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### Research Article

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# Stimulation of Rat Endothelial Cell Transforming Growth Factor- $\beta$ Production by Bleomycin

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## Abstract

This study examines the hypothesis that mediators from lung endothelial cells could promote lung collagen synthesis in pulmonary fibrosis. Since bleomycin induces pulmonary fibrosis in humans and animals, the effects of this drug on endothelial cells were examined. Endothelial cell conditioned media were prepared in the presence of various doses of bleomycin, and tested for their ability to stimulate lung fibroblast collagen synthesis. The results show a dose-dependent stimulation of endothelial cell secretion of collagen synthesis stimulatory activity by bleomycin, which peaked at a dose  $\geq 100$  ng/ml. Stimulation was selective for collagenous protein synthesis. Gel filtration analysis showed most of the activity to reside in fractions with an estimated molecular mass range of 10–27 kD. The activity was inhibited by anti-transforming growth factor- $\beta$  (TGF- $\beta$ ) antibody, but not by nonimmune control IgG. The presence of TGF- $\beta$  was confirmed using the mink lung epithelial cell assay. Northern blotting revealed significant increases in TGF- $\beta$  mRNA in bleomycin-stimulated endothelial cells. Thus in vitro stimulation of endothelial cells by bleomycin upregulates TGF- $\beta$  production, presumably by increased transcription. In view of the chemotactic and matrix synthesis stimulatory properties of this cytokine, such an increase in TGF- $\beta$  production may play an important role in bleomycin-induced pulmonary fibrosis. (*J. Clin. Invest.* 1991. 87:148–154.) **Key words:** bleomycin • endothelial cells • pulmonary fibrosis • transforming growth factor -  $\beta$

## Introduction

Pulmonary fibrosis is characterized by the presence of lung inflammation and increased deposition of lung collagen. Bleomycin-induced pulmonary fibroses in humans and animals also show these changes (1). Hence, to understand the pathogenesis of this disease, the mechanisms underlying regulation of the lung inflammatory response and collagen metabolism need to be clarified. Although many cytokines and other mediators have been shown to have these regulatory effects, the source of such regulators and regulation of their cellular production have not been clearly defined.

The key cellular participants in the pathogenesis of bleomycin-induced pulmonary fibrosis have not been completely de-

finied, although there is good suggestive evidence that the lung macrophage and T cells (2–6), but not the neutrophil (7–9), are important for full expression of the disease. However, these studies have only looked at cells that are easily sampled and have neglected the possible role of other lung cells in regulating lung inflammation and collagen synthesis. Recent studies have demonstrated that the fibroblast and the endothelial cell are important sources of cytokines with inflammatory and fibrogenic properties, owing to either their chemotactic and/or cellular growth and matrix synthesis stimulating properties. Although alveolar macrophages are capable of elaborating many of these cytokines, such secreted macromolecules must traverse the alveolar lining permeability barrier to effect chemotactic recruitment of inflammatory cells and stimulation of interstitial fibroblast collagen production. Thus the possibility of endothelial and interstitial cell production of regulatory cytokines at the immediate sites where their target cells are likely to be located provides a more efficient process by which cells are recruited and activated.

In bleomycin-induced pulmonary fibrosis, animal models have shown early recruitment of inflammatory cells within the 1st wk after disease induction (10, 11), which is promptly followed by significant increases in lung collagen synthesis and deposition over the subsequent 2–3 wk (1). There is recent evidence that tumor necrosis factor- $\alpha$  (TNF- $\alpha$ )<sup>1</sup> is critical for fibrosis in this model (12), and that increases in lung mRNA for this cytokine are accompanied by increases in lung transforming growth factor- $\beta$  (TGF- $\beta$ ) production (13–15). Studies of alveolar macrophage-conditioned media have not unequivocally established the presence of fibroblast collagen synthesis stimulatory activity, except in the presence of added exogenous indomethacin (16). Although fibroblast growth stimulatory activity is present in increased amounts in conditioned media of alveolar macrophages from fibrotic animals (5, 6), the identity of this mediator remains to be unequivocally determined, and direct quantitative data for the production of TNF- $\alpha$  and TGF- $\beta$  by such macrophages remain lacking. Direct in vitro stimulation of alveolar macrophages by bleomycin causes secretion of fibroblast growth promoting and monocyte chemotactic activities (17, 18). Thus there is precedent for the ability of bleomycin to regulate cytokine production by cells, which could play an important role in initiating pulmonary inflammation and fibrosis in this model.

In this article the regulatory effects of bleomycin are further explored to determine whether they are applicable to other cell types as well. This was done to help test the hypothesis that endothelial cells could play an important role in the pathogenesis of bleomycin-induced pulmonary fibrosis by increased production of cytokines with fibroblast collagen synthesis stimulatory

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1. *Abbreviations used in this paper:* TGF- $\beta$ , transforming growth factor- $\beta$ ; TNF- $\alpha$ , tumor necrosis factor- $\alpha$ .

properties. Specifically, the study was designed to determine whether endothelial cells could represent a source of mediators that could promote fibroblast collagen synthesis, and whether production of these mediators could be upregulated by bleomycin.

## Methods

**Cells.** Rat pulmonary artery endothelial cells were isolated from the pulmonary vasculature using microcarrier beads as previously described and characterized (19–22). These cells possess characteristic cobblestone morphology and high levels of angiotensin-converting enzyme. They were also positive for factor VIII antigen and bound acetylated low density lipoprotein. Visual inspection of monolayers showed a uniform population of cells with these properties without morphologic evidence of contamination by elongated or stellate-shaped cells suggestive of fibroblasts. Furthermore, multiple passaging in media favorable to the growth of fibroblasts failed to uncover any contamination by such cells. Endothelial cells were maintained in monolayer culture in minimum essential medium (MEM) supplemented with nonessential amino acids and 10% fetal bovine serum (FBS). Upon reaching confluence, they were passaged either by scraping or by incubation in calcium-free phosphate-buffered saline.

Rat lung fibroblasts were isolated from 150–175-g male Fisher 344 rats by trypsinization of lung mince as previously described (23). Cells were maintained in Dulbecco's modified Eagle's medium (DME) supplemented with 10% FBS, and passaged by trypsinization.

A mink lung epithelial cell line was used for TGF- $\beta$  assay. These cells were obtained from the American Type Culture Collection, Rockville, MD (Mv 1 Lu, CCL 64) and were maintained in DME containing 10% FBS.

**Preparation of conditioned media.** Rat pulmonary endothelial cells were grown to confluence and washed, and the media were replaced with serum-free DME containing 2 mg/ml bovine serum albumin (BSA) (Bovuminar-low endotoxin, Armour Pharmaceutical Co., Kankakee, IL), supplemented without or with the indicated concentrations of bleomycin (Blenoxane, Bristol-Myers, Evansville, IN). They were incubated for 18–24 h except where otherwise indicated. Control media containing the same doses of bleomycin were also placed in cell-free dishes, and referred to as the no-cell conditioned media. All media, BSA, and bleomycin contained undetectable levels of endotoxin as determined using the *Limulus* amoebocyte lysate assay (E-Toxate, Sigma Chemical Co., St. Louis, MO) (17). At the end of the incubation period, all media were dialyzed in tubing with nominal molecular mass cutoff of 3.5 kD (Spectrum Medical Industries, Los Angeles, CA) against either RPMI-1640 or 1 N acetic acid. The conditioned media were stored frozen at  $-70^{\circ}\text{C}$  until assay and characterization. Unless otherwise stated, all conditioned media were adjusted to an equivalent cell concentration of  $10^6/\text{ml}$ . Samples dialyzed against acetic acid were dried using a Speed Vac (Savant Instruments, Inc., Farmingdale, NY) and resuspended in RPMI before assay.

**Collagen synthesis assay.** Endothelial cell conditioned media were assayed for their ability to modulate rat lung fibroblast collagen synthesis. For this assay, rat lung fibroblasts were grown to confluence in 12-well (22 mm diam) tissue culture dishes and treated with the indicated concentrations of conditioned media diluted in serum free DME containing 2 mg/ml BSA. For comparison, some cells were also stimulated with the indicated doses of porcine TGF- $\beta_1$  (R & D Systems, Minneapolis, MN). After 24 h of incubation, the cells were pulsed for 6 h with [ $^3\text{H}$ ]proline (L-[2,3,4,5- $^3\text{H}$ ]proline,  $> 100 \text{ Ci/mmol}$ , New England Nuclear, Boston, MA) in the presence of ascorbate and  $\beta$ -aminopropionitrile as previously described (23, 24). Collagen synthesis was expressed as the incorporated radioactivity in disintegrations per minute per  $10^5$  fibroblasts that were susceptible to digestion by purified bacterial collagenase (23–25). The resistant portion of the radioactivity was defined as noncollagenous protein synthesis.

**Gel filtration.** To estimate the molecular mass of any activity pres-

ent in renal cortical conditioned media, acid-dialysed conditioned media were dried by vacuum centrifugation and resuspended in 1 N acetic acid at 10% of the original volume. The concentrated sample was clarified by centrifugation and injected onto a high-performance liquid chromatograph equipped with a TSK3000SW gel filtration column (Varian Associates, Inc., Palo Alto, CA) plus guard column. The sample was eluted with 1 N acetic acid at  $25^{\circ}\text{C}$  with a flow rate of 0.8 ml/min. Fractions were collected at 1-min intervals. The collected samples were dried by vacuum centrifugation, resuspended in serum-free RPMI containing 0.2% BSA, and stored frozen until assayed for fibroblast collagen synthesis stimulatory activity. The gel filtration column was calibrated using purified protein standards.

**Antibody studies.** Endothelial cell conditioned media containing collagen synthesis stimulatory activity were treated with anti-TGF- $\beta$  antibody to determine whether the activity could be due to TGF- $\beta$ . The IgG fraction of rabbit antibody to porcine TGF- $\beta_1$  was purchased from R & D Systems and used at a concentration of 50  $\mu\text{g}/\text{ml}$ , which was approximately five times the dose required to neutralize completely the activity of 250 pg/ml pure porcine TGF- $\beta$  (product insert from R & D Systems). This antibody cross-reacts with and neutralizes TGF- $\beta_2$ , but does not cross-react with acidic or basic fibroblast growth factors, platelet-derived growth factor, or epidermal growth factor (R & D Systems product insert). It did not inhibit growth factor activity present in conditioned media of endotoxin-stimulated alveolar macrophages (data not shown), suggesting that this antibody does not cross-react with rat growth stimulatory cytokines. Since authentic and pure rat TGF- $\beta$  is not available, the cross-reactivity of this antibody to human TGF- $\beta_1$  with rat TGF- $\beta$  cannot be evaluated directly, although the results of experiments using this antibody were consistent with such cross-reactivity (see Results). Nonspecific or nonimmune rabbit IgG (Sigma Chemical Co.) was used at the same concentration. These IgGs were preincubated with conditioned media for 1 h at room temperature before their use in assays of fibroblast collagen synthesis stimulatory activity as described above.

**TGF- $\beta$  assay.** This assay was performed essentially as previously described (26) with a few modifications. Mink lung epithelial cells were plated at  $5 \times 10^4$  cells per well of a 96-well microtiter plate in DME supplemented with 5% heat-inactivated bovine plasma-derived serum replacement (Controlled Process Serum Replacement-1, Sigma Chemical Co.). This serum replacement was used instead of FBS to minimize substantial background TGF- $\beta$  activity present in FBS. After 1 h, various dilutions of endothelial conditioned media with or without 10  $\mu\text{g}/\text{ml}$  of anti-TGF- $\beta$  or nonimmune IgG were added to the wells. Appropriate negative and positive (50 pg/ml porcine TGF- $\beta$ ) with or without antibodies were also included in every assay. After 24 h of incubation, the cells were pulsed with 1  $\mu\text{Ci}/\text{well}$  of [ $^3\text{H}$ ]thymidine ([methyl- $^3\text{H}$ ]thymidine, 60–90 Ci/mmol, ICN, Costa Mesa, CA) for 2 h. At the end of the pulsing period, the cells were trypsinized and harvested onto filters, and washed with PBS, 5% cold trichloroacetic acid and ethanol. The filters were then counted for radioactivity. Each sample was assayed in triplicate and the difference in disintegrations per minute incorporated by cells in the absence of anti-TGF- $\beta$  antibody vs. that in the presence of antibody were expressed as a percentage of the disintegrations per minute incorporated by cells treated with antibody-neutralized media. Expression in this manner is equivalent to the percentage of radioactivity incorporated that could be specifically inhibited by TGF- $\beta$ . The plot of the dose-response curve was used to determine the dose of conditioned media that caused 50% inhibition of thymidine incorporation, or  $\text{ED}_{50}$ , which was defined as 1 U of activity.

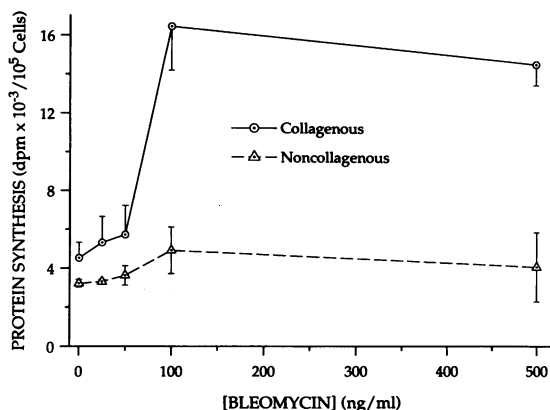
**mRNA analysis.** Endothelial cells were grown to confluence as described above and treated with the indicated doses of bleomycin for the indicated times. After removal of media, total RNA was extracted from the cells using a modification (27) of the method of Chirgwin et al. (28). Equal amounts (11  $\mu\text{g}$ ) were then electrophoresed in 1% agarose gels containing formaldehyde (27, 25). After overnight transfer onto filters (Gene Screen, New England Nuclear), they were baked, prehybridized, hybridized at  $60^{\circ}\text{C}$ , and washed at  $65^{\circ}\text{C}$  as previously described (29, 30). A human TGF- $\beta$  cDNA ( $p\beta\text{as}$ ) probe was generously provided by

Dr. R. Derynck (Genentech, Inc., South San Francisco, CA) (31). The plasmid was inserted in *Escherichia coli* AG1 competent cells (Stratagene, Inc., La Jolla, CA) as described by the manufacturer, and the transformed cells were selected and then grown up to harvest the plasmid. Plasmid DNA was isolated by sodium dodecyl sulfate (SDS)-alkaline lysis and purified by CsCl-ethidium bromide gradient centrifugation. The cDNA insert was cut out by digestion with *EcoRI* and isolated by low melting temperature agarose gel electrophoresis before use (29, 32). The probe was radiolabeled using the random prime labeling method (33) using a kit from Bethesda Research Laboratories, Gaithersburg, MD. Autoradiograms from these blots were quantitated by laser densitometry.

**Statistical analysis.** All data were expressed as means  $\pm$  SE. Comparisons of mean values were undertaken using analysis of variance with Scheffé's test (34) to evaluate statistical significance between any two groups.

## Results

**Effects of bleomycin.** Rat pulmonary artery endothelial cells spontaneously secreted low levels (< 25% stimulation) of fibroblast collagen synthesis stimulatory activity. Upon overnight treatment with bleomycin, the conditioned media of these endothelial cells contained significantly increased levels of such activity as tested on rat lung fibroblasts (Fig. 1). This increase was dependent on the dose of added bleomycin, and reached maximal levels at doses  $\geq$  100 ng/ml. Stimulation by the bleomycin-treated endothelial cell conditioned media was selective for collagenous vs. noncollagenous fibroblast protein synthesis. Analysis by SDS gel electrophoresis revealed significant increases in types I and III collagens without any new collagenous product being synthesized (data not shown). The time course of bleomycin-induced secretion of fibroblast collagen synthesis stimulatory activity is relatively rapid, with significantly in-



**Figure 1.** Bleomycin dose-response curve for secretion of collagen synthesis stimulatory activity. Rat pulmonary artery endothelial cells were treated with the indicated doses of bleomycin for 18 h, and the conditioned media were collected, dialyzed, and analyzed for their ability to stimulate rat lung fibroblast protein synthesis. Confluent rat lung fibroblasts were pulsed for 6 h with [<sup>3</sup>H]proline in the presence of 50% endothelial conditioned media. Synthesis was expressed as disintegrations per minute of radioactive proline incorporated per 10<sup>5</sup> fibroblasts. Results shown were from triplicate flasks of endothelial cell conditioned media produced at each dose of bleomycin. Collagenous protein synthesis refers to collagenase susceptible proteins; the resistant portion is referred to as noncollagenous.

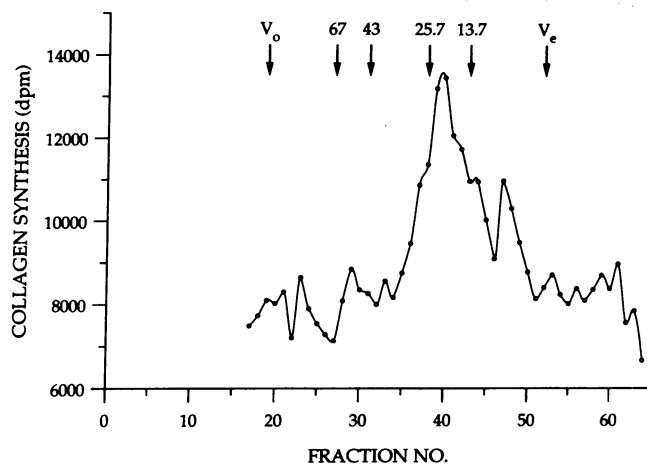
creased activity detected as early as 2 h after bleomycin addition (data not shown).

Assays of control no-cell conditioned media containing equal amounts of bleomycin showed no activity against rat lung fibroblasts, indicating that the stimulatory effect is due to a cellular product whose secretion was stimulated by bleomycin, and not due to carryover of bleomycin used to stimulate the endothelial cells. All doses of bleomycin tested, up to 500 ng/ml, failed to significantly inhibit endothelial or fibroblast DNA synthesis in 10% fetal bovine serum, and did not cause cytotoxicity as assessed by trypan blue exclusion and lactate dehydrogenase assay of conditioned media (data not shown). 1 mg/ml stock solutions of bleomycin contained undetectable amounts of endotoxin by the *Limulus* lysate assay, thus making it unlikely that the observed effects were due to endotoxin contamination, especially at the lower effective doses.

**Characterization of activity.** Initial characterization of this activity in cortical conditioned media was to estimate the molecular mass. Dialysis in tubing with a nominal molecular mass cutoff of 3.5 kD did not significantly diminish the activity present in endothelial cell-conditioned media, suggesting a molecular mass of > 3.5 kD. Before gel filtration analysis, endothelial cell conditioned media were first examined to see if they are stable to 1 N acetic acid, conditions under which the gel filtration analysis was performed and which are preferable for concentration by vacuum centrifugation due to volatility of this solvent. For this experiment, control and bleomycin-treated conditioned media were acidified with 1 N HCl to pH 2.0, and let stand for 30 min at ambient temperature. After this, the samples were dialyzed against 1 N acetic acid and dried by vacuum centrifugation, resuspended to the original volume in assay media, and tested for fibroblast collagen synthesis stimulatory activity. Control media were not treated with acid, but allowed to stand identically and dialyzed against media. The results revealed stability of the activity to acid treatment and without evidence of statistically significant stimulation by such treatment, although occasional samples did show up to 20% stimulation by acidification (data not shown).

Previous studies have indicated that gel filtration under acidic conditions are necessary to disaggregate and/or activate certain mediators and obtain more appropriate estimates of molecular mass (35-37). Since the activity present in bleomycin-treated endothelial cell conditioned media was stable to acidification with 1 N acetic acid they were acidified, dialyzed against acetic acid, and concentrated by drying before being injected into the gel filtration column, and eluted with 1 N acetic acid. Analysis of the eluted fractions found a major peak of activity to be in fractions 36-44, which corresponded to an estimated molecular mass of 20-25 kD (Fig. 2). Minor peaks of activity were also present with retention times of 24, 29, and 47 min, but each of these, with the exception of the 47-min peak, constituted < 10% of the major peak at 39 min.

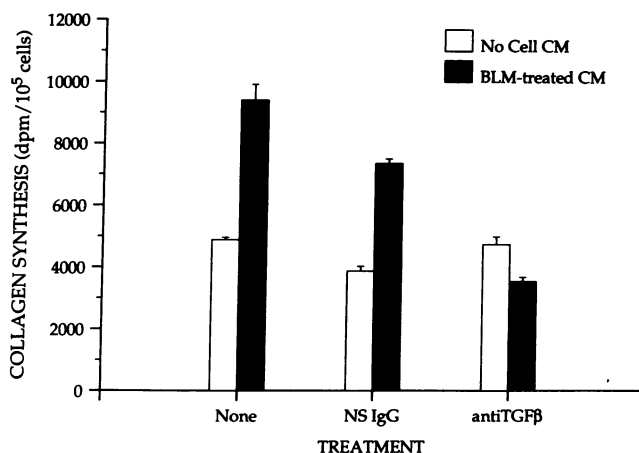
**Effect of anti-TGF- $\beta$  antibody.** Stability of this activity to acidification, its estimated molecular mass, and its selective effect on collagenous protein synthesis are properties consistent with TGF- $\beta$ . To evaluate this possibility further, active and control conditioned media were assayed in the presence of specific anti-TGF- $\beta$  antibody or nonspecific IgG. Preincubation of bleomycin-treated conditioned media with anti-TGF- $\beta$ <sub>1</sub> totally abolished the collagen synthesis stimulatory activity, whereas the same concentration of nonimmune IgG failed to significantly inhibit activity (Fig. 3). Both the anti-TGF- $\beta$  and



**Figure 2.** Gel filtration analysis of bleomycin-treated conditioned media. 1 ml of concentrated bleomycin-treated endothelial cell conditioned media was injected and the column was eluted with 1 N acetic acid. The column was calibrated with the indicated molecular mass standards, which were in descending order of molecular mass: BSA, ovalbumin, chymotrypsinogen A, and ribonuclease A. The void volume ( $V_0$ ) was determined with dextran blue 2000, and void volume + pore volume ( $V_c$ ) with phenol red. 1-min fractions were collected, dried, resuspended in media, and assayed in duplicate at a concentration of 5%. Fibroblast collagen synthesis was measured as described in legend to Fig. 1.

nonimmune IgG did not inhibit basal collagen synthesis in rat lung fibroblasts exposed to control no cell bleomycin-containing conditioned media, indicating that the anti-TGF- $\beta$  antibody was neutralizing activity present in the bleomycin-treated endothelial cell conditioned media, and not by nonspecific or general inhibition of fibroblast collagen or protein synthesis.

**Mink lung epithelial cell assay.** To further confirm the identity of the activity present in bleomycin-treated endothelial cell conditioned media, a standard bioassay for TGF- $\beta$  was used. This assay is based on the ability of TGF- $\beta$  to inhibit proliferation of a mink lung epithelial cell line, Mv 1 Lu (26). Increasing doses of endothelial cell conditioned media were



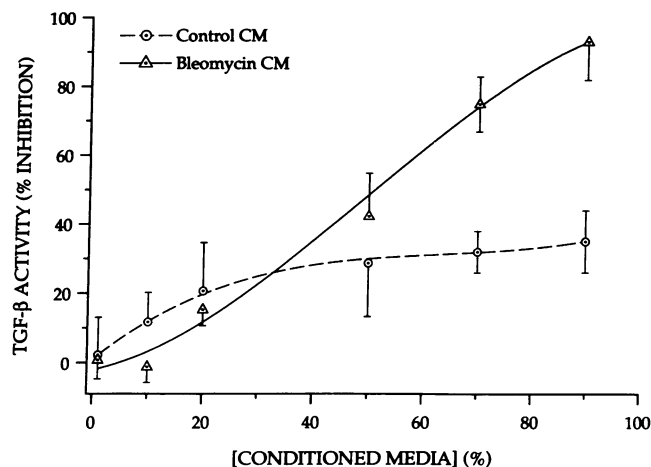
**Figure 3.** Effect of anti-TGF- $\beta$  antibody. No-cell (*No Cell CM*) or bleomycin-treated endothelial cell conditioned media (*BLM-treated CM*) were treated with nothing (*None*), nonspecific (*NS IgG*), or anti-TGF- $\beta$  antibody (*antiTGF $\beta$* ) and then tested in triplicate for their effects on fibroblast collagen synthesis.

found to progressively inhibit thymidine incorporation by mink cells, with the bleomycin-treated media showing markedly more inhibitory activity than the untreated control media (Fig. 4). The inhibition shown in Fig. 4 was completely abolished by specific anti-TGF- $\beta$  antibody, but not by nonimmune IgG. The antibody or nonimmune IgG alone failed to affect mink cell thymidine incorporation. Based on a definition of 1 ED<sub>50</sub> as 1 U of activity, these dilution curves revealed that bleomycin-treated endothelial cell conditioned media contained  $8.5 \pm 1.1$  U/ml of TGF- $\beta$  activity, whereas the untreated control conditioned media contained  $< 5$  U/ml of activity. A precise quantitation of activity in the control media was unobtainable since the dilution curve failed to reach 50% inhibition even at the highest concentration of media that could be tested (Fig. 4).

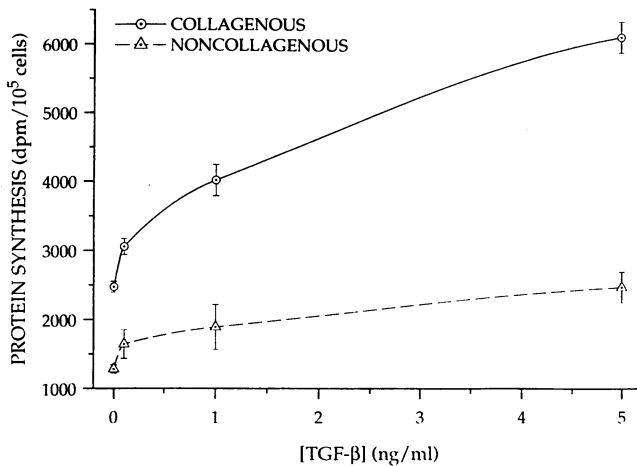
**Effects of pure TGF- $\beta$  on rat lung fibroblast collagen synthesis.** A TGF- $\beta$  dose-response curve is shown in Fig. 5. Rat lung fibroblasts responded to purified porcine TGF- $\beta_1$  in a manner as previously reported for fibroblasts from other species and tissues (38, 39). Significant and selective stimulation of collagenous protein synthesis was evident at doses  $\geq 1$  ng/ml (Fig. 5). These results are again consistent with the presence of TGF- $\beta$  in bleomycin-treated endothelial cell conditioned media, which is responsible for the observed rat lung fibroblast collagen synthesis stimulatory activity detected in these media.

The mechanism of how bleomycin upregulates endothelial cell TGF- $\beta$  production remains to be determined, but may be due to increased transcription, as shown by the following results of TGF- $\beta$  mRNA quantitation.

**mRNA analysis.** Rat pulmonary endothelial cells treated with 100 ng/ml of bleomycin for 18 h, showed upon Northern blot analysis of total cellular RNA, significantly elevated levels of TGF- $\beta$  mRNA species migrating as a 2–2.5-kb species (Fig. 6), consistent with mRNA for TGF- $\beta$  found in tissues of other species (31). Control untreated cells constitutively expressed a low but detectable level of TGF- $\beta$  mRNA, suggesting that there



**Figure 4.** Mink lung epithelial cell assay. Control and bleomycin-treated endothelial cell conditioned media were added to mink lung epithelial cells at the indicated dilutions and pulsed with [<sup>3</sup>H]thymidine. TGF- $\beta$  activity was expressed as percent inhibition of thymidine uptake by the indicated dilutions of conditioned media which was specifically reversible by anti-TGF- $\beta$  antibody. The results were from triplicate determinations at each dose of conditioned media. The ED<sub>50</sub> was defined as 1 U of activity.



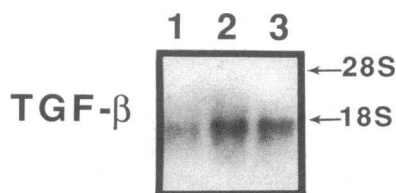
**Figure 5.** Effect of pure TGF- $\beta$  on rat lung fibroblast protein synthesis. Purified TGF- $\beta_1$  at the indicated doses were added to confluent rat lung fibroblasts for 24 h and then pulsed with radioactive proline to measure protein synthesis as described in legend to Fig. 1. The results were from triplicate determinations at each dose of TGF- $\beta$ .

is a low level of expression for this cytokine in isolated endothelial cells under tissue culture conditions.

To determine the time course of this bleomycin-induced increase in steady-state levels of endothelial cell TGF- $\beta$  mRNA, total cell RNA was isolated from cells treated at various times with 100 ng/ml of bleomycin. Northern analysis of the RNA was then undertaken, and the autoradiograms from the blots were then analyzed by laser densitometry to quantitate the intensity of the bands corresponding to TGF- $\beta$  mRNA. The results show an almost linear increase in TGF- $\beta$  mRNA as a function of time of treatment with bleomycin, reaching more than twofold the control (untreated cells) level at 30 h (Fig. 7). These results again are consistent with the induction of increased endothelial cell TGF- $\beta$  production by bleomycin.

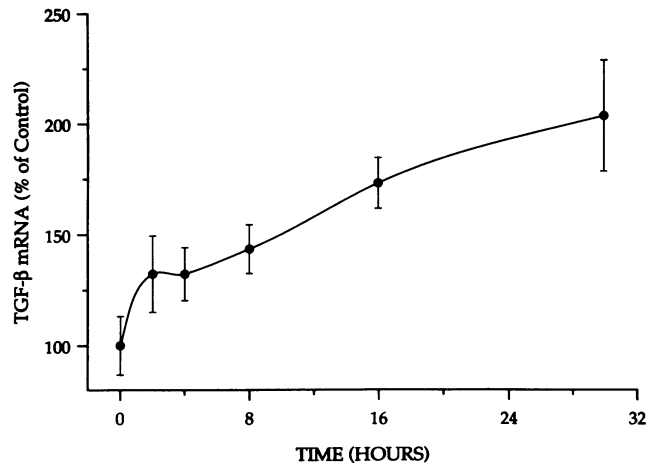
## Discussion

The basis for increased collagen synthesis and deposition in pulmonary fibrosis remains unclear. On the assumption that abnormal regulation of matrix synthesis is somehow involved in this process, this study has examined the possibility that lung endothelial cells may be involved in the production of media-



**Figure 6.** Northern blot analysis of endothelial cell RNA. 11  $\mu$ g of rat pulmonary artery endothelial cell total RNA was loaded onto agarose gels and analysed for steady-state

content of TGF- $\beta_1$  mRNA by hybridization with a human TGF- $\beta_1$  cDNA probe. The arrows on the right indicate the location of 28S and 18S rRNA. Lane 1 represents RNA from control untreated cells; lanes 2 and 3 represent RNA from two separate flasks of cells treated with 100 ng/ml of bleomycin for 18 h.



**Figure 7.** Time course of TGF- $\beta$  mRNA upregulation. Rat pulmonary artery endothelial cells were treated with 100 ng/ml of bleomycin for the indicated times. Total cellular RNA were extracted and analyzed by Northern blotting for TGF- $\beta_1$  mRNA. Autoradiograms were quantitated by laser densitometry and results expressed as relative integration units, and normalized as a percentage of the mean value obtained for the untreated control. Triplicate flasks of cells were studied at each time point.

tors or cytokines with fibrogenic properties, namely the ability to stimulate mesenchymal cell collagen synthesis. Such a possibility could account for the observed stimulation of interstitial lung collagen synthesis in this disease, and represents an especially attractive concept because of the proximity of endothelial cells to the interstitium where the target cells for the increased cytokine production are located. To test this hypothesis within the context of a rodent model of bleomycin-induced pulmonary fibrosis, rat pulmonary artery endothelial cells were analyzed for their ability to secrete fibrogenic cytokines upon stimulation with bleomycin. The results show that these endothelial cells could respond to bleomycin treatment *in vitro* by increased secretion of fibroblast collagen synthesis stimulatory activity, with maximal responses observed at doses of bleomycin  $\geq$  100 ng/ml. These doses of bleomycin were several orders of magnitude lower than those required to cause cytotoxicity or cellular DNA damage (40). The activity was not due to carry-over of bleomycin used to stimulate the endothelial cells, since it was not dialyzable and such activity was absent in control media supplemented with equal doses of bleomycin. The stimulatory effect on endothelial cell production of collagen synthesis stimulatory activity was not due to endotoxin contamination of the bleomycin stock solution, since none was detected using a sensitive assay.

On the basis of its stability in acid, its estimated molecular mass, and selective stimulatory effect on collagenous protein synthesis, the activity in bleomycin-stimulated endothelial cell conditioned media is consistent with that due to TGF- $\beta$  (41). This conclusion was confirmed by four other pieces of evidence. First, the activity was completely neutralized by antibody to TGF- $\beta$  in a specific manner. Secondly, TGF- $\beta$  activity was detected in the active conditioned media using a standard bioassay based on inhibition of proliferation of a mink lung epithelial cell line (26). Thirdly, the same rat lung fibroblasts that responded to the activity in bleomycin-treated endothelial cell conditioned media also responded in a similar manner to

purified TGF- $\beta$ . Finally, endothelial cells stimulated with bleomycin contained significantly higher steady state levels of TGF- $\beta$  mRNA, further suggesting that the increased secretion of this cytokine may be partly, if not wholly, due to increased transcription.

Previous studies have shown TGF- $\beta$  to be ubiquitously produced by all cells that have been studied (41, 42). Recently, however, various subtypes of TGF- $\beta$  have been reported which seem to belong to a large superfamily with conservation of up to nine cysteine residues (42). The subtype composition of the TGF- $\beta$  activity in bleomycin-stimulated endothelial cell conditioned media remains to be determined, although complete neutralization by the antibody to TGF- $\beta_1$  suggests that the activity in endothelial cell conditioned media is mostly due to TGF- $\beta_1$  and perhaps TGF- $\beta_2$ , since the antibody also cross-reacts with the latter form of TGF- $\beta$ . Upregulation of TGF- $\beta_1$  production by bleomycin is further supported by increased steady-state levels of TGF- $\beta_1$  mRNA. Recent studies have shown TGF- $\beta_1$  to be ubiquitously made by all cells, whereas expression of TGF- $\beta_2$  and TGF- $\beta_3$  appears to be more selective, being present mainly in a monkey epithelial cell line and mesenchymal cells, respectively (43). Expression of these latter TGF- $\beta$  types has not been examined in pulmonary endothelial cells. Studies with more selective neutralizing antibodies are required for further delineation of the TGF- $\beta$  composition in rat pulmonary artery endothelial cell conditioned media.

The pathophysiologic significance of these findings are multiple given the diverse biological activities of this cytokine (41, 42). In addition to stimulation of matrix synthesis and inhibition of cell proliferation, TGF- $\beta$  is also known to be chemotactic for monocytes and fibroblasts, to cause cellular morphological changes, to be immunosuppressive, and to promote wound healing (41, 42). Many of these activities are relevant to events associated with pulmonary fibrosis. Of specific relevance is the recent finding that lung TGF- $\beta$  antigen is significantly increased in bleomycin-induced pulmonary fibrosis (14), which is accompanied by significant increases in lung steady-state levels of TGF- $\beta$  mRNA (13, 15). These increases occur relatively early, within the first 2 wk after induction of the model (13–15). The cellular source of this increased TGF- $\beta$  production has not been unequivocally identified, although the alveolar macrophage certainly is a prime candidate (44). However, there are data against such a role for the alveolar macrophage. Conditioned media from alveolar macrophages obtained from animals with bleomycin-induced pulmonary fibrosis fail to stimulate fibroblast collagen synthesis unless the target cells are also treated with a cyclooxygenase inhibitor (16). Thus, even if these macrophages do secrete TGF- $\beta$ , the net activity in their conditioned media actually inhibit collagen synthesis (16), perhaps owing to autocrine inhibition by endogenously produced prostaglandin E<sub>2</sub> as a result of stimulation by interleukin 1 and/or TNF- $\alpha$  present in the macrophage-conditioned media. Hence, another more suitable cellular source for active TGF- $\beta$  in the lung, which could more directly participate in the pathogenesis of pulmonary fibrosis in this model, needs to be identified.

In this study, the lung endothelial cell has been demonstrated to be another potential source of increased TGF- $\beta$  production in the lung in this model. An attractive feature of the endothelial cell as a source of active cytokine is its anatomic location which is in direct contact with the interstitial cells likely to be responsible for the increased collagen deposition.

Furthermore, unequivocal collagen synthesis stimulatory activity is present in the endothelial conditioned media without any need for exogenous drug or other manipulation of the target fibroblasts. This suggests that in vivo the pulmonary endothelial cell could respond to bleomycin and perhaps other cytokines by increasing secretion of active TGF- $\beta$  with its inflammatory and matrix synthetic promoting properties to promote lung inflammation and fibrosis. Early on after initiation of induction, or during intravenous bleomycin therapy in humans, endothelial cells in the pulmonary vasculature are likely to be exposed to doses of bleomycin ( $\geq 100$  ng/ml) found in this study to stimulate TGF- $\beta$  production. Such increases in TGF- $\beta$  production could play significant roles in the observed early recruitment of mononuclear phagocytes into the lung seen in this model (1). They could also be a key element in the upregulation of lung collagen synthesis in this model (1). This upregulation of endothelial cell TGF- $\beta$  production could be sustained in later stages of the disease by other cytokines also known to be elevated in this model of fibrosis (5, 6, 12–15). Autocrine upregulation by the secreted TGF- $\beta$  itself is another possibility. Further studies are necessary to further define these possibilities.

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