Epithelial–Fibroblast Interactions in Bleomycin-induced Lung Injury and Repair

Linda Young and Ian Y. R. Adamson
Department of Pathology, University of Manitoba, Winnipeg, Canada

Interacellular communication between epithelial cells and fibroblasts is important for cellular regulation in a variety of organ systems, particularly during development (1,2). It has been shown that fibroblasts in the lung or their secreted collagen fibers can influence epithelial cell growth and differentiation, whereas the epithelium may control fibroblast growth (3–5).

In some cases, cell–cell interaction occurs through secreted molecules (6,7), but there is also evidence that direct intercellular contact between these different cell types may alter growth characteristics or epithelial cell function (4,8).

These results have largely been obtained from studies of cells isolated from normal, adult lungs or from developing lungs. Changes in this reciprocal epithelial cell–fibroblast interaction may occur in injured lungs and may alter the reparative processes. The pulmonary epithelium is a common target for a variety of environmental toxins as well as circulating drugs, and severe injury to this cell is frequently associated with subsequent fibroblast proliferation (9).

In considering the mechanisms underlying pulmonary fibrosis after epithelial injury, most studies have focused on cytokine production and fibroblast stimulation by macrophages in particular (10). In the pulmonary response to bleomycin, for example, recovered alveolar macrophages have been shown to secrete a fibroblast growth factor and are also positive for transforming growth factor β (TGF-β), which is associated with increased collagen production (11,12). However, bleomycin induces epithelial injury (13,14), which may disrupt the normal epithelial–fibroblast control system. It is not known whether this change in the local microenvironment alters proliferation of either cell type and induces abnormal repair, which is characterized by epithelial proliferation, abnormal differentiation, and extensive underlying fibrosis.

In the present study, type 2 epithelial cells and fibroblasts were isolated from rat lungs 10 days after administration of bleomycin, when epithelial injury is maximal, and after 6 weeks when abnormal repair with fibrosis occurs. This allowed us to determine if there are changes in either cell type at these two stages of injury and repair, which could alter the proliferation of the other cell type, either through a secreted molecule or by a cell-contact-related process.

Materials and Methods
Male Sprague-Dawley rats (about 150 g) were used. In a trial series, we injected animals intratracheally while they were under mild barbiturate anesthesia with various doses from 1 to 15 U of bleomycin (kindly provided by Bristol-Myers Squibb Co., Evansville, IN). Rats were killed by an intraperitoneal barbiturate overdose at intervals to 12 weeks, and the lungs were processed for histologic examination. Based on this trial, we chose a dose of 8 U/kg body weight in 0.3 ml sterile water for further experiments. Two groups of 12 rats were injected, then 1 set was killed at 10 days, the other at 6 weeks. We lavaged the lungs four times with 5 ml saline and counted inflammatory cells by using a hemocytometer. After centrifugation to remove cells, we assayed the lavage fluid for protein content. A sample of lung tissue was taken to measure hydroxyproline (HYP), and the remainder was processed for microscopy (15).

Control groups received the same volume of water and were killed at the same times. This experiment established that the 8 U/kg bleomycin induced acute lung injury at 10 days, followed by pulmonary fibrosis at 6 weeks.

Cell Isolation
We isolated type 2 epithelial cells and fibroblasts from normal rats and killed rats at 10 days and 6 weeks after intratracheal administration of bleomycin or water. We isolated type 2 cells using the method of Dobbs et al. (16). Briefly, the lungs were excised, and after perfusion, 4.3 U/ml elastase was instilled into the trachea. Subsequently, the lungs were minced and filtered through successive nylon meshes down to 7 µm to obtain a cell suspension that was incubated in serum-free media in petri dishes coated with IgG. We collected nonadherent cells and spun them down; viability by trypan blue was >90%. We resuspended the cells in Dulbecco’s modified Eagle’s medium with 10% fetal bovine serum for counting by hemocytometer and seeding into culture dishes. Cells were isolated from two rats, then pooled for each experimental run. Previous experiments using special stains and electron microscopy on cell pellets showed that cells prepared in this way were >94% type 2 cells. We made a cytospin preparation of each starting preparation to ensure purity.

Address correspondence to I. Y. R. Adamson, Department of Pathology, University of Manitoba, 236-770 Bannatyne Avenue, Winnipeg, Canada R3E OW3.

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cells were only used when purity was >90% with <2% macrophages present. There was no morphologic difference between isolated cells from control or bleomycin-treated lungs.

We prepared fibroblasts from rats in the same group using a trypsin digestion method as previously described and cultured the fibroblasts to confluence (17). Cells were passaged once before using them in the experiments described below. They stained >98% positive for vimentin and negatively for factor VIII.

**Culture System**

Fibroblasts were seeded at a density of 2 × 10⁵ per well on a 24-well plastic culture dish and allowed to adhere for 1 hr. We seeded epithelial cells at 4 × 10⁴ per well into a well insert, which had a filter as a base (Millicell-CM, Millipore Corp., Bedford, MA). These filters were coated with 50 μg rat-tail collagen just before using them to improve cell attachment. Other epithelial cells were seeded directly onto a fibroblast layer and co-cultured. Using various combinations, we studied 1) epithelial cells alone; 2) fibroblasts alone; 3) epithelial cells exposed to secretions of underlying fibroblasts; 4) fibroblasts exposed to secretions of overlying epithelial cells; and 5) co-cultures with epithelial cells and fibroblasts in direct contact.

For both bleomycin groups, we cultured isolated epithelial cells and fibroblasts under the five conditions outlined above. We carried out additional experiments in which cells isolated from normal rats were cultured with cells from bleomycin-exposed lungs; for example, normal type 2 cells were cultured over fibroblasts from bleomycin-exposed lungs and vice versa.

**Cell Growth**

Cells were cultured as described for 1 day then changed to serum-free conditions for a further day. In some experiments, we quantitated the growth of each cell type by counting cell numbers after trypsinization of the wells and filters separately, then by using a hemocytometer. In most experiments, we measured DNA synthesis by adding tritiated thymidine (³H) at 0.1 μCi/ml for the final 4 hr of culture, when the cells were rinsed well and scraped off for scintillation counting. The thymidine uptake (dpm) was taken as the mean of four wells for each culture series. In addition, from one additional well, we removed cells and made duplicate cytosin preparations from each suspension. Slides were subsequently dipped in Kodak NTB2 emulsion in the dark room under a red filter. After 2 weeks in total darkness, we developed the slides and stained them with toluidine blue. For each time period, we calculated the percentage of cells labeled after counting 500 cells per slide.

To quantitate DNA synthesis by the individual cell types in the co-cultures, we stained cytosin preparations for cytokeratin and vimentin using standard immuno-peroxidase methods (18), then prepared slides for autoradiography. This allowed us to count separately the thymidine-labeled nuclei of epithelial cells and fibroblasts from the same culture dish. Similarly, slides of "pure" epithelial cells were stained for vimentin so that any labeled, contaminating fibroblasts could be eliminated from the cell counts.

**Growth Factor Studies**

A few additional experiments using cells from different rats were set up to test the effects of selected, known growth factors and their antibodies on epithelial cell proliferation. Agents used at various dose levels were tumor necrosis factor (TNF-α), platelet-derived growth factor (PDGF), TGF-α, TGF-β, epidermal growth factor (EGF), and their respective antibodies (R and D Systems, Minneapolis, MN). Effects were quantitated by counting thymidine-labeled cells in autoradiographs using the same experimental outline described earlier.

**Statistics**

We used isolated cells from at least four different preparations for each of the conditions studied. Means ± SEs were calculated for each experimental group and compared to the corresponding control for significance using the Student’s t test.

**Results**

**In vivo Studies**

The intratracheal injection of water causes a mild inflammation response, which in our experience ceases by 3–5 days. In this experiment, no change in lung cells, lavage protein, or lung HYP over noninjected controls was found at the times studied. The number of alveolar macrophages (AM) recovered by bronchoalveolar lavage (BAL) from bleomycin-injected lungs was more than double control values at 10 days and remained above normal at 6 weeks (Fig. 1). Few polymorphonuclear leukocytes (PMN) were recovered from normal lungs, but significantly higher numbers were present in BAL from bleomycin-injected lungs. This inflammatory response was confirmed by measuring the protein content of BAL fluid (Fig. 2). The protein level was greatly increased 10 days after bleomycin, and, although it fell subsequently, it was still above control levels after 6 weeks. We indexed changes in lung collagen by the HYP level, which was unchanged at 10 days after treatment but was significantly increased after 6 weeks (Fig. 2).

Lung morphology confirmed the general biochemical findings. At 10 days, diffuse injury to the alveolar walls was seen, the tissue was edematous, and the alveoli contained AM and PMN (Fig. 3A). By 6 weeks, fewer cells were seen in alveolar spaces; the main change was in the pulmonary interstitium, which was thicker than normal due to excess fibroblasts and collagen (Fig. 3B). Evidence of epithelial repair was seen, as some alveoli were lined by cuboidal cells rather than the usual mixture of type 1 and 2 cells.

![Figure 1. Number of cells (mean ± SE) lavaged from noninjected control and bleomycin (BLM)-treated rats after 10 days and 6 weeks. (Open bars) Alveolar macrophages; (shaded bars) polymorphonuclear leukocytes. *p < 0.01 compared to water-injected control at the corresponding time.](image1)

![Figure 2. Protein content of bronchoalveolar lavage fluid and lung content of hydroxyproline (HYP) in noninjected controls and in lungs at 10 days and 6 weeks after bleomycin treatment (BLM). *p < 0.01 compared to water-injected control at the corresponding time.](image2)
In vitro Studies: 10-Day Bleomycin Group

We studied growth characteristics of epithelial cells and fibroblasts using \(^{3}\)HT uptake. Type 2 cells isolated from normal lung (N-EP) or from lungs 10 days after bleomycin injection (B-EP) incorporated low levels of \(^{3}\)HT (Fig. 4). When either set of EP cells was cultured over fibroblasts from normal (N-FB) or bleomycin (B-FB)-treated rats in the lower culture chamber, thymidine incorporation of EP cells significantly increased (Fig. 4). Proliferation was confirmed by counting the number of EP cells, which increased about 70% when EP were cultured over FB.

When fibroblasts were grown alone, N-FB and B-FB incorporated \(^{3}\)HT equally (Fig. 5). However, when fibroblasts were cultured in wells beneath EP cells, DNA synthesis was significantly lower than in fibroblasts grown alone. In particular, a secretion of B-EP depressed \(^{3}\)HT uptake in B-FB to a greater extent (Fig. 5).

In vitro Studies: 6-Week Bleomycin Group

Epithelial cells isolated from this older group of rats, normal or treated, showed a slightly lower level of \(^{3}\)HT uptake. However, in each experimental group, N-EP and B-EP cells showed increased DNA synthesis when cultured on inserts over N-FB or B-FB (Fig. 6). However, when using cells derived from bleomycin-treated rats, a lower increase in \(^{3}\)HT uptake by EP cells was observed compared to that seen when only normal cells were used (N-EP, N-FB).

The \(^{3}\)HT uptake of normal fibroblasts was significantly reduced when they were exposed to factors secreted by N-EP or B-EP (Fig. 7). Growth of B-FB was suppressed...
by N-EP cells, but no effect was seen when B-FB were cultured beneath B-EP (Fig. 7).

Most of the experiments were carried out using \(^{3}H\)T uptake (dpm) as the end point. Other experiments were also carried out using this group of rats to determine cell counts to confirm cell division as opposed to DNA synthesis only. We also used autoradiography to establish the identity of cells in DNA synthesis. The results confirm that epithelial cells cultured over, but separated from, fibroblasts show significantly increased labeling and cell numbers compared to cells cultured alone (Table 1). This occurred using normal cells, and to a lesser extent with cells from bleomycin-treated lungs. The autoradiographic study also allowed quantitation of labeled cells in co-cultures. When EP and FB were co-cultured, the stimulatory effect on the EP population was lost. Total cell numbers in the co-cultures were high due to fibroblast growth (data not shown), but only about 2.5–3% of EP cells were labeled in autoradiographs, a value equal to that found when EP cells were cultured alone (Table 1). This contrasts to an EP labeling value of 7–12% when these cells were exposed to a fibroblast secretory product.

**In vitro Growth Factor Studies**

In a preliminary set of studies to identify the fibroblast secretory product responsible for epithelial growth stimulation, antibodies to the well-known growth factors PDGF, EGF, TNF-α, TGF-α, and TGF-β were added at various concentrations to the culture system. The increase in \(^{3}H\)T incorporation by EP cells grown over fibroblasts was not blocked by antibodies to any of these growth factors (data not shown). The antibodies were biologically active because lower growth was seen in the underlying fibroblasts. One surprising result occurred using anti-TGF-β: it seemed to stimulate EP growth. This result was investigated more closely, and results are shown in Table 2.

Addition of pure TGF-β to the epithelial cells cultured over fibroblasts lowered the percentage of labeled EP cells. When the anti-TGF-β was used, \(^{3}H\)T incorporation by EP cells alone, and in particular those maintained over fibroblasts, was significantly increased (Table 2).

**Discussion**

Pulmonary fibrosis is a common sequel to severe or chronic injury to the lung. Most research into the underlying mechanisms of fibroblast stimulation has concentrated on cytokine release by macrophages because these cells secrete a variety of hormone-like molecules that enhance fibroblast growth and collagen production (10). This is also true of alveolar macrophages recovered from fibrotic lungs injured by the antineoplastic drug bleomycin (11). However, it has been previously shown that intravenously or intratracheally injected bleomycin initially damages endothelial and epithelial cells of the lung, and repair is abnormal leading to fibrosis (13). Despite severe epithelial damage, the effects of bleomycin on the local epithelial–fibroblast control system have not been investigated.

The model of intratracheal injection permits a lower dose to be instilled directly to the lung and is a useful model of generating rapid fibrosis (14). We chose a dose level of bleomycin to allow study of these two cell types isolated from lungs at different stages of the injury-repair cycle. At 10 days after injection, there is acute lung injury indexed by the inflammatory response, a high level of alveolar protein, and morphologic changes, all of which indicate diffuse alveolar injury, especially to type 1 cells. From these lungs, isolated type 2 cells, although probably injured to some extent, were certainly viable and could be maintained in culture. At 10 days in vivo, there was no change in collagen levels in the lungs. In contrast, 6 weeks after bleomycin treatment, fibrosis was evident biochemically and by lung morphology. The acute inflammatory reaction had subsided and the blood-air barrier was much less permeable. Previous studies have shown that the peak of epithelial repair is over by this time (19), when epithelial cells and fibroblasts were also isolated for *in vitro* study.

Fibroblasts from each group, cultured alone, proliferated rapidly and approximately equally as measured by cell counts and thymidine incorporation. When normal fibroblasts were cultured in a lower chamber beneath epithelial cells, their growth was slower and a significant decrease in fibroblast DNA synthesis was observed. This was also true using cells from the 10-day bleomycin group; fibroblast growth was particularly suppressed when fibroblasts were exposed to secreted products of epithelial cells from the same rats (Fig. 5). When cells were prepared from the fibrotic lungs, epithelial cells were still able to decrease \(^{3}H\)T uptake by normal fibroblasts, but these cells had no effect on fibroblasts from bleomycin-treated rats. This indicates that a product secreted by epithelial cells, which has some fibroblast inhibitory effects on normal cells, is not secreted in fibrotic lung in which excess fibroblast growth occurs. This suggests that in normal cells, localized fibroblast control is mediated through the overlying epithelium. However, the epithelial surface 6 weeks after bleomycin treatment is not normal *in vivo*, and various cuboidal epithelial cell forms have been described (19). These
Table 1. Growth of epithelial cells (EP) from normal (N) lungs or from lungs 6 weeks after bleomycin treatment (B), cultured alone, or exposed to fibroblast (FB)-
derived factors

|                | N-EP alone | N-EP over N-FB | N-EP with B-FB | B-EP alone | B-EP over N-FB | B-EP with B-FB |
|----------------|------------|----------------|----------------|------------|----------------|----------------|
| % EP cell label| 2.6 ± 0.2  | 11.5 ± 1.1*    | 2.8 ± 0.3      | 8.8 ± 0.8* | 2.3 ± 0.2      | 2.9 ± 0.3      |
| EP cell number x 10^5 | 2.0 ± 0.3  | 3.2 ± 0.3*     | —              | 2.6 ± 0.4  | 1.8 ± 0.2      | 2.9 ± 0.3*     |

* p < 0.05 compared to corresponding EP alone.

may be functionally deficient in producing the fibroblast inhibitor as shown here and so may contribute to the development of fibrosis.

The identity of the inhibitory molecule is not known. Although macrophages are a well-recognized source of cytokines, the low number present in our epithelial cell preparation makes it more likely that the fibroblast inhibitory molecule is produced by type 2 cells. One candidate is TGF-β, which has been shown in lung epithelial cells in bleomycin-treated lungs, though it was associated more with areas of increased collagen deposition (12). Another possible inhibitor is prostaglandin E2 (PGE2), which is secreted by type 2 cells in culture (19) and is secreted in larger amounts by these cells during injury such as exposure to silica (21). Because PGE2 is associated with reduced fibroblast growth in the lung, it may function at a local level in epithelial–fibroblast growth control. It has been shown previously that alveolar epithelial cells secrete a low-molecular-weight factor that inhibits proliferation of interstitial cells (22). Evidence of epithelial control of fibroblast growth has also been found in organ culture studies in which fibroblast growth is promoted in the absence of epithelium and is controlled as the epithelial surface is restored (23,24). The present results suggest that epithelial cells are capable of fibroblast growth control through diffusion of a secreted factor, but in a fibrotic lung, this inhibitory effect is lost.

In a reciprocal interaction, epithelial cell proliferation appears to be promoted by exposure to a diffusible product of lung fibroblasts. Type 2 cells isolated from control or treated rats incorporated a low level of [3H]Tdr and showed little division; as determined by autoradiography, only about 2–3% of cells were labeled. However, when these cells were cultured on a filter insert with fibroblasts in the lower chamber, all parameters of cell growth were significantly increased, whether using cells from normal or bleomycin-injected rats. This indicates that a fibroblast-derived factor is capable of stimulating epithelial growth and so may accelerate repair. It is interesting to note that this stimulation occurred through a secreted, diffusing factor. When co-cultures were studied, fibroblast growth predominated, but when autoradiographs were examined, epithelial cell labeling was low and identical to levels seen in pure epithelial cultures. This suggests that direct epithelial cell–fibroblast contact induces an inhibitor to epithelial growth. Similar results were obtained earlier using fetal cells, whereby direct cell–cell contact between these two cell types was associated with reduced epithelial cell growth but enhanced type 2 cell differentiation (4).

Pure type 2 cells proliferate poorly under normal culture conditions and require addition of specific growth factors to stimulate cell division (7,25). The identity of the fibroblast-derived factor that promotes epithelial growth in the present study is not known; it could be one or a combination of several fibroblast-derived growth factors that promote lung cell proliferation (7). Fibroblast-conditioned media has been shown to produce a competence factor for fetal lung epithelial cells (5), and it has also been shown that lung fibroblasts produce a growth factor for bronchial epithelial cells (26). In preliminary experiments to identify the factor, we were unable to block the increase in epithelial growth using antibodies to PDGF, TNF-α, TGF-β or EGF. When TGF-β was added to type 2 cells in culture over fibroblasts, the usual growth increase was not seen, suggesting that TGF-β may act as an antagonist to the epithelial growth factor. This was supported when the addition of anti-TGF-β to the culture system resulted in a significant increase in type 2 cell labeling. Lung fibroblasts are known to secrete TGF-β (27) and its regulation may play a key role in both epithelial and fibroblast cell behavior during injury and repair. This aspect of the epithelial–fibroblast interaction is now under more detailed study.

The existence of a fibroblast-derived growth factor for epithelial cells could be important in providing a stimulus to lung repair after type I cell necrosis, when rapid regeneration of the epithelial surface by type 2 cell proliferation is a key event in the restoration of normal lung structure (23,28). Because type 1 cell injury is a common event when the lung is exposed to various environmental toxins, the changes in cellular control seen here after bleomycin may represent a general response to air- or bloodborne agents that are capable of inducing fibrosis. Control mechanisms involving epithelial cell–fibroblast interactions may be important in regulating normal repair in a highly localized manner. This system may be effective in repairing acute focal injury. It may be speculated that, in cases of more severe epithelial injury, this localized control process is supplemented by more generalized mechanisms of repair in which chronic inflammation, macrophage activation, and general cytokine networks are involved.

Table 2. Percentage (mean ± SE) of [3H]-thymidine labeled epithelial cells (EP) from normal (N) and bleomycin (B)-injected rats after culture alone or over fibroblasts (FB)

|                | N-EP alone | N-EP over N-FB | N-EP with B-FB | B-EP alone | B-EP over N-FB | B-EP with B-FB |
|----------------|------------|----------------|----------------|------------|----------------|----------------|
| Cells alone    | 2.5 ± 0.2  | 4.5 ± 0.5      | 3.2 ± 0.7      | 4.4 ± 0.7  | 7.2 ± 1.0      |                |
| +1 ng TGF-β    | 2.7 ± 0.2  | 4.0 ± 0.4      | 3.8 ± 0.2      | 3.6 ± 0.4  | 5.4 ± 0.6      |                |
| +5 ng TGF-β    | 2.0 ± 0.1  | 3.8 ± 0.4      | 1.6 ± 0.2**    | 2.9 ± 0.4**| 3.7 ± 0.5**    |                |
| +5 μg anti-TGF-β| 4.8 ± 0.8* | 6.5 ± 0.8      | 3.2 ± 0.6      | 6.7 ± 0.8  | 10.6 ± 1.3     |                |
| +20 μg anti-TGF-β| 4.1 ± 0.4* | 7.8 ± 1.0*     | 4.2 ± 0.7      | 8.8 ± 1.1* | 13.6 ± 1.4*    |                |

In all cases, EP cells grown over FB show a greater labeling percentage (p < 0.05) than EP grown alone. TGF, transforming growth factor.

* p < 0.05, treated cells compared to corresponding untreated cells.

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