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Improved maintenance of adult rat alveolar type II cell differentiation in vitro: effect of hydrocortisone and cyclic AMP

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We have examined the effect of hydrocortisone and cyclic AMP on the maintenance of lipid synthesis in primary cultures of adult rat alveolar type II cells. These hormones were tested in the presence of either 1% or 5% charcoal-stripped rat serum (CS-rat serum). The effect of substratum on responsiveness to these hormones was evaluated by comparing cells cultured for 4 days on tissue culture plastic, on floating type I collagen gels, on rat lung fibroblast feeder layers on floating collagen gels (floating feeder layers), and on Engelbreth-Holm-Swarm (EHS) tumor basement membrane gels. Type II cells cultured on floating feeder layers in medium containing 1% CS-rat serum and 10^{-5} M hydrocortisone plus 0.5 mM dibutyryl cyclic AMP exhibited significantly increased incorporation of [^{14}C]acetate into total lipids (238% of control). The hormone combination also increased the relative percentage of acetate incorporated into phosphatidylglycerol (PG; 7.3% versus 1.9%) and saturated phosphatidylcholine (PC; 43.6% versus 37.6%). The percentage of acetate incorporated into neutral lipids was significantly decreased by the addition of hormones (28.6% versus 70.0%). The addition of hydrocortisone and cyclic AMP to medium containing 5% CS-rat serum resulted in an increase in the relative incorporation of acetate into saturated PC (51.2% versus 46.4%), but had no effect on the relative incorporation of acetate into PG or on the incorporation of acetate into total lipids. Type II cells cultured on EHS gels in medium containing 1% CS-rat serum plus hydrocortisone and cyclic AMP showed increased acetate incorporation into total lipids (204% of control) and a relative decrease in the percentage of acetate incorporated into neutral lipids (16.9% versus 47.0%). The hormone combination also increased the relative incorporation of acetate into PG (4.4% versus 2.5%) and saturated PC (49.9% versus 42.1%). Hydrocortisone and cyclic AMP added to medium containing 5% CS-rat serum concentration increased the relative incorporation of acetate into saturated PC by type II cells on EHS gels, but these additions had no effect on acetate incorporation into PG. No responses to these soluble factors were seen when type II cells were cultured on floating type I collagen gels without feeder layers or on tissue culture plastic. These data indicate that there are positive interactions between substratum, soluble factors and serum in the maintenance of differentiated function of adult rat alveolar type II cells in vitro.

Abbreviations: CS-rat serum, charcoal-stripped rat serum; EHS gel, Engelbreth-Holm-Swarm tumor basement membrane gel; PG, phosphatidylglycerol; PC, phosphatidylcholine, DMEM, Dulbecco's modified Eagle's medium; Bt_2cAMP, dibutyryl cyclic AMP; PBS, phosphate-buffered saline; FBS, fetal bovine serum.

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Introduction

Primary cultures of adult rat alveolar type II cells have been useful for studying the synthesis and secretion of pulmonary surface active material [1,2]. In vitro studies on the regulation of synthesis and secretion of surface active material have been limited by the fact that adult type II cells maintained on tissue culture plastic rapidly lose biological characteristics of functional differentiation. The percentage of radiolabeled palmitate and acetate incorporated into phosphatidylcholine (PC), saturated phosphatidylcholine and phosphatidylglycerol (PG), the major phospholipids of surfactant, fall as early as 1 day in culture [1,4]. The cells flatten soon after attachment and lose many of their lamellar inclusions in 3–5 days [4,5].

Investigators have recently begun to evaluate biological matrices for the maintenance of adult type II cell differentiation. These matrices include rat tail collagen gels [6], fibroblast feeder layers on collagen gels (floating feeder layers) [7], preparations of alveolar basement membrane [8], human amniotic membrane [9,10], extracellular matrix prepared from corneal endothelial cells [3,4], laminin [11] and gels made of Engelbreth-Holm-Swarm [EHS] tumors [7,11]. Even with the use of these biological substrata, attempts to maintain primary cultures of type II cells in the presence of 10% serum have shown only moderate success. Type II cells cultured on hydrated collagen gels [6] or bovine corneal endothelial cell extracellular matrix [4] show improved retention of morphological differentiation, but exhibit a pattern of phospholipid biosynthesis similar to that seen on tissue culture plastic. In addition, soluble factors such as hormones do not significantly enhance differentiation when type II cells are cultured on these biological substrata [4,6]. Type II cells cultured on floating feeder layers [7], preparations of alveolar basement membrane [8], human amniotic basement membrane [9] and EHS gels [7] incorporate a significantly greater percentage of acetate into PC or saturated PC than do cells cultured on plastic. These cells, however, fail to synthesize a significant percentage of PG when compared to freshly isolated type II cells.

We hypothesized that physiological substrata, hormones and other types of soluble factor are required for the maintenance of differentiated function of type II cells, as has been demonstrated previously for several other types of epithelial cell [12–17]. We therefore examined the effect of specific soluble factors and found that cyclic AMP or a combination of hydrocortisone and cyclic AMP helped maintain the synthesis of lipids of surface active material by adult rat type II cells.

In this paper, we report that phenotypic expression of type II cell-differentiated function induced by these soluble factors is affected by both the substratum on which the cells are cultured and by serum concentration. The conditions that appear to be maximal for PG synthesis are not necessarily the same as those for maximal PC synthesis.

Materials and Methods

Animals

Specific-pathogen-free male Sprague-Dawley rats, 180–250 g, were obtained from Bantam-Kingman (Fremont, CA). The rats were housed in a laminar flow hood and given free access to standard laboratory chow and water.

Isolation of alveolar type II cells

Type II cells were isolated by tissue dissociation with porcine pancreatic elastase (Worthington Biochemical Co, Freehold, NJ) and partially purified on discontinuous density gradients of metrizamide as described previously [18]. The viability of the isolated cells was $86 \pm 4\%$ (mean $\pm$ S.E., $n = 18$) as judged by exclusion of the vital dye Erythrosin B. The purity of the freshly isolated type II cell preparations was $84 \pm 3\%$ ($n = 18$) as determined by the modified Papanicolaou stain [19]. The culture medium consisted of Dulbecco's modified Eagle's medium (DMEM) supplemented with 1%, 5% or 10% charcoal-stripped rat serum (CS-rat serum), 2 mM glutamine, 100 U/ml penicillin, 1000 μg/ml streptomycin, 2.5 μg/ml amphotericin B (all from Gibco, Grand Island, NY), and 10 μg/ml gentamicin (Sigma, St. Louis, MO). The medium was gassed with 10% CO_2/90% air.

Preparation of substrata

Collagen gels. Hydrated collagen gels were prepared as described in detail elsewhere [20]. Briefly,

a stock solution of approx. 3 mg/ml rat tail collagen was prepared in 0.1% acetic acid and stored at 4°C. This solution was neutralized on ice with a 2:1 (v/v) solution of 10X medium 199 and 0.34 M NaOH. The collagen solution gels at room temperature when it is neutralized and returns to physiologic ionic strength.

EHS basement membrane gels. Basement membrane extract from the Engelbreth-Holm-Swarm (EHS) tumor was prepared according to the procedure of Kleinman [21], except that the final dialysis was performed against DMEM. This extract contains primary laminin, type IV collagen, heparan sulfate proteoglycan, entactin and nidogen [21,22]. Aliquots of the extract were stored at −20°C. After the extract was thawed on ice, 300 μl/well was pipetted into 24-well culture dishes, then allowed to gel for 30 min at 37°C. Type II cells were seeded onto this substratum at concentrations of $5 \cdot 10^5$ cells/cm^2.

Vitrogen gels. Vitrogen gels were prepared according to the specifications of the manufacturer (Collagen Corp. Palo Alto, CA). Human fibronectin (Collaborative Research, Bedford, MA) was mixed with vitrogen (33 μg/ml). Type II cells were seeded onto the gels at a concentration of $5 \cdot 10^5$ cells/cm^2. After 24 h, the gels were rimmed free of the culture dish and allowed to float free in the medium.

Tissue culture plastic. Cultures on tissue culture plastic were prepared by seeding the cells into 24-well culture dishes at a concentration of $5 \cdot 10^5$ cells/cm^2.

Preparation of fetal lung fibroblast feeder layers

Fetal lung fibroblasts. Fibroblasts were isolated from the lungs of day 19 fetal Sprague Dawley rats (Sasco, Omaha, NE) by the method of Post et al. [24]. They were grown to confluence in 25 cm^2 tissue culture flasks (Costar, Cambridge, MA), removed from the culture dish with 0.05% trypsin plus 0.02% EDTA (Gibco), frozen in DMEM with 10% fetal calf serum (Gibco) plus 10% dimethylsulfoxide (Sigma) and stored in liquid nitrogen. Cells between passage number 4 and 15 were used as feeder layers.

Procedure. Feeder layers were prepared by seeding $5 \cdot 10^4$ fetal lung fibroblasts onto a 0.5 ml rat tail collagen gel in each 18-mm well of a 24-well cluster dish (Costar, Cambridge, MA). After 1 or 2 days of culture, the feeder layer cells were growth-arrested by exposing them to 6000 R of ^{137}Ce irradiation. The feeder layers were rinsed with media after irradiation. Type II cells were seeded onto the irradiated feeder layers at a concentration of $5 \cdot 10^5$/cm^2. After 24 h, the collagen gels were rimmed free of the culture well and allowed to float free in the medium. The purpose of the collagen gel was to provide a flexible substratum that would allow cell shape changes to occur. In some experiments, type II cells were seeded onto the rat tail collagen gels without feeder layers and the gels were treated as above.

Preparation of charcoal-stripped rat serum C

CS-rat serum was prepared as described by Yoshizato te al. [23]. Activated ('Norit' SGI) charcoal (Matheson Coleman and Bell, Norwood, OH) was added to 500 ml of rat serum (Hazelton, Denver, PA). This slurry was stirred gently at 4°C for 48 h and then centrifuged at $10000 \times g$ for 30 min at 4°C. The supernatant was recentrifuged at $10000 \times g$ and stored at −20°C. CS-rat serum was sterilized by filtration through a 0.2-μM Millipore filter immediately prior to use. After carbon stripping, corticosterone in CS-rat serum was not detectable when assayed by high-performance liquid chromatography. The concentration of corticosterone in whole rat serum was 1.2 μM (data not shown).

Incubation of type II cells with hydrocortisone

Hydrocortisone (Sigma) was kept dissolved in ethanol at −20°C. The amount of ethanol added to the cultures with hydrocortisone was always less than 0.05%, a concentration that did not affect any of the parameters tested. Type II cells were cultured on floating feeder layers with 10% CS-rat serum and concentrations of hydrocortisone varying between 10^{-5} and 10^{-9} M from the beginning of culture. The cultures were fed again on day 2 with the same medium.

Incubation of type II cells with hydrocortisone and dibutyryl cyclic AMP

Type II cells were suspended in DMEM with 1% or 5% CS-rat serum and plated onto plastic wells, irradiated feeder layers on collagen gels,

collagen gels without feeder layers or EHS gels. In the case of floating feeder layers or floating collagen gels without feeder layers, collagen gels were rimmed free of the culture dish and allowed to float free in the medium after 1 day of culture. This resulted in contraction of the gel, such that the diameters were reduced by one-third on day 2 of culture. Type II cells were cultured for an additional 2 days with either control medium (DMEM supplemented with 1% or 5% CS-rat serum) or the same medium with the addition of either 10^{-5} M hydrocortisone, or 0.5 mM dibutyryl cyclic AMP (Bt_2cAMP, Sigma), or 10^{-5} M hydrocortisone plus 0.5 mM Bt_2cAMP.

When Bt_2cAMP was added to type II cells at the beginning of culture, the diameter of the collagen gels was not significantly reduced. The reason for this observation is not known, but Bt_2cAMP might have inhibited microfilament-mediated contraction as has been reported in cultured fibroblasts [25]. We therefore added Bt_2cAMP singly or in combination with hydrocortisone after day 2 of culture when the contraction of the collagen gels was almost completed. When type II cells were cultured onto plastic wells or EHS gels, we also added Bt_2cAMP singly or in combination with hydrocortisone after day 2 of culture.

Incubation of type II cells with radioactive acetate

In previous studies it has been shown that the rate of incorporation of radiolabeled acetate into total lipids, PC and saturated PC is constant between 0.5 and 3 h of incubation [26]. Rat alveolar type II cells have been shown to incorporate radiolabeled acetate into fatty acids by de novo synthesis, and the relative distribution of acetate in phospholipids after 24 h of culture is similar to the composition of rat pulmonary surfactant [1,27]. We chose acetate as the substrate for phospholipid synthesis because it is readily incorporated into all lipids and would allow us to evaluate incorporation into neutral lipids as well as phospholipids. We evaluated the relative distribution of radiolabeled acetate into PC, saturated PC and PG as criteria for differentiation of type II cells in vitro. After 4 days of culture, type II cells cultured under various conditions were given fresh medium that contained 10 μCi/ml [1-^{14}C]acetate (spec. act. 25–60 μCi/mmol, ICN, Irvine, CA). The cells were incubated for an additional 4 h in the presence of the additions, washed three times in phosphate-buffered saline (PBS) without calcium or magnesium, and then harvested for lipid extraction. In some experiments, type II cells were given fresh medium with 10 μCi/ml [1-^{14}C]acetate after 3 days of culture and incubated for an additional 24 h in this medium for equilibrium labeling.

Collagen gels were transferred intact to test tubes with forceps. EHS gels were fragmented with a soft plastic pipette, and the fragments were transferred to test tubes. The well was then washed twice with calcium/magnesium-free PBS to ensure that all cells were transferred to the tube. Type II cells on plastic dishes were scraped from the substratum with a soft plastic pipette and transferred to test tubes. Control cultures of irradiated feeder layers of fetal lung fibroblasts that did not have type II cells seeded onto them were also detached from the dish after 1 day of culture and evaluated for acetate incorporation on day 4 of culture. The irradiated feeder layer cells by themselves incorporated less than 7% of the total cpm in all of our experiments. Hence, the results are reported for the individual cultures, and the contribution of the irradiated fibroblasts was not subtracted because it was so small.

Lipid analysis

Lipids were extracted with methanol and chloroform according to the method of Bligh and Dyer [28]. Phopsholipids were separated by two-dimensional chromatography on DC-Fertigplatten Kieselgel 60 plates (E. Merck, Darmstadt, F.R.G.) [29]. The first dimension was run with a solvent system of chloroform/methanol/acetic acid (75:25:10, v/v) and the second dimension was run by a solvent system of chloroform/methanol/90% formic acid (75:25:10, v/v). The chromatography was performed with carrier lipids that included 500 μg dog lung lipids and 500 μg phosphatidylglycerol (Avanti Polar Lipids, Inc., Birmingham, AL). Saturated phosphatidylcholine was measured by first isolating total PC by one-dimensional thin-layer chromatography on 250 μm silica gel H plates (Analtech, Newark, DE) in a solvent system of chloroform/methanol/acetic acid/water (50:25:8:2.5), then reacting the ex-

tracted PC with osmium tetroxide in carbon tetrachloride, and finally separating the saturated from unsaturated species on boric acid-impregnated 500 μm silica gel G plates (Analtech) in a solvent system of chloroform/methanol/ammonium hydroxide/water (75:25:2:1) [30]. This method routinely yields 90% recovery of total PC [30].

The separated lipids were identified by a brief exposure to 8-anilino-1-naphthalene-sulfonic acid aerosol and scarped directly into scintillation counting vials for analysis. Radioactivity was measured in a Beckman LS 1801 liquid scintillation system with samples in Liquiscint (National Diagnostics, Somerville, NJ) and water (10:1, v/v).

Values usually correspond to means of duplicate determinations in at least three separate experiments.

DNA assay

Some data on the total incorporation of [^{14}C]acetate into lipids in type II cells were normalized to the DNA content of the cultures. Duplicate wells were used for DNA determinations. Floating feeder layers with and without type II cells were incubated with 0.25% collagenase (Cooper Biomedical, Malvern, PA) for 1 h at 37°C, rinsed three times with PBS, sedimented and stored at −20°C. DNA content in floating feeder layers was less than 9% of that in floating feeder layers that have type II cells seemed onto them in all of our experiments. Hence, the contribution of the irradiated fibroblasts was not subtracted because it was so small. Type II cells on EHS gels were incubated with Dispase (Collaborative Research, Bedford, MA) for 2 h at 37°C, rinsed three times with PBS, sedimented and stored at −20°C. DNA was assayed by fluorimetry with diaminobenzoic acid [31].

Morphology

Cultured cells were fixed with 2.0% glutaraldehyde and 1% paraformaldehyde in 0.1 M phosphate buffer and the samples were post-fixed in 1.5% osmium. Samples were stained en bloc with uranyl acetate and embedded in Polybed 812 (Polysciences, Warrington, PA) as described elsewhere [32]. Sections of 1 μm thick were stained with Mallory's azure II-methylene blue for light microscopy. Thin sections were stained with lead citrate and uranyl acetate and examined in a Philips 400 electron microscope.

Statistics

Statistical significance was assessed by paired Student's *t*-test or Ryan-Einot-Gabriel-Welsch multiple *F* test for multiple comparisons [33]. Data are expressed as mean ± S.E.

Results

Cott et al. recently reported that rat serum is better than fetal bovine serum (FBS) as a supplement for maintenance of saturated PC synthesis by adult rat alveolar type II cells in primary culture [10]. We also compared 10% FBS (Gibco) to 10% whole rat serum (Hazelton, Denver, PA) for its effects on lipid synthesis. We observed that type II cells cultured for 4 days in the presence of rat serum showed a pattern of phospholipid synthesis more like freshly isolated type II cells than that shown by cells cultured in FBS, regardless of whether the substratum was tissue culture plastic or floating feeder layers. On tissue culture plastic the percentage of saturated was significantly PC was significantly lower ($P < 0.05$) for FBS (27.7 ± 1.4%) vs. rat serum (38.8 ± 1.1%). The percentage of acetate incorporated into PG was not different for FBS (0.9 ± 0.1%) vs. rat serum (1.0 ± 0.1%). Type II cells cultured on floating feeder layers synthesized a significantly greater ($P < 0.05$) percentage of SPC in the presence of rat serum (45.3 ± 1.1%) vs. FBS (37.0 ± 1.2%). The percentage of acetate incorporated into PG was not significantly different for rat serum (1.2 ± 0.2%) vs. FBS (1.1 ± 0.1%). Data are the mean ± S.E. of three independent experiments. We therefore used rat serum instead of FBS in all our experiments.

Effects of hydrocortisone on lipid synthesis in type II cells cultured on floating feeder layers

Hydrocortisone was selected as the first hormone to be tested, because corticosteroids have been reported to stimulate fetal lung fibroblasts to produce fibroblast pneumonocyte factor, which subsequently stimulates choline incorporation into fetal type II cells [24]. This was also the reason for selecting fetal lung fibroblasts as the cell type for the feeder layer, although our previous data indi-

TABLE I

EFFECT OF HYDROCORTISONE ON LIPID SYNTHESIS IN TYPE II CELLS CULTURE ON FLOATING FEEDER LAYERS

Control cells in these experiments were cultured in the presence of 10% charcoal-stripped rat serum (CS-rat serum) alone. Experimental samples were cultured in the presence of 10% CS-rat serum plus the indicated concentrations of hydrocortisone (HC). PI, phosphatidylinositol; PS, phosphatidylserine; PE, phosphatidylethanolamine; SM, sphingomyelin; NL, neutral lipids; *n*, number of experiments.

Condition	cpm/well (% control)	*n*	Distribution of radioactivity (% phospholipid)						Distribution of radioactivity (% total lipid)	% PC that is saturated
			PC	PG	PI	PS	PE	SM	NL	
Control	100	3	78.1±1.4	1.4±0.0	4.2±0.4	2.5±0.2	5.4±1.0	8.5±0.4	60.9±3.5	43.3±0.8
HC (10^{-9} M)	103	2	78.3±1.0	1.2±0.1 *	3.6±0.0	2.4±0.2	6.6±0.0	8.4±0.4	59.3±2.7	42.6±0.3
HC (10^{-8} M)	106	3	78.0±0.5	1.2±0.1 *	4.1±0.3	2.4±0.3	5.8±0.5	8.5±0.3	61.1±4.0	42.8±0.8
HC (10^{-7} M)	135	3	79.5±0.6	1.0±0.0 *	3.7±0.3	2.2±0.1	5.3±0.5	8.4±0.4	54.6±4.0 *	44.4±0.8
HC (10^{-6} M)	185	3	81.4±0.5 *	1.0±0.0 *	3.0±0.2 *	1.8±0.2	4.2±0.3 *	8.6±0.4	47.7±4.8 *	45.3±1.2
HC (10^{-5} M)	195	3	81.6±0.8 *	1.1±0.1 *	3.0±0.1 *	1.8±0.2	4.1±0.4 *	8.6±0.4	45.3±3.2 *	44.4±0.8

*Significantly different from control ($P < 0.05$).

cate that the effect of the feeder layer is not cell type specific [7]. The relative incorporation of [^{14}C]acetate into cellular lipids for different concentrations of hydrocortisone is listed in Table I. Type II cells cultured on floating feeder layers with 10% CS-rat serum containing 10^{-6} M or 10^{-5} M hydrocortisone had a statistically significant relative increase in acetate incorporated into phosphatidylcholine (PC) and a significant decrease in the percent incorporated into phosphatidylinositol (PI), phosphatidylglycerol (PG) and neutral lipid (NL). The variability in the data was very small from experiment to experiment and, thus, relatively small changes were statistically significant. Cells cultured with concentrations of hydrocortisone varying between 10^{-9} M and 10^{-5} M did not produce a higher percentage of saturated PC.

Total incorporation of acetate into cellular lipids per well increased when cells were cultured with hydrocortisone at higher concentrations. The control value for cpm/18-mm culture dish after a 4-h labeling period was 8680 ± 1490 cpm; the ratio (treated/control × 100) was 195 ± 30% when cells were cultured with 10^{-5} M hydrocortisone. We did not, however, measure cellular DNA in these experiments to correct for the relative number of cells in each well. We subsequently compared the number of cells adherent to the feeder layers with and without 10^{-5} M hydrocortisone. The DNA in control wells was 1.61 ± 0.02 μg DNA/well and the values for wells with hydrocortisone were about 40% greater than the control in two separate experiments. Based on these data, the reason for the increase of total incorporation of [^{14}C]acetate into cellular lipids per well with higher concentrations of hydrocortisone was due, in part, to the difference in plating efficiency. In a previous study, dexamethasone increased the plating efficiency of adult rat type II cells to rat tail collagen gels [6]. Although hydrocortisone did not have a dramatic effect on maintaining type II cell differentiation under these culture conditions, we chose a concentration of 10^{-5} M hydrocortisone for further studies, because that was the concentration that produced the greatest effect in these experiments.

Effects of cyclic AMP and the combination of cyclic AMP plus hydrocortisone on lipid synthesis in type II cells cultured on various substrata

Type II cells cultured on floating feeder layers. Because the effect of hydrocortisone in the presence of 10% CS-rat serum was so small, we tested hydrocortisone and cyclic AMP alone and in combination in reduced serum. In pilot experiments, we found that the effect of cyclic AMP plus hydrocortisone was greater in 1% CS-rat serum

than in 5% or 10% CS-rat serum. We therefore selected 5% CS-rat serum as our high serum concentration and 1% CS-rat serum as our low serum concentration for subsequent experiments. We did not carry out experiments under serum-free conditions because we thought it would be impractical to try to get the collagen gels with the feeder layers reproducibly free from traces of serum. A variety of agents were screened singly for their ability to alter acetate incorporation in the presence of 1% CS-rat serum. It was decided to use the irradiated fibroblast feeder layer system to maximize the possibility of a positive effect, since the gels contracted well and fetal lung fibroblasts have been reported to produce paracrine factors for fetal type II cell differentiation [24].

As noted above, gel contraction was reduced when cyclic AMP was present from the beginning of culture. The pattern of phospholipid synthesis was also slightly altered when hormones were present during the entire culture period. There was a small decrease in the percentage of acetate incorporated into PC and saturated PC, but no change in the relative incorporation into PG ($n = 4$). We have previously shown that the contraction is related to cell shape changes, and that cuboidal cell shape is important for maintenance of differentiated function on type II cells [7]. We chose to add hormones on day 2 of culture in order to standardize the amount of gel contraction, thus we were able to evaluate the effects of hormones without complicating variations in cell shape.

Because adult type II cells have been reported to have receptors for glucocorticoids [34], thyroid hormone [35], insulin [36] and epidermal growth factor [37] we chose these hormones as well as some others that have been reported to accelerate the maturation of fetal lung in vivo or to help maintain other epithelial cells in vitro. As a single addition, we tested 10^{-5} M hydrocortisone, 10^{-6} M morphine sulfate, 10 μg/ml insulin, 100 pM transforming growth factor beta, and 5 μg/ml transferrin. Singly and in combination with hydrocortisone, we ested 10^{-7} M triiodothyronine, 0.5 mM dibutylyl cyclic AMP, 1 μg/ml prolactin, 10 ng/ml epidermal growth factor, 10% rat alveolar macrophage-conditioned medium, 50 nM phorbol 12-myristate 13-acetate, 10^{-6} M retinol and 1% dimethylsulfoxide. Except for cyclic AMP alone or a combination of hydrocortisone plus cyclic AMP, these other additions had little effect on lipid synthesis. The differences from control were less than 3% in terms of percent PC and less than 1% in terms of percent PG and percent saturated PC.

The effects of cyclic AMP and the combination of cyclic AMP plus hydrocortisone on the rate of incorporation of acetate into total lipids, total phospholipids and the distribution of [^{14}C]acetate into individual lipid classes and species are shown in Table II. In the presence of 1% CS-rat serum, 0.5 mM cyclic AMP alone produced a significantly higher percentage of saturated PC and PG, a significantly lower percentage of phosphatidylinositol (PI), phosphatidylserine (PS), sphingomyelin (SM) and neutral lipid (NL) and a significantly higher incorporation of acetate into total lipids. The effects of cyclic AMP plus hydrocortisone were about the same as cyclic AMP alone, except that the percentage of phosphatidylinositol and neutral lipid was further reduced and the incorporation of ^{14}C acetate into total lipids was increased (Table II, Expt. 1). Culture of cells with 5% CS-rat serum and a combination of hydrocortisone plus cyclic AMP produced a significantly higher percentage of PC and saturated PC, but did not alter the incorporation into PG or total lipids (Table II, Expt. 2).

Type II cells cultured on floating collagen gels without feeder layers. To eliminate the influence of the feeder layer, type II cells were cultured on floating collagen gels without a feeder layer of fibroblasts. When type II cells were cultured with 1% CS-rat serum on floating collagen gels without the feeder layer, the gels did not contract well after detaching from the dish. This could have been due to the lack of the feeder layer [38], to the lower plating efficiency in 1% CS-rat serum compared to the plating efficiency with 5% CS-rat serum, or to the slow spreading of the type II cells on the gel. For these reasons we were only able to use 5% CS-rat serum in these experiments. The combination of cyclic AMP plus hydrocortisone did not affect the distribution of [^{14}C]acetate into individual phospholipids and neutral lipids, and it did not affect the incorporation of [^{14}C]acetate into total lipids. (Table III, Expts. 1 and 2). These results suggest that the feeder layer contributes in some way to the maintenance of type II cell

TABLE II

EFFECT OF HYDROCORTISONE AND Bt_2cAMP ON LIPID SYNTHESIS IN TYPE II CELLS CULTURED ON FLOATING FEEDER LAYERS

Values of control for total lipid synthesis (cpm/μg DNA) for 4 h are 13638±850 in Expt. 1 and 10936±2673 in Expt. 2. Abbreviations are defined in Table I.

Condition	cpm/μg DNA (% control)	Distribution of radioactivity (% phospholipid)						Distribution of radioactivity (% total lipid) NL	% PC that is saturated
		PC	PG	PI	PS	PE	SM		
Expt. 1 (1% CS-rat serum, $n = 5$)									
Control	100	80.6±1.3	1.9±0.2	6.1±0.2	1.9±0.3	4.0±0.9	5.7±0.3	70.0±2.2	37.7±0.9
HC (10^{-5} M)	89± 8	82.5±1.1	2.0±0.3	4.4±0.2 **	1.4±0.2	3.4±0.6	6.7±0.4	54.3±2.1 **	40.7±1.1 *
Bt_2cAMP	123±30	81.2±1.6	4.9±1.2 *	4.5±0.5 **	1.3±0.2	3.9±0.8	4.4±0.4 **	42.2±4.5 **	42.4±0.6 *
HC+Bt_2cAMP	238±14 *,+	81.0±1.6	7.3±1.3 **	2.5±0.4 **,++	1.1±0.1 **	4.0±0.8	4.2±0.4 **	28.6±4.1 **,++	43.6±1.3 **
Expt. 2 (5% CS-rat serum, $n = 4$)									
Control	100	79.2±1.8	1.2±0.1	4.3±0.3	2.3±0.3	5.8±0.5	7.5±0.8	60.9±0.3	46.4±0.5
HC+Bt_2cAMP	132±12	84.4±1.4 *	1.5±0.3	3.1±0.3 **	1.4±0.3 *	4.1±0.1 *	4.7±0.4	42.5±4.9 *	51.2±0.4 **

Significant difference from control * $P < 0.05$, ** $P < 0.01$; significant difference from type II cells cultured in the presence of either hydrocortisone or cyclic AMP alone, $^{+}$ $P < 0.05$, $^{++}$ $P < 0.01$.

TABLE III

EFFECT OF HYDROCORTISONE PLUS Bt_2cAMP ON LIPID SYNTHESIS IN TYPE II CELLS CULTURED ON FLOATING COLLAGEN GELS WITHOUT FEEDER LAYERS

Values of control for total lipid synthesis (cpm/μg DNA) for 4 h are: 8898±1633 in Expt. 1 and 10275±2677 in Expt. 2. Abbreviations are as defined in Table I.

Condition	cpm/μg DNA (% control)	Distribution of radioactivity (% phospholipid)						Distribution of radioactivity (% total lipid)	% PC that is saturated
		PC	PG	PI	PS	PE	SM	NL	
Expt. 1. (rat tail collagen gels with 5% CS-rat serum, $n = 3$)									
Control	100	81.5±1.6	1.4±0.4	3.8±0.2	2.0±0.6	4.2±1.6	7.0±0.6	51.3±3.2	44.9±2.6
HC (10^{-5} M)+ Bt_2cAMP (0.5 mM)	104±18	79.4±3.5	1.6±0.4	2.9±0.7	1.6±0.2	4.2±0.2	10.3±2.9	43.1±6.5	46.9±1.3
Expt. 2. (vitrogen + fribonectin gels with 5% CS-rat serum ($n = 3$)									
Control	100	82.3±1.1	1.2±0.0	3.7±0.1	1.8±0.2	4.8±0.4	6.3±0.5	52.0±2.5	46.7±0.4
HC+B_2cAMP	85± 5	84.3±0.7	1.0±0.1	2.9±0.2	1.3±0.1	4.0±0.3	6.6±0.2	38.5±0.9	46.1±2.0

differentiation when the type II cells are cultured in the presence of these soluble factors. We have not determined whether this is a paracrine factor produced by the fibroblasts, an extracellular matrix produced by the fibroblasts or some other possibility.

Effects of cyclic AMP and the combination of cyclic AMP plus hydrocortisone on lipid synthesis in type II cells cultured on EHS gels. Because the collagen gels alone or those containing fibronectin were not satisfactory, we chose to evaluate EHS gels, which we have used previously without hormonal supplementation [7]. The effects of cyclic AMP alone or in combination with hydrocortisone on the rate of incorporation of [^{14}C]acetate into total lipids, and the distribution of [^{14}C]acetate into neutral lipids and individual phospholipids are shown in Table IV. Because the absolute incorporation of acetate into lipids cultured on EHS in the presence of 1% CS-rat serum was low (data not shown), we chose to extend the incubation period to 24 h to provide sufficient counts for analysis and to approach equilibrium labeling. When added to medium containing 1% CS-rat serum, cyclic AMP alone increased the incorporation of [^{14}C]acetate into total lipids and the relative incorporation of [^{14}C]acetate into PC, saturated PC and PG, and reduced the relative incorporation into phosphatidylinositol, phosphatidylserine, phosphatidylethanolamine, sphingomyelin and neutral lipid (Table IV, Expt. 1). The effects of cyclic AMP plus hydrocortisone were almost the same as those of cyclic AMP alone. In the presence of 5% CS-rat serum, the combination of hydrocortisone and cyclic AMP increased the relative incorporation into PC and saturated PC, but not PG, and did not enhance the incorporation of acetate into total lipids. These patterns of response of type II cells on EHS gels to cyclic AMP alone or in combination with hydrocortisone (Table IV) were almost the same as those for type II cells cultured on floating feeder layers (Table II). On both substrata, 1% CS-rat serum was preferable to 5% CS-rat serum for stimulation of the relative incorporation into PG.

Because butyrate has significant effects on some cell types, type II cells were incubated with 8-bromo cyclic AMP (8-BrcAMP) instead of Bt_2cAMP to investigate the effect of this cyclic AMP analog. There were no significant differences between the effects of 8-Bromo cAMP and Bt_2cAMP. Furthermore, butyrate itself did not affect the pattern of phospholipid synthesis in these cells (data not shown).

Type II cells cultured on tissue culture plastic. Although we have previously found that type II cells maintained on plastic were less able to maintain differentiated function than those on floating feeder layers or on EHS gels in 10% fetal bovine serum [7], we needed to determine whether tissue culture plastic would support the hormonal effects of cyclic AMP alone, hydrocortisone alone

TABLE IV

EFFECT OF HYDROCORTISONE AND cAMP ON LIPID SYNTHESIS IN TYPE II CELLS CULTURED ON EHS GELS

Value of control for total lipid synthesis (cpm/μ DNA) for 24 h is: 50065 ± 690 in Expt. 1 and the value for 4 h is 7464 ± 1406 in Expt. 3. There were no differences between the groups incubated with Bt_2cAMP ($n = 1$) and those incubated with 8-bromo cAMP ($n = 2$), and the data listed here include a combination of these groups (Expt. 2). Abbreviations are as defined in Table I.

Condition	cpm/μg DNA (% of control)	Distribution of radioactivity (% phospholipid)						Distribution of radioactivity (% total lipid) NL	% PC that is saturated
		PC	PG	PI	PS	PE	SM		
Expt. 1. (1% CS-rat serum and 24 h incubation with [^{14}C]acetate, ($n = 3$)									
Control	100	77.5 ± 1.5	2.5 ± 0.4	5.1 ± 0.1	1.8 ± 0.1	5.2 ± 0.6	7.9 ± 0.8	47.0 ± 5.2	42.1 ± 1.0
Bt_2cAMP (0.5 mM)	139 ± 5	85.6 ± 1.0 **	4.3 ± 0.4 **	2.5 ± 0.2 **	1.1 ± 0.0 *	2.7 ± 0.9 *	3.9 ± 0.6 **	20.5 ± 3.6 *	49.7 ± 1.2 **
HC + Bt_2cAMP	204 ± 24 *	84.3 ± 1.3 **	4.4 ± 0.5 **	2.6 ± 0.2 **	1.0 ± 0.1 *	2.6 ± 0.5 *	5.2 ± 0.5 **,+	16.9 ± 3.1 *	49.9 ± 1.8 **
Expt. 2. (5% CS-rat serum and 24 h incubation with [^{14}C]acetate, ($n = 3$)									
Control	–	71.4 ± 2.7	1.4 ± 0.4	5.8 ± 0.7	2.2 ± 0.4	6.6 ± 3.5	11.9 ± 1.0	52.6 ± 5.3	44.4 ± 1.5
HC (10^{-5} M)	–	68.8 ± 0.3	1.7 ± 0.5	4.3 ± 0.3 **	2.9 ± 0.3 *	7.7 ± 4.0	14.8 ± 4.0	51.8 ± 3.6	47.4 ± 0.3
cAMP (0.5 mM)	–	76.5 ± 3.3	1.5 ± 0.4	4.7 ± 0.8 **	2.2 ± 0.5	6.4 ± 3.0	8.7 ± 0.9	48.6 ± 6.2	47.5 ± 0.7
HC + cAMP	–	81.5 ± 2.5 *	1.2 ± 0.1	4.3 ± 0.5 **	1.5 ± 0.3 *,+	4.6 ± 2.5	7.0 ± 0.1	34.4 ± 9.1	54.5 ± 0.2 **,++
Expt. 3. (5% CS-rat serum and 4 h incubation with [^{14}C]acetate, ($n = 3$)									
Control	100	72.1 ± 3.2	3.2 ± 1.4	4.9 ± 0.1	2.7 ± 0.2	8.4 ± 0.8	8.7 ± 0.7	72.1 ± 1.6	40.9 ± 1.0
HC + Bt_2cAMP	126 ± 7	81.7 ± 1.1 *	1.6 ± 0.2	3.3 ± 0.2 **	1.4 ± 0.1 *	6.5 ± 0.7 *	5.6 ± 0.1 *	54.1 ± 3.0 *	48.5 ± 1.2 **

Significant difference from control, * $P < 0.04$, ** $P < 0.01$; Significant difference from type II cells cultured in the presence of either hydrocortisone or cyclic AMP alone $^{+}$ $P < 0.05$, $^{++}$ $P < 0.01$.

TABLE V

EFFECT OF HYDROCORTISONE AND cAMP ON LIPID SYNTHESIS IN TYPE II CELLS CULTURED ON PLASTIC

Value of control for total lipid synthesis (cpm/18-mm culture dish) for 4 h is 18578±2832 and ratios (treated/control) are: control, 100% HC, 75±11%; Bt_2cAMP, 89±5%; HC±cAMP, 44±10%. Abbreviations are as defined in Table I.

Condition	Distribution of radioactivity (% phospholipid)						Distribution of radioactivity (% total lipid) NL	% PC that is saturated
	PC	PG	PI	PS	PE	SM		
Expt. with 1% CS-rat serum ($n = 3$)								
Control	76.3±4.0	1.0±3.0	5.8±0.3	1.9±0.2	3.6±1.9	11.3±2.6	57.5±6.9	28.6±1.3
HC (10^{-5} M)	78.9±1.7	0.9±0.2	4.7±0.6	1.9±0.5	2.4±1.0	11.4±1.0	50.7±3.8	31.5±0.9 *
Bt_2cAMP (0.5 mM)	76.5±2.8	1.0±0.2	5.1±0.2	1.9±0.1	3.1±1.1	12.5±2.1	60.5±6.2	28.8±1.7
HC+Bt_2cAMP	73.5±2.4	0.9±0.1	4.9±0.4	2.4±0.4	3.3±1.3	15.1±2.2	59.5±3.5	30.5±1.1

* Significantly different from control, $P < 0.05$.

or a combination of these factors in the presence of 1% or 5% CS-rat serum. The data for 1% CS-rat serum are shown in Table V. These additions did not affect the distribution of acetate into individual phospholipid species and neutral lipids except the percentage of saturated PC, which was slightly

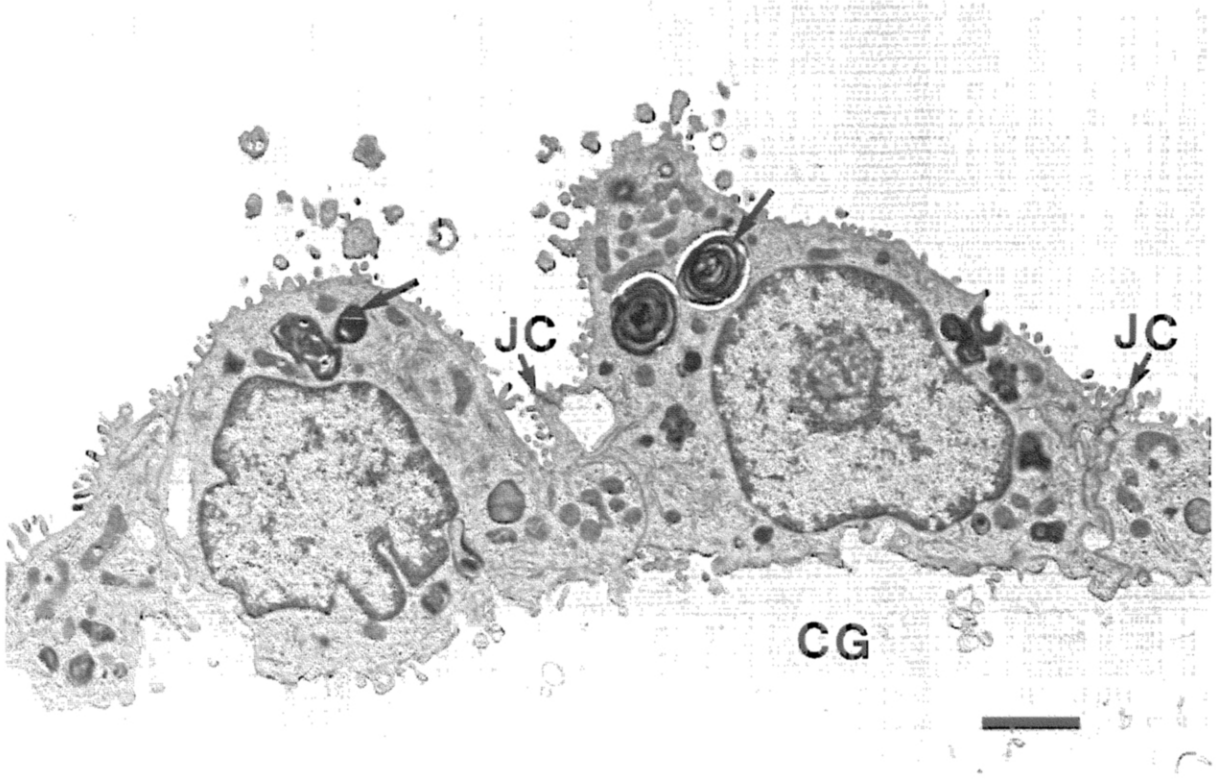

Fig. 1. Transmission electron micrograph of type II cells cultured for 4 days in association with a feeder layer of irradiated fetal rat lung fibroblasts on a detached rat tail collagen gel (CG). The cells were cultured with 1% charcoal-stripped rat serum containing 10^{-5} M hydrocortisone and 0.5 mM Bt_2cAMP. The cytoplasm of the cells contains numerous lamellar bodies (arrows). Type II cells are joined by junctional complexes (JC). Lung fibroblasts were not seen on this micrograph due to their low density on collagen gels. Bar represents 2 μm.

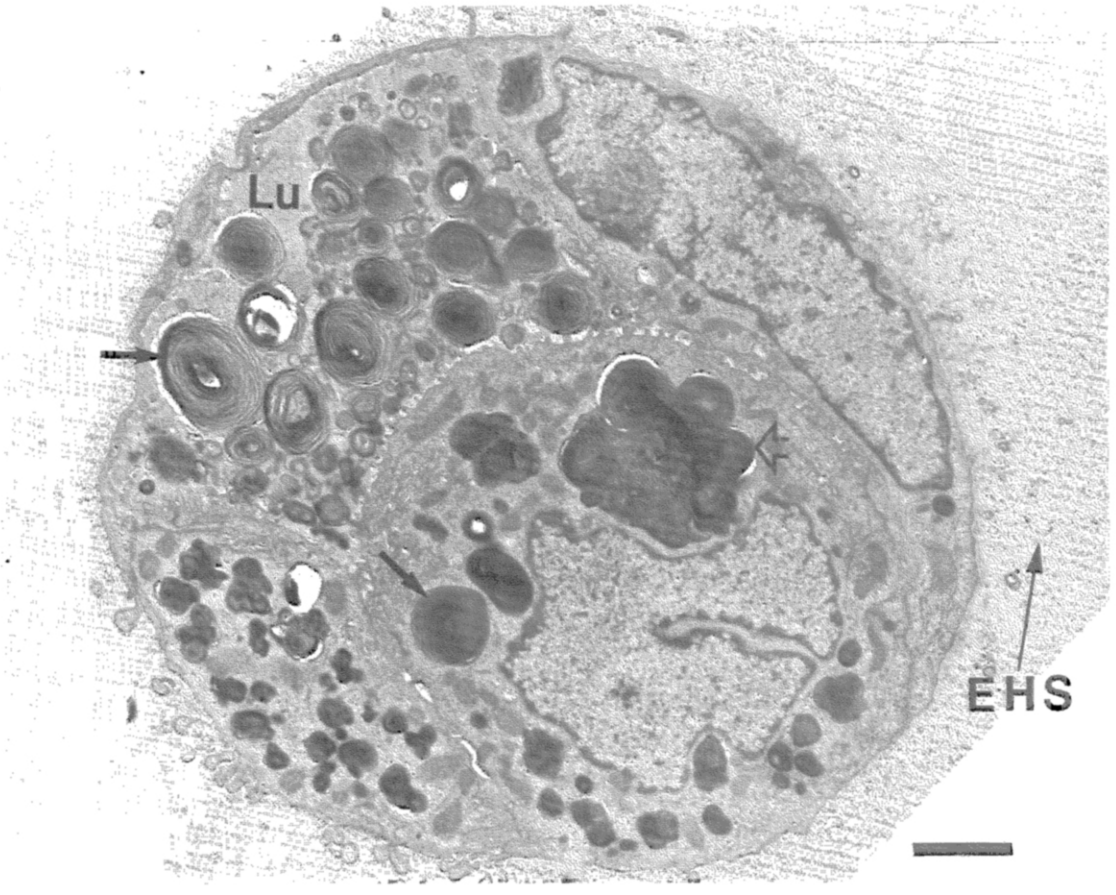

Fig. 2. Transmission electron micrograph of type II cells cultured for 4 days on an EHS gel. The cells were cultured with 1% charcoal-stripped rat serum containing 10^{-5} M hydrocortisone and 0.5 mM Bt_2cAMP. The aggregate that has formed on the surface of the EHS gels (EHS) is composed of low cuboidal cells. Inclusion bodies that possess the typical multi-lamellar structure can be seen (solid arrow), as well as inclusions with an atypical appearance (open arrow). The cells form a lumen (Lu) that is filled with lamellar bodies and an amorphous, densely stained material. Bar represent 2 μm.

higher when cells were exposed to hydrocortisone alone (Table V). In experiments with 5% CS-rat serum, there was no effect of cyclic AMP alone, hydrocortisone alone, or the combination of these factors on the distribution of acetate incorporation (data not shown). The soluble factors reduced total acetate incorporation when expressed as cpm/well, regardless of whether 1% or 5% CS-rat serum was used.

Morphology

The morphologic features of alveolar type II cells cultured for 4 days on both floating feeder layer gels and EHS gels with 10% fetal bovine serum have been described previously [7]. We repeated the experiments as well as experiments with 1% or 5% CS-rat serum in the presence of hydrocortisone and dibutyryl cyclic AMP. Our findings were similar to those of the previous report. Transmission electron micrographs of type II cells cultured for 4 days in the presence of hydrocortisone and cyclic AMP and on a fibroblast feeder layer revealed that the type II cells had maintained their cuboidal shape and contained numerous lamellar bodies in their cytoplasm (Fig. 1). The cells were polarized with the apical portion facing the medium. The type II cells cultured on EHS gels for 4 days in the presence of hydrocorti-

sone and cyclic AMP revealed that the cells had organized to form organoid structures with lumina that were filled with densely stained material that consisted of lamellar bodies, amorphous materials and cell debris. The cells had a cuboidal shape and numerous lamellar bodies in their cytoplasm (Fig. 2). On EHS gels, the basolateral surface of the type II cells faced the culture media. We could not demonstrate apparent morphological differences between type II cells cultured in the presence or absence of additives with either 1% or 5% CS-rat serum on both substrata.

Discussion

Although fetal alveolar type II cells respond to hormones in vivo or in mixed cell culture [39], it has been difficult to demonstrate similar hormonal effects on surfactant production in adult type II cells in vitro [4,6,40]. We wanted to develop a hormone-responsive culture system in order to study the regulation of individual components of surface active material, as well as to determine the rate-limiting steps in the assembly and intracellular transport of the individual components of surface active material. In this study, we examined the effects of corticosteroids and cyclic AMP under conditions designed to be permissive for hormone response (floating feeder layer gels and EHS gels) and under conditions unlikely to be permissive (tissue culture plastic). We have shown previously that the relative synthesis of saturated PC is dependent on serum [10], extracellular matrix and cell shape [7]. The concept that cell shape and substratum are important for maintenance of differentiated function is not new and has been shown for a variety of differentiated functions in other epithelial cell types in vitro [41–43]. In the current studies, we demonstrated the importance of a low serum concentration, and we were able, for the first time, to stimulate the synthesis of phosphatidylglycerol to a level that is close to that found in freshly isolated type II cells (approx. 10–12% [44]).

We chose corticosteroids and cyclic AMP as the main factors to be tested because cyclic AMP has been reported to stimulate mRNA for the surfactant apoprotein in fetal lung organ culture [45,46] and because corticosteroids can accelerate fetal lung maturation in vitro and in vivo [47,48]. In addition, Gross et al. [49] reported an additive interaction between corticosteroids and cyclic AMP on the rate of choline incorporation into saturated PC in explant cultures of fetal rat lungs. We chose fetal lung fibroblasts for the feeder layer because fetal lung fibroblasts have been reported to secrete fibroblast pneumonocyte factor (FPF), which is a paracrine factor for stimulation of fetal type II cells [24]. Fibroblast pneumonocyte factor, however, has not been reported to stimulate adult type II cells and our studies did not directly test for its presence. Our conditions were different from those of Post et al. [24] in that CS-rat serum was present and irradiated fibroblasts were used. In our studies, hydrocortisone alone was less stimulatory than cyclic AMP analogs alone, but a combination of both factors was better than either alone, especially with 1% CS-rat serum.

The floating feeder layer had a positive effect on the ability of type II cells to respond to hormones. This was particularly noteworthy in the case of acetate incorporation into PC. The only confounding variable of this culture system was the presence of the irradiated fibroblasts. We have previously observed [7] that type II cells on floating feeder layers will form acinar structures within the contracted gel. Since apical cell surfaces are sequestered in these structures, we were unable to study secretion by these cells. Although plain collagen gels or collagen gels mixed with fibronectin were ineffective in supporting a hormone response, EHS gels allowed the demonstration of a direct stimulatory effect of cyclic AMP and corticosteroids on type II cells without the need for the feeder layer. For biochemical studies in which the presence of fibroblasts would obscure the results, EHS gels should be the most satisfactory. The major problem with the EHS gels is that the cells form small aggregates with the apical surface on the inside; secreted material [7] is thus trapped in the acinar lumen, thereby preventing the study of secretion. The components of EHS matrix that are conducive to maintenance of differentiated morphology and surfactant phospholipid biosynthesis in cultured type II cells have not been completely defined. A recent report [11] suggests that laminin is an important constituent of this matrix in its support of type II cell differentiation.

The criteria chosen for type II cell differentiation are arbitrary. We chose the relative rate of acetate incorporation, i.e., percent distribution, into PC, saturated PC and PG. Saturated PC is the key phospholipid for reducing surface tension at the air/liquid interface [50]. Saturated PC is not unique to surface active material, however, and even in lung, a significant portion of SPC may not mix with the total surfactant pool [51]. In this series of experiments as well as in others [7,10], we were able to stimulate the synthesis of PC and saturated PC without increasing PG synthesis. The relative incorporation of acetate into PG may be a more specific marker for differentiation of normal adult rat type II cells. With time in primary culture on plastic, PG decreases rapidly and more completely than does saturated PC [1]. In addition, in diseased lungs the relative percentage of phosphatidylglycerol in lavage phospholipids falls without significant changes in the percent of PC that is saturated [52,53]. In our experiments hydrocortisone plus cyclic AMP in the presence of 5% CS-rat serum maintained saturated PC synthesis in type II cells on EHS gels and on floating feeder layers, but not PG synthesis. The incorporation into PG was best with 1% CS-rat serum in the presence of both hydrocortisone and cyclic AMP. The 1% CS-rat serum experiments on EHS gels and on floating feeder layers cannot be directly compared to each other, however, because the incorporation on EHS gels was for 24 h, whereas the acetate incorporation on floating feeder layers was for 4 h. In these studies, as well as in previous studies that compared different sera on the synthesis of saturated PC and PG [10], the conditions that were maximal for the relative incorporation into PG were not necessarily those that were maximal for the relative incorporation into saturated PC. Although we believe that the synthesis of saturated PC and PG can be coordinately regulated, there is still an element of independent regulation; this can be seen in the studies with 5% CS-rat serum or in experiments using other sera [10].

The stimulation by hydrocortisone and cyclic AMP in our experiments were greater than previously reported. Post et al. studied type II cells cultured for 24 h with various hormones in the presence of 10% fetal bovine serum on tissue culture plastic and found a slight stimulation of radioactive precursors incorporated into saturated PC or PG with cortisol or cortisol plus thyroxine, but the maximal stimulation was about 50% [40]. Geppert et al. cultured type II cells on floating rat tail collagen gels and were able to maintain morphologic differentiation but not phospholipid synthesis with dexamethasone and/or insulin [6]. Dobbs et al. were unable to alter differentiation with a variety of soluble factors when rat type II cells were plated on extracellular matrix derived from corneal endothelial cells or on plastic [4].

In summary, the control of differentiation of type II cells in vitro is highly complex. Extracellular matrix, maintenance of cuboidal shape, soluble factors, serum concentration and possibly cell–cell interactions all influence differentiation of adult type II cells. In our studies, floating feeder layers or EHS gels are preferable to collagen gels without feeder layers or tissue culture plastic as substrata, and cyclic AMP or a combination of cyclic AMP plus hydrocortisone are important soluble factors. In addition, a low serum concentration was necessary to demonstrate an effect of cyclic AMP and hydrocortisone on PG synthesis. Thus, it appears that multiple factors are important in maintaining the differentiated state of adult type II cells in vivo.

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