Recovery of rat type II cell surfactant components during primary cell culture

SANDRA R. BATES,1 LINDA W. GONZALES,2 JIAN-QIN TAO,1 PETER RUECKERT,1 PHILIP L. BALLARD,1,2 AND ARON B. FISHER1
1The Institute for Environmental Medicine, University of Pennsylvania; and 2Department of Pediatrics, Children's Hospital of Philadelphia, Philadelphia, Pennsylvania 19104

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Bates, Sandra R., Linda W. Gonzales, Jian-Qin Tao, Peter Rueckert, Philip L. Ballard, and Aron B. Fisher. Recovery of rat type II cell surfactant components during primary cell culture. Am J Physiol Lung Cell Mol Physiol 282: L267–L276, 2002. First published October 12, 2001; 10.1152/ajplung.00227.2001.—A culture system designed to maintain the differentiated characteristics of rat type II cells based on protocols used for human fetal lung pneumocytes was investigated. Type II cells were isolated either from adult rats with elastase (adult type II cells) or from young rats (4–11 days postnatal) with collagenase and trypsin (young type II cells) and were incubated with dexamethasone (Dex, 10 nM) and cAMP (0.1 mM). By day 4 of culture with hormone treatment, the mRNA levels in adult type II cells were less than 3% of day 0 values, whereas surfactant protein (SP)-A protein content was 26%. However, young type II cells maintained lamellar bodies and microvilli and secreted phospholipid in response to ATP. SP-A, -B, and -C mRNA levels were elevated to 159, 350, and 39%, respectively, of day 0 values with a synergistic response to Dex and cAMP, whereas SP-A protein content rose to 119%. Surfactant mRNA and protein did not recover in cells cultured without hormones. This cell culture system restored surfactant components in rat type II cells.

ALVEOLAR TYPE II CELLS, located in the distal portions of the lung airways, synthesize, store, and secrete surfactant. Lung surfactant is a phospholipid and protein mixture that functions to prevent end-expiratory collapse of alveoli and to protect the host against challenge by foreign organisms and substances. To characterize the important elements for control of surfactant metabolism by type II cells, it is necessary to have a well-defined model system. Whole animals, perfused lungs, and lung explant cultures have been used with the limitation that the relative contributions of the numerous cell types present, the extracellular matrix, and various blood components are difficult to delineate.

Primary cultures of type II cells, where experimental variables can be manipulated readily, theoretically represent a more ideal approach to specifically investigate function of these cells. However, it is well known that type II cells rapidly change their phenotype after isolation and culture (16). Several culture conditions have been tested with the goal of retaining type II cells’ differentiated characteristics. Various criteria that have been used to demonstrate the “differentiated state” of the type II cells include morphology, presence of lamellar bodies, synthesis of surfactant phospholipids and proteins (and the associated mRNA), phospholipid content, and a “normal” response to secretagogues. One approach has involved altering the physical environment of the culture system by providing an air-liquid interface directly over the cells by placing the cells on floating collagen gels (22, 42). Another variable, the extracellular matrix, was explored by plating the cells on more “physiological” substrates such as Matrigel or collagen-coated plastic dishes (7, 19, 38, 41, 43, 44) or microporous membranes (10, 13). For example, we have shown that type II cells plated on Transwell microporous membranes retain their unique cellular morphology and show enhanced surfactant protein (SP)-A binding and surfactant uptake (3, 12, 13). Addition of factors or hormones to the culture media, such as dexamethasone (35, 52), thyroxin, fibroblast-pneumocyte factor (45), or keratinocyte growth factor (KGF; Refs. 43, 51), has also been tried. In general, provision of a biologically derived substrate and glucocorticoids promoted retention of normal cuboidal morphology with lamellar bodies and preservation of both the surfactant protein (SP-A) and its specific mRNA (42, 45).

Recently, a protocol that was effective in the prolonged maintenance of the phenotype of isolated type II cells from human fetal lung explants was reported by Alcorn et al. (1) and further characterized by Gonzales et al. (24). The method emphasized the ability of hormone treatment to maintain type II morphology and surfactant metabolism. Alcorn et al. (1) determined that type II cells, isolated from tissue explants with collagenase and plated on a matrix derived from the Madin-Darby canine kidney (MDCK) cells in the presence of a low volume of serum-free media containing

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cAMP, maintained substantial mRNA levels for SP-A, -B, and -C. The surfactant mRNA levels could be hormonally manipulated, and the cells were readily transfected with an adenoviral vector. Gonzales et al. (24) described a modification of this system and utilized a mixture of collagenase and trypsin for the isolation of human fetal type II cells. They found that plating these cells on plastic and incubating them in the presence of glucocorticoid and cAMP resulted in preservation of surfactant lipid metabolism, type II cell morphology, ability to process SP-B and -C from precursor forms, and complete recovery of surfactant protein gene expression. The limited availability of human fetal tissue, difficulty with sterility and viability of samples, and the requirement for organ culture to allow time for differentiation of fetal precursors into type II cells remain obstacles.

Our research has focused on the metabolism of surfactant lipids and proteins and has used the adult rat lung as an experimental model. The goal of this investigation was to determine whether the simplified protocols, which have proven to be so successful for human fetal lung type II cells isolated from explant cultures, could be applied to isolated neonatal or adult rat type II cells from intact lungs. We found that isolation of type II cells from neonatal rat lungs with collagenase plus trypsin and incubation of the cells with dexamethasone plus cAMP for 4 days resulted in the restoration of the pneumocyte phenotype.

**MATERIALS AND METHODS**

**Cell Preparation**

*Adult rats, elastase method.* Type II cells were isolated from the lungs of anesthetized adult (200 g) male Sprague-Dawley rats as described previously (13, 17). Briefly, perfused, lavaged lungs were digested with elastase (3 U/ml porcine pancreas, Worthington, Lakewood, NJ), minced, sequentially filtered through nylon meshes (160, 37, and 10 μm size) and panned on rat IgG (St. Louis, MO)-coated petri dishes for 1 h at 37°C to eliminate macrophages. The isolated cells (*day 0 cells*) were either analyzed or cultured overnight in 10% fetal calf serum (FCS) in Eagle’s minimal essential medium (MEM) at 37°C in a humidified incubator with 5% CO₂ in air. They were plated on 35-mm plastic tissue culture dishes (Costar) or dishes coated with a MDCK cell matrix. The matrix-coated dishes were prepared according to Alcorn et al. (1). In brief, MDCK cells were grown to confluence and treated with 1% deoxycholate for 5 min. The dishes were washed and stored in phosphate-buffered saline (PBS) at room temperature until use. The type II cell preparation was routinely >93% pure by modified Papain-Nicolau stain after 24 h of culture. The day after plating (*day 1 cells*), the cells on plastic were refed with experimental media or without FCS, or serum-free media with or without dexamethasone (10 nM), 8-bromoadenosine 3′,5′-cyclic monophosphate (cAMP, 0.1 mM), and isobutylmethylxanthine (IBMX, 0.1 mM) (DCI). IBMX was used to stabilize the intracellular cAMP levels by inhibiting degradation by phosphodiesterases. The cells were incubated up to *day 4* (3 days of hormone treatment) before harvesting.

*Young rats, elastase method.* The isolation procedure for young rats aged 4–21 days postnatal was identical to that used for adult rats. The cells were plated on fibronectin-coated dishes (25 ng/cm², Ref. 33) or plastic on day 0 in MEM with 10% FCS. On day 1, the purity of the cultures was >90% type II cells as assessed by the presence of Nile red-positive phase-dense organelles. The cells were refed with 1 ml of serum-free Waymouth’s media containing DCI, as described in Adult rats, elastase method.

*Young rats, collagenase and trypsin method.* Lungs were obtained from anesthetized young rats (4–11 days postnatal), and type II cells were isolated essentially according to the methods of Alcorn et al. (1) and Gonzales et al. (24). Briefly, a mixture of collagenase type I (0.5 mg/ml, Sigma), collagenase type IA (0.5 mg/ml, Sigma) (1), and trypsin (0.5%) (24) (Col/Try) was added intratracheally or to pieces of lung that had been minced with a McIlwain tissue chopper. The mode of protease administration did not affect the purity of the cells. Trypsin was added to aid in the dispersion of large clumps of cells (24). The isolated cells were filtered through nylon meshes and plated three times on plastic culture dishes for 30 min each to remove contaminating fibroblasts. The nonadherent cells were plated in 35-mm plastic dishes at 2 × 10⁶ cells/dish in Waymouth’s medium with 10% aymouth’s medium with 10% FCS overnight. Type II cells were identified by positive staining for the type II cell lamellar body membrane-specific monoclonal antibody (mAb) 3C9 (53) and for the anti-cytokeratin antibody (rabbit anti-cytokeratin, PAN, Zymed Laboratories, San Francisco, CA) by microscopy. Six fields (~200 cells/field) from three different experiments were counted. Fibroblasts were the principal contaminating cell as assessed by cell morphology and positive staining with anti-vimentin antibody. There are very few macrophages in rat lungs of this age. Unlike the results with human type II cells (1), treatment of the rat type II cell preparation with DEAE-dextran did not remove the fibroblasts, and its use reduced the yield of type II cells. Approximately 10 × 10⁶ type II cells were obtained per rat lung. On day 1, after removal of nonadherent cells, the type II cells were incubated in 1 ml of serum-free medium without or with DCI. The cells were refed with the hormones daily from *day 1* to *4* without or with added hormones. The percentage of type II cells (mAb 3C9 and/or cytokeratin-positive cells) without or with DCI after 4 days of culture was 43.3 ± 3.6% or 42.0 ± 5.8%, respectively (mean ± SE, n = 3). Isolation, using the protease mixture Col/Try, of type II cells from adult rats yielded cells that did not adhere to the dish during 24 h of culture, and this approach was not studied further.

**Western Blotting**

Type II cells were scraped from the dish with a rubber policeman in ice-cold PBS and centrifuged. Lysis buffer containing 0.1 M EGTA, proteinase inhibitors, and 100 mM phenylmethylsulfonyl fluoride in PBS was added to the cell pellet, and the sample sonicated twice for 15 s. Type II cell proteins were resolved with 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) under reducing (5% β-mercaptoethanol) conditions (30). The proteins were electrophoretically transferred to a nitrocellulose membrane at room temperature overnight (47). The membrane was transiently stained with Ponceau S to monitor the transfer efficiency of proteins, then blocked with 5% Carnation nonfat milk in Tris-buffered saline (TBS), incubated with 1:1,500 dilution of the rabbit anti-rat SP-A antibody (20) for 2 h, washed with Tween 20 TBS (TTBS), and incubated with a 1:3,000 dilution of the goat anti-rabbit antibody. The mem-
brane was washed with TTBS and developed via enhanced chemiluminescent techniques according to the directions of the manufacturer (Amersham, Piscataway, NJ). The membrane then was exposed to Kodak X-ray film for 10–60 s at room temperature.

**RNA Analysis and Northern Blotting**

RNA was isolated from the cells by sonication and acidic guanidium isothiocyanate (14). RNA was quantitated by absorbance at 260 nm. Surfactant protein mRNA was measured by either dot blots or Northern blotting. The dot blot procedure was essentially as described by Gonzales et al. (25). Increasing amounts (0.25–8 μg) of RNA from each sample were applied to nitrocellulose filters (Duralose-UV), baked, and prehybridized with the hybridization solution. Hybridization (18 h at 42°C) used ~7 x 10^6 cpm/ml of 32P-labeled probe, labeled using the Ready-To-Go Kit (Pharmacia). After high stringency wash, the blots were applied to Kodak XAR film with Cronex intensifying screens (DuPont Instruments, Wilmington, DE) for autoradiography at −80°C. Developed films were analyzed by densitometry (25). For Northern analysis, total RNA was electrophoresed on 0.7% agarose gels, transferred to nitrocellulose, and probed with 32P-labeled probe labeled rat SP-A cDNA, SP-B cDNA, or SP-C cDNA (kindly provided by Drs. S. Feinstein, M. Beers, and M. Koval, University of Pennsylvania, Philadelphia, PA) as previously described in detail (20, 25). All gels were stained with ethidium bromide before transfer to assess RNA quality. The mRNA content was expressed as arbitrary chemiluminescent units relative to β-actin cDNA (dot blot) or to the 18S RNA (Northern blots).

**Immunohistochemistry and Electron Microscopy**

Immunofluorescence was performed on type II cells fixed with methanol-acetone (1:1 in volume) for 2 min at 4°C, washed three times with PBS containing 1 mg/ml BSA (PBS-BSA buffer), and incubated at 4°C for 2 h with nonimmune mouse IgG (control) or mouse mAb 3C9 (53) as primary antibodies. The cells were washed thoroughly with PBS-BSA buffer, incubated with rhodamine (tetramethylrhodamine isothiocyanate)-conjugated goat anti-mouse IgG (Sigma, St. Louis, MO) at a dilution of 1:100 in PBS-BSA buffer, and prehybridized with the hybridization solution. Hybridization (18 h at 42°C) used ~7 x 10^6 cpm/ml of 32P-labeled probe, labeled using the Ready-To-Go Kit (Pharmacia). After high stringency wash, the blots were applied to Kodak XAR film with Cronex intensifying screens (DuPont Instruments, Wilmington, DE) for autoradiography at −80°C. Developed films were analyzed by densitometry (25). For Northern analysis, total RNA was electrophoresed on 0.7% agarose gels, transferred to nitrocellulose, and probed with 32P-labeled probe, labeled rat SP-A cDNA, SP-B cDNA, or SP-C cDNA (kindly provided by Drs. S. Feinstein, M. Beers, and M. Koval, University of Pennsylvania, Philadelphia, PA) as previously described in detail (20, 25). All gels were stained with ethidium bromide before transfer to assess RNA quality. The mRNA content was expressed as arbitrary chemiluminescent units relative to β-actin cDNA (dot blot) or to the 18S RNA (Northern blots).

**Phosphatidylcholine Secretion**

After isolation, type II cells were incubated overnight with 0.5 μCi of [methyl-3H]choline (Amersham, Arlington Heights, IL) in media containing 10% FCS to label cellular phospholipids. On day 1, cells were washed six times and either re-fed with the indicated test media for analysis on subsequent days or incubated in MEM to measure phosphatidylcholine (PC) secretion on day 1. For the cells examined on day 1, one set of cells was harvested after 30 min to serve as control for phospholipid secretion, which is stimulated due to the medium change (time 0). The remaining cells were incubated with no additions, 1 mM ATP, or 0.1 mM terbutaline for 2 additional hours. The medium was removed and centrifuged to remove detached cells. Methanol was added to the cell monolayer, and the cells were scraped from the dish. Both cells and media were extracted with chloroform, methanol, and water (8). The same procedure was followed on day 4. The amount of phospholipid secretion was calculated as the percentage of lipid counts per minute in the medium relative to the total lipid counts per minute present in the cells plus the medium. All experiments were performed in triplicate, and the values were averaged.

**RESULTS**

**Characteristics of Type II Cells Isolated From Adult Rats With Elastase**

Microscopic examination of adult rat type II cells plated on plastic with 10% FCS or on MDCK matrix with DCI after 4 days of culture indicated that the cells on plastic or matrix had flattened, and the lamellar bodies appeared larger and fewer in number, although more lamellar bodies were apparent in the cells on matrix (not shown).

The rapid loss of mRNA for surfactants proteins during culture of isolated type II cells is well documented (48). In the next series of experiments, the cells were plated on plastic or MDCK matrix in serum-free media with or without hormones. As shown in Table 1, culture of rat type II cells on MDCK matrix in the presence of hormones did not prevent the loss of surfactant protein gene expression. The mRNA levels for SP-A, SP-B, and SP-C in pneumocytes on MDCK matrix fell to <3% of day 0 levels on day 1 and continued to fall in the absence of hormones (Table 1). Hormone treatment for 2 days (day 3) increased surfactant protein mRNA levels fivefold or greater above untreated cells, but levels remained <3% of day 0 values. Further incubation to day 5 did not significantly change SP-A, -B, and -C mRNA levels.

The SP-A protein content of the cells also fell with time. The total SP-A protein content dropped to 32% on day 1 and continued to fall on day 4 to 19% of day 0 levels as shown in Table 1. Hormone treatment for 3 days (day 4) raised the total SP-A protein levels by 33% over untreated controls to 26% of day 0 values. However, the SP-A content did not recover to day 0 levels.
Table 1. Lack of recovery of rat type II cell surfactant components from cells isolated from adult rat lungs with elastase

<table>
<thead>
<tr>
<th>Component</th>
<th>n</th>
<th>Addition</th>
<th>Day 0 Content, %</th>
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<tbody>
<tr>
<td><strong>MDCK matrix substratum</strong></td>
<td></td>
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<tr>
<td>SP-A, -B, or -C mRNA</td>
<td>3</td>
<td>10% FCS</td>
<td>1</td>
</tr>
<tr>
<td>SP-A mRNA</td>
<td>5</td>
<td>None</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>DCI</td>
<td>3</td>
</tr>
<tr>
<td>SP-B mRNA</td>
<td>6</td>
<td>None</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td>6</td>
<td>DCI</td>
<td>3</td>
</tr>
<tr>
<td>SP-C mRNA</td>
<td>3</td>
<td>None</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>DCI</td>
<td>3</td>
</tr>
<tr>
<td><strong>Plastic substratum</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>SP-A protein</td>
<td>6</td>
<td>10% FCS</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>6</td>
<td>None</td>
<td>4</td>
</tr>
<tr>
<td></td>
<td>6</td>
<td>DCI</td>
<td>4</td>
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Data are the means ± SE in arbitrary units (AU) expressed relative to day 0 cells (=100%). MDCK, Madin-Darby canine kidney; DCI, dexamethasone, cAMP, and IBMX. n, no. of experiments. mRNA levels were determined by dot blot. Similar mRNA data were seen with cells on plastic. Surfactant protein (SP)-A protein (AU)/μg cell protein as a percentage of SP-A content on day 0 (=100%). *Significantly different from no DCI (None), P < 0.05.

In separate experiments, plating the cells on MDCK matrix in the presence of hormones for 3 days (day 4 of culture) did not significantly change the levels of total SP-A protein (29 ± 2% of day 0 values) compared with cells on plastic (23 ± 4% of day 0 values, means ± SE, n = 3). Thus hormone treatment had a limited effect on maintenance of surfactant protein gene expression in adult rat type II cells isolated with elastase.

**Phenotype of Type II Cells Isolated From Young Rats With Col/Try**

In view of the lack of success using elastase as the isolating enzyme, type II cells were isolated from young rats (4–11 days postnatal) with Col/Try and placed in culture on plastic dishes with 10% FCS. The type II cells were refed with 1 ml of serum-free Waymouth’s media with or without DCI for 3 days (day 4 of culture).

**Morphologic characterization of young rat type II cells.** The cultures were ~42% type II cells (see MATERIALS AND METHODS) with islands of pneumocytes surrounded by spindle-shaped fibroblast-like cells. After 1 day of culture, the cells contained numerous phase-dense organelles that labeled with Nile red (indicative of the presence of lipid) and were positive for mAb 3C9 (Fig. 1, day 1). mAb 3C9 recognizes a 180-kDa lamellar body protein in type II cells in rat lungs and in isolated type II cells (6, 40, 53). After 4 days in culture (Fig. 1, day 4), the mAb 3C9-positive vesicles remained with no obvious differences between DCI-treated and untreated cells. By electron microscopy, lamellar bodies and multiple microvilli were visible in the 4-day cultures of type II cells treated with DCI (Fig. 2A). Secreted lamellar bodies (closed arrow) as well as tubular myelin figures (open arrowhead) were found outside the cells (Fig. 2, A and B).

**Time course of restoration of surfactant protein mRNA levels in neonatal type II cells.** The mRNA levels for the surfactant proteins fell after 1 day in culture in type II cells isolated from the young rats with Col/Try (Fig. 3). The mRNA levels for SP-A, -B, and -C were 11, 7, and 12% of day 0 values, respectively. Removal of serum and refeeding with media without hormones resulted in a rise in the quantity of SP-A and -B mRNA to 34 and 31%, respectively, of day 0 values by day 4, whereas SP-C mRNA fell further to 5% of day 0. Exposure to DCI resulted in a 4.7-, 11.6-, or 7.4-fold elevation of the SP-A, -B, or -C mRNA levels over untreated cells, representing 159 ± 28, 360 ± 125, or 39 ± 16% (mean ± SE, n = 11–13), respectively, of the amount found on day 0 (Fig. 3B). Thus by day 4, the amount of surfactant protein mRNA had returned to levels approaching or above those found in freshly isolated cells. To ascertain the relative contribution of the hormones, the cells were incubated in dexamethasone or cAMP alone or in combination. As seen in Table 2, dexamethasone or cAMP alone did not significantly enhance mRNA values over no-treatment values after 4 days of culture. However, when the agents were combined, the effects of dexamethasone and cAMP were synergistic for all three surfactant protein mRNA species.

To determine whether elastase treatment affected the hormonal responsiveness of type II cells, cells were isolated with Col/Try from young rats, plated, and, on day 1, either treated or not treated with 75 μg/ml of elastase for 30 min and washed, and all were incubated until day 4 with hormones. Elastase at this concentration does not remove cells from the dish (12). The SP-A mRNA levels in the elastase-treated type II cells were 36.9 ± 16.1% (mean ± SE, n = 3, P < 0.05 compared with nontreated cells) of nonelastase-treated cells (100%). Such data indicate a detrimental effect of the commercial elastase preparation. Moreover, type II cells isolated from young rats with elastase and incubated under the same conditions with hormones did not significantly change the levels of total SP-A protein (29 ± 2% of day 0 values) compared with cells on plastic (23 ± 4% of day 0 values, means ± SE, n = 3). Thus hormone treatment had a limited effect on maintenance of surfactant protein gene expression in adult rat type II cells isolated with elastase.
not recover surfactant mRNA levels (on day 4, with hormone treatment, SP-A, -B, and -C mRNA levels were 2.0 ± 1.0, 3.9 ± 2.5, and 0.5 ± 0.5% of day 0 values, respectively (mean ± SE, n = 3)) or SP-A protein content (on day 4, the total SP-A protein content was 56 ± 6% (mean ± SE, n = 3) of day 0 values). Thus type II cells isolated from young rats via an elastase protocol showed a seminal lack of retention of type II differentiated characteristics as seen with cells from adult rats.

Recovery of SP-A protein content in neonatal type II cells after 4 days of culture with hormones. Rat SP-A is synthesized as a 28-kDa protein that is modified by glycosylation and sialylation to higher-molecular-mass protein forms, predominantly 32 and 36 kDa (21, 36, 49, 50). To determine the effect of restoration of SP-A mRNA content on SP-A protein levels, a time course examining SP-A protein was performed. By day 1, the total SP-A protein had risen slightly above day 1 values (Fig. 4B), due to 30 and 70% losses of the 36- and 32-kDa forms of SP-A, respectively (Fig. 4, A and C). By day 4, in the absence of hormones, intracellular SP-A protein had risen slightly above day 1 values. With exposure to dexamethasone and cAMP, the SP-A protein content rose 1.7-fold over untreated cells to 120 ± 15% of day 0 cells. There was no significant evidence of the 42-kDa SP-A species. The 32- and 36-kDa forms of SP-A were 1.6- and 2-fold higher in cells exposed to hormones than in untreated cells resulting in values of 81 and 171% of day 0 cells (Fig. 4). The changes in SP-A protein were consistent with the SP-A mRNA data.

Responsiveness of phospholipid secretion to ATP. Although the neonatal type II cells maintained morphologically identifiable lamellar bodies, it remained to be determined whether they were functionally active. Thus the release of phospholipid in response to secretagogues was tested. The freshly isolated cells were labeled on day 0 with [3H]choline. On day 1, ATP-sensitive phospholipid secretion was tested in some cells, while the others were incubated without or with hormones for an additional 3 days (culture day 4) before measurement of phospholipid secretion. On day 1, phospholipid release into the media from the neonatal cells had a low (120% of control) but statistically significant response to ATP (1 mM) (Fig. 5), consistent with the response of neonatal rats to ATP shown previously (26). By day 4, upon stimulation with ATP, phospholipid secretion by the neonatal cells exposed to hormones demonstrated a substantial threefold increase, similar to that seen in day 1 adult rat cells isolated with elastase. Neonatal type II cells incubated without hormones did not respond to the secretagogues (Fig. 5). Similar results were seen upon exposure to terbutaline (0.1 mM) under identical experimental conditions. Terbutaline stimulated secretion by 155 ± 5% over control values in day 1 cells, did not effect secretion in day 4 cells without hormones, and enhanced PC secretion by 197 ± 37% over no agonist controls in day 4 hormone-treated cells (mean ± range, n = 2).

DISCUSSION

The loss of differentiated function by type II cells in tissue culture has limited investigation of the surfactant metabolism by pneumocytes. The present report describes a relatively simple tissue culture procedure for primary culture of type II cells whereby morphologic and biochemical characteristics of differentiated alveolar type II cells are maintained or restored during relatively short-term culture. Pneumocytes, when obtained from the lungs of young rats by enzymatic digestion with Col/Try and cultured with DCI on plastic tissue culture dishes, contained secretagogue-responsive lamellar bodies and synthesized surfactant proteins.

The protocol closely parallels the methods used by Gonzales et al. (24) to isolate type II cells from human fetal lung explants, procedures that were based on those described by Alcorn et al. (1). The principle difference between the human and rat systems is that the 5-day preincubation step required for maturation of type II cells in the human fetal lung explants was not

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Fig. 2. Electron microscopy of type II cells from young rats isolated with collagenase plus trypsin after 4 days in culture and incubated with DCI. Type II cells on day 4 of culture after isolation from young rats with collagenase plus trypsin and incubation with DCI as described in Fig. 1. The cells were scraped from the dish, pelleted, and processed for electron microscopy. A: portion of a type II cell demonstrating multiple microvilli (MV) and a lamellar body (LB). B: two type II cells with material resembling secreted lamellar bodies (closed arrow) and tubular myelin figures (open arrowhead) outside of the cells. C: enlargement of area between arrows demonstrating tubular myelin figures.
necessary in the current procedure. The rat type II cell cultures were less pure (42% type II cells) than the human cultures [93% type II cells, Gonzales, et al. (24)], with fibroblasts the predominant contaminating cell type. Differential adherence steps to remove fibroblasts, a procedure that had proven to be effective for type II cells derived from human lung explants, were not as effective for rat type II cell preparations. Treatment with DEAE-dextran, a procedure that had removed many fibroblasts from human cell cultures (1), reduced the cell yield and did not improve the purity of rat type II cell cultures. Therefore, cells were grown in the absence of serum to keep the fibroblast population to a minimum. Serum-free media also aided in the maintenance of the type II phenotype (1, 28, 29). The role of fibroblasts in determining the cellular charac-

| Table 2. Effect of dexamethasone and/or cAMP on mRNA content of type II cells isolated from young rats with collagenase and trypsin |
|---|---|---|---|
| Additions | n | % of DCI Values |
| None | 5 | 14 ± 5 | 9 ± 4 | 13 ± 4 |
| DCI | 5 | 100* | 100* | 100* |
| Dex | 5 | 24 ± 7 | 26 ± 11 | 18 ± 8 |
| cAMP | 5 | 16 ± 3 | 19 ± 5 | 21 ± 6 |

Data are means ± SE for n number of experiments in AU/18S RNA content and expressed as a percentage of DCI values (=100%). Type II cells were incubated without or with dexamethasone (Dex), cAMP plus IBMX (cAMP), or DCI as described in Fig. 1 and harvested on day 4 of culture. *Significant difference from all other additions, P < 0.05.
characteristics in this system is uncertain. Complete removal of fibroblasts may not be prudent as fibroblast-type II cell interactions have been shown to be important for maintenance of type II cell phenotype (27, 37, 43) and may play a role in the success of this culture system. It is unlikely that the fibroblasts were interfering with the surfactant metabolism of the rat type II cells to any great extent, if at all, in view of the similarities in response of the type II cells isolated from human fetal lung explants [with few fibroblasts (24)] to that of cells from neonatal rat lungs (with many fibroblasts). However, the presence of fibroblasts may be problematic for some applications.

Light and electron microscopy morphologically confirmed the presence of lamellar bodies in the type II cells isolated from young rats. By light microscopy, lamellar bodies were identified as Nile red-positive phase-dense vacuolar organelles that were recognized by mAb 3C9, an antibody to lamellar body membranes (53). The extent of lamellar body preservation in the alveolar cells under all isolation conditions after 4 days of culture was surprising. Under the “worst-case” scenario (type II cells isolated from adult rat lungs with elastase and plated on plastic), where lamellar body genesis was reduced and the rate of PC secretion was slowed (46), remarkably, Nile red-positive phase-dense structures remained visible. In addition, in the neonatal rat cells, whether surfactant protein gene expression and lamellar body function were low (absence of hormones) or high (presence of hormones), there was no visible diminution in the amount of Nile red- and mAb 3C9-positive lamellar bodies. The pool of lamellar bodies must be retained through synthesis or be large enough to remain undepleted after 4 days of culture.

For the alveolar cells isolated with elastase from adult or neonatal rat lungs, there was a rapid decline in the amount of surfactant protein mRNAs to 1% or less of day 0 baseline concentrations after 4 days of culture. Although the elastase-isolated cells retained the ability to respond positively to hormone treatment as measured by surfactant mRNA and SP-A protein levels, the cells did not recover to the extent of the Col/Try-isolated cells. For rat cells obtained by elastase digestion, neither the cell substrate (plastic or MDCK matrix for the adult cells or plastic or fibronectin for the neonatal cells) nor the addition of 10% serum substantially affected gene expression, comparable to results observed with the human fetal type II cells (24).

In contrast to the elastase-isolated rat cells, treatment of the Col/Try-isolated young rat pneumocytes with dexamethasone and cAMP resulted in the successful maintenance of type II cell morphology, surfactant protein mRNA levels, and lamellar body presence and function. These results confirm those found with human fetal lung explants (24). Our findings support the hypothesis that dexamethasone- and cAMP-sensitive factors are important for the maintenance of pneumocyte phenotype, as has been found by others. Glucocorticoids and cAMP analogs promoted and retained differentiation in fetal lung explanted tissue (2, 9, 35, and for review 34). Additional variables found to be important in other culture systems include preservation of a cuboidal shape (for review, see Ref. 48); use of various matrices (1, 13, 41); inclusion of KGF (10, 51); and exposure to an air/aqueous interface (18). For example, levels of SP-A and SP-B mRNA and protein increased (whereas SP-C mRNA decreased) with time in culture of adult rat cells on collagen gels with fibroblasts in the presence of KGF, although the mRNA levels did not reach original values by 8 days (42). In addition, plating human fetal type II cells isolated from explants on a MDCK matrix in a low volume of serum-free media with cAMP alone resulted in an elevation in SP-A protein over the starting tissue in human fetal type II cell cultures isolated from explants (1). In contrast, the present culture system is unique for rat type II cells with regard to full recovery of surfactant protein gene expression in the absence of a matrix.

Due to the similarities in the isolation procedures used for the human fetal (24) and rat neonatal type II cells, some direct comparisons are possible. Pneumocytes isolated with Col/Try from neonatal rat lungs responded to treatment by a complete (SP-A and SP-B) or partial (SP-C) return to day 0 levels of the surfactant protein mRNAs, as did the human fetal cells (24). Rat SP-C mRNA may require a longer incubation time for full recovery. Robust phospholipid secretion in response to secretagogues was seen only for the cells treated with hormones in both species of alveolar cells. Further, both dexamethasone and cAMP were required for the maintenance of SP-B and -C mRNAs. However, in the rat cells, dexamethasone enhanced the stimulatory effect of cAMP on SP-A gene expression but reduced the cAMP effect in human cells (1, 24). This species-specific difference in effect of dexamethasone...
has been previously noted (21, 31). For both human and rat type II cells, differentiation was maintained on tissue culture plastic without a requirement for a specific matrix.

There was a diminished response in PC secretion with exposure to ATP in the type II cells isolated from the newborn rats with Col/Try after 1 day in culture relative to that seen in adult rat cells (26). This is likely due to developmental immaturity in components of the P2Y2 signaling pathway as the response to ATP is decreased in cells from fetal and newborn rats and increases with age (26). After 4 days in culture, the greater rate of PC secretion seen after incubation with ATP or terbutaline is not due to simple aging of the cells in culture, because the 4-day-old cells incubated in the absence of hormones did not respond to secretory cells in culture, because the 4-day-old cells incubated in the absence of hormones did not respond to secreta-

In conclusion, a simplified method for the maintenance of rat type II cell phenotype is described. This procedure isolates type II cells from neonatal rat lungs with Col/Try. After 1 day of culture in 10% serum on plastic dishes, incubation of the cells is continued with serum-free media containing DCI. After 4 days of culture under these conditions, the morphological characteristics of differentiated type II cells were maintained, the mRNA levels for surfactant proteins recovered to near-baseline values or higher, intracellular SP-A protein content was restored, and secretagogue-sensitive phospholipid secretion was present. This procedure facilitates the culture of differentiated rat type II cells and markedly increases the usefulness of the rat type II cell model for the study of surfactant metabolism.

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REFERENCES


39. Rueckert P, Tao J-Q, Gonzales L, Ballard P, Fisher AB, and Bates SR. A culture system for restoration of surfactant...