Inhibition of Invasion of HT1080 Sarcoma Cells Expressing Recombinant Plasminogen Activator Inhibitor 2

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ABSTRACT

Plasminogen activators (PA) elaborated by tumor cells play an important role in the complex process of tissue invasion and metastasis. In the present study the effect of the PA inhibitor type 2 (PAI-2) on tissue invasion in vitro and in vivo was investigated. Clones either expressing (B+) or not expressing the endogenous PAI-2 gene (C) were isolated from the human HT1080 fibrosarcoma cell line and transfected with full-length PAI-2 cDNA. Recombinant PAI-2 (rPAI-2) expressed by these cells completely inhibited receptor-bound urokinase activity and partially neutralized secreted PA activity. Degradation of extracellular matrix proteins by these transfected cells was markedly decreased when compared to mock or untransfected control cells. The rPAI-2-expressing cells did not penetrate a multilayer of rat smooth muscle cells in vitro, which was readily invaded and destroyed by control cells. The PAI-2 transfectants remained tumorigenic in athymic/nude mice, but tumors originating from these cells showed the presence of a thick, collagenous capsule absent in tumors formed by control cells. Thus, expression of rPAI-2 in HT1080 cells resulted in neutralization of receptor-bound urokinase with subsequent inhibition of matrix protein degradation and invasion in vitro and induction of a thick, peritumoral capsule in vivo.

INTRODUCTION

Tumor cell invasion and subsequent metastasis are highly complex, multistep processes partially mediated by a variety of degradative enzymes, including serine proteases, heparinases, and thiol- and metal-dependent enzymes (for reviews, see Refs. 1 and 2). Recent evidence suggests that a cascade of these different enzymes is involved in the invasive process. Thus, PAs,3 highly specific serine proteases, convert plasminogen into plasmin, a trypsin-like enzyme which, in addition to degrading specific matrix proteins, activates procollagenase to its active form (3). There are two genetically distinct PA types, uPA and tPA, which exist in cell surface-associated, receptor-bound, and secreted forms (4). Since plasminogen also has a receptor on the cell surface, plasmin is generated at the cell-matrix interface allowing for localized pericellular proteolysis (5). PA activities in normal cells are tightly controlled by specific PAs, which include PAI-1, PAI-2, and protease nexin-1. These inhibitors covalently bind to the active site of receptor-bound and secreted PA, thus neutralizing their activities (6). This control mechanism is disturbed in some cancer cells resulting, either through increased PA or decreased PAI production, in enhanced plasmin generation. Therefore, inhibition of receptor-bound and/or secreted PA should result in lack of plasmmin generation with inhibition of matrix protein degradation, invasion, and metastasis. This hypothesis is supported by data showing that inhibition of uPA activities with either anticalytic antibodies (7, 8) or exogenously added PAI-1 (9), PAI-2 (10), or protease nexin 1 (11) resulted in decreased matrix degradation and/or inhibition of tumor cell invasion in vitro and metastasis in vivo. Recently, it was shown that expression of mutant uPA cDNA in PC3 cells resulted in suppression of spontaneous metastasis in nude mice (12). This inhibition occurred through competitive displacement of active uPA from its receptor by the mutant uPA, which is enzymatically inactive. Thus, there is ample evidence that inhibition of tumor cell PA prevents invasion and metastasis in certain tumor cell systems. Since most of the studies were done with exogenously added PA, which precludes meaningful in vivo experiments because of distribution problems, we transfected PAI-2 cDNA into HT1080 cells. This inhibitor was chosen because of its high inhibitory activity for uPA expressed in most invasive and metastatic tumor cells (13). Clones either expressing (B+) or not expressing the endogenous PAI-2 gene (C*) were isolated from the highly invasive human sarcoma cell line HT1080 (14) and stably transfected with full-length PAI-2 cDNA. Clones expressing high levels of rPAI-2 were found to have their receptor-bound uPA activity neutralized, resulting in suppression of matrix protein degradation, inhibition of invasion in vitro, and induction of capsule formation in vivo.

MATERIALS AND METHODS

Cell Cultures. The HT1080 fibrosarcoma clones C+ and B+, previously shown to constitutively express (B+) or not express the PAI-2 gene (C*), were used (14). The growth conditions were as previously described. Transfection was done at passage 12. Untransfected cells were used between passages 12 and 20 after isolation with stable PA and PAI expression (14), and transfected cells were used between passages 4 and 10 after transfection with unchanged PA and PAI gene expression.

Serum-free medium conditioned by U937 histiocyte lymphoma cells treated with phorbol 12-myristate 13-acetate (25 ng/ml) was used as a source for the PAI-2 control (15).

PAI-2 Transfection. Full-length PAI-2 cDNA (15) was cloned into the unique EcoRI restriction site of the expression vector pEMSVSCRIBE-a2 containing the MSV-LTR promoter (16). Proper orientation was verified by restriction enzyme mapping and limited sequencing. Cotransfection with pSV-neo was done with the calcium phosphate precipitation method and stable transfectants isolated by neomycin selection (200 µg/ml) (17). Mock transfectants were obtained under identical conditions using pSV2-neo and pEMSVSCRIBE-a2 without insert. Transfectants were grown in neomycin selection medium.

PA and PAI Determination. The conditions were as previously described with the exception that the cells were seeded at 2 x 10⁵/35-mm dish (14). PA activities were determined on ²¹²⁵i-labeled fibrin dishes in aliquots of serum-free conditioned medium (secreted PA), acidic eluates (receptor-bound PA), and cell lysates (cellular PA). The levels of PAI-2 antigen were measured with an enzyme-linked immunosorbert assay (14).

Western Blot. Aliquots (10 µl) of acidic eluate (8-fold concentrated), serum-free conditioned medium (10-fold concentrated), and cell lysates were separated on SDS-polyacrylamide minigels and electroblotted to nitrocellulose membranes (14). The bands were visualized with the alkaline phosphatase reaction using specific antibodies at a concentration of 1:500-1:1000 and goat anti-rabbit, rabbit anti-goat, or rabbit anti-mouse antiserum coupled to alkaline phosphatase.

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2 To whom requests for reprints should be addressed, at Children's Hospital Los Angeles, Division of Hematology-Oncology, 4650 Sunset Blvd., M/S #126, Los Angeles, CA 90027.

3 The abbreviations used are: PA, plasminogen activator; PAI, PA inhibitor; PAI-1, PA inhibitor type 1; PAI-2, PA inhibitor type 2; rPAI-2, recombinant PAI-2; TIMP-2, tissue inhibitor of metalloproteinases type 2; uPA, urokinase; IPA, tissue-type PA; SDS, sodium dodecyl sulfate; uPAR, uPA receptor.

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phosphatase (Bio-Rad, Richmond, CA) at a 1:1000 dilution. The antibodies or
antisera used (all obtained from American Diagnostica, Greenwich, CT) were
rabbit anti-human uPA, goat anti-human PAI-2, rabbit anti-human PAI-1, and
monoclonal anti-human uPA receptor.

Fibrin-Agarose Overlay. Aliquots (10 μl) of 8-fold concentrated acidic
eluate were separated by SDS-polyacrylamide gel electrophoresis as described
above. The gel was then washed in 2.5% Triton X-100, underlaid with a gel
consisting of bovine fibrinogen (10 mg/ml), 2.5% low temperature melting
agarose, thrombin, and plasminogen, and incubated at 37°C in humid air.
Zones of lysis appeared within 4-8 h. The same agarose layer was used to
examine for inhibition of lysis to screen nonconcentrated serum-free condi-
tion medium (5 μl) derived from PAI-2 transfectants (Fig. 1).

Northern Blots. Total RNA was isolated using the ISOBIOTEX method
as described before (18). After electrophoresis on formaldehyde-agarose gels
and blotting to nitrocellulose by capillary transfer, the specific bands were
visualized with the following 32P-labeled probes: uPA nucleotide 623–1039
and tPA 801–1273 (both kindly provided by D. Collen, Leuven, Belgium),
full-length cDNA for PAI-1 (a kind gift from Dr. Loskutoff, La Jolla, CA),
full-length PAI-2 (isolated by E. K. O. Kruithof), and the BamHI fragment of
the uPAR cDNA (a kind gift from F. Blasi, Copenhagen, Denmark).

Matrix Degradation. Degradation of radiolabeled extracellular matrices
produced by R22C1D cells in vitro was done as previously described (19).
Radioactivities released into the supernatant were determined at the time
intervals indicated, and cumulative counts were determined after subtraction of
counts released by medium alone (background). The data on matrix degra-
dation of rPAI-2 on mock transfected cells (C+M and B+M) was determined by
the daily addition of 3300 units rPAI-2/ml growth medium to the cell cultures.

In Vitro Invasion Assay. Rat smooth muscle cells R22C1D (2.5 x 10⁶
cells/35-mm dish) were grown for 10 days and then overlaid with 2.0 x 10⁵
tumor cells/dish and grown for another 12 days. The cultures were then fixed
with buffered formaldehyde, stained with toluidine blue, and examined by light
microscopy. Duplicate cultures were examined for each cell clone, and the
experiment was repeated once.

In Vivo Tumor Growth. Tumor cell suspensions containing 5 x 10⁶ cells/
0.2 ml phosphate-buffered saline were injected s.c. on each side below the
shoulder pad of congenitally athymic nu/nu mice and grown for 28 days. After
the animals were euthanized with CO₂, the tumors were surgically removed
under aseptic conditions and the sizes were determined by measuring the
longest diameter. Part of the tumor was dissected into small pieces and regrown
in tissue culture for Northern blot analysis. The remainder of the tumor was
fixed in buffered formalin mounted in paraffin, cut, and stained with either
trichrome or hematoxylin and eosin. A total of 10 tumors were evaluated for
each cell line.

RESULTS

Isolation of Clones Expressing Recombinant PAI-2. The cells
were transfected with PAI-2 cDNA, and clones originating from single
cells were isolated and grown to subconfluency. They were screened
with a reverse fibrin-agarose plate assay for their PA inhibitory activity
in serum-free conditioned medium. Medium conditioned by U937
cells as the source for PAI-2 and uPA were included as controls (Fig.
1). Clones whose medium prevented uPA-induced lysis (white areas in
Fig. 1) were grown and further evaluated by quantitative methods.
Similar inhibition of lysis was obtained when acidic cell eluates (receptor-bound uPA activity) were taken instead of supernatants (data
not shown). Clones expressing the highest PAI-2 levels were selected
for the present study. Mock-transfected cells (vector without insert)
were designated with subscript M, and PAI-2 transfectants are design-
ated with subscript T followed by the number of clone.

The expression of PAI-2 was evaluated on total cellular RNA by
Northern blot. The densities of the bands were normalized by video
densitometry (Bio-Rad) using α-tubulin as the internal standard.
While C+ and C+M had no PAI-2 specific mRNA, the transfectant
C+T10 had significant levels of mRNA hybridizing with the PAI-2
cDNA (Fig. 2). Untransfected clone B- had moderate PAI-2 mRNA
levels, which increased 2-fold in the mock-transfected (B-M) and
3-fold in the PAI-2-transfected B–T4 cells. The mRNA levels of PAI-1 remained unchanged, while the uPA receptor mRNA levels increased 1.5-fold in the PAI-2-transfected B–T4 wells when compared to the control cells. The uPA mRNA levels decreased by 50% in C*T10 cells (PAI-2 transfectants) relative to the control cells (C+ and C*M), and the levels increased 2-fold in mock-transfected (B–M) and PAI-2-transfected cells (B–T4) when compared to the B– cells. Trace amounts of tPA mRNA were found in the C+ clones and was absent in the B– clones (14). Thus, transfection and expression of PAI-2 cDNA affected the mRNA levels of both uPA and uPAR to a certain degree but without a specific consistency.

The production of PAI-2 protein was investigated by enzyme-linked immunosorbent assay in cell lysates and serum-free medium conditioned by the various cell clones (Table 1). The low level of PAI-2 antigen increased 4-fold in the supernatant of the PAI-2 transfectant compared to mock-transfected and untransfected B– clones. PAI-2 antigen was not detected in the C+ control cells, while high levels were found in the medium of C*T10 cells. Similar increases of PAI-2 antigen levels were found in the lysates of the PAI-2 transfectants when compared to control cells. Thus, the increases of PAI-2 were similar on both the mRNA and protein levels.

**PA Activities.** The influence of PAI-2 on functional PA activities was determined with the fibrin plate assay (Table 2). While the secreted PA activities in the serum-free supernatant increased 1.5- to 2-fold in the mock-transfected cells, the PAI-2 transfectants showed an 80% (B–T4) to 90% (C*T10) decrease when compared to the mock-transfected counterparts. The receptor-bound PA activity increased about 5-fold in the mock-transfected cells, but there was virtually no receptor-bound uPA activity in the PAI-2-transfected cells (C*T10 and B–T4). The absence of receptor-bound uPA activity was confirmed with fibrin overlay after separation by SDS-polyacrylamide gel electrophoresis (data not shown).

Western blots were performed to show that the absence of receptor-bound PA activity was due to inhibition by rPAI-2 and not due to lack of translation of the uPA and/or uPA receptor gene (Fig. 3). Receptor-bound uPA was found in all clones, with more intense bands observed when compared to control cells. Thus, the increases of PAI-2 were similar on both the mRNA and protein levels.

**Table 1** Quantitative determination of PAI-2 in serum-free supernatant and cell lysates determined with an enzyme-linked immunosorbent assay

<table>
<thead>
<tr>
<th>Tumor cells</th>
<th>Secreted PAI-2</th>
<th>Cellular PAI-2</th>
</tr>
</thead>
<tbody>
<tr>
<td>C+</td>
<td>1.4 ± 0.9</td>
<td>1 ± 0.9</td>
</tr>
<tr>
<td>C+M</td>
<td>0.7 ± 0.9</td>
<td>0.7 ± 0.9</td>
</tr>
<tr>
<td>C*T10</td>
<td>3.2 ± 0.9</td>
<td>3.2 ± 0.9</td>
</tr>
<tr>
<td>B–</td>
<td>2.0 ± 0.9</td>
<td>2.0 ± 0.9</td>
</tr>
<tr>
<td>B–M</td>
<td>2.0 ± 0.9</td>
<td>2.0 ± 0.9</td>
</tr>
<tr>
<td>B–T4</td>
<td>1.3 ± 0.9</td>
<td>1.3 ± 0.9</td>
</tr>
</tbody>
</table>

* Control cells.  
* Mock-transfected cells.  
* PAI-2 transfectants.

**Table 2** PA activities in tumor cells

<table>
<thead>
<tr>
<th>Tumor cells</th>
<th>Secreted PA activities</th>
<th>Receptor-bound PA activities</th>
</tr>
</thead>
<tbody>
<tr>
<td>C+</td>
<td>34.9 ± 3.6</td>
<td>1.41 ± 0.25</td>
</tr>
<tr>
<td>C+M</td>
<td>50.6 ± 11.2</td>
<td>6.02 ± 0.97</td>
</tr>
<tr>
<td>C*T10</td>
<td>6.3 ± 1.6</td>
<td>0.2 ± 0.14</td>
</tr>
<tr>
<td>B–</td>
<td>21.8 ± 1.3</td>
<td>1.6 ± 0.3</td>
</tr>
<tr>
<td>B–M</td>
<td>55.9 ± 6.2</td>
<td>8.24 ± 1.16</td>
</tr>
<tr>
<td>B–T4</td>
<td>10.6 ± 1.7</td>
<td>0</td>
</tr>
</tbody>
</table>

* RU/culture.

in the mock- and PAI-2-transfected cells than the untransfected control cells (Fig. 3A). A small part of the uPA formed complexes with PAI, while the remainder was uncomplexed. Since the cultures were kept in serum-free medium, this native uPA remained in the inactive proform, thus escaping reaction with PAI-2. The receptor for uPA, determined in the cell lysate, was found in its glycosylated and unglycosylated forms, with strongest bands observed in the mock-transfected cells (Fig. 3B). These changes were not detected on the mRNA level. PAI-2 was absent in the acidic eluate of C+ and C+M and was present distinctively in B– and B–M and with dense bands in the PAI-2 transfectants (Fig. 3C). Whether this represents PAI-2 specifically or nonspecifically bound to the cell membrane or released from damaged cells during incubation in acidic buffer remains to be determined. Faint bands indicating PA-PAI-2 complexes were detected in the M, range 90,000–100,000. No significant bands staining with anti-PAI-1 were detected in the acidic eluate (not shown). In contrast, the conditioned medium of all clones showed distinct bands of PAI-1, which stained weaker in the PAI-2 transfectant (Fig. 3D). Thus, the PAI-2-transfected cells showed uPA and uPA receptor proteins at a level similar to or higher than the untransfected cells, indicating that the absence of receptor-bound uPA was due to inhibition by rPAI-2. Also, the level of the PAI-1 protein was lower in the transfectants than in control cells, ruling out inhibition of PA by this protein.

**Matrix Degradation.** The uncloned HT1080 cells were previously shown to rapidly degrade the extracellular matrix produced in vitro by R22CID rat smooth muscle cells (19). Similar results were obtained in the present study with the C+ clones, which degraded 71%, and B– clones, which solubilized 27% of the proteins during the 8-day experiment (Fig. 4). The slower degradation rate of B– cells was previously observed with subendothelial matrices (20). Mock transfectants (C+M and B–M) had degradation rates similar to those observed with control cells. In contrast, the PAI-2 transfectants (C*T10 and B–T4) demonstrated a 10-fold reduced matrix degradation when compared to the control cells.
Enzymatic analysis of the residual matrices demonstrated that the mock-transfected and untransfected control cells mainly degraded trypsin-sensitive material, which remained intact in the PAI-2 transfectants (Table 3). The degradation of the collagenous matrix proteins by the transfectants decreased between 3- (B−T4) and 8-fold (C+T10). There was no elastinolytic activity in control or transfected cells. Similar growth rates were observed for all cell lines tested, thus excluding this possibility as a reason for the observed decrease of matrix degradation (data not shown). Furthermore, the addition of rPAI-2 to mock-transfected B− and C+ clones growing on matrices decreased the degradation by >70% during the 2-day experiment, further substantiating that the inhibition was indeed due to the effect of rPAI-2 (Table 4).

**Invasion in Vitro and Tumor Formation in Vivo.** Control and transfected cells were grown for 12 days on a multilayer of rat smooth muscle cells and then fixed, stained, and microscopically examined (Fig. 5). The spindly appearing smooth muscle cells (arrows) are easily identifiable. The untransfected control cells (A and D) invaded and partially destroyed the smooth muscle cell layer and penetrated to the bottom of the culture dishes, suggesting a reactive response of the peritumoral tissue. Dissection of the animals after tumor removal failed to show any spontaneous metastasis. This study shows that transfection and expression of PAI-2 cDNA in HT1080 clones results in inhibition of matrix degradation and invasion in vitro. More than 90% of the recombinant PAI-2 was found in the cell lysates, since the transfected PAI-2 cDNA lacks, like the endogenous PAI-2 gene, a proper signal peptide sequence (21). The distribution of high cellular and low extracellular levels in our PAI-2 transfectants (C and E) left the smooth muscle cells intact with only occasional tumor cells penetrating it. These pictures were taken at random, and the findings remained the same in the various areas of the cultures.

Transfected and untransfected control cells remained tumorigenic when injected s.c. into athymic/nude mice, and tumor growth was similar in all clones tested. The largest diameters of the tumors removed after 27 days of growth were 14.2 ± 2.3 mm for C+, 13.2 ± 1.3 mm for C+M, 14.2 ± 2.1 mm for C+T10, 14.3 ± 1.85 mm for B−, 17.4 ± 2.9 mm for B−M, and 14.0 ± 3.31 mm for B−T4. Striking differences with regard to histopathology were found between the 2-expressing and control cells (Fig. 6). While the tumors induced by control cells showed only minimal reactive organization of surrounding connective tissue, the tumors arising from rPAI-2-expressing cells had a consistently thick capsule around them. This capsule was found with trichrome staining to consist mainly of collagenous material. Whether this capsule is produced by the injected rPAI-2-expressing tumor cells or by the normal tissue surrounding the tumor remains to be determined. No significant matrix protein production was observed by either the control or transfected cells growing for 1 week in tissue culture dishes, suggesting a reactive response of the peritumoral tissue. Dissection of the animals after tumor removal failed to show any spontaneous metastasis.

**DISCUSSION**

This study shows that transfection and expression of PAI-2 cDNA in HT1080 clones results in inhibition of matrix degradation and invasion in vitro. The Northern and Western blot results demonstrate that the absence of cell-associated uPA was not due to transfection-induced alteration of expression of the uPA or uPA receptor gene. Also, the level of secreted PA activity remained. The Northern and Western blot results demonstrate that the inhibition of receptor-bound uPA was due to its neutralization by complexing with rPAI-2.

Complex formation between receptor-bound uPA and PAI-2 as well as PAI-1 with neutralization of the enzymatic activity has been shown in various cell lines (24, 25). The uPA-PAI complexes are subsequent internalized into the cell and degraded in the lysosomal com-

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**Table 3 Analysis of matrix after digestion by the different clones of HT1080 cells**

<table>
<thead>
<tr>
<th>Tumor cell line</th>
<th>Matrix components degraded (% of total)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Trypsin sensitive</td>
</tr>
<tr>
<td>C+</td>
<td>87.0 ± 6.0</td>
</tr>
<tr>
<td>C+M</td>
<td>60.0 ± 5.0</td>
</tr>
<tr>
<td>C+T10</td>
<td>6.0 ± 8.0</td>
</tr>
<tr>
<td>B−</td>
<td>47.0 ± 3.0</td>
</tr>
<tr>
<td>B−M</td>
<td>43.0 ± 4.0</td>
</tr>
<tr>
<td>B−T4</td>
<td>9.0 ± 8.0</td>
</tr>
</tbody>
</table>

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**Table 4 Inhibition of matrix degradation by exogenously added rPAI-2**

<table>
<thead>
<tr>
<th>Cell line</th>
<th>24 h</th>
<th>48 h</th>
</tr>
</thead>
<tbody>
<tr>
<td>C+M</td>
<td>16,140</td>
<td>24,200</td>
</tr>
<tr>
<td>C+M + rPAI-2</td>
<td>3,280</td>
<td>7,100</td>
</tr>
<tr>
<td>B−M</td>
<td>8,080</td>
<td>13,320</td>
</tr>
<tr>
<td>B−M + rPAI-2</td>
<td>3,200</td>
<td>4,840</td>
</tr>
</tbody>
</table>

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**Fig. 4. Matrix degradation by C+ and B− clones.** Cells were seeded (5 × 10⁵/dish) on day 0 on radiolabeled R22CID matrices and grown for 8 days with medium changes on days 1 and 4. Radioactivities released were counted in a scintillation counter, and results are expressed in cumulative counts after subtraction of background counts (matrices incubated with medium alone). SE < 10% for each point. Control cells, C+ and B−; mock transfectants, C+M and B−M; PAI-2 transfectants, C+T10 and B−T4.
clones showed that the control cells degraded mainly glycoproteins, those of Pöllänen et al. (26), who showed with immunohistochemical methods that PAI-2 added to rhabdomyosarcoma cells in culture binds mainly to the cell surface areas expressing receptor-bound uPA. Also, Bruckner et al. (27) reported that addition of rPAI-2 to the human lung carcinoma cell lines A549 and Calu-1 resulted in inhibition of receptor-bound uPA. Our data expand this finding by demonstrating that continuous expression of rPAI-2 in tumor cells inhibits receptor-bound uPA through complex formation.

The changes of expression of the uPA and the uPA receptor genes observed on the mRNA and protein levels, respectively, in both mock- and PAI-2-transfected cells are most likely due to clonal variation. The clones were grown from single cells after transfection of the B~ and C~ cells, which most likely have clonal instability as observed in their parental HT1080 cells (14).

The inhibition of receptor-bound uPA was sufficient to significantly inhibit degradation of matrix proteins, although the PAI-2 transfec tants continued to have functional secreted PA activity. Similarly, Jones and DeClerck (19) found that uncloned HT1080 cells, although secreting large amounts of PA, induced protein degradation only when the cells were in contact with the matrix. Therefore, cell-associated, receptor-bound and not secreted PA is of major importance in HT1080 cells for tissue degradation as previously shown for other cell types (4). A similar inhibition of matrix degradation by colon carcinoma cells was obtained when PAI-2 was added to the culture medium (28). This inhibitory effect was shown to be due to neutralization by PAI-2 of both cell-associated and secreted uPA. Also, inhibition of uPA binding to its receptor in colon carcinoma cells using a specific monoclonal antibody against the A-chain of uPA resulted in decreased extracellular matrix invasion, stressing again the importance of receptor-bound uPA in this process (29). These authors also found that an 8-fold increase of uPA secretion after uPA-cDNA transfection into GEO colon cancer cells did not alter their low invasiveness.

Analysis of the complex matrices exposed to the different HT1080 clones showed that the control cells degraded mainly glycoproteins, which remained unaffected by the PAI-2 transfectants, indicating lack of plasmin generation on the cell surface. Our data also suggest a significant inhibition of collagen degradation by the transfectants. Since the matrices used had a low collagen content, these data have to be interpreted with caution. We have previously shown that removal of the matrix glycoproteins by trypsin treatment prior to seeding HT1080 cells resulted in increased collagen degradation (20). This suggests that the glycoproteins protect the collagens from the action of collagenases. A similar protective function of glycoproteins was shown by Montgomery et al. (10), who demonstrated that rPAI-2 added to human melanoma cells growing on similar matrices prevented both glycoprotein and collagen degradation. This inhibitory effect of rPAI-2 on collagen degradation was abolished when the matrix glycoproteins were removed prior to the initiation of the experiment. Alternatively, since plasmin is a known activator of metalloproteinases (3), one might speculate that inhibition of plasmin generation will result in diminished metalloproteinase activation with decreased collagen digestion.

The uncloned HT1080 cells were previously shown to readily invade and destroy in vitro a multilayer of smooth muscle cells (30). Similar results were obtained in the present study with the control cells. In contrast, the clones expressing rPAI-2 barely invaded and destroyed the multilayer of rat smooth muscle cells in vitro. Similar findings were obtained with daily addition of recombinant TIMP-2 to uncloned HT1080 cells growing on rat smooth muscle cells (31). This suggests a common pathway of suppression of tumor cell invasion in vitro through inhibition of either the PA-plasmin or the metalloproteinase system. This hypothesis is supported by our in vivo data obtained in athymic/nude mice showing the induction of a thick, collagenous capsule around tumors derived from HT1080 clones expressing rPAI-2, which was only minimally present in control cells. A similar fibrotic reaction was observed with rat 4R cells expressing recombinant TIMP-2 and growing s.c. in athymic/nude mice (32). Taking into account our interpretation of inhibition of matrix degradation, we may assume that inhibition of the PA-plasmin system leaves collagen fibers embedded in glycoproteins, which prevents collagenases from degrading their substrate. Similarly, inhibition of
INHIBITION OF TUMOR CELL INVASION

Collagenases with TIMP-2 results in decreased degradation of collagenous material. In both circumstances, the end result is an accumulation of collagen fibers resulting in a peritumoral capsule formation, which may act as a mechanical barrier against invasion. Neither control nor PAI-2-transfected cells showed any signs of invasion in our in vivo test system. In contrast, DeClerck et al. (32) found highly invasive behavior of the control 4R cells, which was absent in the TIMP-2-expressing cells.

No spontaneous metastasis was detected in the nude mice injected with the control cells of the HT1080 clones, and, therefore, the effect of rPAI-2 on this process could not be evaluated. Of interest is the recent observation that inhibition of the uPA receptor in PC3 prostate carcinoma cells resulted in significant suppression of spontaneous metastasis in nude mice (12). A similar inhibitory effect may result from transfection and overexpression of PAI-2 cDNA in metastatic tumor cells.

In conclusion, our data demonstrate that inhibition of tumor cell-derived, receptor-bound PA by transfection and expression of PAI-2 cDNA prevents tissue invasion by HT1080 in vitro and induces a thick peritumoral capsule in vivo. In addition, our studies indicate that receptor-bound rather than secreted PA plays a crucial part in this complex process.

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Fig. 6. Tumor formation in athymic/nude mice. Tumor cells ($5 \times 10^5$) were injected s.c. below the shoulder pad, and tumors formed were removed after 27 days. Pictures were taken after surgical removal of the tumors, fixation, and staining with hematoxylin and eosin. C* (A) and B* (D), untransfected control cells; C*M (B) and B*M (E), mock-transfected cells; C*T10 (C) and B*T4 (F), PAI-2 transfectants. Arrows, capsule in PAI-2 transfectants and border between tumor and stroma in control cells. Original magnification, $\times 100$. 

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