

# Cytokines Induce Urokinase-dependent Adhesion of Human Myeloid Cells

## A Regulatory Role for Plasminogen Activator Inhibitors

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### Abstract

Differentiation of monocytic precursors often results in adhesive properties thought to be important in migration. In this study, the influence of cytokines, known to induce macrophage differentiation, on the adhesiveness of the monocytic cell line U937 was examined *in vitro*. Despite development of a macrophage morphology, < 5% of cytokine-stimulated U937 cells were adherent at 24 h. Addition of 1–10 nM urokinase-type plasminogen activator (uPA) induced adherence in the presence of transforming growth factor type beta-1, 1,25-(OH)<sub>2</sub> vitamin D<sub>3</sub>, granulocyte macrophage colony-stimulating factor, or tumor necrosis factor alpha. uPA-dependent adhesiveness was reversible after 24 h of stimulation with cytokines and uPA as adherence was prevented by the subsequent addition of anti-uPA antibodies. Adherence induced by diisopropylfluorophosphate-inactivated uPA was severalfold greater than that seen with active uPA. This difference was largely due to cell-surface turnover of active uPA complexed with plasminogen activator inhibitor (PAI). These data indicate that cytokines prime monocyte progenitors for uPA receptor-mediated signals leading to adherence, continued uPA receptor occupancy is required for adherence, and PAI decreases adherence by promoting clearance of uPA/PAI complexes. Thus the interaction of uPA and PAI at the cell surface, known to affect extracellular matrix proteolysis and hence myeloid cell migration, also regulates adhesion. The coordinated regulation of these two uPA functions by PAI may enhance the migratory potential of monocytic cells. (*J. Clin. Invest.* 1993. 91:1541–1552.) **Key words:** urokinase receptor • transforming growth factor type beta • differentiation • monocyte • U937 cells

### Introduction

Tissue macrophages are derived from blood-borne monocytes that undergo terminal differentiation during migration from the vascular space (1). A common property of mature monocytes and macrophages is their ability to adhere to endothelial cells (2), plastic surfaces, and components of the extracellular matrix (3). The migration of monocytes and macrophages through the extracellular matrix is thought to be dependent in

part on this property of adhesion (4, 5). For example, defective cell-surface expression of integrin adhesion molecules by myeloid cells is known to abrogate their migratory ability *in vivo* (6).

The urokinase/plasmin system is also involved in cell migration. Cells of the monocytic lineage synthesize and secrete urokinase-type plasminogen activator (uPA)<sup>1</sup> and plasminogen activator inhibitor types 1 (PAI-1) and 2 (PAI-2) and express a high-affinity (low nM) uPA receptor (uPAR) (7–11). Urokinase receptors have been demonstrated on the leading edge of migrating monocytes (12), and endogenously occupied uPA receptors are important in monocyte migration (13). Urokinase, either in solution or bound to uPAR, can be inhibited by PAI-1 and PAI-2 (14–17), and excess PAI-1 has been shown to decrease monocyte migration (18). The presence of active uPA on its cell-surface receptor may provide for focused extracellular proteolysis with dissolution of the extracellular matrix and thus facilitate migration (19, 20).

The process of myeloid cell differentiation has been studied *in vitro* using anchorage-independent human leukemic cell lines, such as HL60, U937, and THP-1, at various stages of maturational arrest (21, 22). A number of cytokines modulate uPA and uPAR expression and induce macrophage characteristics in these cells (23–30). Although cytokine induction of macrophage-like properties in myeloid leukemic cell lines has been well documented, the acquisition of adhesiveness has been less well characterized. One aim of this study was to explore the effect of cytokines on myeloid cell adhesiveness *in vitro*.

Recent evidence indicates that uPA binding to its receptor, independent of its catalytic activity, is critical to the differentiation and adhesive response of phorbol diester-stimulated myeloid cell lines (31). We now extend that observation and show that multiple cytokines prime myeloid cells for uPA-mediated signals leading to adhesion. The presence of cell surface-bound uPA is necessary for adherence in this system, although active enzyme is not required. We further provide evidence that PAI reduces adhesiveness by promoting turnover of uPA/PAI complexes from the surface of cytokine-stimulated cells.

### Methods

**Reagents.** RPMI 1640 was obtained from GIBCO BRL (Gaithersburg, MD); bovine fetal serum from JRH Biosciences (Lenexa, KS); and tissue culture ware from Falcon Labware (Lincoln Park, NJ). The myeloid cell lines U937, THP-1, and HL60 were obtained from the American Type Culture Collection (Rockville, MD). [<sup>3</sup>H]Thymidine,

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1. **Abbreviations used in this paper:** DFP, diisopropylfluorophosphate; D<sub>3</sub>, 1,23-(OH)<sub>2</sub> vitamin D<sub>3</sub>; GPI, glycosyl-phosphatidylinositol; M-CSF, macrophage colony-stimulating factor; PAI, plasminogen activator inhibitor; tPA, tissue plasminogen activator; uPA, urokinase; uPAR, urokinase receptor.

[<sup>32</sup>P]dATP, and Na<sup>125</sup>I were from New England Nuclear (Boston, MA). Recombinant human two-chain high molecular weight uPA and the amino-terminal fragment of uPA, residues 1–143, was a gift of Drs. Jack Henkin and Andrew Mazar, Abbott Laboratories (Abbott Park, IL); a second preparation of uPA was a gift of Dr. Robert Broeze, Collaborative Research (Bedford, MA). 1,25-(OH)<sub>2</sub> vitamin D<sub>3</sub> was a gift of Dr. M. Uskokvic, Hoffmann-LaRoche Laboratories (Nutley, NJ). Human recombinant EGF, IFN-γ, IL-6, and TGF-β1 were from Collaborative Research. Recombinant human GM-CSF and TNF-α were from Genzyme Corp. (Boston, MA). Recombinant human macrophage colony-stimulating factor (M-CSF) was a gift of Dr. Lester Kobzik, Harvard School of Public Health (Boston, MA). Human thrombin was from Enzyme Research Laboratories (South Bend, IN). Boc-valine-leucine-lysine-aminomethylcoumarin was from Peninsula Laboratories, Inc., (Belmont, CA). Affinity-purified human fibrinogen and plasminogen were from KabiVitrum (Stockholm, Sweden). Human PAI-1 purified from HT 1080 fibrosarcoma cell conditioned media was from two sources: a gift of Dr. T. C. Wun of Monsanto Co. (St. Louis, MO); and (#1090) from American Diagnostica Inc. (Greenwich, CT). Sepharose PD-10 columns were from Pharmacia LKB Biotechnology Inc. (Uppsala, Sweden). Other chemical reagents were from Sigma Immunochemicals (St. Louis, MO).

**Cell culture.** Cells were cultured in RPMI 1640 supplemented with penicillin (100 U/ml), streptomycin (100 μg/ml), Hepes (20 mM, pH 7.4), and 10% heat-inactivated fetal bovine serum. Cells were negative for the presence of mycoplasma using an enzymatic detection kit (Mycotect; GIBCO BRL). Culture media were made 48 μM in 2-mercaptoethanol in the case of the THP-1 cells. All incubations were at 37°C, 5% CO<sub>2</sub>, and cells were plated at 10<sup>6</sup>/ml.

**Diisopropylfluorophosphate (DFP) inactivation of uPA.** uPA was inactivated with 5 mM DFP at room temperature for 1 h and exhaustively dialyzed (31). DFP-uPA concentration was determined by absorption at 280 nm and confirmed by ELISA and total protein analysis. DFP treatment resulted in undetectable uPA activity using a plasminogen-dependent fluorometric substrate.

**Assay for adherent cells.** Adherent cells were assayed as previously described (31) with modifications. Cells at 10<sup>6</sup>/ml were labeled by incubation with 1 μCi/ml [<sup>3</sup>H]thymidine for 16–24 h followed by centrifugation at 300 g for 5 min at 4°C. Cells were washed once in serum-free RPMI and resuspended at the original volume in RPMI containing 10% fetal bovine serum. Cytokines and/or uPA reagents were added to cells and 100 μl of cells per well were placed in triplicate in 96-well microtiter plates. After 24 h of incubation, the contents of control wells were removed for determination of total cpm per well, and the plates were immersed in normal saline. After three rinses, non-adherent cells were flicked out and adherent cells were lysed in 50 μl lysis buffer (10% glycerol, 0.2% sodium dodecyl sulfate, 0.2% Triton X-100) and counted in a liquid-scintillation counter. Adherent cells were also visualized by phase-contrast microscopy; inspection consistently correlated with [<sup>3</sup>H]thymidine incorporation.

**Preparation and DFP treatment of active <sup>125</sup>I-uPA.** Urokinase was iodinated by a modification of the chloramine T method (32). 12.5 μg of uPA were incubated with 1 mCi of Na<sup>125</sup>I in 0.012 mg/ml chloramine T in PBS for 45 s at room temperature, and the reaction stopped with sodium metabisulfite. Labeled enzyme was separated from unbound <sup>125</sup>I by gel chromatography in Sepharose PD-10 in PBS containing 0.25 mg/ml BSA as carrier. Active enzyme was then isolated by passage over a 1 ml benzamidine-Sepharose affinity column followed by elution with 0.1 M glycine HCl, pH 4.0, containing 0.25 mg/ml BSA. The resultant iodinated enzyme was almost entirely an active 55-kD form as judged by its ability to bind active PAI-1 and by its migration in nonreducing SDS polyacrylamide gels. <sup>125</sup>I-uPA was inactivated with 5 mM DFP at room temperature overnight to allow unreacted DFP to hydrolyze: SDS-PAGE analysis of <sup>125</sup>I-DFP-uPA revealed a single band at 55 kD under nonreducing conditions. The concentration of iodinated enzyme was determined by ELISA.

**Assay of uPA antigen by ELISA.** Determination of uPA antigen was performed using a sandwich-type ELISA employing a monoclonal

capture antibody and a polyclonal secondary antibody (33). Analysis of uPA antigen in cell lysate was determined by lysis in 0.5% Triton X-100 in PBS, pH 7.4, followed by assay in duplicate of 100 μl of cell lysate representing 10<sup>6</sup> cells.

**Radiolabeled uPA equilibrium-binding studies.** Equilibrium binding of <sup>125</sup>I-uPA was performed as previously described with minor modifications (31). A stock solution of DFP-treated uPA at 300 nM with a specific activity of 864 cpm/fmol was used. Unstimulated U937 cells (2 × 10<sup>5</sup>) or cells stimulated for 24 h with various cytokines were incubated in RPMI containing 1 mg/ml BSA along with iodinated uPA at 4°C for 90 min. Cell-bound radioactivity was separated from unbound label by centrifugation through an oil cushion consisting of a 1:1 mixture of silicone oil and dioctylphthalate and counted in a gamma counter. Two types of binding studies were performed. First, U937 cells were incubated without or with various cytokines and a one-point binding assay employing a final uPA concentration of 1.5 nM was performed for each cell population. Second, saturation-binding curves were obtained with unstimulated cells and cells stimulated with TGF-β1 and vitamin D<sub>3</sub> for 24 h. All assays were performed in triplicate in the absence and presence of 100 M excess unlabeled uPA. Nonspecific binding accounted for < 10% of total binding. The K<sub>d</sub> and number of receptor sites per cell were determined by Scatchard analysis.

**Polymerase chain reaction.** Total cellular RNA was isolated by lysing cells in guanidium isothiocyanate in the presence of sodium sarkosyl and 2-mercaptoethanol according to Chirgwin et al. (34). RNA was precipitated, quantitated by spectroscopy, and stored at -70°C. Total cellular RNA, 10 μg, was reverse transcribed using random hexamer primers in 100 μl of PCR buffer (50 mM KCl, 10 mM Tris-HCl, pH 8.0, 1.5 mM MgCl<sub>2</sub>, 0.001% gelatin, 0.1 mg/ml BSA) containing 1 mM dNTP (Pharmacia, LKB Biotechnology Inc.), 80 U of RNase inhibitor (Promega Corp., Madison, WI), 4.7 μM random hexamers (Amersham Corporation, Arlington Heights, IL), and 1,000 U of murine moloney leukemia virus (GIBCO BRL) at room temperature for 10 min, 42°C for 1 h, 95°C for 5 min. The cDNA was then divided equally and amplified using specific 5' sense and 3' antisense 20-mer cDNA primers for uPA, PAI-1, PAI-2, or β-actin synthesized as previously described (35). PCR was performed for 24 cycles of 94°C for 30 s, 55°C for 30 s, and 72°C for 1 min in a thermocycler (USA Scientific, Ocala, FL), and the reaction products were analyzed by electrophoresis in agarose gels containing ethidium bromide followed by photography under ultraviolet (UV) illumination as described (35).

**Northern analysis.** Northern analysis was performed essentially as previously described (36). Total cellular RNA, 25 μg, from control or stimulated cells prepared as described above for PCR was electrophoresed in a 2% agarose gel containing 1% formaldehyde, transferred to a nylon filter (ICN Pharmaceuticals, Inc., Costa Mesa, CA), and fixed by UV cross-linking. Equivalent amounts of transferred RNA were verified by visualization of 18S and 28S ribosomal RNA bands under UV light. Double-stranded cDNA probes were prepared for uPA, uPAR, PAI-1, and PAI-2 using PCR product as described (35) and labeled by random hexamer primer extension using the Multiprime DNA labeling system (Amersham Corporation) according to the manufacturer's directions. Blots were probed using the <sup>32</sup>P-labeled PCR product followed by autoradiography.

**Demonstration of uPA activity by fibrin gel autoradiography.** The presence of functional uPA activity was determined by the fibrin gel underlay method (37). Samples containing 10<sup>6</sup> cells were lysed in 100 μl nonreduced sample buffer (62.5 mM Tris HCl, pH 6.8, 10% glycerol, 2% SDS, 0.013% bromophenol blue), electrophoresed in 10% polyacrylamide slab gels with molecular weight standards and urokinase under nonreducing conditions, and laid on 1% agarose gels containing fibrin and plasminogen. The underlays were incubated at 37°C and inspected for bands of lysis at 4 and 24 h.

**Fluorometric assay of PAI activity.** PAI activity was measured as the ability to inhibit plasminogen-dependent esterolytic activity produced by purified uPA using the plasmin substrate Boc-val-leu-lys-aminomethylcoumarin (35). Conditioned media from unstimulated

U937 cells or cells cultured in the presence of 1 ng/ml TGF- $\beta$ 1 and 50 nM vitamin D<sub>3</sub> for 24 h in RPMI containing 1 mg/ml BSA were tested in duplicate with or without the addition of 0.5 U/ml uPA and/or 7.5  $\mu$ g/ml plasminogen. Conditioned media were also subjected to acidification to inactivate PAI-2 (35) and then made 4 M in guanidine HCl for 10 min at room temperature to activate PAI-1 (38) followed by successive dialysis against H<sub>2</sub>O and RPMI before testing. Samples were incubated for 30–45 min at 37°C and the reaction stopped by addition of an equal volume of 1 M NH<sub>4</sub> acetate, pH 4.2. Fluorometric activity was determined at an excitation wavelength of 383 nm and an emission wavelength of 460 nm. Standard curves with purified uPA (0.08–2.5 U/ml) were generated with each assay.

**SDS gel electrophoresis and ligand blotting.** SDS gel electrophoresis in 10% polyacrylamide under nonreducing conditions and ligand blotting for demonstration of the uPA receptor were performed as previously described with modifications (32). Total membrane preparations (30  $\mu$ g total protein) prepared by centrifugation at 100,000 *g* of a postnuclear fraction from homogenized stock unstimulated cells or cells stimulated with various cytokines for 24 h at 10<sup>6</sup>/ml were electrophoresed, electroblotted, and exposed to DFP-treated iodinated uPA (0.25 nM) followed by autoradiography.

**Activation of PAI-1.** Purified PAI-1 was activated by acidification in 4 M guanidine HCl for 10 min at room temperature (38), and then dialyzed exhaustively against either 100 mM L-arginine HCl, pH 5.5, or 10 mM sodium acetate, pH 5.5, and stored at –70°C. Activity of purified PAI-1 was assessed by the ability to abrogate the plasminogen-dependent fluorometric activity of active uPA after incubation at room temperature for 60 min.

**Antibodies.** Goat anti-rabbit alkaline phosphatase was from Kirkegaard & Perry Laboratories, Inc. (Gaithersburg, MD). Murine monoclonal anti-uPA directed against the active site (377, 392, and 394) and the A-chain (3921), murine monoclonal anti-tissue plasminogen activator (tPA) (374b), and polyclonal goat anti-PAI-1 (395G) and anti-PAI-2 (375G) were from American Diagnostica (Greenwich, CT). As determined by the manufacturer, 1 mg of goat anti-PAI-1 antibody inhibits ~ 1,000 IU PAI-1. The neutralizing ability of goat anti-PAI-2 antibody was determined by a fibrin plate method (32). Preincubation of PAI-2 with 12.5–100  $\mu$ g/ml of PAI-2 antibodies before placement in the fibrin plate resulted in increasing inhibition of PAI-2 activity. At the 50% inhibition point, 1 mg of PAI-2 antibodies inhibits ~ 2.5 IU PAI-2. Thus PAI-2 antibodies were much less potent than PAI-1 antibodies at neutralizing PAI activity. Polyclonal rabbit anti-uPA was from Alpha Therapeutic Corp. (Los Angeles, CA). Monospecificity of the polyclonal rabbit anti-uPA antibodies was determined by immunoblotting of whole-cell lysates from unstimulated U937 cells or cells stimulated for 24 h with 16 nM PMA or 1 ng/ml TGF- $\beta$ 1 and 50 nM vitamin D<sub>3</sub>. Whole-cell lysates from 8  $\times$  10<sup>6</sup> cells were fractionated by SDS-PAGE, blotted, and exposed to polyclonal uPA antibodies followed by iodinated goat anti-rabbit IgG. Autoradiography revealed the only bands to be consistent with the 55-kD form of uPA in both the PMA- and TGF- $\beta$ 1/D<sub>3</sub>-treated cells.

**Acid treatment of fetal bovine serum.** Fetal bovine serum was titrated to pH 3.0 with 1 M HCl, incubated for 60 min, and neutralized to pH 7.4 with 1 M NaOH to inactivate fibrinolytic inhibitors as previously described (39).

**Determination of <sup>125</sup>I-uPA turnover.** 1-ml aliquots of U937 cells at 10<sup>6</sup>/ml were incubated in RPMI containing 10% heat-inactivated or acid-treated fetal bovine serum along with cytokines and iodinated uPA in 24-well tissue culture plates for 2 or 24 h. Cells and media were removed from the wells by vigorous pipetting and conditioned media were isolated by centrifugation. The cell pellet was washed twice in 250  $\mu$ l cold PBS and resuspended in acid buffer (50 mM glycine HCl, 0.1 M NaCl, pH 3.0) for 3 min followed by the addition of 100  $\mu$ l of neutralizing buffer (0.5 M HEPES, 0.1 M NaCl, pH 7.5) (40). The acid wash supernatant was obtained by centrifugation and the cell pellet was lysed in 200  $\mu$ l nonreduced sample buffer. Aliquots of conditioned media, acid wash supernatant, and cell lysate were analyzed by liquid-scintillation counting and by PAGE under nonreducing conditions followed by

autoradiography. Preliminary experiments demonstrated that specific binding of iodinated uPA and DFP-uPA to U937 cells was roughly equivalent as determined by measurement of cell-associated radioactivity after incubation at 4°C for 1 h in the absence or presence of excess (23 nM) unlabeled uPA.

**Statistical analysis.** Two-tailed Student's *t* test was performed for comparison of paired mean experimental values (41).

## Results

**Cytokines induce uPA-dependent adherence in U937 cells.** Previous reports indicate that the cytokines TGF- $\beta$  and vitamin D<sub>3</sub> promote differentiation of myeloid cells in vitro (23, 29). Our initial experience indicated that although culture of U937 cells for 24 h with 1 ng/ml TGF- $\beta$ 1 and 50 nM vitamin D<sub>3</sub> (TGF- $\beta$ 1/D<sub>3</sub>) resulted in a morphologically differentiated phenotype, virtually none of these stimulated cells were adherent to serum-coated tissue culture plastic. However, the addition of exogenous uPA at the time of stimulation resulted in a marked increase in adherent cells at 24 h. Preincubation of cells with uPA for 24 h followed by washing and stimulation with TGF- $\beta$ 1/D<sub>3</sub> for an additional 24 h resulted in adherence similar to that induced by cytokines alone. Preliminary experiments revealed that catalytically inactive DFP-uPA had a maximal effect at 10 nM, and this concentration was used for the majority of experiments in this study.

In addition to TGF- $\beta$ 1 and vitamin D<sub>3</sub>, IFN- $\gamma$  (25, 28), IL-6 (42), GM-CSF (26, 27), M-CSF (43), and TNF- $\alpha$  (25) have all been shown to promote monocytic cell differentiation. The adherence of U937 cells cultured in the presence of these cytokines with or without uPA for 24 h was determined (Table I). The cytokine concentrations indicated were at least a log over that required to induce a maximal effect, i.e., similar uPA-dependent adherence was seen with a 10-fold lower concentration, with adherence decreasing with diminishing cytokine concentrations below that point. In the absence of uPA there was little adherence noted with any of the cytokines. Adherence of unstimulated cells was modestly enhanced by exogenous uPA alone. The combination of TGF- $\beta$ 1/D<sub>3</sub> was more effective than any other single cytokine or any cytokine combined with vitamin D<sub>3</sub> (data not shown) at inducing uPA-dependent adherence. Stimulation with GM-CSF, TGF- $\beta$ 1, vitamin D<sub>3</sub>, and TNF- $\alpha$  all resulted in adherence above control. IFN- $\gamma$ , IL-6, and M-CSF did not increase uPA-dependent adherence over that of uPA alone. TGF- $\beta$ 1/D<sub>3</sub> stimulation of the myeloid cell lines THP-1 and HL60 also resulted in the induction of uPA-dependent adherence (data not shown), demonstrating generalization of this phenomenon to other myeloid cell lines. The uPA-dependent response was not due to contamination by endotoxin, as minimal adherence was seen at 24 h in the presence of TGF- $\beta$ 1/D<sub>3</sub> and 100 ng/ml LPS.

Equilibrium-binding studies using radiolabeled uPA were performed to determine if cytokine-induced increases in uPA-dependent adherence correlated with increases in cell-surface uPA binding. Preliminary studies indicated that, as previously reported, U937 cells express specific saturable uPA receptors (data not shown). Relative uPA binding to U937 cells after 24 h of stimulation with cytokines as determined by a one-point assay method are listed in Table I. TGF- $\beta$ 1, TGF- $\beta$ 1/D<sub>3</sub>, GM-CSF, and TNF- $\alpha$  all induced an increase in uPA binding as well as uPA-dependent adherence. However, the relative magnitude of increase in uPA binding did not strictly correlate with

Table I. Effect of Cytokines on U937 Cell uPA Binding and uPA-dependent Adherence

Cytokine	Concentration	Percent maximal adherence				uPA binding	SD
		-uPA	SD	+uPA	SD		
None	—	0.8	(0.2)	15.5	(8.4)	1	
Vitamin D <sub>3</sub>	50 nM	1.2	(0.6)	37.7	(22.7)	0.9	(0.01)
TGF-β1	1 ng/ml	1.0	(0.4)	41.2	(22.1)	1.6	(0.05)
TGF-β1/D <sub>3</sub>	1 ng/ml	1.9	(0.9)	100	(34.5)	3.4	(0.76)
IFN-gamma	1,000 U/ml	0.6	(0.1)	12.9	(5.8)	0.9	(0.12)
IL-6	100 ng/ml	0.7	(0.1)	17.8	(6.3)	ND	
GM-CSF	1,000 U/ml	1.4	(0.5)	60.2	(12.0)	1.6	(0.16)
M-CSF	100 ng/ml	0.7	(0.1)	9.8	(5.2)	0.9	(0.43)
TNF-α	10 U/ml	0.9	(0.1)	36.4	(14.2)	1.9	(0.81)

U937 cells prelabeled with [<sup>3</sup>H]thymidine were incubated at 10<sup>6</sup>/ml without or with cytokines in the absence or presence of 10 nM DFP-uPA for 24 h at 37°C. Adherence was quantified by measuring residual radioactivity in wells after washing to remove nonadherent cells. Results from three separate experiments each performed in triplicate (two experiments in the case of IL-6) are expressed as the mean adherence relative to TGF-β1/D<sub>3</sub>/DFP-uPA. For measurement of uPA binding, unstimulated U937 cells or cells stimulated with cytokines for 24 h at the indicated concentrations were incubated for 90 min at 4°C with 1.5 nM <sup>125</sup>I-DFP-uPA in the absence and presence of 100 M excess cold uPA. Cells were then spun through oil to separate free ligand and cell-associated label was counted in a gamma counter. Data are expressed as specific uPA binding relative to unstimulated cells. ND, not done.

the magnitude of increase in adherence, as seen by examining the values for TGF-β1 and GM-CSF. Indeed, although vitamin D<sub>3</sub> increased uPA-dependent adherence, it did not alter uPA binding over control. IFN-gamma and M-CSF had no effect on either adherence or uPA binding.

In general < 5% of control unstimulated cells or cells incubated with cytokines alone were adherent after washing. Adherence increased to an average of 50% with a range of 25–90% in wells containing cells stimulated with TGF-β1/D<sub>3</sub> and uPA. In part this variability appeared to be due to a gradual loss of responsiveness with time in culture and for this reason cells having undergone between 2 and 11 weekly passages were used for the subsequent work.

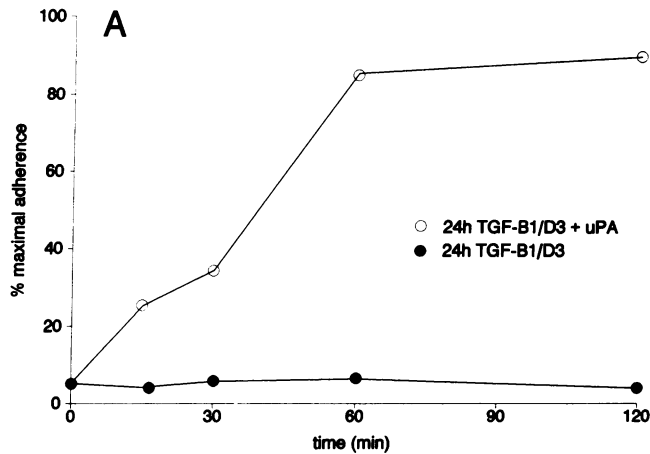
We next sought to determine if morphologically differentiated yet nonadherent myeloid cells 24 h after stimulation with cytokines would respond to uPA by becoming adherent. U937 and THP-1 cells were cultured in the presence of TGF-β1/D<sub>3</sub> and then 10 nM uPA was added to the cell culture at 24 h. Adhesion occurred within minutes of the addition of uPA in these cytokine-stimulated cells (Fig. 1 A). Adhesion was accompanied by changes in cellular morphology (Fig. 1 B). 60 min after the addition of uPA, cells were spread and had numerous cytoplasmic projections. The morphologic changes were more striking in THP-1 cells (shown in Fig. 1 B) than in U937 cells, whereas adherence was similar (data not shown).

*Examination of the uPA axis in cytokine-stimulated U937 cells.* U937 cells are known to constitutively express uPA (7), cell-surface uPA receptor (10), and the uPA-specific inhibitor PAI-2 (7). No information on the effect of TGF-β1 on expression of these proteins by U937 cells is currently available. Because the uPA-dependent adherent response was most pronounced in TGF-β1/D<sub>3</sub>-stimulated U937 cells (Table I) we explored the regulation of uPA, its inhibitors, and its receptor in these cells. The presence of messenger RNA transcripts for uPA, PAI-1, and PAI-2 was determined by PCR (Fig. 2 A). The presence of PCR product for reverse-transcribed uPA

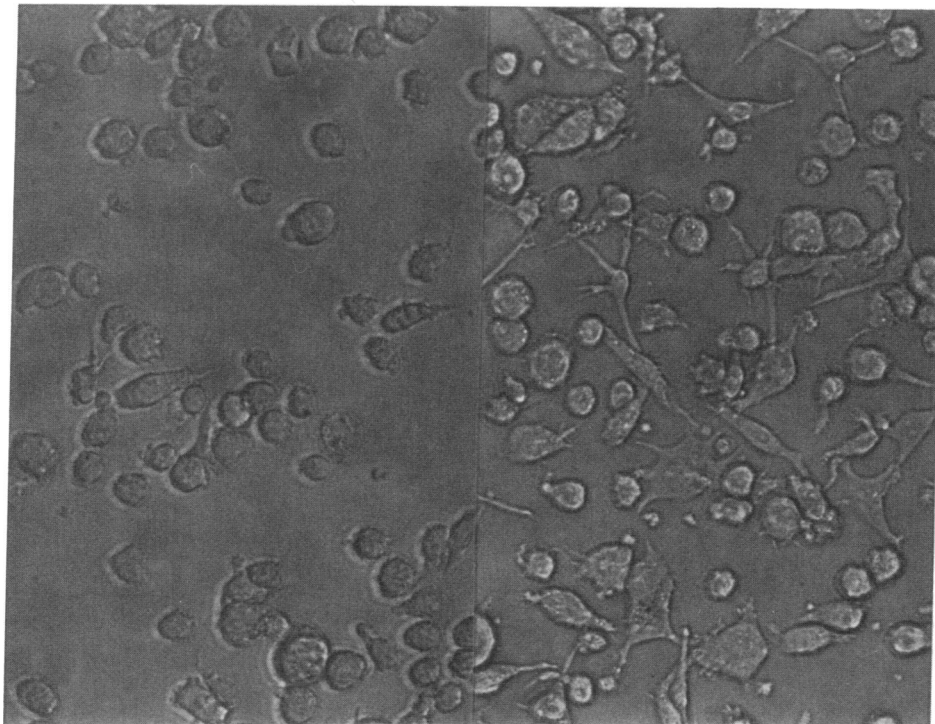
mRNA was demonstrated under all conditions. Although PAI-1 mRNA was undetectable in control cells and in response to vitamin D<sub>3</sub>, mRNA was clearly evident in response to TGF-β1 and TGF-β1/D<sub>3</sub>. In other experiments, PAI-1 mRNA could be detected by 4 h after stimulation. These findings were also seen in THP-1 cells stimulated in a similar manner: PAI-1 mRNA was not detected by PCR in unstimulated cells, whereas a strong signal was evident after TGF-β1/D<sub>3</sub> stimulation (data not shown). PAI-2 mRNA was present in control cells and in cells treated with vitamin D<sub>3</sub> or TGF-β1 and was clearly reduced in TGF-β1/D<sub>3</sub> cells at 24 h: Indeed, reduced or absent PAI-2 mRNA was also noted by 4 h after stimulation.

Quantitation of messenger RNA for uPAR, uPA, and PAI-1 by Northern analysis is shown in Fig. 2 B. Phorbol ester, a known inducer of uPAR mRNA in U937 cells (44), was included for comparison (lane 2). uPAR mRNA was present in stock unstimulated cells (lane 1) and vitamin D<sub>3</sub>-stimulated cells (lane 3) in similar amounts, and increased by PMA, TGF-β1 (lane 4), and TGF-β1/D<sub>3</sub> (lane 5). Stimulation with TGF-β1/D<sub>3</sub> had a modest effect in upregulating uPA mRNA whereas both known transcripts for PAI-1 were strongly upregulated by TGF-β1/D<sub>3</sub>, confirming the PCR data shown in Fig. 2 A. PAI-2 mRNA was undetectable by Northern analysis after TGF-β1/D<sub>3</sub> stimulation (data not shown), verifying the PCR results.

Assays were performed to confirm the presence of functional uPA, PAI, and uPAR. Urokinase antigen levels detected by ELISA in lysates of cells stimulated with TGF-β1/D<sub>3</sub> were similar to those seen in unstimulated cells (data not shown). The presence of functional urokinase activity in cell lysates of cytokine-primed cells was confirmed by fibrin gel underlay. In this assay TGF-β1/D<sub>3</sub>-stimulated cells had functional uPA activity comparable to that seen in unstimulated cells. As discussed below, endogenously produced uPA is actively turned over in cytokine-stimulated cells; thus measurement of uPA antigen or functional activity at 24 h may not accurately reflect



**B**



24h control      60 min after addition of uPA

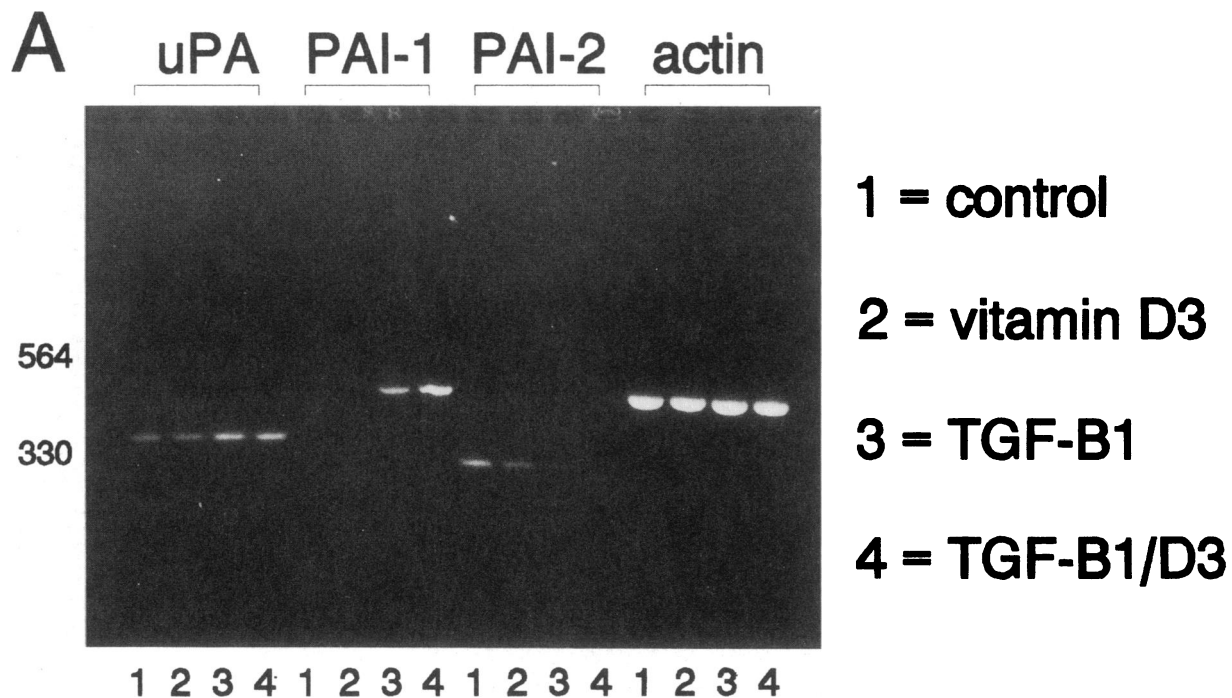
*Figure 1.* Priming effect of cytokines for uPA responsiveness in myeloid cells. Prelabeled U937 and THP-1 cells were cultured at  $10^6$ /ml in the presence of 1 ng/ml TGF- $\beta$ 1 and 50 nM vitamin D<sub>3</sub>. At 24 h, 10 nM DFP-uPA was added to the cell culture. (A) Adherence after washing at various times in a representative experiment is indicated. Adherence reflects the mean of triplicate determinations of residual radioactivity in wells from a representative experiment and is expressed relative to maximal adherence, the amount of radioactivity present in wells containing cells cultured concurrently with 1 ng/ml TGF- $\beta$ 1, 50 nM vitamin D<sub>3</sub>, and 10 nM DFP-uPA for 24 h. Adherence of U937 cells stimulated with TGF- $\beta$ 1/D<sub>3</sub> but without addition of DFP-uPA are given for comparison. (B) The photograph illustrates the morphology seen in THP-1 cells cultured for 24 h in the presence of 1 ng/ml TGF- $\beta$ 1 and 50 nM vitamin D<sub>3</sub> before and 60 min after the addition of 10 nM DFP-uPA.

uPA production. This fact may explain the apparent discrepancy of upregulation of uPA mRNA despite no change in uPA antigen or functional activity in TGF- $\beta$ 1/D<sub>3</sub>-stimulated as opposed to unstimulated cells. For this reason, no attempt was made to further quantitate uPA expression by these cells.

The presence of functional inhibitor was confirmed by plasminogen-dependent fluorometry. In the absence of exogenous uPA, conditioned media from unstimulated cells or cells stimulated with TGF- $\beta$ 1/D<sub>3</sub> for 24 h had no plasminogen-dependent fluorometric activity. When incubated with active uPA, both sets of media substantially inhibited urokinase activity. Inhibition was dose dependent. Acidification of conditioned

media containing PAI inactivates PAI-2 (35) whereas detergent treatment activates PAI-1 (38). The inhibitor activity of unstimulated conditioned media was completely abrogated by acidification and detergent treatment, whereas that of stimulated cells was resistant to these treatments. These physical properties are consistent with the inhibitory activity of control medium being PAI-2 and that in TGF- $\beta$ 1/D<sub>3</sub> medium being mostly PAI-1, as was also suggested by the mRNA analyses (Fig. 2). Because of the tendency of PAI-1 to become latent at 37°C, the inhibitor activity was not further quantified.

The presence of functional uPA receptor in cytokine-stimulated myeloid cells was confirmed by ligand blotting (Fig. 3).



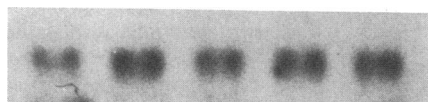
**B**

uPAR



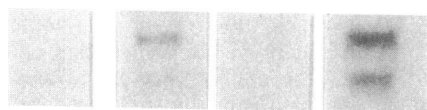
1 2 3 4 5

uPA



1 2 3 4 5

PAI-1



1 2 3 5

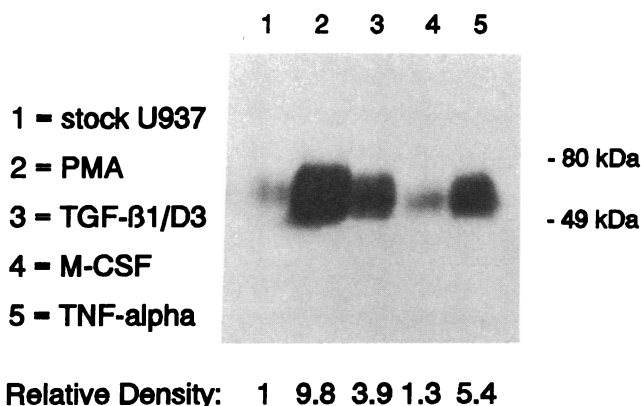
1 = stock U937  
2 = PMA  
3 = vitamin D3  
4 = TGF-β1  
5 = TGF-β1/D3

*Figure 2.* Effects of TGF-β1/D<sub>3</sub> on uPA, PAI-1, PAI-2, and uPA-R mRNA in U937 cells. (A) PCR. Total cellular RNA from stock U937 cells and cells cultured in the presence of 50 nM vitamin D<sub>3</sub>, 1 ng/ml TGF-β1, and a combination of TGF-β1 and vitamin D<sub>3</sub> for 24 h was reverse transcribed using random primers. The resultant cDNA was amplified by PCR using specific primers for uPA, PAI-1, PAI-2, and β-actin. The predicted sizes of the PCR transcripts were 360, 497, 299, and 493 bp for uPA, PAI-1, PAI-2, and β-actin, respectively. The size of DNA standards (in bp) is given to the left of the figure. (B) Northern analysis. Total cellular RNA (10 μg) from unstimulated cells or cells cultured in the presence of 16 nM PMA or vitamin D<sub>3</sub>, TGF-β1, or TGF-β1/D<sub>3</sub> as indicated above for 24 h was electrophoresed, blotted, and probed with radiolabeled cDNA for uPAR, uPA, and PAI-1. Equivalent loading of RNA was verified by direct visualization under UV light.

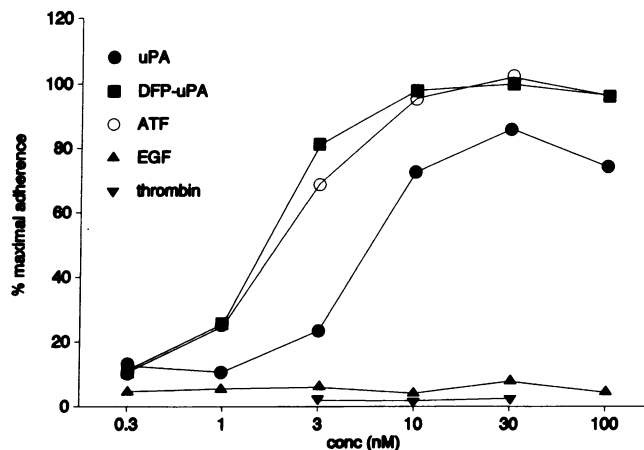
The amount of membrane-associated uPA receptor in stock unstimulated cells can be seen in lane 1. After PMA stimulation, uPAR expression is markedly increased (lane 2); phorbol ester stimulation has previously been shown to strongly up-regulate uPAR (44, 45). The amount of uPAR was also increased by stimulation with TGF- $\beta$ 1/D<sub>3</sub> (lane 3) and TNF- $\alpha$  (lane 5) but essentially unchanged by M-CSF (lane 4). This observation parallels the adherence assay data shown in Table I in that TGF- $\beta$ 1/D<sub>3</sub> and TNF- $\alpha$  but not M-CSF increase uPA-dependent adherence over that seen in control cells. Relative uPAR expression as determined by densitometry correlated well with relative uPA binding (Table I) in the case of TGF- $\beta$ 1/D<sub>3</sub>, although a greater increase in functional receptor was seen by ligand blot than by binding in TNF- $\alpha$ -stimulated cells. This may reflect the fact that the ligand blot measures total cellular uPAR whereas the binding studies only examine unoccupied cell-surface receptors. In summary, by 24 h, TGF- $\beta$ 1/D<sub>3</sub> stimulation of U937 cells increased uPA receptor and PAI-1 expression, downregulated PAI-2 expression, and had no detectable effect on uPA expression.

*Urokinase-dependent adherence is specific and reversible.* We explored the uPA-dependent adherence of TGF- $\beta$ 1/D<sub>3</sub>-stimulated U937 cells and sought to determine the specificity of this response for uPA receptor binding. Both uPA and DFP-inactivated uPA induced adherence in a dose-dependent manner (Fig. 4). DFP-uPA was severalfold more potent than active uPA at inducing half-maximal adherence. An amino-terminal fragment of uPA (ATF), comprising amino acid residues 1–143 and containing the receptor-binding domain but lacking the catalytic domain, was as potent as DFP-uPA at inducing adherence. In contrast, the inclusion of EGF, which has a receptor binding domain homologous to that of uPA (46), or thrombin with TGF- $\beta$ 1/D<sub>3</sub> had no effect on adherence.

Scatchard analyses of saturation-binding studies employing radiolabeled DFP-inactivated uPA were performed to determine the affinity and number of cell-surface uPA binding sites.



**Figure 3.** Demonstration of functional uPA receptor as determined by ligand blotting. Total membrane preparations (30  $\mu$ g per lane) from stock U937 cells or cells cultured for 24 h in the presence of 16 nM PMA; 1 ng/ml TGF- $\beta$ 1 and 50 nM vitamin D<sub>3</sub>; 100 ng/ml M-CSF; or 10 U/mL TNF- $\alpha$  were electrophoresed, electroblotted, and exposed to 0.25 nM DFP-treated <sup>125</sup>I-uPA followed by autoradiography. The migration of molecular weight standards is indicated along the right edge of the figure. Relative binding as reflected by computer-aided densitometry is shown under each lane.



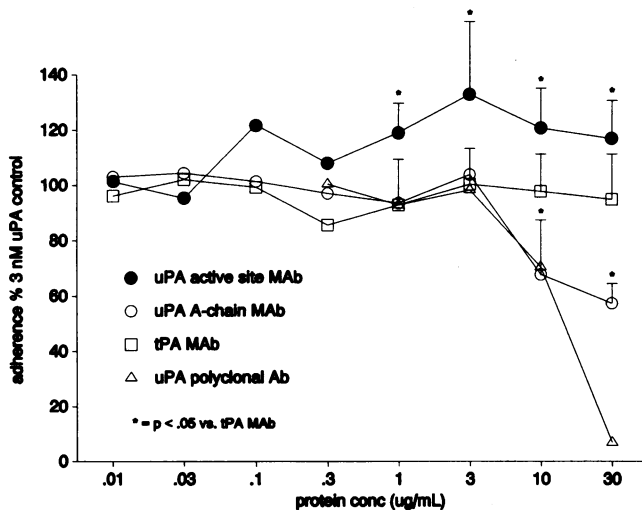
**Figure 4.** Dose–response relationship of uPA-induced adherence in TGF- $\beta$ 1/D<sub>3</sub>-stimulated U937 cells. U937 cells were cultured in the presence of 50 nM vitamin D<sub>3</sub> and 1 ng/ml TGF- $\beta$ 1 along with uPA, DFP-uPA, ATF, EGF, or thrombin at the indicated concentrations for 24 h and adherence was determined. Adherence is expressed relative to the adherence of cells cultured in the presence of 30 nM DFP-uPA along with TGF- $\beta$ 1/D<sub>3</sub> (maximal adherence) and results reflect the mean of triplicate determinations from a representative experiment.

Both stock unstimulated U937 cells and cells cultured for 24 h in the presence of 1 ng/ml TGF- $\beta$ 1 and 50 nM vitamin D<sub>3</sub> had a  $K_d$  of 0.33 nM for uPA binding. Stock cells had  $\approx$  11,000 binding sites per cell; this rose to  $\approx$  41,000 after TGF- $\beta$ 1/D<sub>3</sub> stimulation. The concentration of DFP-uPA needed for half-maximal adherence, 2 nM as seen in Fig. 4, was higher than the  $K_d$  for binding at 24 h: This may be due in part to cell-surface turnover of the enzyme over the course of the 24-h stimulation as presented below. Indeed, half-maximal adherence of cells stimulated with 1 ng/ml TGF- $\beta$ 1 and 50 nM vitamin D<sub>3</sub> for 24 h and then exposed to DFP-uPA as in Fig. 1 was induced by 0.1–0.3 nM DFP-uPA (data not shown), correlating well with the measured binding affinity.

As previously reported (31), polyclonal anti-uPA antibodies known to block receptor binding blocked the adherent response to uPA (Fig. 5). A murine mAb directed against the A-chain of uPA (which contains the receptor-binding domain) also blocked adherence. In contrast, uPA-induced adherence was increased by a neutralizing mAb directed against the active site of uPA that recognizes receptor-bound uPA and thus may not interfere with uPA binding to uPAR. A mAb against tPA, included as a control, had no effect on adherence.

Taken together, the data from Figs. 4 and 5 indicate that the adherent response is specific for uPA binding to its receptor and does not involve active uPA-dependent proteolysis. This conclusion supports earlier work showing an autocrine role for the receptor-binding domain of uPA in phorbol diester-mediated myeloid cell differentiation (31).

The possibility that uPA-dependent adhesiveness is reversible was considered. U937 cells were cultured in the presence of 1 ng/ml TGF- $\beta$ 1, 50 nM vitamin D<sub>3</sub>, and 3 nM uPA for 24 h on Teflon surfaces to prevent adhesion. 60 min after placement on tissue culture plastic, cells were adherent (data not shown). Addition of 0.25 mg/ml polyclonal rabbit anti-uPA or 30  $\mu$ g/



**Figure 5.** Effect of anti-uPA antibodies on uPA-dependent adherence of TGF- $\beta$ 1/D<sub>3</sub>-stimulated U937 cells. U937 cells were cultured in the presence of 1 ng/ml TGF- $\beta$ 1, 50 nM vitamin D<sub>3</sub>, and 3 nM uPA without or with antibodies for 24 h and adherence was determined. Murine mAbs directed against the active site of uPA (a neutralizing antibody), the A-chain of uPA (containing the receptor binding domain), and tPA as well as rabbit polyclonal anti-uPA were used in the indicated concentrations. Adherence is expressed relative to TGF- $\beta$ 1/D<sub>3</sub>/uPA-treated cells cultured in the absence of antibodies and reflects the mean of triplicate determinations from two representative experiments. Error bars representing the standard deviation are given for uPA mAb concentrations with adherence significantly different ( $P < 0.05$ ) than tPA mAb.

ml A-chain mAb at the time of placement on plastic prevented adherence whereas 0.25 mg/ml nonimmune rabbit serum and 30  $\mu$ g/ml active-site mAb had no effect. Prevention of adherence by anti-uPA could be overcome by the subsequent addition (1 h later) of 100 nM uPA. Similar findings were observed in cells cultured for 24 h in the presence of TGF- $\beta$ 1/D<sub>3</sub> and uPA and then subjected to acid treatment to remove cell-surface uPA (40). Although initially nonadherent 60 min after placement on plastic, these surface uPA-depleted cells became adherent upon addition of 10 nM uPA (data not shown). These results demonstrate that the adherence of cytokine-primed cells is dependent on the continued presence of cell surface-bound uPA.

*PAI affects uPA-dependent adherence by increasing clearance of uPA from its receptor.* DFP-inactivated uPA was consistently severalfold more effective than active uPA at inducing adherence of cytokine-stimulated cells. In addition, a neutralizing mAb directed against the active site of uPA increased uPA-dependent adherence in these cells. We explored three possible mechanisms for this difference. Plasmin activation by active enzyme may result in proteolytic processing of active uPA or another molecule resulting in decreased adherence. Alternatively, the presence of serum inhibitors in the culture media may bind active uPA and prevent its binding to the uPA receptor. Finally, PAI-1 is known to bind to receptor-bound uPA (15), the uPA PAI-1 complex has been shown to be internalized and degraded in unstimulated U937 cells (47), uPA/PAI-1 turnover is regulated by uPAR (48), and DFP-uPA does not bind PAI-1. As shown above, PAI-1 production is increased in

U937 cells stimulated with TGF- $\beta$ 1/D<sub>3</sub>. Because adherence requires continued occupancy of the uPA receptor, turnover of uPA/PAI-1 complexes from the cell surface may decrease receptor-mediated signals leading to adherence.

To determine the importance of plasmin activation and serum inhibitors in uPA-dependent adherence, adherence assays analogous to those shown in Fig. 4 were performed in the presence of serum depleted of inhibitors by acid treatment (39). U937 cells stimulated with TGF- $\beta$ 1/D<sub>3</sub> along with DFP-inactivated uPA (3 nM) under these conditions were adherent at 24 h whereas active uPA did not induce adherence (data not shown). The addition of the plasmin inhibitor aprotinin (6  $\mu$ M) partially restored the ability of active uPA to promote adherence, although DFP-inactivated uPA remained more effective than active uPA with dose-response curves similar to those shown in Fig. 4. These data indicate that although serum inhibitors and/or a plasmin-dependent process may account for differences between uPA and DFP-uPA under some conditions, DFP-inactivated uPA remains severalfold more effective than active uPA under conditions in which neither mechanism is operative. Thus the possible role of PAI-1 in modulating adhesion was explored.

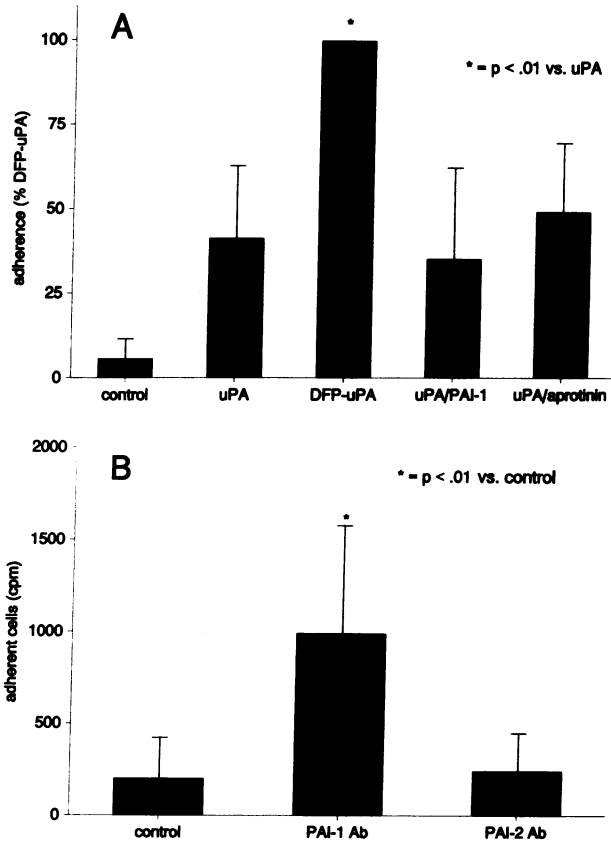
The effect of PAI-1 complexed with uPA on the adherence of TGF- $\beta$ 1/D<sub>3</sub>-stimulated U937 cells is shown in Fig. 6 A. In contrast to DFP treatment of uPA, preincubation of uPA with PAI-1, while also completely abrogating the proteolytic activity of uPA, did not enhance the ability of uPA to induce adherence of TGF- $\beta$ 1/D<sub>3</sub>-stimulated U937 cells. In a similar fashion, uPA incubated with aprotinin was only as effective as uPA alone at inducing adherence, although plasmin activation was blocked as demonstrated by a fluorometric assay.

The effect of anti-PAI-1 antibodies on the adherence of TGF- $\beta$ 1/D<sub>3</sub>-stimulated U937 cells is shown in Fig. 6 B. As stimulation results in downregulation of PAI-2 (Fig. 2 A), anti-PAI-2 antibodies were used as a control IgG. The addition of anti-PAI-1 resulted in increased adherence as compared with control cells or cells treated with control antibody. The maximal effect of PAI-1 antibodies shown in Fig. 6 B with 50  $\mu$ g/ml was not exceeded by  $\leq 400$   $\mu$ g/ml of antibodies whereas PAI-2 antibodies had no effect in this concentration range. The magnitude of the response was small, with  $< 5\%$  of cells adhering.

Clearance of uPA/PAI-1 complexes from the cell surface has been documented in unstimulated U937 cells, and this clearance is abrogated by chloroquine, an inhibitor of lysosomal protein degradation (47). We sought to determine if a clearance mechanism is also operative in TGF- $\beta$ 1/D<sub>3</sub>-stimulated cells. The effect of NH<sub>4</sub>Cl, which blocks acidification of the lysosomal compartment (49), on the uPA-dependent adherence of U937 cells was determined (Fig. 7). Adherence of control cells and cells incubated with TGF- $\beta$ 1/D<sub>3</sub> in the absence or presence of uPA or DFP-inactivated uPA were all enhanced by the addition of NH<sub>4</sub>Cl. The enhancing effect seen with active uPA was greater than that seen with DFP-inactivated enzyme, suggesting that lysosomal degradation modulates uPA-dependent adherence. The modest increase in adherence observed in control and DFP-uPA-treated cells may reflect NH<sub>4</sub>Cl-induced decreases in turnover of endogenously produced uPA.

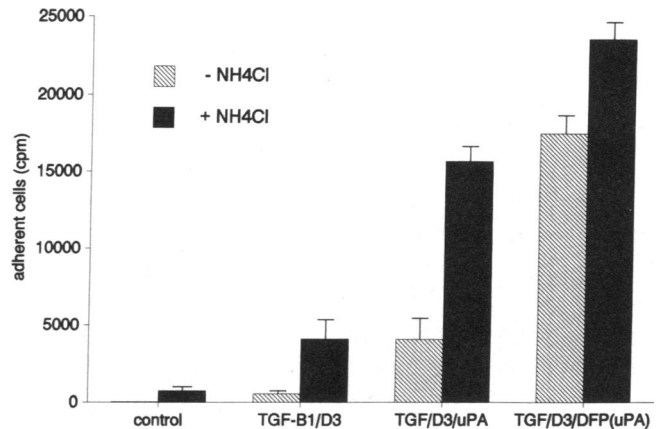
We next compared clearance of active and DFP-inactivated uPA in cytokine-stimulated myeloid cells (Fig. 8). Iodinated uPA or DFP-uPA, in concentrations lower than that required





**Figure 6.** Effects of uPA and plasmin inhibitors and PAI-1 and PAI-2 antibodies on TGF- $\beta$ 1/D<sub>3</sub> U937 cell adherence. (A) PAI-1 was activated as described and 6 nM uPA incubated in the presence or absence of active PAI-1 (1,250 U/ml) or aprotinin (12  $\mu$ M). The uPA samples were then added to U937 cells to yield a final concentration of 3 nM uPA and 6  $\mu$ M aprotinin. DFP-uPA, 6 nM, was sham incubated and added to cell culture under similar conditions. Cells were cultured for 24 h and adherence was determined. The mean ( $\pm$ SD) adherence of eight separate experiments (four experiments in the case of aprotinin) each performed in triplicate are expressed relative to the adherence of cells cultured with TGF- $\beta$ 1/D<sub>3</sub>/DFP-uPA. (B) Neutralizing goat anti-PAI-1 or anti-PAI-2 antibodies at 50  $\mu$ g/ml were added to U937 cell culture at the time of stimulation with 1 ng/ml TGF- $\beta$ 1 and 50 nM vitamin D<sub>3</sub> and adherence determined at 24 h. The results shown represent the mean ( $\pm$ SD) from three separate experiments each performed in triplicate.

to induce adherence at 24 h (Fig. 3), was added to U937 cells along with TGF- $\beta$ 1/D<sub>3</sub> and conditioned media and cell-surface elutable enzyme subsequently examined by SDS-PAGE. The clearance of uPA and DFP-uPA from conditioned media at 2 and 24 h of culture is illustrated in Fig. 8 A. The 55-kD purified form of iodinated uPA incubated in cell-free medium is shown in lane 9. In the presence of cells, active uPA forms a complex of  $\approx$  90 kD at 2 h (lane 1); the formation of this complex is blocked by excess unlabeled uPA (lane 2). A much higher molecular weight band at the top of the gel may represent binding of uPA to inhibitors produced by the cells themselves. This point was not explored further. In contrast, in the presence of cells, iodinated DFP-uPA remained a single 55-kD band. By 24 h, active 55-kD uPA was no longer present in the conditioned media (lane 5) unless excess unlabeled uPA was added (lane 6). A small amount of residual iodinated enzyme



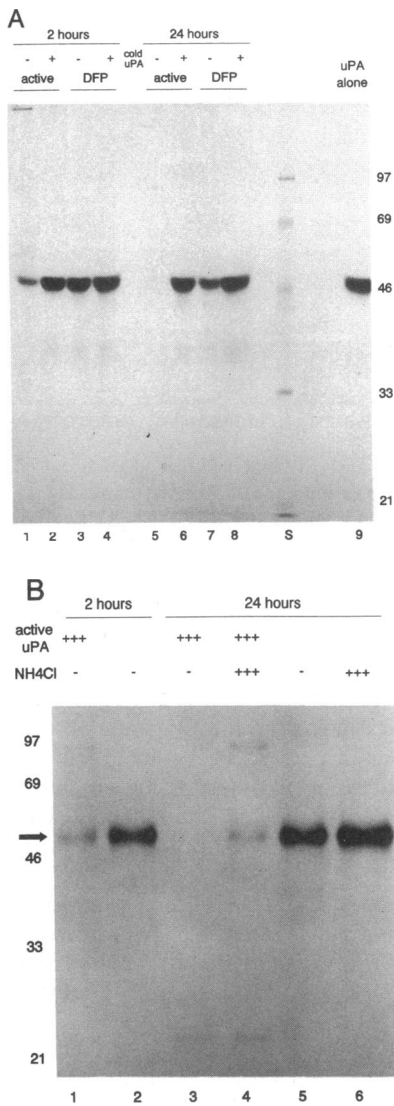
**Figure 7.** Effect of NH<sub>4</sub>Cl on TGF- $\beta$ 1/D<sub>3</sub> U937 cell adherence. Cells were incubated in the absence or presence of 50 nM vitamin D<sub>3</sub>, 1 ng/ml TGF- $\beta$ 1, 3 nM uPA or DFP-uPA, and 10 mM NH<sub>4</sub>Cl as indicated in the figure for 24 h and adherence was determined. The mean values  $\pm$  the SD of three separate experiments each performed in triplicate are given.

can be seen at  $\approx$  90 kD in lane 5; this most likely represents uPA/PAI complex. The amount of DFP-uPA in the absence of excess cold uPA has also decreased (lane 7). These observations were paralleled by an increase in TCA soluble counts at 24 h in media without excess unlabeled uPA: The amount of TCA soluble counts was greater in media containing active <sup>125</sup>I-uPA than in media containing DFP-<sup>125</sup>I-uPA (data not shown).

The amount of iodinated uPA on the cell surface under the various culture conditions is shown in Fig. 8 B. The presence of 55-kD active uPA and DFP-uPA can be seen at 2 h (lanes 1 and 2); active uPA but not DFP-uPA is also present in a higher molecular mass band  $\approx$  90 kD. By 24 h (lane 3) there is no active enzyme detectable on the cell surface; the presence of NH<sub>4</sub>Cl (lane 4) partially restored surface-elutable uPA. The amount of DFP-uPA on the cell surface at 24 h is also relatively increased by NH<sub>4</sub>Cl (lanes 6 vs. 5). These data at 24 h parallel the adherence data seen with the addition of NH<sub>4</sub>Cl in Fig. 7, although the increase in adherence (1.2 $\times$ ) is less than the increase in cell-surface DFP-uPA as determined by computer-assisted densitometry (1.8 $\times$ ). This observation may be explained by the fact that the concentration of <sup>125</sup>I-DFP-uPA added for determination of turnover was less than that required for adherence. In additional experiments it was observed that clearance of iodinated uPA precomplexed with PAI-1 before co-culture with cells is virtually the same as that seen with active uPA alone. These results are similar to the adherence data given in Fig. 6 A in which the addition of active uPA and uPA precomplexed with PAI-1 were functionally equivalent in promoting adhesion, indicating that active uPA when added to these cells becomes complexed with PAI and ultimately cleared from the cell surface.

## Discussion

Previous reports have outlined two distinct functions of uPA bound to its receptor in monocyte/macrophage biology: a catalytic role important in generating pericellular proteolysis and a noncatalytic signaling function operative in the differentiation and adhesion of phorbol diester-stimulated monocytic pro-



**Figure 8.** Fate of iodinated uPA in TGF- $\beta$ 1/D<sub>3</sub> U937 cell culture. U937 cells ( $10^6$ /ml) were cultured for either 2 or 24 h along with  $\approx 100,000$  cpm ( $10$  pM) iodinated uPA or DFP-uPA (specific activity 7,000 cpm/fmol, stock solution 3 nM) in the absence or presence of excess (23 nM) unlabeled active uPA. Conditioned media were collected by centrifugation. (A) Conditioned media (50  $\mu$ l) was examined by SDS-PAGE under nonreducing conditions followed by autoradiography. In lane 9 incubation of  $\approx 25,000$  cpm iodinated uPA in cell-free culture medium for 60 min was followed by SDS-PAGE. The molecular weight of the standards (S lane) are listed to the right of the figure expressed in kD. (B) After removal of conditioned media, cells were washed in PBS and cell-surface proteins eluted via acid washing as described in the text. Eluted protein was lyophilized, resuspended in nonreduced

sample buffer, and analyzed by SDS-PAGE followed by autoradiography.

genitors (31). Data presented here not only confirm these observations but also extend them in several important respects. First, we show that physiological cytokines such as vitamin D<sub>3</sub>, TGF- $\beta$ 1, and GM-CSF also induce uPA-dependent adhesiveness in human leukemic cell lines (Table I). Further, cells prestimulated with cytokines become adherent rapidly upon addition of uPA (Fig. 1) and the concentration of uPA inducing half-maximal adherence is very near the  $K_d$  for uPA binding to uPAR. Finally, this potential for adhesion is reversible and dependent on the continued occupation of the uPA receptor. The need for continued receptor occupation suggests that cytokine-induced adhesiveness is regulated not only by the expression of uPA receptor and the availability of ligand, but also by factors that modulate uPA receptor turnover. One such factor identified in our study is the presence of PAI-1, which not only abrogates proteolytic activity but also decreases adhesiveness. The ability of a single molecule to regulate both proteolysis and adhesiveness makes it likely that the two apparently distinct functions of uPA in monocyte/macrophage biology are in fact intrinsically linked.

None of the cytokines tested, namely vitamin D<sub>3</sub>, TGF- $\beta$ 1, IFN- $\gamma$ , IL-6, GM-CSF, M-CSF, and TNF- $\alpha$ , induced strong adherence at 24 h in the absence of exogenous uPA. This was true in TGF- $\beta$ 1/D<sub>3</sub>-primed cells despite expression of uPA and upregulation of uPA receptor (Figs. 2 and 3). Several lines of evidence indicate that the interaction of endogenous uPA with endogenously produced PAI followed by turnover of the uPA/PAI complex may account for part or all of this lack of adherence. DFP-uPA or an amino-terminal fragment of uPA, which do not bind PAI, were severalfold more effective than active uPA at inducing adherence of cytokine-stimulated cells (Fig. 4) and a mAb directed against the active site of uPA, which may interfere with uPA/PAI complex formation, enhanced uPA-dependent adherence (Fig. 5). Further, addition of anti-PAI-1 antibodies to cytokine-stimulated cells in the absence of exogenous uPA increased the adherence of these cells, although the amount of adherence seen was small (Fig. 6 B). Finally, disruption of lysosomal acidification promoted adhesion of TGF- $\beta$ 1/D<sub>3</sub>-stimulated cells and this correlated with the appearance of both 55-kD uPA and uPA/PAI complex on the cell surface (Figs. 7 and 8). It is also possible that insufficient local concentrations of uPA are produced by the cells in culture, as adhesion required low nM concentrations of uPA. In either case, the fact that none of the tested cytokines induced strong adherence in the absence of exogenous uPA indicates that paracrine sources of uPA may be important for the in vivo adherence of monocytic cells under the influence of these cytokines.

Although it appears that PAI-1 is the major regulator of uPA turnover and cellular adherence in TGF- $\beta$ 1/D<sub>3</sub>-stimulated U937 cells, we cannot exclude a role for PAI-2. It is possible, even likely, that there is some PAI-2 present at 24 h in conditioned medium from these cells and the anti-PAI-2 antibodies, having only weak neutralizing activity, may not have blocked PAI-2 activity in Fig. 6 B. However, they had no effect over a wide concentration range. Even if PAI-2 does not play a major role in TGF- $\beta$ 1/D<sub>3</sub>-stimulated U937 cells, uPA/PAI-2 complex turnover has been documented in other myeloid cells (12) and it is possible that PAI-2 may be the dominant regulator of uPA turnover in other settings. Regulation of uPA/PAI turnover is incompletely understood, although a recent report indicates a role for the alpha-2-macroglobulin receptor in controlling the turnover of the uPA/PAI-1 complex bound to uPAR of monocytes (50).

It is interesting that cytokine-primed cells, despite nonadherence at 24 h, are capable of rapid adherence and morphological changes upon exposure to uPA (Fig. 1). Indeed, the concentration of uPA required for half-maximal adherence when added at 24 h (0.1–0.3 nM) correlates well with the measured cell-associated binding affinity for uPA under these conditions (0.33 nM). These data suggest the presence of a uPA-dependent adhesive mechanism that is upregulated by cytokine priming. This mechanism is likely a complex phenomenon involving factors such as adhesive membrane proteins, the cytoskeleton, etc. Indeed, a strict correlation between cell-associated uPA binding and uPA-dependent adherence was not seen (Table I). Although stimulation with vitamin D<sub>3</sub> did not increase uPA binding, it did increase uPA-dependent adherence over that of stock unstimulated cells. In addition, although TGF- $\beta$ 1 and GM-CSF induced similar increases in uPA binding, uPA-dependent adherence after GM-CSF stimulation was 1.5 times that of TGF- $\beta$ 1.

The nature of the uPA-dependent adhesive signaling mechanism is unclear. The only known uPA receptor is linked to the membrane by a glycosyl-phosphatidylinositol (GPI) membrane anchor (51). Ciliary neurotrophic factor receptor, another GPI-linked receptor, has been shown to participate in signaling despite the lack of an intracytoplasmic domain. This receptor has partial sequence homology with the IL-6 receptor (52), which may transduce its signal by coupling to a nonligand-binding glycoprotein, gp130 (53, 54). Further, GPI-linked receptor proteins have been shown to associate with protein kinases (55). These reports provide precedence for a possible mechanism by which GPI-linked receptors such as the uPA receptor may participate in signal transduction.

Of note at 24 h is a low molecular mass cell-surface protein present at  $\approx 22$  kD in cells exposed to active uPA (Fig. 8 B). This band may represent an amino-terminal fragment of uPA known to exist as a naturally occurring protein in human urine (56). Its presence may explain at least in part the ability of active uPA to induce a signal for adherence despite active turnover. Amino-terminal portions of the uPA molecule containing only the receptor-binding domain induce adherence in cytokine-primed cells. The absence of this protein on DFP-uPA-exposed cells suggests that processing of uPA, possibly during turnover in association with PAI, results in production of this fragment. It is not known if U937 cells possess an endogenous pathway for generation of an amino-terminal fragment of uPA and, if so, what the role of such a fragment in cellular adherence may be. This is a subject for future study.

This study provides evidence for clearance of both active and DFP-inactivated uPA from the cell surface (Fig. 8). This contrasts with an earlier report (47), where little degradation of active uPA or DFP-uPA was seen in U937 cells. Two significant differences in methodology exist between the present report and the previous one. The present experiments used cytokine-stimulated rather than unstimulated cells and the time period of observation was extended to 24 h rather than 3 h. Either or both of these differences may explain the apparent discrepancy in results. The previous report also presented data that lysosomal degradation was important in the turnover of uPA/PAI-1 complexes from the uPA receptor: The present study extends these observations by confirming an increase in cell-surface-bound uPA and DFP-uPA (Fig. 8 B) under conditions in which lysosomal degradation is impeded, and by showing a functional correlate of changes in surface-bound uPA.

The observations presented here expand on the known functions of uPA and PAI in localized extracellular proteolysis by providing evidence of a regulatory role for the interaction of uPA and PAI in cellular adherence. Although we have not specifically studied migration and the adhesive mechanisms are undefined, possible implications of these findings for cellular migration through the extracellular matrix are as follows. The production of uPA by the cell and subsequent binding to its cell surface receptor focuses proteolytic activity at the leading edge of the cell and also results in local cellular adherence while proteolysis of the local extracellular matrix takes place. PAI-1, released from binding to vitronectin in the extracellular matrix (57) or produced by the cell under the influence of cytokines, and/or PAI-2, complexes with uPA, effectively abrogating proteolytic activity. The turnover of the uPA/PAI complex then occurs, simultaneously removing the stimulus for strong adherence, thus allowing migration and clearing the uPA receptor so that new or recycled receptors can again bind active uPA to

continue extracellular matrix proteolysis at a new site. An implication of this hypothesis is that receptor-binding fragments of the urokinase molecule that are neither proteolytically active nor rapidly cleared from the uPA receptor could interfere with migration of monocytic cells and thus be used to modulate inflammatory processes in which monocyte/macrophage accumulation is prominent. Future studies are necessary to test directly the influence of receptor-bound but inactive uPA on cellular migration.

## Acknowledgments

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