lymerases in chloroplasts. If the two polymerases contain polypeptides of close molecular weights, the analytical tools described in this paper would not distinguish them. The solubilization and purification of RNA polymerase from chloroplasts has provided us with a method to study the detailed transcription mechanism of the chloroplast genome.

Registry No. RNA polymerase, 9014-24-8.

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Granular Pneumocytes in Primary Culture Secrete Several Major Components of the Extracellular Matrix†

Helene Sage,* Federico M. Farin, Gary E. Striker, and Aron B. Fisher

ABSTRACT: Primary cultures of rat alveolar type II epithelial cells (granular pneumocytes) produced several components of the pulmonary extracellular matrix. Fractionation by ion-exchange chromatography of radiolabeled protein secreted into the culture medium resulted in the partial purification of two of these components: fibronectin and type IV procollagen. Identification of these proteins by sodium dodecyl sulfate–polyacrylamide gel electrophoresis was confirmed by radioimmune-precipitation studies with affinity-purified antibodies. Thrombospondin, a platelet α-granule protein that was recently shown to be secreted by endothelial and other mesenchymally derived cells and may be involved in platelet aggregation, was, in addition, purified by elution from diethylaminoethylcellulose with 0.5 M NaCl. The levels of these secreted proteins were measured by radioimmune precipitation. Of the total radiolabeled culture medium protein secreted during a 24-h period by the granular pneumocytes, fibronectin, type IV procollagen, and thrombospondin represented 3–15%, 2%, and 3%, respectively. The biosynthesis, by alveolar epithelial cells, of proteins that constitute or are closely associated with the alveolar basement membrane implies that this structure is at least partially derived from the cells themselves. Furthermore, it suggests that the type II epithelial cell is involved in pulmonary cytodifferentiation, in lung morphogenesis and repair, and in certain interstitial lung disorders in which derangement of the extracellular matrix occurs.

More than 40 different cell types have been described in adult human lung [for a review, see Kuhn (1976)]. This cellular heterogeneity reflects not only the structural diversity of the lung architecture in toto but also the metabolic and functional properties of this organ as well. In recent years, an increased understanding of the primary gas-exchanging unit, the pulmonary alveolus, has been achieved by approaches at the cellular level. Studies dealing with alterations in cell populations and connective tissue proteins in the interstitial lung disorders have, in particular, established that an important relationship exists between alveolar function and its cellular/extracellular matrix composition [for a review, see Crystal et al. (1978)].

The normal adult alveolus contains two major cellular populations: parenchymal and inflammatory/immune effector cells. The latter group is comprised mainly of macrophages (80–90%) and lymphocytes (Crystal et al., 1978). There are four major types of alveolar parenchymal cells. Interstitial mesenchymal connective tissue ("fibroblast-like") cells are found principally in alveolar septae and comprise approximately 39% of the total parenchymal cell population (Kuhn, 1976; Crystal et al., 1978). The pulmonary alveolus is lined with type I and type II epithelial cells, representing 8% and 14% of the parenchymal cell types, with the remainder contributed by capillary endothelial cells (Crystal et al., 1978).

† From the Departments of Biochemistry (H.S.) and Pathology (F.M.F. and G.E.S.), School of Medicine, University of Washington, Seattle, Washington 98195, and the Department of Physiology, University of Pennsylvania School of Medicine, Philadelphia, Pennsylvania 19104 (A.B.F.). *Received December 10, 1982.* This work was supported in part by National Institutes of Health Grants HL 18645, AM 11248, HL 27218, GM 29853, GM 07187, GM 21797, and HL 19737 and by Juvenile Diabetes Foundation Grant 79R134. H.S. is an Established Investigator of the American Heart Association, with funding contributed in part by the Washington Heart Association.

* Address correspondence to this author at the Department of Biological Structure, School of Medicine, University of Washington, Seattle, WA 98195.

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Alveolar epithelial cells manifest several structural and biosynthetic specializations that have been correlated with their respective roles in permeability, repair and morphogenesis, and lung disease. Both type II cells (also referred to as granular pneumocytes) and type I cells (membranous pneumocytes) are derived from the endoderm of the embryonic foregut. In the normal adult lung, they occur in a ratio of 1.5–2:1 (type II:type I), but the squamous type I cell covers approximately 95% of the alveolar epithelial surface (Kuhn, 1976; Crystal et al., 1978). Although relatively few studies have been performed on type I cells to date, most investigators agree that these cells are highly specialized for efficient gaseous exchange, since they are flattened and contain few organelles, exhibit reduced metabolic activity and oxygen consumption, and do not proliferate (Kuhn, 1976). In contrast, type II epithelial cells are cuboidal, metabolically active, and capable of undergoing cell division. A distinguishing feature of type II cells is the presence of lamellar bodies, the cytoplasmic organelles that store surfactant.

Advances in cell-culture techniques have enabled several investigators to establish essentially pure cultures of type II granular pneumocytes (GP)[1] from rat (Fisher et al., 1980; Dobbs et al., 1980), fetal cat (Kniazeff et al., 1976), rabbit (Diglio & Kikkawa, 1977; Finkelstein & Shapiro, 1982), and fetal human (Tanswell & Smith, 1980). These studies have resulted in rigorous criteria for the identification of GP in vitro and, in addition, have provided important data on the metabolic pathways, oxygen consumption, various enzyme levels, and fatty acid/phospholipid biosynthesis of these cells. Investigation of extracellular matrix components produced by GP in vitro, however, has not been performed.

It has been shown that lung fibroblasts in culture produce primarily types I and III collagen (Crystal et al., 1978; Alitalo, 1980). Other proteins that are secreted by cells and incorporated into an extracellular matrix in vitro include fibronectin and thrombospondin (Alitalo et al., 1980; Raugi et al., 1982). Fibronectin has also been identified as a component of the epithelial fluid of the normal lower respiratory tract (Rennard & Crystal, 1982). Elevated levels of this protein in the bronchopulmonary lavage fluid of patients with interstitial lung disease support the postulate that disruption of the pulmonary extracellular matrix is associated with some aspect of this group of heterogeneous disorders (Rennard & Crystal, 1982). In addition, glycoproteins containing both collagenous and noncollagenous sequences that have been recovered from the lavage fluid of patients with alveolar proteinosis are most likely released from the alveolar extracellular matrix and, possible, from the granular pneumocytes as well (Bhattacharyya, 1981; Sahu et al., 1980).

Because the alveolar and capillary basement membranes in lung are at least partially derived from the epithelial and endothelial cells that are adjacent to them, we have studied the biosynthetic activity and interactions of these cell types in vitro. A preliminary report described the synthesis of certain basement membrane components by rat alveolar type II epithelial cells (GP) in culture (Farin et al., 1981). We now have defined a secretory phenotype for these cells with respect to certain components of the extracellular matrix. In addition to fibronectin and basement membrane collagens, GP also secreted thrombospondin, a high molecular weight glycoprotein previously characterized in platelet α-granules and endothelial cells (McPherson et al., 1981; Mosher et al., 1982).

Materials and Methods

Cell Culture and Metabolic Labeling. Granular pneumocytes (GP) were isolated from the lungs of pathogen-free Sprague-Dawley rats, as described by Fisher et al. (1980). The procedure entailed serial trypsinization of minced tissue, followed by filtration through Nylon, to produce a crude cell mixture. GP were purified from this suspension by sequential plating in plastic flasks for 3 and 21 h, respectively. After 21 h, the attached cells were removed by EDTA/trypsin treatment and were plated at approximately 5×10^6 cells/75-cm^2 flask in modified MEM containing 10% fetal calf or newborn calf serum. All the GP used in these experiments were prepared according to this method and did not undergo further passage.

The only cells recognized as contaminants in these preparations of GP were alveolar macrophages—controls for the biosynthetic contribution of these cells were performed as indicated below. Other cell types to be considered as possible contaminants are fibroblasts, endothelial cells, ciliated bronchiolar epithelium, and nonciliated bronchiolar epithelium. The presence of fibroblasts was excluded due to the absence of types I and III collagen in the culture media. On morphologic grounds, the presence of ciliated bronchiolar cells can similarly be excluded. Nonciliated bronchiolar epithelial cells, if present, would occur with a very low frequency; their rate of collagen secretion would not be expected to exceed that of the alveolar epithelial cells (GP). Endothelial cells were not detected by morphologic criteria and would a priori not be expected to be retained under the culture conditions used in these experiments. In addition, the endothelial collagen type EC (Sage, 1982) was not observed in the GP culture medium.

Alveolar macrophages were isolated from the crude lung cell mixture as previously described (Fisher et al., 1980).

Nearly confluent 75-cm^2 flasks of GP were preincubated in serum-free DMEM (Grand Island Biological Co.) containing 50 μg/mL sodium ascorbate and 64 μg/mL β-APN. After 1 h, fresh preincubation medium containing 50 μCi/mL L-[2,3-$^3H_2$]proline (35 Ci/mmol; New England Nuclear) or L-[5-^{3}H]proline (29 Ci/mmol; Amersham/Searle Co.) was added (4 mL/flask), and the cells were incubated for 18–24 h at 37 °C in an atmosphere of 95% air/5% CO_2 (Sage et al., 1979). Alternatively, a mixture of L-[2,3-$^3H_2$]proline and L-[2-^{3}H]glycine (23 Ci/mmol; Amersham/Searle Co.) (25 μCi/mL of each isotope) was used for metabolic labeling in preparations that were performed on a larger scale. In all cases, these amino acids were omitted from the labeling media prior to introduction of the isotope.

Fractionation of Culture Medium Protein. Culture medium was removed from cells directly into protease inhibitors, resulting in a final concentration of 0.2 mM $PhCh_2SO_2F$, 10 mM MalNEt, and 2.5 mM EDTA, and was subsequently clarified by centrifugation according to previously described procedures (Sage et al., 1979). Radiolabeled culture medium proteins were then analyzed by precipitation with 10% trichloroacetic acid in the presence of 50 μg/mL pepstatin (Peninsula Laboratories, San Carlos, CA), followed by $NaDodSO_4$–polyacrylamide gel electrophoresis (Sage & Bornstein, 1982). Alternatively, proteins were precipitated with 50% ammonium sulfate (w/v) and were fractionated by chromatography on

[1] Abbreviations: MEM, minimal essential medium; $NaDodSO_4$, sodium dodecyl sulfate; DTT, dithiothreitol; MalNEt, *N*-ethylmaleimide; $PhCH_2SO_2F$, phenylmethanesulfonyl fluoride; EDTA, ethylenediaminetetraacetic acid; β-APN, β-aminopropionitrile fumarate; w/v, weight to volume ratio; DEAE, diethylaminoethyl; GP, granular pneumocytes; BSA, bovine serum albumin; Tris, tris(hydroxymethyl)aminomethane; Tris–saline, 0.15 M NaCl and 50 mM Tris-HCl, pH 7.5; CIG, cold-insoluble globulin (plasma fibronectin); TS, thrombospondin; DMEM, Dulbecco's modified Eagle's medium; ELISA, enzyme-linked immunosorbent assay; IgG, immunoglobulin G.

DEAE-cellulose (DE-52, Whatman) at 4 °C, as previously described (Sage et al., 1979). Elution of bound material was performed in 6 M urea/50 mM Tris-HCl buffer, pH 8.0, containing 0.2 mM $PhCH_2SO_2F$ and 2.5 mM EDTA with a linear gradient from 0 to 200 mM NaCl over a total volume of 400 mL. Appropriate column fractions were pooled in the presence of 0.5 μg/mL pepstatin, dialyzed against 0.1 N acetic acid, lyophilized, and analyzed by $NaDodSO_4$–polyacrylamide gel electrophoresis.

Radiolabeled cell layers were sonicated in 0.5 N NH_4OH containing 0.2 mM $PhCH_2SO_2F$. After centrifugation (20000*g*), soluble proteins were precipitated in 10% trichloroacetic acid and were analyzed by $NaDodSO_4$–polyacrylamide gel electrophoresis (Sage et al., 1979). Protein concentration in the cell layers was determined by the method of Lowry et al. (1951).

Immunochemistry. Samples of culture medium or fractions pooled from DEAE-cellulose chromatography were dialyzed into Tris–saline containing 0.2 mM $PhCH_2SO_2F$ and 2.5 mM EDTA at 4 °C. Radioimmune precipitation utilizing a double-antibody technique was performed as previously described (Sage et al., 1979). Radioimmune titration curves were generated by adding increasing amounts of the particular antisera to equal aliquots of radiolabeled samples, followed by 200 μL of sheep anti-rabbit IgG (Miles Laboratories, Inc., Elkhart, IN). In all instances, the radioimmune precipitate was analyzed by $NaDodSO_4$–polyacrylamide gel electrophoresis to determine the specificity of the reaction.

Antibodies to human CIG (Sage et al., 1979), human platelet TS (Raugi et al., 1982), and human placental type IV collagen (Sage et al., 1981) were raised in rabbits, and serum IgG fractions were affinity purified by selective absorption on Sepharose CL-4B to which the purified antigen had been coupled. These antibodies were shown to be specific by sensitive, quantitative ELISA and were free of contaminating activities toward other serum or matrix proteins (Raugi et al., 1982). In all cases, $NaDodSO_4$–polyacrylamide gel electrophoresis analysis of the remaining radioimmune supernate showed essentially complete removal of the antigenic protein. Control incubations with preimmune sera were also performed, and the cpm precipitated nonspecifically were subtracted from those obtained with specific antisera. These background levels were consistently less than 1% of the total cpm in the starting material.

Digestion of Cell Layers with Pepsin. All procedures were conducted at 4 °C. Flasks (14) of [^{3}H]proline- and [^{3}H]-glycine-labeled cells and their associated extracellular matrix were scraped with a rubber policeman into 0.5 N acetic acid containing 0.5 μg/mL pepstatin (2 mL/flask). Each flask was rinsed with an additional 2 mL of 0.5 N acetic acid, and the pooled extracts were homogenized in a Dounce homogenizer. The dispersed material was dialyzed against 0.5 N acetic acid and was lyophilized.

To the lyophilized cell layers (13 mg) was added 1.3 mL 0.5 N acetic acid and 1.3 mg of pepsin (Worthington No. 3319PM-30B955, 2828 units/mg), and the solution was stirred gently for 24 h. The digest was subsequently clarified in a microfuge (Beckman). Solid NaCl was added to the supernate to a final concentration of 10% (w/v); the solution was stirred gently overnight, and the precipitate was collected by centrifugation.

The 10% NaCl precipitate was subsequently resuspended in and dialyzed against Tris–saline. The cloudy precipitate that formed at this point was collected by centrifugation, dissolved in and dialyzed against 0.1 N acetic acid, and lyophilized. The components that were soluble in Tris–saline, as well as those remaining soluble after the 10% NaCl precipitation step, were dialyzed against 0.1 N acetic acid, lyophilized, and examined by $NaDodSO_4$–polyacrylamide gel electrophoresis.

Other Procedures. For determination of the level of synthesis of collagenous protein, bacterial collagenase digestion assays were performed on soluble fractions of culture media and cell layers (Sage et al., 1979). The percent of insoluble cell layer protein that was collagenous was estimated by hydroxyproline and proline analyses, assuming an equal content of these amino acids in collagen.

Hyp and Pro contents were determined by amino acid analysis of radiolabeled culture medium and cell layer protein after hydrolysis in 6 N HCl at 110 °C for 24 h, as previously described (Sage et al., 1979). Protein labeled with [5-^{3}H]-proline was used for measurement of 3-Hyp.

Proteins were analyzed by electrophoresis on discontinuous methylenebis(polyacrylamide) slab gels containing 0.5 M urea in the presence of $NaDodSO_4$, as described by Sage & Bornstein (1982). Protein-containing bands were detected by fluorescence autoradiography in dimethyl sulfoxide and 2,5-diphenyloxazole, followed by exposure to sensitized X-ray film (Sage et al., 1979). Bands were scanned in the linear range on a Joyce-Loebl scanning densitometer.

Results

Collagen and Total Protein Synthesis by GP in Vitro. Six separate preparations of GP were analyzed by metabolic labeling in vitro. These cultures contained from 85% to 95% GP, with the remainder comprised of alveolar macrophages. Neither capillary endothelial cells nor type I epithelial cells are retained under these particular culture conditions. Previous studies on GP prepared according to this methodology have established that the cells contain prominent lamellar bodies and exhibit >90% viability by erythrosin B exclusion (Fisher et al., 1980). In culture, these cells synthesized and secreted disaturated phosphatidylcholine and phosphatidylglycerol (Chander & Fisher, 1982). In addition, metabolic studies revealed active glycolytic and oxidative pathways that were indicative of intact cell membranes and the retention of cellular function during short-term culture (Fisher et al., 1980).

The incorporation of [^{3}H]proline into protein by GP was linear for up to 18 h. The levels of total protein synthesis over a 24-h period have been summarized in Table I. In comparison to several types of endothelial cells in vitro, on a per cell basis, GP exhibited significantly higher rates of [^{3}H]proline incorporation into protein that was subsequently secreted into the culture medium [25×10^6 dpm/10^6 GP (Table I) vs. $(1.2–2.4) \times 10^6$, 2.8×10^6, 0.4×10^6, and 4.6×10^6 dpm/10^6 aortic, venous, corneal, and capillary endothelial cells, respectively (Sage et al., 1981)]. It is often difficult to compare levels of protein synthesis among different cell types on the basis of ^{3}H dpm/mg of cell protein, however, as this number is a function of cellular age, degree of confluence, growth rate, and accumulation of extracellular matrix. Moreover, in this paper, we have analyzed only that fraction of cellular protein that was solubilized in 0.5 N NH_4OH. In the case of GP, there were few, if any, mitotic figures, and the cells represented primary cultures that were present in tissue-culture flasks for not more than 3–4 days from the time of initial plating to the termination of the labeling experiment.

Collagen comprised between 1% and 2% of the total protein synthesis in the medium and the cell layer (Table I). This estimate was based on a bacterial collagenase assay or, in the case of insoluble cell layer protein, on Hyp to Pro ratios ob-

Table I: Collagen and Total Protein Synthesis by Rat Alveolar Granular Pneumocytes in Vitro[a]

	μg of total protein[e]	^{3}H dpm (×10^{-6}) in total protein[f]	% collagen synthesis	Hyp/Pro (×100)[i]	3-Hyp/4-Hyp (×100)[i]
medium[b]	ND[j]	25.2	1.2[g]	2.1	7.7
cell layer[c]					
soluble	13.8	35.16	1.99[g]	1.2	ND
insoluble			0.82[h]	2.07	ND
cell layer[d]					
soluble	16.6	39.5	1.09[g]	0.43	12.3
insoluble			0.42[h]	1.07	23.3

[a] Measured by incorporation of [^{3}H]proline; cells were incubated with [5-^{3}H]proline or [2,3-$^3H_2$]proline (50 μCi/mL) for 24 h in serum-free DMEM supplemented with ascorbate and β-APN, as described under Materials and Methods. [b] Cells were labeled with [5-^{3}H]proline. [c] Cells were labeled with [2,3-$^3H_2$]proline. Cell layers were dispersed in and dialyzed against 0.5 N acetic acid, and the suspension was separated into soluble and insoluble fractions. [d] Cells were labeled with [5-^{3}H]proline. Cell layers were sonicated in 0.5 N NH_4OH and were separated into soluble and insoluble fractions. The soluble fraction was analyzed after trichloroacetic acid precipitation. [e] Per 10^6 cells, on the basis of Lowry determination of soluble cell layer protein (see Materials and Methods). [f] Per 10^6 cells, on the basis of trichloroacetic acid precipitation. [g] Based on bacterial collagenase digestion assay. [h] Based on Hyp and Pro analysis, assuming an equal content of these amino acids in collagenous proteins. [i] Calculated from amino acid analyses of radiolabeled proteins. [j] ND, not determined.

tained after hydrolysis and amino acid analysis of this fraction. The latter method, however, tends to be an underestimate of the actual proportion of collagenous protein, because it does not make provision for the lower Hyp to Pro ratios that are characteristic of procollagen. In comparison to other cell types in vitro, GP synthesized relatively low levels of collagen; e.g., bovine aortic endothelial cells secrete from 3% to 8% collagenase-sensitive protein into the culture medium when metabolically labeled under similar conditions as those described for GP (Sage, 1982), and bovine capillary endothelial cells secreted 49% collagenase-sensitive protein (Sage et al., 1981). This latter figure reflects more the secretory phenotype of fibroblasts and smooth muscle cells, which in culture often produce as much as 10-fold more collagen relative to total protein synthesis, than do epithelial cells (Sage & Bornstein, 1982).

In the GP culture medium, the ratio of 3-Hyp to 4-Hyp was found to be 0.077 (Table I). This level of 3-prolyl hydroxylation was similar to those that have been reported for type IV procollagen; however, it is not in itself a sufficiently rigorous criterion for identification of the collagen type secreted by GP. It was of interest that, in the cell layer, the 3-Hyp to 4-Hyp ratio (0.23) was significantly higher than that found for the medium procollagen, especially in the fraction that remained insoluble after sonication in 0.5 M NH_4OH (Table I). These ratios were very similar (±10% SEM) in three separate experiments with different preparations of GP. Duplicate or triplicate analyses within a given set produced variabilities of 2–4%, and the recovery after amino acid analysis ranged from 79% to 95% of initial cpm. Analysis of the isotope L-[5-^{3}H]proline alone showed a single peak in the position corresponding to Pro (data not shown).

Both the culture medium and cell layer proteins were initially analyzed by precipitation in trichloroacetic acid, followed by $NaDodSO_4$–polyacrylamide gel electrophoresis. As shown in Figure 1A, GP secreted into the culture medium two high molecular weight, disulfide-bonded components that, after reduction, exhibited electrophoretic mobilities similar to those

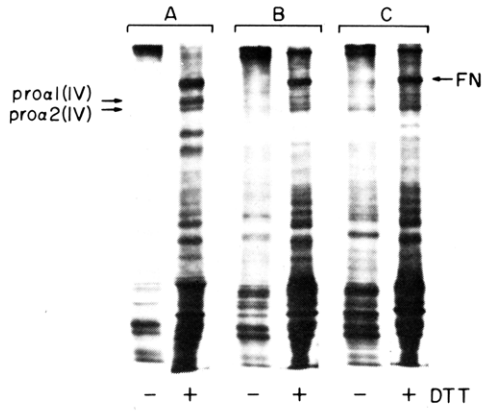

FIGURE 1: Synthesis and secretion of radiolabeled protein by granular type II pneumocytes from rat lung in vitro. Cells were incubated for 21 h in serum-free medium supplemented with sodium ascorbate and β-APN, containing [^{3}H]proline, as described under Materials and Methods. Protein was precipitated with 10% trichloroacetic acid. Radiolabeled proteins were resolved by $NaDodSO_4$–polyacrylamide gel electrophoresis under both reducing (+DTT) and nonreducing (–DTT) conditions and were visualized by fluorescence autoradiography. (A) Culture medium protein; (B) cell layer protein, labeled with [2,3-$^3H_2$]proline and solubilized in ammonium hydroxide prior to trichloroacetic acid precipitation; (C) as in (B) but label was [5-^{3}H]proline. Fibronectin (FN) and two type IV procollagen chains [proα1(IV) and proα2(IV)] are indicated.

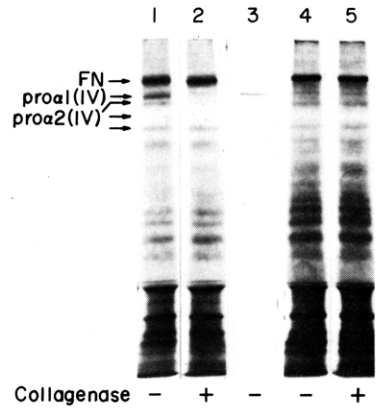

FIGURE 2: Synthesis of collagenous protein by granular pneumocytes in vitro. Cells were incubated under conditions described in the legend to Figure 1, with [2,3-$^3H_2$]proline. Identical aliquots of culture medium or cell layer protein were incubated with bacterial collagenase or in buffer without enzyme, as described under Materials and Methods. Radioimmune precipitation was performed on culture medium protein with affinity-purified rabbit IgG. Proteins were resolved by $NaDodSO_4$–polyacrylamide gel electrophoresis in the presence of 50 mM DTT and were visualized by fluorescence autoradiography. (Lane 1) Culture medium protein, incubated control; (lane 2) lane 1 after collagenase digestion; (lane 3) radioimmunoprecipitate of protein as shown in lane 1, with antibodies to type IV collagen; (lane 4) cell layer protein, incubated control; (lane 5) lane 4 after collagenase digestion. Fibronectin (FN) and two chains of type IV procollagen [proα1(IV) and proα2(IV)] are indicated. The positions of migration of the type I procollagen chains, proα1(I) and proα2(I), are shown by the two unlabeled arrows on left.

of fibronectin (M_r 225 000) and type IV procollagen (M_r 185 000 and 170 000). These tentative identifications were subsequently confirmed in further experiments that are described below. Fibronectin was also present in the cell layer (Figure 1B,C); however, identification of collagenous components in this fraction was difficult.

For facilitation of detection of (pro)collagens, both the culture medium and solubilized cell layer components were incubated with bacterial collagenase (Figure 2). The two reducible radiolabeled bands in the culture medium (see Figure 1A) were sensitive to this enzyme (Figure 2, lanes 1 and 2);

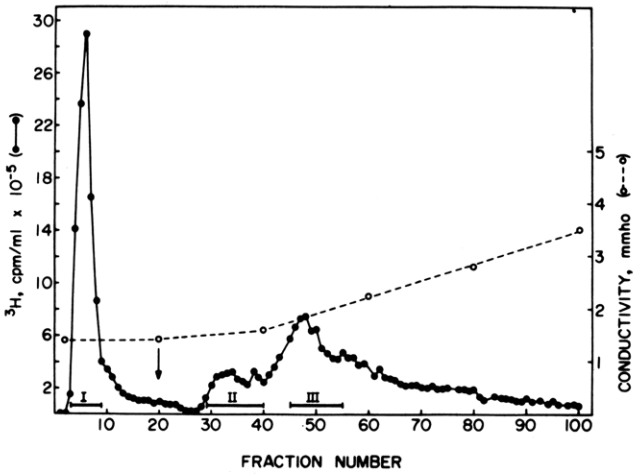

FIGURE 3: Fractionation of culture medium protein by chromatography on DEAE-cellulose. GP were incubated with [^{3}H]proline and [^{3}H]glycine for 18 h, and the culture medium proteins were initially precipitated with 50% ammonium sulfate. This fraction was chromatographed on DEAE-cellulose at 4 °C in 6 M urea, 50 mM Tris-HCl, 0.2 mM $PhCH_2SO_2F$, and 2.5 mM EDTA buffer, pH 8.0. Linear gradient elution (arrow) was from 0 to 200 mM NaCl in a total of 400 mL of buffer. Roman numerals indicate pooled fractions.

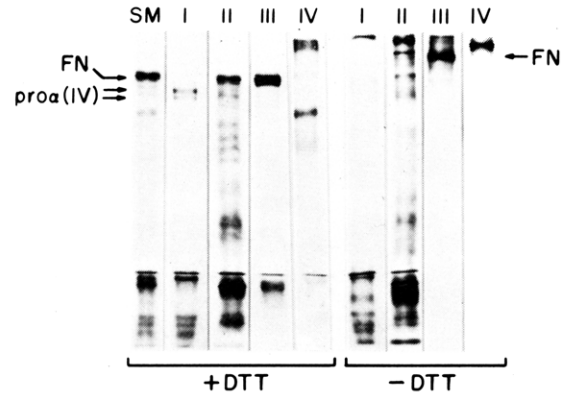

FIGURE 4: Analysis by $NaDodSO_4$–polyacrylamide gel electrophoresis of culture medium proteins after DEAE-cellulose chromatography. Fractions obtained from ion-exchange chromatography, as shown in Figure 3, were analyzed on 6%/10% composite slab gels in both the presence and absence of 50 mM DTT. Radiolabeled proteins were visualized by fluorescence autoradiography. Roman numerals refer to column fractions as shown in Figure 3. (IV) Material eluted with 0.5 M NaCl. (SM) Starting material (50% ammonium sulfate precipitate of culture medium proteins, prior to chromatography on DEAE-cellulose). Fibronectin (FN) and type IV procollagen chains have been identified.

no other proteins appeared to be digested in this fraction. When the culture medium was incubated with affinity-purified antibodies to procollagen or collagen types I, III, IV, and V, these two collagenase-sensitive bands were specifically precipitated only by rabbit anti-human type IV collagen IgG (lane 3). In addition, antisera to mouse type IV procollagen (a gift from Dr. G. R. Martin, NIH, Bethesda, MD) also precipitated this protein with very similar recoveries. These data therefore provide strong evidence that primary cultures of GP secrete type IV procollagen.

In contrast to the culture medium, collagenase-sensitive protein was not readily apparent in the GP cell layer fraction (Figure 2, lanes 4 and 5). Even though by both hydroxyproline content and collagenase assay some collagen was present in the cell layer (Table I), it would appear that $NaDodSO_4$–polyacrylamide gel electrophoresis analysis was not sufficiently sensitive to identify this protein.

Further examination of GP culture medium proteins was carried out by ammonium sulfate precipitation, followed by chromatography on DEAE-cellulose. As shown in Figure 3, a major proportion of incorporated radioactivity did not bind to the column (peak I). In addition, two peaks were eluted within the gradient (II and III). Analysis of these pooled fractions by $NaDodSO_4$–polyacrylamide gel electrophoresis is presented in Figure 4. Under the conditions used in these experiments, type IV procollagen does not bind to DEAE-cellulose (Sage & Bornstein, 1982). A high molecular weight component from GP culture medium was observed in the unbound fraction [Figure 4, lane I (−DTT)]; in the presence of DTT, two bands, corresponding in mobility to the proα1 and proα2 chains of type IV procollagen, were apparent [Figure 4, lane I (+DTT)]. Peak III (Figure 4) contained principally a component with mobilities corresponding to those of fibronectin [M_r 450000 (−DTT) and M_r 225000 (+DTT)]. Some of this protein was also present in peak II.

When the remainder of the applied cpm was eluted from the DEAE-cellulose with 0.5 M NaCl, a single component was recovered that migrated near the top of a 6% separating gel [Figure 4, lane IV (−DTT)]. Reduction using 50 mM DTT produced a protein of M_r 190000, although approximately 60% of the cpm remained in the higher molecular weight form [Figure 4, lane IV (+DTT)].

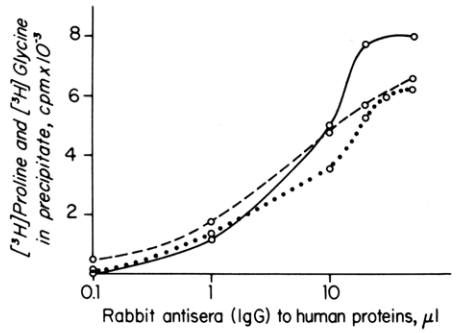

FIGURE 5: Radioimmune precipitation of proteins derived from GP culture medium. Fractions I, III, and IV from DEAE-cellulose chromatography (Figure 4) were incubated with increasing concentrations of rabbit antibodies to human type IV collagen, CIG, and thrombospondin, respectively. The radioimmune complex was subsequently precipitated with sheep anti-rabbit IgG and was measured by scintillation counting. (--) Fraction I, incubated with affinity-purified antibodies to human type IV collagen; (···) fraction III, incubated with affinity-purified antibodies to human CIG; (—) fraction IV, incubated with affinity-absorbed anti-human thrombospondin IgG.

Some of the proteins that were recovered after DEAE-cellulose chromatography were characterized further by radioimmune precipitation. Titration curves for DEAE-cellulose fractions I, III, and IV (see Figure 3 and 4) were produced from 0.01–50 μL of rabbit IgG directed against human type IV collagen, CIG and TS, respectively, as shown in Figure 5. These precipitates were analyzed by $NaDodSO_4$–polyacrylamide gel electrophoresis under both reducing and nonreducing conditions. In fractions I and IV, a single radioactive band was obtained that comigrated with CIG and TS protein standards, respectively (data not shown). The radioimmune precipitate obtained with antibodies to type IV collagen migrated as a doublet after reduction, as was shown in Figure 2 (lane 3).

The levels of type IV procollagen, fibronectin, and thrombospondin secreted by GP were estimated from radioimmune-precipitation data and from recoveries of radiolabeled protein after fractionation in ammonium sulfate and DEAE-cellulose chromatography (Table II). Approximately 2% of the protein in the culture medium that was precipitated by 50% ammonium sulfate corresponded to type IV procollagen (Table

Table II: Identification and Quantitation of Granular Pneumocyte Culture Medium Proteins by Ion-Exchange Chromatography and Radioimmune Precipitation[a]

fraction	total dpm ($\times 10^{-7}$)	% of starting material[f]	% of dpm in radioimmune precipitate[g]	% of protein in starting material[h]
(1) 50% ammonium sulfate precipitate[b]	32.2			
(2) DEAE, I[c]	10.9	34	5.5	1.86
(3) DEAE, II	3.2	10	ND[i]	ND
(4) DEAE, III[d]	8.1	25	11.0	2.75
(5) DEAE, IV[e]	6.4	20	15.5	3.10

[a] 60×10^6 cells were incubated with 25 μCi/mL each of [2,3-$^3H_2$]proline and [2-^{3}H]glycine for 18 h, in serum-free DMEM containing ascorbate and β-APN, as described under Materials and Methods. [b] Culture medium proteins were precipitated with 50% ammonium sulfate. This material was subsequently dissolved in and dialyzed against DEAE-cellulose chromatography buffer, prior to chromatography, as described in Figure 3. $NaDodSO_4$-polyacrylamide gel electrophoresis analysis of this fraction is shown in Figure 4 (SM). [c] As shown in Figure 4 (I) (contains type IV procollagen). [d] As shown in Figure 4 (III) (contains fibronectin). [e] As shown in Figure 4 (IV) (contains thrombospondin). [f] Based on dpm in 50% ammonium sulfate precipitate. Total recovery from DEAE-cellulose column was 89%. [g] See Figure 5 for details of radioimmune precipitation. [h] [(Column 4) × (column 3)] × 100. [i] ND, not determined.

II, column 5); this value agreed closely with that obtained from the experiments in which collagenase digestion was used to estimate total procollagen production (Table I, column 4). From the data in Table II, thrombospondin comprised approximately 3% of the radiolabeled culture medium protein (the average value from three separate preparations of GP was 3.75%). These values were in close agreement with the levels of type IV procollagen and thrombospondin in unfractionated culture medium that were estimated by scanning densitometry after $NaDodSO_4$–polyacrylamide gel electrophoresis (data not presented). However, a discrepancy was noted in the case of fibronectin (FN). By radioimmune precipitation, FN comprised 2.75% of the culture medium protein (Table II, column 5) (the average value from three separate preparations of GP was 3.0%). However, on the basis of scanning densitometry of the 50% ammonium sulfate precipitate before DEAE-cellulose chromatography (as shown in Figure 4, SM), fibronectin was approximately 15% of the total protein. Three explanations for this apparent difference are (1) incomplete precipitation of rat cellular FN by affinity-purified antibodies to human plasma CIG, (2) immunologic differences between CIG (plasma fibronectin) and cellular fibronectin, and (3) selective losses of fibronectin during chromatography, dialysis, and resolubilization after lyophilization. Because of this variability, we consider 3% to be the lower limit of fibronectin production by rat GP cultured under these conditions and 15% to be the upper limit.

An additional consideration in assessing the levels of certain proteins secreted by GP was the contribution from alveolar macrophages, which, depending on the preparation, comprised from 5% to 15% of the total cell population. Accordingly, pure cultures of alveolar macrophages were incubated with [^{3}H]-proline and [^{3}H]glycine under similar conditions as described for GP. Analysis of culture medium proteins after trichloroacetic acid precipitation or DEAE-cellulose chromatography did not reveal any type IV procollagen (data not shown). Although thrombospondin was not identified by radioimmune precipitation, bands migrating in the area of 180K and 160K were observed after $NaDodSO_4$–polyacrylamide gel electrophoresis of culture medium proteins in the presence of DTT (data not shown). By scanning densitometry, it was estimated that not greater than 2% of the total secreted protein could be thrombospondin.

Fibronectin, however, comprised from 2% to 12% of the total protein in macrophage culture medium, on the basis of radioimmune precipitation and scanning densitometry, respectively. The percent contribution of alveolar macrophage FN to the total FN that was estimated to be secreted by GP would therefore be maximally 15% (since GP and macrophages contribute nearly equally to extracellular protein, on the basis of their respective numbers). However, we do not believe that this level was approached, because we were unable to detect any alveolar macrophages in the cultures used in these experiments after the cell layers were washed and preincubated in the absence of serum.

The relatively high ratio of 3-Hyp to 4-Hyp in GP cell layers, as was shown in Table I (column 6), suggested the presence of basement membrane collagen types in the extracellular matrix synthesized by the cells in culture. This fraction was accordingly analyzed after pepsin digestion, a procedure used to release collagen selectively from cell layers. Pepsin treatment solubilized principally a high molecular weight, disulfide-bonded aggregate. Preliminary structural studies indicated that GP cell layer collagen was present in a highly aggregated form that was at least partially stabilized by disulfide bonding to noncollagenous proteins. Further characterization of this fraction is in progress.

Discussion

Primary cultures of rat alveolar type II epithelial cells (granular pneumocytes) secrete several proteins that have been shown to be components of extracellular matrices produced by other cell types. The proteins identified in this study included FN, type IV procollagen, and TS in the culture medium.

The culture medium proteins were initially fractionated by chromatography on DEAE-cellulose and were further characterized and quantitated by radioimmune precipitation. Type IV procollagen was observed as the only (pro)collagen type in the culture medium and comprised approximately 2% of the total radiolabeled protein in this compartment (Tables I and II). From 3% to 15% of ^{3}H cpm incorporated over the 18–24-h labeling period into culture medium protein were attributed to FN (Table II). This range reflects a lower limit approached by incomplete interspecies cross-reactivity in radioimmune precipitation and an upper limit estimated by scanning densitometry of $NaDodSO_4$ gels, on the basis of the assumption that all the protein in the M_r 225 000 band corresponded to FN. Studies by Villiger et al. (1981) have shown that human alveolar macrophages secreted FN; it was not associated with the extracellular matrix, however, but was located in the culture medium and at cell-membrane binding sites for gelatin-coated latex beads. In view of these results, we have estimated that a maximum of one-sixth of the FN recovered from GP cultures could be derived from alveolar macrophages. However, our studies on alveolar macrophages isolated from rat lungs demonstrated that these cells did not secrete any procollagen, including type IV procollagen. The

source of the procollagen analyzed from GP culture medium was therefore exclusively the GP.

GP, a cell type derived from embryonic endoderm, represent the first epithelial cells that have been reported to secrete TS. This protein, which is released from platelet α-granules upon exposure of platelets to thrombin or other activating agents, has recently been described as an endogenous lectin responsible for platelet aggregation (Jaffe et al., 1982). The protein is also a constituent of normal human serum (10–30 μg/mL) (Raugi et al., 1982; Saglio & Slayter, 1982) and is a prominent secretion product of several different types of endothelial cells in vitro (McPherson et al., 1981; Mosher et al., 1982; Sage, 1982). Recently, TS was shown by ELISA and immunofluorescence to be secreted into the culture medium and also incorporated into the cell layer of smooth muscle cells and fibroblasts (Raugi et al., 1982). This is perhaps more pertinent to the biosynthesis of TS by GP, as both studies suggest a function for the protein that is independent of platelet interactions or the coagulation cascade.

Characterization of collagen biosynthesis by lung epithelial cells has been limited to a single study by Fulmer et al. (1977). Using fetal cat lung epithelial cells, these authors described the synthesis of the interstitial collagen types I and III, which comprised approximately 3% of the total protein synthesis. Interstitial mesenchymal cells (fibroblasts) isolated from the same tissue source also secreted types I and III collagen, although in a different ratio and at a slightly higher level than was observed for the presumptive epithelial cells. These results differ from those in our study, in which rat GP were shown to synthesize exclusively basement membrane collagens. A possible explanation for this discrepancy is that the feline epithelial cells, originally isolated by Kniazeff et al. (1976) (American Type Culture Collection, CCL 150), contained either some fibroblastic cells or a significant proportion of type I cells (Fulmer et al., 1977). This latter possibility raises some interesting questions regarding the secretory phenotype of type I cells and modulation of matrix protein production by the two types of alveolar epithelial cells.

A cloned line of human fetal lung type II epithelial cells secreted a glycoprotein of M_r 250 000, which in some respects resembled FN (Sahu et al., 1980). However, unlike FN, this protein did not bind to gelatin–Sepharose and appeared different immunologically and by amino acid composition. It is indeed possible that part of the material observed with the apparent mobility of FN after $NaDodSO_4$–polyacrylamide gel electrophoresis of GP culture medium protein (see Figures 1, 2, and 4) was the alveolar glycoprotein described by Sahu et al. (1980). This explanation would account for the discrepancy observed between the scanning densitometry and radioimmune-precipitation data for the levels of FN secreted by rat GP in vitro.

The role of epithelial cells in the formation of basement membrane is not completely understood. Studies on epithelial cells have been limited because serial culture techniques have been established for only a few specialized types. Alitalo et al. (1980) described the synthesis of several extracellular matrix components by primary cultures of human amniotic epithelial cells. These proteins, which included FN and type III procollagen, with smaller amounts of the basement membrane proteins laminin and types IV and V procollagen, were also identified in amniotic membrane by tissue immunofluorescence. It was therefore of interest that GP synthesized several components that have been described in or adjacent to the alveolar basement membrane, such as types IV and V collagen (Madri & Furthmayr, 1980), fibronectin, and heparan sulfate rich proteoglycans (Villiger et al., 1981; Farin et al., 1981). Type IV procollagen in alveolar basement membrane could be identical with the glycoprotein isolated from the bronchial lavage fluid of patients with alveolar proteinosis. Sequence studies have shown that several of the lavage protein fragments have hydroxyproline and Gly-X-Y repeats, in addition to apparently noncollagenous domains that could correspond to nontriple helical procollagen propeptides (Bhattacharyya, 1981).

The significance of extracellular matrix components in the maintenance of the alveolar wall with an intact epithelium can best be appreciated by examining the derangements of this structure that occur in several types of fibrotic lung disorders. Kawanami et al. (1982) have described the behavior of alveolar epithelial cells in response to lung damage. In areas of severe injury, cuboidal epithelial cells (probably derived from bronchiolar cells) underwent multilayering and squamous metaplasia, while type II cells proliferated without multilayering in areas where fibrosis was less advanced. Redistribution of collagen types is also known to occur in pulmonary fibrosis (Madri & Furthmayr, 1980), and elevated levels of FN have been detected in bronchopulmonary lavage fluid from individuals with interstitial lung disease (Rennard & Crystal, 1982).

An important direction in the study of alveolar pneumocytes will be the subculture, and possibly cloning, of both type I and type II cells under conditions that stabilize their morphologic, metabolic, and biosynthetic phenotype. Diglio & Kikkawa (1977) have described a progressive loss of several of these phenotypic properties in primary cultures of rabbit GP after 5 days. The use of suitable substrata and growth factors will undoubtedly be necessary for the propagation of viable strains of these cells that retain the critical in vivo characteristics.

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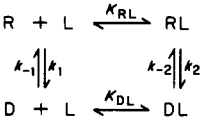
Conformational Effects of Volatile Anesthetics on the Membrane-Bound Acetylcholine Receptor Protein: Facilitation of the Agonist-Induced Affinity Conversion†

Anthony P. Young‡ and David S. Sigman*

ABSTRACT: The rate of the carbamylcholine-induced affinity conversion of the membrane-bound acetylcholine receptor protein from *Torpedo californica* is enhanced by pretreatment of the membranes under an atmosphere of 3% halothane or 1% chloroform. The enhancement is much more pronounced in the presence of low rather than high concentrations of carbamylcholine since the volatile anesthetics alter the apparent dissociation constant for carbamylcholine from 17 to 3 μM without affecting the first-order rate constant for the ligand-induced conversion (0.07 s^{-1}). These results indicate that the acetylcholine receptor is assuming a conformational form with intermediate affinity for carbamylcholine in addition to the previously described low- and high-affinity forms. The dissociation constants for carbamylcholine obtained from kinetic studies of the carbamylcholine-induced transition are 3–15-fold lower than those obtained as inhibition constants from the rate of ^{125}I-labeled α-bungarotoxin binding to the low-affinity conformer of the acetylcholine receptor protein. This pattern, observed in both the presence and absence of anesthetic, provides further evidence that the acetylcholine receptor has nonequivalent ligand binding sites for carbamylcholine.

It is generally accepted that structural transitions of the acetylcholine receptor protein (AcChR) mediate the rapid agonist-induced increase in cation permeability of nicotinic postsynaptic membranes and the slower loss of permeability (desensitization) which occurs upon prolonged exposure to agonists. *Torpedo* electroplaques provide an enriched source of the AcChR, and membrane fractions prepared from this source have been used extensively to investigate the conformational properties of the AcChR (Heidmann & Changeaux, 1978; Conti-Tronconi & Raftery, 1982). Studies of agonist inhibition of the rate of radiolabeled snake α-neurotoxin binding to the AcChR (Weber et al., 1975; Weiland et al., 1976; Lee et al., 1977) as well as direct binding studies using radiolabeled (Boyd & Cohen, 1980) or fluorescent agonists (Heidmann & Changeaux, 1979a) have established that the membrane-bound AcChR from *Torpedo* undergoes a slow (seconds to minutes time scale) agonist-induced structural transition from a weak-binding to tight-binding conformer. It is likely that this structural transition is the in vitro correlate of the densensitization of receptors of postsynaptic membranes (Weber et al., 1975; Lee et al., 1977; Sine & Taylor, 1979).

Scheme I



The two-state cyclic model shown as Scheme I has been used to characterize the slow affinity conversion observed in vitro (Boyd & Cohen, 1980; Heidmann & Changeaux, 1979a; Quast et al., 1978; Weiland et al., 1977) and desensitization observed by electrophysiological procedures in situ (Katz & Thesleff, 1957; Rang & Ritter, 1970). In this scheme, R and D signify the conformers of low and high affinity of the

† From the Molecular Biology Institute and the Department of Biological Chemistry, School of Medicine, University of California, Los Angeles, California 90024. *Received October 7, 1982.* The research was supported by U.S. Public Health Service Grant 21199.
‡ Present address: Department of Pharmacology, Stanford University School of Medicine, Stanford, CA.

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