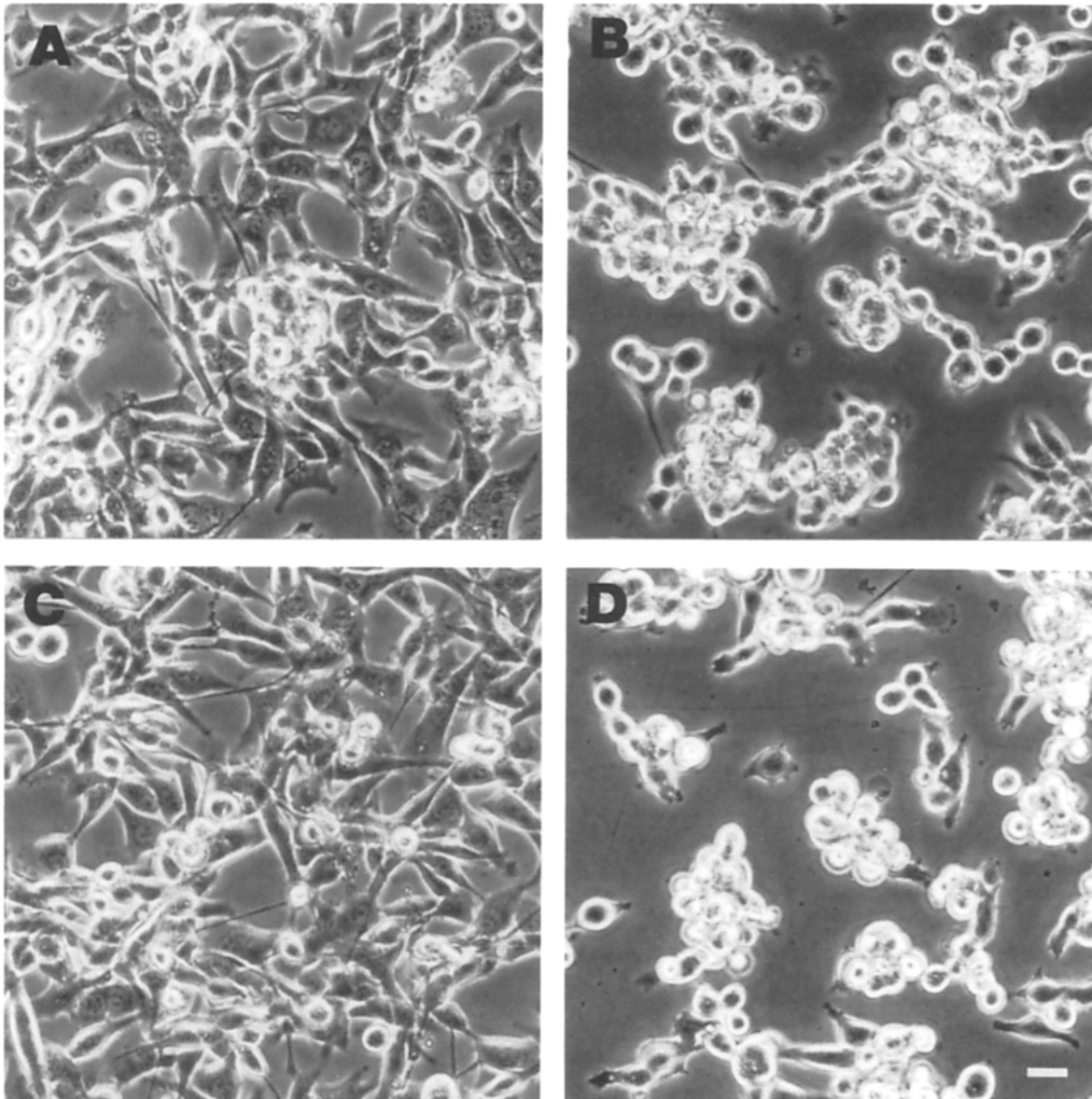
Figure 5. Effect of anti-PAI-1 on HT-1080 cells plated onto fibronectin-or vitronectin-coated wells. Wells were coated with 5 μg/ml of vitronectin (*A* and *B*) or fibronectin (*C*) either individually or in combination. (*D*). Cells (5 × 10⁵) in MEM containing 1% BSA were plated into these wells and allowed to incubate for 5 h. The medium was then removed and new medium containing 100 μg/ml of normal or anti-PAI-1 IgG was added to the wells. Photographs were taken 5 h after addition of the antibodies. (*A*) Normal IgG; (*B–D*) anti-PAI-1 IgG. Bar, 25 μm.

(lane *1*). These findings indicate that HT-1080 cells deposit PAI-1 into vitronectin but not fibronectin-containing adhesion sites.

PAI-1 Deposited on Vitronectin by HT-1080 Cells Is Active

To determine whether PAI-1 was inhibiting cell-associated urokinase, HT-1080 cell monolayers were tested for the ability to activate exogenous plasminogen. Cells were seeded into wells coated with purified human vitronectin or fibronectin and allowed to adhere for 7 h. In some wells, an mAb directed against the active site of PAI-1 was used to block PAI-1 activity. Unlike the polyclonal antibodies (Fig. 5 *B*), monoclonal anti-PAI-1 did not disrupt cell adherence to vi-

tronectin (Fig. 7 *A*). Monolayers containing active and inactive PAI-1 were then compared for their susceptibility to plasminogen-dependent retraction of cells from the substrate. 12 h after the addition of either 5 or 15 μg of plasminogen, cell layers containing anti-PAI-1 antibody showed evidence of active plasmin (Fig. 7, *C* and *D*). Cells have retracted from each other and from the substrate. Plasminogen had no effect on cell layers that did not receive anti-PAI-1 antibody (Fig. 7 *B*).

Similar results were obtained when PAI-1 activity was modulated by the substrate rather than by the mAb. Cell layers plated on fibronectin-coated wells were disrupted after the addition of plasminogen to the medium (Fig. 7 *F*), while cells adherent to vitronectin were unaffected (Fig. 7 *B*). To
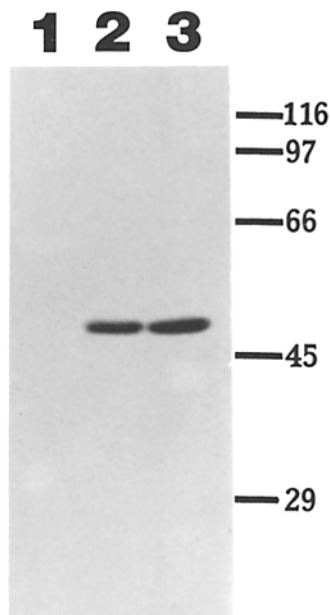
Figure 6. Western immunoblot of substrate-attached material. 24-well tissue culture plates were coated with 5 μg/ml of fibronectin or vitronectin either individually or in combination. 5 × 10⁵ cells in MEM containing 1% BSA were then plated into these wells and allowed to adhere for 7 h. SAMs were made as described in Materials and Methods. Substrate-attached material was solubilized in reducing gel sample buffer and electrophoresed into a polyacrylamide gel. Proteins were transferred to nitrocellulose and PAI-1 was detected using mAb against human PAI-1. (lane *1*) Fibronectin-coated wells. (lane *2*) Vitronectin-coated wells. (lane *3*) Fibronectin- and vitronectin-coated wells. Position of molecular weight markers is shown on the right.

verify that the changes in cell morphology were due to increased plasmin activity, the cell-associated plasmin was measured using the chromogenic substrate, spectrozyme-PL. Fig. 8 *A* shows that after the addition of plasminogen, a twofold increase in plasmin activity could be detected on cells plated onto fibronectin, whereas plasmin levels were increased only onefold on cells plated onto vitronectin. After the inactivation of PAI-1, vitronectin adherent cells could activate plasminogen to the same extent as fibronectin adherent cells. Fig. 8 *B* shows that the activation of plasminogen by cells adherent to vitronectin can be modulated by neutralizing antibodies against both PAI-1 and urokinase. These data suggest that PAI-1 bound to substratum vitronectin is active and can inhibit surface-bound urokinase on HT-1080 cells, preventing plasmin generation in the vicinity of the vitronectin molecule.

Fibronectin Is Degraded at a Faster Rate than Vitronectin by HT-1080 Cells

To test whether HT-1080 cells preferentially degrade substratum fibronectin, cells were plated into wells coated with either ¹²⁵I-fibronectin or ¹²⁵I-vitronectin. Conditioned medium from the wells was then analyzed at various times for release and fragmentation of labeled proteins. Fig. 9 shows the cell-mediated release of radioactivity from the substrate. After an initial 15-min lag period, radioactivity began to accumulate in the medium. Over a 9-h time course, the rate of fibronectin release from the substrate was threefold greater than the rate of vitronectin release. When analyzed by SDS gel electrophoresis and autoradiography, both conditioned media showed evidence of protein degradation (data not shown). Thus, these cells degrade both adhesion molecules, but the degradation of fibronectin is much more rapid.

### Acquisition of Sensitivity to Anti-PAI-1 by HT-1080 Cells

To determine how soon after plating the cells become sensitive to the adhesion disrupting activity of the polyclonal anti-PAI-1, anti-PAI-1 IgG was added at various times after plating and the cells observed 3.5 h later. The addition of anti-PAI-1 IgG to the medium at the time of plating had no effect on either cell attachment or spreading when the cells were observed 3.5 h later (Fig. 10 *B*). However, if the cells were allowed to attach and spread for 1 h before the addition of antibody, retraction of cells from the substrate could be seen within the 3.5-h time frame (Fig. 10 *D*). These results indicate that although the antibody had no effect during the initial stages of cell adhesion, the cells soon became sensitive to the antibody. The 1-h delay before the cells acquire sensitivity to the anti-PAI-1 IgG no doubt reflects, in part, the time required for sufficient PAI-1 deposition. In addition, the antibody-mediated disruption of adhesion may require the dissolution of fibronectin-dependent contact points.

To determine whether the degradation of fibronectin containing contacts was a prerequisite to anti-PAI-1 IgG–mediated detachment, serine protease inhibitors were added to the cultures at the time of plating. If the proteolytic removal of fibronectin-dependent contacts facilitated the antibody-mediated detachment, the addition of protease inhibitors should extend the 1-h lag period during which the cells are insensitive to the anti-PAI-1 IgG. Cells plated without inhibitors round up within 4 h in the presence of anti-PAI-1 IgG (Fig. 11 *B*). The antibody had no effect on the cells which were plated with inhibitors (Fig. 11 *D*). However, by 8 h, these cells were as rounded as control cultures treated with anti-PAI-1 (data not shown). Although the protease inhibitors could extend the lag time during which the cells became responsive to the antibody, antibody-mediated detachment was not prevented.

We propose that during initial plating in serum the cells attach and spread on both vitronectin and fibronectin, and that anti-PAI-1 IgG has no effect on this process. As the cells secrete and deposit PAI-1 into vitronectin containing adhesions, local urokinase activity is inhibited and vitronectin is spared from plasmin degradation. Fibronectin-dependent adhesions are preferentially degraded. Eventually, the cells become primarily dependent on vitronectin-PAI-1 complexes for adhesion. It is at this time that the cell adhesion can be disrupted with anti-PAI-1 IgG.

Discussion

Previous studies have indicated that the changes in cell morphology which accompany glucocorticoid treatment of HT-1080 cells (Oliver et al., 1983; McKeown-Longo and Etzler, 1987) are correlated with an increase in PAI-1 production (Andreasen et al., 1987; Pollanen et al., 1987). To probe the function of PAI-1 in HT-1080 cell layers, polyclonal antibodies against PAI-1 were prepared. When added to confluent cultures of HT-1080 cells, antibodies caused rapid retraction and subsequent detachment of cells from the substrate. This effect was specific for anti-PAI-1 antibodies as IgG from normal serum and antiurokinase antibodies did not cause retraction. The disruption of cell adhesion by the anti-PAI-1 antibodies was not dependent on the activation of urokinase or
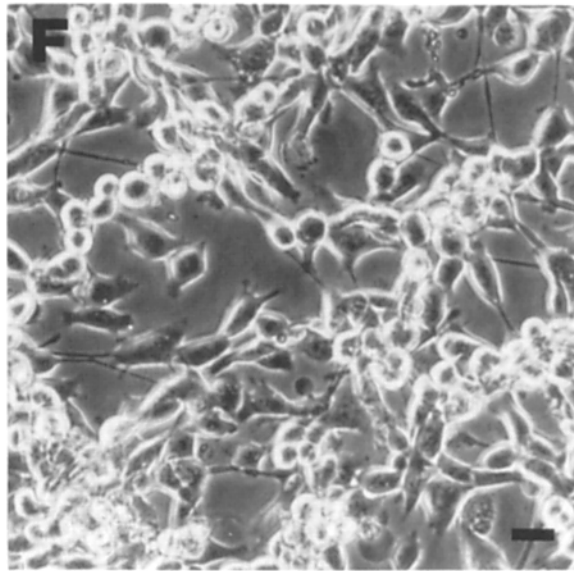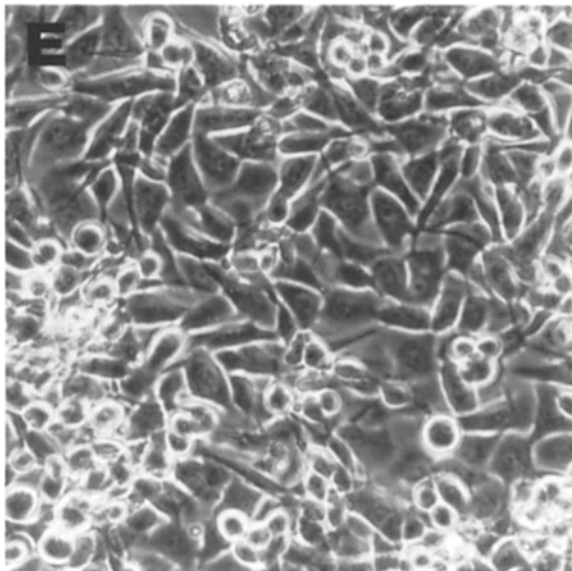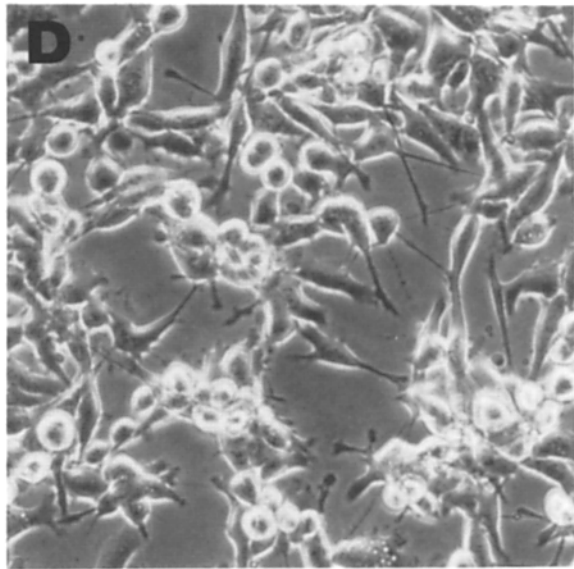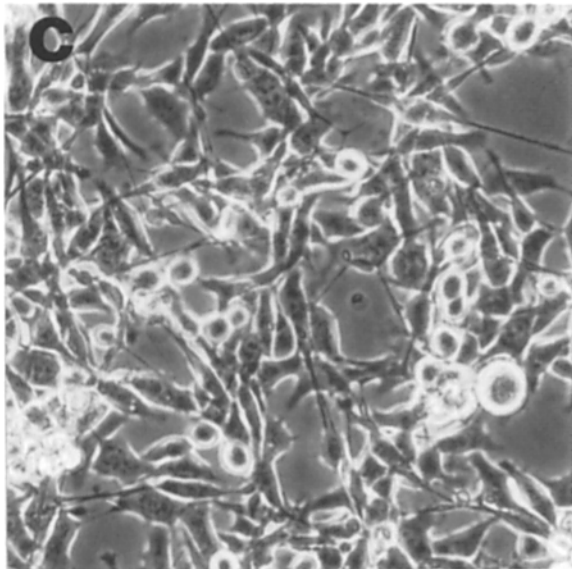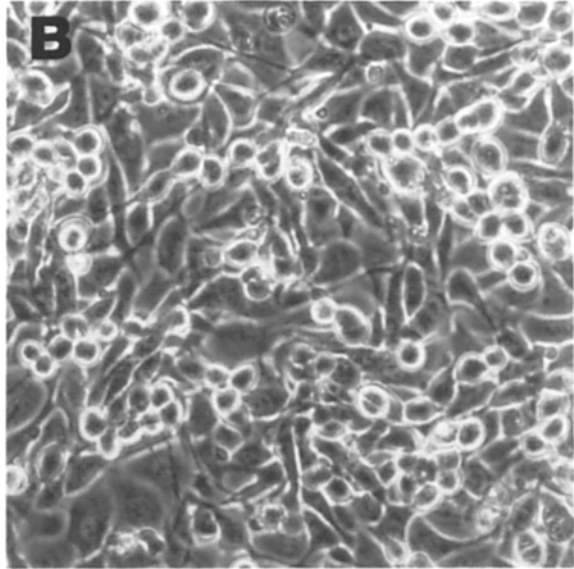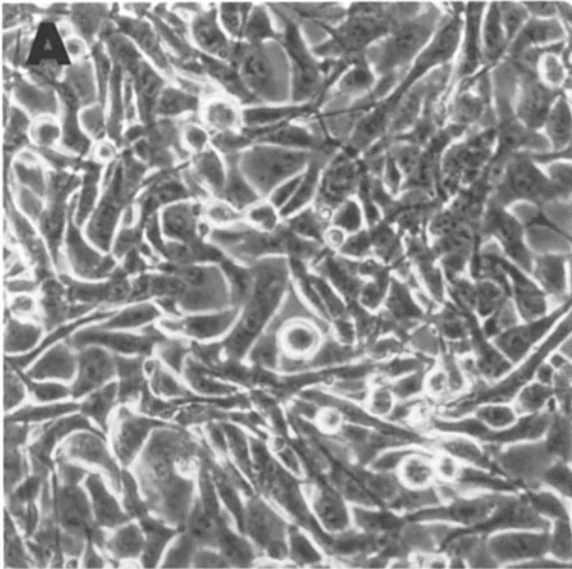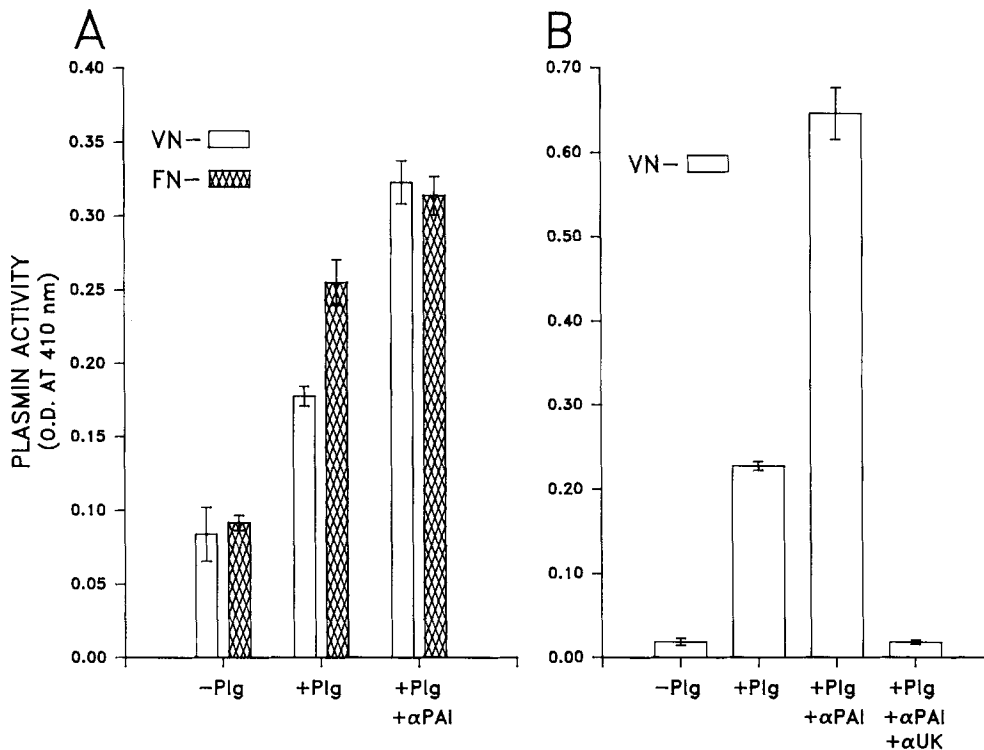
Figure 8. (*A*) Cell-associated plasmin activity. Parallel cultures to those shown in Fig. 7 were rinsed three times and incubated with Spectrozyme PL in MEM containing 0.1% albumin for 90 min at 37°C. Plasmin activity was determined by reading the OD at 410 nm. The open bars represent cells plated onto vitronectin. The hatched bars represent cells plated onto fibronectin. (*B*) Total plasmin activity. Cells were plated as described in Fig. 7 except that one set of wells was incubated with both an mAb against the active site of PAI-1 and an mAb against the active site of urokinase (10 μg/ml). After the 12-h incubation with exogenous plasminogen, Spectrozyme PL was added to the medium and the OD at 410 was measured after 25 min. The data were plotted as the mean ± SEM of four determinations.

plasminogen. Inactivation of urokinase, plasminogen depletion and serine protease inhibitors had no effect on the ability of antibodies to disrupt adhesion of confluent monolayers. The ability of the polyclonal antibodies to inhibit the active site of PAI-1 was not tested, but an mAb that does inhibit PAI-1's ability to block urokinase did not elicit cell detachment (Fig. 3), suggesting that the adhesion disrupting ability of the anti-PAI-1 antibodies did not result from disinhibition of urokinase. However, the mAb could elicit cell detachment if a second antibody, directed against the monoclonal antibody, was added to the cell layer (data not shown). In fact, the adhesion disrupting activity of the monoclonal was enhanced when experiments were performed on cells grown in plasminogen-depleted serum. These data suggest that the cell detachment resulted from a steric disruption of adhesion and not from the active degradation of adhesion proteins or receptors.

In vitro, cell adherence is thought to be initiated by the interaction of RGD-dependent receptors for the serum proteins fibronectin and vitronectin (Ruoslahti, 1988). The addition of RGD-containing peptides also caused rapid rounding and detachment of HT-1080 cells (data not shown), indicating that the cells were not adhering directly to PAI-1. Recently, purified PAI-1 has been shown to bind to both soluble and immobilized vitronectin (Declerck et al., 1988; Mimuro and Loskutoff, 1989; Salonen et al., 1989). Results presented here provide the first in situ evidence that cells specifically deposit PAI-1 on vitronectin. The sensitivity of HT-1080 adhesion to Fab fragments prepared against PAI-1 suggests that the binding site on vitronectin for PAI-1 is close to, but does not overlap, the binding site for the vitronectin receptor. This is in agreement with previous studies showing that PAI-1-vitronectin complexes support cell adhesion (Salonen et al., 1989). Consistent with these observations, subcellular localization of newly synthesized PAI-1 was dependent on vitronectin (Fig. 6). Cells plated onto fibronectin-coated substrates did not accumulate detectable quantities of PAI-1 on the substrate. Whether the absence of PAI-1 from the fibronectin substratum was due to a redistribution of secreted PAI-1 or a decreased rate of PAI-1 synthesis is not known. It is possible that the synthesis of PAI-1 may be regulated by interaction of the vitronectin receptor with vitronectin. Antibodies against the fibronectin receptor have been shown to trigger an increase in the synthesis of collagenase and stromelysin by cultured fibroblasts (Werb et al., 1989). However, preliminary data (Ciambrone and McKeown-Longo, unpublished observations) indicate that vitronectin determines the localization and not the synthesis rate of PAI-1.

The PAI-1 bound to the vitronectin was active. Inactivation of cellular PAI-1 either by using an inhibitory monoclonal (Fig. 7, *C* and *D*) or by removing vitronectin from the substrate (Fig. 7 *F*), resulted in increased plasmin activity and in subsequent disruption of cell adhesion by a plasminogen-dependent mechanism. The increase in plasmin activity after

Figure 7. Effect of PAI-1 inactivation on plasminogen-dependent cell detachment. HT-1080 cells, (5 × 10⁵) in MEM containing 0.1% BSA, were plated into vitronectin (*A–D*) or fibronectin (*E* and *F*)-coated wells and allowed to adhere for 7 h. All wells received mAb against tPA (25 μg/ml). mAb (50 μg/ml) directed against the active site of PAI-1 was added to three wells (*A*, *C*, and *D*). 1 h later, 5 μg/ml (*B*, *C*, and *F*) or 15 μg/ml (*D*) of human plasminogen was added to wells. After an additional 12 h of incubation, cells were photographed and plasmin assays (see Fig. 8) were done. Bar, 25 μm.
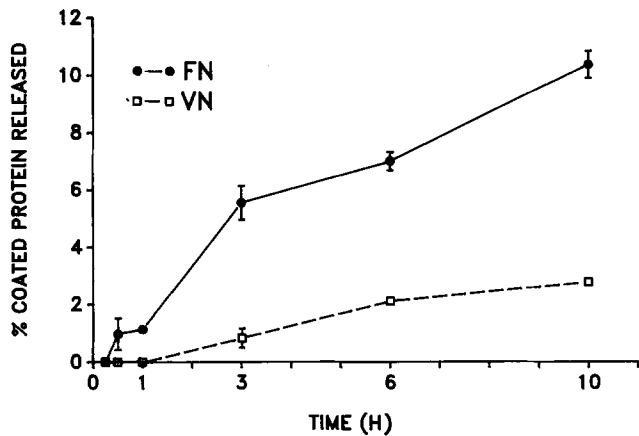
Figure 9. Release of bound protein by HT-1080 cells. Wells were coated with ^{125}I-fibronectin or ^{125}I-vitronectin at 10 μg/ml as described in Materials and Methods. Cells (5 × 10^{5}) in MEM containing 1% BSA, were plated into these wells and allowed to incubate for various periods of time. Coated control wells received

neutralization of PAI-1 was completely dependent on urokinase. We have done experiments both in the presence and absence of neutralizing antibodies against tPA, and found no tPA activity associated with the surface of these cells. This is consistent with previous results showing that these cells do not secrete tPA (Stephens et al., 1989). These data indicate that the active site of vitronectin bound PAI-1 is available to

1 ml/well of MEM containing 1% BSA and were incubated for the same periods of time. Radioactivity released into the medium of wells without cells was subtracted from the radioactivity released into the medium of wells with cells. The result was divided by total cpm of coated protein at zero time to yield the percentage of coated protein released. Fibronectin-coated (●-●). Vitronectin-coated (□-□). Data shown is mean ± SEM and is a representative experiment with triplicate determinations for each time point and protein. Degradation rates were calculated during the last 9 h of incubation using the slopes of each line. Slopes were calculated by linear regression analysis.
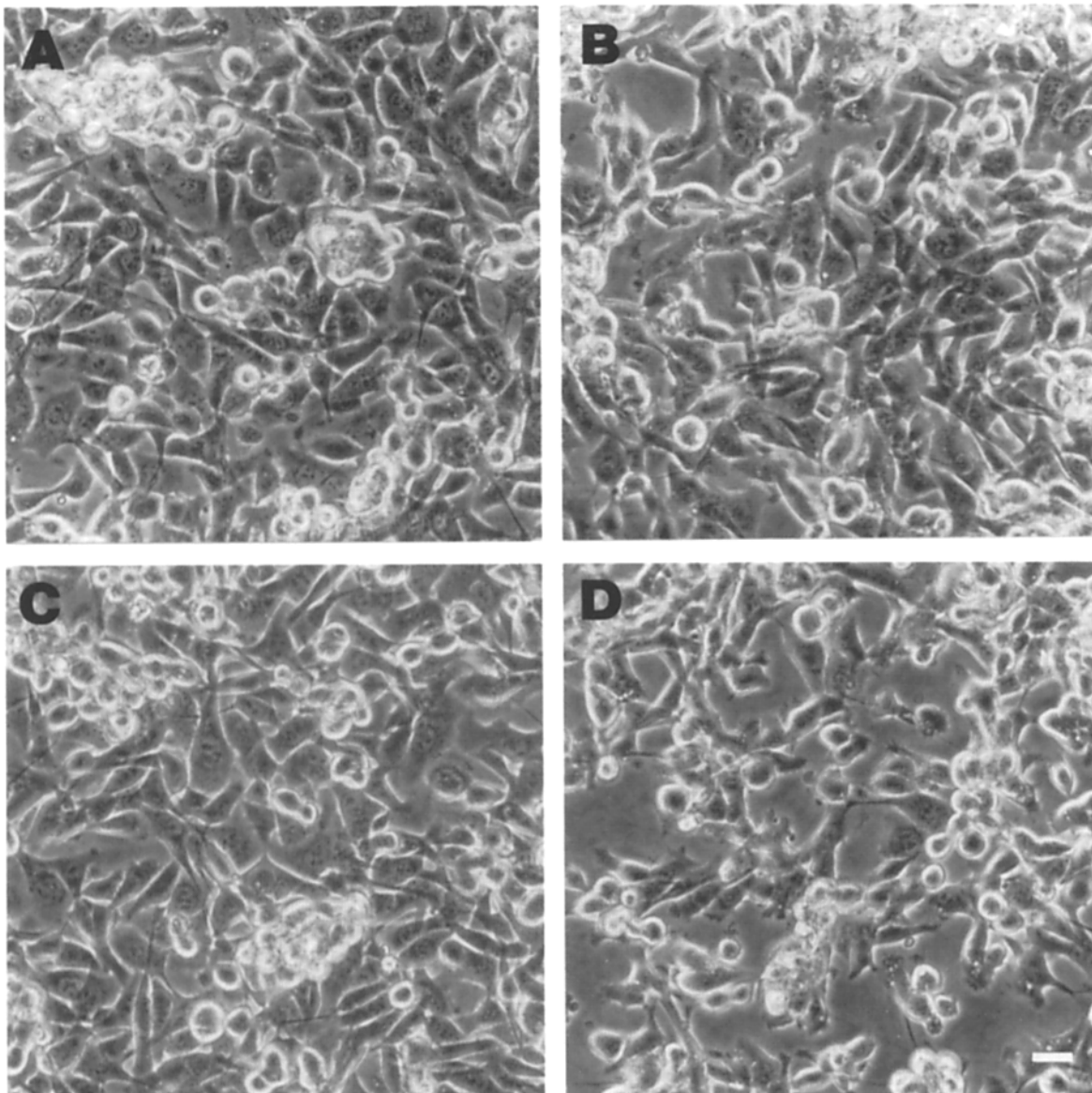
Figure 10. Effect of anti-PAI-1 on initial attachment and spreading. Cells (5 × 10^{5}) in complete medium were plated into 12-well tissue culture plates and incubated in the presence of anti-PAI-1 IgG (*B* and *D*) or normal IgG (*A* and *C*). Antibodies, 50 μg/ml, were added either at the time of plating (*A* and *B*) or 1 h after plating (*C* and *D*). Photographs were taken 3.5 h after the addition of antibodies. Bar, 25 μm.
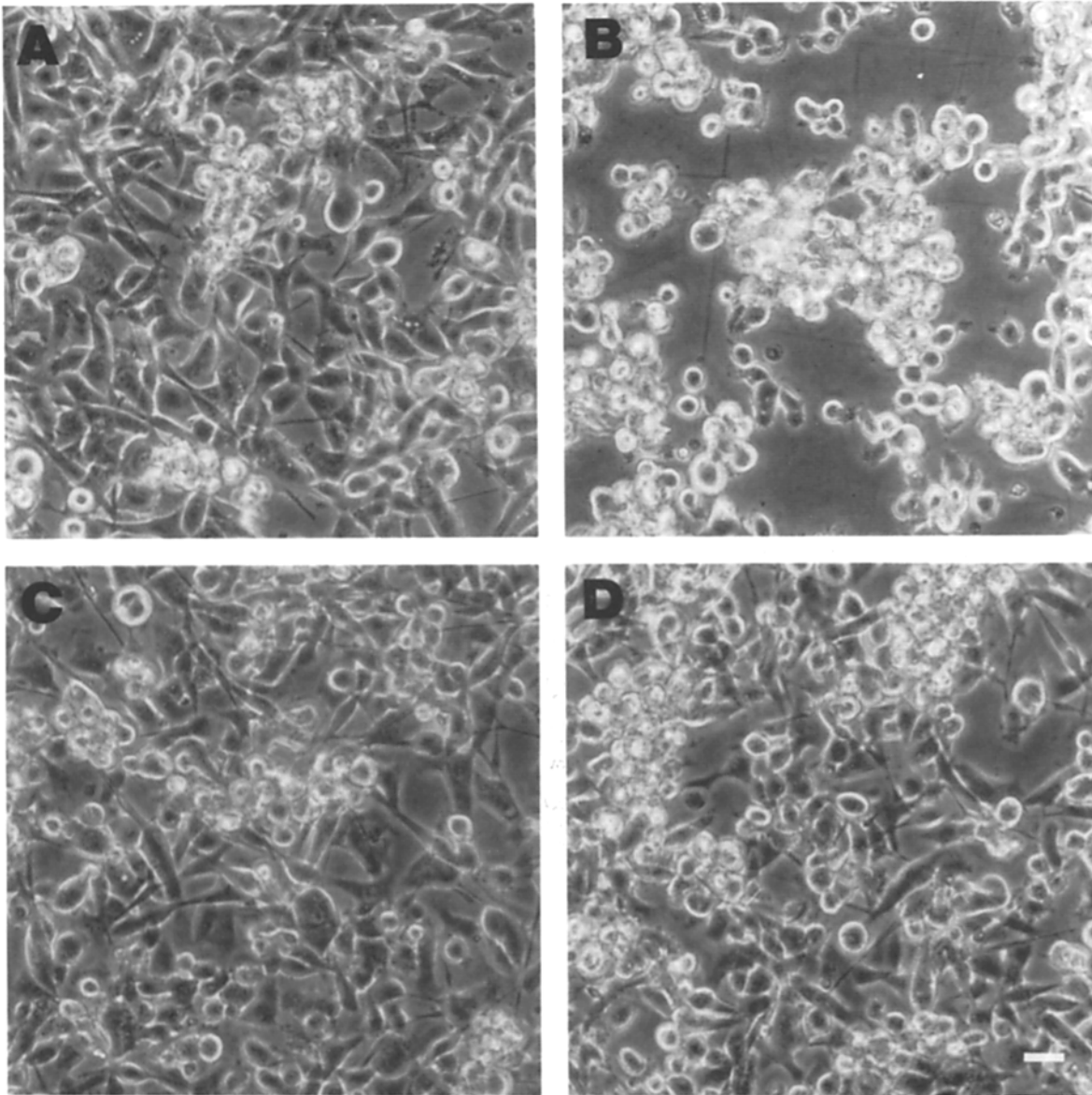
Figure 11. Effect of plating HT-1080 cells with serine protease inhibitors on the anti-PAI-1 mediated detachment response. Cells (5 × 10⁵) in complete medium were plated into 12-well tissue culture plates in the presence (*C* and *D*) or absence (*A* and *B*) of the serine protease inhibitors p-nitrophenyl-guanidino-benzoate (40 μM) and diisopropylfluorophosphate (250 μM). 1 h after seeding, normal or anti-PAI-1 IgG was added directly to the wells to a final concentration of 50 μg/ml. The photomicrographs were taken 4 h after antibody addition. (*A* and *C*) Normal IgG. (*B* and *D*) Anti-PAI-1 IgG. Bar, 25 μm.

inhibit cell-surface urokinase when the cells are adherent to vitronectin. This is consistent with a previous report showing that purified PAI-1-vitronectin complexes can inhibit urokinase activation of plasminogen (Salonen et al., 1989), however, the present report represents the first demonstration that substrate-associated PAI-1 regulates urokinase activity and that this regulation can be modulated by the nature of the substrate.

Anti-PAI-1 antibodies did not block initial attachment and spreading of newly plated cells, suggesting that the vitronectin in the FBS is not complexed with PAI-1 to a significant extent. Time course studies indicated that there was a 1-h lag period before the cells became sensitive to the anti-PAI-1 antibodies. This lag probably reflects in part the time required

for synthesis and secretion of PAI-1. The lag period could be extended several hours by the addition of serine protease inhibitors. The presence of fibronectin in the serum and the fact that HT-1080 cells are known to secrete fibronectin (Oliver et al., 1983), suggests that the cells may establish some initial contacts on fibronectin. Since fibronectin-dependent contacts are not disrupted by anti-PAI-1 antibody, the removal of substrate fibronectin would be expected to facilitate the antibody response. The removal of fibronectin-dependent contacts during initial spreading would explain why cells plated onto substrates coated with both fibronectin and vitronectin responded to the anti-PAI-1 IgG as rapidly as cells plated on vitronectin alone (Fig. 5).

Endothelial cells also secrete PAI-1 into the subcellular

matrix (Mimuro et al., 1987; Levin and Santel, 1987). However, we were unable to disrupt endothelial cell monolayers using anti-PAI-1 antibodies (data not shown). Endothelial cells produce an extensive subcellular matrix, which would be expected to provide alternate adhesion sites. As only cultured hepatoma cells have been shown to synthesize vitronectin (Barnes et al., 1985), the previously described binding site for PAI-1 in the endothelial matrix (Mimuro and Loskutoff, 1989) is probably serum vitronectin. In vitro, binding sites in the matrix for serum vitronectin could include heparan sulfate proteoglycans (Suzuki et al., 1984) and collagen (Gebb et al., 1986). In vivo, vitronectin has been detected in the matrix of a number of tissues by immunocytochemical techniques (Hayman et al., 1983; Simonton et al., 1985). However, plasma vitronectin interacts poorly with heparin (Barnes et al., 1985; Hayoshi et al., 1985) and collagen (Gebb et al., 1986). Therefore, the mechanism of vitronectin incorporation into tissue matrix remains to be clarified.

Previously, urokinase has been localized to focal contact areas in HT-1080 and human fibroblasts (Pollanen et al., 1988; Hebert and Baker, 1988). These experiments could not distinguish inactive proenzyme from active enzyme. In other studies, antibodies against urokinase have been shown to inhibit HT-1080 cell-mediated destruction of extracellular matrix indicating the presence or some active enzyme (Bergman et al., 1986; Reich et al., 1988). The experiments presented here represent the first demonstration that PAI-1 is directly regulating urokinase activity in cell adhesion sites. Distribution of urokinase to sites of cell–substrate adhesion would help confine proteolytic activity to discrete areas. Local proteolysis, by releasing a limited number of adhesion sites, could facilitate directed cell migration and tumor invasion. Local deposition of PAI-1 would permit reestablishment of contacts by decreasing urokinase activity. The ability of vitronectin to modulate plasmin activity suggests a role for matrix components in regulating the invasive properties of a tumor.

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