

## Plasminogen activator inhibitor type 2: a regulator of monocyte proliferation and differentiation

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We have explored the role of plasminogen activator inhibitor type 2 (PAI-2) in THP-1 monocyte-like cells. These cells possess a mutation in the *PAI-2* gene and do not produce an active PAI-2 protein. Transfection of THP-1 cells with plasmids expressing active PAI-2 reduced the cells' inherent adhesive properties and decreased the rate of cell proliferation. THP-1 cells expressing active PAI-2 also displayed an altered phenotype in response to phorbol ester-induced differentiation

that was concomitant with a reduction in CD14 expression. THP-1 cells transfected with a variant PAI-2 containing a mutation in the reactive center (PAI-2<sub>Ala380</sub>) displayed no noticeable change in any of these parameters, suggesting the involvement of a PAI-2-sensitive serine protease(s). The antiproliferative effect of PAI-2 was attenuated by treating the PAI-2-expressing THP-1 cells with recombinant urokinase (u-PA), suggesting that PAI-2 was disruptive of a u-PA/u-PA receptor signal-

ing pathway initiated on the cell surface. Consistent with this, treatment of wild-type THP-1 cells with recombinant PAI-2 also caused a reduction in cellular proliferation. These results implicate endogenous PAI-2 as a modulator of monocyte adhesion, proliferation, and differentiation. (Blood. 2002;99:2810-2818)

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### Introduction

The maintenance of the cellular microenvironment relies on controlled proteolytic cleavage and degradation of extracellular matrix molecules.<sup>1</sup> One of the most potent catalysts of extracellular proteolysis is the plasminogen activating (PA)/plasmin system. Cleavage of plasminogen to plasmin is achieved by the plasminogen activators: tissue-type PA (t-PA), which functions mainly in the context of intravascular fibrinolysis<sup>2</sup> and in turnover of the extracellular matrix<sup>3</sup>; and urokinase-type PA (u-PA), which, in conjunction with its specific cell surface receptor, uPAR, plays a role in the regulation of pericellular proteolysis.<sup>4</sup> The activities of the plasminogen activators are regulated by at least 2 specific serine protease inhibitors. Plasminogen activator inhibitor (PAI) type 1 is a major inhibitor of t-PA, whereas plasminogen activator inhibitor-2 (PAI-2) exhibits inhibitory activity mainly toward u-PA and is less effective against t-PA.<sup>5</sup>

Among the PA family members, PAI-2 is considered the most enigmatic.<sup>6</sup> Based on gene structure and amino acid sequence homology, PAI-2 belongs to the Ov-serpin subfamily.<sup>7-10</sup> Like all Ov-serpins, PAI-2 lacks a typical N-terminal secretory signal peptide and contains a relatively inefficient internal secretory signal sequence.<sup>11</sup> Although a variable fraction is secreted as a 60- to 70-kd glycosylated protein, most PAI-2 appears to be retained in the cytoplasm in a nonglycosylated (47 kd) form that is capable of spontaneous polymerization, particularly at high expression levels.<sup>12</sup> The functional significance of this large intracellular pool of PAI-2 is unknown, though several studies suggest that it may contribute a cellular resistance to tumor necrosis factor- $\alpha$ -mediated apoptosis.<sup>13-15</sup> In a recent study, transgenic mice overexpressing PAI-2 in keratinocytes facilitated the generation of

papillomas in response to treatment with tumor promoters. These authors also presented evidence to suggest that the overexpressed PAI-2 was inhibiting apoptosis.<sup>16</sup> In this context, it is also interesting that exogenous PAI-2 reduces the proliferation rate of keratinocytes<sup>17</sup> while an increase in PAI-2 expression correlates with the process of keratinocyte differentiation.<sup>18</sup>

PAI-2 has also been shown to confer protective effects against viral infections and to modulate the expression of intercellular adhesion molecular-1, decay-accelerating factor, and p53<sup>19</sup> and to inhibit u-PA/uPAR-mediated cell motility.<sup>20</sup> In the context of tumor biology, increases in PAI-2 levels have been shown to reduce tumor progression and metastasis.<sup>21</sup> The *PAI-2* gene is highly expressed in monocytes and macrophages.<sup>22,23</sup> The presence of PAI-2 in these cells has prompted the use of a variety of human monocytic cell lines (U-937, K562, HL-60) to investigate *PAI-2* gene regulation and cell biology. Although most monocytic cell lines express and regulate PAI-2, there is one notable exception. THP-1 monocytes contain a unique defect in the production of PAI-2.<sup>24</sup> In these cells, the PAI-2 transcript is truncated while no u-PA inhibitory activity can be found. Recently, we reported that the THP-1-derived PAI-2 transcript was missing the first 6 exons but instead contained a 180 nucleotide fragment of the intron 5 of the *PAI-2* gene followed by all of exons 7 and 8 of the normal *PAI-2* transcript.<sup>25</sup> This transcript does not contain an in-frame ATG and cannot therefore synthesize a PAI-2 protein. Interestingly, despite the absence of PAI-2 in these cells, THP-1 cells have been used to investigate cell surface-associated plasminogen activation and the signaling pathways initiated by engagement of u-PA with its receptor (uPAR).<sup>26</sup>

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THP-1 cells can be considered as a human PAI-2<sup>-/-</sup> cell system. Here we have exploited the THP-1 cells to explore the biology of PAI-2 by introducing into these cells an active PAI-2 or a mutant PAI-2 that possessed an amino acid substitution in the reactive center (P1 residue) of the PAI-2 protein. Our results indicate that the presence of wild-type PAI-2 in THP-1 monocytes reduces cell-cell adhesion and cell proliferation in a process that is dependent on the presence of the P1 residue. Evidence is provided to suggest that PAI-2 interferes with a u-PA/uPAR-dependent adhesion and signal transduction processes that are initiated on the cell surface. Finally, the presence of active PAI-2 also markedly altered the ability of THP-1 cells to differentiate in response to phorbol ester treatment. Taken together, these results implicate an important regulatory role for PAI-2 in monocyte cell proliferation and differentiation.

## Materials and methods

### Reagents

Human recombinant PAI-2 was a gift from Delta Biotechnology (Nottingham, United Kingdom) and recombinant u-PA from ARES Serono (Frensch Forest, NSW, Australia). Anti-u-PA, anti-uPAR, and anti-PAI-2 antibodies were purchased from American Diagnostica (Greenwich, CT). Fluorescein isothiocyanate-conjugated mouse anti-human CD14 monoclonal antibody was purchased from BD Biosciences (San Diego, CA). Methyl-<sup>3</sup>H-thymidine (662.3 GBq/mM [100 Ci/mM]) was obtained from NEN Life Science Products (Boston, MA). Phorbol 12-myristate 13-acetate (PMA) was purchased from Sigma (Saint Louis, MO). RPMI 1640 culture medium was obtained from Life Technologies (Rockville, MD).

### Construction of wild-type and mutant PAI-2 expression vectors

To construct the wild-type PAI-2 expression vector, plasmid pJ7 containing the 1872-base pair PAI-2 complementary DNA (cDNA)<sup>27</sup> was digested with *EcoRI* and the 1872 base pair fragment removed and inserted into the *EcoRI* site of the expression plasmid pCI-neo (Promega, Madison, WI), creating pCI-PAI-2. The mutant PAI-2 expression vector was created by the site-specific mutagenesis using standard techniques using pCI-PAI-2 as a template. The P1 arginine residue at position 380 was replaced with an alanine residue to create plasmid pCI-PAI-2<sub>Ala380</sub> (Figure 1A). The mutagenic primer used for this purpose had the following sequence: 5'-CATGACAGGCGCCACTGGACATG-3'; the underlined residues are the mutations introduced into the PAI-2 sequence.

### Cell culture

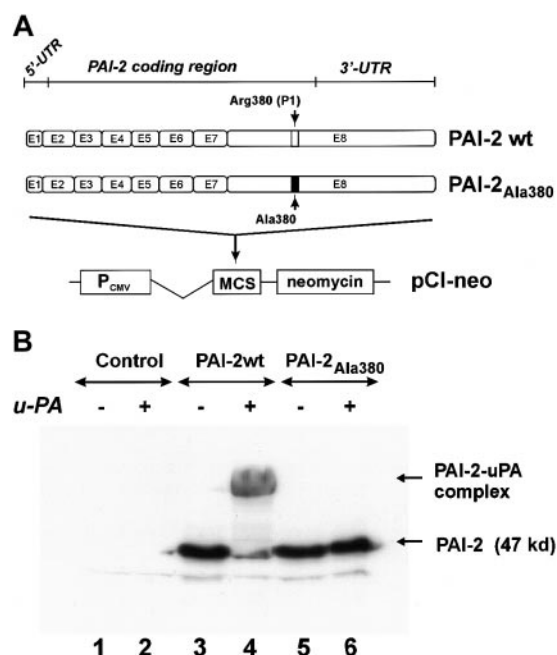
Human THP-1 monocytic leukemia cells were maintained in RPMI 1640 medium containing 10% heat-inactivated fetal calf serum as previously described.<sup>25</sup> COS cells were maintained under similar conditions but using Dulbecco modified Eagle medium.

### Transient transfection and preparation of cellular extracts

COS cells were transiently transfected with the PAI-2 expression plasmids by the calcium phosphate method<sup>28</sup> using 5 μg plasmid DNA. Cells were subjected to a 15% glycerol shock (2 minutes) and then washed twice with phosphate-buffered saline (PBS) (0.9% NaCl in 10 mM sodium phosphate pH 7.0). Fresh Dulbecco modified Eagle medium containing 10% fetal calf serum was then added and cells were maintained for up to 48 hours. Cytoplasmic protein was extracted using the Nonidet P-40 (NP-40) lysis method<sup>29</sup> as described below.

### Generation of stably transfected THP-1 cells

Plasmids pCI-PAI-2 and pCI-PAI-2<sub>Ala380</sub> were linearized with *BamHI* and transfected into THP-1 cells by electroporation. THP-1 cells (1 × 10<sup>7</sup>) were



**Figure 1. Generation of wild-type and mutant (Ala380) PAI-2 expression plasmids.** (A) Schematic representation of the full-length wild-type (wt) and mutant (Ala380) PAI-2 cDNAs cloned into the multiple cloning site (MCS) of plasmid pCI-neo. The mutant PAI-2 expression plasmid contains an alanine substitution for arginine at the P1 residue at position 380 and is denoted as pCI-PAI-2<sub>Ala380</sub>. E indicates exon. (B) PAI-2 expression and u-PA binding activity in COS cells transfected with either pCI-neo, pCI-PAI-2wt, or pCI-PAI-2<sub>Ala380</sub> was assessed by Western blot analysis using an anti-PAI-2 antibody. Extracts (100 μg) were incubated with u-PA (10 U) as indicated. As shown, cells transfected with the pCI-PAI-2wt plasmid produce a protein of 47 kd (lane 3) that forms SDS-stable complex with recombinant u-PA (lane 4). Cells expressing mutant PAI-2, although producing a protein of a similar molecular weight (lane 5), cannot form SDS-stable complexes with u-PA (lane 6). Lanes 1 and 2: extracts prepared from COS cells transfected with the control pCI-neo plasmid.

washed in 10 mL PBS containing 10 mM MgCl<sub>2</sub> and then centrifuged and resuspended in 800 μL of the same solution; 20 μg plasmid DNA was then added and incubated for 10 minutes at room temperature. Electroporation was performed using a Cell-Porator (300 V, 800 microfarads; Bethesda Research Laboratories, Life Technologies). After discharging, cells were put on ice for 2 minutes and then transferred into a 50-mL Falcon tube containing 20 mL serum-free medium and further incubated for 20 minutes at room temperature. Cells were centrifuged then resuspended in 6 mL RPMI medium, and 1 mL aliquots were dispensed into the wells of a 24-well plate and incubated for 2 days to recover. Selection was achieved by the addition of geneticin (G418; 1 mg/mL). After 5 days, individual cells were selected and removed using a pipette viewed using a microscope and grown separately to establish the stably transfected THP-1 cell clones. Seventy-two stably transfected (G418-resistant) cell lines were initially established expressing either PAI-2 variant while 10 clones transfected with the pCI-neo control plasmid were selected as controls. PAI-2 antigen levels in cytoplasmic extracts prepared from each clone were assessed by Western blot analysis. Twelve clones from each group of THP-1 cells transfected with either wild-type or mutant PAI-2 expression plasmids selected from this initial screen were assessed more closely. From these, 4 clones from each PAI-2-transfected group and 4 clones expressing pCI-neo were selected for experimentation.

### Western blot analysis for PAI-2

For Western blot assays, cells were rinsed twice with ice-cold PBS and lysed in NP-40 lysis buffer (10 mM Tris-HCl [pH 7.4], 10 mM NaCl, 3 mM MgCl<sub>2</sub>, 0.5% NP-40, 5 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride) for 30 minutes on ice. After centrifugation, the protein content of the supernatants was measured using the Bio-Rad protein reagent (Bio-Rad, Hercules, CA) using bovine serum albumin as a protein standard. Total

cellular extract (20–100  $\mu\text{g}$ ) was subjected to 10% sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) and blotted onto a polyvinylidene fluoride membrane. After blocking for 2 hours at room temperature with TBST buffer (10 mM Tris-HCl [pH 8.0], 150 mM NaCl, 0.05% Tween 20) containing 5% nonfat dry milk, the membrane was washed and incubated with a primary anti-PAI-2 antibody (1:1000 dilution) at 4°C overnight. The blots were then washed 3 times in TBST buffer and incubated with appropriate horseradish peroxidase–conjugated secondary antibody (1:10 000 dilution) for 1 hour at room temperature. Immunoreactive proteins were detected by the enhanced chemiluminescence system (ECL reagents, NEN Life Science Products).

To determine whether the expressed PAI-2 protein retained u-PA binding activity, cytoplasmic extracts (30–100  $\mu\text{g}$ ) were incubated with recombinant u-PA (0.1 to 10 U) for 15 minutes at 37°C and samples were electrophoresed through a 10% SDS–PAGE gel under reducing conditions. Samples were transferred to polyvinylidene fluoride membranes using standard techniques, and PAI-2 antigen and formation of PAI-2/u-PA complexes were assessed by Western blot analysis using a monoclonal anti-PAI-2 antibody.

### Measurement of PAI-2 antigen

Quantification of PAI-2 antigen in cytoplasmic extracts prepared from the THP-1 cells transfected with pCI-PAI-2 and pCI-PAI-2<sub>Ala380</sub> was performed by enzyme-linked immunosorbent assay (ELISA) using a commercial kit (TintElize PAI-2; Biopool, Umea, Sweden) following the manufacturer's instructions.

### Assessment of cell proliferation and morphology

Cell suspensions (1 mL) were seeded into 24-well plates at a starting density of  $1 \times 10^5$  cells per well in RPMI containing 10% fetal calf serum. Cell proliferation was measured by counting cells at 24-hour intervals (in triplicate) over a 5-day period. <sup>3</sup>H-thymidine incorporation (described below) was also used to assess DNA synthesis. Where indicated, recombinant human PAI-2 (1  $\mu\text{M}$ ) or antibodies directed against uPAR (anti-uPAR; American Diagnostica) were added (5  $\mu\text{g}/\text{mL}$  final concentration) to cultures of wild-type THP-1 cells for up to 5 days and the rate of cell proliferation was assessed. The degree of cell-cell adhesion was assessed by determining the number of free cells as a percentage of all cells in at least 5 separate fields.

### <sup>3</sup>H-thymidine incorporation

Cells were seeded in triplicate at a density of  $1 \times 10^5$  cells per well in RPMI containing 10% fetal calf serum. Cells were cultured for 12 hours and then pulsed with 18.5 kBq/mL <sup>3</sup>H-thymidine for a further 16 hours. After washing in ice-cold PBS, cells were fixed with 100  $\mu\text{L}$  acetic acid/methanol (3:1) for 10 minutes. The soluble radioactivity was extracted with 500  $\mu\text{L}$  ice-cold 10% trichloroacetic acid at 4°C for 30 minutes. After centrifugation, the precipitates were washed with 10% trichloroacetic acid and dissolved in 200  $\mu\text{L}$  of 0.2 M NaOH at 37°C for 2 hours. The incorporated radioactivity was quantified by liquid scintillation counting.

### Fibrin zymography

The u-PA activity in cellular extracts and in subcellular fractions was detected by fibrin zymography according to the method of Granelli-Piperno and Reich.<sup>30</sup> The preparation of subcellular fractions was performed as described<sup>31</sup> with modifications. Cells ( $10^7$ ) were lysed in a hypotonic buffer (10 mM Tris-HCl [pH 7.4], 3 mM MgCl<sub>2</sub>) by dounce homogenization (30 strokes) and the nuclear fraction pelleted by centrifugation at 1000 rpm for 10 minutes at 4°C. The nuclear pellets were resuspended in 100  $\mu\text{L}$  NP-40 lysis buffer, vortexed, and left on ice for 30 minutes and then finally centrifuged at 14 000 rpm for 20 minutes at 4°C. The supernatant containing the soluble nuclear fraction was kept and stored at –80 degrees until use.

For the preparation of the cytosolic and particulate (membrane) fractions, supernatants obtained after the initial dounce homogenization (above) were centrifuged at 45 000 rpm for 1 hour to separate the cytosolic

fraction from the particulate fraction. The supernatant containing the cytosolic fraction was kept for analysis. The pellet containing the particulate fraction was resuspended in 100  $\mu\text{L}$  NP-40 lysis buffer, left on ice for 30 minutes, and then centrifuged at 14 000 rpm for 20 minutes and the supernatant collected and kept at –80 degrees prior to analysis. The protein content of the subcellular fractions was measured using the Bio-Rad protein assay system.

To perform the zymographic analyses, conditioned medium (20  $\mu\text{L}$ ) or protein extract (100  $\mu\text{g}$ ) was subjected to 10% SDS–PAGE. The gel was washed twice in 2.5% Triton X-100 to remove SDS, layered onto a 0.8% low melting agarose gel containing polymerized fibrinogen and plasminogen, and then incubated at 37°C in a humidified chamber.

### Flow cytometric analysis

Flow cytometry was used to quantitate the level of CD14 expression on the surface of THP-1 cells expressing either active or mutant PAI-2. CD14, a monocyte differentiation marker, is constitutively expressed on THP-1 cells, and levels are further increased following exposure to PMA.<sup>32,33</sup> Suspensions of THP-1 cells (3 mL) transfected with either pCI-neo, pCI-PAI-2, or pCI-PAI-2<sub>Ala380</sub> were seeded in triplicate into 6-well plates at a starting density of  $1 \times 10^6$  cells per well in RPMI medium containing 10% fetal calf serum. Cells were left either untreated or treated with 25 nM PMA for 3 days. Untreated and PMA-treated cells were washed in diluent (0.01 M PBS [pH 7.4] containing 1% bovine serum albumin and 0.1% sodium azide) and then incubated with fluorescein isothiocyanate–conjugated anti-human CD14 antibody (10  $\mu\text{L}$  antibody in 300  $\mu\text{L}$ ) for 30 minutes at room temperature. After washing with 2 mL diluent, cells were resuspended in 0.5 mL diluent and the percentage of CD14<sup>+</sup> cells was assessed using a Becton Dickinson FACSCalibur flow cytometer.

### Statistical analyses

Data are presented as mean  $\pm$  SEM of at least 3 separate experiments performed in triplicate. Statistical analysis was performed by analysis of variance.  $P < .05$  was considered statistically significant.

## Results

### Expression analysis of wild-type and mutant PAI-2 protein in COS cells

To confirm that the plasmids harboring the wild-type and mutant PAI-2 (PAI-2<sub>Ala380</sub>) cDNAs were indeed able to express functional or nonfunctional PAI-2 protein, respectively, plasmids pCI-PAI-2 and pCI-PAI-2<sub>Ala380</sub> (Figure 1A) were transiently transfected into COS cells. Transfected cells were harvested after 2 days and PAI-2 protein expression and u-PA binding ability in total cell extracts was assessed by Western blotting. As shown in Figure 1B, cells transfected with both the wild-type and mutant PAI-2 expression plasmid produced a 47 kD PAI-2 protein (lanes 3 and 5, respectively), whereas PAI-2 was not produced in mock-transfected cells (lanes 1 and 2). The expressed wild-type PAI-2 protein was active: incubation with exogenous 2-chain u-PA resulted in the formation of a SDS-stable complex (Figure 1B, lane 4). In contrast, addition of u-PA to extracts containing the mutant PAI-2 did not result in the formation of a SDS-stable complex (Figure 1B, lane 6). This is consistent with previous reports that have demonstrated the dependence of Arg380 for u-PA inhibitory activity. These results confirm that PAI-2 expressed from wild-type PAI-2 cDNA can form SDS-stable complexes with u-PA and that this is abolished by the substitution of arginine at the P1 position for alanine.

### Generation of THP-1 cell clones expressing wild-type or mutant PAI-2

To study PAI-2–dependent changes on monocyte cell function, plasmids pCI-PAI-2 and pCI-PAI-2<sub>Ala380</sub> were stably transfected into THP-1 cells and 4 individual clones of each group were established (clones a-d expressing wild-type PAI-2 and clones e-h expressing PAI-2<sub>Ala380</sub> [mutant PAI-2]). Western blot analysis was performed to determine the levels of PAI-2 protein in cytoplasmic extracts (100  $\mu$ g). As shown in Figure 2 (upper panel), PAI-2 expression was absent in THP-1 cells transfected with pCI-neo (“neo”; lane 1) but was detected in all 4 clones of THP-1 cells expressing wild-type PAI-2 (wt PAI-2; lanes 2 to 5) and in all 4 clones expressing the mutant PAI-2 (mut PAI-2; lanes 6 to 9). Some variation in the level of PAI-2 expression was observed.

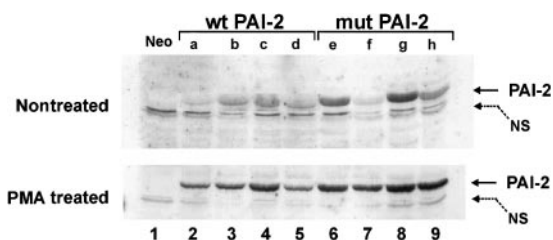
Interestingly, treatment of all PAI-2–expressing cells (both wild-type and mutant) with 25 nM PMA for 24 hours resulted in a further increase (minimum 5-fold) in PAI-2 expression (Figure 2, lower panel; only 20  $\mu$ g protein loaded per lane). This increase in PAI-2 protein expression following PMA treatment is most likely due to inducible stabilization of PAI-2 messenger RNA occurring under these conditions.<sup>34,35</sup> The ability to further increase PAI-2 expression was exploited for various functional studies (below).

### Quantitation of PAI-2 antigen in transfected THP-1 cells

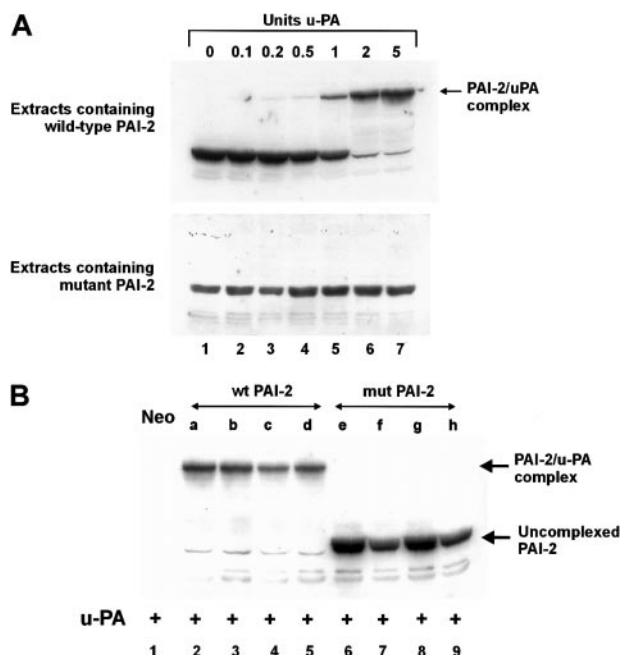
Quantitation of PAI-2 antigen in the cytoplasmic extracts from all clones expressing wild-type and 2 clones expressing mutant PAI-2 clones under constitutive conditions was determined by ELISA. Results indicated that PAI-2 expression levels in these clones varied from 7.64 to 23.22 ng/mg protein for cells expressing wild-type PAI-2 and 22.2 to 35 ng/mg protein for cells expressing mutant PAI-2. These concentrations compare favorably to the levels of PAI-2 detected in normal human monocytes (approximately 20 ng/mg protein<sup>36</sup>).

### PAI-2 expressed in THP-1 cells retains u-PA binding activity

To verify that the PAI-2 expressed in THP-1 cells also retained u-PA binding activity, cytoplasmic extracts prepared from one of the THP-1 clones expressing either wild-type PAI-2 (clone c) or mutant PAI-2 (clone g) were incubated with increasing amounts of recombinant u-PA (0.1 to 5 U) and incubated at 37°C for 15



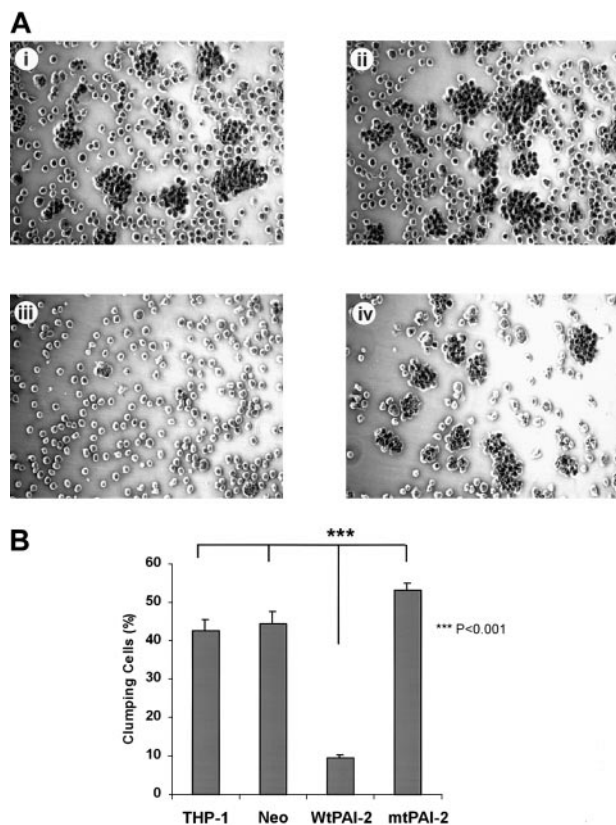
**Figure 2. Expression of wild-type and mutant PAI-2 in THP-1 cell clones.** Cytoplasmic extracts (100  $\mu$ g) prepared from nontreated THP-1 cells (clones a-d) expressing wild-type PAI-2 (wt PAI-2, lanes 2-5) and from cells expressing mutant PAI-2 (mut PAI-2, clones e-h) (lanes 6-9) were subjected to SDS-PAGE analysis. Relative levels of PAI-2 protein were assessed by Western blot analysis using a monoclonal anti-PAI-2 antibody. As shown in the upper panel, PAI-2 protein is present in all clones expressing either wild-type or mutant PAI-2. Lane 1: extracts prepared from pCI-neo–transfected cells. The broken arrow to the right of the figure indicates nonspecific (NS) binding. To determine whether PMA could further increase PAI-2 protein, all clones of THP-1 cells expressing active or mutant PAI-2 were treated with 25 nM PMA for 24 hours. Cytoplasmic extracts (20  $\mu$ g) were prepared and subjected to SDS-PAGE and Western blot analysis as described above. As shown in the lower panel, PMA treatment caused at least a 5-fold increase in PAI-2 protein in all clones of cells expressing either wild-type or mutant PAI-2.



**Figure 3. Functional assessment of PAI-2 expressed in THP-1 cells.** (A) To determine whether the wild-type PAI-2 expressed in THP-1 cells retained u-PA binding activity, cytoplasmic extracts (30  $\mu$ g) prepared from THP-1 cells expressing wild-type PAI-2 (clone c) were incubated with increasing concentrations of u-PA (0-5 U) as indicated at the top of the figure. PAI-2 protein was detected by Western blot analysis using a monoclonal anti-PAI-2 antibody. As shown, addition of u-PA resulted in complex formation that was evident when using 1 U u-PA (lane 5). Complete displacement of PAI-2 was obtained with 5 U u-PA (lane 7). The same procedure was performed using cytoplasmic extracts (30  $\mu$ g) prepared from THP-1 cells expressing mutant PAI-2 (clone g). No complex formation is with u-PA even at the highest concentration of u-PA added (lower panel). (B) Cytoplasmic extracts (30  $\mu$ g) prepared from all clones of THP-1 cells expressing wild-type PAI-2 (clones a-d) and all clones of cells expressing mutant PAI-2 (clones e-h) were incubated with 2 U u-PA. Samples were then applied to SDS-PAGE and PAI-2 protein detected by Western blot analysis as described above. As shown, all clones of cells expressing wild-type PAI-2 contained active inhibitor, as evidenced by the formation of SDS-stable complex formation (lanes 2-5). In contrast, all clones expressing mutant PAI-2 failed to produce a complex with u-PA (lanes 6-9). Lane 1: extracts prepared from cells transfected with pCI-neo.

minutes. In this experiment, extracts were obtained from PMA-treated cells to increase the levels of PAI-2 protein. Samples (30  $\mu$ g) were subjected to SDS-PAGE and transferred to nylon membranes. PAI-2 antigen and PAI-2/u-PA complexes were detected by Western blot analyses using a monoclonal anti-PAI-2 antibody. THP-1 cells expressing active PAI-2 produced SDS-stable complexes with u-PA (Figure 3A, upper panel). In contrast, THP-1 cells expressing mutant PAI-2 failed to generate SDS-stable complexes with u-PA (Figure 3A, lower panel).

To verify that this was a consistent feature of all clones of THP-1 cells expressing active or mutant PAI-2, extracts prepared from all clones of THP-1 cells (PMA treated) were incubated with u-PA and the formation of SDS-stable u-PA/PAI-2 complexes was determined by Western blotting as described above. As shown in Figure 3B, all 4 clones of THP-1 cells expressing active PAI-2 contained u-PA binding activity because the addition of u-PA complexed all available PAI-2 (lanes 2-5). In contrast, the presence of u-PA to extracts of cells expressing mutant PAI-2 failed to generate complexes (Figure 3B, lanes 6-9). These data confirm that all 4 clones of THP-1 cells expressing wild-type PAI-2 produce an active protease inhibitor, while the mutant PAI-2 expressed in all THP-1 clones cannot associate with u-PA and is therefore functionally inactive.



**Figure 4. THP-1 cells stably expressing wild-type PAI-2 display a loss of cell-cell adhesion.** (A) Representative images of nontransfected THP-1 cells (i), cells transfected with pCI-neo (ii), pCI-PAI-2wt (clone c) (iii), or pCI-PAI-2<sub>Ala380</sub> (clone g) (iv). Phase contrast photomicrographs (magnification  $\times 200$ ). Cells transfected with wild-type PAI-2 do not grow as clumps but instead grow as individual cells in suspension. (B) Quantitation of results presented in panel A. Percentage of single cells compared with total cell number was determined in 5 separate fields.

#### Expression of active PAI-2 reduces cell-cell adhesion

THP-1 cells typically grow as a cell suspension in clumps. As shown in Figure 4 Ai,ii, both nontransfected THP-1 cells and cells transfected with plasmid pCI-neo displayed this classic phenotype. However, THP-1 cells transfected with wild-type PAI-2 (clone c) did not display this clumping phenotype and instead grew as single-cell suspensions (Figure 4Biii). In contrast, cells transfected with the mutant PAI-2 (clone g) exhibited normal morphology (Figure 4Biv); clump formation was maintained as seen with both the wild-type THP-1 cells and the control pCI-neo-transfected cells. Representative results from one clone of THP-1 cells expressing either wild-type or mutant PAI-2 are presented in the figure. However, all clones of THP-1 cells expressing active PAI-2 displayed substantially reduced clump formation, whereas all clones expressing the mutant PAI-2 maintained the normal clumping phenotype. To quantitate this effect, the percentage of single cells relative to total cells was assessed in 5 separate fields by microscopy (Figure 4B). As shown, the presence of active PAI-2, but not mutant PAI-2, reduced the inherent cell-cell adhesive properties of these cells by approximately 80%.

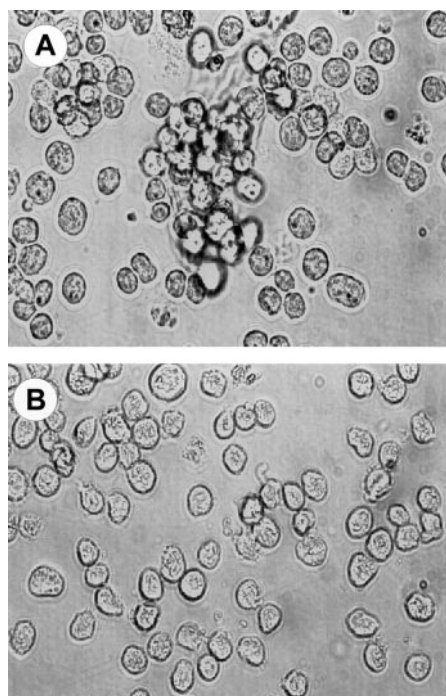
The lack of effect of the expressed mutant PAI-2 to inhibit cell-cell adhesion suggests that the antiadhesive properties mediated by PAI-2 are associated with the inhibition of a serine protease.

#### Reduction in cell-cell adhesion of normal THP-1 cells by anti-uPAR antibodies

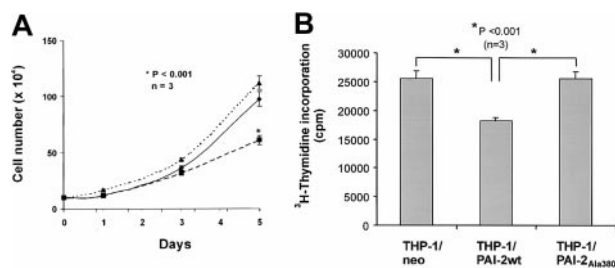
The u-PA/uPAR on the cell surface has previously been shown to play a role in the adhesiveness of monocytes.<sup>37,38</sup> Because u-PA is the only known target for PAI-2, we considered the possibility that some of the expressed PAI-2 was being released from cells and binding to u-PA and, in doing so, altered the adhesive capacity of uPAR. To determine whether disruption of the uPAR would produce a similar reduction in cell-cell adhesion, anti-uPAR antibodies were added to suspensions of wild-type THP-1 cells for 3 days (final concentration 5  $\mu\text{g}/\text{mL}$ ). As shown in Figure 5A, cells cultured in the absence of antibody grew in clumps ( $46\% \pm 2\%$  clumped cells,  $n = 5$  randomized microscopic fields), while cells cultured in the presence of the anti-u-PAR antibody (Figure 5B) remained mostly as individual cells ( $9\% \pm 1\%$  clumped cells,  $n = 5$ ,  $P < .001$ ). This is very similar to the effect of active PAI-2 in transfected THP-1 cells and is consistent with the possibility that the antiadhesive effect of PAI-2 involves inhibition of u-PA binding to uPAR on the cell surface.

#### PAI-2 reduces the rate of cell proliferation and DNA synthesis

Experiments were conducted to determine whether the presence of PAI-2 altered the proliferation rate of THP-1 cells. As shown in Figure 6A, cells expressing wild-type PAI-2 (clones b and c) displayed a significant reduction in their proliferation rate compared with cells transfected with pCI-neo ( $P < .001$ ,  $n = 3$  on day 5). In contrast, cells expressing PAI-2<sub>Ala380</sub> (clones e and g) did not demonstrate any significant change in cell proliferation compared with control pCI-neo-transfected cells. These results indicate that the expression of PAI-2 in the THP-1 cells reduces cell proliferation and that this effect depends on the P1 residue in the PAI-2 protein, again suggesting the involvement of a serine protease.



**Figure 5. Cell-cell adhesion of wild-type THP-1 cells is inhibited by anti-uPAR antibodies.** Wild-type (nontransfected) THP-1 cells were incubated with normal RPMI medium (control) (A) or with 5  $\mu\text{g}/\text{mL}$  anti-uPAR antibodies (B) for 3 days. As shown, the presence of the anti-uPAR antibody reduces the ability of the cells to clump.



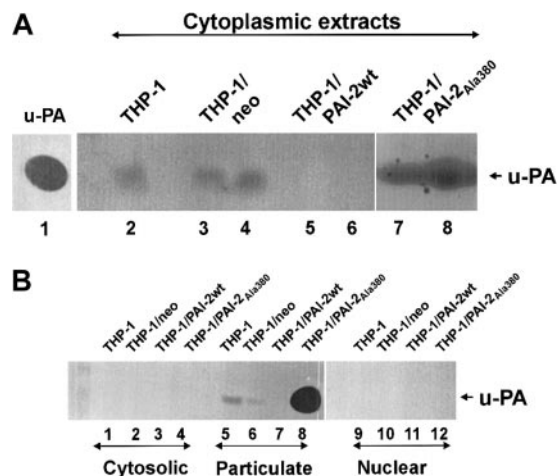
**Figure 6. Effect of PAI-2 on THP-1 cell proliferation and DNA synthesis.** (A) The proliferation rate of 2 clones of THP-1 cells transfected with either pCI-neo (◆), pCI-PAI-2wt (■; clones b and c), or pCI-PAI-2Ala380 (▲; clones e and g) was assessed over a 5-day period. As shown, the presence of wild-type PAI-2 (PAI-2wt) but not mutant PAI-2 (PAI-2Ala380) significantly reduced the rate of cell proliferation ( $P < .001$ ). This experiment was performed on 3 separate occasions using triplicate cultures at each time point. (B) The rate of DNA synthesis in THP-1 cells transfected with the control pCI-neo plasmid ("neo") or expressing wild-type PAI-2 ("PAI-2wt"; clones a and c) or mutant PAI-2 ("PAI-2Ala380"; clones f and g) was determined by <sup>3</sup>H-thymidine incorporation. Cells expressing active PAI-2 displayed a significant reduction in DNA synthesis. Data presented are the average results of 3 separate experiments performed in triplicate using 2 clones from each group.

To provide further evidence that PAI-2 had reduced cell proliferation, the rate of DNA synthesis in clones b and c of THP-1 cells expressing wild-type PAI-2 and clones a and c of cells expressing mutant PAI-2 was assessed by <sup>3</sup>H-thymidine incorporation. As shown in Figure 6B, PAI-2-transfected cells displayed a 30% reduction in <sup>3</sup>H-thymidine incorporation compared with the cells transfected with either pCI-neo or pCI-PAI-2Ala380 ( $P < .001$ ,  $n = 3$ ). These results indicate that the suppressive effect of PAI-2 on the cell proliferation is associated with inhibition of DNA synthesis.

#### Altered u-PA activities in PAI-2-transfected THP-1 cells

Binding of u-PA to its receptor, uPAR, has also been shown to initiate an intracellular signaling pathway that can increase proliferation of monocytes and other cells.<sup>39-41</sup> We tested the hypothesis that the antiproliferative effect of PAI-2 was associated with changes in u-PA expression on the cell surface. To this end, the u-PA activities in the conditioned medium and cellular extracts were examined by fibrin zymography. No u-PA activity could be detected in the conditioned medium from wild-type THP-1 cells or any of the transfected THP-1 cells (data not shown). However, a moderate level of u-PA activity was detected in cytoplasmic extracts prepared from wild-type THP-1 cells (Figure 7A, lane 2) and in cytoplasmic extracts from 2 clones of THP-1 cells transfected with the control pCI-neo plasmid (lanes 3 and 4). In contrast, u-PA activity was not detected in protein extracts prepared from THP-1 cells transfected with pCI-PAI-2wt but was found in cells overexpressing the mutant (PAI-2Ala380) protein (Figure 7A, lanes 5 and 6). Curiously, relatively high levels of u-PA activity were found in the THP-1 cells expressing mutant PAI-2.

To determine which cell compartment contained the u-PA activity, zymographic analyses were performed using extracts prepared from the cytosolic, particulate, and nuclear fractions of THP-1 cells. As shown in Figure 7B, u-PA activity was restricted to the particulate fraction (lanes 5-8), suggesting that u-PA activity was confined to the cell surface. Consistent with the results shown in Figure 7A, u-PA activity was not at all detected in the particulate fraction cells expressing wild-type PAI-2 (lane 7), whereas relatively high u-PA activities were again present in cells transfected with the mutant PAI-2 (lane 8). These data suggest that the presence of active PAI-2 inhibits cell surface-located u-PA. The reason for the particularly high increase in u-PA in cells expressing the mutant



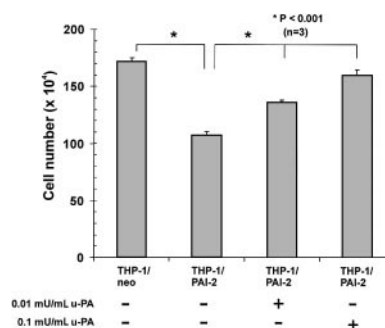
**Figure 7. Endogenous u-PA activity is inhibited in THP-1 cells expressing wild-type PAI-2.** (A) Zymographic analyses of u-PA activity in cytoplasmic extracts prepared from nontransfected THP-1 cells (lane 2) or 2 representative clones of THP-1 cells transfected with pCI-neo (THP-1/neo; lanes 3 and 4), THP-1 clones (a and c) expressing wild-type pCI-PAI-2 (THP-1/PAI-2wt; lanes 5 and 6), and THP-1 clones (f and h) expressing mutant PAI-2 (THP-1/PAI-2Ala380; lanes 7 and 8). Recombinant u-PA was used as a positive control (lane 1). (B) Subcellular localization of the u-PA activity in the various THP-1 cell lines. The u-PA activity was localized to the particulate fraction (lanes 5, 6, 8) but was not detected in the particulate fraction of cells transfected with wild-type PAI-2 (THP-1/PAI-2wt; lane 7). No u-PA activity was detected in either the cytosolic (lanes 1-4) or nuclear fraction (lanes 9-12) of any THP-1 cell line.

PAI-2 is presently unknown. Western blot analyses exclude that the increase in u-PA activity is due to an increase in u-PA protein (data not shown). However, it is possible that the mutant PAI-2 may act to protect u-PA from inhibition by other inhibitors, but this remains to be determined.

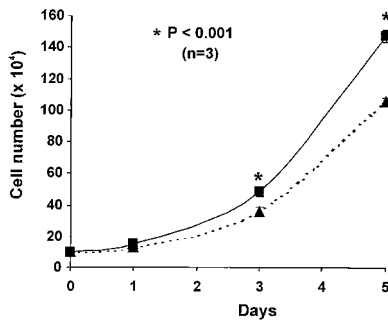
The loss of u-PA activity in the PAI-2-expressing cells is not surprising because u-PA is the natural target for PAI-2. Also, because activation of uPAR by its ligand u-PA has been shown to elicit changes in cell proliferation and migration, these findings are consistent with the possibility that the antiproliferative effect of PAI-2 involves inhibition of a u-PA-dependent signaling event.

#### Effects of exogenous u-PA on the proliferation rate of PAI-2-expressing THP-1 cells

If PAI-2 is inhibiting cell proliferation via a u-PA/uPAR-dependent process, it is predicted that the restoration of u-PA activity to the cell



**Figure 8. Addition of recombinant u-PA attenuates the antiproliferative effect of PAI-2-expressing THP-1 cells.** The proliferation rate of THP-1 cells expressing wild-type PAI-2 (THP-1/PAI-2) was determined in the absence and in the presence of recombinant u-PA (0.01 and 0.1 mU/mL) as indicated ("+"). Addition of u-PA increased the proliferation rate of the PAI-2-expressing THP-1 cells. Cell number was determined after 5 days. THP-1/neo represents THP-1 cells transfected with pCI-neo. This experiment was performed in triplicate on 3 separate occasions.



**Figure 9. Addition of recombinant PAI-2 to wild-type THP-1 cells inhibits proliferation.** Normal (nontransfected) THP-1 cells were grown in the presence (▲) or absence (■) of 1  $\mu$ M recombinant PAI-2 (rPAI-2) and cell growth assessed after 5 days. As shown, addition of exogenous PAI-2 significantly inhibited THP-1 cell proliferation ( $P < .001$ ;  $n = 3$ ). This experiment was performed in triplicate on 3 separate occasions.

surface would override the antiproliferative effect of PAI-2 on THP-1 cells. To test this possibility, increasing concentrations of recombinant u-PA (0.01 and 0.1 mU) were added to pCI-PAI-2-transfected cells, and the rate of cell proliferation was assessed over a 5-day period. As shown in Figure 8, the PAI-2-transfected cells demonstrated significant lowering in the rate of cell proliferation compared with the pCI-neo-transfected control, which is consistent with the data shown in Figure 6. However, addition of recombinant u-PA to the PAI-2-expressing cells significantly increased the rate of cell proliferation in a dose-dependent manner. In fact, addition of 0.1 mU u-PA to the PAI-2-expressing THP-1 cells virtually restored the proliferation rate to that seen in the control (pCI-neo-transfected) cells. These findings support the hypothesis that PAI-2 exerts its cellular effects through modulation of u-PA activity.

#### PAI-2 reduces cell proliferation when added to wild-type THP-1 cells

To provide further evidence that PAI-2 affects cell proliferation by modulating proteolytic activity at the cell surface, we investigated the effect of exogenous recombinant PAI-2 on the rate of cell proliferation of wild-type THP-1 cells. As shown in Figure 9, addition of recombinant PAI-2 (1  $\mu$ M) to the THP-1 cells reduced cell proliferation by approximately 30%. This inhibitory effect was evident on day 3 but became more significant after day 5 ( $P < .001$ ,  $n = 3$ ). Hence, PAI-2 added exogenously produces an antiproliferative effect on THP-1 cells.

#### Effects of PAI-2 on PMA-induced differentiation of THP-1 cells

To determine whether the presence of wild-type or mutant PAI-2 altered the ability of THP-1 cells to differentiate, THP-1 cells transfected with pCI-neo, pCI-PAI-2, or pCI-PAI-2<sub>Ala380</sub> were left untreated (Figure 10A-C) or were treated with 25 nM phorbol ester

(PMA) (Figure 10D-F) for 5 days and changes in cell morphology were assessed. THP-1 cells transfected with the empty pCI-neo plasmid became spindlelike after the 5-day period (Figure 10D). However, cells expressing wild-type PAI-2 (Figure 10E) did not adapt to this phenotype and instead maintained a rounded appearance. Again, the ability of PAI-2 to alter the differentiation process was dependent on its active form because cells expressing mutant PAI-2 (PAI-2<sub>Ala380</sub>) differentiated in a manner similar to that seen for the control transfected cells (Figure 10F).

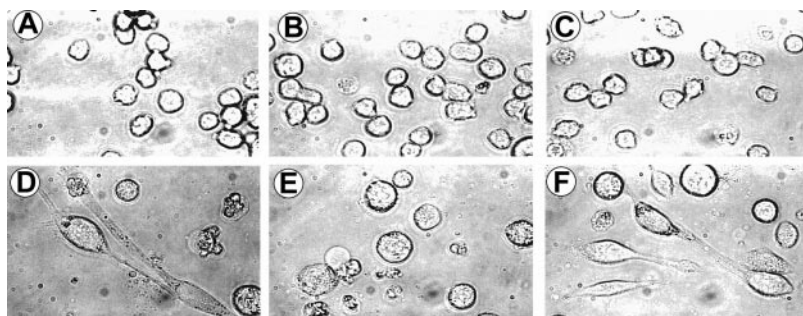
#### CD14 expression is reduced in cells expressing wild-type PAI-2

The effect of PAI-2 on the differentiation of THP-1 cells was investigated by quantitating the level of expression of the cell surface antigen CD14 on the cell surface by flow cytometry. As shown in Figure 11, approximately 10% of THP-1 cells transfected with pCI-neo are CD14<sup>+</sup>; however, this increases 23% following PMA treatment for 3 days ( $P < .01$ ,  $n = 3$ ). Interestingly, cells expressing active PAI-2 (clone c) displayed a significant decrease in the percentage of CD14<sup>+</sup> cells compared with cells transfected with pCI-neo or mutant PAI-2 (clone g) ( $3.6\% \pm 0.5\%$  vs  $7.3\% \pm 1.0\%$  and  $6.6\% \pm 0.9\%$ , respectively,  $n = 3$ ,  $P < .05$ ). However, although PMA treatment still resulted in a similar relative increase in CD14 expression in PAI-2-transfected cells, the percentage of induced CD14<sup>+</sup> cells remains significantly lower in cells expressing wild-type PAI-2 ( $7.2\% \pm 0.9\%$ ) compared with cells expressing mutant PAI-2 ( $11.8\% \pm 2.4\%$ ) or the pCI-neo-transfected cells ( $11.1\% \pm 1.0\%$ ). These data indicate that the presence of PAI-2 reduces constitutive levels of CD14.

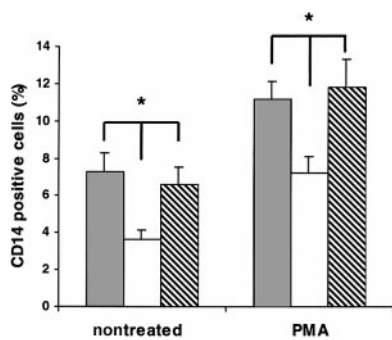
## Discussion

We have taken advantage of the THP-1 monocytic cell line to explore PAI-2 function. These cells were chosen because they are the only human monocytic cell line known that does not express a functional PAI-2 protein due to the aberrant production of the PAI-2 messenger RNA.<sup>25</sup> The complete absence of a functional PAI-2 protein essentially classifies the THP-1 cell as a PAI-2<sup>-/-</sup> monocytic cell system. We reasoned that the introduction of active PAI-2 into THP-1 cells would allow us to explore novel roles for PAI-2 in a relevant cell system not burdened with an endogenous supply of this protein.

Four independent clones of THP-1 cells expressing either wild-type or mutant PAI-2 were established. Cells expressing active PAI-2 displayed a number of characteristics. First, we noted that the presence of PAI-2 resulted in the THP-1 cells growing as single-cell suspensions rather than as clumps. The presence of PAI-2 also reduced the rate of THP-1 cell proliferation and DNA synthesis and altered the ability of the cells to differentiate in



**Figure 10. PMA-induced differentiation is altered in THP-1 cells expressing wild-type PAI-2.** THP-1 cells stably transfected with pCI-neo (A,D), expressing wild-type PAI-2 (THP-1/PAI-2wt; [B,E]), or mutant PAI-2 (THP-1/PAI-2<sub>Ala380</sub> [C,F]) were cultured in the absence (A-C) or presence (D-F) of 25 nM PMA. Phase contrast photomicrographs (magnification  $\times 400$ ) were taken after 5 days of treatment.



**Figure 11. Cells expressing wild-type PAI-2 have reduced expression of CD-14.** The level of expression of the monocytic marker CD14 in THP-1 cells transfected with pCI-neo (□) or in cells expressing wild-type (■) or mutant PAI-2 (▨) was assessed by flow cytometry using an anti-CD14 antibody. Levels of CD14 expression in nontreated cells expressing wild-type PAI-2 were 50% lower than in control cells (pCI-neo-transfected) or in cells expressing mutant PAI-2. PMA treatment of cells resulted in an increase in CD14 expression in all cell groups; however, CD14 levels remained lower in cells expressing wild-type PAI-2 compared with control and mutant PAI-2-expressing cells.

response to PMA. For the latter, this was associated with a reduction in CD14 expression. Importantly, all of the effects observed for PAI-2 on the THP-1 cell were directly related to its protease inhibitory property because mutation of the P1 arginine residue in the PAI-2 protein caused no effect on THP-1 cell adhesion, proliferation, or differentiation. This result suggested that PAI-2 was inhibiting the activity of a serine protease(s) central to these cellular processes. The effect of PAI-2 is unlikely to be due to nonphysiologic levels of PAI-2 expression because ELISA data indicated that the concentration of PAI-2 detected in cytoplasmic extracts of THP-1 cells was similar to levels reported in normal human monocytes.<sup>36</sup>

Because u-PA is, at present, the only bona fide target for PAI-2 and via its receptor, uPAR, has also been associated with cellular adhesion and proliferation, we investigated the involvement of uPAR and u-PA in these events. Addition of anti-uPAR antibodies inhibited cell-cell adhesion in a manner similar to that seen in cells expressing active PAI-2, suggesting that there might be a relationship between PAI-2 and uPAR on the cell surface and the inhibition of cell adhesion. Zymographic studies demonstrated that u-PA activity was detected only within the particulate (membrane) fraction of normal THP-1 cells but was not at all detected in cells expressing active PAI-2. This provided further evidence to suggest a role for cell surface-bound u-PA. Consistent with this, treatment of the PAI-2-expressing cells with increasing concentrations of recombinant u-PA attenuated the antiproliferative effect of PAI-2, while addition of recombinant PAI-2 to wild-type THP-1 cells also significantly reduced cell proliferation. Exogenous addition of PAI-2 has previously been shown to reduce proliferation of keratinocytes.<sup>17</sup> It therefore appears likely that the antiprolifera-

tive effect of PAI-2 on THP-1 cells is not unique to this particular cell system.

Although these data clearly point to a cell surface-mediated event, it still remains to be directly proven that PAI-2 and uPAR are colocalized on the cell surface. However, immunofluorescence studies on whole cells and immunoprecipitation and Western blot experiments using the anti-PAI-2 or anti-uPAR antibodies could be performed to address this issue.

Binding of u-PA to uPAR initiates an intracellular signaling cascade that stimulates mitogenesis.<sup>42</sup> The means by which u-PA/uPAR transmits such a signal has been the subject of much research. The uPAR-dependent signaling involves the physical engagement with a number of integrins, including the  $\beta 1$  and  $\beta 2$  integrins and  $\alpha v \beta 5$ , and matrix proteins, including vitronectin. The signaling process itself involves tyrosine as well as serine phosphorylation of a number of substrates, including components of the JAK/STAT pathway.<sup>43</sup> It is reasonable to speculate that the inhibition of u-PA activity by PAI-2 would block the ability of u-PA to bind to uPAR and/or result in a conformational change of the u-PA/uPAR complex. This in turn might alter the ability of uPAR to engage with other integrins and modulate the signaling process.

The extracellular concentration of u-PA could be used as a sensing mechanism for cells to modulate their rate of proliferation. It is interesting that addition of u-PA to HT-1080 cells has also been shown to increase the expression of the endogenous PAI-2 gene.<sup>43,44</sup> When considering the results of this present study, we speculated that u-PA-mediated induction of PAI-2 may provide an autocrine-negative feedback loop designed to attenuate proliferative signals transmitted by the u-PA/uPAR complex.

Another significant finding of this study was that the presence of active PAI-2 altered PMA-mediated differentiation of THP-1 cells. An effect of PAI-2 in this capacity has been suspected because PAI-2 levels are increased during differentiation of a number of cells, including monocytes<sup>27</sup> and keratinocytes.<sup>18</sup> Experiments were not conducted to determine whether this effect of PAI-2 involved disruption of a u-PA/uPAR signaling event on the cell surface. However, we suspect that the mechanism by which PAI-2 implements this effect on differentiation is independent of its antiproliferative actions. This then predicts that PAI-2 is inhibiting a different protease (probably intracellular) involved in the differentiation process.

In summary, evidence is provided to implicate PAI-2 in 3 critical aspects of monocyte biology: as a modulator of cell-cell adhesion, proliferation, and differentiation. Further studies are required to explore the mechanism by which PAI-2 influences these processes, whether the presence of PAI-2 alters other cellular functions, including apoptosis, and whether other proteins that alter u-PA/uPAR interaction (ie, PAI-1) perform similar functional effects on THP-1 monocytes.

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