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Research Article

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Spontaneous Production of Transforming Growth Factor-β2 by Primary Cultures of Bronchial Epithelial Cells

Effects on Cell Behavior In Vitro

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Abstract

The ability of airway epithelial cells to produce transforming growth factor-β (TGF-β) may be an important mechanism for the control of growth, differentiation, and repair of the airway epithelium. To determine whether airway epithelial cells are capable of producing TGF-β, we examined primary cultures of bovine bronchial epithelial cells. Using a bioassay, TGF-β activity was detected readily in media conditioned by bovine bronchial epithelial cells. Neutralizing antisera to TGF-β1 and TGF-β2 were used to demonstrate that the majority of the activity was of the TGF-β2 isoform. Interestingly, some of the TGF-β activity was present in the conditioned media as "active" TGF-β, not requiring acid activation. The production of TGF-β was variable, depending on cell density and the presence of retinoic acid. The presence of endogenously produced active TGF-β in the culture media was shown to modulate the behavior of the cell cultures as evidenced by the effects of TGF-β–neutralizing antisera on cell size and fibronectin production. Our results suggest that active TGF-β produced by airway epithelial cells may function in an autocrine or paracrine manner to modulate epithelial cell behavior. (*J. Clin. Invest.* 1992. 90:1379–1385.) Key words: TGF-β • fibronectin • cell density • growth inhibition • epithelium

Introduction

A number of growth factors appear to be important for the growth and differentiation of airway epithelial cells (1–3). Transforming growth factor-β (TGF-β),[1] often described by a transformation assay (4), has a broad range of effects on growth and differentiation of many cell types and has the striking effect of causing terminal squamous differentiation of tracheobronchial cells in vitro (5, 6). Concentrations of TGF-β as low as 1 pM inhibit the growth of primary cultures of tracheal epithelial cells with the cells accumulating in the G0–G1 phases of the cell cycle and tending to become squamous with formation of cross-linked envelopes (5). Three isoforms of TGF-β(1-3) have been described in mammals and they appear to play important roles in the developing lung as they have been shown to colocalize with matrix proteins in the developing lung of the mouse embryo (7, 8). TGF-β is also likely to play important roles in the repair of injured epithelium since it is known to influence wound healing in several systems (9) and has marked effects on the regulation of extracellular matrix proteins (10, 11).

TGF-β is present in the epithelial lining fluid of the normal human lower respiratory tract at a concentration estimated between 200 and 300 pM (12). Since this value is ~ 15-fold greater than the concentration of TGF-β in plasma, the majority of TGF-β present in the epithelial lining fluid may be produced by cells present in the respiratory tract. Several potential sources of TGF-β exist. Alveolar macrophages are one potential source of TGF-β in the lower respiratory tract as they are known to constitutively express the gene (13, 14). In a recent study of the normal adult mouse lung, mRNA transcripts for TGF-β1, -β2, and -β3 were found in smooth muscle cells and fibroblasts (15). One immunohistochemistry study of lung tissue from subjects with advanced pulmonary fibrosis demonstrated increased localization of TGF-β in bronchiolar epithelium (16). Thus, epithelial cells may be a source of TGF-β in some conditions. Other normal mammalian cell types and tissues have been shown to produce TGF-β in culture, including keratinocytes (17).

The ability of airway epithelial cells to produce TGF-β would be an important mechanism for autocrine or paracrine control of epithelial growth and differentiation. Several observations suggest that cultured bronchial cells are capable of expressing TGF-β. Human bronchial epithelial cells in vitro have a tendency to progressively assume squamous morphology (5, 6). Bovine bronchial epithelial cells also can spontaneously produce fibronectin in culture, an effect that can be augmented by the addition of exogenous TGF-β (18). We therefore evaluated bovine bronchial epithelial cells for the production of TGF-β. In this report, we demonstrate that bronchial epithelial cells express TGF-β, primarily of the isoform TGF-β2. In addition, a physiologically significant portion of the secreted TGF-β activity is in the active form and can modulate the behavior of the primary cultures of airway epithelial cells.

Methods

Reagents. Laboratory of Human Carcinogenesis (LHC) basal medium, FCS, triiodothyronine, hydrocortisone, and epinephrine were purchased from Biofluids Inc. (Rockville, MD). RPMI 1640 (RPMI), PBS, DME, MEM, streptomycin–penicillin, and fungizone were obtained from Gibco (Chagrin Falls, OH). Bovine insulin, bovine transferrin, bacterial type XIV protease, and retinoic acid were purchased

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1. *Abbreviations used in this paper:* CM, conditioned media; LHC, Laboratory of Human Carcinogenesis; TGF-β, transforming growth factor-β.

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from Sigma Chemical Co. (St. Louis, MO). Phosphoethanolamine/ethanolamine, trace elements (selenium, manganese, molybdenum, vanadium, nickel, tin), and calcium were obtained from Fisher Scientific Co. (Pittsburgh, PA). Frozen bovine pituitaries were obtained from Pel-Freez Biologicals (Rogers, AR); bovine type I collagen (Vitrogen 100) was obtained from Collagen Corp. (Palo Alto, CA).

Isolation of bovine bronchial epithelial cells. Bovine bronchial epithelial cells were prepared by a modification of a method described by Wu and colleagues (19, 20). Bronchi were removed from fresh bovine lungs, cut into pieces, and trimmed of connective tissue. The bronchi were then incubated at 4°C overnight in sterile MEM containing 0.1% protease. The bronchial lumens were then gently rinsed several times with MEM containing 10% FCS to detach the epithelial cells. The collected cells were washed once with MEM containing 10% FCS, filtered through 250-μm sterile mesh (Tetko, Elmsford, NY), and washed twice with MEM. The cell count was manually determined using a standard hemocytometer. Nonviable cells (trypan blue dye exclusion test) and ciliated cells were not included in the total cell number. Ciliated cells rarely attached to tissue culture plates. Extensive evaluation of cells prepared by these methods in our laboratory has documented that > 95% of the harvested cells are epithelial cells (1).

Culture of bronchial epithelial cells in vitro. The cells were plated on 60-mm tissue culture plates or in 96-well plates (Becton Dickinson, Lincoln Park, NJ) previously coated with bovine type I collagen (Vitrogen 100; Collagen Corp.). The bronchial epithelial cells were cultured, at 37°C in 5% CO_2, in LHC basal medium (2) supplemented with 5 μg/ml bovine insulin, 10 μg/ml bovine transferrin, 10 nM T_3, 0.2 μM hydrocortisone, 0.33 nM retinoic acid, 5 μM phosphoethanolamine/ethanolamine, trace elements, 0.11 mM calcium, bovine pituitary extract (2) containing 10 mg/ml of protein (0.5%), 50 μg/ml penicillin-streptomycin, and 2 μg/ml fungizone. The LHC-supplemented medium (LHC-9) was mixed 1:1 with RPMI, a mixture that has been found to optimize bronchial epithelial cell growth in our laboratory (21). The percentage of bronchial epithelial cells able to attach to the dish and to proliferate varies from preparation to preparation, ranging from 10 to 50% of the cells plated. In the average cell preparation, ~ 20–30% of the viable cells plated at the start of the culture are attached to the tissue culture plate after 24 h. In some experiments, the bronchial epithelial cells were plated at different densities in 60-mm dishes so as to become confluent at different times after the start of the culture: 0.5×10^6 cells/dish for "low density" cells, 2×10^6 cells/dish for "middensity" cells, and 8×10^6 cells/dish for "high density" cells. Unless stated otherwise, the dishes were rinsed once with Dulbecco's PBS 1 d after plating (day 1) to remove the nonattached cells and 1.5 ml of new medium was added. All the experiments were repeated at least twice with reproducible results. Different conditions were compared for statistical significance using Student's *t* test and analysis of variance (22).

Preparation of conditioned media (CM) from bronchial epithelial cells for TGF-β bioassay. CM collected from dishes with identical culture conditions were pooled, centrifuged, and immediately frozen at −20°C. CM were then lyophilized, reconstituted in distilled water (1/10–1/20 of the original volume), dialyzed against repeated changes of PBS, and filtered through 0.2-μm filters (Acrodisc; Gelman Sciences, Inc., Ann Arbor, MI). The CM processed in this way were denoted "neutral CM." Some aliquots of CM were briefly acidified by a modification of the acidic ethanol procedure ("acidified CM") to activate any precursor TGF-β present (23). 10 μl of 1 N HCl was added to 1 ml of neutral CM to reduce the pH from 7.2 (neutral CM) to 1.6–1.8 (acidified CM). After 15 min at 4°C, the pH was neutralized with 10 μl of 1 N NaOH.

Assay for TGF-β activity. TGF-β activity was determined by measuring the inhibition of [^{3}H]thymidine incorporation by CCL-64 (ATCC) cells (24–27). CCL-64 mink lung epithelial cells were maintained in DME supplemented with 5% FCS. Cells were kept at 37°C in 5% CO_2 and passaged at a seed density of 2×10^5 cells/25 cm^2 tissue culture flask at 3–4-d intervals. Cells were trypsinized, washed twice in DME, resuspended in DME with 5% FBS, and seeded at 6×10^3/well in 96-well tissue culture plates. 3 d later the concentrated CM were added directly to triplicate wells at different dilutions (1:4–1:16 for neutral CM; 1:16–1:128 for acidified CM). After 24 h the media were replaced with DME containing 5% FCS and [^{3}H]thymidine (0.5 μCi/ml). After 2 h the wells were washed three times with ice-cold 5% TCA, solubilized with 100 μl/well of 1 N NaOH, and neutralized after 30 min at room temperature with 100 μl/well of 1 N HCl. Thymidine incorporation was determined by adding 180 μl of solubilized material from each well to 5 ml of scintillation cocktail (Scintiverse BD; Fisher Scientific Co.) and counting in a scintillation counter (Tri-Carb 4530; Packard Instrument Co. Inc., Downers Grove, IL). The average counts per minute values were determined from triplicate samples that varied by < 10% within each condition. The thymidine incorporation in the wells with the CM was plotted against reference values, obtained by incubating the CCL-64 cells with increasing amounts (25–2000 pg/ml) of purified TGF-β1 or TGF-β2 (R&D Systems, Minneapolis, MN). This bioassay was able to detect a level of TGF-β2 activity as low as 12.5 pg/ml (final dilution in the well), corresponding to 50 pg/ml of active TGF-β in the concentrated CM. An example of a standard curve with TGF-β2 is shown in Fig. 1.

Use of TGF-β–neutralizing antibodies to characterize the TGF-β activity. Two rabbit polyclonal antibodies, one neutralizing both TGF-β1 and TGF-β2 and one specific for TGF-β2, were purchased from R&D Systems. Two different turkey antisera, one specific for TGF-β1 and the other neutralizing TGF-β2 (27), were a generous gift from Anita Roberts (National Institutes of Health/National Cancer Institute, Bethesda, MD). To show that the factor(s) interfering with the incorporation of [^{3}H]thymidine by CCL-64 cells were member(s) of the TGF-β family, the CM were incubated with serial dilutions of the neutralizing antibodies for 1 h at room temperature. After this preincubation period, the samples were then transferred to wells containing the CCL-64 cells and the assay of the residual TGF-β activity was performed as described above.

Fibronectin ELISA. An indirect inhibition ELISA for bovine fibronectin was used to quantitate the fibronectin present in the unconcentrated bronchial epithelial cell CM. Bovine fibronectin was purified from bovine serum according to the technique of Engvall and Ruoslahti (28). The purified bovine fibronectin was diluted in Voller's buffer (0.5 μg/ml) and used to coat 96-well flat-bottom plates (Immulon 2; Dynatech Labs, Chantilly, VA) with 200 μl/ml. Samples and duplicate fibronectin standards were diluted 1:1–1:2187 in 96-well round-bottom polystyrene plates (Linbro/Titertech, McLean, VA)

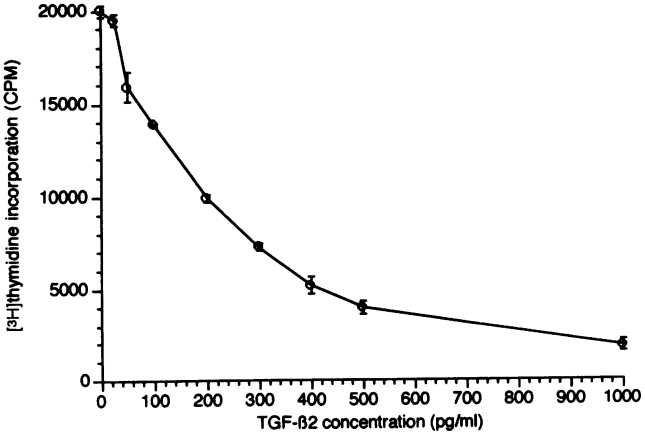

Figure 1. Growth inhibition of CCL-64 cells by TGF-β2. CCL-64 mink lung epithelial cells were seeded into wells of 96-well tissue culture plates as described in Methods. The cells were cultured for 3 d and then exposed to varying concentrations of TGF-β2 for 24 h. [^{3}H]Thymidine (0.5 μCi/ml) was added for an additional 2 h before harvesting for scintillation counting. The TGF-β2 dilutions and test media were assayed in triplicate.

with PBS-Tween (0.08% Tween). The samples were incubated overnight at 4°C in a humidified chamber with 1:400 dilution of rabbit anti-bovine fibronectin serum. The following day the samples were transferred to the flat-bottom plates, which had been previously washed three times with PBS-Tween. After a 30-min incubation, the flat-bottom plates were again washed three times with PBS-Tween and 200 μl/well of a 1:1,000 dilution of goat anti-rabbit IgG conjugated with horseradish peroxidase (ICN ImmunoBiologicals, Lisle, IL) were added. After 90 min the plates were again washed three times with PBS-Tween followed by the addition of 200 μl/well of *o*-phenylenediamine substrate (10 mg dissolved in 1 ml methanol/100 ml H_2O) with 10 μl of 30% of H_2O_2. The enzymatic reaction was stopped with 28 μl/well of 4 N H_2SO_4. The absorbance was then measured at 492 nm using an ELISA reader (model 2550 EIA Reader; Bio-Rad Laboratories, Richmond, CA). Data were analyzed using the algorithm of Rodbard (29) by a Macintosh computer, which was interfaced to the ELISA reader.

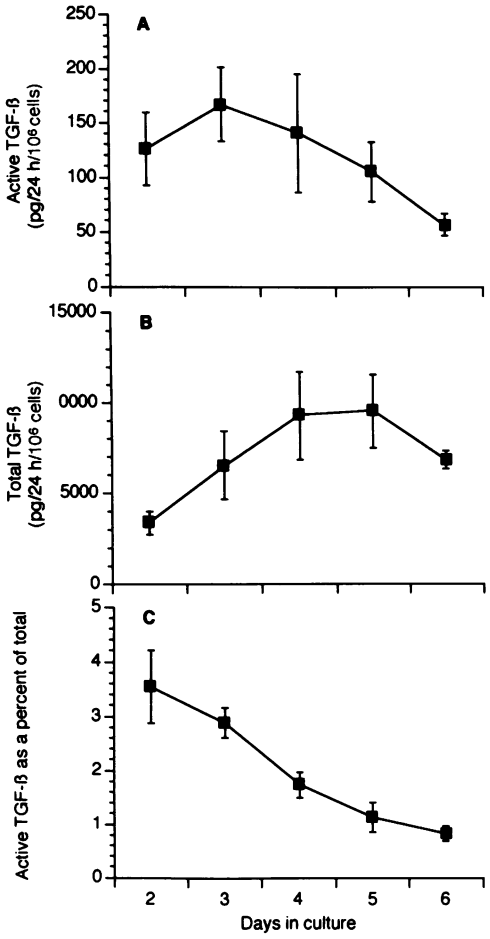

Figure 2. Detection of TGF-β-like activity in CM from bronchial epithelial cells. The bovine bronchial epithelial cells were plated at middensity (2×10^6 cells/60 mm dish) in LHC-9/RPMI (see Methods). Medium containing unattached cells was removed and replaced with 1.5 ml of fresh medium 24 h after plating. Every 24 h three dishes were trypsinized for cell counts with a Coulter counter (ZM; Coulter Electronics Inc., Luton, Beds, England) and the medium (1.5 ml/dish) was changed. The harvested CM was processed for TGF-β bioassay as specified in Methods. The values represent the means and SEM for five separate experiments. (*A*) Vertical axis, TGF-β-like activity of the neutral bronchial epithelial cell CM (TGF-β naturally activated) expressed as pg produced every 24 h and normalized for 1×10^6 cells. (*B*) The TGF-β-like activity found after transient acidification of the CM and expressed as the total amount of TGF-β produced per 24 h by 10^6 cells. (*C*) TGF-β-like activity present in the neutral CM is expressed as the percentage of the total TGF-β-like activity present in the acidified CM.

Results

Release of TGF-β-like activity by bronchial epithelial cells. When the bronchial epithelial cells were cultured at middensity in serum-free conditions, TGF-β-like activity was detected in CM (Fig. 2 *A*). Thus, active TGF-β appears to be produced by the bronchial epithelial cell cultures. To quantify both the active and inactive latent TGF-β present in the CM, total TGF-β was assayed by subjecting aliquots of the same CM to the acid-activation procedure (23). Approximately 5–10-fold more TGF-β activity was detected (Fig. 2 *B*), demonstrating that the majority of TGF-β produced was in the latent form. The quantity of TGF-β activity in the CM was roughly similar to that reported for another cell system, bovine aortic endothelial cells (30). The ratio of active TGF-β versus total (active plus latent) TGF-β was highest on day 2 and gradually decreased because of increasing total TGF-β production by the cultured bronchial epithelial cells.

To confirm that the TGF-β-like activity observed in bronchial epithelial cell CM was due to a member(s) of the TGF-β family rather than to any other factor(s) interfering with the incorporation of [^{3}H]thymidine by CCL-64 cells, TGF-β-neutralizing antibodies were used (Table I). The rabbit polyclonal antiserum that recognizes both TGF-β1 and TGF-β2 was able to completely neutralize all of the activity in neutral CM and nearly all of the activity in the acidified CM. Evaluation of the TGF-β activity present in the bronchial epithelial cell culture supernatants with the specific neutralizing antibodies that

Table I. Antibody Neutralization of the TGF-β-like Activity in Conditioned Media From Bronchial Epithelial Cells

Factors added*	Relative incorporation of [^{3}H]thymidine
No addition (control)	1.00
Neutral CM	0.56
Neutral CM + rabbit TGF-β1 and -β2–neutralizing antibody	1.03
Neutral CM + turkey TGF-β1 antiserum	0.63
Neutral CM + rabbit TGF-β_2 antibody	0.99
Acidified CM	0.31
Acidified CM + rabbit TGF-β1 and -β2–neutralizing antibody	0.88
Acified CM + turkey TGF-β1 antiserum	0.33
Acidified CM + rabbit TGF-β2 antibody	0.87

The CCL-64 cell bioassay was performed as described in Methods.
* Neutral and acidified conditioned media were treated as described in Methods and added at a concentration capable of causing a 30–40% inhibition of [^{3}H]thymidine incorporation (equal to 200–300 pg/ml of TGF-β1 or TGF-β2. All of the neutralizing antibodies were added at concentrations equivalent to the amount that will neutralize 1 ng/ml of the specific TGF-β activity (rabbit TGF-β2–specific antibodies were added at 16 μg/ml and turkey TGF-β1–specific antiserum was added at 0.5 μl/ml). Relative [^{3}H]thymidine incorporation was determined as described in Methods. The control was designated as 1.00 and represented 15,166 cpm.

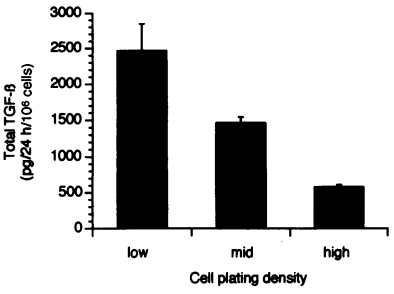

Figure 3. Release of TGF-β by bronchial epithelial cells plated at different densities. Bronchial epithelial cells were plated in 60-mm dishes at low (0.5×10^6 cells/dish), mid (2×10^6 cells/dish), and high density (8×10^6 cells/dish). Every 24 h three dishes were trypsinized for cell counts with a Coulter counter (ZM; Coulter Electronics) and the medium (1.5 ml/dish) changed. The harvested CM was processed for TGF-β bioassay as specified in Methods. The total TGF-β–like activity present in the acidified CM is expressed as pg of TGF-β produced every 24 h and normalized for 1×10^6 bronchial epithelial cells. The differences in peak production of TGF-β between the three conditions were significant at $P < 0.05$, analysis of variance.

distinguish between TGF-β1 and TGF-β2 revealed that a turkey antiserum to TGF-β1 had negligible effect in neutralizing the TGF-β activity in both neutral and acidified CM (Table I). In contrast, a rabbit antibody to TGF-β2 was effective in neutralizing the TGF-β activity present in bronchial epithelial cell CM. In the neutral CM, all of the TGF-β–like activity was neutralized by the anti-TGF-β2 antibody. In the acidified CM, the anti-TGF-β2 antibody was effective in neutralizing the majority, but not all of the activity measurable in the CCL-64 bioassay. A turkey anti-TGF-β2 antiserum gave similar results (data not shown). The residual growth inhibitory activity in acidified CM is unlikely to be entirely due to active TGF-β1, since the rabbit TGF-β1-2 neutralizing antibody showed no better performance in neutralizing the TGF-β–like activity of the acidified CM. To confirm the activity of the antisera used to evaluate the bronchial epithelial cell CM in our assay systems, the same serial dilutions of the antibodies were tested against purified TGF-β1 and -β2 at 250 pg/ml. The neutralizing antisera were effective as expected against the relevant TGF-β type. Thus, the majority of TGF-β activity produced by bronchial epithelial cells in vitro appears to be TGF-β2.

Effect of cell density on release of TGF-β by bronchial epithelial cells. It was noted above (Fig. 2) that production of TGF-β decreases at or after the cells reach confluence (usually from day 4 to day 5). Since TGF-β is thought to function as an autocrine mediator of epithelial differentiation and its production can be density dependent (31), we evaluated the effect of cell density on TGF-β production by bronchial epithelial cells. Primary cultures of bronchial epithelial cells were cultured at three different densities as described in Methods. We determined that cells cultured at low density produced more TGF-β on a per cell basis than cells that were plated at higher densities (Fig. 3).

Effect of retinoic acid on production of TGF-β. Since our usual growth media for bronchial epithelial cells (LHC-9/RPMI) contains retinoic acid, and retinoic acid has been shown to induce the expression of TGF-β2 by keratinocytes (32) and during early mammalian development (25), we investigated its role in the production of TGF-β by bronchial epithelial cells. The presence of retinoic acid (0.33 nM) increased the total TGF-β produced by the cells (Fig. 4). The percent increase in TGF-β production caused by retinoic acid ranged from 27% early in the time course to 57% later in the time course, but the differences in TGF-β production narrowed by day 6 of the cultures. In most cases, this occurred when TGF-β production began to decrease as seen in Fig. 2. The differences in TGF-β production were significant at $P < 0.01$ (paired t test). The effect of retinoic acid on TGF-β production did not appear to be directly due to an alteration in cell density, as the results were normalized for cell number. Furthermore, retinoic acid at the concentration used did not appear to alter cell proliferation as determined by direct measurements of cell number. In addition, retinoic acid does not appear to influence the TGF-β isoform produced by the cultured bronchial epithelial cells. Using the neutralizing antibodies specific for TGF-β1 and TGF-β2 as described above, we found that in the absence of retinoic acid the TGF-β–like activity present in the neutral bronchial epithelial cell CM still appears to be TGF-β2 (data not shown).

Evaluation of the role of TGF-β in squamous differentiation of bronchial epithelial cells. TGF-β induces tracheobronchial epithelial cell to undergo squamous differentiation (6) and this effect is particularly evident on low density cell cultures. Therefore, we tested the effect of native bronchial epithelial cell CM on one measure of squamous differentiation, cell surface area (33). TGF-β–neutralizing antibodies added to the culture medium attenuated the frequency of bronchial epithelial cells cultured at low density to acquire a squamous morphology (Fig. 5). The anti-TGF-β1 and -β2 antibody decreased the mean planar area per cell from 1,322±160 μm^2 (control cells) to 847±78 ($P < 0.05$). Whereas the control nonimmune rabbit IgG preparation at the same concentration (32 μg/ml) showed no effect on the mean cell surface. Bronchial epithelial cell CM,

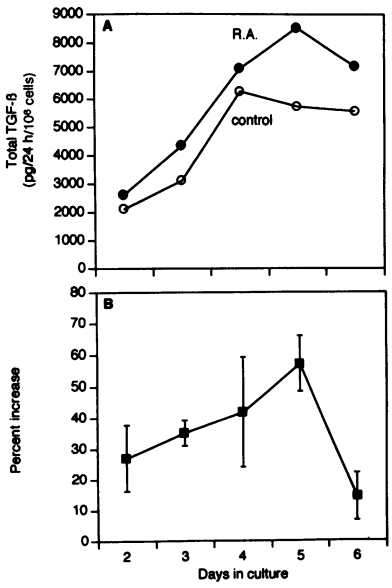

Figure 4. Effect of retinoic acid on production of TGF-β by bronchial epithelial cells. Bronchial epithelial cells were plated in 60-mm dishes at 1×10^6 cells/dish in LHC-9/RPMI medium with retinoic acid (0.33 nM) or without retinoic acid (LHC-8E/RPMI). The next day, the medium was removed and changed with 1.5 ml/dish of fresh medium (+ or − retinoic acid), which was then replaced and harvested every 24 h. Each day, three dishes were trypsinized and cells counted on a Coulter counter. The CM was processed for TGF-β bioassay as in Fig. 2. The total TGF-β activity present in the acidified CM is expressed as pg of TGF-β produced every 24 h and normalized for 1×10^6 bronchial epithelial cells. In *A*, the total TGF-β activity is shown for a representative experiment (with retinoic acid, *R.A.*; without retinoic acid, *control*). In *B*, the means and SEM of three separate experiments are shown for the percent increase in TGF-β production.

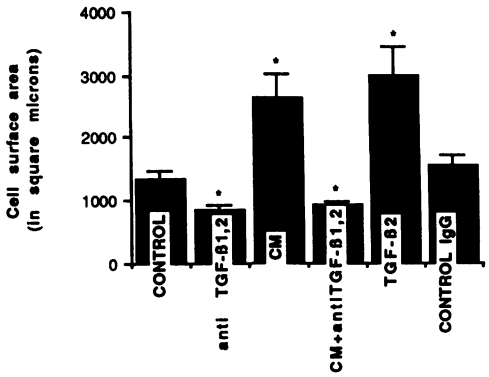

Figure 5. Effect of anti-TGF-β antibodies and TGF-β on squamous differentiation of bronchial epithelial cells. The bronchial epithelial cells were plated at 24×10^3 cells/chamber (equal to low density cells in 60-mm dishes) in LHC-9/RPMI in four-chamber, tissue culture glass slides (Lab-Tek, Nunc, Naperville, IL) that were coated with collagen. The following day the medium was replaced with fresh medium, with or without anti-TGF-β1 and two antibodies and bronchial epithelial cell CM. Each neutralizing antibody concentration was high enough to neutralize 1 ng TGF-β/ml. The bronchial epithelial cell CM was neutral CM used at a concentration to achieve 250 pg/ml of TGF-β. This preparation of bronchial epithelial cell CM was split after dilution and one half was incubated with rabbit anti-TGF-β1 and- β2-neutralizing antibody at a concentration able to neutralize 1.25 ng of TGF-β/ml. As a positive control, 250 pg/ml of purified TGF-β2 was used. As a negative control, an equivalent amount of a nonimmune rabbit IgG was used. Every 24 h the medium was replaced with fresh medium with the same condition for each chamber. After 5 d in culture the cells were fixed and stained (Leukostat; Fisher Scientific Co., Orangeburg, NY). To determine the surface covered in each field by the cells, an Optomax V Image analyzer (AMS; Saffron Walden, Essex, England) was used. For each condition, 12 fields were analyzed with 20× magnification. The cell count was determined manually for each field. At least 1,000 total cells were measured for each condition. The bars represent the mean±SEM for 12 fields. The asterisk (*) denotes conditions statistically different from control.

which contains active TGF-β, further increased the cell surface area to 2,655±358 ($P < 0.01$; compared with control). The same preparation of bronchial epithelial cell CM, preincubated with the TGF-β1 and -β2–neutralizing antibody lost its ability to induce squamous differentiation, yielding a mean surface area per cell of 931±63. Purified TGF-β2 used at the same concentration as the active TGF-β in the CM, 250 pg/ml, had a similar effect. The mean surface area was increased to 3,004±453 ($P < 0.01$; compared with control).

Evaluation of the role of TGF-β in the growth of bronchial epithelial cells. TGF-βs inhibit proliferation of almost all nonneoplastic epithelial cells in culture (34, 35). Therefore, we tested the growth effect of neutral CM from confluent bronchial epithelial cells (Fig. 6). Neutral bronchial epithelial cell CM had an inhibitory effect on the growth of bronchial epithelial cells ($P < 0.001$ compared with control on days 5, 7, and 9). The growth inhibition shown by the TGF-β–like activity present in the neutral CM was comparable to the degree of inhibition produced by a theoretically equal amount of purified TGF-β (250 pg/ml). Preincubation of the neutral CM with the TGF-β1 and -β2–neutralizing antibody blocked the growth inhibitory effects, confirming that TGF-β in the neutral CM was responsible for the growth inhibition seen (Fig. 6).

Evaluation of the role of TGF-β on production of fibronectin by bronchial epithelial cells. TGF-β is a well-known inducer for the expression of the fibronectin gene (36). TGF-β induces an increase in production of fibronectin by primary bronchial epithelial cells after 24 h of exposure, and fibronectin production peaks at 48 h (18). We thus investigated whether differences in the production of endogenous active TGF-β could affect the spontaneous production of fibronectin by bronchial epithelial cells.

To evaluate this question, bronchial epithelial cells were incubated with increasing amounts of TGF-β1 and -β2–neutralizing antibody. The antibody at 24 μg/ml reduced the production of fibronectin by the bronchial epithelial cells compared with control cells incubated with an equivalent amount of control rabbit IgG (Fig. 7). The differences in fibronectin production were significant at $P < 0.01$ (paired t test).

Discussion

Our studies demonstrate that primary cultures of bovine bronchial epithelial cells are capable of producing both active and inactive TGF-β and that the majority of TGF-β produced is TGF-β2. The results also suggest that TGF-β may contribute to the modulation of squamous differentiation and the production of fibronectin.

The amount of active and inactive TGF-β produced was affected primarily by the time in culture, the plating cell density, and the presence of retinoic acid in the medium. Primary bronchial epithelial cells exhibit a very low basal production of latent TGF-β at the beginning of the culture, which progressively increased until cell confluency. Our data also suggest that cell density regulates the production of active TGF-β by primary cultures of bronchial epithelial cells. This is of further

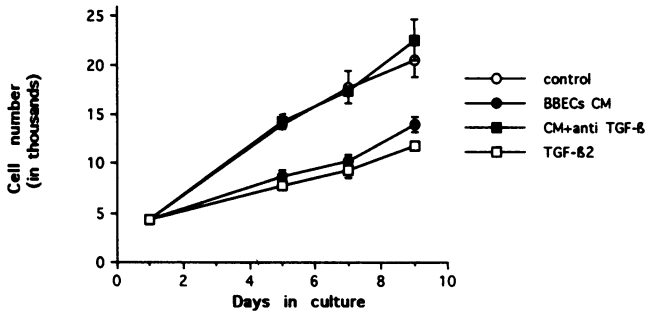

Figure 6. Effect of neutral CM on growth of bronchial epithelial cells. Bronchial epithelial cells were plated in 96-well plates, 8×10^3 cells/well (equal to low density cells in 60-mm dishes). The following day the wells were rinsed with DPBS and incubated with 80 μl/well of fresh medium with or without bronchial epithelial cell CM and anti-TGF-β antibody. Media used, as for the experiment of squamous differentiation, were neutral CM at a final calculated concentration of TGF-β activity equal to 250 pg/ml. This preparation of bronchial epithelial cell CM was split after dilution and one-half was incubated with rabbit anti TGF-β1 and -β2–neutralizing antibody at a concentration able to neutralize 1.25 ng of TGF-β/ml. As a positive control, 250 pg/ml of purified TGF-β2 (R&D Systems) was used. Each day the cells were refed with fresh medium (80 μl) with the same composition. On days 5, 7, and 9, three wells were trypsinized for each condition and the cells counted twice on a Coulter counter. The figure shows the mean cell count/dish±SEM.

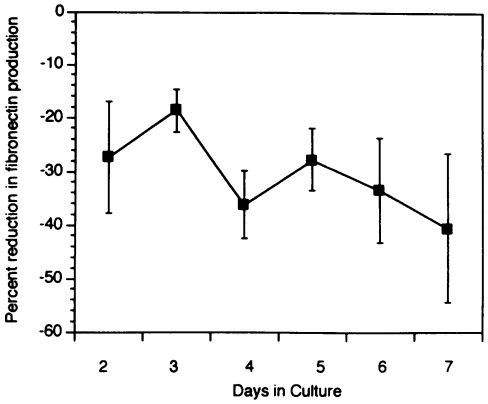

Figure 7. Effect of anti-TGF-β antibodies on spontaneous fibronectin production by bronchial epithelial cells. The bronchial epithelial cells were plated in 96-well plates, 8×10^3 cells/well (equal to low density cells in 60-mm dishes). The following day the wells were rinsed with Dulbecco's PBS and incubated with 80 μl/well of fresh medium with or without rabbit anti-TGF-β1 and -β2-antibody. We used 24 μg/ml of antibody, which is able to neutralize 750 pg/ml of TGF-β. A non-immune rabbit IgG preparation was used as negative control. Each day, the medium was replaced and the harvested CM was tested for fibronectin using an ELISA technique as described in Methods. Each day three wells for each condition were trypsinized for cell counts. The values represent the mean and SEM of the percent reduction in fibronectin production, normalized for cell number, (anti-TGF-β1 and -β2 antibody vs. control antibody) for three separate experiments.

interest because cell density also regulates TGF-β receptor expression. TGF-β receptors are down-regulated as cell density increases (37), and high density human (33) and bovine (21) bronchial epithelial cells are less responsive to the differentiation effects of TGF-β. Retinoic acid, present as a standard component in the serum-free supplemented medium (LHC-9/RPMI) for respiratory epithelial cell cultures (2) and a known inducer of the expression of TGF-β2 in embryonal carcinoma cells and keratinocytes (25, 32), also increased the secretion of TGF-β by bronchial epithelial cells.

The physiological mechanisms by which latent TGF-β is activated in vivo are unclear. The ability of the cells to produce active TGF-β in situ is probably an important mechanism for regulation of the affects of TGF-β and may be under the control of specific hormones. In addition, proteolytic enzymes may play an important role in the physiological activation of TGF-β. Purified preparations of the protease plasmin activate a portion of the inactive TGF-β present in fibroblast-conditioned medium (38). Furthermore, plasmin inhibitors were shown to block the activation of inactive TGF-β described in coculture of endothelial cells and pericytes in serum-free conditions (39). It is also known that TGF-β induces plasminogen activator inhibitor type 1 in bronchial epithelial cells (40). Thus, the induction of protease inhibitors as the cells accumulate more TGF-β may be an explanation for the decrease in the percent of TGF-β that is active (Fig. 2). It is reasonable to speculate that bronchial epithelial cells secrete proteases or have cell proteases that activate TGF-β and can also produce inhibitors of proteases. Currently, we have no direct evidence for proteolysis in activating TGF-β in our system. Thus far, the addition of the plasmin inhibitors e-amino-*n*-caproic acid and aprotinin to bronchial epithelial cell cultures has had no effect on the production of active TGF-β (data not shown).

The antibodies used in the present study suggest that most of the TGF-β activity released by the bovine bronchial epithelial cells is TGF-β2 and not TGF-β1. These results do not exclude, however, the production of other isoforms of TGF-β by these cells. The significance of bronchial epithelial cell production of TGF-β2 in excess of other TGF-β isoforms (like TGF-β1) is unclear. At least five different isoforms of TGF-β have been described (41) with an extraordinary conservation of sequence in organisms as disparate as mammalians and amphibians. This evolutionary conservation underlines the universality of TGF-β action, but the reasons for so many isoforms are puzzling. TGF-β1 and -β2, the two most studied isoforms, are nearly identical in their biological activity, although there is evidence for differences in binding to different TGF-β receptors (42, 43).

The production of active TGF-β by bronchial epithelial cells at sites of injury may be an important event in healing processes in vivo. TGF-β is a powerful chemoattractant for neutrophils, mononuclear cells, and fibroblasts (44, 45). In another study, application of TGF-β1 to the chicken chorioallantoic membrane resulted in early accumulation of endothelial cells, fibroblasts, and epithelial cells, despite the observation that all the cell types showed growth inhibition to TGF-β (46). An important role for TGF-β in pulmonary inflammation was also shown in a model of bleomycin-induced lung injury (47). In addition, modulation of fibronectin production by TGF-β may be an important modifier of cell accumulation and healing at sites of injury. In this regard, fibronectin is a chemoattractant for both fibroblasts and bronchial epithelial cells (48). Thus, increases in local fibronectin production induced by TGF-β might amplify the effect of active TGF-β in recruiting more cells at the site of mucosal injury.

In conclusion, cultures of primary bronchial epithelial cells produce TGF-β and release some in an active form. This potent cytokine can then function in an autocrine or paracrine manner to modulate epithelial cell behavior. Additional studies are needed to identify all TGF-β targets and to investigate the mechanisms by which TGF-β is activated and how the activation mechanisms themselves are controlled.

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References

1. Takizawa, H., J. D. Beckmann, S. Shoji, L. R. Claassen, R. F. Ertl, J. Linder, and S. I. Rennard. 1990. Pulmonary macrophages can stimulate cell growth of bovine bronchial epithelial cells. *Am. J. Respir. Cell Mol. Biol.* 2:245–255.

2. Lechner, J. F., and M. A. LaVeck. 1985. A serum-free method for culturing normal human bronchial epithelial cells at clonal density. *J. Tissue Culture Methods.* 9:43–48.

3. Leslie, C. C., K. McCormick-Shannon, J. L. Cook, and R. J. Mason. 1985. Macrophages stimulate DNA synthesis in rat alveolar type II cells. *Am. Rev. Respir. Dis.* 132:1246–1252.

4. Roberts, A. B., M. A. Anzano, L. C. Lamb, J. M. Smith, and M. B. Sporn. 1981. New class of transforming growth factors potentiated by epidermal growth factor: isolation from non-neoplastic tissue. *Proc. Natl. Acad. Sci. USA.* 78:5339–5343.

5. Jetten, A. M., J. E. Shirley, and G. Stoner. 1986. Regulation of proliferation and differentiation of respiratory tract epithelial cells by TGF-β. *Exp. Cell Res.* 167:539–549.

6. Masui, T., L. M. Wakefield, J. F. Lechner, M. A. LaVeck, M. B. Sporn, and C. C. Harris. 1986. Type β transforming growth factor is the primary differentiation-inducing serum factor for normal human bronchial epithelial cells. *Proc. Natl. Acad. Sci. USA.* 83:2438–2442.

7. Heine, U. I., E. F. Munoz, K. C. Flanders, A. B. Roberts, and M. B. Sporn. 1990. Colocalization of TGF-β1 and collagen I and III, fibronectin and glycosaminoglycans during lung branching morphogenesis. *Development (Camb.).* 109:29–36.

8. Pelton, R. W., B. Saxena, J. Jones, H. L. Moses, and L. I. Gold. 1991. Immunohistochemical localization of TGFβ1, TGFβ2, and TGFβ3 in the mouse embryo. Expression patterns suggest multiple roles during embryonic development. *J. Cell Biol.* 115:1091–1105.

9. Pierce, G. F., T. A. Mustoe, J. Lingelbach, V. R. Masakowski, G. L. Griffin, R. M. Senior, and T. F. Deuel. 1989. Platelet-derived growth factor and transforming growth factor-β enhance tissue repair activities by unique mechanisms. *J. Cell Biol.* 109:429–440.

10. Rizzino, A. 1988. Transforming growth factor-β: multiple effects on cell differentiation and extracellular matrices. *Dev. Biol.* 130:411–422.

11. Ignotz, R. A., and J. Massagué. 1986. Transforming growth factor-β stimulates the expression of fibronectin and collagen and their incorporation into the extracellular matrix. *J. Biol. Chem.* 261:4337–4345.

12. Yamauchi, K., Y. Martinet, P. Basset, G. A. Fells, and R. G. Crystal. 1988. High levels of transforming growth factor-β are present in the epithelial lining fluid of the normal human lower respiratory tract. *Am. Rev. Respir. Dis.* 137:1360–1363.

13. Yamauchi, K., P. Basset, Y. Martinet, and R. G. Crystal. 1987. Normal human alveolar macrophages express the gene coding for transforming growth factor-β, a protein with a capacity to suppress fibroblast growth. *Am. Rev. Respir. Dis.* 135:A66.

14. Assoian, R. K., B. E. Fleurdelys, H. C. Stevenson, P. J. Miller, D. K. Madtes, E. W. Raines, R. Ross, and M. B. Sporn. 1987. Expression and secretion of type β transforming growth factor by activated human macrophages. *Proc. Natl. Acad. Sci. USA.* 88:6020–6024.

15. Pelton, R. W., M. D. Johnson, E. A. Perkett, L. I. Gold, and H. L. Moses. 1991. Expression of transforming growth factor-β1, -β2, and -β3 mRNA and protein in the murine lung. *Am. J. Respir. Cell Mol. Biol.* 5:522–530.

16. Khalil, N., R. N. O'Connor, H. W. Unruh, P. W. Warren, K. C. Flanders, A. Kemp, O. H. Bereznay, and A. H. Greenberg. 1991. Increased production and immunohistochemical localization of transforming growth factor-β in idiopathic pulmonary fibrosis. *Am. J. Respir. Cell Mol. Biol.* 5:155–162.

17. Patridge, M., M. R. Green, J. D. Langdon, and M. Feldmann. 1989. Production of TGF-α and TGF-β by cultured keratinocytes, skin and oral squamous cell carcinomas—potential autocrine regulation of normal and malignant epithelial cell proliferation. *Br. J. Cancer.* 60:542–548.

18. Romberger, D. J., J. D. Beckmann, L. Claassen, R. F. Ertl, and S. I. Rennard. 1992. Modulation of fibronectin production of bovine bronchial epithelial cells by transforming growth factor-beta. *Am. J. Respir. Cell Mol. Biol.* 7:149–155.

19. Wu, R., and D. Smith. 1982. Continuous multiplication of rabbit tracheal epithelial cells in a defined, hormone supplemented medium. *In Vitro (Rockville).* 18:800–812.

20. Wu, R., J. W. Groelke, L. Y. Chang, M. E. Porter, D. Smith, and P. Nettesheim. 1982. Effects of hormones on the multiplication and differentiation of tracheal epithelial cells in culture. *In* Growth of Cells in Hormonally Defined Media. D. Sirbasku, G. H. Sato, and A. Pardee, editors. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY. 641–656.

21. Beckmann, J. D., H. Takizawa, D. Romberger, M. Illig, L. Claassen, K. Rickard, and S. I. Rennard. 1992. Serum-free culture of fractionated bovine bronchial epithelial cells. *In Vitro Cell Dev. Biol.* 28A:39–46.

22. Godfrey, K. 1985. Statistics in practice. Comparing the means of several groups. *N. Engl. J. Med.* 313:1450–1456.

23. Roberts, A. B., L. C. Lamb, D. L. Newton, M. B. Sporn, J. E. Delarco, and G. J. Todaro. 1980. Transforming growth factors: isolation of polypeptides from virally and chemically transformed cells. *Proc. Natl. Acad. Sci. USA.* 77:3494–3498.

24. Tucker, R. F., G. D. Shipley, H. L. Moses, and R. W. Holley. 1984. Growth inhibitor from BSC-1 cells closely related to platelet type β transforming growth factor. *Science (Wash. DC).* 226:705–707.

25. Kelly, D., W. J. Campbell, J. Tiesman, and A. Rizzino. 1990. Regulation and expression of transforming growth factor type-β during early mammalian development. *Cytotechnology.* 4:227–242.

26. Cheifetz, S., J. A. Weatherbee, M. L.-S. Tsang, J. K. Anderson, J. E. Mole, R. Lucas, and J. Massague. 1987. The transforming growth factor-β system, a complex pattern of cross-reactive ligands and receptors. *Cell.* 48:409–415.

27. Danielpour, D., L. L. Dart, K. C. Flanders, A. B. Roberts, and M. B. Sporn. 1989. Immunodetection and quantitation of the two forms of transforming growth factor-beta (TGF-beta 1 and TGF-beta 2) by cells in culture. *J. Cell Physiol.* 138:79–86.

28. Engvall, E., and E. Ruoslahti. 1977. Binding of soluble form of fibroblast surface protein, fibronectin, to collagen. *Int. J. Cancer.* 20:1–5.

29. Rodbard, D. 1971. Statistical aspects of radioimmunoassay. *In* Competitive Protein Binding assays. W. D. Daughaday, and W. A. Odell, editors. J. B. Lippincott, Philadelphia. 204–259.

30. Sato, Y., R. Tsuboi, R. Lyons, H. Moses, and D. B. Rifkin. 1990. Characterization of the activation of latent TGF-β by co-cultures of endothelial cells and pericytes or smooth muscle cells. A self-regulating system. *J. Cell Biol.* 111:757–763.

31. van Zoelen, E. J. J., I. Koornneef, J. C. M. Holthuis, Th. M. J. Ward-van Oostwaard, A. Feijen, T. L. de Poorter, C. L. Mummery, and S. C. van Buul-Offers. 1989. Production of insulin-like growth factors, platelet-derived growth factor, and transforming growth factors and their role in the density-dependent growth regulation of a differentiated embryonal carcinoma cell line. *Endocrinology.* 124:2029–2041.

32. Glick, A. B., K. C. Flanders, D. Danielpour, S. H. Yuspa, and M. B. Sporn. 1989. Retinoic acid induces transforming growth factor-β2 in cultured keratinocytes and mouse epidermis. *Cell Regul.* 1:87–97.

33. Ke, Y., B. I. Gerwin, S. E. Ruskie, A. M. A. Pfeifer, C. C. Harris, and J. F. Lechner. 1990. Cell density governs the ability of human bronchial epithelial cells to recognize serum and transforming growth factor beta 1 as squamous differentiation-inducing agents. *Am. J. Pathol.* 137:833–843.

34. Sporn, M. B., A. B. Roberts, L. M. Wakefield, and R. K. Assoian. 1986. Transforming growth factor-β: biological function and chemical structure. *Science (Wash. DC).* 233:532–534.

35. Massagué, J. 1987. The family of growth and differentiation factor. *Cell.* 49:437–438.

36. Dean, D. C., R. F. Newby, and S. Bourgeois. 1988. Regulation of fibronectin biosynthesis by dexamethasone, transforming growth factor-β and cAMP in human cell lines. *J. Cell Biol.* 106:2159–2170.

37. Rizzino, A., P. Kazakoff, E. Ruff, C. Kuszynski, and J. Nebelsick. 1988. Regulatory effects of cell density on the binding of transforming growth factor-β, epidermal growth factor, platelet-derived growth factor, and fibroblast growth factor. *Cancer Res.* 48:4266–4271.

38. Russette, M. L., J. Keski-Oja, and H. L. Moses. 1988. Proteolytic activation of latent transforming growth factor-β from fibroblast-conditioned medium. *J. Cell Biol.* 106:1659–1665.

39. Sato, Y., and D. B. Rifkin. 1989. Inhibition of endothelial cell movement by pericytes and smooth muscle cells: activation of a latent transforming growth factor-β1–like molecule by plasmin during co-culture. *J. Cell Biol.* 109:309–315.

40. Gerwin, B. I., J. Keski-oja, M. Seddon, J. F. Lechner, and C. C. Harris. 1990. TGF-β1 modulation of urokinase and PAI-1 expression in human bronchial epithelial cells. *Am. J. Physiol.* 259(*Lung Cell Mol. Physiol. 3*):L262–L269.

41. Roberts, A. B., and M. B. Sporn. 1990. The transforming growth factors-β. *In* Handbook of Experimental Pharmacology. Vol. 95.1. Peptid Growth Factors and Their Receptors. M. B. Sporn, and A. B. Roberts, editors. Springer-Verlag, Heidelberg, FRG, 419–472.

42. Wakefield, L. M., D. M. Smith, T. Masui, C. C. Harris, and M. B. Sporn. 1987. Distribution and modulation of the cellular receptor for transforming growth factor-beta. *J. Cell Biol.* 105:965–975.

43. Massagué, J., S. Cheifetz, F. T. Boyd, and J. L. Andres. 1990. TBF-β receptors and TGF-β binding proteoglycans: recent progress in identifying their functional properties. *Ann. NY Acad. Sci.* 593:59–72.

44. Lee, T. C., L. I. Gold, B. Cronstein, and J. Reibman. 1990. Transforming growth factor-β: a potent neutrophil chemoattractant. *Am. Rev. Respir. Dis.* 141:A681.

45. Postlethwaite, A. E., J. Keski-Oja, H. L. Moses, and A. H. Kang. 1987. Stimulation of the chemotactic migration of human fibroblasts by transforming growth factor β. *J. Exp. Med.* 165:251–256.

46. Yang, E. Y., and H. L. Moses. 1990. Transforming growth factor β1-induced changes in cell migration, proliferation and angiogenesis in the chicken chorioallantoic membrane. *J. Cell Biol.* 111:731–741.

47. Khalil, N., O. Bereznay, M. Sporn, and A. H. Greenberg. 1989. Macrophage production of transforming growth factor β and fibroblast collagen synthesis in chronic pulmonary inflammation. *J. Exp. Med.* 170:727–737.

48. Shoji, S., K. A. Rickard, R. F. Ertl, J. Linder, and S. I. Rennard. 1989. Lung fibroblasts produce chemotactic factors for bronchial epithelial cells. *Am. J. Physiol.* 257 (*Lung Cell Mol. Physiol. 1*):L71–L79.