Plasmin cleaves betaglycan and releases a 60 kDa transforming growth factor-β complex from the cell surface

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Plasmin regulates the activity and distribution of transforming growth factor β (TGF-β) and other growth factors. The purpose of the present investigation was to determine the effects of plasmin on cellular receptors for TGF-β. AKR-2B fibroblasts were affinity-labelled with ^{125}I-TGF-β1 and ^{125}I-TGF-β2, demonstrating betaglycan, the type-I TGF-β receptor and the type-II TGF-β receptor. Treatment of TGF-β-affinity-labelled cells with plasmin (10–100 nM) for 1 h profoundly and selectively decreased recovery of TGF-β–betaglycan complex. The type-I and type-II receptors were not plasmin substrates. A radiolabelled complex with an apparent mass of 60 kDa was detected by SDS/PAGE in both the medium and cell extracts of plasmin-treated affinity-labelled cells. In order to demonstrate that plasmin cleavage of betaglycan did not require prior exposure of the betaglycan to cross-linking agent, AKR-2B cells were treated with plasmin first and then affinity-labelled. Markedly decreased TGF-β binding to cellular betaglycan was observed. Although plasmin treatment of AKR-2B cells decreased overall binding of ^{125}I-TGF-β1 and ^{125}I-TGF-β2, the rate at which the cells degraded bound ^{125}I-TGF-β at 37 °C was not changed. AKR-2B cells treated with plasmin demonstrated slightly increased [^{3}H]thymidine incorporation; the plasmin-treated cells retained their ability to respond to TGF-β. Conditioned medium from plasmin-treated AKR-2B cells contained increased amounts of active TGF-β as determined in Mv 1 Lu epithelial-cell-proliferation assays. Specific cleavage of betaglycan represents a novel mechanism whereby plasmin may regulate the assortment of receptors available for TGF-β. In addition, plasmin may facilitate transfer of active TGF-β between neighbouring cells by releasing the active growth factor from the cell surface.

INTRODUCTION

Plasmin is a multifunctional serine proteinase which plays a pivotal role in fibrinolysis and has been strongly implicated in cell growth regulation [1,2]. Plasminogen activation is mediated by urokinase-type plasminogen activator (u-PA) and tissue-type plasminogen activator; the efficiency of plasminogen activation can be significantly increased when both zymogen and activator are bound to cell surfaces [3]. Once generated in the pericellular spaces, plasmin may cleave glycoprotein components of the extracellular matrix, activate metalloproteinases and generate growth factors from their precursors. Plasmin affects the activity and distribution of transforming growth factor-β1 (TGF-β1) in at least two ways: by activating the latent form of secreted TGF-β1 and by releasing latent TGF-β1 from binding sites in the pericellular matrix [4–7].

TGF-β1 and TGF-β2 belong to a superfamily of signalling polypeptides which influence cell growth, wound repair, the inflammatory response and tissue development [5,8,9]. Both growth factors interact with multiple cell receptors and binding proteins [10]. In many cell types, the most abundant cell-surface TGF-β-binding protein is the type-III receptor, also known as betaglycan [11]. This receptor provides up to 120000 TGF-β-binding sites per cell [11].

Betaglycan is a large cell-surface proteoglycan [12,13]. The core protein of betaglycan, which has been cloned, includes 853 amino acids, a transmembrane domain and the binding site for TGF-β [12,14–16]. As determined by SDS/PAGE, the apparent mass of the core protein is 110–130 kDa. The glycosaminoglycan (GAG) chains of betaglycan bind basic fibroblast growth factor (bFGF) [17]. Betaglycan apparently does not mediate signalling events for either TGF-β or bFGF [14,15]. Instead, TGF-β signal transduction is mediated in most cells by the type-I and type-II receptors [18,19]. Betaglycan may enhance the cellular response to TGF-β by binding the growth factor and presenting it to the type-II receptor [20,21].

In addition to betaglycan and the type-I and type-II receptors, prominent TGF-β-binding proteins in the 50–60 kDa range have been identified on the surfaces of some cell types [20,22,23]. The same protein(s) may be associated with the extracellular matrix [22]. The function and biochemical nature of these protein(s) remain incompletely characterized.

In the present investigation, binding of TGF-β1 and TGF-β2 to AKR-2B fibroblasts was analysed by affinity-labelling [24]. Plasmin selectively decreased TGF-β binding to cellular betaglycan without decreasing binding to the type-I and type-II receptors. After plasmin treatment, a new 60 kDa complex containing radioiodinated TGF-β was recovered in the culture medium and associated with the cells. Conditioned medium from AKR-2B cells, which were pulse-exposed to plasmin, contained increased TGF-β activity as determined in bioassays with Mv 1 Lu epithelial cells. On the basis of these studies, we propose a novel mechanism whereby plasmin may regulate TGF-β activity and distribution.

Abbreviations used: TGF-β, transforming growth factor β; bFGF, basic fibroblast growth factor; GAG, glycosaminoglycan; DSS, disuccinimidyl suberate; PMSF, phenylmethanesulphonyl fluoride; EBSS, Earle's balanced salts solution; u-PA, urokinase plasminogen activator; PNPGB, *p*-nitrophenyl *p'*-guanidinobenzoate hydrochloride; S-2251, D-Val-L-Leu-L-Lys-*p*-nitroanilide; DMEM, Dulbecco's modified Eagle's medium; FBS, fetal bovine serum; DFP, di-isopropyl phosphorofluoridate; DIP, di-isopropylphosphoryl; SBTI, soya-bean trypsin inhibitor.

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MATERIALS AND METHODS

Proteins and reagents

Disuccinimidyl suberate (DSS) was purchased from Pierce Chemical Co. (Rockford, IL, U.S.A.). Chloramine-T, Hepes, phenylmethanesulphonyl fluoride (PMSF), benzamidine hydrochloride, leupeptin, soya-bean trypsin inhibitor (SBTI), diisopropyl phosphorofluoridate (DFP), *p*-nitrophenyl *p'*-guanidinobenzoate hydrochloride (PNPGB), ε-aminohexanoic acid, aprotinin, heparitinase, chondroitinase ABC and Ham's F12 medium were from Sigma Chemical Co. (St. Louis, MO, U.S.A.). D-Val-L-Leu-L-Lys-*p*-nitroanilide hydrochloride (S-2251) was from Kabi Vitrum (Franklin, OH, U.S.A.). Earle's balanced salts solution (EBSS) and Dulbecco's modified Eagle's medium (DMEM) were from Gibco (Grand Island, NY, U.S.A.). Fetal bovine serum (FBS) was from Biowhittaker (Walkersville, MD, U.S.A.).

[Glu^1]plasminogen was purified from fresh-frozen human plasma and dialysed against 10 mM Hepes/50 mM sodium acetate, pH 7.4 [25]. Plasminogen (5.0 μM) was activated with low-molecular-mass u-PA (50 nM) (Calbiochem, La Jolla, CA, U.S.A.) for 15 min at 37 °C. The concentration of active plasmin was determined by active-site titration with PNPGB or by S-2251 hydrolysis using previously reported kinetic parameters: $k_{cat.}$, 11.7 s^{-1}; K_m, 180 μM [26]. Di-isopropylphosphoryl (DIP)-plasmin was prepared by incubating plasmin with 10 mM DFP for 1 h at 22 °C. Excess DFP was removed by dialysis. Final DIP-plasmin preparations retained less than 0.5 % of the original amidase activity, as determined by S-2251 cleavage.

TGF-β1 was purified from human platelets by the method of Assoian et al. [27], or purchased from R&D Systems (Minneapolis, MN, U.S.A.). TGF-β2 was purchased from Genzyme (Cambridge, MA, U.S.A.). TGF-β1 and TGF-β2 were radioiodinated by the chloramine-T method as previously modified [28]. Specific radioactivities ranged from 50 to 100 μCi/μg.

Cell culture

AKR-2B (subclone 84A) fibroblasts were kindly provided by Dr. Harold Moses (Vanderbilt University, Nashville, TN, U.S.A.). Mv 1 Lu cells were obtained from the American Type Culture Collection (Rockville, MD, U.S.A.). Both lines were maintained in DMEM with 10 % FBS in T-75 flasks (Costar, Cambridge, MA, U.S.A.) and passaged by trypsin treatment every 3 days.

Affinity-labelling of TGF-β receptors

Cellular TGF-β receptors were affinity-labelled by the method of Massagué [24] with the following minor modifications. AKR-2B cells were plated at 2.5×10^5 cells/well in 35 mm wells (Costar). After being cultured for 48 h, the cells were washed three times with EBSS/25 mM Hepes, pH 7.4, containing 2 mg/ml BSA (EHB) and then incubated for 3 h with 100 pM ^{125}I-TGF-β1 or ^{125}I-TGF-β2 at 4 °C. After five washes with EHB, 10 μl of the cross-linking agent, DSS (final concentration 0.167 mM in 1.0 ml), was added for 15 min. The cells were then washed once for 5 min in 50 mM glycine/100 mM NaCl, pH 5, and once in EHB.

Plasmin treatment of AKR-2B cells

^{125}I-TGF-β-affinity-labelled AKR-2B cells were incubated with 10–100 nM plasmin for 1 h at 37 °C. The supernatants overlying the cells were collected and treated with aprotinin (20 μM). The cultures were washed for 5 min with EHB containing 20 μM aprotinin and then with 10 mM Tris/HCl, pH 7.4, containing 0.25 M sucrose, 1 mM EDTA and 0.3 mM PMSF. Cells were detached by scraping, pelleted by centrifugation for 2 min at 12000 *g*, and then resuspended for 1 h in 100 μl of detergent-extraction buffer [10 mM Tris/HCl, 125 mM NaCl, 1 mM EDTA, 1.0 % (w/v) Triton X-100, 1 mM PMSF, 10 μg/ml leupeptin, 50 μg/ml aprotinin, 100 μg/ml SBTI, 100 μg/ml benzamidine hydrochloride, pH 7.4). Insoluble debris was removed by centrifugation (12000 *g* × 15 min). Cell extracts and plasmin-conditioned medium samples were subjected to SDS/PAGE on 4–10 % polyacrylamide gradient slabs using the Laemmli buffer system [29]. Affinity-labelling of TGF-β receptors was not affected when the AKR-2B cells were incubated for 1 h at 37 °C in the absence of plasmin subsequent to DSS treatment.

In some experiments, AKR-2B cells were treated with plasmin at 37 °C before affinity-labelling. The cells were then washed extensively in buffers that included aprotinin to inactivate the plasmin and affinity-labelled with ^{125}I-TGF-β1 or ^{125}I-TGF-β2. Plasmin treatment (100 nM) of AKR-2B cells for 1 h at 37 °C did not affect cell viability as determined by Trypan Blue exclusion.

GAG digestion experiments

Monolayers of AKR-2B cells were treated with heparitinase (2 units/ml) and chondroitinase ABC (0.5 unit/ml) for 3 h, as described by Chiefetz et al. [12]. The cells were then washed in EHB, treated with plasmin and affinity-labelled. Medium samples (from affinity-labelled and plasmin-treated cultures) were also incubated with heparitinase (2 units/ml) and chondroitinase ABC (0.5 unit/ml) for 18 h at 37 °C before analysis by SDS/PAGE and autoradiography.

Binding and degradation studies

Binding and degradation of TGF-β1 and TGF-β2 were evaluated using a modification of previous methods [30]. AKR-2B fibroblasts were plated at a density of 2.5×10^4 cells/well in 48-well plates in DMEM with 10 % FBS and cultured for 48 h. The cells were then washed three times with EHB and exposed to 100 nM plasmin for 1 h. Control (untreated) and plasmin-treated cells were washed twice with EHB containing 20 μM aprotinin for 5 min and twice with cold EHB. The cultures were then incubated with 150 pM ^{125}I-TGF-β1 or ^{125}I-TGF-β2 at 4 °C for 3 h. After being washed five times with cold EHB, the cell cultures were equilibrated in DMEM containing 25 mM Hepes and 1 mg/ml BSA, pH 7.4, and warmed to 37 °C. At various times, the medium was collected and the cells were fixed in 10 % trichloroacetic acid. Trichloroacetic acid (10 %) was also added to the medium samples; soluble and precipitated fractions were separated by microcentrifugation for 10 min. Fixed-cell preparations were lysed in 2 % SDS/0.1 M NaOH. Radioactivity in the cell lysates and in medium samples (trichloroacetate-precipitable and soluble) was determined in a γ counter.

Bioassay for TGF-β

The mitogenic activity of TGF-β1 and TGF-β2 in AKR-2B fibroblasts was determined by a modification of the method of Shipley et al. [31]. Cells were plated at a density of 1.0×10^4/well in 48-well culture dishes in DMEM with 10 % FBS and incubated for 12 h. The medium was then replaced with serum-free medium [1:1 formulation of DMEM and Ham's F12 medium, L-glutamine (0.68 mM), transferrin (5 μg/ml), ascorbate (0.2 mM) and selenium (38 nM)]. After 48 h, the cells were washed with DMEM, and then incubated with 0–100 nM plasmin for 1 h at 37 °C. To inactivate the plasmin, the cultures were washed twice

for 5 min with 20 μM aprotinin in DMEM. The cells were then pulse-exposed to TGF-β1 or TGF-β2 for 1 h, washed twice with serum-free medium and cultured in this medium for an additional 36 h. [^{3}H-*methyl*]Thymidine (1.0 μCi/ml; NEN, Boston, MA, U.S.A.) was added to each culture for 1 h. The cultures were washed twice with cold PBS, fixed for 1 h in methanol/acetic acid (3:1, v/v) and lysed. Thymidine incorporation was determined in a liquid-scintillation counter.

Analysis of TGF-β activity in plasmin-conditioned medium

Confluent monolayers of AKR-2B fibroblasts were incubated with TGF-β1 or TGF-β2 (150 pM) in sterile EHB for 3 h at 4 °C. The cells were washed four times with EHB and once with DMEM containing 25 mM Hepes and 0.25 mg/ml BSA, pH 7.4. The fibroblasts were then incubated in the same medium with or without 100 nM plasmin at 37 °C for 5 min. Medium samples were collected into aprotinin (final concentration, 20 μM). Active TGF-β in the medium was determined by inhibition of [^{3}H]thymidine incorporation into Mv 1 Lu cells [32]. Briefly, Mv 1 Lu cells were plated at a density of 2.5×10^4 cells per well in DMEM containing 10% FBS (48-well culture plates). After 12 h, the medium was replaced with serum-free DMEM containing various amounts of AKR-2B cell plasmin-conditioned medium. As a control, the Mv 1 Lu cells were also incubated in serum-free DMEM supplemented with known concentrations of TGF-β1 or TGF-β2. The cultures were incubated for 24 h, pulse-exposed to 0.5 μCi/well [^{3}H]thymidine for 1 h, washed twice with PBS at 4 °C, and then fixed for 1 h in ice-cold methanol/acetic acid (3:1, v/v). The cells were lysed in 2% SDS/0.1 M NaOH. Thymidine incorporation was determined in a liquid-scintillation counter.

RESULTS

Plasmin treatment of TGF-β-affinity-labelled fibroblasts

AKR-2B fibroblasts were affinity-labelled with ^{125}I-TGF-β1. The three expected TGF-β receptors (betaglycan, type I and type II) were demonstrated (Figure 1a). A 200-fold molar excess of unlabelled TGF-β1 completely inhibited ^{125}I-TGF-β1 binding to the receptors, confirming the specificity of these interactions (lane 1). Treatment of ^{125}I-TGF-β1-affinity-labelled fibroblasts with plasmin (10–100 nM) significantly decreased recovery of intact (cross-linked) ^{125}I-TGF-β1–betaglycan complex in the cell extracts. The extent of the decrease was plasmin-concentration-dependent. In contrast, recovery of ^{125}I-TGF-β1 associated with the type-I and type-II receptors was minimally affected. A novel band with an apparent mass of approximately 60 kDa was detected in lysates of plasmin-treated cells (arrow). Assuming that the cross-linked ^{125}I-TGF-β1 is monomeric (12.5 kDa), the mass of the TGF-β1-associated product is about 50 kDa.

Affinity-labelling experiments were performed with ^{125}I-TGF-β2. As shown in Figure 1(b), betaglycan was clearly demonstrated; however, the type-I and type-II receptors were barely visible. Other investigators have also demonstrated highly selective labelling of betaglycan by ^{125}I-TGF-β2 [12]. ^{125}I-TGF-β2 binding was specific, because a 200-fold molar excess of unlabelled TGF-β1 substantially inhibited it (lane 7). Treatment of the TGF-β2-affinity-labelled cells with plasmin markedly decreased recovery of intact cross-linked ^{125}I-TGF-β2–betaglycan complex. The novel 60 kDa band was again apparent in the cell extracts.

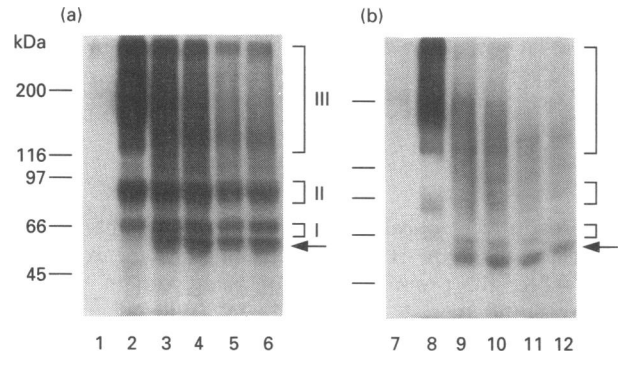

Figure 1 Plasmin treatment of TGF-β-affinity-labelled AKR-2B fibroblasts

Confluent monolayers of AKR-2B fibroblasts were affinity-labelled with 100 pM ^{125}I-TGF-β1 (**a**) or ^{125}I-TGF-β2 (**b**). The cultures were then treated with plasmin for 1 h. Cell extracts were prepared and analysed by SDS/PAGE and autoradiography. Lanes 1 and 7 show ^{125}I-TGF-β binding to cell receptors in the presence of a 200-fold molar excess of unlabelled TGF-β1. Lanes 2 and 8 show ^{125}I-TGF-β binding without subsequent plasmin treatment. Cells treated with 10 nM plasmin are shown in lanes 3 and 9; 25 nM plasmin in lanes 4 and 10; 50 nM plasmin in lanes 5 and 11; and 100 nM plasmin in lanes 6 and 12. The arrow indicates the position of a 60 kDa radiolabelled band present in cell lysates of plasmin-treated cells. The mobilities of known TGF-β receptors and molecular-mass markers are shown.

Analysis of plasmin-conditioned medium

AKR-2B fibroblasts were affinity-labelled with ^{125}I-TGF-β1. The cultures were then treated with plasmin for 1 h and the medium analysed by SDS/PAGE and autoradiography. In addition to free ^{125}I-TGF-β1 (which migrated near the dye front), a single novel band was detected in the medium. The mobility of this band suggested a molecular mass of 60 kDa, identical with the product detected in association with plasmin-treated cells (Figure 2a). We speculate that the 60 kDa complexes recovered from the medium and cell extracts are equivalent. No other cross-linked complexes containing ^{125}I-TGF-β1 were detected in the medium. Small amounts of the 60 kDa species were present in the medium collected from control cultures that were not treated with plasmin. DIP-plasmin did not increase the amount of the 60 kDa complex recovered in the medium, indicating that plasmin activity is required.

Total radioactivity released from ^{125}I-TGF-β1-affinity-labelled cells was quantified over a range of plasmin concentrations (Figure 2b) and exposure times (Figure 2c). Approximately 25% of total cell-associated ^{125}I-TGF-β1 was released into the medium by 100 nM plasmin in 1 h at 37 °C. When cells were incubated for 1 h in the absence of plasmin, 5% of the radioactivity was released. Similar studies were performed using cells that were affinity-labelled with ^{125}I-TGF-β2 instead of ^{125}I-TGF-β1. Incubation with 100 nM plasmin for 1 h released 51 ± 7% of the total cellular radioactivity into the medium (compared with 7% in the absence of plasmin) (mean $\pm$ S.E.M.; $n = 3$).

The release of TGF-β1 into the medium may reflect cleavage of substrate by plasmin or a second proteinase that is activated by plasmin. In order to distinguish between these two possibilities, affinity-labelled AKR-2B cells were treated with 100 nM plasmin. After 10 min, 50 μM aprotinin was added to half of the cultures. The aprotinin terminated the rapid plasmin-dependent release of cellular radioactivity (Figure 3). This result indicates that a second plasmin-activated proteinase is probably not involved unless this enzyme is an aprotinin-sensitive serine proteinase.

In separate studies, we examined the possibility that plasmin which is intrinsic to the AKR-2B cell cultures is responsible for the slow release of radioactivity from the affinity-labelled cells in the control experiments (no plasmin added). The cells were incubated for 1 h in EHB or EHB supplemented with 50 μM

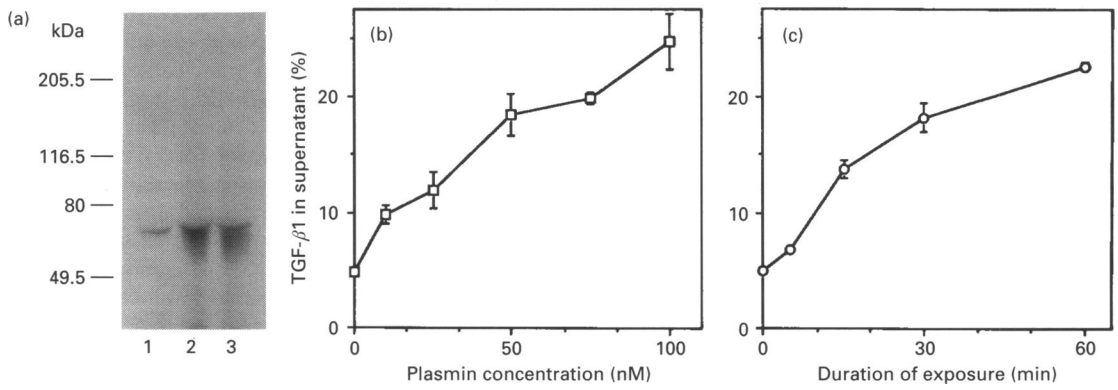

Figure 2 Plasmin-dependent release of ^{125}I-TGF-β1 from affinity-labelled fibroblasts

Confluent monolayers of AKR-2B fibroblasts were affinity-labelled with ^{125}I-TGF-β1 and then treated with plasmin. The medium was collected and subjected to SDS/PAGE. In (**a**) the time of incubation with plasmin was 1 h and the plasmin concentration was 0 (lane 1), 50 nM (lane 2) or 100 nM (lane 3). In (**b**) and (**c**) radioactivity released into the medium was quantified in a γ counter. Radiolabelled TGF-β1 in the medium after treatment with different concentrations of plasmin for 1 h (**b**) or with 100 nM plasmin for different times (**c**) was expressed as a percentage of the total radioactivity that was initially cell-associated (typically 60000–80000 d.p.m.). For the first time point in (**c**) (0 min), 100 nM plasmin was added to the cultures; the plasmin-containing medium was then immediately removed. Experiments were performed in triplicate (means ± S.E.M.).

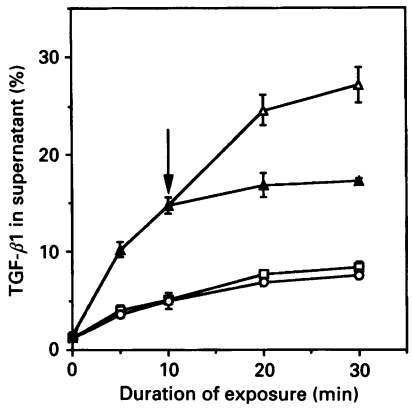

Figure 3 Effect of aprotinin on plasmin-dependent release of radioactivity from ^{125}I-TGF-β-affinity-labelled AKR-2B cells

Confluent monolayers were affinity-labelled and incubated with EHB alone (□), EHB supplemented with 50 μM aprotinin (○) or 100 nM plasmin (▲, △). After 10 min (arrow), aprotinin (final concentration 50 μM) was added to some of the plasmin-treated cultures (▲). Other plasmin-treated cultures did not received aprotinin (△). At the specified times, media and cells were separated. Radioactivity in each fraction was determined.

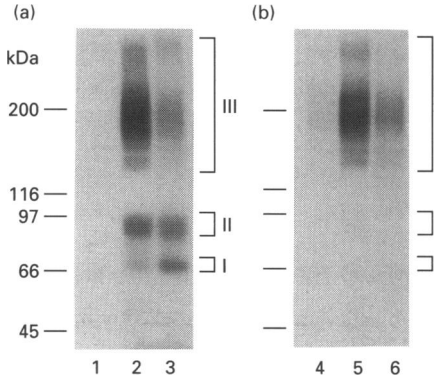

Figure 4 TGF-β-affinity-labelling of plasmin-treated AKR-2B cells

Confluent monolayers of AKR-2B cells were treated for 1 h with 100 nM plasmin or were incubated in buffer without plasmin for the same time period. The cells were then affinity-labelled with ^{125}I-TGF-β1 (**a**) or ^{125}I-TGF-β2 (**b**) and subjected to SDS/PAGE on 4–10% polyacrylamide gradient gels. ^{125}I-TGF-β receptors were detected by autoradiography. Lanes 1 and 4, ^{125}I-TGF-β binding in the presence of a 200-fold molar excess of unlabelled TGF-β1; lanes 2 and 5, binding to untreated cells; lanes 3 and 6, binding after plasmin treatment.

aprotinin (Figure 3). The aprotinin did not affect the slow release of radioactivity.

TGF-β binding to plasmin-treated fibroblasts

As TGF-β or DSS might alter the conformation and proteinase susceptibility of cell TGF-β receptors, the order of plasmin treatment and affinity-labelling was reversed. AKR-2B cells were treated with plasmin for 1 h and then affinity-labelled with ^{125}I-TGF-β1. Binding of ^{125}I-TGF-β1 to betaglycan was significantly decreased (Figure 4a). Therefore plasmin cleavage of betaglycan does not require prior exposure to TGF-β or DSS. In five separate experiments, plasmin treatment either slightly decreased or had no effect on ^{125}I-TGF-β1 labelling of the type-II receptor. In contrast, increased ^{125}I-TGF-β1 labelling of the type-I receptor (or a protein of identical mass) was consistently observed. The slight decrease in type-II receptor labelling in some experiments might reflect the proposed role for betaglycan in presenting TGF-β to the type-II receptor [14,24]. With regard to the increased labelling of type-I receptor in plasmin-treated cells, we cannot rule out the possibility that plasmin unmasks a novel TGF-β-binding protein of equivalent molecular mass.

Plasmin-treated AKR-2B cells were also affinity-labelled with ^{125}I-TGF-β2 (Figure 4b). Substantial loss of functional betaglycan was once again observed. This change was not accompanied by visibly altered labelling of the type-I or type-II receptors. The 60-kDa band was not observed in cells that were plasmin-treated before affinity-labelling with TGF-β1 or TGF-β2. This result could indicate that the affinity of ^{125}I-TGF-β for the 50 kDa protein (which remained associated with the cell-layer) is decreased. Alternatively, the protein may be further

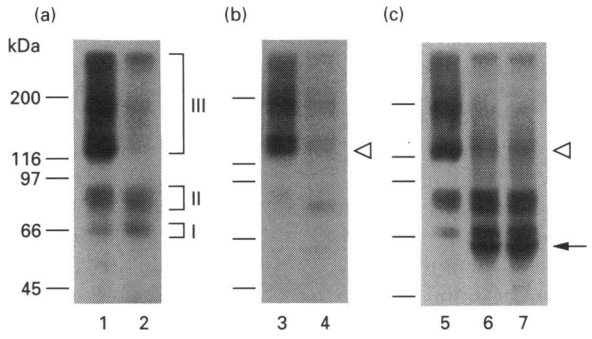

Figure 5 Cleavage of betaglycan core protein by plasmin

Confluent monolayers of AKR-2B cells were incubated for 3 h with heparitinase (2 units/ml) and chondroitinase ABC (0.5 unit/ml). The cells were then treated with 100 nM plasmin (lanes 2 and 4) or buffer only (lanes 1 and 3) for 1 h and affinity-labelled with ^{125}I-TGF-β1 (**a**) or ^{125}I-TGF-β2 (**b**). Cell lysates were subjected to SDS/PAGE on 4–10% polyacrylamide gels. In (**c**), cells were affinity-labelled first with ^{125}I-TGF-β1 and then treated with buffer only (lane 5), 50 nM plasmin (lane 6) or 100 nM plasmin (lane 7). The arrowhead indicates the position of the core protein.

digested by plasmin when not complexed to TGF-β. In control experiments, prior exposure of cells to plasminogen (100 nM), DIP-plasmin (100 nM) and low-molecular mass u-PA (10 nM) had no effect on subsequent ^{125}I-TGF-β-affinity-labelling.

Effect of GAG digestion on plasmin-dependent TGF-β release

AKR-2B fibroblasts were treated with heparitinase and chondroitinase ABC (Figure 5). By affinity-labelling with ^{125}I-TGF-β1 (Figure 5a) and ^{125}I-TGF-β2 (Figure 5b), betaglycan core protein was identified as a 110–130 kDa band. The diffuse band (centred at 200 kDa), which is usually attributed to intact betaglycan, was significantly decreased in intensity but not eliminated. This result was reported previously by Cheifetz et al. [12] and may reflect incomplete enzymic digestion of the betaglycan-associated GAG. Plasmin treatment (100 nM) for 1 h substantially decreased subsequent binding of ^{125}I-TGF-β to betaglycan core protein. Again, increased amounts of ^{125}I-TGF-β1 were identified at the position of the type-I receptor (Figure 5a). When cells were affinity-labelled with ^{125}I-TGF-β1 before plasmin treatment, loss of cellular ^{125}I-TGF-β1–betaglycan core protein was demonstrated (Figure 5c). The 60 kDa band was also present (arrow). The GAG-digesting enzymes did not change the mobility of the 60 kDa complex.

Degradation of TGF-β by plasmin-treated cells

As plasmin specifically decreases TGF-β binding to betaglycan, degradation of TGF-β by plasmin-treated cells was studied. AKR-2B fibroblasts were treated with 100 nM plasmin for 1 h. The cells were then cooled to 4 °C and incubated with ^{125}I-TGF-β1 or ^{125}I-TGF-β2. Total binding was decreased by 27% and 52% respectively. When the temperature was increased to 37 °C, TGF-β1 and TGF-β2 were internalized and digested by both the plasmin-treated and untreated cells, as indicated by the loss of cell-associated radioactivity and the accumulation of trichloroacetate-soluble radioactivity in the medium (Figure 6). When the results in Figure 6 were replotted so that cell-associated radioactivity at each time was expressed as a percentage of that

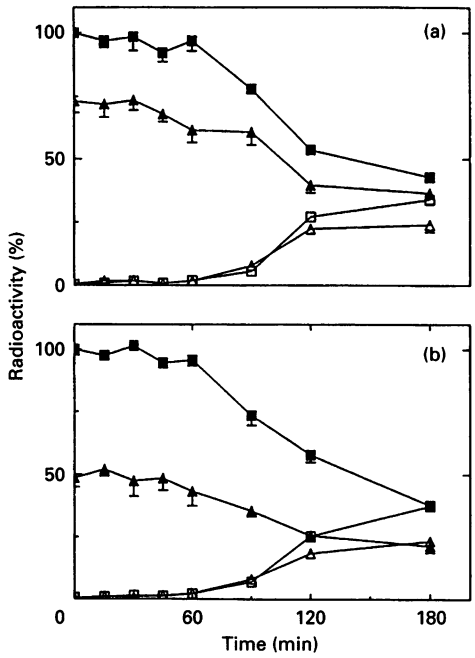

Figure 6 Cellular binding and degradation of TGF-β after plasmin treatment

AKR-2B fibroblasts were treated with buffer only (squares) or 100 nM plasmin (triangles) for 1 h at 37 °C. Cells were then washed with buffer containing aprotinin and incubated at 4 °C with 150 pM ^{125}I-TGF-β1 (**a**) or ^{125}I-TGF-β2 (**b**). After 3 h, the cultures were washed and warmed to 37 °C. At various times, the medium was collected and precipitated with trichloroacetic acid. The cells were fixed in trichloroacetic acid and lysed. Radioactivity in the cell lysates (solid symbols) and trichloroacetate-soluble radioactivity in the medium (open symbols) was determined. Values are expressed as a percentage of the radioactivity in the lysates of untreated cells at time 0 (means $\pm$ S.E.M.; $n = 3$).

present at time zero, plasmin-treated cells and untreated cells were not significantly different. These results suggest that plasmin-mediated cleavage of betaglycan does not alter the overall rate of degradation of TGF-β by AKR-2B cells.

Mitogenesis in response to TGF-β

TGF-β1 and TGF-β2 caused dose-dependent increases in [^{3}H]-thymidine incorporation in quiescent AKR-2B fibroblasts 36 h after exposure (Figure 7). This was an expected result for this stromal cell line [31,33]. Cells that were pulse-exposed to 100 nM plasmin for 1 h (no TGF-β treatment) also demonstrated increased [^{3}H]thymidine incorporation. The intrinsic mitogenic activity of plasmin may have reflected effects on proteins/receptors other than (or in addition to) cellular betaglycan and/or activation of growth factors produced endogenously by the cultures (see the Discussion section). When AKR-2B cells were exposed to plasmin and TGF-β in sequence, the effect on [^{3}H]thymidine incorporation was additive. The additive responses to TGF-β and plasmin were observed in separate experiments in which the TGF-β pulse-exposure time was varied (results not shown). These studies demonstrate that plasmin treatment of AKR-2B cells, and the resulting effects on betaglycan structure, do not eliminate the ability of the cells to respond to TGF-β1 or TGF-β2. As plasmin may have affected other undefined proteins involved in cell growth, we cannot conclude from these experiments whether plasmin cleavage of betaglycan (as an isolated event) enhances or decreases cellular responsiveness to TGF-β.

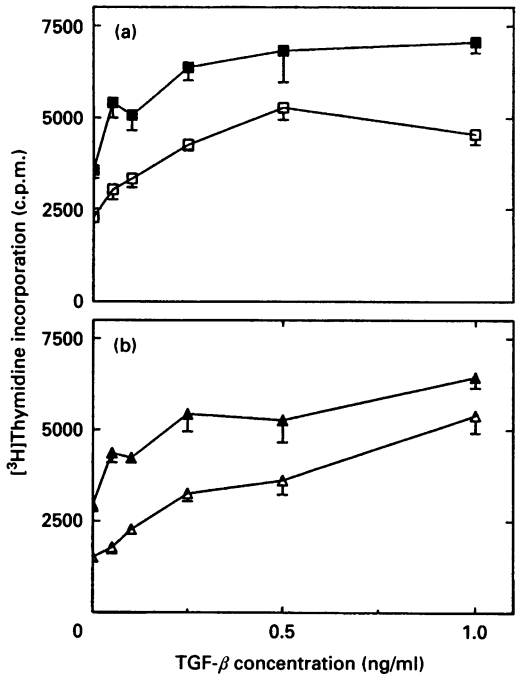

Figure 7 TGF-β response in plasmin-treated AKR-2B cells

Quiescent AKR-2B fibroblasts were incubated in serum-free medium, with or without 100 nM plasmin, for 1 h. The cultures were then treated with aprotinin and pulse-exposed to TGF-β1 or TGF-β2 for 1 h. (**a**) TGF-β1 dose–response in control (□) and plasmin-treated (■) cells. (**b**) TGF-β2 dose–response in control (△) and plasmin-treated (▲) cells. Values represent means (±S.E.M.) ($n = 4$ in a representative experiment).

Table 1 Plasmin-dependent transfer of TGF-β activity from AKR-2B cells to Mv 1 Lu cells as determined by [3H]thymidine incorporation into the Mv 1 Lu cells

TGF-β1 or TGF-β2 were incubated with AKR-2B cells for 3 h at 4 °C. The cells were then washed and incubated with 100 nM plasmin or medium alone for 5 min at 37 °C. Conditioned-medium samples were recovered from the AKR-2B cell cultures, treated with aprotinin (20 μM) and assayed for TGF-β activity in the Mv 1 Lu cell bioassay. [3H]Thymidine incorporation into Mv 1 Lu cells is shown (means±S.E.M., $n = 3$) for the different percentages of conditioned medium indicated.

TGF-β	Plasmin concn. (mM)	10^{-3} × [3H]Thymidine incorporation (d.p.m.)				
		0%	0.5%	1.0%	2.0%	4.0%
TGF-β1	0	25±4	19±5	19±2	21±2	17±3
TGF-β1	100	25±4	11±4	11±2	8±2	3±1
TGF-β2	0	23±1	17±3	15±3	14±2	10±1
TGF-β2	100	23±1	3±1	3±1	1±0	1±0

Activity of TGF-β released from cells by plasmin

The affinity-labelling studies revealed plasmin-dependent release of TGF-β1 and TGF-β2 from the cell surfaces (primarily in association with the 60 kDa complex). To determine whether plasmin might facilitate transfer of active TGF-β from one cell to another, AKR-2B cells were preincubated with TGF-β (150 pM), washed extensively and then treated with plasmin for 5 min (cross-linking agent was not added at any step in this protocol). The plasmin-conditioned medium was tested for TGF-β activity by adding the medium to cultures of Mv 1 Lu cells and measuring [3H]thymidine incorporation. In control experiments, purified TGF-β1 and TGF-β2 caused dose-dependent inhibition of [3H]-thymidine incorporation in the Mv 1 Lu cells (results not shown), as previously reported [32]. [3H]Thymidine incorporation was also inhibited by the conditioned medium from AKR-2B cells which were plasmin-treated after TGF-β preincubation (Table 1). When plasmin was omitted, TGF-β was still detected in the AKR-2B cell conditioned medium; however, the level of activity was substantially lower. Medium from cultures that were treated with plasmin, but not preincubated with TGF-β1 or TGF-β2, did not inhibit [3H]thymidine incorporation.

DISCUSSION

Proteinases of the fibrinolytic system modulate growth factor/cytokine activity and alter the concentration of active and latent growth factors in the extracellular environment [2]. In turn, growth factors regulate expression of fibrinolytic proteins [1,5]. In this investigation, we demonstrated a novel mechanism by which plasmin may regulate distribution of TGF-β in the pericellular microenvironment. The ability of plasmin to specifically decrease the binding of TGF-β to betaglycan indicates that plasmin can change the assortment of TGF-β receptors/binding proteins available on the cell surface. In addition, since plasmin is active against betaglycan with bound TGF-β, plasmin can release a complex containing active growth factor from the cell surface and promote transfer of the growth factor to neighbouring cells.

Betaglycan is an integral membrane proteoglycan with growth-factor-binding activity but no apparent signalling activity [11–17]. The binding site for TGF-β on betaglycan is known to involve the core protein and not associated GAG [12]. Plasmin treatment of ^{125}I-TGF-β1- or ^{125}I-TGF-β2-affinity-labelled fibroblasts profoundly decreased the radiolabel associated with betaglycan and generated a novel 60 kDa radiolabelled complex. Although we have not at this time definitively identified the 60 kDa complex, it is quite likely that it includes a plasmin-released fragment of betaglycan. A similar complex was identified in trypsin-treated cultures by Cheifetz et al. [12]; however, there are a number of important differences in the results obtained with plasmin and trypsin. First, tryptic digestion resulted in significant loss of both type-I and type-II receptors, particularly when the cells were treated with trypsin before TGF-β binding. Furthermore, trypsin treatment yielded several additional cell-associated fragments which were not observed with plasmin (although this may reflect differences in duration of exposure to proteinase). Finally, plasmin, unlike trypsin, is a physiologically significant proteinase known to be present in the pericellular spaces of various tissues [3]. Therefore we can propose that the results reported here may reflect a reaction which occurs *in vivo*.

In recent studies, novel 50–60 kDa TGF-β-binding protein(s) have been identified in association with the surfaces of certain cells and in the extracellular matrix [20,22,23]. The function of these protein(s) in TGF-β regulation and distribution is unclear; however, it is interesting that the 60 kDa TGF-β-binding protein from Hep-G2 cells can be released from the cell surface [22]. The relationship between the 60 kDa TGF-β–protein complex released by plasmin and the previously described TGF-β-binding proteins remains to be determined.

Although plasmin decreased the TGF-β-binding activity of betaglycan in AKR-2B cells, we could not detect a decrease in cellular responsiveness to TGF-β1 or TGF-β2 (as determined by

[^{3}H]thymidine incorporation). This result was unexpected as it has been proposed that betaglycan promotes TGF-β activity by sequestering and delivering TGF-β to the type-II receptor [14,20,21]. It is possible that a decrease in cellular responsiveness to TGF-β in our experiments was obscured by the independent mitogenic activity of plasmin. We have demonstrated that plasmin expresses independent mitogenic activity in other cell lines as well (J. LaMarre, A. M. Weaver and S. L. Gonias). Possible explanations for this activity include: plasmin modification of the growth-factor-binding properties of the extracellular matrix; plasmin-mediated activation of endogenous growth factors; and release of latent growth factors from the extracellular matrix [6,7,34].

When AKR-2B cells were briefly exposed to plasmin, active TGF-β was released. The activity may reflect TGF-β that is part of the intact 60 kDa complex (without cross-linking agent) or TGF-β that dissociates from the 60 kDa complex after release from the cell surface. In either case, plasmin supports transfer of TGF-β from one cell surface to another. Therefore we propose that plasmin plays an important role in intercellular trafficking of TGF-β. A similar hypothesis has been presented by Falcone et al. [35]. These investigators demonstrated that the release of TGF-β and bFGF from pericellular matrix depends on plasminogen activation. The cleavage of betaglycan by plasmin suggests a second general conclusion about the role of plasmin in the regulation of cell function. Several studies have demonstrated that high concentrations of plasmin may be generated at or near the cell surface [3]. Given the proximity of cell-associated plasmin to integral membrane proteins and proteoglycans, plasma-membrane receptors represent an important class of potential plasmin substrates.

We thank Nancy Figler and Tara Atkins for excellent technical assistance and Dr. John Humphries for helpful discussions. This work was supported by grants CA-53462 and HL-45786 from the National Institutes of Health. S.L.G. is the recipient of NIH Research Career Development Award HL-02272. J.L. was supported by a fellowship from the Medical Research Council of Canada.

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Received 26 November 1993/28 February 1994; accepted 15 March 1994