Characterization of the Activation of Latent TGF-β by Co-cultures of Endothelial Cells and Pericytes or Smooth Muscle Cells: A Self-regulating System

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Abstract. The conversion of latent transforming growth factor beta (LTGF-β) to the active species, transforming growth factor beta (TGF-β), has been characterized in heterotypic cultures of bovine aortic endothelial (BAE) cells and bovine smooth muscle cells (SMCs). The formation of TGF-β in co-cultures of BAE cells and SMCs was documented by a specific radioreceptor competition assay, while medium from homotypic cultures of BAE cells or SMCs contained no active TGF-β as determined by this assay. The concentration of TGF-β in the conditioned medium of heterotypic co-cultures was estimated to be 400–1,200 pg/ml using the inhibition of BAE cell migration as an assay. Northern blotting of poly A+ RNA extracted from both homotypic and heterotypic cultures of BAE cells and SMCs revealed that BAE cells produced both TGF-β1 and TGF-β2, while SMCs produced primarily TGF-β1. No change in the expression of these two forms of TGF-β was apparent after 24 h in heterotypic cultures. Time course studies on the appearance of TGF-β indicated that most of the active TGF-β was generated within the first 12 h after the establishment of co-cultures. The generation of TGF-β in co-cultures stimulated the production of the protease inhibitor plasminogen activator inhibitor-1 (PAI-1). The inclusion of neutralizing antibodies to TGF-β in the co-culture medium blocked the observed increase in PAI-1 levels. The increased expression of PAI-1 subsequent to TGF-β formation blocked the activation of the protease required for conversion of LTGF-β to TGF-β as the inclusion of neutralizing antibodies to PAI-1 in the co-culture medium resulted in prolonged production of TGF-β. This effect was lost upon removal of the PAI-1 antibodies. Thus, the activation of LTGF-β appears to be a self-regulating system.

Transforming growth factor β (TGF-β) is a homodimeric protein with a molecular weight of 25,000. TGF-β is produced by a variety of cells in vitro (16, 34) and has been isolated from a number of tissue sources (2, 8, 24). The purified protein displays a variety of activities in a cell specific manner. These include the induction of matrix macromolecule synthesis (3, 6, 12), decreases in the synthesis of the proteases collagenase and plasminogen activator (PA) (5, 15, 27) and stimulation of the synthesis of inhibitors of both collagenase and PA (5, 14, 30). TGF-β is a strong chemoattractant and mitogen for some cell types (3, 25, 36), although it blocks the migration and growth of other cells (7, 10, 21, 26, 28, 35). These activities suggest that a major physiological role of TGF-β may be in wound healing.

Although TGF-β has a widespread distribution, the molecule usually is isolated as an inactive high molecular mass complex (16, 37). This inactive complex has been shown to contain the 25,000-D TGF-β homodimer in noncovalent association with the amino-terminal region of the TGF-β precursor (17a, 19, 38). The latent TGF-β complex isolated from platelets contains an additional, unrelated molecule that is covalently bound (19, 38). The latent form of TGF-β (LTGF-β) does not bind to the TGF-β receptor (16, 22). However, LTGF-β can be converted into the active form by strong acid (pH 2) treatment. Although mildly acidic environments occur in vivo, activation of LTGF-β by acid pH has not been demonstrated to occur in either in vivo or in vitro biological systems. Purified LTGF-β can be activated by plasmin or cathepsin D (17). Recent studies suggest that proteases, specifically plasmin, act by cleaving within the amino-terminal region of the TGF-β precursor, thereby destabilizing the latent complex and releasing active TGF-β (17a).

Recently, two reports described the conversion of LTGF-β to the active species in co-cultures of endothelial cells and pericytes or smooth muscle cells (SMCs) (1, 29). Antonelli-
Orlidge et al. (1) showed that the inhibition of endothelial cell mitosis by pericytes which they had described previously (20) resulted from the formation of TGF-β in heterotypic co-cultures. In a similar set of experiments, Sato and Rifkin (29) found that pericytes or SMCs blocked endothelial cell movement and that this effect also depended upon the formation of TGF-β from its inactive precursor. All three of the cell types examined appeared to secrete only LTGF-β in homotypic cultures, while in heterotypic cultures activation occurred. Activation appeared to require cell-cell contact or the very close apposition of the two different cell types. The activation reaction also required plasmin as inclusion of inhibitors of plasmin in the heterotypic culture medium blocked the formation of TGF-β (29). This observation agreed with the previous results of Lyons et al. (17) that plasmin will convert LTGF-β to TGF-β.

Since most cultured cells appear to produce only LTGF-β, the activation reaction may be a critical regulatory step. To better understand the mechanism of activation, we have extended our analysis of the activation reaction. We have found that both the heterotypic and homotypic cultures produce both LTGF-β1 and LTGF-β2 and that there is no significant increase in TGF-β mRNA abundance in heterotypic cultures. Approximately 1-10% of the inactive species is converted to the active form in co-cultures. The activation reaction is dependent upon the length of time the two cell types are co-cultured and the activation product, TGF-β, induces the synthesis of plasminogen activator inhibitor 1 (PAI-1). PAI-1 blocks subsequent conversion of plasminogen to plasmin, thereby suppressing further activation of LTGF-β. Thus, the system appears to self-regulate.

Materials and Methods

Cell Culture

Bovine aortic endothelial (BAE) cells and bovine pericytes were grown in alpha minimal essential (αMEM) and bovine SMCs were isolated and grown in DME as described previously (29). Cells were used between passages 7 and 15.

Preparation of Conditioned Medium

BAE cells and SMCs were passaged using 0.25% trypsin 0.1 mM EDTA, and the trypsin neutralized with αMEM plus 10% calf serum. Cells were centrifuged and resuspended in serum-free αMEM containing 0.1% BSA. BAE cells and SMCs were seeded separately at 5 x 10⁴ cells/cm² in αMEM plus 0.1% BSA. For co-culture experiments, 4 x 10⁴ BAE cells plus 1 x 10⁴ SMCs/cm² were seeded in the same dish in αMEM plus 0.1% BSA. Cells were incubated at 37°C for 2 h to allow them to attach to the surface, and then changed to fresh αMEM plus 0.1% BSA (0.1 ml/cm²). After the appropriate incubation, the medium was collected and centrifuged to remove debris. For time course experiments, the medium was collected at the indicated times and replaced with the same volume of fresh medium.

Concentration of Conditioned Medium

Conditioned medium from homo- or heterotypic cultures was collected after 24 h of incubation and 20 µg/µl of aprotinin (Sigma Chemical Co., St. Louis, MO) was added. The conditioned medium was concentrated 50-fold by ultrafiltration (molecular weight cut off at 10,000). All procedures were carried out at 4°C, and the concentrated medium was stored at 4°C before assay.

Wound Assays and Cell Migration in the Presence of a Second Cell Type

Each of these techniques was performed exactly as described (29).

TGF-β Radioreceptor Assay

This assay was performed as described by Lyons et al. (17) except that the AKR-2B (clone 84A) cells were preincubated for 1 h at room temperature in binding buffer. The binding buffer consisted of 128.0 mM NaCl, 50 mM KCl, 5.0 mM MgSO₄, 1.2 mM CaCl₂, 500 mM Hepes pH 7.5, and 2.0 mg/ml BSA. The buffer was stored at 4°C and filtered through a 0.5-µm filter before use. The solubilization buffer was 1.0% Triton X-100, 0.1% BSA, 0.02% sodium azide in PBS. Nonspecific binding was determined in the presence of a 400-fold excess of unlabeled TGF-β and was <25%.

Northern Blots

RNA was extracted by the method of Schwab et al. (31). Oligo (dT) selected RNA was separated by electrophoresis in a 1.2% agarose-formaldehyde gel. Transfer and hybridization of RNA was performed as previously described (33). Northern blots were probed with cDNA inserts isolated from plasmids containing the following genes: TGF-β1 (4), TGF-β2 (18). cDNAs were labeled using a random primer extension method (32). All Northern blots were hybridized with a control mouse GAP probe (5) and signals normalized by laser densitometry. All hybridizations and washes were performed at 42°C.

Antisera

Rabbit anti-porcine TGF-β IgG was purchased from R & D Systems (Minneapolis, MN). The antibody neutralizes both porcine and human TGF-β1 (13). The antibody does not cross-react with either acidic or basic FGF, PDGF, or EGF, and neutralizes the TGF-β stimulation of anchorage independent growth of rabbit kidney cells. Neutralizing rabbit anti-bovine PAI-1 serum was a generous gift from D. Loskutoff (Scripps Clinic and Research Foundation, La Jolla, CA). IgG fractions from anti-PAI-1 sera were prepared by chromatography on protein A-Sepharose (Pharmacia Fine Chemicals, Piscataway, NJ).

Immunoprecipitations

Quantitative immunoprecipitations of PAI-1 were carried out in the following manner. Conditioned medium containing [³⁵S]methionine (40 µCi/ml, 100 µCi/mMol, New England Nuclear, Boston, MA) was collected from 60-mm dishes of either homo- or heterotypic cultures of BAE cells and SMCs after 16 h of incubation. The conditioned medium was centrifuged in an Eppendorf tube (made by Brinkmann Instruments, Inc., Westbury, NY) at 10,000 rpm for 10 min. 500 µl of the supernatant was then incubated...
Table 1. TGF-β Content of Homotypic and Heterotypic Cultures

| Sample          | Treatment | TGF-β (ng/ml) |
|-----------------|-----------|---------------|
| BAE             | None      | ND            |
| BAE + SMCs      | None      | 0.02          |
| BAE             | pH 1.5    | 8             |
| Pericytes       | pH 1.5    | 4             |
| BAE + Pericytes | pH 1.5    | 12.5          |
| BAE             | pH 1.5    | 5             |
| BAE + SMC       | pH 1.5    | 11            |

Conditioned medium from homotypic or heterotypic cultures of BAE cells and pericytes was prepared and concentrated as described in Materials and Methods. The medium was divided in half; one part was treated with acid (pH 1.5, 1 h), while the other was held as a control. The amount of TGF-β in each sample was then computed by using the receptor inhibition assay. The values for TGF-β represent the concentrations in the original medium. ND, not detectable.

with 20 μl of either immune or nonimmune serum for 1 h at room temperature. At this time 40 μl of protein A-Sepharose beads were added and a further 1-h incubation at room temperature carried out. The solution was centrifuged at 10,000 rpm for 10 min. The beads were washed three times with a 1:1 solution of PBS:RIPA buffer (10 μM Tris hydrochloride [pH 7.4]; 0.15 M NaCl; 1% sodium deoxycholate; 1% NP-40; 0.1% SDS; 1 mM EDTA; 10 mM KCl; 20 μg aprotinin/ml) and centrifuged at 10,000 rpm for 10 min after each wash. The beads were then washed once with distilled water and centrifuged at 10,000 rpm for 10 min. 40 μl of 4X sample buffer was added to the beads and the sample boiled for 10 min. The contents of the tube were then subjected to SDS-PAGE. The gel was then analyzed by autoradiography followed by densitometry scanning (23). Different exposure times were used to assure that the density of the spot on the autoradiography film was in the linear range of the densitometer used to quantify the PAI-1 bands. Subsequent incubation of the initial supernatant with additional antiserum or protein A-Sepharose beads failed to reveal additional radioactivity which could be immunoprecipitated. Control experiments with nonimmune rabbit serum also failed to demonstrate any immunoprecipitation of PAI-1.

Results

Our previous identification of the migration inhibiting molecule formed upon co-cultivation of ECs with SMCs or pericytes as TGF-β relied primarily upon the specificity of neutralizing TGF-β1 IgG (29). Since the antibodies used in these studies were polyclonal and might have had secondary reactivities, we attempted to demonstrate the presence of TGF-β by a specific radio-receptor competition assay. In this assay 125I-TGF-β1 binding to its receptor is measured in the presence of the test solution and the concentration of TGF-β present computed by the degree of inhibition of 125I-TGF-β1 binding to AKR-2B cells. Pure porcine TGF-β1 is used in this assay to generate a standard curve for comparison and concentration estimates. LTGF-β1 does not compete for binding to the TGF-β receptor and, therefore, does not interfere with the assay. Our initial attempts to measure TGF-β in the medium from co-cultures of ECs and SMCs were negative indicating that the TGF-β concentration was below the detection limit of the assay (500 pg/ml). However, if the conditioned medium was concentrated 50-fold before being assayed, competing material was detected in the co-culture conditioned medium (Fig. 1).

The degree of inhibition corresponded to a TGF-β concentration of 20 pg/ml in the original co-culture medium. No competing activity was observed in the conditioned medium from the homotypic cultures of BAE cells (Fig. 1). If the conditioned medium from either homo- or heterotypic cultures was treated with acid followed by reneutralization to convert the LTGF-β to the active form (16, 17), ∼4–12 ng/ml of TGF-β was found in medium conditioned either by BAE cells, SMCs, pericytes, or co-cultures. Therefore, active TGF-β accounted for ∼1% of the total LTGF-β. This value may well be an underestimate since measurements of TGF-β generated in cell culture systems of this type may be low because active TGF-β will be removed from the medium by high affinity receptors, while LTGF-β will not. While both ECs and SMCs appeared to produce LTGF-β, active TGF-β was found only in conditioned medium of heterotypic cultures. Although a somewhat greater total amount of TGF-β was found in heterotypic culture medium than in homotypic culture medium (Table 1), it is unlikely that this difference accounted for the active TGF-β.

Figure 2. Inhibition of BAE cells migration by increasing TGF-β1 concentrations. Confluent monolayers of BAE cells were wounded as described in Materials and Methods. The cells were allowed to migrate overnight, and the monolayers were fixed, stained, and the number of cells that had crossed the original wound margin counted. Data are expressed as a percent of migration observed in a control untreated wound. (●) TGF-β1 added postwounding; (▲) TGF-β1 added 3 h prewounding and removed postwounding; (○) TGF-β1 present 3 h prewounding and throughout postwounding.

Figure 3. Effect of acid treatment on co-culture conditioned medium. Confluent monolayers of BAE cells were wounded as described in Materials and Methods. The medium was replaced with varying concentrations of conditioned medium from co-cultures of BAE cells and SMCs or the same medium that had been treated at pH 2 for 1 h at 4°C. The co-culture conditioned medium was collected after a 24-h incubation with the cells. The cultures were incubated overnight, and the cells fixed, stained, and counted. The wounded BAE cell number of cells that had migrated across the original wound margin is expressed as a percentage of the number of cells that had migrated in a control culture which received normal medium. (●) Co-culture conditioned medium; (○) acid-treated conditioned medium.
seen in heterotypic cultures. This requirement for heterotypic co-culture for activation of LTGF-β1 as established by the radioreceptor inhibition assay confirms the previous results employing either cell migration (29) or cell division (1) to detect LTGF-β1 activation.

When the concentration of exogenous TGF-β1 required to block cell migration was measured, concentrations as low as 30 pg/ml were found to be effective (Fig. 2). The inhibitory response was independent of whether the exogenous, pure TGF-β1 was added 3 h prewounding or was added immediately postwounding. If the TGF-β1 was added 3-h prewounding and kept in the cultures throughout the experiment, inhibition was observed between 3 and 10 pg/ml (Fig. 2).

If different dilutions of medium from heterotypic cultures were tested (Fig. 3), strong inhibitory activity was apparent at a 1:10 dilution. If the co-culture medium was acid-treated and assayed for its migration inhibitory activity (Fig. 3), it was apparent that acid treatment resulted in an approximate 10-fold increase in inhibitory activity. If the total concentration of LTGF-β1 was 4–12 ng/ml (Table I), the 10-fold increase indicates that the active TGF-β concentration in heterotypic co-cultures was 400–1,200 pg/ml rather than the 20 pg/ml calculated from the receptor competition assay. The simplest explanation for these apparently contradictory results is that the actual concentration of TGF-β in the co-culture medium may be higher than that revealed by the receptor competition assay and that some TGF-β was lost during the preparation of the co-culture medium for the receptor competition assay. A control experiment in which [125I]-TGF-β1 was added to the conditioned medium before concentration indicated that only 20% of the added radioactivity was recovered in the retentate (data not shown).

Since several forms of TGF-β exist, we attempted to identify by Northern blotting the species of TGF-β produced in both homo- and heterotypic cultures. Northern blotting of poly A⁺ RNA extracted from BAE cells, SMCs and co-cultures of BAE cells and SMCs revealed that both cell types produced TGF-β1 mRNA (Fig. 4). The BAE cells also contained TGF-β2 mRNA while the SMCs expressed little if any TGF-β2. The expression of TGF-β3 mRNA was not measured. Laser densitometric analysis of Northern blots followed by normalization to the MGAP constitutive transcript for each lane independently indicated that message levels were equivalent for all conditions examined at 24 h. These results agree with the measurements of TGF-β protein in homo- and heterotypic cultures (Table I) in that there is no significant increase in the level of TGF-β expression observed after co-culture.

We next measured the time course of inhibition of migration after addition of TGF-β1 or SMCs to wounded BAE cell monolayers (Fig. 5). For the first 4-h after initiation of the experiment, BAE cells in control cultures migrated at the same rate as cells in cultures to which either TGF-β1 or SMCs had been added after wounding. After 4 h, cell migration in the co-cultures or TGF-β1-treated cultures decreased. Between 4 and 8 h after initiation of the experiment, a significant difference between the rates of migration of treated and untreated cells was observed. By 20-h after wounding, there was a 40–50% difference between treated and untreated
and the number of cells that had migrated across the wound edge were counted. (A; ●) Conditioned medium from BAE cells; (●) conditioned medium from SMCs; (○) conditioned medium from co-cultures of BAE cells and SMCs plus nonimmune IgG (400 μg/ml); (●) conditioned medium from cultures of BAE cells and SMCs plus anti-PAI-1 IgG (400 μg/ml); (♦) conditioned medium from cultures of BAE cells and SMCs plus anti-PAI-1 IgG (400 μg/ml) that was removed and replaced at 24 h with medium containing nonimmune IgG (400 μg/ml); (large *) control BAE cell migration in the presence of anti-PAI-1 IgG; (*) control BAE cell migration in the presence of nonimmune IgG. The results are expressed as a percentage of cell movement in control wounded monolayers incubated overnight.

cultures. These results implied that active TGF-β is formed rapidly in co-cultures since the time course of inhibition was similar in wounded co-cultures or TGF-β1 supplemented cultures.

Therefore, we next characterized the appearance of TGF-β in co-cultures of BAE cells and SMCs as a function of time after mixing of the two cell types. In this experiment, co-cultures of BAE cells and SMCs were established and incubated for 36 h. The medium was replaced every 12 h and at the end of the experiment the presence of TGF-β in the samples was assessed by the wound migration assay. The results (Fig. 6) indicated that the production of TGF-β was transient. Thus, there was significant inhibitory activity in the medium collected 12 h after the initiation of co-culture, but a rapid loss of inhibitory activity occurred during the subsequent 12-h period. By 24–36 h, no active TGF-β was detectable in the medium. This result was predicted by Lyons et al. (1), who hypothesized that if LTGF-β was activated in a plasmin-mediated reaction, the product, TGF-β, would block further activation via the increase in the production of the plasminogen activator inhibitor of PAI-1. TGF-β is a potent stimulator of the synthesis of PAI-1. Since plasminogen activator converts the zymogen plasminogen to the active protease plasmin, the production of PAI-1 will decrease plasmin formation. Lyons et al. (17) proposed that TGF-β would regulate its own activation if plasmin is responsible for the conversion of LTGF-β to TGF-β.2

The following experiments were performed to verify this hypothesis. First, we measured the ability of anti-PAI-1 antibodies to prolong the period of TGF-β generation. When neutralizing antibodies to PAI-1 were added to heterotypic co-cultures, TGF-β continued to be formed for 36 h (Fig. 6), while in control heterotypic cultures, the formation of TGF-β effectively ceased after 12 h (Fig. 6). If the antibody to PAI-1 was removed after 24 h, a rapid loss of inhibitory activity in the cultures was observed, presumably because of the increase in PAI-1 in the medium. These results indicate that even though much of the PAI-1 normally found in the culture medium is inactive (11) sufficient active inhibitor must exist, perhaps transiently, to block the activation reaction.

Second, we measured the levels of PAI-1 in co-cultures of BAE cells and SMCs as a function of time after plating. PAI-1 synthesis was low in both BAE cells and SMCs but increased appreciably during the 16 h after co-culture (Table II). If the observed induction of PAI-1 resulted from the formation of TGF-β, inclusion of neutralizing antibodies to TGF-β in the co-culture medium should block PAI-1 induction. When this was tested, the increase of PAI-1 synthesis was almost completely blocked (Table II). The small increase in the PAI-1 levels observed in the presence of antibodies to TGF-β may indicate that the antibodies were not totally effective in neutralizing all of the TGF-β formed.

Discussion

The experiments presented in this paper characterize the in vitro activation of LTGF-β by plasmin in co-cultures of ECs and SMCs. The radio-receptor inhibition assay and Northern blots indicated that both TGF-β1 and 2 are transcribed and that a small percentage of the LTGF-β produced is activated. Purified TGF-β1 is extremely active in this system with inhibitory effects on cell motility observed at concentrations as low as 10–20 pg/ml. Activation of LTGF-β occurs rapidly after co-culture and reaches a maximum by 12 h. After that point, decreasing amounts of TGF-β are formed. The decrease in TGF-β formation results from the stimulation of PAI-1 synthesis by TGF-β. The secreted PAI-1 inhibits the

2. It should be noted that although our experiments were carried out in the absence of serum, which is the source of the plasminogen, the cells were exposed to serum to inhibit the trypsinization reaction. This step presumably provides the necessary plasminogen. Furthermore, the activation of LTGF-β can be blocked by the inhibition of antibodies to plasminogen and by inhibiting the trypsinization with serum depleted of plasminogen (our unpublished observations). All of these procedures would be expected to inhibit a plasmin-dependent reaction.

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plasminogen activator catalyzed generation of plasmin that activates LTGF-β. Neutralizing antibodies to TGF-β block PAI-1 induction in heterotypic co-cultures, while neutralizing antibodies to PAI-1 extend the period of LTGF-β activation.

These experiments verify the hypothesis that TGF-β formation may be a self-regulating system. At low concentrations of TGF-β, the level of PA synthesis and activity will be sufficient to convert plasminogen to plasmin. This will result in the enhanced conversion of LTGF-β to TGF-β. As the level of TGF-β increases, PAI-1 synthesis will rise and plasmin formation decrease resulting in a decrease in the conversion of LTGF-β to TGF-β. During extended periods, it is possible that this reaction will oscillate between activation and inhibition. The description by Antonelli-Olridge et al. (1) of long-term (14 d) generation of TGF-β in co-cultures of endothelial cells may represent such a phenomenon.

Several important questions remain unanswered with respect to TGF-β formation in heterotypic co-cultures. First, our co-culture experiments indicate that only a fraction of the LTGF-β is activated. This may in part reflect the rapid inhibition of the activation system combined with the continued production of LTGF-β. However, Antonelli-Olridge et al. (1) have reported that in their co-culture system 100% of the LTGF-β is activated. The reasons for this difference are not clear but may reflect differences in the experimental methodology since Antonelli-Olridge et al. (1) used serum containing medium in some of their experiments. This may result in higher plasmin levels. It is also possible that additional mechanisms or requirements for activation exist. It should be noted, however, that in many proteolytic reactions, an excess of the substrate is present.

Second, why does activation occur only with heterotypic co-cultures rather than homotypic cultures? Heterotypic cultures produce similar amounts of LTGF-β as do homotypic cultures. One possibility is that changes in the plasminogen activator (PA) plasmin system occur upon heterotypic coculture. An obvious change would be the induction of PA. However, assays of PA levels in homotypic and heterotypic cultures have not revealed a stimulation of PA production or activity upon co-culture (unpublished observations). It appears more likely that any changes in the PA-plasmin system that may occur involve modifications of the level of pericellular proteolysis and not total proteolysis. We are currently exploring this possibility. A recent paper by Glick et al. (9) described the production of active TGF-β2 by keratinocytes treated with retinoic acid. It is possible that retinoic acid induces the synthesis of all of the cellular components required for the activation of LTGF-β. We are currently exploring this possibility.

Finally, the in vivo significance of these observations should be considered. Thus far, there are no data available on the in vivo activation of LTGF-β or even on the in vivo role(s) of endogenous TGF-β. It is appealing to speculate that the in vivo regulation of LTGF-β activation is mediated via cell–cell contact and the PA-plasmin system in a manner similar to the in vitro activation. Since TGF-β acts as a negative regulator, the contact of two different cell types would generate a molecule that blocks further cell movement and division. In the normal case, this might maintain tissue cytoarchitecture. The sustained pathological interaction of cell types might lead to continued production of low levels of TGF-β. This may in turn result in the abnormal production of matrix.

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Table II. Induction of PAI-1 Synthesis in Co-cultures

| Cells | Additions | PAI-1 level |
|-------|-----------|-------------|
| BAE   | –         | 20          |
| SMC   | –         | 22          |
| BAE + SMC | + anti-TGF-β IgG | 126     |
| BAE + SMC | + nonimmune IgG | 404     |

Culture of BAE cells, SMCs, or co-cultures of BAE cells and SMCs were incubated for 16 h. The medium was collected and immunoprecipitated with anti-PAI-1 IgGs. The immunoprecipitates were then analyzed by SDS-PAGE followed by autoradiography. The autoradiographs were quantitated by densitometry and the results integrated and presented as relative units.
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