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Epidermal Growth Factor Increases Antioxidant Enzyme and Surfactant System Development during Hyperoxia and Protects Fetal Rat Lungs *In Vitro* from Hyperoxic Toxicity

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ABSTRACT. Epidermal growth factor (EGF) has been shown to accelerate fetal lung maturation in rabbits, lambs, and rhesus monkeys *in vivo* and increase surfactant synthesis *in vitro*. Its effect on the maturation of the lung antioxidant enzyme system, however, is unknown. We studied the effect of EGF (10 nM) on 19-d fetal rat lung explant cultures in serum-free medium in air/5% CO_2 or >90% O_2/5% CO_2 compared with similarly grown control cultures in air or hyperoxia at 72 h. Fetal lung activities of superoxide dismutase and catalase were unchanged by EGF in air, whereas glutathione peroxidase activity was significantly decreased ($p < 0.05$ *versus* air control). However, in hyperoxia, EGF-treated fetal lung cultures had significantly elevated superoxide dismutase and catalase activities ($p < 0.01$) *versus* O_2-exposed controls, and glutathione peroxidase activity similar to that of controls. The mRNA levels for all the antioxidant enzymes showed patterns similar to the enzyme activities except in the case of Cu,Zn-superoxide dismutase mRNA, which increased in EGF-air cultures. EGF decreased the rate of ^{3}H-choline incorporation into disaturated phosphatidylcholine in air ($p < 0.01$ *versus* air control), but increased disaturated phosphatidylcholine synthesis in response to hyperoxia ($p < 0.01$ *versus* O_2 control). The histologic appearance of EGF-treated cultures in O_2 was superior to that of O_2-exposed controls, which showed thickened septal walls, decreased surfactant in the air spaces, and epithelial cell mitochondrial swelling. EGF therefore accelerates antioxidant enzyme and disaturated phosphatidylcholine maturation under hyperoxic conditions and protects fetal rat lung cultures from hyperoxic injury. This accelerated O_2-dependent maturation by EGF occurs at the pretranslational level. These findings could have clinical implications for premature infants requiring O_2 therapy and at risk for bronchopulmonary dysplasia because of immature pulmonary antioxidant defenses. (*Pediatr Res* 34: 577–585, 1993)

Abbreviations

EGF, epidermal growth factor
AOE, antioxidant enzyme
DSPC, disaturated phosphatidylcholine
SOD, superoxide dismutase
GP, glutathione peroxidase
CAT, catalase
GAPDH, glyceraldehyde 3-phosphate dehydrogenase
RDS, respiratory distress syndrome
LDH, lactate dehydrogenase
TNA, total nucleic acid

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In all mammalian species studied to date, there is a late gestational increase in surfactant and a parallel increase in the AOE system of the lungs (1, 2). These late gestational biochemical changes prepare the fetal lungs for the transition from a fluid-filled state in a relatively O_2-poor intrauterine environment to air breathing in a relatively O_2-rich environment at birth (3). The prematurely born infant with a poorly developed surfactant system is prone to develop severe RDS and requires early mechanical ventilation combined with O_2 therapy. Prolonged O_2 therapy is associated with the development of chronic lung disease or bronchopulmonary dysplasia believed to be due in part to a poorly developed AOE defense system to counteract toxic O_2 radical species generated by hyperoxia (3, 4).

Lung maturation is known to be regulated or modulated by a number of hormones, among them the polypeptide EGF (5). *In vivo*, EGF has been shown to enhance maturation of alveolar type II cells and increase surfactant production in fetal rabbits (6, 7) and rhesus monkeys (8). Similarly, EGF increased morphologic maturation of the fetal lung and decreased RDS in fetal lambs (9). EGF also caused similar effects on surfactant system maturation *in vitro* in rat fetal lung explant cultures (10) and isolated fetal alveolar type II cells (11). There is no information to date on the effects of EGF on fetal lung AOE system maturation. We investigated whether EGF accelerates the normal late gestational maturation of the AOE in parallel with the surfactant system in fetal rat lung cultures under serum-free conditions. Because the AOE response may be modulated by hyperoxia (12, 13), and because fetal lung exposure to hyperoxia is essentially what occurs clinically in O_2-requiring very premature infants, we also investigated the effect of hyperoxia plus EGF on the AOE and surfactant system in these lung explant cultures.

MATERIALS AND METHODS

Animals. Timed pregnant Sprague-Dawley albino rats were obtained by placing two females and one male together overnight and checking vaginal smears for the presence of sperm the next morning (considered d 0). On the 19th d, pregnant females were anesthetized with an i.p. injection of ketamine:xylazine, 90 mg/kg:10 mg/kg (Ketalar, Parke-Davis, Morris Plains, NJ, and Gemini, Rugby Laboratories, Inc., Rockville Center, NY). Fetuses were obtained by hysterotomy under sterile conditions. Fetuses were given an i.p. overdose of pentobarbital and their lungs were perfused with cold sterile 0.9% NaCl and transferred to chilled

serum-free Waymouth MB 752/1 medium (Media Facility, Department of Microbiology, University of Miami School of Medicine) containing 100 U/mL penicillin and 100 μg/mL streptomycin. Lungs from three to four litters were pooled for each experiment. All animal use protocols were preapproved by the University of Miami Animal Welfare Committee.

Lung explant culture method. The lung explant culture technique described by Gross *et al.* (14) was followed with modifications. Briefly, perfused lungs were chopped into 0.1- to 0.2-mm-thick sections with a McIlwain tissue chopper (Brinkman Instruments, Westbury, NY) and suspended in chilled serum-free Waymouth medium. Cultures were grown on 60 × 15-mm plastic dishes (Falcon, Oxnard, CA) whose surfaces were scratched with a sterile scalpel. Approximately 100 mg of wet tissue in 0.5 mL of medium were placed in each dish and incubated at 37°C in a humidified CO_2 incubator (Bellco Glass Inc., Vineland, NJ) in 95% air/5% CO_2 for 2 h. After 2 h of incubation, the unattached tissue was aspirated off and 2 mL of medium at 37°C was added.

O_2 exposure and treatment with EGF. Explants from 19-d fetal lungs were grown in the presence or absence of 10 nM EGF (human recombinant EGF, United States Biochemicals, Cleveland, OH) in serum-free Waymouth's medium. At 24 h of incubation, half the cultures from control and EGF-treated groups were transferred to an atmosphere of 90 ± 2% O_2/5% CO_2 in a tri-gas incubator (Nuaire Inc., Plymouth, MN) and incubation was continued until 72 h, with fresh medium ± EGF added daily. Oxygen and CO_2 percentages were monitored with Beckman model OM-11 and LB-2 gas analyzers (Beckman Instruments, Inc., Schiller Park, IL). Earlier studies in our laboratory (15) and others (16) have reported medium Po_2 of 150 and 600 mm Hg (20 and 80 kPa) in cultures exposed to 95% air and 95% O_2, respectively.

Preparation of tissue for lung biochemical analyses. At the different time intervals indicated (see Results), medium was aspirated and cultures were rinsed with 0.9% NaCl and gently scraped off into preweighed 12 × 15-mm test tubes. Tissue from two to three dishes was combined for each sample. The tissue was pelleted by centrifugation at 4°C (≈1000 × *g*, 10 min) and the pellet was dried under vacuum overnight (Speed Vac SC100, Savant Instruments Inc., Farmingdale, NY). The dried pellet was weighed and resuspended in 0.9% NaCl and homogenized for 30 s in an Omni 2000 model homogenizer (Omni International Inc., Waterbury, CT). Aliquots were taken for lipid extraction and DSPC analysis. The remaining homogenate was diluted in 50 mM potassium phosphate buffer, pH 7.5, and centrifuged at 27 000 × *g* for 45 min, and the supernatants were kept frozen at −20°C until EGF-treated and control samples were analyzed at the same time for activities of SOD, CAT, GP, and LDH. Aliquots were also analyzed for DNA (17) and protein content (18) before centrifugation.

AOE activities. SOD activity was measured by the xanthine-xanthine oxidase assay (in the presence of 0.015 mM cyanide). The rate of reduction of cytochrome *c* at 550 nm (19) measured the total activities of Cu, Zn, and MnSOD. CAT activity was assayed by the rate of reduction of H_2O_2 at 240 nm (20), and GP activity was measured by the rate of oxidation of NADPH at 340 nm using cumene hydroperoxide as substrate (21). Both Se-dependent and Se-independent GP activities are measured by this method. AOE activities were expressed as U/mg DNA.

LDH assay. LDH activity was measured in both the media and tissue homogenates of cultures using a spectrophotometric assay kit (cat. no. 340-UV, Sigma Chemical Co., St. Louis, MO). Results were expressed as percent of total LDH activity in the medium.

Lipid extraction and DSPC analysis. Lipids were extracted from aliquots of tissue homogenates according to Bligh and Dyer (22). DSPC was separated from the total lipids (23) and analyzed for inorganic phosphorus (24). A known quantity of ^{14}C-dipalmitoyl phosphatidylcholine (New England Nuclear Research Products, Boston, MA) was added during extractions to estimate recovery. Results were expressed per mg dry tissue weight.

Rate of ^{3}H-choline incorporation into DSPC. Explants were grown for 67 h as described above. At 67 h, medium was aspirated and fresh medium containing 2 μCi/mL [methyl-^{3}H]choline chloride (New England Nuclear Research Products), sp act 81 Ci/mmol, was added and incubation was continued until 72 h. At 72 h, medium was collected in silanized glass tubes and stored at −20°C until lipid extraction. The tissue was rinsed with 0.9% NaCl, scraped off into silanized glass tubes, and vacuum-dried overnight. Lipid extraction and DSPC separation were performed separately in the medium and tissue homogenates as described just above. The DSPC extract was counted for ^{3}H/^{14}C radioactivity using a dual-label program in a Tri-Carb model 1900 TR liquid scintillation analyzer (Packard Instrument Co., Meriden, CT). The ^{3}H counts were corrected for losses during extraction using ^{14}C counts, and total ^{3}H counts in the medium and tissue were calculated. Results were expressed as dpm incorporated per mg protein.

^{3}H-thymidine incorporation into DNA. Cultures were grown for 48 h in the presence or absence of EGF in air or 90% O_2 as described earlier. At 48 h, medium was replaced by fresh medium containing 1 μCi [methyl-^{3}H]thymidine (New England Nuclear Research Products, sp act = 80.5 Ci/mmol) and 10 μg of unlabeled thymidine (Sigma Chemical Co.) per mL and incubated for 24 h. At the end of incubation, cultures were rinsed twice with cold PBS and scraped off in the same buffer and homogenized as mentioned earlier. The DNA was precipitated with 10% cold trichloroacetic acid, redissolved in 0.8 N NaOH, and neutralized with 1.0 N HCl. Aliquots were counted for ^{3}H dpm. Protein was quantitated in aliquots of 0.8 N NaOH-soluble precipitate by Lowry's method. Results were expressed as ^{3}H dpm incorporated per mg protein.

cRNA preparations. Rat lung Cu,ZnSOD cRNA was prepared as described previously (25). For preparation of CAT cRNA (26), a 1.1-kb *Pst*I fragment of rat CAT cDNA clone, PM J1010 (27), was subcloned into pGEM-3Z (Promega Corporation, Madison, WI). After transformation in *Escherichia coli* JM109 and linearization of the recombinant plasmid, a ^{35}S-labeled antisense cRNA probe was synthesized using SP6 RNA polymerase and [^{35}S]-uridine triphosphate (New England Nuclear Research Products, Boston, MA). An unlabeled sense cRNA was synthesized using T7 RNA polymerase.

For GP cRNA preparation (26), the plasmid DNA pkS-cGP-1, carrying a ≈0.9-kb fragment of GP cDNA (28), was transformed directly in *E. coli* JM109. After linearization of the recombinant plasmid, ^{35}S-labeled antisense cRNA was prepared using T7 RNA polymerase and [^{35}S]-uridine triphosphate, and unlabeled sense cRNA was synthesized using T3 polymerase.

The GAPDH cDNA clone used for preparation of GAPDH cRNA was a kind gift from Dr. Ph. Fort, Laboratoire de Biologie Moleculaire, Universite des Sciences et Techniques du Languedoc, Montpellier Cedex, France. The *Pst*I fragment of rat cDNA clone (29) was subcloned into pGEM-3Z and ^{35}S-labeled sense cRNA and unlabeled antisense cRNA strands were synthesized as detailed above for CAT cRNA preparation.

AOE mRNA quantitations. mRNA for Cu,ZnSOD, GP, CAT, and GAPDH were quantitated in the TNA extracts of explant culture homogenates by solution hybridization according to Durnam and Palmiter (30). Tissue was homogenized in 1 × SET buffer (1% SDS, 10 mM Tris, pH 7.5, and 5 mM EDTA) in the presence of 200 μg/mL proteinase K (GIBCO-BRL, Gaithersburg, MD). A known amount (≈3500 cpm) of [^{3}H]-α-actin cRNA was added during TNA extractions to estimate mRNA recoveries. The homogenates were incubated at 45°C for 1 h, extracted with chloroform/phenol, and precipitated with ethanol. The ethanol-precipitated pellet was redissolved in RNase-free H_2O at 68°C and stored at −70°C until solution hybridization. Aliquots of TNA extracts were allowed to hybridize with the labeled cRNA probes in the hybridization mixture at 68°C for

16 h. The unhybridized cRNA probes were digested at 55°C for 1 h with 8 U/mL S_1 nuclease and 100 U/mL T_1 ribonuclease (GIBCO-BRL) in the presence of 100 μg/mL salmon testes DNA (Sigma). The hybridized cRNA were precipitated with trichloroacetic acid, filtered on Whatman GF/C filters, and counted in Optifluor-O (Packard, Providence, RI). Unlabeled sense cRNA were used to construct standard curves for each AOE mRNA and for the GAPDH mRNA. mRNA were expressed as molecules per mg DNA.

Light microscopy and transmission electron microscopy. At different time intervals, explant cultures were fixed in a commercial fixative (Decal Chemical Corporation, Congers, NY) overnight. Paraffin-embedded tissues were sectioned to 3- to 4-μm thickness and stained with hematoxylin and eosin, and coded slides were observed under a light microscope for evidence of lung maturation.

For morphometric assessment of the hematoxylin and eosin-stained lung explants at 72 h, coded slides were examined using a standard integrating morphometric eyepiece with seven equal lines and 42 intercept bars (Carl Zeiss, Inc., Thornwood, NY). Random fields were examined at ×400 magnification, with 15–20 fields (explants) counted per slide. A field was counted as long as there were some patent air spaces present. We counted the number of times the intercept bars fell on an air space per field and the number of times the lines were crossed by tissue septa per field. Percent air space was calculated by:

$$\% \text{ air space} = P_A/(P_A + P_T) \times 100$$

where P_A is the number of intercept bars hitting air and P_T the number of intercept bars hitting tissue. L_M or mean linear intercept (= average air space diameter) was calculated by:

$$L_M = \frac{\text{length of line} \times \text{no. of lines} \times \text{no. of fields}}{\text{no. of tissue interceptions}}$$

where length of line = 0.21 mm, number of lines = 7, and number of fields = 15–20/slide (31, 32).

For electron microscopic examination, explants were fixed overnight in 2% glutaraldehyde in 0.1 M cacodylate buffer, pH 7.4. They were then postfixed in a 2% solution of osmium tetroxide in cacodylate buffer and dehydrated by passing through grades of ethyl alcohol. The dehydrated tissue was embedded in Araldite 502 and ultrathin sections were cut with an ultramicrotome (Sorvale Porter-Blum model MT-2, DuPont Co., Wilmington, DE), stained with uranyl acetate and lead citrate, and observed under transmission electron microscopy (model CX-100, Jeol USA, Inc., Peabody, MA).

Statistical analysis. For each parameter measured, the mean ± SD or SEM was calculated using the pooled n value from two to three separate experiments with three to four samples per experiment (n = 6–12). Because each sample in an experiment was derived from different fetal animals, each represents the response of different fetal rat lungs to experimental conditions, and thus each sample was treated independently as a separate n for statistical analysis. Also, because variation between the mean values from the separate experiments was in general no greater than the intrasample variation for each separate experiment, a total n value reflecting the number of individual samples tested for each parameter was considered justifiable.

Comparison of data for the four test groups was by analysis of variance. Intergroup differences were detected by Duncan's multiple range test with Kramer's extension (33, 34). Statistical significance between group values was set at $p < 0.05$.

RESULTS

AOE activities and AOE mRNA in lung explant cultures. The control 19-d fetal rat lung cultures grown in serum-free medium in an atmosphere of air/5% CO_2 showed significant increases in AOE activities of SOD, CAT, and GP from 0 to 72 h (Fig. 1) similar to the late gestational increases reported *in vivo* (1, 2).

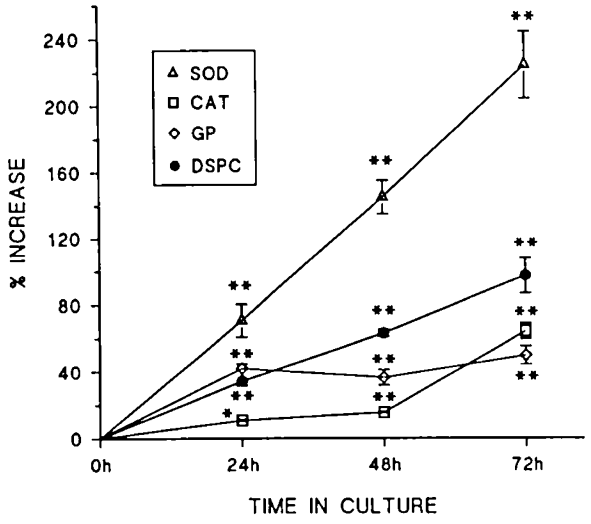

Fig. 1. Increases in AOE activities and DSPC content of 19-d fetal rat lung explant cultures. Explants were grown in serum-free Waymouth MB 752/1 medium for 72 h in an atmosphere of 95% air/5% CO_2. Values are percent increases compared with values for 0-h cultures, which were (in enzyme U/mg DNA) SOD = 25.8 ± 2.1, CAT = 73.8 ± 5.6, GP = 0.175 ± 0.02, and DSPC (μg/mg dry tissue) = 21.3 ± 0.3. Statistical significance at $p < 0.05$ (*) and at $p < 0.01$ (**) *vs* 0-h cultures was determined by analysis of variance and Duncan's multiple range test (n = 6–8 per time point). The error bars in this and subsequent figures indicate SEM.

Untreated rat lung cultures exposed to 90% O_2 (from 24 to 72 h) had increased activities of SOD (↑19%, $p < 0.05$), CAT (↑117%, $p < 0.01$), and GP (↑40%, $p < 0.01$) at 72 h compared with (untreated) air-controls (Fig. 2). EGF treatment of lung cultures in air did not result in any changes in SOD or CAT activities, but a decrease in GP activity (↓30%, $p < 0.05$) occurred *versus* control-air cultures (Fig. 2). Upon O_2 exposure of EGF-treated cultures, significant increases ($p < 0.01$) in SOD (↑48% *versus* air-control; ↑51% *versus* air-EGF; ↑24% *versus* O_2-control) and CAT (↑198% *versus* air-control; ↑216% *versus* air-EGF; ↑37% *versus* O_2-control) activities were observed (Fig. 2). Enzyme activity of GP was not significantly different from O_2-control cultures but was elevated when compared with air-grown cultures (↑26% *versus* air-control, $p < 0.05$; ↑63% *versus* air-EGF, $p < 0.01$; Fig. 2).

The mRNA levels for the three enzymes Cu,ZnSOD, CAT, and GP at 72 h under various experimental conditions are shown in Figure 3. The specific mRNA levels for CAT and GP followed a similar trend as their enzyme activities (*i.e.* an increase in mRNA level consistent with increased activity and *vice versa*). However, the increases in CAT and GP mRNA observed in O_2-control cultures were not statistically significant *versus* control-air cultures, whereas those observed in O_2-EGF cultures were statistically significant *versus* the other three groups (CAT) or *versus* EGF-air cultures (GP). The mRNA levels for Cu,ZnSOD, however, followed a different pattern: although the total SOD activity was unchanged in EGF-air cultures compared with control-air, the Cu,ZnSOD mRNA levels were significantly higher (↑35%, $p < 0.01$). In 90% O_2, as with SOD activity, Cu,ZnSOD mRNA levels were significantly elevated compared with air-grown cultures in both the control-O_2 and EGF-O_2 groups, the mRNA level in EGF-O_2 being further elevated compared with control-O_2 cultures (↑31%, $p < 0.05$).

In contrast to the AOE mRNA, the mRNA content of GAPDH (a marker enzyme generally used for measuring nonspecific gene expression) was unaltered in control-O_2 and EGF-O_2 cultures, whereas the GAPDH mRNA levels in EGF-air cultures were significantly elevated (GAPDH mRNA molecules × 10^{12}/mg DNA, mean ± SD, n = 6: control-air = 2.77 ± 0.30 *versus*

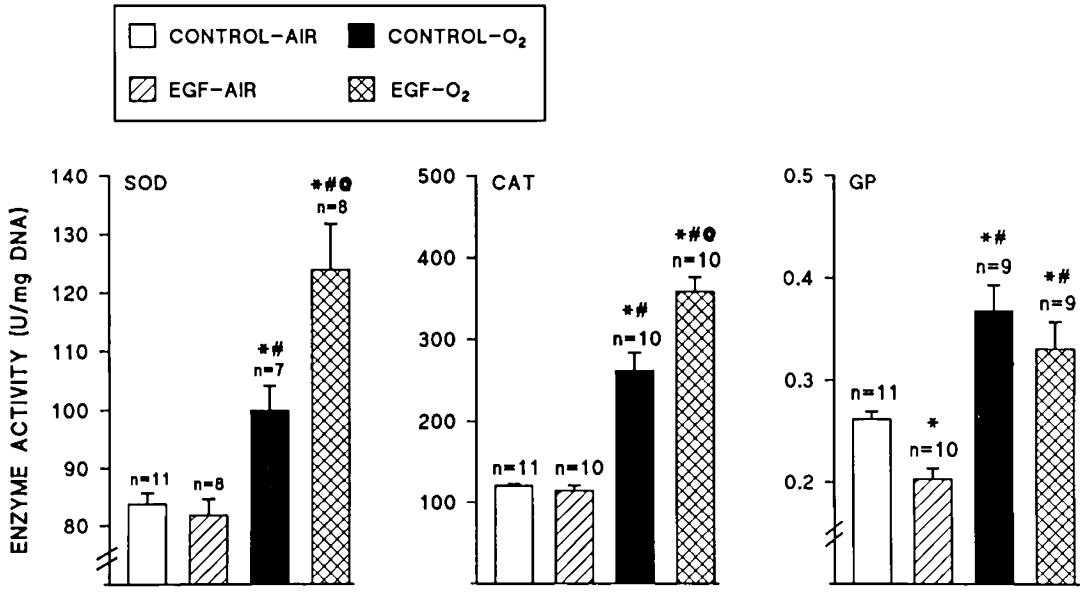

Fig. 2. Effect of EGF on AOE activities of 19-d fetal rat lung explant cultures in air or 90% O_2 at 72 h. Explant cultures treated with EGF (10 nM) or untreated (control) were incubated for 24 h in 95% air/5% CO_2. Half of the explant cultures were then exposed to 90% O_2/5% CO_2 and the other half maintained in air until 72 h. Other details are described in Materials and Methods. Statistical significance at $p < 0.05$ *vs* control-air (*), *vs* EGF-air (#), and *vs* control-O_2 (@) was determined by Duncan's multiple range test.

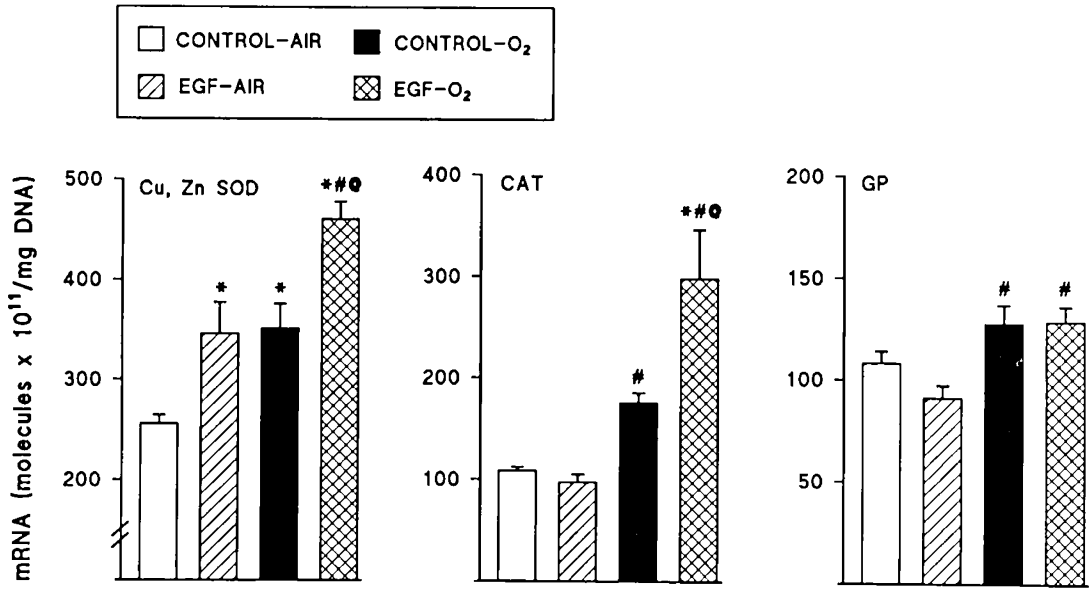

Fig. 3. Effect of EGF on AOE mRNA content of 19-d fetal rat lung explant cultures in air or O_2 at 72 h. See legend to Figure 2 for experimental details. Statistical significance at $p < 0.05$ *vs* control-air (*), *vs* EGF-air (#), and *vs* control-O_2 (@) was determined by Duncan's multiple range test. $n = 6$ per group.

control-O_2 = 2.14 ± 0.64 *versus* EGF-O_2 = 2.58 ± 0.61, $p >$ 0.05; EGF-air = 4.28 ± 0.80, $p < 0.01$, *versus* all the other groups).

DSPC synthesis and DSPC content. The DSPC content of 19-d control lung culture grown in serum-free medium in air/5% CO_2 progressively increased from 0 to 72 h in culture in parallel with the AOE activities (Fig. 1), similar to *in vivo* findings (1, 2). The rate of DSPC synthesis at 72 h as measured by ^{3}H-choline incorporation (dpm incorporated/mg protein) was significantly decreased by EGF treatment in cultures maintained in air [control-air = 15 035 ± 795; EGF-air = 10 725 ± 1130; (↓40%), n = 6, $p < 0.01$]. This was accompanied by a similar decrease in DSPC content (μg/mg tissue) [control-air = 33.3 ± 8.0, $n = 6$ *versus* EGF-air = 25.6 ± 4.0, $n = 9$; (↓23%), $p < 0.05$]. O_2 exposure of untreated cultures resulted in a small but significant increase in the rate of DSPC synthesis [control-O_2 = 16 485 ± 980; (↑10% *versus* control-air), $n = 6$, $p < 0.05$] whereas the increase observed in EGF treated cultures was much greater [EGF-O_2 = 19 880 ± 1105, $n = 6$, (↑85% *versus* EGF-air; ↑32% *versus* control-air; ↑21% *versus* control-O_2); $p < 0.05$ or less in all cases]. These DSPC synthesis rates appear to correlate with the electron microscopic observations on lamellar body contents and air space surfactant (see below).

Histologic observations. We examined the tissue explants by light microscopy as well as electron microscopy to see whether the observed biochemical changes were associated with identifiable morphologic changes. Under a light microscope, the control cultures grown in air for 72 h showed morphologic changes such as an increase in air space, a decrease in mesenchymal tissue, and thinning of interstitial septa (Fig. 4*A*). There was no micro-

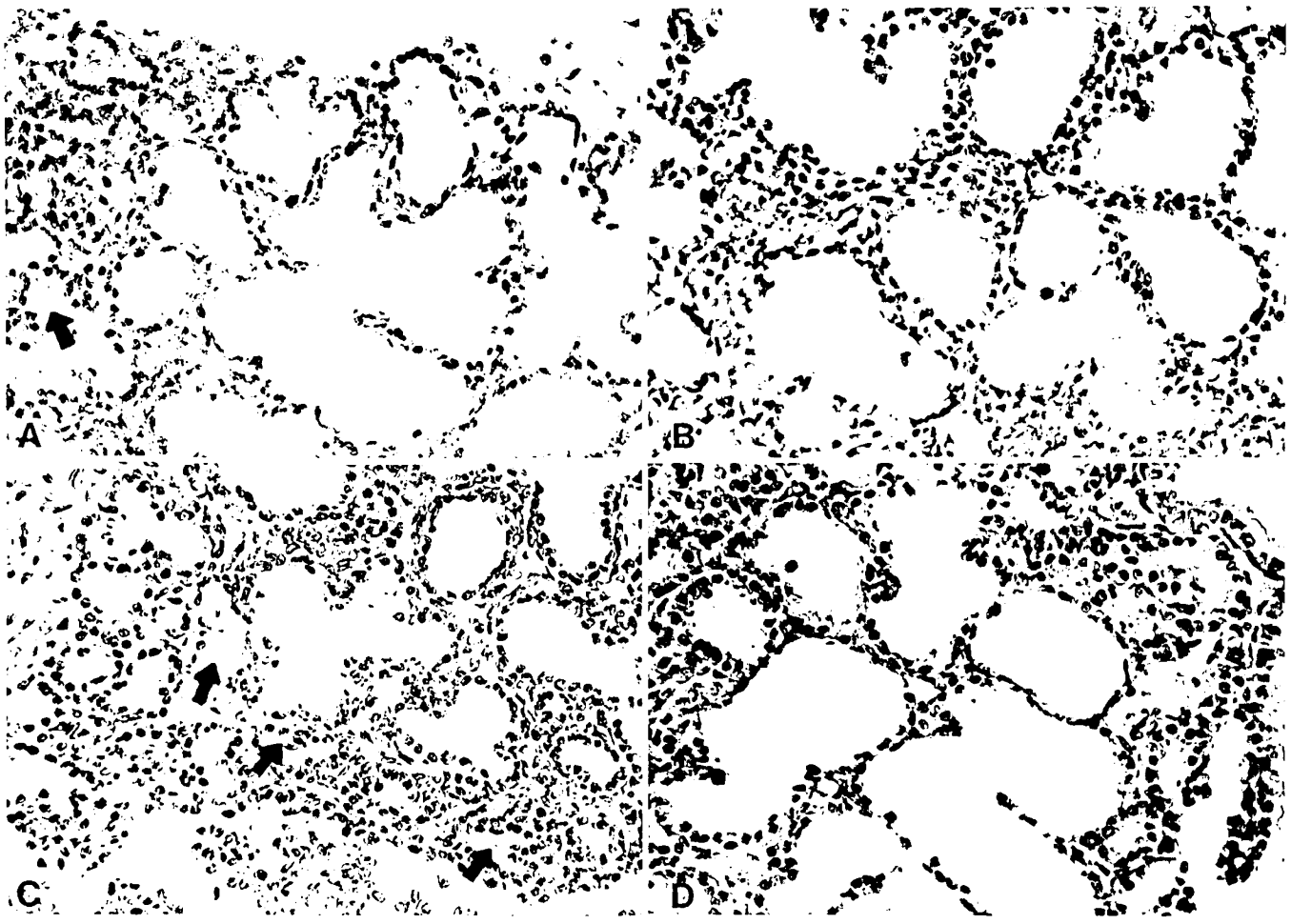

Fig. 4. Light microscopic appearance of 19-d fetal rat lung explant cultures treated with 10 nM EGF or untreated (control), grown in air or 90% O_2, at 72 h. *A*, Control-air; *B*, EGF-air; *C*, control-O_2; *D*, EGF-O_2. Control-air (*A*) and EGF-air (*B*) cultures appear quite similar by light microscopy except for the more homogeneously sized air spaces in the EGF-air lungs and typical small areas of air space collapse in the control-air explants (*arrow*). Note the increased thickness of interstitial septa in the O_2-exposed controls (*C*) compared with the EGF-O_2 cultures (*D*), which closely resemble the air cultures (*A* and *B*). Note also the areas of collapsed and partially collapsed air spaces in the control-O_2 lung explants (*arrows*). Hematoxylin and eosin stain. Magnification ×250.

scopic evidence of gross tissue necrosis up to 72 h in any of the explants. However, the control-O_2 cultures revealed significant morphologic differences such as a large proportion of collapsed air spaces and much thicker interstitial septa (Fig. 4*C*). In contrast, EGF-O_2 cultures had comparatively few collapsed air spaces and consistently thinner interstitial septa (Fig. 4*D*).

The percentage of LDH activity in the medium was high at 24 h (average for all cultures ≈35%) but decreased consistently reaching 4–12% at 72 h (Table 1). This initial high LDH activity is likely due to a large proportion of cut edges resulting from thin sectioning of tissue (14), which seemed to repair during the culture period. The percentage of LDH release in control-O_2-exposed cultures was not significantly different from control-air cultures, suggesting that 90% O_2 for 48 h was not extremely cytotoxic for these cultures. The percentage of LDH activity in the medium was substantially lower in the EGF-O_2 group compared with the other three groups (EGF-O_2 = 3.7 ± 0.7%, $n = 6$; $p < 0.01$), consistent with the improved histologic appearance.

Table 1. *Effect of EGF (10 nM) on LDH release and morphometry in 19-d fetal rat lung explant cultures grown in air or O_2 at 72 h**

Parameter	Control-air	EGF-air	Control-O_2	EGF-O_2
LDH in medium (%)	11.5 ± 2.4	8.5 ± 1.2†	9.7 ± 2.2	3.7 ± 0.7†‡§
n	7	3	10	6
Morphometry				
% Air space	38.2 ± 2.7	51.3 ± 4.2§‖	36.6 ± 4.2	49.5 ± 1.0§‖
n	3	2	3	3
L_M (μm)	49.3 ± 3.1	71.2 ± 3.6§‖	49.2 ± 0.8	65.0 ± 4.1§‖
n	3	2	3	3

* Values are mean ± SD. L_M, mean linear intercept or mean air space diameter. For morphometry, *n* = no. of experiments, with 15–20 fields (coded lung explants) examined per experiment.

† $p < 0.05$ or less *vs* control-air.

‡ $p < 0.01$ *vs* EGF-air.

§ $p < 0.01$ *vs* control-O_2.

‖ $p < 0.01$ *vs* control-air.

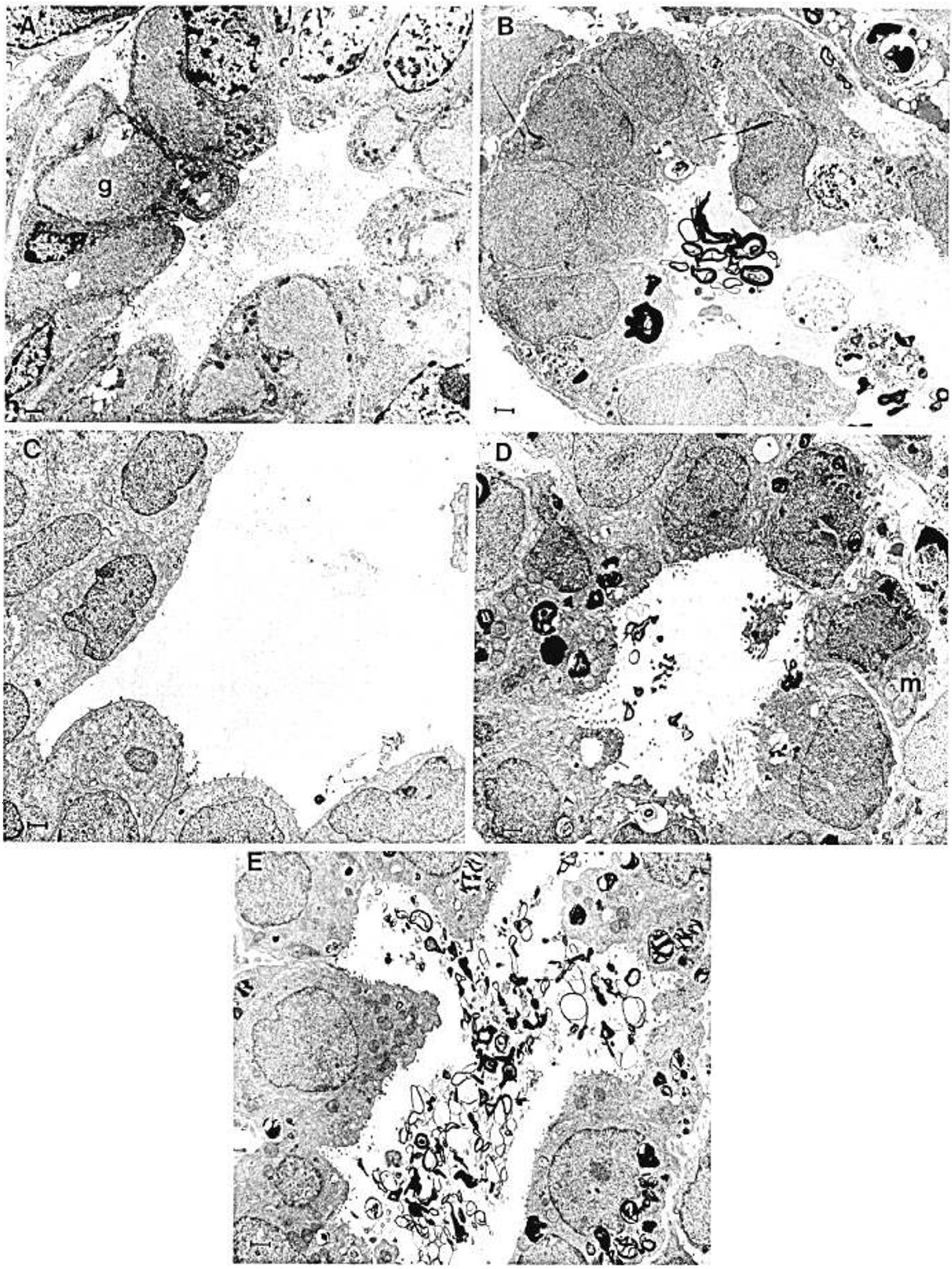
A
g
B
C
D
m
E

Table 1 also summarizes the morphometric data in fetal rat ung cultures at 72 h under various experimental conditions. Morphometrically, EGF-air cultures at 72 h had an increased ercentage of air space (↑34%, $p < 0.01$) and larger mean air space diameter (↑44%, $p < 0.01$) *versus* control-air cultures. The morphometric parameters (percentage of air space and mean air space diameter) of patent air spaces in control-O_2 cultures did not differ significantly from those in control-air cultures, whereas EGF-O_2 cultures had a significantly increased percentage of air space (↑35%, $p < 0.01$) and mean air space diameter (↑32%, $p < 0.01$) compared with control-O_2 cultures.

Electron microscopic observations provided further support that the 19-d lung cultures underwent histologic maturation during the culture period. At 0 h, the cells lining the air spaces appeared columnar, with large dense nuclei, abundant glycogen stores, and only a few nascent lamellar-type bodies (Fig. 5*A*). At 72 h, the control-air cultures had considerably less dense nuclei, much smaller glycogen stores, increased lamellar body content, and considerable osmiophilic material in the air spaces (Fig. 5*B*). The ultrastructural appearance of EGF-air cultures (Fig. 5*C*) indicated poorer maturation of epithelial cells with very few lamellar bodies and proportionately more glycogen stores in the cells (consistent with the DSPC findings noted above). The O_2-exposed cultures at 72 h showed improved maturation of type II cells, with increased lamellar body content (Fig. 5*D* and *E*), again consistent with the increased rate of DSPC synthesis described earlier. However, in control-O_2 cultures, only a small amount of surfactant was observed in the open air spaces, whereas the lamellar bodies in type II cells were comparatively enlarged and dense in appearance (Fig. 5*D*). The mitochondria in O_2-exposed control cultures were considerably swollen in comparison with air-grown cultures, and many of the mitochondria also showed aberrant appearance of cristae. The EGF-treated cultures exposed to 90% O_2 showed improved maturation of type II cells and greater lamellar body content than the other three groups. The air spaces in EGF-O_2 cultures were filled with abundant surfactant material (Fig. 5*E*), consistent with the significantly increased DSPC synthesis. The appearance of mitochondria in EGF-O_2 cultures at 72 h was comparable with that in air-exposed cultures.

DNA and protein. Table 2 summarizes the effects of EGF treatment on fetal explant lung DNA synthesis, DNA and protein content, and protein to DNA ratio in air and in hyperoxia. The DNA synthesis rate (measured by ^{3}H-thymidine incorporation) between 48 and 72 h was significantly higher in EGF-treated cultures both in air and in O_2 compared with untreated cultures. No significant O_2 effect was observed for either the control or the EGF-treated explants. Similarly, EGF treatment markedly increased the DNA and protein content of the explant cultures in air and in O_2. The only significant O_2 effect observed was in the elevated protein content of the O_2-EGF explants.

DISCUSSION

By decreasing the thickness of the lung explants, we were able to avoid the high O_2 requirement previously reported for fetal rat lung cultures (14). This has enabled us to study the effect of EGF on biochemical and morphologic maturation of fetal lungs in air as well as in hyperoxia under serum-free conditions.

The effects of EGF on lung surfactant phospholipids, surfactant apoproteins, and morphologic maturation have been previously studied in various species (6–11). To our knowledge, this is the first report of EGF's effect on the maturation of the fetal lung AOE system. Our study also shows that the effects of EGF are strongly modulated by hyperoxia (and *vice versa*).

We found that under lower O_2 concentrations EGF had essentially no positive effect on the normal late gestational increase in fetal lung AOE activities (Fig. 2). Similarly, EGF did not increase the content or the synthesis of DSPC, which normally has a time course of maturation very similar to the chronology of AOE system maturation both *in vitro* and *in vivo* (1–3). However, EGF was able to substantially stimulate both lung biochemical systems under hyperoxia, resulting in increased AOE activities and an augmented DSPC synthesis rate in response to high O_2 challenge.

Gross *et al.* (10) have reported increases in DSPC synthesis by EGF in fetal rat lung cultures grown in 95% O_2 similar to the increases observed in the present study. However, the absence of definitely increased histologic maturation (Fig. 5*C*) and a clear decrease in DSPC synthesis in our EGF-air cultures is in contrast to the reported effects of *in vivo* administration of EGF in rats (35) and other fetal animal species (6–9). The differences observed between the above *in vivo* studies and our *in vitro* cultures may have been due to the absence of systemic hormonal interactions in our serum-free culture conditions. Similar to our present findings, others (36) have reported depressed numbers of lamellar bodies and retarded histologic maturation in fetal rat lung epithelial cells chronically exposed to EGF under normoxic, serum-free conditions. The above observations suggest that the effects of EGF on lung maturation under *in vitro* and *in vivo* conditions may be regulated by different mechanisms.

The ability of EGF to induce increased AOE activities under hyperoxic conditions appears similar to the AOE response to hyperoxia recently reported in newborn rats prenatally treated with dexamethasone (37). In that study, dexamethasone did not alter lung AOE levels in air-exposed neonatal rats, but resulted in significantly increased AOE responses to hyperoxic challenge and improved survival of the newborns in hyperoxia. The effect of EGF on fetal lung AOE in our explant cultures also appears similar to the effects reported in lungs of adults treated with low doses of bacterial endotoxin. When adults are maintained in air, no effect of endotoxin on pulmonary AOE activities is noted, whereas the endotoxin-treated adult lungs demonstrate significantly increased AOE activities (and AOE mRNA) on challenge with hyperoxia and become remarkably O_2 tolerant (38). Whether or not the response of lung cells to dexamethasone, endotoxin, or EGF under hyperoxia is mediated by a common mechanism needs to be further studied.

Under hyperoxic conditions, EGF's stimulatory effect on AOE induction may have directly resulted in improved morphologic and histologic appearance of the lung cultures. The ability to augment lung AOE activities under hyperoxia, rather than the basal AOE levels *per se*, has been repeatedly shown to be a key factor associated with the development of resistance to O_2-induced lung damage (3, 4, 13, 37–40). Even though fetal rat lungs grown in 90% O_2 in the present study were found to be able to increase their AOE activities in the absence of EGF, the AOE response was significantly greater when EGF was present in cultures, thus strongly suggesting an enhanced beneficial effect

Fig. 5. Transmission electron micrographs showing the effect of 10 nM EGF on the histologic maturation and epithelial (type II) cell appearance of 19-d fetal rat explant cultures grown in air or 90% O_2. *A*, 0-h explants showing columnar epithelial cells filled with glycogen granules (*g*) and very few and immature lamellar bodies compared with cultures at 72 h; *B*, 72-h control-air; *C*, 72-h EGF-air; *D*, 72-h control-O_2; and *E*, 72-h EGF-O_2. Note that the control-air explants at 72 h (*B*) have more lamellar bodies in type II cells plus more osmiophilic material (surfactant) in the air space compared with the more immature appearance of the EGF-air explants (*C*). The control-O_2 cultures have more prominent and very dense-staining lamellar bodies in type II cells but few secreted into the air space (*D*), whereas the lamellar bodies of EGF-O_2 explants have a less dense and more lamellar appearance and the air space of EGF-O_2 explants (*E*) is filled with surfactant secretions. Note also the swollen appearance of mitochondria (*m*) in control-O_2 explants compared with others and the aberrant cristae within many of the mitochondria. Magnification ×9000. Scale bar = 10 μm.

Table 2. *Effect of EGF (10 nM) on tissue growth parameters in 19-d fetal rat lung explant cultures grown in air or O_2 at 72 h**

Parameter	Control-air	EGF-air	Control-O_2	EGF-O_2
^{3}H-thymidine incorporated (dpm/mg protein)	44 952 ± 5952 (9)	104 760 ± 9000†‡ (8)	53 088 ± 5880 (10)	107 736 ± 16 104†‡ (10)
DNA (mg/plate)	0.31 ± 0.08 (10)	0.53 ± 0.16†‡ (10)	0.37 ± 0.14 (10)	0.53 ± 0.08†‡ (9)
Protein (mg/plate)	2.00 ± 0.58 (10)	2.97 ± 0.91† (10)	2.56 ± 0.52 (10)	4.08 ± 1.07†‡§ (9)
Protein/DNA ratio	6.45 ± 1.17 (10)	5.67 ± 0.61 (10)	7.45 ± 1.75§ (10)	7.71 ± 1.25†§ (9)

* Values are mean ± SD for number of observations in parentheses. Statistical significance by Duncan's multiple range test.
† $p < 0.05$ *vs* control-air.
‡ $p < 0.05$ *vs* control-O_2.
§ $p < 0.05$ *vs* EGF-air.

of EGF in the premature lung challenged with hyperoxia. It is also conceivable that increases in AOE activities (and DSPC synthesis) observed in control-O_2 cultures in serum-free medium are mediated by a possible EGF-related effect due to local induction of EGF and/or synthesis of other active paracrine factors under hyperoxic conditions. The interesting observation by Tanswell *et al.* (41) that medium from fetal lung cell cultures exposed to hyperoxia is capable of increasing AOE activity of lung cells grown in the absence of high O_2 concentrations would tend to support such a possibility.

The AOE activity changes in fetal lung explant cultures produced by EGF under hyperoxia were in general accompanied by changes in AOE mRNA levels of similar magnitude. Our study did not definitely elucidate whether the changes were regulated at the transcriptional level or were due to posttranscriptional mechanisms such as changes in mRNA stability. Clerch *et al.* (42, 43) have recently shown that the normal developmental increases in fetal rat lung CAT activity during late gestation as well as the accelerated rise in fetal lung CAT activity stimulated by prenatal dexamethasone treatment are mediated at the transcriptional level. In contrast, increases in CAT, Cu,ZnSOD, and GP in newborn animal lungs under hyperoxic exposure are brought about primarily by increased stability of AOE mRNA. Chen *et al.* (26) have found that prenatal thyroid releasing hormone treatment of late gestation rats produced concomitant decreases in fetal lung AOE activities and mRNA with no changes in mRNA half-lives, suggesting again that changes in fetal lung AOE activities are transcriptionally regulated under control and hormone treatment conditions.

In addition to the augmented AOE activities, the increase in DSPC synthesis accompanied by an increase in the surfactant released into the air space in EGF-O_2 cultures (compared with O_2-controls) may have contributed to the protective effect of EGF under hyperoxia. The recent work by Baker *et al.* (44) and Matalon *et al.* (45) has strongly suggested that natural lung surfactant itself may have important antioxidant (O_2 radical-scavenging) properties.

Another possible explanation for the protection offered by EGF against hyperoxia in our fetal lung cultures is obtained by indirect evidence from the present study as well as direct evidence by others. EGF has been found to increase lactate levels and decrease total O_2 consumption *in vivo* (46), as well as to increase lactate production *in vitro* in human breast cancer cells (47). In our explant system, total LDH activity in EGF-treated cultures was elevated 2- to 3-fold *versus* nontreated cultures (control-air = 2865 ± 461, $n = 7$; EGF-air = 6413 ± 537, $n = 3$; control-O_2 = 2585 ± 588, $n = 10$; EGF-O_2 = 8253 ± 2273 U/plate, $n = 6$). These marked LDH activity increases indirectly suggest that increases in lactate levels (not measured) may have occurred. Taken together, these facts suggest that EGF treatment may increase glycolysis and decrease O_2 consumption at the mitochondrial level, thereby decreasing the production of toxic O_2 free radicals under hyperoxia.

Our data on ^{3}H-thymidine incorporation and DNA content (Table 2) indicate that EGF induces cell proliferation in lung explant cultures under both normoxic and hyperoxic conditions. Inhibition of DNA synthesis is a frequently reported concomitant of hyperoxic exposure of lung tissue *in vitro* (41, 16) and *in vivo* (see ref. 4 and references cited therein). Increased cell proliferation is likely to be critical not only for prenatal lung growth but also for repair of O_2-induced lung damage. Thus, EGF's ability to induce both greater cell proliferation and cell maturation under hyperoxia may have been an important factor in protecting fetal lung cells exposed to high O_2. This dual effect of EGF on lung cell proliferation and maturation contrasts with the well known antiproliferative effect of dexamethasone, whose biochemical effects on fetal lung maturation are quite similar to those of EGF.

Finally, the observation that EGF can induce fetal lung AOE and surfactant increases under hyperoxic conditions may be of particular significance to the O_2-requiring premature infant who is susceptible to chronic lung disease (bronchopulmonary dysplasia). EGF could potentially offer a method of postnatal therapy in premature infants with RDS to stimulate an immature AOE defense system to respond protectively to hyperoxic treatment. Similarly, EGF's effect on DSPC synthesis (and secretion) during hyperoxia would be another important potential benefit of EGF treatment. Because EGF is also reported to induce maturation of intestinal mucosa under experimental conditions (48), this could be an added benefit to premature infants in whom early enteral feeding to stimulate gastrointestinal tract maturation is often withheld. Obviously, more studies on these potential benefits as well as possible negative side effects of EGF need to be done before recommending EGF treatment for the respiratory distressed premature infant, but as a natural component of maternal breast milk it could be a very tempting treatment for future clinical consideration.

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