Alveolar type II cell-fibroblast interactions, synthesis and secretion of surfactant and type I collagen

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SUMMARY

During alveolar development and alveolar repair close contacts are established between fibroblasts and lung epithelial cells through gaps in the basement membrane. Using co-culture systems we have investigated whether these close contacts influence synthesis and secretion of the principal surfactant apoprotein (SP-A) by cultured rat lung alveolar type II cells and the synthesis and secretion of type I collagen by fibroblasts.

The alveolar type II cells remained cuboidal and grew in colonies on fibroblast feeder layers and on Matrigel-coated cell culture inserts but were progressively more flattened on fixed fibroblast monolayers and plastic. Alveolar type II cells cultured on plastic released almost all their SP-A into the medium by 4 days. Alveolar type II cells cultured on viable fibroblasts or Matrigel-coated inserts above fibroblasts accumulated SP-A in the medium at a constant rate for the first 4 days, and probably recycle SP-A by endocytosis. The amount of mRNA for SP-A was very low after 4 days of culture of alveolar type II cells on plastic, Matrigel-coated inserts or fixed fibroblasts. Alveolar type II cells cultured on viable fibroblasts accumulated SP-A in the medium at a constant rate for the first 4 days, and probably recycle SP-A by endocytosis. The amount of mRNA for SP-A was very low after 4 days of culture of alveolar type II cells on plastic, Matrigel-coated inserts or fixed fibroblasts.

Co-culture of alveolar type II cells with confluent human dermal fibroblasts stimulated by 2- to 3-fold the secretion of collagen type I into the culture medium, even after the fibroblasts’ growth had been arrested with mitomycin C. Collagen secretion, by fibroblasts, also was stimulated 2-fold by conditioned medium from alveolar type II cells cultured on Matrigel. The amount of mRNA for type I collagen increased only modestly when fibroblasts were cultured in this conditioned medium. This stimulation of type I collagen secretion diminished as the conditioned medium was diluted out, but at high dilutions further stimulation occurred, indicating that a factor that inhibited collagen secretion also was being diluted out. The conditioned medium contained low levels of IGF-1 and the stimulation of type I collagen secretion was abolished when the conditioned medium was pre-incubated with antibodies to insulin-like growth factor 1 (IGF-1).

There are important reciprocal interactions between alveolar type II cells and fibroblasts in co-culture. Direct contacts between alveolar type II cells and fibroblasts appear to have a trophic effect on cultured alveolar type II cells, increasing the levels of mRNA for SP-A. Rat lung alveolar type II cells appear to release a factor (possibly IGF-1) that stimulates type I collagen secretion by fibroblasts.

Key words: alveolar type II cell, fibroblasts, surfactant, collagen type I, insulin-like growth factor

INTRODUCTION

The alveolar surface of the lung, which provides the large area required for efficient gas exchange, is lined by two distinct epithelial cells. The thin alveolar type I cells overlie the capillaries, whilst cuboidal type II cells are found in the corner of the alveolus. The alveolar type II cell has several important metabolic roles, including regulation of alveolar ion transport, surfactant production and alveolar repair after injury (Adamson and Bowden, 1974; Mason et al., 1982; Phelps and Floros, 1988). Since these alveolar type II cells constitute only about one sixth of the total lung cell population their properties have been investigated in isolated, cultured cells. Alveolar type II cells rapidly lose their phenotypic characteristics when maintained in culture (Digglio and Kikkawa, 1977; Dobbs, 1990). In the lung these epithelial cells have communication with other epithelial cells, the basement membrane and the interstitial cells beneath the basement membrane. Contacts with the fibroblasts and other interstitial cells beneath the basement membrane are probably limited, except during alveolar development and repair, when epithelial cell processes and fibroblasts are in close contact through the gaps in the basement membrane (Adamson and King, 1984, 1985; Adamson et al., 1990; Carrington and Green, 1970). It is not known whether these close contacts influence either alveolar type II cell or fibroblast metabolism.

Fibroblasts are recognized to influence surfactant syn-
thesis through paracrine interactions. Prior to parturition, lung fibroblasts start to secrete a polypeptide that stimulates cytidylyltransferase, the rate-limiting enzyme for surfactant synthesis by alveolar type II cells (Smith and Post, 1989). It is the secretion of surfactant into the alveolar lining that reduces the surface tension to permit the regular inflation and deflation of the alveoli during respiration. Four apoproteins have been characterized in this phosphatidylylcholine-rich surfactant: SP-A, SP-B, SP-C and SP-D. The properties and functions of these apoproteins have recently been reviewed (Hagwood and Clements, 1990). SP-A is the most abundant and traditionally has been used to monitor surfactant synthesis in tissue culture systems. The gene for SP-A has been cloned and the primary translation product is a 28 kDa protein. The protein is secreted, after extensive post-translational modification, as large oligomers with a molecular mass of about 700 kDa. Many components of surfactant are taken back into the alveolar type II cells and reutilized locally. In vitro studies indicate that SP-A may regulate the reutilization of surfactant through its interaction with specific receptors on the surface of the alveolar type II cell (Hagwood and Clements, 1990).

Here we investigate how the synthesis and secretion of this SP-A protein by alveolar type II cells, and the synthesis and secretion of the principal fibrillar collagen of the lung, type I collagen, by fibroblasts, are modulated in alveolar type II cell-fibroblast co-culture systems.

MATERIALS AND METHODS

Materials

Tissue culture reagents were obtained from Gibco-BRL. Collagen type I and antibodies to collagen type I were obtained from Southern Biotechnology Inc. Polyclonal antibodies to IGF-1 and recombinant IGF-1 were a gift from G. Carter, Endocrinology, Charing Cross Hospital. Matrigel was obtained from Flow and Cyclopore™ inserts from Millipore.

 Fibroblast cultures

Human dermal fibroblasts were grown from explants in 25 cm² tissue culture flasks. Explants, 1 mm³ pieces, were adhered in a small volume of culture medium (DMEM containing 10% FCS, 2 mM L-glutamine, 20 μg/ml penicillin and 50 μg/ml streptomycin (Gibco) for several hours and then supplemented with 5 ml culture medium, which, after the first week, was replenished every 3 days. The cells had reached confluence by 2-3 weeks, after culture at 37°C in an atmosphere of 95% air, 5% CO₂.

Human lung fibroblasts in early passage were a kind gift from Lynn Bingle, Department of Medicine, Charing Cross and Westminster Medical School.

 Culture and characterization of type II cells

Alveolar type II cells were isolated from the lungs of adult rats (~150 g) and cultured and quantitated as previously described (Bhandari and Powell, 1987). Cytosine preparations of freshly isolated alveolar type II cells were stained for alkaline phosphatase using the method of Miller et al. (1987). Cells were stained with freshly prepared fast red (Sigma, 0.1%) and naphthol phosphoric acid (Sigma, 0.1%) in propanol buffer (0.125 M 2-amino-2-methyl propanol buffer, pH 8.9, containing 0.625 mM MgCl₂) at room temperature for 15 min. Stained slides were washed briefly with distilled water and counterstained with methylene green (Sigma, 1%) at room temperature for 30 s. Slides were washed with water and viewed using an aqueous mounting medium. Cells cultured on plastic, fibroblast monolayers or Cyclopore™ inserts (Millipore) were fixed with precooled methanol (~20°C) for 5 min before immunocytochemical staining using either rabbit anti-SP-A or mouse anti-cytokeratin (LabSystems) as the first antibody. Conditioned medium was harvested from cells cultured on Matrigel (Flow)-coated Cyclopore™ inserts for 4 days.

Co-culture systems

Freshly isolated rat alveolar type II cells were plated, for various periods of time, directly on top of confluent viable or methanol-fixed fibroblast monolayers. Rat type II cells were also plated, at various densities, onto Cyclopore™ inserts (Millipore) coated with Matrigel (Flow; diluted 1:7 with water), which were then placed into multwell plates containing viable fibroblast monolayers.

 Purification of surfactant SP-A

Pulmonary surfactant was purified from rat lung lavage by a modification of the procedure of Whitsett et al. (1985). Briefly, the lungs from 25 adult rats (100-300 g) were removed and lavaged three times with ice-cold saline. Each lung was fully inflated, via the trachea, with the saline and the lavage fluid collected and maintained on ice. The lavage was then centrifuged twice at 250 g for 10 min at 4°C to remove cells and debris. The pellet was discarded and the supernatant centrifuged at 15,000 g for 45 min at 4°C. The pellet from this step was resuspended in 0.9% saline (22 ml) and surface-active material was further purified from this pellet using salt density gradient ultracentrifugation: the surfactant, in 12 ml saline, was layered over 3.8 M KBr (5 ml) and centrifuged at 380,000 g for 2 h in a Sorvall T865V vertical rotor. Surfactant was isolated as a single white flocculent band, density 1.04 g/cm³, which was recovered and resuspended in saline.

Antiserum to SP-A

Antiserum to surfactant protein was raised in English rabbits by carrying out 5 subcutaneous injections with 80 μg of protein in Freund’s incomplete adjuvant for each injection. At 5, 6 and 7 weeks after injection approx. 10-20 ml of blood was collected from an ear vein and the serum was collected after clotting. After 8 weeks, the rabbits were anaesthetized and bled from the abdominal aorta and the serum from this bleeding was also collected and stored at −20°C. IgG was purified from the immune serum by ammonium sulphate precipitation followed by chromatography on a DEAE-cellulose column (Johnstone and Thorpe, 1982).

ELISA for surfactant

An enzyme-linked immunosorbence assay was used to assay tissue culture supernatants for surfactant protein levels (Floros et al., 1989). The ELISA was performed using a competitive assay protocol. An Immulon 96-well plate (Dynatech) (plate 1) was coated with 60 μg/ml rat surfactant in coating buffer (50 mM ammonium carbonate, pH 9.6), 100 μl/well, and left at 4°C overnight.

A second multiwell plate (Sterilin) with conical wells was used to prepare standard amounts of purified surfactant. To 100 μl amounts of purified surfactant and samples on plate 2, an equal volume of purified anti-surfactant IgG (1:500 in PBS, 5% NP-40) was added and the plate was left at 4°C overnight to allow binding of antigen and antibody. The next day, plate 1 was washed three times with phosphate buffered saline (PBS) containing 0.05% Tween-20 and allowed to dry. The antigen-antibody mixture of the samples and the standards from plate 2 (100 μl) were transferred to separate wells on plate 1 and left to incubate at 37°C for 60 min. Unbound antibody from plate 1 was removed by washing (3 times with PBS/0.05% Tween-20) and the plate was then incubated at 37°C for 60 min with alkaline phosphatase conjugate
of goat anti-rabbit IgG (Sigma) diluted 1:1000 with PBS/0.05% Tween-20. Plate 1 was washed as before and then incubated with freshly prepared p-nitrophenyl phosphate (1 mg/ml in glycine buffer: 0.1 M glycine, 0.001 M MgCl₂, 0.001 M ZnCl₂, pH 10.4; 200 µl/well). The reaction was allowed to proceed until a coloured product could be seen and then stopped by adding 30 µl of 6 M NaOH per well and then the absorbance at 405 nm was monitored. Amounts of surfactant were determined using a linear-log plot of absorbance versus surfactant concentration.

**Synthesis of oligonucleotide probes**

Two 24mer oligonucleotides were synthesized, complementary to the mRNA encoding the amino acid sequence of SP-A (Sanò et al., 1987). Oligonucleotide no. 1, which was complementary to the mRNA encoding amino acids 129-136, hybridized to mRNA from dermal fibroblasts and type II cells and was not used further. Oligonucleotide no. 2 was complementary to the mRNA encoding amino acids 206-213 and hybridized to mRNA from type II cells but not to mRNA from fibroblasts. Northern blot analysis demonstrated that this second oligonucleotide hybridized to a 1.6 kb and 0.9 kb mRNA from rat lung and to a 1.5 kb mRNA from isolated alveolar type II cells. The oligonucleotide was labelled using the Klenow fragment of DNA polymerase I to incorporate [³²P]labelled nucleotides to a specific activity of 10⁸ d.p.m./µg DNA.

**Quantitation of mRNA in cultured cells**

Total RNA was extracted from cells using guanidinium chloride according to the method of Grimes et al. (1988). The DNA content of the guanidinium hydrochloride extracts was quantitated using the enhancement of fluorescense observed when bisbenzimizadole (Hoechst 33258) binds to DNA (Labarca and Paigen, 1980). The cDNA probes for the 1.8 kb complement of human α₁(I) collagen (Wilson et al., 1989), galaptin (Murphy et al., 1987) and IGF-1 (Chu et al., 1982) were labelled by the incorporation of [³²P]dCTP using DNA polymerase Klenow fragment (Feinberg and Vogelstein, 1984). The mRNA was serially diluted onto nylon membranes (GeneScreen Plus) and hybridized with the labelled cDNA and autoradiograms prepared according to a published procedure (Kafatos et al., 1979). The density of the RNA dots was determined with a Joyce-Loebl Chromoscan 3 densitometer.

**Hybridization conditions**

The filters were prehybridized for 3 h at 60°C in a plastic bag containing 10 ml of 1% SDS, 1 M NaCl, 10% dextran sulphate. Hybridization was carried out overnight at 60°C in prehybridization solution containing sonicated salmon sperm DNA (300 µg/ml) and radioactive probe (10 ng/ml). The filters were washed in 2x SSC for 5 min at room temperature; 2x SSC, 0.1% SDS for 20 min at 60°C; 0.1x SSC for 30 min at room temperature. The filters were air dried and exposed to X-ray film at 4°C. The radioactivity of the dots was determined by scanning the autoradiogram in a densitometer.

**Amino acid analysis**

Cell culture supernatants were prepared for amino acid analysis by adding 100 µl of SSA (5-sulphosalicylic acid, 0.05%) to 1 ml of supernatant. This was left on ice for 1 h and centrifuged at 250 g for 5 min. Samples were analysed on an LKB amino acid analyser.

**Collagen ELISA**

Type I collagen in cell culture supernatant was quantitated using a competitive ELISA protocol. The wells of a 96-well plate (Sterilin) were coated at 4°C overnight with 200 µl of type I collagen solution, 200 µg/ml in coating buffer (50 mM calcium carbonate, pH 9.6). A second multiwell plate, with conical wells, was used to prepare standard amounts of type I collagen. To 110 µl amounts of standards and samples on plate 2, an equal volume of goat anti-type I collagen antibody (1:1000, in PBS, 5% Tween-20) was added and left overnight at 4°C. Plate 1 was washed three times with PBS containing 0.5% Tween-20 and allowed to dry. Portions of 200 µl of standards and samples were transferred from plate 2 to the collagen-coated plate and left at room temperature for 2 h. Plate 1 was washed as before, air dried, and incubated with freshly prepared phosphatase substrate (1 mg/ml p-nitrophenyl phosphate in glycine buffer (0.1 M glycine, 0.001 M MgCl₂, 0.001 M ZnCl₂), 200 µl per well). The reaction was allowed to proceed until a coloured product could be seen and the reaction was stopped by adding 30 µl of 6 M NaOH per well. The absorbance at 490 nm was read on a multiwell plate reader and collagen concentration was determined using a log linear plot of type I collagen concentration versus absorbance.

**TGFβ bioassay**

Conditioned medium from rat type II cell preparations was dialysed at 4°C successively against 1 M acetic acid for 24 h, 10 mM acetic acid for 24 h and PBS for 24 h. The dialysed medium was lyophilized and made up to its original volume with DMEM containing 10% FCS, 20 mM L-glutamine, 100 µg/ml penicillin-streptomycin solution. The conditioned medium was then added to confluent mink lung epithelial cells (ML1Vu cells) in a 96-well plate, and left for 20 h. Doubling dilutions of the dialysed conditioned medium prepared with 10% serum-containing DMEM were added. After 24 h the cells were washed and DMEM containing 1 mM/ml ³H-thymidine used to culture ML1Vu cells at 37°C for 4 h. The [³H]thymidine DMEM was then removed and the cells were washed three times in serum-free, ice-cold Hanks’ Balanced Salt Solution (HBSS, without Ca²⁺ or Mg²⁺; Gibco). The cells were then incubated in ice-cold 10% trichloroacetic acid (TCA) for 10 min. The TCA was removed and the cells washed as before in HBSS. Then 2 M perchloroacetic acid (PCA) was added to the cells at 37°C for 30 min. Equal volumes of solubilized sample were added to scintillant (Ecoscint) and the radioactivity of these samples was counted in a liquid scintillation counter.

**IGF-1 assay**

Conditioned medium was assayed for IGF-1 using an immunoradiometric assay (IRMA) designed to detect human IGF-1. This was performed in the Department of Endocrinology, Charing Cross and Westminster Medical School.

**IL-6 assay**

Conditioned medium was assayed for interleukin 6 (IL-6) by radioimmunoassay (RIA; Amersham). This was performed by Philip Worrell, Department of Surgery, Charing Cross and Westminster Medical School.

**Mitomycin C treatment**

Confluent dermal fibroblasts were treated with mitomycin C (5 µg/10⁶ cells) in serum-containing DMEM, overnight at 37°C. The cells were then washed three times with culture medium and allowed to recover for 48 h before being used in an experiment. Growth arrest was confirmed by the incorporation of [³H]thymidine, as described in the TGFβ bioassay procedure above.

**Heparin-Sepharose and Sepharose chromatography**

A 1 ml column of heparin-Sepharose or Sepharose CL 4B was washed with 5 ml 0.5 M NaCl. Rat type II cell-conditioned medium (5 ml) was passed drop-wise through the column and 5 ml of flow through collected. The conditioned medium was ster-
pared with cells cultured on fixed or viable fibroblasts, cells were immunostained using anti-SP-A immunoglobulin, flattened and vacuolated (Fig. 1B) whilst cells on viable fibroblasts remain cuboidal in packed colonies (Fig. 1C). The cells on Matrigel-coated inserts (Fig. 1D) also grow in colonies but these are more open and the cells less cuboidal than those on viable fibroblasts. The morphology of cells cultured on Matrigel-coated plastic was similar to alveolar type II cells cultured on fixed fibroblasts. After 4 days in culture only cells grown on Matrigel-coated inserts or viable fibroblasts stained with the polyclonal antiserum to SP-A.

**RESULTS**

**Morphology of alveolar type II cells**

The morphology of alveolar type II cells after 4 days in culture on plastic, on a monolayer of fixed and viable dermal fibroblasts and on Matrigel-coated inserts, above confluent fibroblasts, is shown using anticytokeratin staining in Fig. 1. On plastic (Fig. 1A) the cells have become flattened and vacuolated. Cells on fixed fibroblasts are less flattened and vacuolated (Fig. 1B) whilst cells on viable fibroblasts remain cuboidal in packed colonies (Fig. 1C). The cells on Matrigel-coated inserts (Fig. 1D) also grow in colonies but these are more open and the cells less cuboidal than those on viable fibroblasts. The morphology of cells cultured on Matrigel-coated plastic was similar to alveolar type II cells cultured on fixed fibroblasts. After 4 days in culture only cells grown on Matrigel-coated inserts or viable fibroblasts stained with the polyclonal antiserum to SP-A.

**Surfactant SP-A secretion**

Freshly isolated alveolar type II cells (approximately 80% pure) contained 345 ± 63 ng of SP-A/10^6 cells. When alveolar type II cells were cultured on plastic nearly all of this SP-A was released into the culture medium within the first 48 h and SP-A could not be detected immunocytochemically in the adhered cells. When alveolar type II cells were cultured on fixed fibroblast monolayers 108 ± 26 ng SP-A/10^6 cells plated was released into the medium in the first 24 h, 68 ± 14 ng/10^6 cells between 24 h and 48 h and a further 116 ± 23 ng/10^6 cells between 48 h and 96 h. After 96 h SP-A could only be detected immunocytochemically in a minority of cells. In contrast after 4 days of culture on viable fibroblasts or Matrigel-coated inserts alveolar type II cells were immunostained using anti-SP-A immunoglobulin. For these conditions SP-A accumulated in the medium at a fairly constant rate for the first 4 days in culture, 70 ± 24 ng/10^6 cells per 24 h on viable fibroblasts and 38 ± 8 ng/10^6 cells per 24 h on Matrigel-coated inserts (Table 1).

These changes in surfactant secretion tended to be paralleled by the release of acid phosphatase into the culture medium, after even 24 h in culture cells on plastic had secreted twice as much acid phosphatase per 10^6 cells compared with cells cultured on fixed or viable fibroblasts (Table 1). After one week in culture cells on plastic had secreted ten times as much acid phosphatase as cells cultured on viable fibroblasts, and on fixed and viable fibroblasts. In the presence of fibroblasts there is no purpose in comparing mRNA levels for SP-A and a housekeeping gene. Rather we compared the amount of mRNA for SP-A to the total number of alveolar type II cells, quantitated by cytokeratin immunostaining in replicate experiments (Bhandari and Powell, 1987). Alveolar type II cells cultured on viable fibroblasts had significantly higher levels of mRNA for SP-A than any other condition; P < 0.02 (Table 1).

**Collagen type I synthesis by dermal fibroblasts**

Cultured fibroblasts synthesize and secrete type I collagen, the amount varies with the cell source, passage and presence of ascorbate. Rat alveolar type II cells do not secrete type I collagen and did not give a positive signal for type I collagen mRNA. For confluent human dermal fibroblasts, passage 4, the secretion of type I collagen, 46 ± 8 ng/ml per 24 h, increased to 72 ± 13 ng/ml per 24 h when these cells were co-cultured with alveolar type II cells (5×10^5 cells per 10 cm^2 well). For the first 4 days in culture confluent dermal fibroblasts, at passage 7, the secretion of type I collagen was 72 ± 10 ng/ml per 24 h, increasing to 150 ± 6 ng/ml per 24 h when these cells were co-cultured with alveolar type II cells (2×10^6 cells per 10 cm^2 well); P < 0.01. After the fourth day in culture the rate of collagen secretion decreased in basal medium and more rapidly in conditioned medium.

To investigate whether the stimulation of type I collagen secretion resulted from an effect of rat alveolar type II cells on fibroblast proliferation the experiments were repeated using dermal fibroblasts treated with mitomycin C, growth arrest being confirmed by[^H]thymidine-uptake studies. When alveolar type II cells (2×10^6 cells per 10 cm^2 well) were cultured on confluent, growth-arrested fibroblasts, the accumulation of type I collagen in the medium increased, from 32 ± 9 ng/ml per 24 h for fibroblasts alone, to 61 ± 25 ng/ml per 24 h; P < 0.01 (Table 2). From the experiments to quantitate mRNA levels for SP-A (above), DNA was measured. Assuming that alveolar type II cells do not proliferate in culture, there was no difference in fibroblast DNA levels when confluent cells were cultured for 4 days in the presence or absence of alveolar type II cells. Therefore, the stimulation of fibroblast collagen secretion by alveolar type II cells did not appear to be dependant on fibroblast proliferation.

The stimulation of type I collagen secretion by coculture with alveolar type II cells was not dependant on direct cell-cell contact alone, since rat alveolar type II cells (2×10^6) cultured on Matrigel-coated Cycloplure™ inserts above fibroblasts in 10 cm^2 wells also effected a 2-fold increase in type I collagen secretion by dermal fibroblasts (Table 2). Conditioned medium prepared from rat alveolar type II cells cultured on Matrigel also stimulated collagen secretion by confluent dermal fibroblasts (Table 2). (Conditioned medium from rat alveolar type II cells cultured on plastic or from co-cultures of alveolar type II cells and fibroblasts did not stimulate type I collagen secretion by dermal fibroblasts.) The increased secretion of type I collagen in conditioned medium was not accompanied by an increase in total protein synthesis or collagen turnover (release of hydroxyproline into the medium) (Table 2).
Fig. 1. The morphology of alveolar type II cells cultured on different substrata. After 4 days of culture the cells were fixed and intermediate filaments immunostained with monoclonal antibodies to cytokeratin. (A) Cells cultured on plastic are flat and vacuolated. (B) Cells cultured on fixed fibroblast monolayers are more rounded than in (A). (C) Cells cultured on viable fibroblasts are cuboidal and grow in colonies or aggregates, with their apical surfaces pointing inwards. (D) Cells cultured on Matrigel-coated Minicell inserts also remain cuboidal but grow in large aggregates. Fibroblasts have been counterstained with haematoxylin. ×91.
The purity of the preparations of alveolar type II from which conditioned medium was prepared varied from 60 to 90%. Conditioned medium from the purest preparations resulted in the greatest stimulation (up to 3-fold) of type I collagen secretion by dermal fibroblasts (Fig. 2). The principal cells contaminating the isolated alveolar type II cells are macrophages. Most of these are removed in the differential adherence step during the isolation procedure (Bhandari and Powell, 1987). The conditioned medium prepared from these macrophages, isolated by differential adherence, did not increase significantly type I collagen secretion by confluent dermal fibroblasts.

The conditioned medium prepared from alveolar type II cells cultured on Matrigel-coated inserts stimulated (~2-fold) collagen secretion by confluent dermal fibroblasts cultured in the presence of ascorbate (Table 3). Again the stimulation of collagen secretion was not accompanied by a general increase in protein synthesis (Table 3). Neither was the stimulation of collagen secretion accompanied by an increased amount of hydroxyproline in the culture medium (Table 3).

Finally, dot-blot analysis for the levels of mRNA for type I collagen demonstrated only a modest increase when fibroblasts were cultured in alveolar type II cell-conditioned medium (Tables 2 and 3).

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the conditioned medium with a 20-fold dilution of antiserum to human IGF-1 completely abolished the stimulatory effect on type I collagen secretion by dermal fibroblasts, reducing collagen synthesis to below basal levels (Table 4). This same dilution of antiserum did not affect type I collagen secretion by fibroblasts cultured in unconditioned medium. Supraphysiological levels of insulin (5 µg/ml) or physiological levels of recombinant IGF-1 (35 ng/ml) effected a similar increase in type I collagen secretion to the alveolar type II cell-conditioned medium (Table 4).

The preparation of RNA from alveolar type II cells cultured on Matrigel yielded only low amounts of RNA, sufficient to detect a housekeeping probe (galaptin) and a low intensity signal for the mRNA for IGF-1 in dot blots, but insufficient to detect the mRNA for IGF-1 by northern blot analysis.

**Collagen type I synthesis by lung fibroblasts**

The secretion of type I collagen from lung fibroblasts (passage 7) increased from 546 ± 50 ng/ml per 24 h to 833 ± 30 ng/ml per 24 h when these cells were cultured in conditioned medium for 4 days; P < 0.02. However, after 24 h in culture the secretion of type I collagen had increased from 488 ± 53 ng/ml to 1055 ± 69 ng/ml in conditioned medium. After 48 h in culture the secretion of type I collagen was 522 ± 54 ng/ml per 24 h in basal medium and 986 ± 63 ng/ml per 24 h in conditioned medium. The stimulatory effect of conditioned medium on collagen secretion by lung fibroblasts appears to be of shorter duration than for dermal fibroblasts. Preincubation of the conditioned medium with antiserum to human IGF-1 completely abolished the stimulatory effects on collagen secretion at 1, 2 and 4 d, reducing collagen secretion to <480 ng/ml per 24 h. The preincubation of antiserum to human IGF-1 with basal medium reduced collagen secretion from 522 ± 54 ng/ml per 24 h to 467 ± 69 ng/ml per 24 h; P not significant, after 2 days in culture. The addition of supraphysiological levels of insulin (5 µg/ml) or physiological levels of recombinant IGF-1 (35 ng/ml) to basal medium effected similar increases in type I collagen secretion to the alveolar type II cell-conditioned medium at 1 and 2 days (data not shown).

**DISCUSSION**

The metabolic functions of cells are controlled by numerous factors including hormones, cell-cell and cell-matrix interactions.
interactions. Some of these influences can be most elegantly studied in tissue culture systems. The lung is a particularly complex organ, with more than 40 different cell types. For this reason in many previous studies organ culture has been used to study the development and function of alveolar type II cells. Since we were interested particularly in how the close contacts between epithelial cell processes and fibroblasts, found in developing and repairing lung, influenced the metabolism of each cell type, we developed three different alveolar type II cell-fibroblast coculture systems. The three coculture systems used were alveolar type II cells on a feeder layer of fibroblasts, alveolar type II cells on a monolayer of fixed fibroblasts and alveolar type II cells cultured on a Matrigel-coated insert above a fibroblast monolayer. The morphology and organization of alveolar type II cells were different in each condition. The most cuboidal alveolar type II cells were observed growing in colonies on fibroblast monolayers, cells on Matrigel-coated inserts were cuboidal but did not grow in tight colonies whilst cells on fixed fibroblasts were flattened. Cells cultured on plastic were very flattened and vacuolated. In the presence of such different morphologies, interpretation of differing metabolic functions becomes very difficult.

Cell-extracellular matrix interactions and cuboidal shape appear to influence the pattern of phospholipid synthesis and secretion in rat lung alveolar type II cells (Shannon et al., 1987). More recently, the accumulation of mRNA for SP-A, SP-B and SP-C, together with the intracellular accumulation of SP-A, has been compared in rat alveolar type II cells cultured on plastic, laminin and Engelbreth-Holm Swarm (EHS) tumour basement membrane (Shannon et al., 1990). Only on the EHS matrix-coated plastic did alveolar type II cells accumulate significant amounts of surfactant apoprotein mRNAs and SP-A protein, retain a cuboid shape and grow in aggregates (Shannon et al., 1990). Cell morphology probably has a dominating effect on the metabolic functions of alveolar type II cells.

Our results support some of these findings. The tight colonies of alveolar type II cells cultured on fibroblast feeder layers have significantly higher levels of SP-A mRNA than cells cultured on Matrigel-coated inserts above fibroblasts, and this seems likely to provide the explanation for their increased release of SP-A into the culture medium as compared with cells grown on inserts. Matrigel is prepared from EHS tumour and is rich in laminin, type IV collagen and other basement membrane constituents. Since cells cultured on Matrigel have similar levels of SP-A mRNA to those in cells cultured on fixed fibroblasts but contain more SP-A, cells cultured on colonies on Matrigel probably continue to function in receptor-mediated endocytosis and recycling of SP-A. Similarly, cells cultured on fibroblast feeder layers probably function in the recycling of SP-A. The high levels of SP-A mRNA and SP-A secretion by alveolar type II cells directly in contact with fibroblasts indicate that this cell-cell interaction has trophic effects on cultured alveolar type II cells. These trophic effects may not be independent of effects on cell morphology.

Cultured rat lung alveolar type II cells have recently been reported to secrete factors that both stimulate and inhibit fibroblast proliferation, depending upon the age and culture conditions of the alveolar type II cells (Adamson and King, 1991). Our experimental conditions did not show these effects on fibroblast proliferation, but suggested that cultured alveolar type II cells secrete paracrine factors capable of influencing type I collagen secretion by fibroblasts.

The direct culture of alveolar type II cells on fibroblasts, or the culture of fibroblasts in conditioned medium prepared from cuboidal alveolar type II cells cultured on Matrigel, resulted in a 2-fold increase in secretion of type I collagen, which was not accompanied by a parallel increase in fibroblast DNA or protein synthesis. Most of our experiments were performed using confluent layers of human dermal fibroblasts in early passage (4-7), cultured in a medium where foetal calf serum provided the only source of ascorbate. Similar results were obtained if the medium was supplemented with ascorbate or if human lung fibroblasts were used. We were also concerned that we were observing an effect from contaminating cells amongst the alveolar type II cells. These concerns were allayed when we observed that macrophages, the principal contaminants, and their conditioned medium did not support increased collagen secretion by fibroblasts. Further, the purest preparations of alveolar type II cells provided the greatest stimulation to fibroblast collagen secretion (Fig. 2).

The doubling of type I collagen secretion by fibroblasts, when cultured in alveolar type II cell-conditioned medium, was accompanied by only modest (1.2 to 1.5-fold) increase in collagen type I mRNA levels and a trivial increase in hydroxyproline concentrations in the medium. Previous studies have associated increased collagen synthesis with an increase in mRNA levels or with an increase in the stability or polysome association of the mRNA for type I collagen (Raghow et al., 1985; Sterling et al., 1983; Tolstoshev et al., 1981). We did not investigate such factors but proceeded to try and identify the paracrine factor associated with stimulation of type I collagen secretion by fibroblasts.

The synthesis and secretion of type I collagen by cultured cells can be stimulated by a number of potential mediators including interleukins, transforming growth factor-β, insulin and insulin-like growth factors (Adams, 1989). Macrophage-conditioned medium (a source of interleukin 1) did not stimulate collagen secretion by fibroblasts and the alveolar type II cell-conditioned medium did not contain interleukin 6 or transforming growth factor-β. The paracrine factor in alveolar type II cell-conditioned medium stimulating collagen synthesis was heat stable and not a member of the heparin-binding growth factor family.

The stimulatory factor present in alveolar type II cell-conditioned medium was diluted out rapidly (Fig. 3). At much higher dilutions of conditioned medium a renewed stimulation of collagen synthesis was observed, so that the presence of both stimulatory and inhibitory factors can be inferred. The possibility that the stimulatory factor was an insulin-like growth factor (IGF-1) was strengthened when preincubation of alveolar type II cell-conditioned medium with antibodies to human IGF-1 completely ablated the stimulatory effect on type I collagen secretion. Pre-incubation of conditioned medium with antibodies to IGF-1 reduced the secretion of collagen to below basal levels, again providing evidence for the presence of inhibitory factors in the conditioned medium. There is a 96% homology
between the rat and human IGF-1 sequences, the cross-species reactivity of a polyclonal antibody being anticipated (Murphy et al., 1987). Low levels of rat IGF-1 were detected in the conditioned medium but mRNA preparations from alveolar type II cells on Matrigel provided insufficient quantities of RNA for a convincing identification of IGF-1 mRNA by northern blot analysis. However, when either dermal or lung fibroblasts were cultured in the presence of physiological levels of recombinant IGF-1, type I collagen synthesis again was stimulated 2-fold (Table 4). Similarly, supraphysiological levels of insulin mimicked these effects: there is substantial sequence homology between insulin and IGF-1 and at high concentrations insulin may cross-react with IGF-1 receptors (Gruppuso et al., 1988). In the absence of proof that cultured alveolar type II cells synthesize IGF-1, the source of IGF-1 in alveolar type II cell-conditioned medium is not clear. Alveolar type II cells may cease to synthesize IGF-1 in culture or they may have endocytosed IGF-1 in vivo, which is released later into the conditioned medium. Although fibroblasts and other cells of mesenchymal origin are considered to synthesize IGF-1, immunocytochemical studies have demonstrated IGF-1 in a wide variety of cells, including epithelial cells, that are in close proximity to mesenchymal cells (Han et al., 1987; Kelly, 1990).

In the healthy lung the connective tissue, of which type I collagen is an important component, is subject to continuous, but highly regulated, turnover (Adams, 1989; Kelly, 1990; Laurent, 1986). Such collagen turnover is controlled by an extensive cytokine network, specific cytokines influencing the synthesis of type I collagen whilst other cytokines stimulate the enzymes involved in collagen degradation (Adams, 1989; Fine and Goldstein, 1987; Kelly, 1990; Matrisian, 1992; Rossi et al., 1988). The transcription of collagen genes also is regulated by glucocorticoids and other hormones (Cockayne et al., 1986). In the context of such complex regulation of collagen turnover, the secretory products of many cell types are likely to influence collagen turnover by lung fibroblasts both in vitro and in vivo.

The close contacts between alveolar type II cells and fibroblasts through gaps in the basement membrane, which are observed during lung development and during lung repair after inhalation injury, could influence the metabolic functions of these cells. In vitro co-culture experiments clearly demonstrate such reciprocal interactions between alveolar type II cells and fibroblasts. Such in vitro experiments also underscore the difficulty of understanding the molecular events where reciprocal interactions between many cell types and cytokine cascades contribute to a pathological process such as pulmonary fibrosis.

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REFERENCES


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