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GROWTH OF DISTAL FETAL RAT LUNG EPITHELIAL CELLS IN A DEFINED SERUM-FREE MEDIUM

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SUMMARY

Fetal rat distal lung epithelial cells, in contrast to adult type II pneumocytes, will divide readily in culture in the presence of 10% (vol:vol) fetal bovine serum. The presence of serum makes purification of uncontaminated cell-derived growth factors difficult and modifies cellular responses to oxidant injury. We report the development of a defined serum-free medium that will support growth of fetal distal lung epithelial cells in primary culture. Initial studies used a low-serum (2%; vol:vol) to determine the effect of basal media, substrata, and various additives. Subsequent studies demonstrated growth on a poly-D-lysine substratum under serum-free culture conditions in Dulbecco's modified minimal essential medium with insulin (50 μg/ml), endothelial cell growth supplement (20 μg/ml), bovine pituitary extract (100 μg/ml), bovine serum albumin (50 μg/ml), selenous acid (4 ng/ml), reduced glutathione (500 ng/ml), soybean trypsin inhibitor (100 μg/ml), transferrin (5 μg/ml), HEPES buffer (2.6 mg/ml), and cholera toxin (5 μg/ml). Growth was enhanced by reducing the gas phase oxygen concentration from 21 to 3%. The undefined components of this medium, bovine pituitary extract and endothelial cell growth supplement, could be replaced by platelet-derived growth factor (20 ng/ml) with prostaglandin E_1 (25 n*M*). The response of fetal distal lung epithelial cells to known growth factors differs substantially from that observed with type II pneumocytes from adult lung and is similar in many, though not all, respects to the responses reported for proximal airway cells from adult lung.

Key words: lung epithelium; growth factors; defined medium; substrata.

INTRODUCTION

Serum has been recognized to inhibit growth and modify differentiated functions of cultured epithelial cells from a number of tissues (10,43,45). The presence of serum, which contains a variety of factors that influence cell growth, also complicates isolation and identification of cell-derived autocrine and paracrine growth factors. The origin of such factors may also be difficult to determine since some serum-derived growth factors can be stored by the cell, in subcellular matrix, for later release (41). Attempts to minimize serum-contamination, for collection of serum-free conditioned medium (31), frequently result in growth inhibition. Inasmuch as the release of growth factors has been related to their growth state (17), this growth arrest may inhibit the release of the paracrine or autocrine growth factors being collected.

Distal fetal lung epithelial cells, in contrast to pneumocytes isolated from the adult lung, will readily divide in culture in the presence of serum. We have previously utilized this property for in vitro studies of cellular interactions during fetal lung development (30), circulating growth factors following oxidant lung injury (34), and effectiveness of exogenous antioxidants (35). As previously reported (30), distal fetal lung epithelial cells, as well as fibroblasts, release paracrine growth factors into their culture medium. Paracrine and autocrine growth factors are also released by cultured lung cells after oxidant injury (31,38). Use of distal fetal lung epithelial cells, for further in vitro studies of growth-controls during normal lung development and after lung injury, would be enhanced by use of a serum-free and defined cell culture medium for the reasons described above, as well as because of specific problems associated with toxicity studies.

Sublethal pulmonary oxygen toxicity in vivo is associated with an early and rapid pneumocyte hyperplasia (9). In vitro, however, isolated fetal lung epithelial cells, grown in a medium containing serum, show a concentration-dependent inhibition of cell division when exposed to elevated oxygen concentrations (35). This disparity is compatible with the hypothesis that pneumocyte hyperplasia in vivo is mediated by such external factors as paracrine growth factors (31), or changes in basement membrane composition (12). It is also possible that the serum present in the culture systems used for in vitro studies modifies the cells' normal response to oxidant injury. In previous studies with mixed fetal lung cell cultures (32,33) we observed that the presence of serum in the culture medium modified the cytotoxic effects of elevated oxygen tensions and prevented any increase in cellular antioxidant enzyme activity in response to oxidant stress.

For these reasons, a defined culture medium has been developed which supports the growth of distal fetal lung epithelial cells in the absence of serum. Established procedures allow very pure cultures

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of distal fetal lung epithelial cells to be obtained (5,25). These procedures have been further modified to minimize potential contamination of conditioned medium by growth factors derived from non-epithelial cells.

Materials and Methods

Materials. All cell culture media, reduced glutathione, HEPES buffer, and porcine trypsin (1:250 wt:wt) were obtained from GIBCO Canada (Burlington, Ontario) and heat-inactivated fetal bovine serum (FBS) from Flow Laboratories (McLean, VA). CLS 1 collagenase (131 U/mg) and type I DNAase (2367 U/mg) were from Worthington (Freehold, NJ). Cell culture flasks were from Falcon (Becton Dickinson, Lincoln Park, NJ) and multiwell plates from Costar (ESBE Laboratories, Markham, Ontario).

Insulin, glucagon, epidermal growth factor (EGF), endothelial cell growth supplement (ECGS) and factor (ECGF), selenous acid, transferrin, triiodothyronine, bovine pituitary extract (BPE), type I collagen, and laminin were supplied by Collaborative Research (Bedford, MA). Fibronectin was from Chemicon (Temecula, CA), poly-D-lysine from Boehringer Mannheim (Mannheim, W. Germany), recombinant insulinlike growth factor I (IGF-I) from Imcera Bioproducts (Terre Haute, IN), and catalase from Cooper Biomedical (Malvern, PA). Linoleic acid, prostaglandins E_1(PGE_1), and $F_{2\alpha}$ ($PGF_{2\alpha}$), bovine serum albumin (BSA), dihydrotestosterone, prolactin, and somatostatin were from Sigma (St. Louis, MO). Cholera toxin was from Calbiochem (La Jolla, CA). Porcine platelet derived growth factor (PDGF), acidic fibroblast growth factor (aFGF), and basic fibroblast growth factor (bFGF) from R&D Systems (Minneapolis, MN).

Monoclonal anti-cytokeratin and anti-vimentin were from Labsystems (Chicago, IL). Fluorescein isothiocyanate-goat anti-mouse IgG was from Boehringer Mannheim. Anti-fading mounting solution (AF-1 solution) was from City-fluor (London, UK).

Fibronectin- and laminin-coated culture surfaces were prepared according to the manufacturers specifications. Poly-D-lysine and type I collagen-coated culture surfaces were prepared as previously described (7).

Preparation of epithelial cell cultures. Primary cultures of epithelial cells were prepared by modifications of previously described methods (5,25). Timed-gestation, pathogen-free Wistar rats were killed with an excess of ether at 19 days of gestation (term = 22 days). The fetuses were removed under sterile conditions and placed on ice until removal of the thoracic contents en bloc. The combined lungs from each litter were placed in calcium and magnesium-free Hanks' balanced salt solution (HBSS). The heart, major vessels, and airways were carefully trimmed from the lungs, and discarded. The lungs were washed twice in cold HBSS, then finely minced with scissors and suspended in 20 ml cold HBSS/litter in a 50-ml centrifuge tube. This suspension was vortexed gently to enhance red cell removal from the tissue fragments, and the fragments allowed to settle by gravity. The supernatant was discarded and the fragments washed with 20 ml/litter aliquots of HBSS until the supernatant was clear. The tissue pieces were then pelleted at 420 $\times g$ for 5 min at 4° C for suspension in 30 ml/litter HBSS containing 0.1% (wt:vol) trypsin and 0.001% (wt:vol) DNAase. This suspension was stirred for 20 min at 37° C. The supernatant was transferred to a centrifuge tube and proteolytic activity neutralized by the addition of an equal volume of Eagle's minimal essential medium (EMEM) with 10% (vol:vol) FBS at 4° C, and the supsension triturated through a serologic pipette to break up cell clumps. This procedure was repeated, if necessary, with any undispersed tissue fragments remaining after the initial exposure to trypsin. The dispersed cells were filtered through 100-μm mesh nylon bolting cloth into centrifuge tubes and pelleted at 420 $\times g$ for 5 min. The supernatant was discarded and the cells suspended in EMEM with trituration once more before pelleting at 420 $\times g$ for 5 min. The pellet was suspended in 10 ml/litter HBSS containing 0.1% (wt:vol) collagenase and 0.001% (wt:vol) DNAase and placed in a 37° C water bath for 15 min, during which time it was vortexed intermittently. The proteolytic activity was again neutralized with an equal volume of EMEM with 10% (vol:vol) FBS and the cells pelleted at 420 $\times g$ for 5 min. The pellet was resuspended in 20 ml/litter EMEM with 10% (vol:vol) FBS and placed in culture flasks (150 cm^2/litter) for 1 h at 37° C to allow fibroblast attachment. Unattached cells were removed and the culture flasks washed with EMEM. The washings were combined with the suspension of unattached cells and the cells pelleted at 420 $\times g$ for 5 min. This pellet was resuspended in EMEM with 10% (vol:vol) FBS at 20 ml/litter and plated at 75 cm^2/litter for a further 1-h differential adherence step at 37° C. Unattached cells from two litters were combined and pelleted at 420 $\times g$ for 5 min, then suspended in 35 ml of EMEM for centrifugation at 120 $\times g$ for 3 min to pellet epithelial cells. The fibroblast-enriched supernatant was discarded. This step was repeated a total of 4 times. The final pellet was suspended in EMEM to obtain a cell count before dilution for plating in multiwell plates. Adherent cells were washed at 24 h to remove any unattached cells or cell debris. Unless otherwise stated, cells were grown in a gas phase of 21% O_2:5% CO_2:74% N_2. Growth in this gas phase was compared with growth in 3% O_2:5% CO_2:92% N_2.

Assessment of population homogeneity. Confluent cultures of distal fetal lung epithelial cells were assessed by phase contrast microscopy. Less dense cultures were used for indirect immunofluorescent staining for intermediate filaments. Cells were fixed for 10 min in methanol cooled to −20° C. After washing in phosphate buffered saline (PBS) containing 5% (vol:vol) normal goat serum (NGS) and 1% (vol:vol) BSA. This was followed by a 20-min incubation with 1:10 (vol:vol) monoclonal anti-cytokeratin or anti-vimentin. The cells were rinsed in cold PBS with 0.1% (vol:vol) Tween 20 and incubated with 1:40 (vol:vol) FITC-conjugated goat anti-mouse IgG, followed by three washes with cold 0.1% (vol:vol) Tween 20 in PBS. The cells were mounted with AF-1 solution and examined under a Leitz fluorescent microscope. All incubation steps were conducted in a humidified chamber, and all primary and secondary antibodies diluted in 5% (vol:vol) NGS with 1% (vol:vol) BSA in PBS. All negative controls slides were incubated as above, but with the primary antibody omitted.

Measurements of growth response. Cell counts were performed using an automated cell counter (Coulter Electronics, Hialeah, FL). Accuracy of cell counts was regularly validated against hemacytometer counts. Assay of [^{3}H]thymidine (1.0 μCi/ml) incorporation into DNA was conducted using the method of Greenstein et al. (11). The isotope was added at 48 h from plating and the duration of the incubation was 24 h, to achieve a gross assay of the integrated response to media manipulations (27).

Statistical analysis. All values are shown as the mean ± stan-

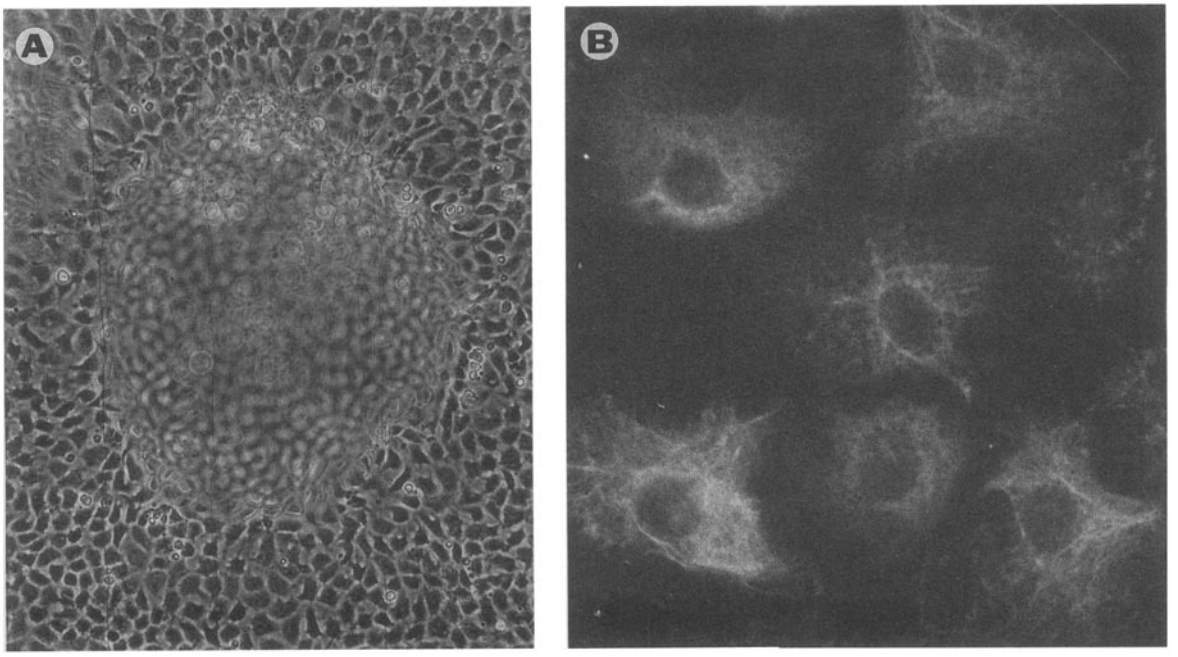

FIG. 1. Plating of fetal lung epithelial cells at high density results in a characteristic dome formation, as observed by phase contrast microscopy (*A*). Homogeneous nature of the cell population is demonstrated by immunofluorescent staining of cytokeratin, a characteristic epithelial cell marker (*B*).

dard error of the mean. In some instances cell number values have been shown as percent values at 24 h from plating to correct for plate-to-plate variability in plating efficiency. Statistical significance ($P < 0.05$) was determined by an analysis of variance followed by assessment of differences using Dunnet's two-sided test (28) or Duncan's multiple range test (29).

RESULTS

Homogeneity of population. The extreme purity and homogeneity of the population, obtained using the modified purification procedure described above, is shown in Fig. 1. The epithelial cells have a homogenous appearance when plated at high cell density and form characteristic domes (Fig. 1 *a*). The epithelial origin of the cell preparations as assessed by immunocytochemical analysis of intermediate filaments in the monolayer cultures. The cell exhibit specific binding of monoclonal antibodies to cytokeratin (Fig. 1 *b*), the cell-specific intermediate filament of epithelial cells. No binding of monoclonal antibodies directed against vimentin was observed (not shown), indicating that the cultures were not contaminated with cells of mesenchymal origin.

Effect of culture medium and substratum variation. The growth of cells initially plated in EMEM with 2% (vol:vol) FBS on plastic for 24 h, then changed to various commercially available media with 2% (vol:vol) FBS for the next 6 days is shown in Table 1. Cell counts were conducted at 1, 2, 4, and 7 days after plating, but only 7-day results are shown for brevity. The best growth at this low serum concentration was observed with Dulbecco's modified Eagle medium containing 4.5 mg/ml D-glucose, 584 μg/ml L-glutamine, and 110 μg/ml sodium pyruvate (DMEM), which was used as the basal medium for all subsequent studies. When various substrata were examined for effect on growth rate with DMEM + 2% (vol:vol) FBS, the best growth rates were observed with laminin and poly-D-lysine, which resulted in similar growth velocities whereas plating on poly-D-lysine resulted in the better plating efficiency of these two substrata (Fig. 2).

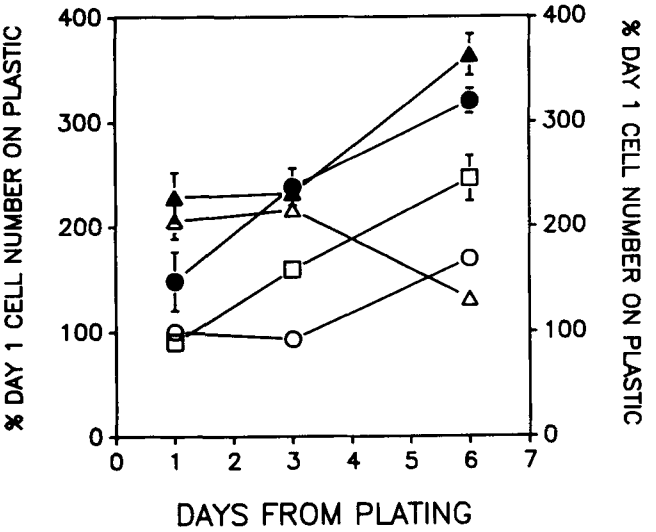

FIG. 2. Growth of fetal lung epithelial cells plated at 5×10^4 cells/cm_2 on various substrata in DMEM with 2% FBS over 6 days in culture. All values are M ± SEM for four wells. Results have been expressed as percent cell number at 24 h of culture. Substrata: fibronectin (*solid triangles*); poly-D-lysine (*solid circles*); laminin (*open squares*); type I collagen (*open triangles*); plastic (*open circles*).

TABLE 1

EFFECT OF VARIOUS CULTURE MEDIA ON GROWTH OF FETAL LUNG EPITHELIAL CELLS OVER 7 DAYS IN CULTURE WITH 2% FBS

Medium	Day 7 Cell No. (% Day 1),[a] M ± SEM	Medium	Day 7 Cell No. (% Day 1),[a] M ± SEM
EMEM	119 ± 5	HF12	74 ± 2
DMEM	198 ± 30	DMEM:HF12	94 ± 7
IMDM	102 ± 6	NCTC	108 ± 2
RPMI	122 ± 2	M199	113 ± 9

[a] All values are expressed as percent cell number on Day 1 of culture (IMDM = Iscove's modified Dulbecco's medium; RPMI = RPMI medium 1640; HF12 = Ham's F12 nutrient mixture; HF12:DMEM = 1:1 (vol:vol) HF12:DMEM; NCTC = Medium NCTC-135; M199 = Medium 199).

Effect of insulin, IGF-I and BPE. Both laminin and poly-D-lysine substrata were used for studies of insulin and IGF-1 effects on epithelial cell DNA synthesis in the presence of 2% (vol:vol) FBS and in the presence or absence of BPE (Table 2) A significant effect of BPE was noted on both substrata, although basal and BPE-stimulated rates of DNA synthesis were greater on poly-D-lysine. Insulin had an effect in the absence of BPE only on poly-D-lysine, whereas both IGF-I and insulin had BPE-mediated effects only on poly-D-lysine. All subsequent studies were therefore conducted with a poly-D-lysine substratum.

Other medium supplements. Various additives previously observed to enhance DNA synthesis by adult rat type II pneumocytes (7) were tested for effect on distal fetal lung epithelial cells in DMEM with 50 μg/ml insulin, 20 ng/ml IGF-I, and 2% (vol:vol) FBS for the first 24 h after plating, then 0.2% (vol:vol) FBS (Table 3). Those supplements that enhanced DNA synthesis by ≥10% of control values were retained for subsequent experiments. HEPES buffer was also retained in the expectation that eventual complete removal of serum would lead to a more evident requirement for a buffer supplement. Also retained was BSA, to act as a binding protein for growth factors, although at a reduced concentration of 50 μg/ml which, when tested separately, had no inhibitory effect on epithelial cell DNA synthesis. Subsequent deletion experiments, with the full medium, did not confirm a beneficial effect of somatostatin on epithelial cell DNA synthesis in the presence of the other supplements, and this was also deleted from the medium.

TABLE 2

EFFECT OF INSULIN, IGF-1, AND BPE ON DNA SYNTHESIS BY FETAL LUNG EPITHELIAL CELLS IN DMEM ON TWO SUBSTRATA[a]

	Poly-D-Lysine		Laminin	
	No BPE	+ BPE	No BPE	+ BPE
No additives	445 ± 63	1461 ± 204[b]	89 ± 13	792 ± 48[b]
Insulin				
5 μg/ml	438 ± 69	1317 ± 236	91 ± 13	753 ± 92
25 μg/ml	637 ± 124	1769 ± 220	126 ± 8	979 ± 98
50 μg/ml	2867 ± 248[b]	5811 ± 434[c]	90 ± 18	656 ± 70
IGF-I				
10 ng/ml	583 ± 117	1450 ± 308	84 ± 11	824 ± 132
20 ng/ml	615 ± 94	2652 ± 339[c]	118 ± 17	734 ± 47
30 ng/ml	843 ± 104	2057 ± 170	87 ± 15	483 ± 69

[a] Values are M ± SEM for [^{3}H]thymidine incorporation into DNA (dpm/cm^2) of 3 to 12 wells. [b] $P < 0.05$ compared with group with no additives and no BPE on same substratum. [c] $P < 0.05$ compared with group with no additives but with BPE on same substratum.

TABLE 3

EFFECT OF VARIOUS MEDIUM SUPPLEMENTS ON DNA SYNTHESIS BY FETAL LUNG EPITHELIAL CELLS ON A POLY-D-LYSINE SUBSTRATUM[a]

Supplement	Concentration	Percent Basal Medium Value
Basal medium[b]		100 ± 6
+ Catalase	700 U/ml	11 ± 6[c]
+ Glucagon	2 ng/ml	26 ± 3[c]
+ Linoleic acid	80 ng/ml	31 ± 1[c]
+ BSA	1 mg/ml	33 ± 4[c]
+ $PGF_{2\alpha}$	25 ng/ml	34 ± 2[c]
+ EGF	50 ng/ml	37 ± 5[c]
+ Triiodothyronine	500 pg/ml	101 ± 4
+ HEPES buffer	2.6 mg/ml	109 ± 6
+ Reduced glutathione	500 ng/ml	111 ± 8
+ Selenous acid	4 ng/ml	124 ± 6
+ ECGS	20 μg/ml	133 ± 16[c]
+ Somatostatin	20 ng/ml	162 ± 10[c]
+ Soybean trypsin inhibitor	100 μg/ml	176 ± 8[b]
+ Transferrin	5 μg/ml	333 ± 23[c]

[a] Values are M ± SEM for [^{3}H]thymidine incorporation into DNA of four to eight wells. [b] DMEM + 50 μg/ml insulin, 20 ng/ml IGF-I, 100 μg/ml BPE, and 0.2% FBS. [c] $P < 0.05$ compared with group with basal medium.

Growth on poly-D-lysine in this serum-free medium (DMEM with insulin 50 μg/ml, BPE 100 μg/ml, BSA 50 μg/ml, ECGS 20 μg/ml, GSH 500 ng/ml, selenous acid 4 ng/ml, soybean trypsin inhibitor 100 μg/ml, transferrin 5 μg/ml, and HEPES buffer 2.6 mg/ml) after a 24-h period in 2% (vol:vol) FBS was compared with continued growth in 2% (vol:vol) FBS or 10% (vol:vol) FBS (Fig. 3). Based on preliminary studies (data not shown) indicating better growth at a reduced initial plating density, this study was conducted at a plating density of 3×10^5 cells/cm^2. A three- to four-fold increase in cell number from 1 to 4 days in culture was noted with the serum-free medium, whereas there was only a two-fold increase with 2% (vol:vol) FBS and no significant increase with 10% (vol:vol) FBS. This same medium, with insulin and IGF-I deleted, was used to study the relative needs for insulin or IGF-I (Table 4) in the presence of the other additional medium supplements. A 2% (vol:vol) FBS supplement was retained for the first 24-h plating period only. Insulin, IGF-I, or a combination of both agents significantly increased epithelial cell DNA synthesis, but no additive effect was observed. Although either factor could have been deleted from the medium, we elected to delete IGF-I for economic reasons. When the basal medium was supplemented with PDGF (10 ng/ml), in addition to both IGF-I and insulin, there was a small effect on DNA synthesis, but this did not achieve statistical significance. In the same experiment, the effect of supplementing the medium with cholera toxin (5 μg/ml) was explored and a significant effect observed (Table 4). Cholera toxin was added to the medium in subse-

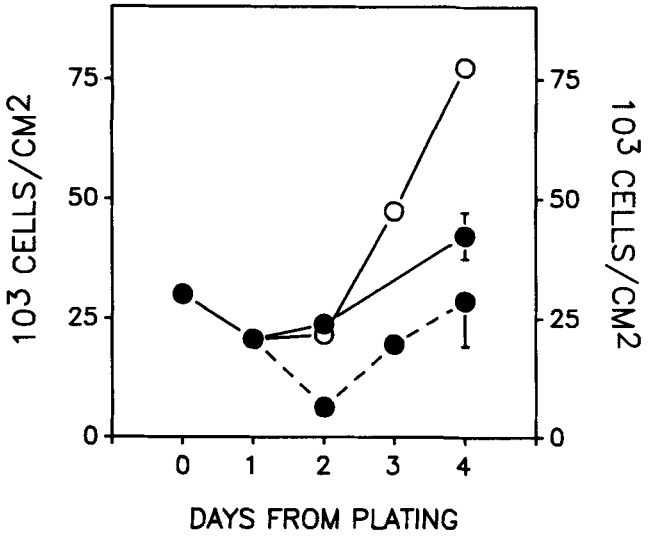

FIG. 3. Growth of fetal lung epithelial cells plated at 3×10^4 cells/cm^2 on poly-D-lysine in supplemented DMEM (*see* text) with 2% FBS for 24 h. Cells were then maintained in the same medium with no serum (○—○, 2% FBS (●—●), or 10% FBS (●--●) until Day 4 of culture. All values are M ± SEM for four wells.

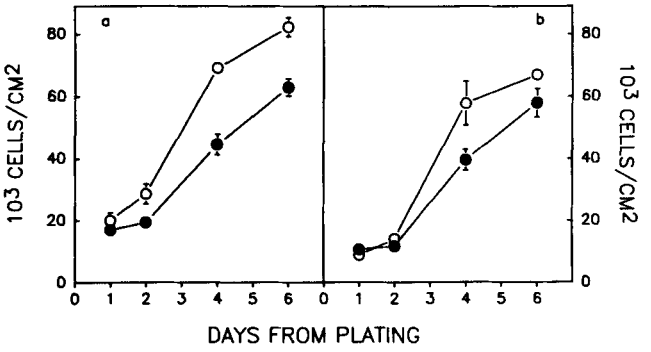

FIG. 4. Comparison of fetal lung epithelial cell growth from 1–6 days (*a*) in culture with supplemented medium (*see* text) after 24 h in 2% FBS (*open circles*) or no exposure to FBS (*solid circles*), or (*b*) with serum-free supplemented medium and a gas phase of 3% O_2:5% CO_2:92% N_2 (*open circles*), or a gas phase of 21% O_2: 5% CO_2:74% N_2 (*solid circles*). All values are M ± SEM for four wells.

quent experiments. In parallel experiments no effect of 100 n*M* dihydrotestosterone on DNA synthesis was observed.

The need for a 24-h plating period in the presence of serum was conducted with the above medium less IGF-1 (Fig. 4 *a*). Both plating efficiency and cell number by 6 days in culture were enhanced by a plating period in serum, but serum was not essential for cell growth.

Effect of oxygen concentration. The potential benefit of growth at a reduced PO_2 was examined by obtaining growth curves in gas phases of 3% O_2: 5% CO_2: 92% N_2 or 21% O_2: 5% CO_2: 74% N_2. After 6 days in culture, cell number was greater in a gas phase containing 3% O_2 than in the gas phase containing 21% O_2 (Fig. 4 *b*).

TABLE 4

EFFECT OF INSULIN, IGF-I, PDGF, AND CHOLERA TOXIN ON DNA SYNTHESIS BY FETAL LUNG EPITHELIAL CELLS IN SUPPLEMENTED MEDIUM[a]

Supplement	dpm/cm²
Basal medium	4952 ± 1008[b]
+ Insulin (50 μg/ml)	9000 ± 732[c]
+ IGF-I (20 ng/ml)	8232 ± 856[c]
+ Insulin (50 μg/ml) + IGF-I (20 ng/ml)	8855 ± 1468[c]
+ Insulin (50 μg/ml) + IGF-I (20 ng/ml) + PDGF (10 ng/ml)	10 240 ± 1440[c]
+ Cholera toxin (5 μg/ml)	28 800 ± 5440[d]

[a] Values are M ± SEM for [^{3}H]thymidine incorporation into DNA of 5–8 wells. DMEM + BPE 100 μg/ml, BSA 50 μg/ml, ECGS 20 μg/ml, GSH 500 ng/ml, selenous acid 4 ng/ml, soybean trypsin inhibitor 100 μg/ml, transferrin 5 μg/ml, and HEPES buffer 2.6 mg/ml. [b,c,d] Like superscripts are not significantly different.

Growth in a defined serum-free medium. The two undefined components of this medium, bovine pituitary extract and endothelial cell growth supplement, were deleted and a competence growth factor provided in the form of either a aFGF or bFGF at 20 ng/ml. Neither competence factor could compensate for the removal of BPE and ECGS. They did not enhance DNA synthesis over basal values (Table 5) or support cell growth (data not shown).

A study of PDGF, as a competence factor to replace ECGS, was repeated using an increased concentration of 20 ng/ml, while BPE was replaced by PGE_1 (25 n*M*) with or without prolactin (1 μg/ml). These replacements, for the undefined factors BPE and ECGS, did allow serum-free cell growth of fetal lung epithelial cells (Fig. 5 *a*). There was no enhancement of cell growth, over that observed with PGE_1 alone, in the presence of prolactin. In separate studies of [^{3}H]thymidine incorporation into epithelial cell DNA there was also no stimulation of DNA synthesis by prolactin alone, but prolactin did reduce the effect of PGE_1 (data not shown). The substitution of PGE_1 for ECGS did not lead to an equivalent enhancement of DNA synthesis. The effect of PGE_1 was approximately 50% of that observed with ECGS, suggesting the presence of an additional mitogen in the crude preparation. When the effect of ECGF (20 ng/ml) was assessed using a poly-D-lysine substratum and a serum-free me-

TABLE 5

EFFECT OF aFGF AND bFGF ON DNA SYNTHESIS BY FETAL LUNG EPITHELIAL CELLS IN SUPPLEMENTED MEDIUM[a]

Supplement	dpm/cm²
Basal medium	3109 ± 96[b]
+ aFGF (20 ng/ml)	1780 ± 269[c]
+ bFGF (20 ng/ml)	2570 ± 327[bc]
+ BPE (100 μg/ml) + ECGS (20 μg/ml)	9938 ± 360[d]

[a] Values are M ± SEM for [^{3}H]thymidine incorporation into DNA of five to eight wells. DMEM + insulin 50 μg/ml, BSA 50 μg/ml, GSH 500 ng/ml, selenous acid 4 ng/ml, soybean trypsin inhibitor 100 μg/ml, transferrin 5 μg/ml, cholera toxin 5 μg/ml, and HEPES buffer 2.6 mg/ml. [b,c,d] Like superscripts are not significantly different.

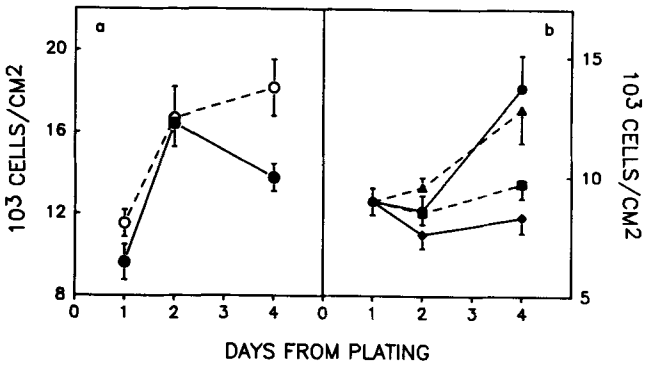

FIG. 5. *a*, Comparison of fetal lung epithelial growth from 1 to 4 days in culture in a defined serum-free medium with (*solid circles*), or without (*open circles*), prolactin. Cells were plated at 2.5×10^4 cells/cm^2 in the absence of serum, and were maintained in a gas phase of 3% O_2: 5% CO_2: 92% N_2. All values are M ± SEM for four wells. *b*, Comparison of fetal lung epithelial growth from 1 to 4 days in culture in a defined serum-free medium at various oxygen tensions. Cells were plated at 3.5×10^4 cells/cm^2 in the absence of serum and were maintained in a gas phase of 3% O_2:5% CO_2:92% N_2 for the first 24 h of culture. Oxygen concentration was then maintained at 3% (*solid circles*), or changed to 21% (*solid triangles*), 50% (*solid squares*), or 95% (*solid diamonds*). All values are M ± SEM for 4 to 16 wells.

dium consisting of insulin (50 μg/ml), BSA (50 μg/ml), GSH (500 ng/ml), selenous acid (4 ng/ml), soybean trypsin inhibitor (100 μg/ml) transferrin (5 μg/ml) HEPES buffer (2.6 mg/ml), cholera toxin (5 μg/ml), PGE_1 (25 mM), and PDGF (20 ng/ml) there was no increase in cell number on Days 4 or 6 of culture compared with cells cultured in the absence of ECGF.

The serum-free defined medium above was also used to examine the effect of elevated oxygen concentrations on cell growth (Fig. 5 *b*). A dose-dependent inhibition of cell growth was evident by 72 h from the gas change.

DISCUSSION

The term distal fetal lung epithelial cell has been used throughout the text, in preference to pneumocyte, because differentiation to characteristic type I and type II pneumocytes is not complete at this early canalicular stage of lung development (22). This time of gestation was selected for cell isolation because of our previous observations, which suggested that isolated epithelial cells from the fetal lung at 19 days of gestation release paracrine growth factors into their culture medium (30). Purification and characterization of such factors would best be conducted from cells that had never been exposed to potential contamination by growth factors in serum. A culture medium that allowed growth in the absence of serum would also potentially overcome serum-mediated difficulties with in vitro models of oxidant tolerance and injury.

The approach taken to develop a defined serum-free medium for this specific cell type was based on established techniques (3,4,42), although no attempt has been made to optimize the concentration of all individual component because growth was obtained without the need to go through this process. In previous studies with this cell type, EMEM was an adequate growth medium in the presence of 10% (vol:vol) FBS (30,34,35). This was not the case with a reduced serum concentration as used in the initial studies reported here, and only DMEM supported growth with 2% (vol:vol) FBS. Using this medium, epithelial cell growth was significantly improved by the use of either laminin or poly-D-lysine substrata, but a response to insulin and IGF-I was only observed on poly-D-lysine. Such a modification of cellular responses to growth factors by substrata has been recognized for other cell types (15,45). The response to IGF-I and to insulin, presumably acting through the IGF-I receptor (40), was anticipated from immunocytochemical studies localizing IGF-I to the epithelium of the developing lung (14). Inasmuch as the epithelium does not contain mRNA for IGF-I (14) it is reasonable to assume that the epithelium has receptors for IGF-I. Of the other factors that enhance distal fetal lung epithelial cell growth, selenium and transferrin are components of most serum-free media (3,4,42), whereas the use of anti-proteases and cholera toxin have been reported for a variety of serum-free media for epithelial cells (24,45). Improved cell growth has been recognized for a wide variety of cell types at a reduced oxygen tension in vitro (2). The selection of a gas mixture containing 3% O_2 was based on an attempt to achieve an oxygen tension close to the ≈20 mmHg observed in fetal blood (26).

Endothelial cell growth supplement is a crude preparation (20) of ECGF, a competence factor related to the EGF family (21). We had anticipated that ECGS could be replaced by aFGF or bFGF in a serum-free system, based on recent studies using adult type II pneumocytes (19), although we had not observed a response to bFGF in earlier studies using a serum-containing medium (30). Upon direct testing with ECGF it was evident that the observed response to ECGS was not attributable to its ECGF content, and was perhaps due to another growth factor present in the crude preparation.

Bovine pituitary extract has been reported to enhance growth of other epithelial cell types in serum-free medium (23), perhaps due to the presence of other pituitary-derived competence factors (37). Our earlier studies with serum-containing medium had shown distal fetal rat lung epithelial cells to be responsive to PDGF. We speculated that PDGF had failed to have a significant effect when first tested in these studies because we had used an inadequate concentration. PDGF was therefore retested at the same time as we attempted to replace BPE with PGE_1 and prolactin, as described by Hammond et al. (13). The combination of PDGF and PGE_1 was able to support cell growth, in the absence of BPE and ECGS, thus constituting a defined serum-free medium. We have subsequently observed, in a separate series of studies, that rat fetal lung epithelial cells in vitro express the gene for both A- and B-type PDGF receptors (Buch et al. unpublished data).

Serum-free culture media have been developed which allow proximal airway epithelial cells from a variety of species to divide in culture (8,16,18,39,44,45). Less success has been achieved with epithelial cells from the distal adult lung. It is only recently that media formulations have been developed which allow one or two population doublings of adult type II pneumocytes (1,36). In contrast to adult type II pneumocytes, distal fetal lung epithelial cells will divide readily, either in the presence of serum or in the defined medium described above. Epithelial cells from distal fetal rat lung share with both adult type II pneumocytes (1,36) and proximal airway cells (8,16,18,39,44,45) a response to insulin and transferrin, but unlike these cell types (1,16,18,36,44,45) do not re-

spond to EGF by enhanced DNA synthesis. The response of distal fetal lung epithelial cells to cholera toxin is opposite to the effect we observed with adult type II pneumocytes (7), although an enhancement of DNA synthesis by cholera toxin has been observed for some (39,45), but not all (18), proximal airway epithelial cells. These differences in the response of distal fetal lung epithelial cells, from the canalicular stage of lung development, could simply reflect differences unique to a fetal cell type. Alternatively, this pattern of responses to growth factors may represent a transitional profile from the growth of airway epithelium during the pseudoglandular stage of lung maturation, and pneumocyte division during the saccular stage of lung development.

We were able to use this defined serum-free medium to test the hypothesis that the presence of serum in culture medium during exposure to elevated oxygen concentrations is responsible for the growth inhibition previously observed (35). Similar degrees of inhibition were observed in the absence of serum as had been observed in the presence of serum. This medium can now be used for the collection of cell-secreted growth factors for the study of epithelial-mesenchymal interactions under basal and oxidant-stressed conditions without concerns that the collected material may contain serum-derived material.

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