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CO-CULTURE OF PRIMARY PULMONARY CELLS TO MODEL ALVEOLAR INJURY AND TRANSLOCATION OF PROTEINS

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SUMMARY

Primary rat alveolar type II cells and early passage rat lung fibroblasts were co-cultured on opposite sides of a collagen-coated polycarbonate filter. This is an approach to "model", in part, an alveolar wall to study mechanisms of cytotoxicity and translocation of bioactive materials from the alveolar space to the lung interstitium. Type II cells were recovered from adult rat (Fischer 344) lungs by enzyme digestion and "panning". Lung fibroblasts were separated from the same species, cultured initially in 10% fetal bovine serum and used in the co-culture system at early passage. The type II cells formed a monolayer of dedifferentiated epithelium which provided a barrier on the upper side of the collagen (human type IV)-coated filter. The fibroblasts on the bottom of the filter replicated logarithmically in the presence of serum, could be rendered quiescent in defined medium and then returned to rapid growth phase with the reintroduction of serum. The intact epithelial monolayer excluded trypan blue, albumin, platelet-derived growth factor, and alpha$_2$-macroglobulin from the lower compartment of the culture chamber. Altering the integrity of the monolayer by a variety of means allowed translocation of these materials through the collagen-coated filters. Particularly interesting was the effect of taurine chloramine which caused subtle changes in the alveolar epithelium and allowed subsequent translocation of albumin. In addition, we showed that rat alveolar macrophages remain viable with some spreading on the surface of the epithelial monolayer. This co-culture system will have future application in the study of how reactive oxygen species might affect the epithelial barrier, and whether macrophage-derived growth factors can influence fibroblast proliferation if the monolayer is intact or injured.

Key words: alveolar type II cells; lung fibroblasts; co-culture; cell culture; growth factors.

INTRODUCTION

The multiple cell types which compose the lung contribute to the complexity of the mechanisms of injury which may result from exposure to a variety of agents. Interstitial pulmonary fibrosis is a prominent pathophysiologic reaction of the lung to injury and is believed to be mediated by undefined interactions among alveolar epithelial cells, fibroblasts, and the products of inflammatory cells (16). During the course of alveolar fibrogenesis interstitial fibroblasts become activated to proliferate and synthesize extracellular matrix components, including collagen, which result in a "stiff" lung (19). Regulation of this fibroblast population is presently the subject of numerous studies (25).

Alveolar macrophages are secretory cells which have the ability to release a variety of mediators of cell growth collectively termed "cytokines" (24). Several of these are known to affect mesenchymal cells by enhancing or inhibiting proliferation or by stimulating the production of matrix components (26). Although macrophages are known to secrete many of these factors that modulate fibroblast biology in vitro, essentially no information is available regarding the role of the alveolar epithelial lining in modulating the translocation of potential mediators of inflammation and fibrosis from the alveolar space to the interstitium. A role for the epithelial layer in the pathophysiology of pulmonary fibrosis has been strengthened by evidence which suggests that the presence of an epithelium is necessary to prevent a fibrogenic response (6,21,29).

The objective of the present experiments is to develop a co-culture system that would allow in vitro modeling of the complex cellular interactions that take place at the alveolar level in vivo. This paper details the model system we have developed. We chose to investigate the proliferation of rat lung fibroblasts co-cultured with alveolar epithelial cells inasmuch as interstitial fibrosis is one of the more common pulmonary responses to injury (16). This model also is consistent with our long-standing interest in fibrogenic lung disease (6,7,10,12). The findings reported here suggest that this is a reasonable model system to study lung injury because an intact monolayer of alveolar type II cells blocked the translocation of trypan blue, albumin, platelet-derived growth factor, and α_2-macroglobulin. When the epithelial monolayer was perturbed with increasing concentrations of taurine chloramine, a biologically relevant long-lived oxidant, albumin flux increased accordingly. Our goal is to develop a co-culture system in which the role of alveolar

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epithelial cells and lung macrophages can be established in the context of fibroblast growth control and matrix production.

Materials and Methods

Preparation of cell culture chambers. Culture chambers were prepared by affixing a 0.2-μm Nuclepore filter (Nuclepore Corporation, Pleasanton, CA) between 6- and 4-mm lengths of $\frac{1}{2}$-inch polycarbonate tubing with GE 361 Silicone Seal (General Electric Company, Waterford, NY). The filters were coated with a solution of human placental (type IV) collagen (Sigma, St. Louis, MO) (3 mg/ml) using a modification of the method of Cereijido et al. (10). The collagen was attached to the filter by precipitation with ammonium hydroxide and crosslinked with 2% glutaraldehyde. The culture chambers were rinsed twice in sterile distilled water and 3 times in Dulbecco's modified Eagle's medium (DMEM) (GIBCO, Grand Island, NY) before a final rinse in DMEM overnight at 37° C.

Isolation of alveolar type II pneumocytes. Type II alveolar epithelial cells (ATII) were isolated from male, specific pathogen-free Fischer 344 rats (Charles River, Raleigh, NC) by the method of Dobbs and colleagues (14). Rats were anesthetized and anticoagulated by an intraperitoneal injection of pentobarbital and heparin. Lungs were perfused free of blood via the portal vein with 20 ml of solution II [140 m*M* NaCl, 5 m*M* KCl, 2.5 m*M* sodium phosphate buffer, 10 m*M* HEPES (*N*-2-hydroxyethylpiperazine-*N*'-2-ethanesulfonic acid), 2.0 m*M* $CaCl_2$, 1.3 m*M* $MgSO_4$, pH 7.4, 37° C]. The lungs were excised and repeatedly lavaged with a total of 80 ml of solution I [140 m*M* NaCl, 5 m*M* sodium phosphate buffer, 10 m*M* HEPES, 6 m*M* glucose, 0.2 m*M* EGTA (ethylene glycol-bis-(β-amino ethyl ether), *N*,*N*'-tetra-acetic acid), pH 7.4] to remove macrophages. The lungs were then lavaged twice with solution II followed by one final lavage with porcine pancreatic elastase (Worthington Biochemical, Freehold, NJ) solution (4 U/ml in solution II). The lungs were expanded with elastase solution at 5 cmH_2O pressure and incubated for 20 min at 37° C. Each lung was minced into pieces of 1 mm^3, sequentially filtered, and washed in solution II. Cells were suspended in DMEM (2 to 3 × 10^6 cells/ml) and incubated on rat IgG (Sigma)-coated, 100-mm bacteriological plastic Petri dishes for 1 h at 37° C in 10% CO_2 and air. The dishes were "panned" as described (14), and the nonadherent alveolar type II enriched cells were collected and centrifuged at 130 ×*g* for 8 min. The cell pellet was gently resuspended at 4 × 10^6 cells/ml in DMEM containing 10% fetal bovine serum (FBS) (GIBCO). The viability of the isolated cells was 88 ± 6% (mean ± SD, n = 9) as determined by trypan blue exclusion. The purity of the freshly isolated alveolar type II cell preparations was 84 ± 5% (mean ± SD, n = 9) as judged by a modified Papanicolau stain (20).

Isolation of rat lung fibroblasts. Adult rat lung fibroblasts (RLF) were isolated by a modification of the method of Shannon et al. (27). Briefly, rat lungs were perfused with phosphate buffered saline (PBS) as previously described above, and the cells dissociated by incubation with collagenase (Worthington) (300 U/ml) and trypsin (GIBCO) (2.5 mg/ml). The dissociated cells were grown in DMEM containing 10% FBS and used at Passage 2.

Isolation of rat alveolar macrophages. Alveolar macrophages (AM) were isolated from the rats by a modification of the method previously described (4,22). In brief, rats were anesthetized and anticoagulated with an intraperitoneal injection of pentobarbital and heparin. The lungs were perfused via the portal vein with calcium- and magnesium-free PBS, pH 7.2 at 37° C, until they blanched. The lungs were removed and lavaged with five equal volumes (10 ml) of warm (37° C) sterile PBS. The bronchoalveolar cells were obtained by centrifugation at 130 ×*g* for 8 min and resuspended in DMEM. Cell counts were determined on a hemacytometer and viability (98 ± 1%) was assessed by the trypan blue exclusion method.

Morphologic studies of co-cultured lung cells. The upper compartments of the co-culture chambers were seeded with 8 × 10^5 ATII cells and cultured in DMEM with 10% FBS for 5 d, at which time intact epithelial monolayers had formed. On Day 5 of culture, the lower compartments of some of the chambers were seeded with RLF (4 × 10^3) which grew to confluence. Other chambers were used to add alveolar macrophages (5 × 10^5) directly to the apical surface of the confluent epithelial cells.

Lung cells cultured on the collagen-coated filters were fixed with 0.35% piperazine-*N*,*N*'- bis[2 -ethane sulfonic acid]-buffered glutaraldehyde-paraformaldehyde solution. After rinsing in buffer, samples were dehydrated in graded series of ethanol to absolute. The samples prepared for scanning electron microscopy were critical point dried in a Samdri-790 (Tousimis, Rockville, Maryland), mounted on stubs, and sputter-coated with gold in a Polaron E 5100 coating unit (Polaron Equipment Limited, Watford, Hertfordshire, UK). Filters with cells prepared for transmission electron microscopy were postfixed with 1% osmium tetroxide for 1 h and embedded in plastic. Ultrathin sections were cut and stained with uranyl acetate and lead citrate for examination in a JEOL 100CX electron microscope.

Growth curves. The upper compartment of the co-culture chamber was seeded with ATII cells (8 × 10^5). After 5 d of culture in DMEM with 10% FBS, a confluent epithelial monolayer was present and the lower compartment was seeded with early passage RLF (4 × 10^3). Cultures were maintained for a period of 13 d with fresh media changes occurring every other day. Growth was monitored daily by counting cells using a hemacytometer.

Fibroblast proliferation assay. Co-culture chambers were seeded with ATII cells and RLF as described above for the growth curves. Twenty-four hours after seeding the RLF, some of the cultures were changed to serum-free, 1% HL-1 (Ventrex, Portland, ME) supplemented DMEM. After 2 d in serum-free conditions, a number of the co-cultures were returned to DMEM with 10% FBS. The proliferative response of the RLF to FBS was monitored daily by counting cells using a hemacytometer.

Trypan blue in vitro barrier function assay. The barrier function of the confluent monolayers of pulmonary cells cultured on collagen-coated filters was assessed using a modification of the method of Boiadjieva et al. (3). The assay measured the rate of transfer of trypan blue (TB) across the rat lung cell monolayers. The upper compartment of the culture chambers were seeded with either ATII cells (4 × 10^5) or RLF (1 × 10^4). Intact confluent monolayers were formed within 4 or 5 d of culture, at which time the chambers were rinsed twice in Hanks' balanced salt solution (HBSS) (GIBCO) supplemented with 1 mg/ml bovine serum albumin (BSA) (Sigma). A solution of TB (200 μl of 0.2% TB in HBSS with 1 mg/ml BSA) was added to the upper compartment of the culture chamber, and the lower compartment was filled with HBSS with 1 mg/ml BSA. Samples were collected from the lower compartment of the culture

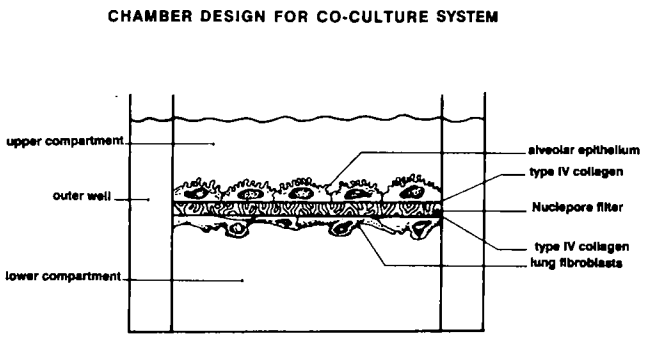

FIG. 1. Diagram depicting the chamber design for the co-culture of rat lung cells. A collagen-coated 0.2-μm Nuclepore filter separates the epithelial monolayer in the upper compartment from the underlying rat lung fibroblasts.

chambers at 1, 2, 5, 10, 20, and 30 min. The amount of TB transfer was determined spectrophotometrically at A_{580}.

Assay for transepithelial permeability of albumin. Chambers were seeded with 8×10^5 ATII cells, and confluent epithelial monolayers were formed after 5 d of culture in DMEM with 10% FBS. The culture chambers were rinsed twice in DMEM and the upper compartment was filled with 500 μl of DMEM containing 10 mg/ml BSA and doses of taurine chloramine (TC) which ranged from 200 μM to 1 mM. TC was prepared within 15 min of use by dissolving taurine (Sigma) in PBS (pH 7.4) and adding NaOCl (Fisher Scientific, Pittsburg, PA) (28). The lower compartment of the culture chamber was filled with 500 μl of protein-free DMEM. After 1 h, the albumin concentration in the upper and lower compartments were determined by the method of Doumas (15). Transepithelial albumin transfer was expressed as:

$$\text{Fraction flux} = \frac{\text{albumin concentration in lower compartment}}{\text{albumin concentration in upper compartment}}$$

Lactate dehydrogenase release assay. Cytotoxicity was assessed by measuring lactate dehydrogenase (LDH) activity released from

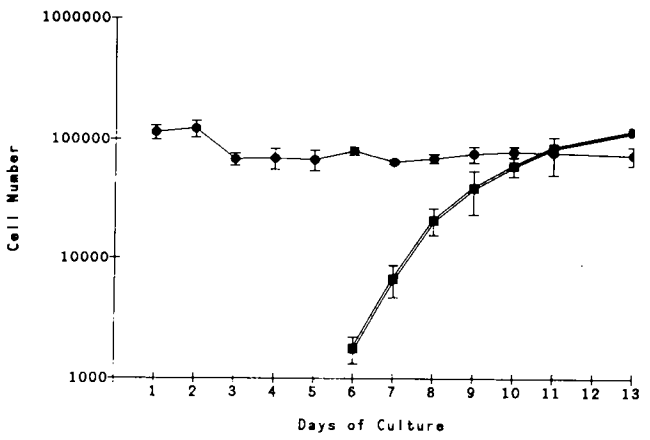

FIG. 2 Maintenance of the alveolar epithelial cell monolayers (*closed circles*) and growth of rat lung fibroblasts (*closed squares*) in DMEM medium containing 10% FBS. Data are from a single experiment typical of three performed in triplicate.

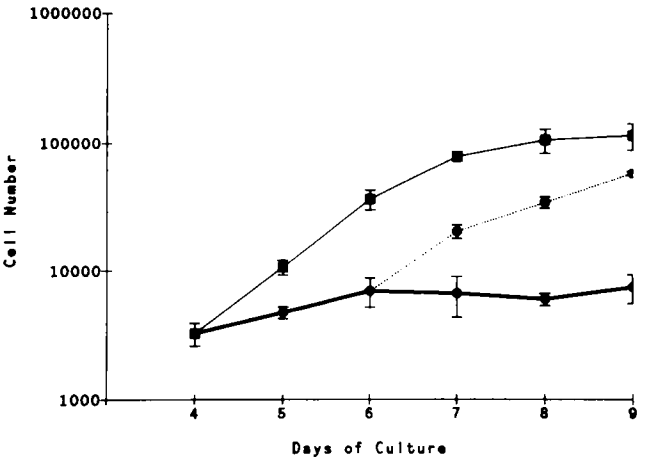

FIG. 3. Effect of serum on the growth of RLFs when co-cultured with confluent alveolar epithelial cells (*see* Fig. 2). RLFs exhibited log growth when maintained in DMEM with 10% FBS (*closed squares*), whereas RLFs switched to serum-free DMEM failed to show a significant increase in cell number (*closed circles*). When quiescent RLFs are returned to DMEM with 10% FBS, they rapidly demonstrate their capacity to again proliferate in the presence of serum (*dotted line*). Data are from a single experiment performed in triplicate.

the cultured alveolar epithelial cells treated with chlorinated amines (28). Briefly, monolayers were exposed to TC for 1 h in DMEM with 10 mg/ml BSA. This medium was then removed and replaced with DMEM alone for an additional 3 h. The 3-h incubation medium was removed and pooled with the earlier 1-h incubation media. The monolayers were then exposed to 0.1% Triton X-100 in H_2O for 30 min. The LDH activity released in the combined media and in

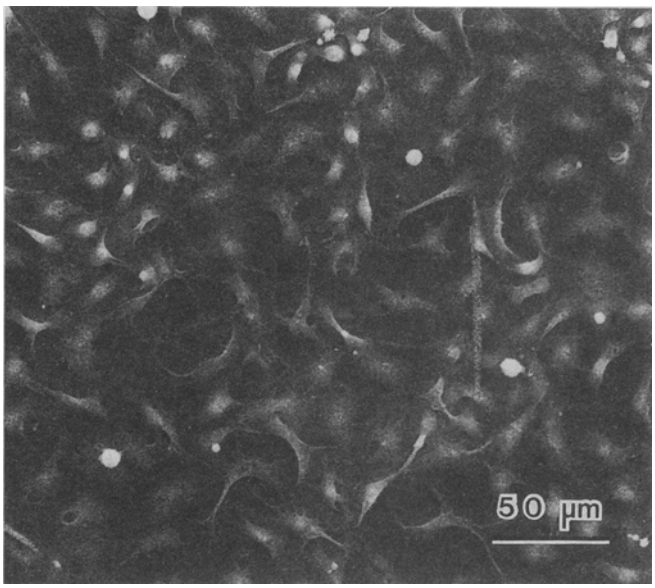

FIG. 4. Scanning electron micrograph of intact monolayer of rat alveolar epithelial cells grown on the upper surface of a collagen-coated Nuclepore filter.

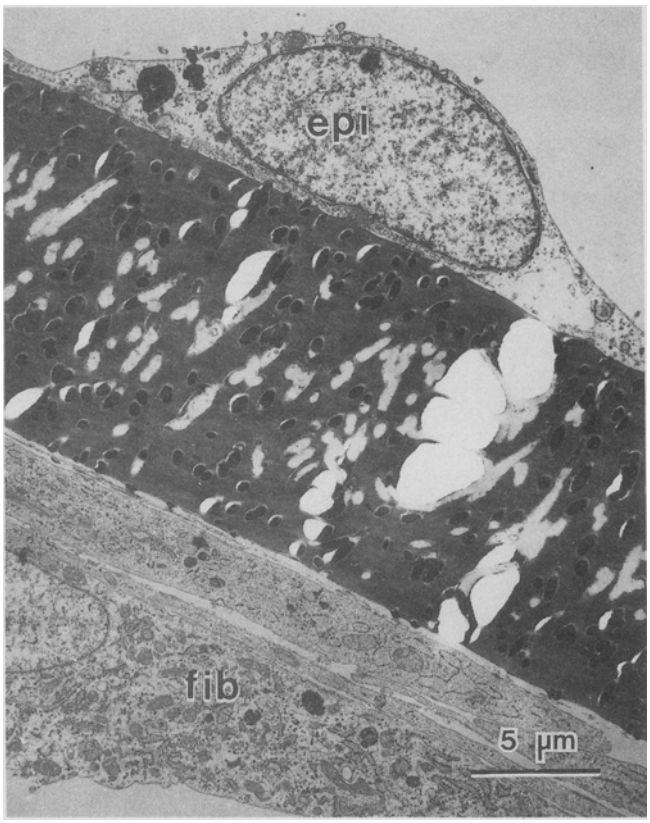

FIG. 5. Transmission electron micrograph of a cross section of the collagen-coated filter displaying an epithelial cell (*epi*) on the upper surface and overlapping fibroblasts (*fib*) below.

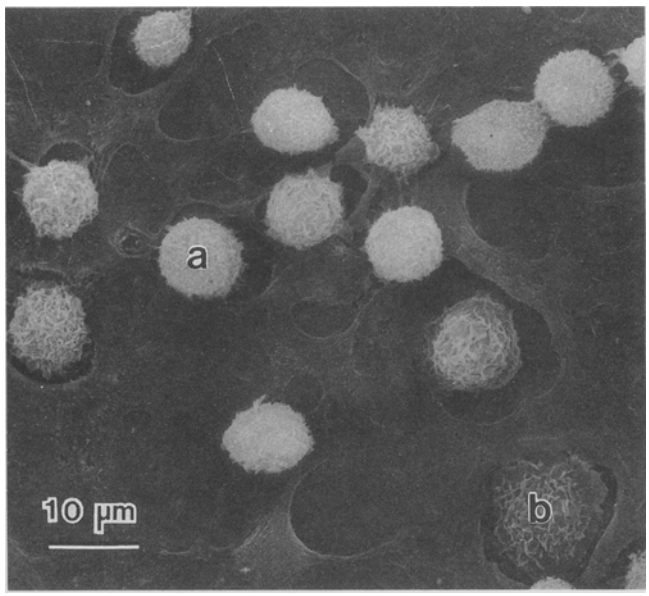

FIG. 6. Scanning electron micrograph of alveolar macrophages co-cultured on an intact epithelial monolayer. Macrophages display morphologic features ranging from rounded cells with relatively smooth surfaces (*a*) to flattened ruffled cells (*b*) that spread over the epithelial surface.

the Triton solution was determined separately using the UV-kinetic method described by Wacker et al. (31).

Preparation of alpha$_2$-macroglobulin. All human alpha$_2$-macroglobulin (α_2-M) (Calbiochem, San Diego, CA) preparations were treated with methylamine (Sigma) to obtain the "fast" form or receptor-recognized form of the molecule (4). Native α_2-M (1 ng/ml) was converted to the "fast" form by reaction with 25 m*M* methylamine (Tris-HCl, 50 m*M*, pH 8.0) overnight at 25° C. Excess methylamine was removed from α_2-M-methylamine complexes by dialysis against 100 vol 50 m*M* Tris-HCl (pH 8.2) overnight at 4° C.

Translocation of platelet-derived growth factor and alpha$_2$-macroglobulin across alveolar epithelial monolayers. To examine the translocation of platelet-derived growth factor (PDGF) and its binding protein alpha$_2$-M (4) across the alveolar epithelial barrier, we compared collagen-coated Nuclepore filters, confluent and subconfluent epithelial monolayers, and physically and chemically injured monolayers. Chambers were seeded with either 4×10^4 or 8×10^5 ATII cells and cultured in DMEM with 10% FBS for 5 d. Culture chambers originally seeded with 4×10^4 cells remained subconfluent, whereas the chambers seeded with 8×10^5 cells formed confluent monolayers. Some of the confluent epithelial monolayers were physically damaged by gently rubbing the filter surface several times with a plastic micropipette tip. Washed monolayers were chemically injured by a 5-min exposure to 20 m*M* ammonium hydroxide followed by three rinses with PBS.

To measure the translocation of PDGF and α_2-M, culture chambers were rinsed twice in PBS, then transferred into a serum-free, defined growth medium (SFDM) that consisted of Ham's F12 containing HEPES, 0.5% BSA and $CaCl_2$ supplemented with 10

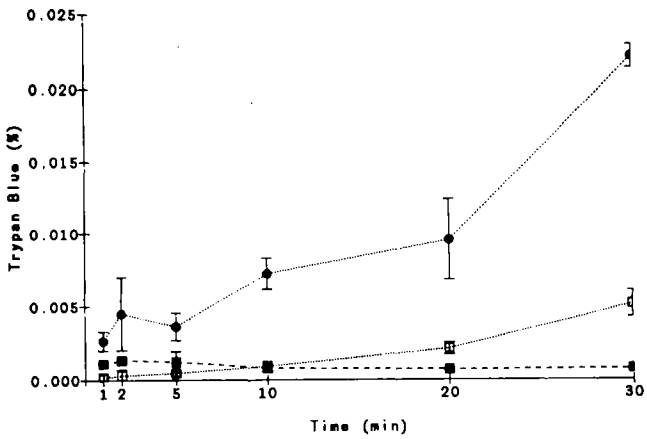

FIG. 7. The transfer of trypan blue across monolayers of rat lung cells cultured on collagen-coated filters was measured to determine the ability of pulmonary cells to form functional barriers. Dye was added to the upper compartment of the culture chamber and the percent concentration of trypan blue in the lower compartment was measured over a 30-min period. Intact epithelial monolayer (*closed squares*) completely blocked translocation, but the fibroblasts (*open squares*) only delayed movement of the dye. Collagen-coated filter (*closed circles*) provided little obstruction to trypan blue. Data are from a single experiment performed in triplicate.

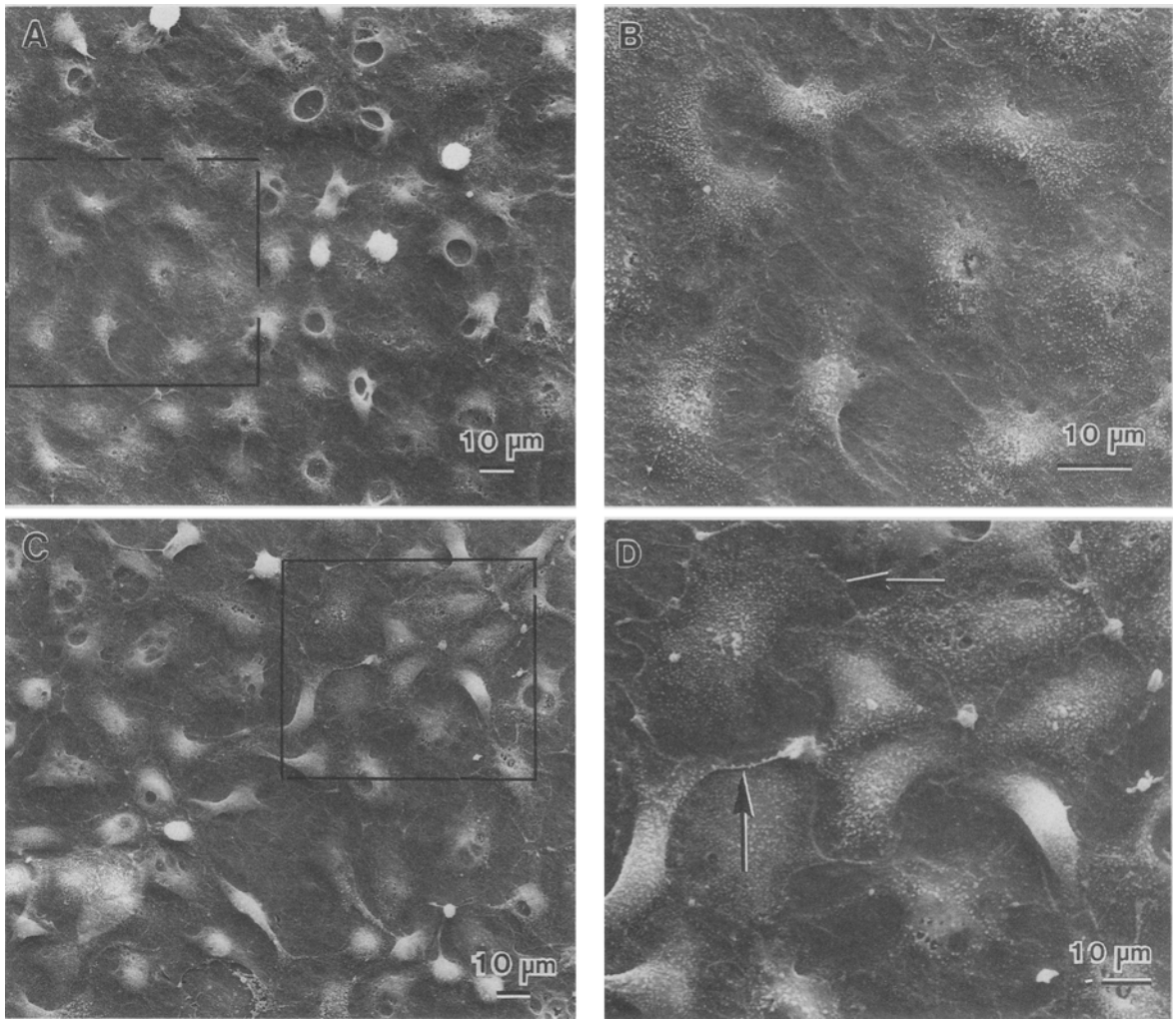

FIG. 8. Scanning electron micrographs showing control (*A,B*) and TC (*C,D*)-treated epithelial monolayers. When alveolar epithelial cultures were exposed to 1 m*M* TC for 1 h before fixation, the intercellular borders became more prominent (*arrows*). Areas of the micrographs delineated are enlarged as indicated (*A,B* = PBS control; *C,D*, = 1 m*M* TC).

ng/ml selenium (Sigma), 10 μg/ml bovine pancreatic insulin, and 10 μg/ml bovine serum transferrin (Calbiochem). Volumes were adjusted so that 1 ml of SFDM was present in the outer well, and the lower compartment of the culture chamber was filled with 500 μl of medium. The medium in the upper compartment of the culture chamber was replaced with 200 μl of SFDM containing either 125 ng/ml of human PDGF (R & D Systems, Minneapolis, MN) or 10 μg of human α_2-M. The medium from the upper and lower compartments of the co-culture chambers was collected 3 d later and assayed for PDGF or α_2-M by enzyme immunoassay.

Platelet-derived growth factor immunoassay. Human PDGF was measured by the enzyme immunoassay method of Kumar et al. (22). Highly purified human PDGF was used to determine a standard reference curve (0.25 to 32 ng/ml PDGF). The assay utilizes a monospecific goat antihuman PDGF antibody (Collaborative Research, Lexington, MA) that reacts with human PDGF.

α_2-M Immunoassay. Immulon 2 Removawell flat-bottomed wells (Dynatech, Chantilly, VA) were coated 100 μl/well with a 1:1000 dilution of rabbit anti-α_2-M in PBS (DakoPatts, Santa Barbara, CA) and incubated overnight at 4° C. The following day the wells were washed 5 times with PBS containing 0.05% Tween-20 (PBST), and 200 μl/well of 1% BSA in PBS were added followed by incubation for 5 h at 4° C. The wells were washed 5 times with PBST and 100 μl/well of standard α_2-M or unknown sample diluted in 1% BSA-PBS were added and incubated overnight at 4° C. Zero antigen controls and horseradish peroxidase (HRP) antibody blanks received only 1% BSA-PBS. The following day, the wells were washed 5 times with PBST and 100 μl/well of HRP conjugated, sheep anti-human α_2-M (Serotec, Kidlington, Oxford, UK) diluted in 1% BSA-PBS (1:5000) was added and incubated at room temperature for 5 h. The wells were washed 5 times with PBST, then developed for 15 to 30 min with the diammonium salt of ABTS (2,2′-azinobis-3-ethylbenzothiazoline-6-sulfonic acid) (Sigma) containing 0.003% hydrogen peroxide. The absorbance (405 nm) was measured on a Titertek Multiskan 96-well plate reader (Flow Laboratories, McLean, VA). Doubling dilutions of α_2-M (2 to 1000 ng/

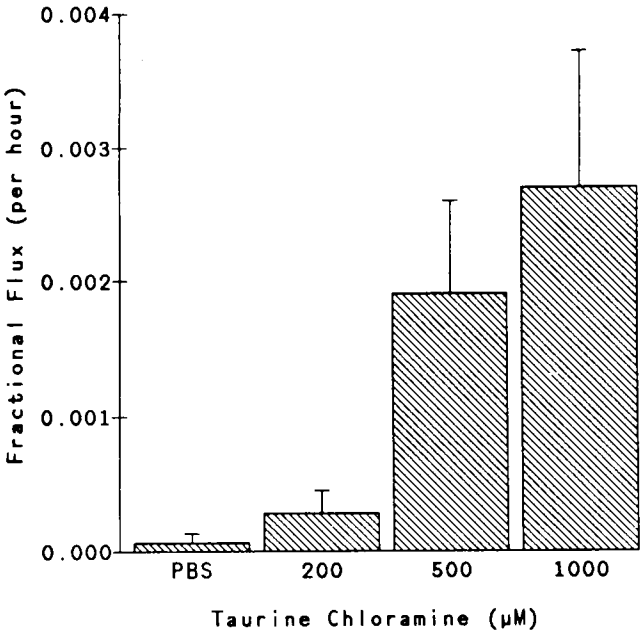

FIG. 9. Effect of TC on the permeability of albumin across alveolar epithelial monolayers cultured on collagen-coated Nuclepore filters. Monolayers were exposed to either PBS or increasing concentrations of TC, which caused a dose-dependent increase of albumin flux. Data are from a single experiment typical of three performed in quadruplicate.

ml) were used and the linear portion of the standard curve typically ranged between 5 and 500 ng/ml.

Statistical analysis. Differences between TC-exposed groups and corresponding PBS-treated control groups were assessed by Dunnett's multiple comparison test. All other data were analyzed using the one-way analysis of variance ANOVA test. Probability values of 0.05 or less were considered significant. All results are reported as the mean ± the standard error of the mean.

RESULTS

Growth curves. The co-culture chambers were seeded with 8×10^5 ATII cells and cultured in DMEM with 10% FBS for 5 d before 4000 RLF were seeded on the opposing side of the collagen-coated polycarbonate filters as depicted diagrammatically in Fig. 1. The ATII cells quickly covered the entire filter surface and by Day 3 of culture the cell number remained approximately 7×10^4 per chamber. The RLF demonstrated log growth characteristic of fibroblasts cultured in medium containing 10% FBS (Fig. 2). By Day 11 of culture, the RLF had reached confluence with a cell count of approximately 1×10^5 cells per chamber.

Fibroblast proliferation assay. The growth of rat lung fibroblasts co-cultured with confluent alveolar epithelial cells was modulated by the presence or absence of FBS in the culture medium. The RLF maintained in DMEM with 10% FBS exhibited log growth, whereas RLF switched to serum-free culture conditions ceased to show a significant increase in cell number (Fig. 3). When quiescent RLFs were returned to DMEM containing 10% FBS, the cells rapidly returned to log phase growth.

Morphology. Electron microscopic examination revealed that alveolar epithelial cells grown on Nuclepore filters coated with type IV collagen spread to cover the entire surface (Fig. 4) and formed a flattened monolayer of polygonal cells. The flattened cells consisted of a dedifferentiated type II epithelium with attenuated cytoplasm containing few lamellar bodies (Fig. 5). Rat lung fibroblasts grown on the lower surface of the collagen-coated filters proliferated to form a layer of overlapping cells (Fig. 5). When rat alveolar macrophages were co-cultured on confluent epithelial monolayers they exhibited variable morphology, ranging from relatively smooth rounded cells to flattened cells with highly ruffled surfaces (Fig. 6).

Translocation assays. Intact monolayers of alveolar epithelial cells formed a barrier to translocation of a variety of materials:

Trypan Blue. The ability of the cultured alveolar epithelial cells to form intact confluent monolayers was demonstrated by their effectiveness in preventing the transfer of trypan blue across the collagen-coated filters. The effects of confluent RLF and ATII cells on the transfer of TB are shown in Fig. 7. It appears that the RLF delayed but did not block the transfer of TB across the Nuclepore filter. In contrast, the epithelial monolayer served as a functional barrier by preventing the transfer of dye over the 30-min period tested.

Albumin. Control and TC-treated epithelial monolayers were examined by scanning electron microscopy (SEM) (Fig. 8). When TC (1 m*M*) was added to the culture medium for 1 h before fixation, the intercellular borders clearly were more prominent compared to PBS-treated cultures. The exposure of the alveolar epithelial monolayers to TC caused a dose-dependent increase in the transepithelial

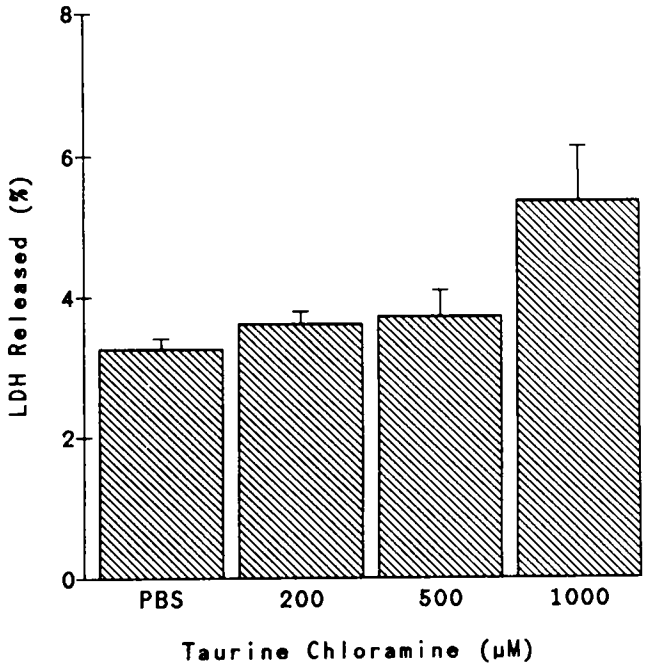

FIG. 10. Monolayers of alveolar epithelial cells were exposed to TC for 1 h in DMEM medium with 10 mg/ml BSA and incubated for an additional 3 h in DMEM without BSA. Percent of LDH activity released from the monolayers during the 4-h period was within the range considered to be noncytotoxic. Data are from a single experiment typical of three performed in quadruplicate.

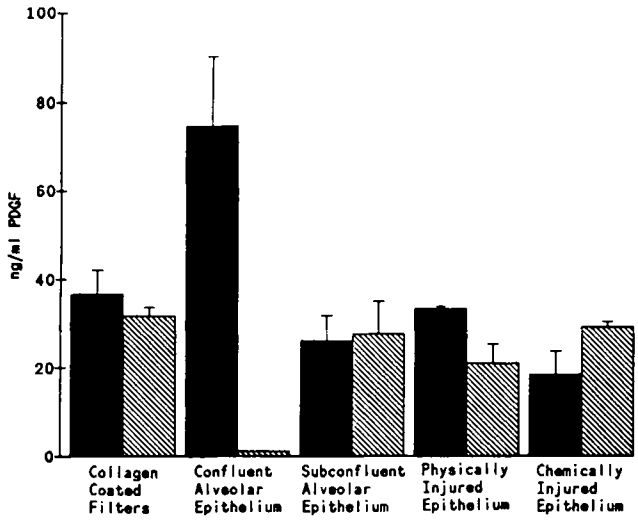

FIG. 11. Histogram depicts the ability of intact and injured alveolar epithelium to allow translocation of PDGF across the collagen-coated filters in the co-culture system. PDGF was added to the upper compartment and quantified by immunoassay 3 d later. Medium from the upper and lower compartments of the culture chamber exhibited an equilibration of PDGF except in the presence of an intact epithelial monolayer, which maintained ~80% of the growth factor in the upper compartment. *Solid bar* represents the PDGF amount still present in the upper compartment; *hatched bar* represents the PDGF concentration in the medium collected from the lower compartment. Data are from a single experiment typical of three performed in triplicate.

permeability to albumin (Fig. 9). LDH levels were assessed in the treated cultures to determine if the increased alveolar permeability was due to cytotoxicity. None of the doses of TC which allowed albumin flux caused LDH release (Fig. 10) above levels considered to be noncytotoxic (9,28).

Platelet-derived growth factor and α-$_2$-Macroglobulin. PDGF and α_2-M placed in the upper compartment of the co-culture chamber readily diffused through the collagen-coated filter (Figs. 11 and 12). In contrast, a confluent monolayer of alveolar epithelial cells maintained about 80% of the PDGF or α_2-M in the upper compartment for 3 d. (Figs. 11,12). In one experiment, the confluent monolayer was set up on the underside of the filter and PDGF again was added to the upper compartment. This configuration made no difference inasmuch as ~80% of the PDGF again remained above the filter after 3 d (data not shown). On the other hand, subconfluent monolayers injured physically or by chemical treatment allowed translocation of PDGF and α_2-M (Figs. 11 and 12). Inasmuch as PDGF and the "fast" form of α_2-M are a potent mitogenic complex (5), we studied the translocation of this complex and found the same pattern of movement as reported above (data not shown).

DISCUSSION

We have shown that an intact epithelial monolayer of type II cells blocks the translocation of a variety of materials including a potent mesenchymal cell growth factor (PDGF) with a molecular weight of ~30 kDa and a high molecular weight (~700 kDa) antiprotease (α_2-M). Interestingly, both of these molecules are synthesized and secreted by rat lung macrophages (4,22). In addition, the α_2-M serves as a specific binding protein for the macrophage-derived PDGF and modulates many of its biological activities (4,5). Whether these biologically relevant proteins are secreted in the alveolar space and can affect events in the lung interstitium are not known. However, it is reasonable to expect that these and similar molecules are present in the alveolar lining fluid. Indeed, PDGF has been detected in fluids lavaged from patients with interstitial lung disease (2), α_2-M reaches high concentrations in serum (30) and albumin is a well characterized component of lavaged lung fluids (1). The role of the alveolar epithelium in controlling movement of such proteins is not clear, but there is a large body of data on solute and protein translocation (13,23,33). Our data support the view that an intact type II monolayer could prevent bioactive materials in the alveolar spaces from affecting cells in the lung interstitium. On the other hand, an epithelium disrupted by macrophage or neutrophil-derived oxygen radicals would probably provide pathways to the interstitium.

Ultrastructural examination of the alveolar epithelium after 5 d of

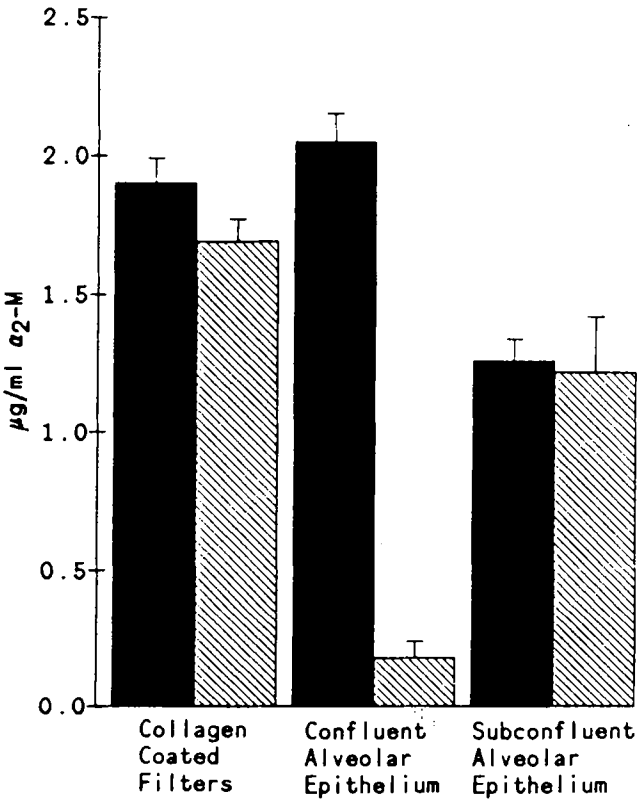

FIG. 12. Histogram depicting the ability of subconfluent and confluent alveolar epithelial monolayers to allow translocation of α_2-M across collagen-coated filters in the co-culture system. α_2-M was added to the upper compartment and quantified by enzyme immunoassay after 3 d. The α_2-M was in equilibrium in medium collected from the upper and lower compartments of the culture chamber except in the presence of an intact monolayer, which blocked its movement during the 3-d period studied. *Solid bar* represents the α_2-M amount still present in the upper compartment; *hatched bar* represents the α_2-M concentration in the medium collected from the lower compartment. Data are from a single experiment typical of three performed in triplicate.

culture revealed a dedifferentiated type II cell monolayer similar to that noted by other investigators (13,27). The cells, although flattened with attenuated cytoplasm and few lamellar bodies, had tight junctions and formed a functional barrier to the transfer of both vital dye and albumin across the collagen-coated filter (Figs. 7 and 9). The morphologic and functional aspects of the epithelial monolayer on the top of the filter were not impaired by the growth of rat lung fibroblasts on the lower side. We were able to effectively culture rat lung fibroblasts in the presence of the intact epithelial monolayer, switch the co-culture to a serum-free medium to bring the lung fibroblasts into quiescent growth, and then return them to log phase growth by replacing serum in the medium (Fig. 3). The ability to co-culture alveolar type II epithelial cells and fibroblasts and measure changes in fibroblast proliferation should prove useful for studies that examine epithelial-mesenchymal interactions at the alveolar level. The role of the alveolar epithelium is postulated to be important because after certain types of oxidant-induced pulmonary injury, intra-alveolar fibrosis results unless there is proliferation of alveolar type II cells (17).

Macrophages have been shown to produce several cytokines and growth factors which affect fibroblast proliferation (16,22,24). Inasmuch as we will be studying these factors in future experiments, alveolar macrophages were co-cultured on intact epithelial monolayers (Fig. 6). The results were similar to macrophages co-cultured on type II epithelial cells grown on plastic (11). The culture system we have described should enable investigators to examine the role of the alveolar epithelial layer in modulating the effects of macrophage-derived growth factors on target fibroblast populations. Such information is essential to understand the mechanisms through which intra-alveolar events such as alveolar macrophage particle interactions lead to interstitial fibrosis which occurs within the alveolar wall. Our data suggest that the alveolar epithelium may play an important modulatory role in these events.

The integrity of epithelial barriers can be compromised by inflammatory responses. For example, Welsh et al. (33) recently showed that reactive oxygen intermediates increase the permeability of an epithelial cell line. Oxidants released from inflammatory cells are believed to contribute to alveolar injury in part through direct effects on epithelial cells as well as by inactivating endogenous antiinflammatory molecules (18). We examined the effect of a chlorinated amine on the integrity of the epithelium in our co-culture model because these oxidants are believed to be important at inflammatory sites (32). Previous studies have shown that TC had the ability to alter endothelial and epithelial barrier permeability (28). Here we demonstrated that noncytotoxic concentrations of TC caused a dose-dependent increase in albumin flux (Fig. 9) in conjunction with morphologic alterations in the epithelial monolayer (Fig. 8). These data indicate that the alveolar epithelial monolayer in our in vitro system can be effectively compromised by biologically relevant agents. The next logical step will be to extend these studies to examine the responses of lung fibroblasts after pertubations of the epithelial layer and introduction of the appropriate growth factors.

There have been significant advances in pulmonary cell biology since epithelial cells have been isolated and cultured (20). The ability to study biologically relevant events which may be significant at the alveolar level can now be expanded by the use of this co-culture system, which more closely models the cellular composition and cell-to-cell interactions of the distal lung.

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