Identification of specific proteins synthesized by type II pneumocytes in primary culture

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Proteins from primary cultures of type II granular pneumocytes have been examined by sodium dodecyl sulphate/polyacrylamide-gel electrophoresis to identify type II cell-specific proteins. The distribution of Coomassie Blue-stained bands in preparations of cellular proteins, culture medium, lavage and lamellar bodies have been compared. The most prominent stained band in the serum-free medium from type II cell cultures (HS1; M_r 39 900) corresponds to a major protein in acellular sedimentable (20 000 g for 30 min) crude surfactant obtained from rat lungs by saline (0.9% NaCl) lavage. A second protein (HS2; M_r 12 000) is also found both in type II cell-conditioned medium and in lavage. Neither rat serum nor donor calf serum (used in the isolation of the type II cells) contains a protein co-migrating with HS1 or HS2 proteins. HS1 is also found in Coomassie Blue-stained gels of cellular proteins and of lamellar bodies isolated from whole lungs. Cultures of type II cells incorporate [^{14}C]phenylalanine into HS1 and HS2 as shown by autoradiography of sodium dodecyl sulphate/polyacrylamide gels of culture medium. Rat lungs perfused *in situ* incorporate [^{35}S]methionine into HS1 in the lamellar body fraction. A third protein (HS3; M_r 47 000) is observed only in autoradiographs of cell culture medium; no corresponding Coomassie Blue-stained band can be identified in medium, in cells or in lung lavage. No protein bands corresponding to HS1, HS2 or HS3 are found in conditioned media from pulmonary alveolar macrophages, rat fibroblasts or bovine aorta endothelial cells. Two-dimensional gel electrophoresis of HS1 shows a single polypeptide with an isoelectric point of 6.3; HS3 appears as a chain of spots with a range of isoelectric points from 6.3 to 6.6. HS2 has not been identified on two-dimensional gels. The amino acid composition of HS1 does not differ significantly from that of surfactant apoproteins studied previously; however, HS1 is not detected by glycoprotein stains, nor does it appear to be a subunit of a thiol-linked multimer.

Protein metabolism (Massaro & Massaro, 1974; Chiang *et al.*, 1979; Rannels *et al.*, 1979; Watkins & Rannels, 1979, 1980) and various other aspects of lung biochemistry have been studied primarily in the intact tissue. The well known diversity of cell types in the lung (Haies *et al.*, 1981), however, makes interpretation of these results difficult; each cell type may respond to experimental manipulation independently of, or interactively with, other cells. For example, the response of cultured type II granular pneumocytes to glucocorticoids (Smith, 1979) ap-

pears to depend on the presence of fibroblasts, an interaction observed only in cell culture. On the other hand, the effect of ventilation on surfactant metabolism (Oyarzun & Clements, 1978; Klass, 1979; Hildebran *et al.*, 1981) can be examined only in intact lungs. Thus investigations of cells in culture in conjunction with experiments in intact lungs represent complementary research approaches.

Identification of cell-specific functions is the most difficult aspect of this integrated approach. Phospholipid metabolism has been extensively investigated in isolated type II cells (Smith & Kikkawa, 1978, 1979; Post *et al.*, 1980; Brown & Longmore, 1980; Mettler *et al.*, 1981), but expansion of these studies to the intact lung presents a serious problem. Although type II cells are more active in the synthesis of phospholipids than are other lung cells

Abbreviations used: MEM-J Joklik's minimal essential medium; MEM-D, Dulbecco's modified minimal essential medium; DCS, donor calf serum; SDS, sodium dodecyl sulphate; p.a.g.e., polyacrylamide-gel electrophoresis; i.e.f., isoelectric focusing; BSA, bovine serum albumin; PAS, periodic acid/Schiff's reagent.

(Finkelstein & Shapiro, 1982), they are not unique in this function (King *et al.*, 1974). Furthermore, alveolar type II granular pneumocytes are most often identified by the presence of lamellar inclusion bodies in electron micrographs or through the use of lamellar body stains (Kikkawa & Smith, 1977; Mason *et al.*, 1977). A protein (or proteins) specific to type II cells would provide a definitive marker for cell identification as well as a probe for studies of the physiological regulation of protein metabolism in these cells in primary culture and in intact lungs. Our initial efforts to identify type II cell-specific molecules have centred on surfactant-associated proteins, because surfactant production is the best known metabolic specialization of type II pneumocytes. Here we report several proteins that appear to provide specific markers for this important cell type.

Materials and methods

Cell preparation and culture

All type II cell cultures used were primary isolated material from male Sprague-Dawley rats of approx. 200 g obtained from Charles River Laboratories; the rats were provided Agway RMH3000 chow and water *ad libitum*. Type II cells were isolated by a modification of the methods of Dobbs & Mason (1979). Rats were anaesthetized by intraperitoneal injection of Nembutal (50 mg/kg body wt.). A thoractomy was performed, and the exposed lungs were perfused with 0.15 M-NaCl (saline) at room temperature until free of blood. After lavage (three times with 5 ml of saline), 10 ml of a solution of trypsin (twice-crystallized; 0.02%, w/v) and BaSO₄ (0.05%, w/v) in MEM-J, containing gentamycin (50 µg/ml) and Fungizone (2.5 µg/ml), were instilled via the tracheal cannula into the excised lungs. The intact lungs were incubated at 37°C for 30 min in MEM-J, with a second portion of 5 ml of trypsin/BaSO₄ instilled after 15 min. The 10 ml of a solution of soya-bean trypsin inhibitor (0.02%, w/v) and deoxyribonuclease (0.017%, w/v) in MEM-J were then instilled and the lungs were removed and placed on ice. The lungs were dissected free of the heart, large airways and associated structures and minced finely. The pieces were agitated vigorously, by using a Vortex mixer, to free single cells, which were separated from undigested tissue by filtration through HC 3-160 nylon bolting cloth (Tetco). Portions (20 ml) were layered on to two-step Ficoll gradients, consisting of 8 ml each of 8.3% (w/v) Ficoll-400 in MEM-J (with a density of 1.040 g/ml) and 20.5% (w/v) Ficoll-400 in MEM-J (density of 1.080 g/ml). The gradients were centrifuged for 20 min at 200g (1200 rev./min; SW-27 rotor in Beckman L2-65B ultracentrifuge). Cells that collected at the interface between the densities of 1.040 and 1.080 g/ml were removed and washed free of

Ficoll with MEM-D, containing gentamycin (50 µg/ml) and 10% DCS. Two sequential 15 min incubations of the resulting cell suspension in T-75 tissue culture flasks substantially reduced the contamination of the preparation with macrophages and other non-type II cells. The cell suspension remaining after differential adherence was distributed to 60 mm tissue culture dishes at a plating density of $(1-2) \times 10^7$ cells/plate in MEM-D + 10% DCS. Plates were incubated at 37°C in humidified CO₂/air (1:9) for 36 h. Non-adherent cells were removed by gently rinsing the plate surface with fresh medium. Plating efficiency was $62.3 \pm 3.9\%$ ($n = 4$), as determined by the amount of applied cellular protein that was retained on the plate. The viability of the adhered cells as judged by erythrocin B exclusion (Phillips & Terryberry, 1957) was $96.2 \pm 3.5\%$. Type II cells constituted $74.5 \pm 3.3\%$ of the final cells in culture as determined by haematoxylin/LiCO₃ staining of the monolayers (Kikkawa & Smith, 1977). The presence of lamellar inclusion bodies in the isolated cells was confirmed by electron microscopy. Estimates based on the morphometric analysis of Brumley *et al.* (1979) indicated recovery of approx. 50% of the type II cells from the lungs by using the present preparatory procedures; a typical isolation from 10–12 rats yielded 30 plates of 2×10^7 cells per plate.

Pulmonary alveolar macrophages were obtained by lavage of rat lungs with MEM-J. Macrophages were collected by centrifugation at 200g for 10 min, washed once with MEM-D + 10% DCS and plated in 60 mm tissue culture dishes. Cultures were handled as described for type II cells.

Lung perfusion

Lungs of male Sprague-Dawley rats (200 g) were perfused *in situ* with Krebs-Henseleit bicarbonate buffer containing 4.5% bovine serum albumin (fraction V), 5.6 mM-glucose and plasma levels of 20 amino acids (except as indicated) as described by Watkins & Rannels (1979).

Estimates of [¹⁴C]phenylalanine incorporation

[¹⁴C]Phenylalanine incorporation into protein by cell monolayers was measured in the absence of serum in MEM-D modified to contain 8.6 µM-phenylalanine; the final specific radioactivity of phenylalanine in the medium was 115 Ci/mol. Incorporation of [¹⁴C]phenylalanine into cellular proteins was quantified by precipitating the cell monolayer, after removal of the medium, with ice-cold 10% (w/v) trichloroacetic acid. The precipitated protein was collected by centrifugation, resuspended in 5% (w/v) trichloroacetic acid containing 1 g of phenylalanine/l, heated at 100°C for 15 min, cooled and collected on Millipore HA filters (0.45 µm pore size). Filters were washed extensively with 5%

(w/v) trichloroacetic acid, and transferred to scintillation vials, where the protein was dissolved in 0.7 ml of 88% formic acid and counted for radioactivity in 10 ml of ACS II (New England Nuclear).

Incorporation of [^{14}C]phenylalanine into proteins in the medium was measured after extensive dialysis against distilled water at 4°C. Dialysed medium was dried *in vacuo*, dissolved and sampled for radioactivity. All determinations of radioactivity were corrected for quench by the external-standard method.

Other preparative methods

Samples of crude surfactant were prepared by centrifugation of cell-free rat lung lavage at 20000g for 30 min at 4°C (Metcalf *et al.*, 1980). In some cases, lavage was dialysed and freeze-dried directly after removal of cells by low-speed centrifugation. Lamellar bodies were prepared by the method of Duck-Chong (1978). Protein was determined by the method of Lowry *et al.* (1951).

Gel electrophoresis

SDS/p.a.g.e. was performed on 0.75 mm-thick slab gels (9 cm $\times$ 14 cm) in the discontinuous Tris/glycine system of Laemmli (1970). The resolving gels contained 3.5 cm of 15% acrylamide, 3.5 cm of 10% acrylamide and 2.0 cm of 5% acrylamide, with a 2.0 cm 4.5% stacking gel. Samples were dissolved in SDS/polyacrylamide-gel sample buffer containing 5% (w/v) SDS, 5% (v/v) β -mercaptoethanol and 10% (v/v) glycerol in 62.5 mM-Tris/HCl, pH 6.8, by boiling for 3 min. When non-reduced samples were examined, the β -mercaptoethanol was omitted. Molecular-weight markers were included in each gel. Molecular-weight determinations were performed both on 7.5% and on 10% acrylamide resolving gels.

Gels were simultaneously fixed and stained for protein by immersion in 0.1% Coomassie Brilliant Blue G-250 in methanol/acetic acid/water (4:1:5, by vol.) and were destained by diffusion in methanol/acetic acid/water (3:1:6, by vol.). PAS staining for glycoprotein was performed by the method of Fairbanks *et al.* (1971). Alcian Blue staining for glycoproteins (Wardi & Michos, 1972) was used in some studies.

Autoradiography was performed on Kodak X-Omat R film. Both autoradiographs and wet gels were scanned with a Zeineh soft-laser scanning densitometer (LKB Instruments). In some experiments, gels were sliced and dissolved in 30% H_2O_2 at 50°C for 3 h to quantify [^{14}C]phenylalanine incorporation into specific bands. Control incubations showed no loss of radioactivity from [^{14}C]phenylalanine-labelled proteins under these conditions. When a profile of radioactivity in 1 mm gel slices was compared with a densitometer tracing of an auto-

radiograph of a parallel channel, no differences in the profiles could be discerned.

I.e.f. was performed as described by O'Farrell & O'Farrell (1976) in cylindrical gels (3 mm $\times$ 115 mm). pH profiles were obtained by slicing a gel into 0.5 cm slices and extracting the ampholines into 1.0 ml of degassed distilled water; the pH of each slice was then determined with a Corning pH meter. These i.e.f. gels were used as the first dimension for a second-dimension electrophoresis (O'Farrell & O'Farrell, 1976) by the SDS/p.a.g.e. system described above.

Amino acid composition was determined on samples of single bands cut from SDS/polyacrylamide gels. Proteins were extracted into 70% formic acid at -20°C from bands cut from stained gels (Wada & Snell, 1972). Samples for amino acid analysis were dried *in vacuo* in a Speed-Vac centrifuge (Savant), then hydrolysed in 6 M-HCl at 105°C in an N_2 atmosphere for 24 h. Analysis of amino acid composition were performed on a Beckman 119C Amino Acid Analyzer. Background values were determined on slices of gels that contained no protein; such samples were processed and analysed in parallel with each unknown sample.

Reagents

All materials for electrophoresis were obtained from Bio-Rad, except for carrier ampholines, which were purchased from LKB. Molecular-weight markers were obtained from Sigma and from Boehringer. Media were purchased from Gibco, and DCS and Fungizone were from Flow Laboratories. All sterile tissue culture plastic ware was from Corning or Falcon. All solutions used in the isolation and culture of cells were sterilized by filtration through 0.22 μm pore size Millipore filters. [^3S]Methionine was purchased from New England Nuclear. Sigma Chemical Co. was the source of trypsin, gentamycin, Ficoll, Schiff's reagent and deoxyribonuclease. Soyabean trypsin inhibitor and deoxyribonuclease were obtained from Worthington.

Results

Observations of surfactant-associated proteins HS1 and HS2

Because alveolar type II granular pneumocytes synthesize and secrete pulmonary surfactant (Batenburg & van Golde, 1979), a surfactant-associated protein is a prime candidate for a type II cell specific marker. Therefore, proteins of lung lavage were compared with medium and cellular proteins from type II cell cultures using SDS/polyacrylamide gels. Examination of the cell-free saline lavage of rat lungs

Fig. 1. Coomassie Blue-stained SDS/polyacrylamide gel of cell medium, lavage, lamellar bodies and sera. SDS-PAGE was performed on discontinuous polyacrylamide gels as described in the Materials and methods section. Gels (a) and (i) contain the following molecular weight markers: *E. coli* RNA polymerase β' subunit, M_r 165 000 and β subunit, M_r 155 000 (β, β'); bovine serum albumin, M_r 68 000 (BSA); soya-bean trypsin inhibitor, M_r 21 500 (TI); cytochrome *c*, M_r 12 500 (cyt.). Gel (b) contains serum-free medium from a primary culture of type II cells. At 36 h after the cell isolation, MEM-D/10% DCS medium was replaced with serum-free MEM-D. After a further 24 h, this medium was removed, freeze-dried, dissolved in sample buffer and electrophoresed. Gel (c) contains a sample of crude surfactant prepared from lavage as described in the Materials and methods section. Gel (d) is the material that collects at the organic/aqueous interface when lavage is extracted with chloroform. Gels (e) and (f) are two separate lamellar body preparations obtained by the method of Duck-Chong (1978). Gel (g) contains rat serum proteins. Gel (h) contains DCS. The positions of HS1 and HS2 are indicated by the upper and lower arrows respectively.

disclosed several Coomassie Blue-stained protein bands in addition to albumin (Fig. 1, gel c). Type II cell extracts (Fig. 2) and serum-free medium from Type II cell cultures (Fig. 1, gel b; Fig. 2) contain at least two proteins (HS1 and HS2) also found in lavage. The proteins common to lavage and cultured cells, which are hypothesized to be lung-specific, are not found in rat serum (Fig. 1, gel g) or in calf serum (Fig. 1, gel h). Thus these proteins are probably not contaminants derived from serum.

Lamellar inclusion bodies are considered an identifying characteristic of type II cells and are the site of storage of pulmonary surfactant before its secretion (Williams & Mason, 1977). Association of the presumptive type II cell-specific proteins with lamellar bodies would thus lend further support to the supposition that they are products of type II pneumocytes. Lamellar bodies isolated from intact lungs contain the protein bands in question (Fig. 1,

Fig. 2. SDS/polyacrylamide gel of type II cells and medium

Samples of cells (C) and conditioned medium (M) were electrophoresed on gradient gels as described in the Materials and methods section. At 36 h after cell isolation, MEM-D + 10% DCS was removed and replaced with serum-free MEM-D modified to contain $8.6 \mu\text{M}$ -[^{14}C]phenylalanine (sp. radioactivity 115 Ci/mol). After a further 24 h the medium was removed, dialysed and freeze-dried. The cell monolayer was rinsed several times with saline, then dissolved in SDS sample buffer. The positions of molecular-weight standards are indicated, as are the positions of HS1 and HS3. (a), Coomassie Blue-stained gel; (b), autoradiograph of gel in (a).

gels e and f). The distribution of these molecules is thus consistent with that expected for surfactant-associated apoproteins.

Synthesis of HS1 and HS2, and HS3 proteins

A demonstration that the proteins shown in Fig. 1 are synthesized by type II cells in culture would preclude a serum source and would further support the conclusion that these molecules are specific markers for type II cells. As shown in the autoradiograph in Fig. 2(b), [^{14}C]phenylalanine is incorporated by type II cells into a medium protein that corresponds to the Coomassie Blue-stained band designated HS1 (Fig. 2a). HS1, with an apparent M_r of $39\,900 \pm 2100$, contains 10–15% of the radioactivity incorporated into proteins in the medium. HS2 (apparent M_r of 12 000) is not consistently detected; when present, it contains radioactivity. In addition, a highly radioactive band of M_r 47 000

(HS3) is present in the autoradiograph (Fig. 2b). This protein contains between 15 and 25% of the total radioactivity in the gel; no distinct corresponding band is observed in Coomassie Blue-stained gels (Fig. 2a), suggesting that this protein has an unusually high specific radioactivity. A newly synthesized protein, tentatively identified as HS1 (see below), is also present in type II cellular proteins. HS2 cannot be detected in cell lysates because of the many proteins in its molecular-weight range, nor is a radioactive band comparable with HS3 observed (Fig. 2).

The incorporation of [^{14}C]phenylalanine into monolayer-associated (cellular) proteins and into total proteins free in the culture medium is linear with time for at least 10h, suggesting that the cells are stable under these conditions. The radioactivity in medium proteins amounts to $3.47 \pm 0.34\%$ ($n=34$; four separate isolations) of the radioactivity found in cellular proteins. In the absence of information about precursor specific radioactivity, the rate of synthesis of these proteins cannot be calculated (Rannels *et al.*, 1982). Militating against cell death as the source of proteins in the medium is the dissimilarity between the SDS/p.a.g.e. profiles either of total proteins or of newly synthesized proteins (Fig. 2) from the cell monolayer and from the medium.

Because a major purpose of these studies was to identify a marker of type II cell function in the intact lung, we sought to demonstrate synthesis of HS1, HS2 and HS3 in perfused lungs. After preliminary experiments with cultured type II cells confirmed that [^{35}S]methionine was incorporated into HS1 and HS2 (results not shown), rat lungs were perfused for 3h with Krebs/Henseleit bicarbonate buffer containing $1.34 \mu\text{M}$ -methionine at a specific radioactivity of 15 Ci/mmol. HS1 is clearly detected in SDS/polyacrylamide gels (Fig. 3) of whole-lung extracts and of lamellar bodies from these lungs. After 3h of perfusion, the specific radioactivity of HS1 recovered from the lungs by saline lavage remained below the level of detection.

Fig. 3. Incorporation of [^{35}S]methionine into lung homogenate and lamellar bodies

Rat lungs were perfused *in situ* with Krebs–Henseleit bicarbonate buffer containing 4.5% (w/v) BSA, 5.6 mM-glucose, normal plasma levels of 19 amino acids and $1.34 \mu\text{M}$ -[^{35}S]methionine (sp. radioactivity 15 Ci/mmol) for 3h (Watkins & Rannels, 1979). Lamellar bodies were prepared by the methods of Duck-Chong (1978), and samples of the initial lung homogenate (H) and lamellar-body fraction (LB) were electrophoresed on a discontinuous-gradient SDS/polyacrylamide gel as described in the Materials and methods section and autoradiographed. The autoradiographs were then scanned with a Zeineh densitometer. The position of HS1 was identified on the basis of molecular-weight standards and a type II cell lysate.

cells were labelled with [^{14}C]phenylalanine. HS1 and HS3 are not synthesized and secreted by any of these cell types (Fig. 4), as shown by the absence of [^{14}C]phenylalanine-containing proteins of the appropriate molecular weight in samples of medium taken from these cultures.

Characterization of HS1

The principal evidence presented thus far that the Coomassie Blue-stained protein bands from type II cells, their medium and lung lavage are the same molecule lies in the similarity of their apparent molecular weights. HS1 from these sources was therefore compared by assessing several of its biochemical characteristics.

The amino acid composition of HS1 from cells, medium and lavage does not differ by Spearman rank correlation, χ^2 analysis or linear regression tests (Siegal, 1956). The composition observed, in residues per 1000 residues recovered, is: Asx, 97.7 ± 9.7 ; Thr, 49.1 ± 5.8 ; Ser, 81.9 ± 34.4 ; Glx,

Specificity of HS1 to type II cells

The cell cultures used in the present experiments are enriched for type II pneumocytes, but remain contaminated by other cell types. Macrophages, which constitute 5–15% of the total cell number, are the major identifiable contaminant. Fibroblasts generally represent less than 1–2% of the total, and some ciliated cells (up to 1%) can be identified by phase-contrast microscopy; other epithelioid cells cannot be distinguished readily from lamellar body-depleted type II cells. To determine whether HS1, HS2 or HS3 might be synthesized by cells other than type II, cultures of rat fibroblasts, rat pulmonary alveolar macrophages and bovine aorta endothelial

Fig. 4. Incorporation of [^{14}C]phenylalanine into proteins in medium from various cell types

Cultures of cells were incubated in serum-free MEM-D containing [^{14}C]phenylalanine ($8.6\mu\text{M}$; sp. radioactivity 115Ci/mol) for 24 h. The medium was dialysed to remove free [^{14}C]phenylalanine and other low-molecular-weight solutes, freeze-dried and analysed by SDS/p.a.g.e. as described in the Materials and methods section. Autoradiographs prepared from the gels were scanned with the Zeineh densitometer to generate these profiles. The positions of HS1 and HS3 are indicated. (A), primary type II cell culture obtained as described in the Materials and methods section; (B), rat fibroblasts; (C), confluent monolayer of freshly prepared bovine aorta endothelial cells (Stalcup *et al.*, 1979); (D), rat

125.0 ± 20.2 ; Gly, 79.7 ± 26.9 ; Pro, 67.7 ± 4.6 ; Ala, 86.0 ± 5.6 ; Val, 49.4 ± 4.7 ; Cys, 6.4 ± 3.0 ; Met, 2.6 ± 2.6 ; Ile, 44.4 ± 8.9 ; Leu, 81.8 ± 8.8 ; Tyr, 29.1 ± 5.2 ; Phe, 41.2 ± 6.8 ; Lys, 64.1 ± 9.8 ; His, 23.1 ± 1.9 ; Arg, 54.7 ± 8.9 ($n = 6$). The amino acid composition of bovine serum albumin eluted from these gels (results not shown) corresponds well with published values (Peters, 1975).

Fig. 5. Two-dimensional-gel electrophoresis of medium from type II cells

Type II cells were cultured in serum-free MEM-D containing $8.6\mu\text{M}$ -[^{14}C]phenylalanine (sp. radioactivity 115Ci/mol) for 21 h. The medium was then subjected to isoelectric focussing in cylindrical gels ($3\text{mm} \times 115\text{mm}$) by the methods of O'Farrell & O'Farrell (1976). The focused gel was equilibrated for several hours with three changes of SDS-gel sample buffer, then mounted in agarose across the top of a discontinuous-gradient SDS/polyacrylamide gel (see the Materials and methods section) and electrophoresed. The gel was stained with Coomassie Blue (a) and autoradiographed (b).

pulmonary alveolar macrophages, obtained by saline lavage of rat lungs as described in the Materials and methods section.

SDS/polyacrylamide gels were treated with several stains in addition to Coomassie Blue. Autoradiographs of the stained gels allowed unambiguous identification of the locations of HS1, HS2 and HS3. Neither PAS (Fairbanks *et al.*, 1971) nor Alcian Blue (Wardi & Michos, 1972) stains a band that corresponds to HS1 or HS3. Gels were also treated with Gelcode reagent (Upjohn Diagnostics AgNO₃ stain), which stains related proteins a similar colour (Sammons *et al.*, 1981). HS1 from all three sources appears as a deep rust band in these gels.

Additional evidence that HS1 is a single protein, rather than a composite of several proteins of similar molecular weight, was obtained from two-dimensional gel electrophoresis by the method of O'Farrell & O'Farrell (1976). In cell lysates, culture medium preparations and lung lavage material, a single Coomassie Blue-stained spot of M_r 39 900 is observed; the absence of other detectable stained spots at this molecular weight rules out significant contamination of HS1 with other proteins in one-dimensional gels. Furthermore, when two-dimensional gels of [¹⁴C]phenylalanine-labelled cell preparations are autoradiographed, the protein of M_r 39 900 corresponds to the most intense spot of film darkening. Thus, HS1 appears to be a single polypeptide of apparent M_r 39 900, with an isoelectric point of 6.3 (Fig. 5). In contrast, HS3, which is more diffuse on autoradiographs of SDS/polyacrylamide gels (Fig. 2), appears as a chain of spots after electrophoresis in the second dimension.

Table 1. *Effect of reduction on apparent molecular weights of the major proteins in lung lavage*

A crude surfactant preparation, obtained by centrifugation of saline lavage of rat lungs as described in the Materials and methods section, was dissolved in 5% (w/v) SDS/10% (v/v) glycerol/62.5 mM-Tris/HCl (pH 6.8) at 100°C for 3 min. Samples were electrophoresed (see the Materials and methods section) on 7.5% acrylamide resolving gels. After staining and destaining of the gels, four bands were cut out, reduced by incubation with β -mercaptoethanol and re-electrophoresed on 7.5% acrylamide gels. In both gels, the same set of reduced molecular-weight standards was used to determine the apparent molecular weights of the unknown proteins. Re-electrophoresis in the presence of β -mercaptoethanol of HS1 and BSA from reduced samples does not alter their migration.

Protein band	Apparent molecular weight	
	Non-reduced conditions	Reduced conditions
A	270 000 $\pm$ 21 500	54 700 $\pm$ 1100
B	71 100 $\pm$ 5000	87 800 $\pm$ 1900
C (BSA)	52 900 $\pm$ 1700	72 000 $\pm$ 1000
D (HS1)	33 700 $\pm$ 5100	37 200 $\pm$ 3300

The gels shown in Figs. 1–5 contain samples that were reduced with β -mercaptoethanol in the presence of SDS before electrophoresis. Several workers (King & MacBeth, 1979; Sueishi & Benson, 1981) reported that the unreduced surfactant apoprotein is a dimer of M_r 72 000. To determine whether unreduced HS1 is a dimer, portions of lavage dissolved in SDS sample buffer lacking β -mercaptoethanol were electrophoresed. The major Coomassie Blue-stained bands were cut from the gel, and the individual proteins were eluted into sample wells containing β -mercaptoethanol on a second gel. The eluted proteins, now reduced, were re-electrophoresed. The apparent molecular weights of these proteins before and after reduction are given in Table 1. The M_r 71 100 protein (B) in the unreduced samples (the presumptive dimer of HS1) appears as a band of M_r 87 800 after reduction. An M_r 52 900 protein (C) from the unreduced sample corresponds to BSA (M_r 72 000) after reduction. The apparent molecular weight of HS1 (D) is not altered by reduction. The effectiveness of this technique to dissociate multimers is demonstrated by the first protein (A) in Table 1. HS1 thus does not appear to arise from reduction of a disulphide-bonded dimer. The apparent molecular weights of HS2 and HS3 are also unaffected by reduction (results not shown).

Discussion

Previous studies of protein metabolism in lung examined regulation at the whole-tissue level. In contrast, studies of the turnover of individual proteins allow investigation of protein metabolism in different cell types *in vitro* and *in vivo*. Proteins for such studies should (1) be specific to a single cell type; (2) be readily identified and quantified; (3) have a relatively rapid rate of turnover; and (4) have a known function, which would suggest physiological regulatory mechanisms. Surfactant-associated proteins appear to meet these criteria for type II cells. Three proteins, designated HS1, HS2 and HS3 have been identified for further study.

The isolated type II pneumocyte preparation used here is a primary culture enriched to 60–95% type II cells. Cultures were studied at 36–72 h after isolation for several reasons. First, it was important to permit the cells to recover from proteinases used in the isolation. Finkelstein & Mavis (1979) reported that exposure to trypsin disrupts the distribution of marker enzymes in lung cells. Trypsin is also known to remove cell surface proteins (Kono, 1969). Comparison of cells prepared using dispase (a bacterial neutral proteinase) or elastase (Dobbs & Mason, 1979), two proteinases reported to be less toxic than trypsin, with those prepared by trypsin treatment revealed no differences in yield, morphology, viability, purity or rates of protein

synthesis in our hands (results not shown). These criteria are, however, less sensitive than the sub-cellular enzyme distribution examined by Finkelstein & Shapiro (1982); further studies of the effects of different tissue digestion procedures on HS1 synthesis and secretion are, therefore, required. Second, the apparent purity of the type II cell preparations (determined by light microscopy) increases after 24–36 h in culture. This may reflect differential survival or attachment of cells to the culture vessels, or an increase in size and/or number of cytoplasmic granules with time (Mason & Williams, 1977).

The evidence presented suggests that HS1, HS2 and HS3 are type II cell-specific. These proteins are not synthesized and secreted by the cell types that are the most likely contaminants of the primary cultures. Although King & Martin (1980) reported finding surfactant apoproteins in macrophages, we found no evidence for secretion of newly synthesized HS1, HS2 or HS3 to the medium by cultures of lavage-derived rat pulmonary alveolar macrophages. This difference may reflect a role for macrophages in the metabolism of surfactant after its secretion (King & Martin, 1980). Similarly, media from cultures of fibroblasts and endothelial cells did not contain newly synthesized protein of the molecular weight of HS1.

Evidence that 'HS1' found in lavage, conditioned medium and cell monolayers is the same protein is not conclusive. Proteins from these sources migrate identically in one-dimensional SDS/polyacrylamide gels (see, e.g., Figs. 1 and 2) and appear to have the same isoelectric point in two-dimensional gels. None of proteins stain with PAS or Alcian Blue. Gelcode AgNO₃ staining (Upjohn Diagnostics) reveals a deep rust band of M_r 39 900 in all three preparations. Amino acid composition data did not confirm the identity of HS1 from the three sources because, although statistical analysis shows them to be similar, they are indistinguishable in composition from total lung proteins or from bovine serum albumin. Preliminary peptide mapping analysis of chymotryptic digests (Cleveland *et al.*, 1977) suggests that the primary sequences of these proteins are similar (results not shown). Further confirmation is being sought by use of additional proteinases for peptide mapping and by immunological means. Co-migration of a single Coomassie Blue-stained and heavily-labelled protein of M_r 39 900 on two-dimensional electrophoresis renders significant contamination of this band on one-dimensional gels unlikely; the correspondence of both molecular weight and isoelectric point of HS1 from lavage, cells and medium further supports the conclusion that they are the same protein.

HS1 was initially selected for study because it met the criteria outlined for a useful type II cell-specific

protein. During subsequent characterization of HS1, it became clear that it may correspond to the surfactant apoprotein reported by several other investigators (King *et al.*, 1977; Sawada & Kasaiwamata, 1977; Katyal & Singh, 1979; Sueishi & Benson, 1981). The apparent molecular weights of these proteins are similar, as is their distribution. The amino acid composition of HS1 is similar to those reported by King *et al.* (1977) for dog, by Sueishi & Benson (1981) for dog and rat, and by Bhat-tacharyya & Lynn (1979) for the 36 000- M_r protein from lavage of pulmonary alveolar proteinosis patients. Hydroxyproline, however, is not detected in HS1, distinguishing it from the proteins of pulmonary-alveolar-proteinosis lavage. Although the amino acid composition of HS1 is not significantly different from that of these other surfactant-associated proteins, no conclusions may be drawn from this comparison, in the light of their similarity to total lung proteins and to BSA. HS1 is not extracted into organic solvents; rather, it concentrates at the aqueous/organic interface in chloroform/water extracts. HS2, which is similar in molecular weight to apoprotein B of King *et al.* (1977) and the surfactant protein of Phizackerley *et al.* (1979), does not partition into the organic solvent phase, nor does HS3. In a number of features, HS1 differs from other surfactant-associated proteins. It does not appear to be a glycoprotein, as determined by staining with PAS or Alcian Blue, nor does it appear to be a disulphide-bonded dimer. The extent of glycosylations of HS1, HS2 and HS3 may be too low, however, to be detected on gels that have not been overloaded.

The protein most likely to represent a dimer of HS1 (protein B, Table 1) is not dissociated by treatment with reducing agents in contrast with the observations of King & MacBeth (1979) and Sueishi & Benson (1981). It is possible that the M_r -36 000 protein is the product of proteolytic cleavage of a larger precursor; if the M_r -72 000 material from the unreduced sample is intact, reduction might be without effect on its apparent molecular weight. Thus, confirmation of the identity or lack thereof between HS1 and apoprotein A (King & Martin, 1981) and other similar surfactant apoproteins and of HS2 with apoprotein B (King & Martin, 1981) must await immunological testing.

Although the present report focuses primarily on HS1, there are at least two other secreted proteins (HS2 and HS3) the metabolism of which can be studied and compared with HS1. A precursor-product relationship may exist among these proteins, although King & Martin (1981) report that there is no simple relationship between apoproteins A and B. HS3 is particularly interesting because of its high specific radioactivity. Although it is the most dense band on autoradiographs of pulse-labelled medium

samples, no corresponding Coomassie Blue-stained band can be located in the gels. This high specific radioactivity could be due to an exceptionally high phenylalanine content, to some characteristic that renders it non-reactive to Coomassie Blue staining or to a rapid fractional rate of synthesis. Further experiments are required to distinguish among these alternatives.

It is important to recognize that HS1, as well as HS2 and HS3, are secreted proteins; the regulation of their synthesis and turnover may differ from that of proteins retained in the cells. Furthermore, in studies of their accumulation in the medium, regulation of their secretion must be taken into account. Peavy *et al.* (1978) showed that alloxan-induced diabetes reduces albumin synthesis in rat livers to a greater extent than synthesis of retained proteins or other secreted proteins. Future experiments might compare the metabolism of retained proteins with total secreted proteins and with HS1, HS2 and HS3.

A marker of differentiated function for the type II pneumocyte provides a monitor for the metabolic integrity of type II cells in culture. The effects of culture conditions and other factors can now be compared with respect to their effects on HS1 synthesis, for example, to evaluate their effectiveness for maintenance of differentiation. In this way, culture conditions can be optimized and the processes of differentiation and de-differentiation may be followed.

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