Transforming Growth Factor-β Induction of Type-1 Plasminogen Activator Inhibitor

PERICELLULAR DEPOSITION AND SENSITIVITY TO EXOGENOUS UROKINASE*

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The human tumor cell line HT-1080 was used as a model system to study the effects of transforming growth factor-β (TGFβ) on polypeptide synthesis and proteolytic activity of malignant cells. Confluent cultures were exposed to TGFβ under serum-free conditions, and alterations in the production of proteins were examined by metabolic labeling and polypeptide analysis. TGFβ induced the synthesis and secretion of the M47,000 endothelial type plasminogen activator inhibitor (PAI-1) as shown by reverse zymography, immunoblotting, and immunoprecipitation analyses. TGFβ-induced PAI-1 was rapidly deposited in the growth substratum of the cells as shown by metabolic labeling and extraction of the cultures with sodium deoxycholate. Using pulse-chase experiments, we found a relatively fast turnover of substratum-associated PAI-1. Exogenously added urokinase released PAI-1 from the substratum even in the presence of the plasmin inhibitor aprotinin, suggesting a direct effect of urokinase. Immunoreactive complexes of higher molecular weight were subsequently detected in the medium. Epidermal growth factor, transforming growth factor-α, platelet-derived growth factor, and insulin did not elicit similar effects on the amount of PAI-1. TGFβ also inhibited the anchorage-independent growth of HT-1080 cells at the same concentrations at which it induced PAI-1. These results indicate that TGFβ can modulate the extracellular proteolytic activity of cultured cells by enhancing the secretion and deposition of PAI-1 into their microenvironment. It remains to be established whether TGFβ inhibition of anchorage-independent growth of these cells is associated with the induction of PAI-1.

The extracellular matrix of cultured cells is composed of a fibrillar network of proteins such as fibronectin, collagen, elastin, laminin, and several other glycoproteins and proteoglycans (1). The matrix mediates the attachment of cells to their substratum as well as participates in the regulation of cell differentiation, proliferation, and morphogenesis (cf. Ref. 2). Although the role of the extracellular matrix is often restrictive and adhesive in nature, certain cell types are able to degrade actively the matrix by solubilizing their matrix components. This matrix breakdown clearly also occurs in vivo and is particularly prominent in connection with malignant growth (cf. Ref. 3).

The degradation of extracellular proteins is largely mediated by plasmin, a proteolytic enzyme capable of degrading most glycoprotein components of the matrix (cf. Refs. 4 and 5). Plasmin activity is created by conversion of the abundant proenzyme plasminogen to plasmin by plasminogen activators (PAs). The production of PAs is frequently enhanced in cultured malignant cells (cf. Ref. 4). Mammalian tissues contain two well-characterized PAs: the tissue-type PA and the urokinase-type PA (u-PA) (6, 7). u-PA is secreted from cells as an inactive proactivator (8, 9), which can be converted to active u-PA by catalytic amounts of plasmin in vitro. This autocatalytic cascade reaction is effectively controlled by PA inhibitors (PAs) secreted by the cells. The following distinct types of PAs have been described: endothelial type PA1 (PAI-1) (10, 11), placental type PA1 (PAI-2) (12), protease nexin I (13, 14), and urinary PAI (15).

Malignantly transformed cells frequently produce transforming growth factors, originally characterized as polypeptides capable of reversibly inducing the transformed phenotype in nonmalignant cells (Ref. 16; for review, see Refs. 17 and 18). Two different transforming growth factor molecules have been characterized so far. TGFα is an epidermal growth factor (EGF)-like growth factor, whereas TGFβ is a distinct molecule and mediates its effects via specific receptors (19, 20). TGFβ appears to be one of the most ubiquitous growth regulators (21). Almost all cell types studied so far have receptors for it (22–24) and also express TGFβ mRNA (25, 26). TGFβ has both stimulatory and inhibitory properties on cellular growth in vitro (21, 27). Alone, it is mitogenic for cells of mesenchymal origin (19), which seems to result from induction of the c-sis-protooncogene (28) encoding the B-chain of platelet-derived growth factor (PDGF).

TGFβ inhibits the growth of cells of epithelial origin such as normal epithelial cells, keratinocytes, hepatocytes, and various hematopoietic cells (21, 29). It is also a major serum-derived differentiation inducer of human bronchial epithelial cells (30). TGFβ enhances wound healing and the formation of granulation tissue (31, 32). Based on these data, it appears

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1 The abbreviations used are: PAs, plasminogen activators; ConA, concanavalin A; ECM, extracellular substratum-attached material; EGF, epidermal growth factor; NaDodSO₄-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; PAI, plasminogen activator inhibitor; PAI-1 and PAI-2, endothelial type and placental type plasminogen activator inhibitors, respectively; PBS, phosphate-buffered saline; PDGF, platelet-derived growth factor; TGFα and TGFβ, transforming growth factors-α and -β, respectively; u-PA, urokinase-type plasminogen activator.
that TGFβ, secreted either in an autocrine or endocrine manner, regulates the growth characteristics of many cell types, depending on the presence of other growth factors or growth inhibitors.

We have studied here the effects of TGFβ on the production of a plasminogen activator inhibitor and the anchorage-independent growth of cultured HT-1080 tumor cells. The secretion, extracellular deposition, and PA sensitivity of TGFβ-induced proteins were studied from radiolabeled cell culture medium and extracellular matrix preparations. We find that TGFβ induces the production and rapid ECM deposition of PAI-1 in these cultures. In addition, at similar concentrations, TGFβ inhibited the anchorage-independent growth of HT-1080 cells. These data suggest that an important effect of TGFβ on malignant cells is an alteration of their extracellular proteolytic activity which may be associated with the growth regulatory properties of TGFβ.

**EXPERIMENTAL PROCEDURES**

**Growth Factors and Reagents—**TGFβ was the kind gift of Drs. H. L. Moses and R. M. Lyons (Department of Cell Biology, Vanderbilt University, Nashville, TN). Its purification has been described in detail (21, 33). Under our assay conditions, the EDB for inhibiting the growth of A549 human lung carcinoma cells was 1 ng/ml. EGF and insulin were purchased from Sigma, TGFα from Peninsula Laboratories, Inc., and PDGF was from Bethesda Research Laboratories. Aprotinin (Trasylol) and p-aminobenzamidine were obtained from Sigma. Urokinase (60,000 IU/mg, M, 54,000) was purchased from Behring Diagnostics, and plasminogen and plasmin were from American Diagnostica.

**Antibodies—**PAI-1 was purified from culture medium of dexamethasone-treated HT-1080 fibrosarcoma cells, and antisera against this protein were raised in rabbits as described by others (34). Our rabbit anti-PAI-1 antibodies gave an identical blotting result with monoclonal anti-PAI-1 antibodies (not shown). Monoclonal antibodies against PAI-1 were a kind gift of Drs. L. Nielsen and K. Danş (Finsen Institute, Copenhagen, Denmark) or were obtained from American Diagnostica Inc. Monoclonal antibodies against PAI-2 and human urokinase were purchased from American Diagnostica.

**Methods**

**Cell Cultures—**HT-1080 fibrosarcoma cells, from the American Type Culture Collection (ATCC, 121), were cultured on plastic tissue culture dishes (35-mm diameter, Falcon Labware) or on Linbro tissue culture wells (16-mm diameter, Flow Laboratories, Inc.) in medium 199 containing 10% fetal calf serum (GIBCO), 100 IU/ml penicillin, and 50 μg/ml streptomycin. The cultures were incubated at 37 °C in a humidified 5% CO2 atmosphere. Upon confluency, the cultures were washed with serum-free medium 199 and then incubated for 6–8 h under serum-free conditions. At the onset of each assay, fresh medium 199 was changed, growth factors were added, and the cultures were incubated for an additional 0.5–48 h as shown in individual experiments. All experiments were carried out under serum-free conditions.

**Radioactive Labeling of the Cell Cultures, Polypeptide Analysis, and Quantitation of Secreted Proteins—**Confluent cultures of HT-1080 cells were labeled with [35S]methionine (40 μCi/ml, 1130 Ci/mmol, Amersham Corp.) in the presence of TGFα and aprotinin (100 IU/ml) for 24 h as indicated. The media were collected and centrifuged at 800 x g for 10 min. One ml aliquots of the media were adsorbed with concanavalin A (ConA)-Sepharose (Pharmacia Biotechnology, Inc.) (50 μl of a 50% (v/v) suspension in phosphate-buffered saline (PBS) (0.15 M NaCl, 0.01 M P04, pH 7.4)). ConA-Sepharose was washed three times with PBS (1% Tween 80 (0.01%), and the bound proteins were dissolved in Laemmli’s sample buffer (35) containing 10% 2-mercaptoethanol. The ConA-binding proteins were subjected to polypeptide analysis by discontinuous 5.5-7% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (NaDodS04-PAGE) (35) followed by fluorography. The radioactive molecular weight markers used were myosin (M, 200,000), phosphorylase b (M, 92,500), bovine serum albumin (M, 69,000), ovalbumin (M, 46,000), carbonic anhydrase (M, 30,000), and lysozyme (M, 14,300) (Amersham Corp.).

The amounts of [35S]methionine-labeled proteins were estimated from the fluorograms of the polyacrylamide gels by densitometric scans using an LKB 2202 ultracson laser densitometer with LKB 2202 integrator. The relative amounts of all proteins or the M, 47,000 protein were quantitated from several separate experiments as described in detail and compared to the amount of the total labeled protein, or to the amount of the inducible protein in untreated control cultures, or to the highest amount of the inducible M, 47,000 protein as indicated in each experiment.

**Preparation of ECM—**After collection of the media, the cultures were briefly rinsed with PBS. Subsequently, the cultures were extracted three times for 5-min periods each with 10 mM Tris-HCl buffer, pH 8.0, containing 0.5% sodium deoxycholate and 1 mM phenylmethylsulfonyl fluoride in an ice bath (36). The cell-free ECM preparations were washed three times with 2 mM Tris-HCl buffer, pH 8.0. ECM was either extracted with Laemmli’s sample buffer and analyzed by NaDodSO4-PAGE and fluorography or used immediately for further experiments. The sodium deoxycholate extraction procedure yields a reproducible pattern of polypeptides consisting of both extracellular matrix proteins (36) and substratum-associated proteins (40).

**Zymographic Analyses—**The culture medium and ECM were analyzed for PA and PAI activity using zymography and reverse zymography assays. These procedures were performed as described in detail earlier (37–40).

**Soft Agar Assay—**Colony formation of HT-1080 cells in soft agar was assayed as described earlier (41) with minor modifications. Briefly, 2.7 x 105 cells were suspended in 0.34% agar (Difco) in 10% fetal calf serum (GIBCO) with increasing concentrations of TGFβ. This suspension was applied to a 1-ml base layer of 0.5% agar in Linbro wells. The cultures were incubated at 37 °C in a humidified 5% CO2 atmosphere for 7 days, and cell colonies composed of over 90 cells were scored.

**Immunoblotting and Immunoprecipitation Analyses—The ConA-Sepharose-binding and ECM proteins were separated by 5-7% NaDodSO4-PAGE under reducing and nonreducing conditions followed by immunoblotting analysis (42). Bound rabbit or mouse anti-PAI-1 antibodies were detected using peroxidase-conjugated anti-immunoglobulins, purchased from Dako (Copenhagen, Denmark). The molecular weight markers used were phosphorylase b (M, 94,000), bovine serum albumin (M, 67,000), ovalbumin (M, 43,000), carbonic anhydrase (M, 30,000), soybean trypsin inhibitor (M, 20,100), and α-lactalbumin (M, 14,400) (Pharmacia Biotechnology, Inc.).

Immunoprecipitation was performed as described previously (40). Briefly, 1 ml of medium from TGFβ-treated culture was precipitated with 50 μl of protein-A-Sepharose (50% (v/v) Pharmacia Biotechnology, Inc.,) at 4 °C for 1 h. After centrifugation, the supernatant was collected and incubated with 5 μl of rabbit anti-PAI-1 antibodies and 50 μl of fresh protein A-Sepharose at 4 °C for 2 h. The immunoprecipitates bound to protein A-Sepharose were collected by centrifugation and washed three times with 10 mM Tris-HCl buffer, pH 8.0, 1 mM NaCl, 0.1% TWEEN 20, 0.1% TWEEN 80, 0.5% sodium deoxycholate, (0.05% sodium dodecyl sulfate), 0.5% sodium deoxycholate, 100 IU/ml aprotinin), and once with PBS. The samples were dissolved in Laemmli’s sample buffer and analyzed by 7% NaDodSO4-PAGE under reducing conditions.

**RESULTS**

**TGFβ Increases the Production and ECM Deposition of PAI-1 in HT-1080 Cells**

**Stimulation of Overall Protein Synthesis of TGFβ—Polypeptide synthesis of HT-1080 cells was studied by [35S]methionine labeling of confluent serum-free cultures in the presence of TGFβ. The changes in the secretion of proteins into the culture medium were observed from fluorograms after NaDodSO4-PAGE. The total stimulation of ConA-Sepharose-binding proteins in 24 h was about 3-fold, consisting of several induced proteins (Fig. 1A). However, the most prominent change was seen in the amount of an M, 47,000 protein (Fig. 1A and Table I). This protein represented about 48% of the total radiolabeled ConA-Sepharose-binding protein in the conditioned medium of TGFβ-treated culture, whereas its amount was 24% of the respective control cultures (Table I). The effect of TGFβ on the extracellularly deposited proteins
Fig. 1. Characterization of TGFβ-induced PAI-1 in HT-1080 cell cultures. A, secretion and ECM deposition of TGFβ-induced proteins. Confluent cultures of HT-1080 cells were labeled with [35S]methionine in the presence of TGFβ (1 ng/ml) for 24 h. The media were collected, and ConA-Sepharose-binding radiolabeled proteins were isolated (see "Methods"). ECM was then prepared from the respective cultures using the sodium deoxycholate extraction procedure. All samples were analyzed by 7% NaDdSO₄-PAGE under reducing conditions followed by fluorography. B, reverse zymography of TGFβ3-induced medium and ECM proteins. Conditioned medium (40 μl) and ECM samples from TGFβ3-treated cultures were analyzed by 7% NaDdSO₄-PAGE under reducing conditions followed by the caseinolysis-overlay assay in the presence of u-PA (2 IU/ml). The gels were then incubated at 37°C until a complete lysis of the caseinolysis gel occurred, at which point the gel was photographed. PAI activity shows as a lysis-resistant opaque band. C, zymography of TGFβ3-induced ECM secretory products in the presence of TGFB. The sensitivity of cultures also slightly decreased the PA activity-containing bands of higher molecular weight were detected in the ECM preparations by zymography. The majority of PA activity of the medium and ECM co-migrated with the M, 52,000 u-PA marker. A minor higher molecular weight complex of M, 94,000 containing active u-PA was detected in the medium by zymography only after prolonged incubation times (not shown). In addition, some faint PA activity-containing bands of higher molecular weight were detected in the ECM preparations by zymography. None of these proteins gave lysis zones in a caseinolysis gel in which plasminogen was omitted, indicating that the activity was specific for PA (gels not shown).

Table I

<table>
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<th>Proportions of TGFβ-induced PAI-1 of the total medium and ECM protein in HT-1080 cell cultures</th>
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| Confluent cultures of HT-1080 cells were labeled with [35S]methionine in the presence of TGFβ3 (1 ng/ml) for 24 h. The media were collected and adsorbed with ConA-Sepharose, and ECM was prepared from cell layers. The samples were analyzed by 7% NaDdSO₄-PAGE and fluorography followed by densitometric scanning. The amount of total radiolabeled ConA-Sepharose-binding medium protein in TGFβ3-treated cultures was increased 3-fold over the control. Accordingly, the TGFβ3 stimulation of ECM deposition of proteins was 2.5-fold. The relative amounts of labeled M, 47,000 protein (PAI-1) are expressed as percent of total labeled protein in the respective medium or ECM samples. The specific TGFβ3-induced increase of PAI-1 was thus 2-fold in the medium and 4-fold in ECM.

<table>
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<th>Component</th>
<th>Control</th>
<th>TGFβ3</th>
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<tr>
<td>Medium</td>
<td>24 ± 8</td>
<td>48 ± 7</td>
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<tr>
<td>ECM</td>
<td>14 ± 10</td>
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* Each value shown represents the mean of five separate experiments.

Dose Response of TGFβ Stimulation of PAI-1 in HT-1080 Cells

A dose dependence study with TGFβ was performed to analyze the response of HT-1080 cells in the stimulation of deposition of PAI-1. Analysis of the ECM preparations showed that half-maximal stimulation of the deposition of PAI-1 in ECM occurred at a concentration of 0.7 ng/ml TGFβ as judged from the scanning curve (Fig. 3).

TGFβ has previously been found to inhibit the anchorage-independent growth of certain malignant cell lines (21, 27). We therefore studied whether TGFβ was inhibitory for the growth of HT-1080 cells. The cells were seeded in soft agar in the presence of increasing concentrations of TGFβ. The cultures were incubated for 7 days, and the colonies containing over 90 cells were scored. It was found that TGFβ inhibited the growth of HT-1080 cells with maximal effect at 5 ng/ml.
TGFB-induced Pericellular PAI-1 Is Sensitive to u-PA

FIG. 2. Immunological characterization of TGFB-induced proteins with anti-PAI-1 antibodies. A, immunoblotting analysis. The ConA-Sepharose-binding proteins and ECM samples were prepared as described for Fig. 1 and analyzed by 7% NaDodSO4-PAGE followed by immunoblotting analysis with polyclonal anti-PAI-1 antibodies. Lane 1, ConA-Sepharose-binding medium proteins; lane 2, ECM preparations. B, immunoprecipitation analysis. Medium (1 ml) from TGFB-treated cultures was preadsorbed with protein A-Sepharose, and the protein A-binding proteins were analyzed (lane 1). After preadsorption, the supernatant was subjected to immunoprecipitation with polyclonal anti-PAI-1 antibodies and fresh protein A-Sepharose (lane 2). Subsequently, the supernatant from the immunoprecipitation was adsorbed with ConA-Sepharose (lane 3). As a positive control, a medium sample from TGFB-treated culture was incubated directly with ConA-Sepharose (lane 4). Protein A- and ConA-Sepharose particles or immunoprecipitates were washed and dissolved in Laemmli's sample buffer and analyzed by 7% NaDodSO4-PAGE followed by fluorography. TGFB treatment of the cultures is indicated (− or +). PAI-1 and the migration of the immunoreactive M, 76,000 protein are indicated on the right.

FIG. 3. Deposition of PAI-1 in ECM of HT-1080 cells by TGFB: dose dependence. Confluent cultures of HT-1080 cells were labeled with [35S]methionine under serum-free conditions in the presence of different growth factors for 24 h. ECM was prepared and analyzed by NaDodSO4-PAGE and fluorography. Lane 1, untreated control; lane 2, TGFB (5 ng/ml); lane 3, EGF (5 ng/ml); lane 4, TGFα (5 ng/ml); lane 5, PDGF (25 ng/ml); lane 6, insulin (500 ng/ml) (Fig. 4). An effective inhibition was obtained at similar concentrations as needed for the deposition of PAI-1.

Induction of PAI-1 into ECM of HT-1080 Cells Is Specific for TGFB

The specificity of TGFB in the induction of PAI-1 synthesis was studied using some growth-promoting polypeptides and comparing their effects on the HT-1080 cell ECM to those of TGFB. Confluent cultures of HT-1080 cells were labeled with [35S]methionine in the presence of different growth factors for 24 h. ECM was prepared and analyzed by NaDodSO4-PAGE and fluorography. Lane 1, untreated control; lane 2, TGFB (5 ng/ml); lane 3, EGF (5 ng/ml); lane 4, TGFα (5 ng/ml); lane 5, PDGF (25 ng/ml); lane 6, insulin (500 ng/ml). The data suggest that TGFB is the major factor which is able to induce PAI-1 synthesis and ECM deposition in HT-1080 cells.
Kinetics of the Deposition and Degradation of TGFβ-induced PAI-1 in HT-1080 Cell ECM and Culture Medium

Time dependence studies were performed to investigate the synthesis and turnover of TGFβ-induced PAI-1 both in the medium and ECM. HT-1080 cells were incubated with TGFβ (1 ng/ml) for 0–48 h under serum-free conditions. The media were collected and adsorbed with ConA-Sepharose, and ECM was prepared from the respective cultures. The amount of M, 47,000 PAI-1 was estimated from fluorograms after NaDodSO4-PAGE by densitometric scanning. PAI-1 appeared at 47,000 protein in the medium and ECM.

PAI-1 appeared at 47,000 protein in the medium and ECM. The results are plotted as percentages of the highest amounts of M, 47,000 protein in either ECM preparations (time 0) or medium (time 14 h), which are indicated as 100%.

The release of PAI-1 from the cell layers was next studied by following its appearance in the culture medium. On the basis of a time dependence study (Fig. 6), the cultures were first labeled with [35S]methionine for 6 h in the presence of TGFβ (1 ng/ml). These conditions were sufficient for ECM deposition of PAI-1 (Fig. 6 and Fig. 7, time 0). The cultures were then washed, and new serum-free medium was added without TGFβ. The media were collected at various times, and ECM was prepared. Analysis of the fluorograms by NaDodSO4-PAGE indicated that PAI-1 was removed rapidly from ECM. About 60% of PAI-1 disappeared within 14 h, and it was almost totally lost by 24 h (Fig. 7). The amount of PAI-1 increased in the medium during the incubation, suggestive of accumulation of ECM-released PAI-1 (Fig. 7).

TGFβ-induced PAI-1 in ECM and the Medium Is Sensitive to Removal by Exogenous u-PA

The accessibility of the TGFβ-induced ECM-associated PAI-1 to purified exogenous u-PA was studied. The cultures were labeled with [35S]methionine in the presence of TGFβ (1 ng/ml) and aprotinin (100 IU/ml) for 6 h. Increasing concentrations of u-PA were then added to the culture medium as indicated, and the cultures were further incubated without removing the label for 18 h. After the incubation, the media were collected and adsorbed with ConA-Sepharose, and ECM was prepared from respective cultures. Analysis of the radiolabeled proteins of the medium and ECM by NaDodSO4-PAGE and fluorography indicated that moderate concentrations of u-PA (30–150 IU/ml) were able to degrade or complex ECM-associated and medium PAI-1 (Fig. 8, A and B). The relative amount of PAI-1 decreased about 90% in the TGFβ-treated cultures by 150 IU/ml u-PA (Fig. 8, A and B). Similar analysis of untreated control cultures indicated that virtually all PAI-1 was removed from the ECM preparations, as judged by fluorograms after NaDodSO4-PAGE (Fig. 8, A and B).

When the amount of M, 47,000 PAI-1 decreased in the medium due to the effect of u-PA, the amount of an M, 62,000 ConA-Sepharose-binding protein increased (Fig. 8B). Immunoblotting analysis with monoclonal and polyclonal antibodies showed that this M, 62,000 protein contained immunoreactive PAI-1 (Fig. 9A) as well as immunoreactive u-PA (Fig. 9B). The ECM samples, on the contrary, contained little if any u-PA or PAI-1 immunoreactive M, 62,000 protein as judged by several separate experiments (Fig. 9, A and B). These effects of u-PA could be prevented by adding polyclonal anti-u-PA antibodies or 50–500 nM p-aminobenzamidine to the cultures, indicating that the removal of PAI-1 was u-PA-specific (not shown).

We also analyzed the u-PA sensitivity of isolated ECM proteins. ECM was isolated from cultures labeled with [35S]methionine with [35S]methionine in the presence of TGFβ (1 ng/ml) and aprotinin (100 IU/ml) for 6 h. Increasing concentrations of u-PA were then added to the culture medium as indicated, and the cultures were further incubated without removing the label for 18 h.
polyclonal anti-PAI-1 antibodies were analyzed by NaDodSO₄-PAGE followed by immunoblotting with u-PA (150 IU/ml) under serum-free conditions. u-PA was added to the culture media, and the cultures were further incubated for 18 h. The media were then collected, and the radiolabeled proteins were adsorbed with ConA-Sepharose. ECM was prepared from the respective cell layers. The samples in the medium and ECM preparations.

The media were then collected and adsorbed with ConA-Sepharose, and ECM was prepared from the respective cultures. All samples were analyzed by NaDodSO₄-PAGE and fluorography. A, ECM preparations; B, ConA-binding medium proteins. The presence of TGFβ during the 24-h incubation and the amounts of added urokinase at 6 h are indicated. The migration of PAI-1 and M, 62,000 and 76,000 proteins are indicated on the right, and molecular weight markers on the left.

Fig. 8. Sensitivity of TGFβ-induced ECM and medium proteins of HT-1080 cells to exogenous u-PA. Confluent cultures of HT-1080 cells were labeled with [35S]methionine in the presence of TGFβ (1 ng/ml) and aprotinin (100 IU/ml). After a 6-h incubation, increasing concentrations of u-PA were added to the culture media, and the cultures were further incubated for 18 h. The media were then collected, and the radiolabeled proteins were adsorbed with ConA-Sepharose. ECM was prepared from the respective cell layers. All samples were analyzed by NaDodSO₄-PAGE and fluorography. A, ECM preparations; B, ConA-binding medium proteins. The presence of TGFβ during the 24-h incubation and the amounts of added urokinase at 6 h are indicated. The migration of PAI-1 and M, 62,000 and 76,000 proteins are indicated on the right, and molecular weight markers on the left.

Fig. 9. Immunoblotting analyses of u-PA-sensitive proteins in the medium and ECM preparations. Confluent cultures of HT-1080 cells were incubated with TGFβ (1 ng/ml) and aprotinin (100 IU/ml) under serum-free conditions. u-PA (200 IU/ml) was added after a 6-h incubation, and the cultures were further incubated for 18 h. The media were then collected and adsorbed with ConA-Sepharose, and ECM was prepared from the respective cell layers. The samples were analyzed by NaDodSO₄-PAGE followed by immunoblotting with polyclonal anti-PAI-1 antibodies (A) and monoclonal u-PA antibodies (B). Lane 1, ConA-Sepharose-binding proteins; lane 2, ECM preparations. The addition of u-PA during cultivation is indicated (− or +). Immunoreactive PAI-1, M, 62,000 and 76,000 proteins, and molecular weight markers are indicated.

Methionine for 6 h in the presence of TGFβ and then incubated with u-PA (150 IU/ml) for 1 h at 37 °C. The incubation medium was collected and analyzed directly by NaDodSO₄-PAGE followed by immunoblotting analysis. ECM was analyzed as described above. Also in this experiment, u-PA released PAI-1 from the ECM preparations, and an anti-PAI-1 immunoreactive M, 62,000 protein was subsequently detected in the incubation fluid. Neither M, 76,000 protein nor any smaller immunoreactive fragments of PAI-1 were detected (not shown). The results strongly suggest that PAI-1 deposited by the cells is a direct substrate of u-PA.

DISCUSSION

Growth factors participate in the regulation of the proteolytic activity of cultured cells. Enhanced PA activity by EGF has been observed in a number of studies (40, 44, 45). The ability of tumor cells to produce growth factors that function in an autocrine or paracrine manner (16, 46) suggests that some malignant cells might produce an activity that could also affect the PA levels in cultured cells. We have earlier detected an efficient cell-secreted modulator of PA activity in the conditioned culture medium of a human fibrosarcoma cell line (47). Subsequently, we found that purified TGFβ had both stimulatory and inhibitory effects on the PA activity of fibroblastic cells (39, 40). Enhanced production of an M, 48,000 protein in TGFβ-treated BSC-1 cells has also been reported (48). This protein has been shown to be immunologically related to PAI-1 (49).

An important mechanism of TGFβ regulation of PA activity in fibroblastic cells is the induction of PAI-1 (40). In this work, we have extended this observation and find that TGFβ can induce significant amounts of PAI-1 in malignant cells. Plasminogen activator-sensitive molecules have been proposed and observed in cultured cells, in their extracellular matrices, and as secreted molecules (50–52). We therefore addressed the question of the significance of the induction and ECM deposition of PAI-1 by metabolic labeling and immunological assays and studied its sensitivity to removal and degradation by urokinase. The term ECM used here refers to the extracellularly substrate-attached proteins, which partly consist of extracellular matrix proteins.

The human fibrosarcoma cell line, HT-1080 secretes high
amounts of PAs and collagenases (53–55). When exposed to TGFβ, a rapid increase in the amount of the metabolically labeled PAI-1 occurs. The inhibitor is secreted into the culture medium and deposited in the substratum of the HT-1080 cells. The deposited inhibitor is removed from ECM by moderate concentrations of u-PA. Time course and pulse-chase experiments suggested that a part of the secreted PAI-1 is directly deposited in the immediate vicinity of the cells in a functionally active form capable of reacting with u-PA, resulting in the release of the inhibitor into the culture medium. The remarkable sensitivity of HT-1080 cells to TGFβ suggests that this growth factor may in part function by modifying the proteolytic balance expressed by malignant cells.

Impaired ability to form organized extracellular matrices is a characteristic feature of different malignant cells grown in vitro (1). The reason for this defect is probably multifunctional. Increased proteolytic activity of malignant cells evidently contributes to the metastatic potential and invasive growth of the cells (cf. Ref. 56), and the production of elevated amounts of PA correlates with the transformed phenotype (51). Since most matrix glycoproteins are sensitive to degradation by plasmin (Ref. 57; cf. Ref. 4), PAs may have a central role in the regulation of extracellular proteolysis. Native collagens are resistant to the action of plasmin, but it can activate latent collagenases (53, 58, 59). On the other hand, invasion of tumor cells into collagen-rich tissue can be prevented both by inhibitors of collagenases and primarily by PA inhibitors (80). Furthermore, the morphological changes resulting from elevated levels of PA activity in virus-transformed cells can be inhibited by plasmin and PA inhibitors and anti-u-PA antibodies (51, 61). Anti-u-PA antibodies have also been shown to inhibit the metastatic ability of a carcinoma cell line in chicken embryos (62). Interestingly, protease nexin I inhibits effectively the ECM degradation of HT-1080 cells, which also leads to a transient arrest of the proliferation of these cells (63).

PAI-1 has been immunocytochemically found to be distributed as a homogenous carpet on the substratum of the cells, with localization distinct from that of fibronectin and u-PA (64). This together with other data on the ECM distribution of PAI-1 (40) or other closely related proteins possibly identical to the inhibitor (65) is suggestive of a regulatory role for PAI-1 in local PA activity. The crucial step leading to enhanced pericellular proteolytic activity and localized degradation thus appears to be the modulation of the activity of PAs either by regulation of proenzyme activation or deposition of specific inhibitors into the microenvironment of the cells. When HT-1080 cells were exposed to u-PA, several events took place indicating that ECM PAI-1 was biologically active. The amount of PAI-1 decreased in ECM during u-PA treatment. In addition to the M, 47,000 form, immunoactive, but not functionally active, PAI-1 complexes of M, 62,000 and 76,000 were found in the conditioned medium, evidently representing inactive complexes between PAI-1 and u-PA (40). These complexes were barely detectable in ECM by immunological analysis. TGFβ-induced PAI-1 is thus at least in part deposited directly in the pericellular area upon secretion. After restoration of the proteolytic activity of the cells, the pericellular inhibitor is rapidly lost, which appears to be a direct effect of u-PA.

According to Nielsen et al. (52), the complex between PAI-1 and u-PA is unstable and leads to the release of active u-PA and an irreversibly inactivated and degraded form of PAI-1 of slightly lower molecular weight. However, we did not detect truncated forms of PAI-1, except very small amounts in the ECM preparations. Although the mechanism of the loss remains unclear, our data suggest that the pericellularly deposited PAI-1 is sensitive to a direct action of u-PA.

TGFβ enhances the synthesis and extracellular deposition of fibronectin and procollagen into the extracellular matrix of cultured fibroblasts (66). TGFβ also induces desmoplastic reactions including collagen formation and angiogenesis when injected subcutaneously in mice (32). In this work, the amount of fibronectin was slightly increased in ECM of HT-1080 cells after TGFβ treatment. This finding is in agreement with the hypothesis that controlled pericellular proteolytic activity, which here may be achieved by excessive ECM deposition of PAI-1 in the microenvironment of the cells, is a prerequisite for enhanced matrix deposition.

The relationships among decreased pericellular proteolysis, PAI-1 induction, and the growth control of cells remain to be established. TGFβ is an efficient growth regulator which decreases the local proteolytic activity of the HT-1080 cells. However, the effects of TGFβ on proteolytic balance and cellular growth may be modulated by other exogenous or autocrine growth factors (18). For example, a role for interleukin-1 in the regulation of PAI-1 has been proposed (67, 68). In accordance with previous results (21, 27), we find here that the same concentrations of TGFβ induce PAI-1 in HT-1080 cells and inhibit the growth of this tumor cell line in soft agar. Whether the inhibition of anchorage-independent growth of HT-1080 cells is related to the decreased proteolytic activity at the cell-substratum contact areas remains unclear. However, we assume that part of the growth inhibitory effects of TGFβ may reflect alterations in the proteolytic activity of the cells which further modify the interactions of cells with the growth substratum.

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