Proteolytic Activation of Latent Transforming Growth Factor-β from Fibroblast-conditioned Medium

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Abstract. Transforming growth factor-β (TGFβ) is produced by most cultured cells in an inactive form. Potential activation mechanisms of latent TGFβ were studied using fibroblastic (NRK-49F and AKR-MCA) cell-conditioned medium as a model. Active TGFβ was monitored by radioreceptor and soft agar assays as well as by antibody inhibition and immunoprecipitation. Little or no TGFβ was detected in untreated conditioned medium. Treatment of the medium with extremes of pH (1.5 or 12) resulted in significant activation of TGFβ as shown by radioreceptor assays, while mild acid treatment (pH 4.5) yielded only 20–30% of the competition achieved by pH 1.5. In an effort to define more physiological means of TGFβ activation, the effects of some proteases were tested. Plasmin and cathepsin D were found to generate 25-kD bands corresponding to the active form of TGFβ as shown by immunoprecipitation analysis of radiolabeled cell-conditioned medium. Plasmin treatment of the medium resulted in activity that was quantitatively similar to that of mild acid treatment as measured by radioreceptor and soft agar assays. In addition, the plasmin-generated activity was inhibited by anti-TGFβ antibodies. Sequential treatments of AKR-MCA cell-conditioned medium with mild acid followed by plasmin or plasmin followed by mild acid gave activation comparable to either treatment alone. The data suggest that conditioned medium may contain at least two different pools of latent TGFβ. One pool is resistant to mild acid and/or plasmin and requires strong acid or alkali treatment for activation. A second pool is activated by mild pH change and/or plasmin. Activation of this form of latent TGFβ may take place by dissociation or proteolytic digestion from a precursor molecule or hypothetical TGFβ-binding protein complex.

TRANSFORMING growth factors (TGF)[1] are defined as proteins which stimulate the anchorage-independent growth of nontumorigenic cells (Todaro, 1982; Moses et al., 1981, Roberts et al., 1981). Two classes of TGF (TGFα and TGFβ) have been sequenced and molecularly characterized. TGFα exhibits sequence homology to epidermal growth factor (Marquardt et al., 1984), binds to the epidermal growth factor receptor, and is weakly mitogenic in soft agar. TGFβ, however, is structurally and functionally different from TGFα. TGFβ is a highly ubiquitous protein and has been purified from several normal tissues (Roberts et al., 1982). Unlike TGFα, TGFβ has its own specific cell surface receptors (Frolik et al., 1984; Tucker, et al., 1984; Massague and Like, 1985; Cheifetz et al., 1986; Cheifetz et al., 1987) and is a growth inhibitor for most cell types (Tucker et al., 1984; Moses et al., 1985). The purified proteins elicit a variety of biological effects in target cells (Moses et al., 1985; Keski-Oja et al., 1987).

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1. *Abbreviations used in this paper*: PA, plasminogen activator; TGF, transforming growth factor.

Platelets, which contain large amounts of TGFβ, are the major source of TGFβ found in normal serum (Childs et al., 1982). Purification by acid–ethanol extraction of platelets yields an intact active TGFβ molecule with a molecular mass of 25 kD, which is comprised of two 12.5-kD chains linked by disulfide bonds (Assoian et al., 1983). The dimeric 25-kD form is biologically active while no activity can be detected after dissociation of the chains by reducing agents. The TGFβ present in human platelets has been termed TGFβ1 while two homodimeric forms (TGFβ1 and TGFβ2) and a heterodimeric form (TGFβ1.2) have been observed in porcine platelets. (Cheifetz et al., 1987). TGFβ2 has been shown to be the same as the growth inhibitor isolated from BSC-1 African green monkey kidney epithelial cells, now termed polyergin (Hanks et al., 1988).

While most cultured cells secrete TGFβ, it is usually in an inactive form (Lawrence et al., 1984). Extremes of pH have been shown to irreversibly activate TGFβ in cell-conditioned media (Lawrence et al., 1985). Although acid activation has provided information regarding the production and secretion of TGFβ from a variety of cell types, it is unlikely to be a physiological activation mechanism. Cell-associated proteases were considered likely candidates in the activation since proteolytic processing of a high molecular mass

precursor evidently occurs, which has been suggested by cDNA sequencing studies (Derynck et al., 1985). However, since the molecular character of inactive TGFβ present in cell-conditioned medium (precursor or complex with a binding protein) has not been elucidated, we use here the terms inactive or latent TGFβ.

We find that a fraction of inactive TGFβ in cell-conditioned medium can be proteolytically activated by plasmin, a wide-spectrum serine protease. Our data suggest the existence of two different inactive pools of this growth modulator secreted by cells in culture. The local activation of TGFβ in the vicinity of cells is expected to play an important role in the regulation of cellular growth, migration, and extracellular matrix formation. Because TGFβ can modulate the plasminogen activator activity of cultured cells (Laiho et al., 1986*a*, *b*), a physiological feedback mechanism for plasmin-mediated activation of latent TGFβ is proposed.

Materials and Methods

Cell Culture and Collection of Conditioned Medium

Continuous clonal cell lines, AKR-MCA, shown previously to produce TGFβ (Moses et al., 1981), and NRK-49F (DeLarco and Todaro, 1978), were used for the production of conditioned medium. The AKR-MCA cell line was obtained by transformation of parent AKR-2B cells with methylcholantrene (Getz et al., 1977; Moses et al., 1978). Stock cultures of AKR-MCA and NRK-49F (CRL-1570; American Type Culture Collection, Rockville, MD) cells were maintained in McCoy's 5a medium supplemented with 5% FBS (Gibco, Grand Island, NY) without antibiotics. For the production of conditioned medium, AKR-MCA cells were seeded in 490-cm^2 plastic roller bottles with 25 ml McCoy's 5a medium with 5% FBS. NRK-49F cells were seeded in 75-cm^2 tissue culture flasks with 15 ml McCoy's 5a medium with 5% FBS. The medium was changed every 3–4 d until the cultures became confluent. Upon confluency, the cultures were rinsed three times with serum-free MCDB 402 medium and incubation was continued in fresh serum-free MCDB 402 medium. The medium was replaced every 24 h and conditioned medium was collected from day 3 through day 7 of serum-free culture. The medium was centrifuged for 30 min in an RC-3B (Sorvall Instruments Div., part of DuPont Co., Wilmington, DE) at 4,000 rpm to remove cell debris. Conditioned medium from each cell line was pooled separately, aliquoted, and stored at 4°C for subsequent activation studies.

All TGFβ activity assays were performed on AKR-2B (clone 84A) cells. Stock cultures of AKR-2B cells were maintained in McCoy's 5a medium supplemented with 5% FBS.

Treatment of Cell-conditioned Medium

The pH of the conditioned medium was adjusted with HCl or NaOH and incubated at 22°C for 1 h followed by neutralization with either HCl or NaOH unless otherwise indicated. Human plasmin from two suppliers (Sigma Chemical Co., St. Louis, MO; Boehringer Mannheim Biochemicals, Indianapolis, IN) was used. Plasmin obtained from Sigma Chemical Co. was solubilized in PBS (0.14 M NaCl, 10 mM Na_2HPO_4, pH 7.4). The conditioned medium was incubated with plasmin at concentrations ranging from 0.01 to 3.0 U/ml. The incubations were performed at 37°C for 2 h. Protease activity was then inhibited by the addition of phenylmethylsulfonyl fluoride (PMSF) and aprotinin (final concentrations: 2 mM and 0.55 TIU/ml, respectively). BSA (Sigma Chemical Co.) was subsequently added to a final concentration of 1 mg/ml. Sequential treatments with acid and plasmin were performed as indicated. Immediately after the treatments, the conditioned medium was analyzed in the biological assays indicated. As a control, MCDB 402 medium not exposed to cells was incubated with plasmin (0.01–3.0 U/ml) in an identical manner to that described above for cell-conditioned medium. Control samples were assayed in parallel experiments.

Radioreceptor Assay

AKR-2B cells were plated into 6-well culture dishes at a density of 2×10^5 cells/well in McCoy's 5a medium with 5% FBS. The following day, cells were washed three times with PBS and then incubated for 2 h at 22°C in the presence of 0.25 ng of ^{125}I-labeled TGFβ in a 1-ml total volume. Nonspecific binding was determined by the addition of 1 μg of 50% pure unlabeled TGFβ (Tucker et al., 1984) or by the addition of 10 ng/ml pure porcine TGFβ (Research & Diagnostic Systems, Inc., Minneapolis, MN). AKR-MCA cell-conditioned medium was assayed for TGFβ-competing activity under neutral conditions, after acidification or alkalinization followed by neutralization, and after plasmin treatments. Results of TGFβ radioreceptor assays are represented as percent inhibition of binding after correction for nonspecific binding. Nonspecific binding was <25% of the total ^{125}I-TGFβ bound.

Soft Agar Assay

Soft agar assays using AKR-2B cells were performed as previously described (Moses et al., 1981; Childs et al., 1982). Briefly, base layers of 0.8% agar in McCoy's 5a medium supplemented with 10% FBS were prepared in 35-mm culture dishes. Upper layers of 0.4% agar in McCoy's 5a medium contained 10% FBS, 7.5×10^3 cells, and the conditioned medium after acid or plasmin treatment as indicated. In parallel experiments, AKR-MCA–conditioned medium after acid or plasmin treatment was incubated with anti-TGFβ IgG or normal rabbit IgG overnight at 4°C before use in soft agar assays. The number of colonies >50 μm that developed after 7–10 d was quantitated using an Omnicon Facs III image analyzer (Bausch & Lomb Inc., Rochester, NY).

Immunoprecipitation of TGFβ from NRK-49F Cell-conditioned Medium

Antibodies raised against native TGFβ were produced in rabbits as described elsewhere (Keski-Oja et al., 1988*b*). Radiolabeling and immunoprecipitation analyses were performed using NRK-49F cells. Confluent cultures were incubated with [^{35}S]cysteine (50 μCi/ml, 1,000 μCi/mmol; New England Nuclear, Boston, MA) in cysteine-free MEM for 48 h. The conditioned medium was treated with plasmin (0.4 U/ml) for 2 h at 22°C. Immunoprecipitation was performed by incubation with 5 μg anti-TGFβ IgG or normal rabbit IgG for 4 h at room temperature or overnight at 4°C (Keski-Oja et al., 1987). Immune complexes were adsorbed with protein A-Sepharose and analyzed by SDS-PAGE followed by fluorography. Radiolabeled molecular mass markers were purchased from Amersham Corp. (Arlington Heights, IL). Reduced and nonreduced ^{125}I-labeled TGFβ was also subjected to electrophoresis as a specific mobility marker for TGFβ.

Results

Activation of TGFβ by Extremes of pH

AKR-MCA cells have been shown previously to release TGFβ into the medium (Moses et al., 1981; Tucker et al., 1983; Moses et al., 1987). However, the proportion of the TGFβ in the active or latent form before acid treatment was not determined in the previous studies. This was examined by testing AKR-MCA and NRK-49F cell-conditioned medium in a TGFβ radioreceptor assay. There was little if any active TGFβ in untreated conditioned medium (Fig. 1). However, repeated freezing and thawing appeared to result in some degree of activation (Lyons, R. M., and H. L. Moses, unpublished data). Therefore, untreated conditioned medium was used extensively in this study as a control, usually without prior freezing. Acidification at pH 1.5 for 1 h followed by neutralization resulted in the appearance of significant amounts of active TGFβ in accordance with previous observations (Lawrence et al., 1985; Moses et al., 1987). Similar results were obtained with NRK-49F cell-conditioned medium as suggested by the earlier results of Lawrence et al. (1984) in which soft agar assays were used. It has also been demonstrated that acid treatment of BSC-1 cell-conditioned medium results in the appearance of a 25-kD band which is specifically immunoprecipitated by anti-TGFβ antibodies (Keski-Oja et al., 1987) verifying that the TGFβ-

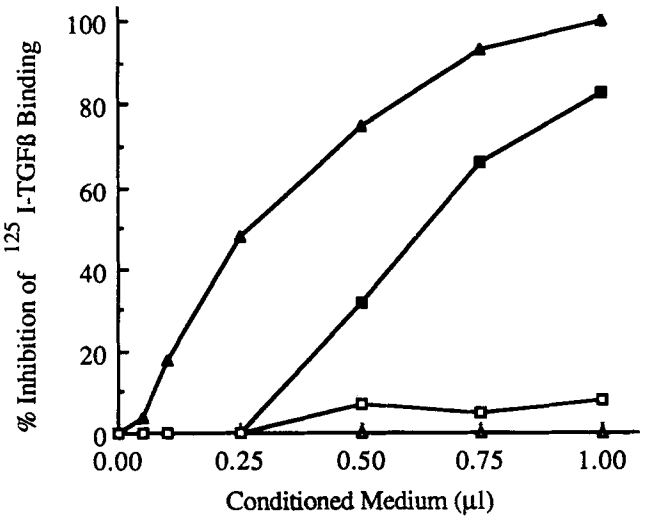

Figure 1. Acid activation of TGFβ in NRK-49F and AKR-MCA cell-conditioned medium. The cell-conditioned medium (NRK-49F, ▲; AKR-MCA, ■) was acidified to pH 1.5 for 1 h with HCl and reneutralized with NaOH before use. Untreated cell-conditioned medium (NRK-49F, △; AKR-MCA, □) was assayed at the same time. Increasing volumes of the medium were then assayed in triplicate for TFGβ-competing activity.

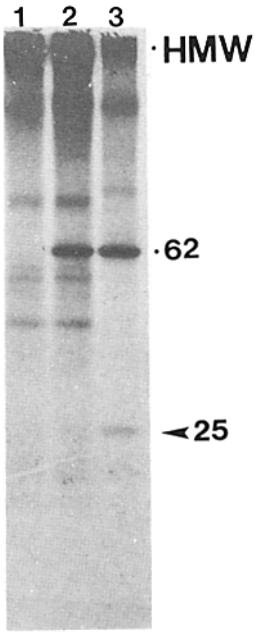

Figure 3. Immunoprecipitation of TGFβ from NRK-49F cell-conditioned medium. Cell cultures were incubated with [^{35}S]cysteine for 48 h and labeled conditioned medium was treated with plasmin as described in Materials and Methods. (Lane *1*) Untreated medium precipitated with normal rabbit IgG; (lane *2*) untreated medium precipitated with anti-TGFβ IgG; (lane *3*) plasmin-treated medium precipitated with anti-TGFβ IgG. The migration of radiolabeled proteins was visualized by autoradiography of a 10% SDS–polyacrylamide gel.

competing activity generated by acid treatment is due to the production of active TGFβ. The two cell lines, however, secreted different amounts of acid-activatable TGFβ (Fig. 1). NRK-49F–conditioned medium contained 4 ng/ml and AKR-MCA–conditioned medium contained 2 ng/ml with 50% inhibition of ^{125}I-TGFβ binding, representing 1 ng/ml of TGFβ.

An analysis of pH dependency for the activation of TGFβ in AKR-MCA and NRK-49F cell-conditioned medium was performed (Fig. 2). It was found that both high and low pH yielded nearly complete inhibition of ^{125}I-TGFβ binding. A plateau in activation occurred with decreasing pH (4–5) resulting in 20–30% inhibition of binding. Physiologic pH (between 5.5 and 7.5) produced little or no TGFβ-competing activity in these assays. The results of these radioreceptor assays confirmed previous reports that extremes of pH result in the activation of TGFβ (Lawrence et al., 1984; Lawrence et al., 1985; Pircher et al., 1986). To examine the possibility that the plateau in the pH curve between 4 and 5 represented activation of a pool of latent TGFβ separate from that activated at lower pH, a time course study of TGFβ activation in AKR-MCA cell-conditioned medium at pH 4.75 was performed using receptor assays. The degree of activation increased over 1.5 h to 30%, but little additional activation was produced with prolonged mild acid treatment (data not shown) further suggesting that the mild acid activatable pool may be a form of inactive TGFβ distinct from that activated at lower pH.

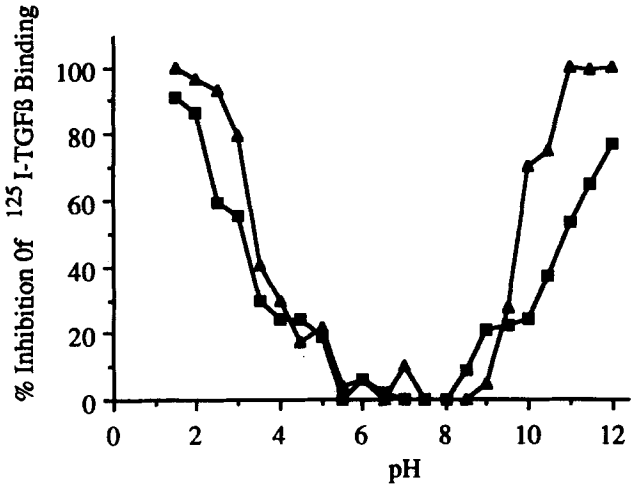

Figure 2. Effect of pH on TGFβ activation in cell-conditioned medium. Cell-conditioned medium (NRK-49F, ▲; AKR-MCA, ■) was treated at the indicated pH by addition of HCl or NaOH and incubated at room temperature for 1 h. The medium was then neutralized and assayed in triplicate (1 ml). Data represent specific binding after subtraction of background.

Activation of TGFβ by Proteolysis

Although mild acid or base treatment did generate TGFβ-competing activity, the biological significance of this finding is unclear. Proteolytic activation of a high molecular mass precursor, suggested from cDNA sequence data (Derynck et al., 1986), was considered as a possible method for cellular processing of inactive TGFβ. Rabbit anti-TGFβ antibodies were initially used to immunoprecipitate radiolabeled NRK-49F cell-conditioned medium treated with plasmin, cathepsin D, elastase, or thrombin. Both plasmin (Fig. 3) and cathepsin D (data not shown) treatment yielded a faint but specific 25-kD band which was sensitive to reduction with 2-mercaptoethanol. No immunoreactive TGFβ-like polypeptides were immunoprecipitated after treatment with elastase or thrombin (data not shown). Plasmin was chosen for further study of TGFβ activation in immunoprecipitation analyses and biological assays because its effect in immunoprecipitation studies was most clear, and because of the wide distribution of plasminogen in vivo and the association of

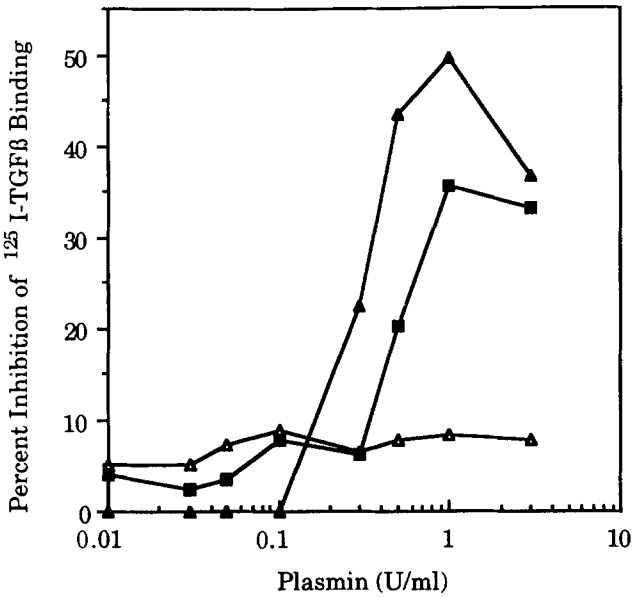

Figure 4. Plasmin activation of TGFβ. The cell-conditioned medium (NRK-49F, ▲; AKR-MCA, ■) was incubated with the indicated concentrations of plasmin at 37°C for 2 h. Protease activity was inhibited by PMSF and aprotinin (final concentrations of 2 mM and 0.55 TIU, respectively). BSA was added at a final concentration of 1 mg/ml. 1 ml of treated medium was assayed in triplicate for TGFβ receptor-competing activity. MCDB 402 medium, not exposed to cells (△), was treated with plasmin in an identical manner and assayed as a control.

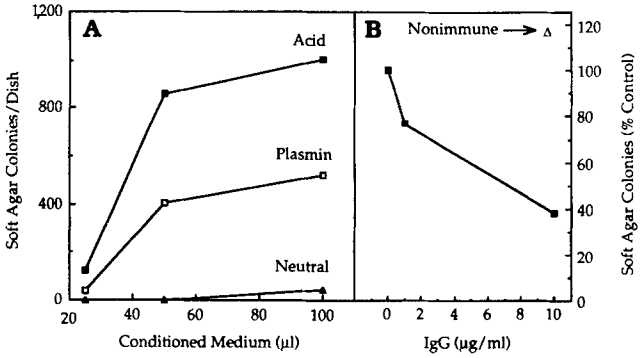

Figure 5. (*A*) Soft agar assay of acid and plasmin activation of latent TGFβ. The conditioned medium was acid (pH 1.5) or plasmin (0.2 U/ml) treated as previously described in Materials and Methods. Indicated volumes of treated and untreated medium were assayed in triplicate for the ability to stimulate the formation of colonies in soft agar. Variation between triplicate determinations was <10%. (*B*) Inhibition of plasmin-generated TGFβ activity by anti-TGFβ antibodies. AKR-MCA-conditioned medium was treated with plasmin (0.2 U/ml) as described in Materials and Methods. After the addition of protease inhibitors, plasmin-treated conditioned medium was incubated with anti-TGFβ antibodies at the indicated concentrations at 4°C overnight. The medium (500 µl) was then assayed for stimulation of soft agar colony formation. MCDB 402 medium, not exposed to cells, was treated identically and the number of colonies appearing under these conditions were subtracted as background. Data are represented as percent control: number of colonies formed by plasmin-treated conditioned medium in the absence of anti-TGFβ antibodies.

plasminogen activation with cell growth and transformation (Reich, 1980; Dano et al., 1985).

A 62-kD band was also observed in immunopreciptation studies of NRK-49F cell-conditioned medium (Fig. 3). This polypeptide was present before and after acid treatment of cell-conditioned medium and was not sensitive to reduction with 2-mercaptoethanol. In addition, immunoprecipitation of this polypeptide could not be blocked by the addition of unlabeled TGFβ, suggesting nonspecific cross-reactivity with anti-TGFβ antibodies (Keski-Oja et al., 1988*b*).

A dose-response study of AKR-MCA and NRK-49F cell-conditioned medium exposed to plasmin was carried out at 37°C for 2 h. Increasing TGFβ-competing activity, as measured by radioreceptor assay, was obtained with increasing doses of plasmin (Fig. 4). The maximum inhibition observed using AKR-MCA-conditioned medium was 20–30%, while 50–60% inhibition could be achieved with NRK-49F-conditioned medium. The lowest dose of plasmin that resulted in inhibition of binding was 0.1 U/ml. Maximal inhibition was achieved with 0.5 U/ml with no additional inhibition at higher doses. The difference in the amount of maximal inhibition obtained by plasmin treatment of the different cell-conditioned media may be due to the difference in total secreted inactive TGFβ as suggested earlier (Fig. 1). Plasmin treatment of serum-free MCDB 402 medium not exposed to cells did not create any competing activity in the assay, demonstrating that plasmin itself did not interfere in the assay as performed. Treatment of ^{125}I-TGFβ with plasmin concentrations used here did not appreciably degrade the protein or generate lower molecular mass fragments as determined by autoradiography of ^{125}I-TGFβ on SDS–polyacrylamide gels (data not shown). This indicates that TGFβ is relatively resistant to digestion by plasmin.

Plasmin and acid activation of AKR-MCA cell-conditioned medium were also compared in soft agar assays. Increasing amounts of acid-treated (pH 1.5) conditioned medium resulted in a dose-dependent increase in the number of colonies formed (Fig. 5 *A*). A similar response to plasmin-treated conditioned medium was observed, although fewer colonies were formed at each concentration of plasmin-treated conditioned medium, in accord with the results of radioreceptor assays.

As further immunologic characterization, we studied the effects of anti-TGFβ antibodies on plasmin-generated TGFβ-like activity in soft agar. The antibodies inhibited plasmin-generated soft agar growth of AKR-2B cells (Fig. 5 *B*) indicating that activation of a TFGβ-immunoreactive protein was responsible for colony formation. Anti-TGFβ antibodies were also able to inhibit colony formation associated with acid-treated AKR-MCA-conditioned medium in a dose-dependent manner (data not shown). These results suggest that plasmin is able to activate a fraction of the latent TGFβ from conditioned medium, while extremes of pH will yield greater activation.

Sequential Effects of pH and Plasmin

To investigate the possibility that the pool of latent TGFβ in AKR-MCA cell-conditioned medium activated by mild acid was the same as that activated by plasmin, various combinations of pH and plasmin treatments were examined for their

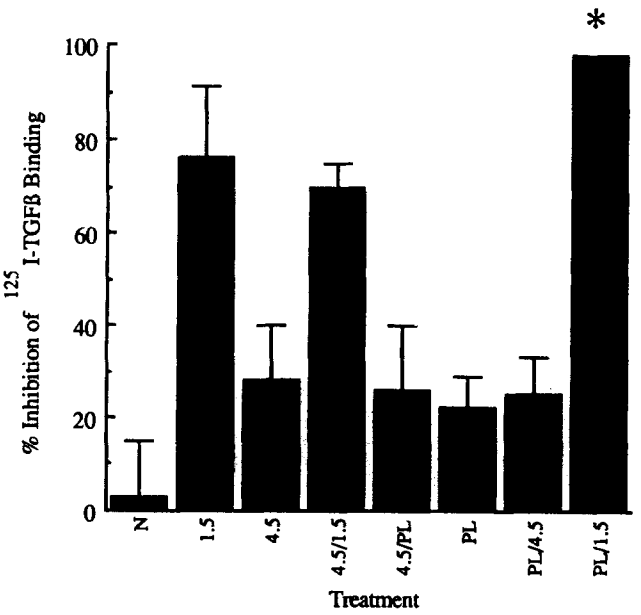

Figure 6. Sequential acid and/or plasmin treatment of AKR-MCA cell-conditioned medium. The indicated treatments of cell-conditioned medium were performed as described. The concentration of plasmin (0.3 U/ml) remained constant in these assays. Radioreceptor assays were performed on 1-ml aliquots of treated conditioned medium. Treated serum-free MCDB 402 medium was assayed and subtracted as background. Data represents the means of three independent experiments performed in triplicate with error bars indicating the standard deviation. (*) One experiment performed in triplicate.

possible additive effect in TGFβ activation (Fig. 6). Mild acid (pH 4.5) or plasmin (0.3 U/ml) treatments of the conditioned medium alone resulted in 20–30% inhibition of ^{125}I-TGFβ binding. Mild acid followed by plasmin, or plasmin followed by mild acid treatment, did not increase the inhibition of ^{125}I-TGFβ binding. Both mild acid followed by strong acid and plasmin followed by strong acid produced TGFβ-competing activity comparable to strong acid alone. These results suggest that there may be two distinct forms of inactive TGFβ in AKR-MCA cell-conditioned medium: an easily activatable pool which represents 20–30% of total inactive TGFβ, and a second pool which requires extremes of pH or some unknown conditions for activation.

Discussion

A variety of cell types are known to secrete TGFβ into the culture medium in an inactive form (Moses et al., 1985; Keski-Oja et al., 1987; Lawrence et al., 1984). In addition, most cells appear to have receptors for TGFβ (Tucker et al., 1984) indicating that activation of the latent form may be a major step in the regulation of TGFβ action. Acidification of cell-conditioned medium is frequently used to monitor TGFβ activity in either radioreceptor or soft agar assays. The physiological mechanisms of activation of the latent form(s) of TGFβ are poorly understood. In the present study, we demonstrate the ability of plasmin, a wide spectrum cell-associated serine protease, to activate a portion of the latent TGFβ secreted by AKR-MCA mouse fibroblasts and NRK-49F rat fibroblasts.

Acidification of cell-conditioned medium at pH 1.5 converts high molecular mass latent TGFβ to lower molecular mass active material as measured by both radioreceptor and soft agar assays (Moses et al., 1985; Lawrence et al., 1984). To determine whether a change in pH might be a physiological means of activating latent TGFβ, a study of the effects of pH on measurable TGFβ activity was performed. In accordance with the studies of Lawrence et al. (1985) on NRK-49F cells, we found that strong acid or base was capable of activating the maximum amount of the latent TGFβ present in both NRK-49F and AKR-MCA cell-conditioned medium. There was a twofold difference in the amount of acid-activatable TGFβ secreted by NRK-49F and AKR-MCA cells. Mild acid treatment (pH 4.5–5.5) of the conditioned medium resulted in only 20–30% of the maximal activity obtained. The possibility that this range of pH might be present in local microenvironments at the cell surface or extracellular matrix of specific cell types under physiologic or pathologic circumstances cannot be excluded. However, such a low pH is known to exist physiologically only in lysosomes (Okhuma and Poole, 1978). A lysosomal mechanism of activation of a polypeptide ligand that binds to cell surface receptors seems unlikely.

The cell-associated serine protease, plasmin, was also studied as a potential biological activator of latent TGFβ. Plasmin was found to activate the same pool of latent TGFβ as that activated at pH 4.5. Such mild acid conditions would not be expected to cleave at the Arg–Arg site that must be cleaved for processing of the TGFβ precursor (Derynck et al., 1985). Therefore, plasmin activation is probably mediated through cleavage at another site. Alternatively, the mild acid conditions could activate an acid protease present in the conditioned medium which in turn activates latent TGFβ by precursor cleavage. We favor the speculation that latent TGFβ released into conditioned medium has already been proteolytically processed at the Arg–Arg site and that plasmin cleaves an associated polypeptide causing an irreversible conformational change which allows the release of the active 25-kD TGFβ. The mild acid conditions could also result in denaturation of this proposed associated polypeptide.

The high molecular mass of latent TGFβ suggests the presence of a binding protein (Lawrence et al., 1985; Pircher et al., 1986). Previous studies have shown that treatments known to dissociate hydrogen-bonded proteins activate latent TGFβ (Lawrence et al., 1985; Pircher et al., 1986; Lyons, R. M., and H. L. Moses, unpublished data). Whether the putative binding protein is the non-TGFβ portion of the precursor molecule or the product of a separate gene is not known. However, recent studies by Gentry et al. (1987) would suggest that TGFβ remains associated with the cleaved portion of its precursor molecule. These investigators have transfected Chinese hamster ovary (CHO) cells with the full length TGFβ gene and amplified the expression of TGFβ by 500-fold. Conditioned medium from these transfected cells required acid dialysis for detection of active TGFβ, indicating that the overexpressing transfected CHO cells secrete TGFβ in a latent form. If a separate gene was responsible for providing a TGFβ-binding protein, coordinate overexpression of this gene would also be required to bind all secreted TGFβ. A more plausible explanation would be that the cleaved 25-kD TGFβ molecule remains associated with the precursor portion of the molecule and thus

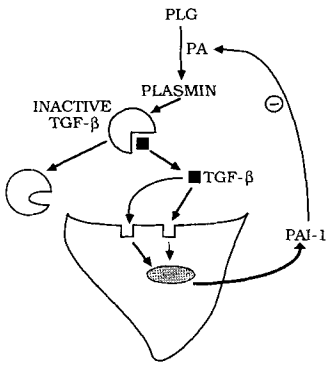

Figure 7. Proposed model of TGFβ activation by plasmin. Plasmin is activated from inactive plasminogen by PA. Plasmin may then proteolytically activate inactive TGFβ at or near the cell surface allowing its binding to the receptor. Binding of TGFβ to the cells may then lead to production of the endothelial-type PA inhibitor (Laiho et al., 1986*a*) which could inhibit further activation of plasminogen resulting in a negative feedback of plasmin-mediated TGFβ activation.

TGFβ remains inactive until dissociation occurs. Since activation is irreversible, there is probably a conformational change of the precursor portion upon dissociation of the complex such that the 25-kD active molecule can no longer bind to the precursor portion. The molecular nature of the putative binding protein can be satisfactorily determined only by purification and sequencing. The pool of latent TGFβ in cell-conditioned medium requiring harsher conditions of acid or base which is not activated by plasmin treatment may require a more complex series of events clearly different from that required for the more easily activated pool.

The possibility that plasmin may function as a physiological regulator of TGFβ action by activation of latent material is strengthened by the observations that TGFβ regulates extracellular plasminogen activator (PA) activity. TGFβ enhances the production of the endothelial-type PA inhibitor (Laiho et al., 1986*a*) and decreases the mRNA levels and extracellular activity of urokinase-type PA in cultured lung fibroblasts (Keski-Oja et al., 1988*a*). This could provide a negative feedback control of TGFβ action (Fig. 7). The sequence of events could be as follows. Cell-derived plasminogen activators convert plasminogen to plasmin which in turn activates TGFβ allowing binding to specific cell surface receptors. Among other subsequent biological effects, TGFβ increases the production of the endothelial-type PA inhibitor and decreases PA levels resulting in lower PA activity, less plasmin production, and diminished activation of TGFβ. Proof of this or related mechanisms will require purification of the latent forms of TGFβ as well as examination of cell membrane and extracellular matrix preparations for TGFβ-activating capability followed by purification of molecule(s) responsible for such activity.

The possibility that local membrane or extracellular matrix components regulate TGFβ activity is also attractive from the standpoint that TGFβ appears to be released in latent form by many cell types, most of which have TGFβ receptors and are capable of responding in a variety of ways to active TGFβ. Therefore, proteolytic activation of latent TGFβ at the cell surface or in the pericellular space could represent a major regulatory step in the action of this important regulatory molecule.

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