DEVELOPMENTAL BIOLOGY 159, 462-473 (1993)

Retinoic Acid Stimulates Mouse Lung Development by a Mechanism Involving Epithelial–Mesenchymal Interaction and Regulation of Epidermal Growth Factor Receptors

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Accepted May 14, 1993

Retinoic acid (RA) stimulated proliferation of both epithelial and mesenchymal cells in cocultures isolated from developing mouse lungs. There was a corresponding increase in epithelial branching activity in organ culture of embryonic lungs exposed to similar doses of RA. Stimulation was maximal with concentrations of 1 μM and progressively decreased with either lower or higher concentrations. However, when lung cell monocultures of isolated epithelial and mesenchymal cells were exposed to RA, the mitogenic effect was observed only in the mesenchymal population. This suggests that RA may not have a direct mitogenic effect on epithelial cells but rather functions indirectly through the mesenchyme. The cellular response to RA was correlated with an increase in the expression of epidermal growth factor receptor (EGFR). Epidermal growth factor (EGF) also stimulated terminal branch formation in the developing lung. Unlike RA, EGF stimulated proliferation in both epithelial cells and mesenchymal cells in monoculture. In comparison, transforming growth factor-α, which also binds to the EGFR, elicited no response. We conclude that RA stimulates cell proliferation and branching activity in the developing mouse lung by a mechanism involving epithelial–mesenchymal interactions. The effect is, in part, produced by stimulation of EGFR expression, with the resulting amplification of the cellular response to EGF or other EGFR ligands. In this process the mesenchyme provides a paracrine support to the epithelium, otherwise unresponsive to RA. Further studies identified the mesenchyme as a major source of EGF in the embryonic lung, suggesting that mesenchymal EGF may represent a paracrine factor involved in the epithelial response to RA.

INTRODUCTION

Retinol (vitamin A) and its active derivative, retinoic acid (RA) play an important role in cell differentiation (Strickland *et al.*, 1980; Shapiro, 1986; Wuarin and Sidell, 1991) and vertebrate morphogenesis (Tickle *et al.*, 1982; Eichele and Thaller, 1987; Abbott *et al.*, 1990). RA exerts its effect on cells in part through a family of nuclear receptors (Petkovich *et al.*, 1987; Krust *et al.*, 1989; Zelent *et al.*, 1989) which act as transcriptional factors (Vasios *et al.*, 1989, de The *et al.*, 1990). Although many RA-responsive genes have been described in a variety of cell types, RA-responsive elements have thus far been identified on the 5′ flanking regions of only few genes. One of these is the murine laminin B1 gene, encoding a subunit of the extracellular matrix protein laminin (Vasios *et al.*, 1989).

RA has been shown to stimulate epidermal growth factor receptor (EGFR) synthesis (Jetten, 1980; Komura *et al.*, 1986; Thompson and Rosner, 1989) thereby enhancing the cellular response to epidermal growth factor (EGF). By binding to its receptor and inducing tyrosine autophosphorylation (Carpenter, 1987; Heisermann and Gill, 1988) EGF exerts a mitogenic effect on a variety of cells derived from epithelial and mesenchymal sources (Carpenter and Cohen, 1979; Gospodarowicz, 1981; Brown *et al.*, 1986; Schreiber *et al.*, 1986).

In the lung, RA is known to affect the differentiation of tracheobronchial epithelium as well as surfactant production (Sporn *et al.*, 1975; Whitsett *et al.*, 1987). However, the high level of expression of nuclear RA receptors and cytoplasmic cellular binding proteins since early lung organogenesis (Ong and Chytil, 1976; Dolle *et al.*, 1990) suggests a far wider involvement of the molecule in the development of this organ.

In the present study, we have examined the effects of RA on mouse lung organogenesis in primary cultures of lung cells and in an organ culture model. When cultures of mixed lung cell populations were prepared from developing lungs and treated with RA, proliferation of both epithelial and mesenchymal cells was observed. Similarly, in embryonic lung explants, RA stimulated cell proliferation in both epithelium and mesenchyme, resulting in an increase in branching activity. However,

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when the two cell populations were separated prior to treatment and treated in monoculture, only the mesenchymal cells responded. These data are consistent with the suggestion that RA has a direct effect on lung mesenchymal development whereas its effect on epithelial morphogenesis depends on a mesenchymal paracrine support. Our studies further suggest that the mesenchymal effect on the epithelium is mediated by the EGFR.

MATERIALS AND METHODS

Mesenchymal and epithelial cell monocultures and cocultures. CD-1 strain (Charles River) mice were mated and the day of finding a vaginal plug was designated as Day 0 of embryonic development (total gestation period is 19 days; lung development begins on late Day 9). Pregnant females were sacrificed and the embryos were collected and immediately decapitated. The lungs were then dissected under a dissecting microscope. Lungs from Gestational Days 12 to 17 (embryonic and fetal periods) were used to isolate mesenchymal cells, and lungs from Days 15 to 17 (fetal period only) were used to generate cocultures or to isolate epithelial cells (we found that epithelial cells from developmental stages earlier than Days 14 and 15 do not survive in the absence of mesenchyme).

Lung tissue was minced and placed in phosphate-buffered saline (PBS) containing 0.3% trypsin and 0.1% EDTA for 10 min at 37°C. A single cell suspension was obtained by forcing cell aggregates and pieces of tissue through a micropipete several times. The cells were then filtered through a 100-μm pore mesh and resuspended in minimal essential medium (MEM) (Gibco, Grand Island, NY) with 10% fetal calf serum (FCS) (Hyclone Lab, Logan, UT), nonessential amino acids (Gibco), 0.29 mg/ml L-glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin, and 0.25 μg/ml amphotericin B (Irvine Scientific, Santa Ana, CA).

Epithelial-mesenchymal cocultures were generated by plating mixed cell populations and culturing them in medium with 10% FCS for a period of 12 to 48 hr. Cocultures were additionally generated by plating together equal numbers of epithelial and mesenchymal cells obtained from monocultures (described below).

To generate monocultures, cell suspensions were plated and incubated for 30 min at 37°C to allow the mesenchymal cells to attach (Scott *et al.*, 1983). After the initial incubation, the nonattached cells were removed and the attached mesenchymal cells were cultured in medium containing 10% FCS until semiconfluency was reached (approximately 24 hr). The nonattached cells were then replated for an additional 1 hr to allow the remaining mesenchymal cells to attach. The cells that were still not attached, essentially epithelial, were collected and cultured in medium containing 10% FCS until semiconfluency was reached (approximately 48–72 hr).

All the cultures were switched to serum-free medium to carry out the experiments. To assess cell composition, culture samples were fixed in alcohol for 10 min and immunostained with a rabbit polyclonal anti-keratin antibody (Dako, Carpinteria, CA) to identify epithelial cells or anti-vimentin (Dako) to identify mesenchymal cells. Only mesenchymal monocultures with less than 1% keratin-positive cells and epithelial monocultures with 10% or less mesenchymal cell contamination were used to carry out experiments.

Lung organ cultures. The lower right lobes of lungs from Days 12 and 13 of gestation were isolated and cultured at the air–medium interface on the upper surface of polycarbonate filter membrane inserts, 0.4-μm pore size (Millipore, Bedford, MA) in medium containing 2% delipidized FCS (Cocalico Biologicals, Reamstown, PA). Preliminary studies demonstrated that a FCS concentration of 2% resulted in the arrest of branching activity (more than 75% inhibition over a period of 2 days compared to 10% FCS) without damaging the lung explants as determined by a ^{51}Cr release assay (Varani *et al.*, 1985). Lower FCS concentrations sharply increased ^{51}Cr release from the organ cultures and were considered detrimental. Organ cultures were incubated at 37°C in 5% CO_2 for 48 hr and occasionally for periods of up to 1 week. HPLC analysis demonstrated no measurable levels of RA or retinol in the lung explant after 12 hr in culture. However, detectable levels of retinyl esters were still found after culturing the explants for 18 hr or more.

RA, EGF, transforming growth factor-α (TGF-α), and antibodies. RA was obtained from Sigma (St. Louis, MO) and dissolved in dimethyl sulfoxide (DMSO) (Sigma). Mouse EGF and rabbit polyclonal antibody to mouse EGF were obtained from Collaborative Biomedical (Bedford, MA). Rat recombinant TGF-α was purchased from Calbiochem (La Jolla, CA) and from Peninsula Laboratories (Belmont, CA). Affinity-purified mouse monoclonal antibody to the mouse EGF receptor was purchased from Calbiochem. An affinity-purified rabbit polyclonal antibody to murine EHS laminin was purchased from Collaborative Biomedical. An additional polyclonal antibody against murine laminin was a gift from Dr. Amy Skubitz. Rabbit and mouse sera and mouse IgG (Cappel, Malvern, PA) were used as controls.

Cell proliferation assays. Semiconfluent epithelial and mesenchymal monocultures and cocultures were exposed in serum-free medium to RA at concentrations ranging from 0.1 to 3 μM or equivalent volumes of DMSO. Additional wells were left untreated as control for possible DMSO effects. After 24 and 48 hr of treat-

ment the cells were trypsinized and counted in a Coulter Counter. In additional studies, lung cell monocultures were exposed to EGF at concentrations ranging from 2 to 100 ng/ml for the same period of time. Cell proliferation was then determined by direct cell counting. All the experiments were repeated at least three times.

Tritiated thymidine incorporation and autoradiography were used as a second means to assess proliferation. Lung explants and cell monolayers were cultured for 24 hr in the presence of RA or DMSO, or left untreated. Then 1 μCi/ml of [^{3}H]thymidine was added to the cultures. After 4 to 24 hr the cultures were lysed and incorporation of [^{3}H]thymidine was estimated in a β-scintillation counter. In additional studies, cell monolayers and frozen sections of lungs treated with RA and exposed to [^{3}H]thymidine were fixed in 1% gluteraldehyde, dipped in NTB2 emulsion (Eastman Kodak Co., Rochester, NY), and exposed for 48 hr at 4°C. Autoradiographies were then developed and the percentage of labeled nuclei was determined. A different approach was used to determine [^{3}H]thymidine incorporation in cocultures. Since epithelial cells cluster and repolarize in cocultures (Schuger *et al.*, 1992; and Results section), the average number of labeled nuclei was calculated per cluster. Incorporation of [^{3}H]thymidine by the mesenchymal compartment of the cocultures was estimated as the average number of labeled nuclei per field, in a field outlined on a projection screen.

Immunolocalization of the EGF receptor. Sections from embryonic and fetal lungs were fixed in 4% paraformaldehyde followed by absolute methanol, exposed to 3% hydrogen peroxide and incubated with a 1:50 dilution of either anti-EGFR or controls (i.e., normal mouse serum, mouse IgG, or PBS) for 2 hr at room temperature. The sections were then washed in PBS and the EGFR/anti-EGFR immunoreaction was detected using a streptavidin-biotin system kit (Dako).

EGFR binding assays. Monocultures and cocultures were exposed in serum-free medium to various concentrations of RA or to equivalent dilutions of DMSO for periods of up to 24 hr. The plates were then washed and incubated for 1 hr at 4°C in serum-free medium with the addition of 0.1% bovine serum albumin (BSA; Sigma) and 1 ng/ml of [^{125}I]EGF (95.5 mCi/mg; ICN, Costa Mesa, CA). Each well received approximately 10^5 cpm. At the end of this incubation the cells were either lysed and analyzed in a γ-counter or counted. Nonspecific binding was determined in parallel cultures which received a 200-fold excess of unlabeled EGF. Nonspecific binding was 4–10% of the total binding and was subtracted from all points.

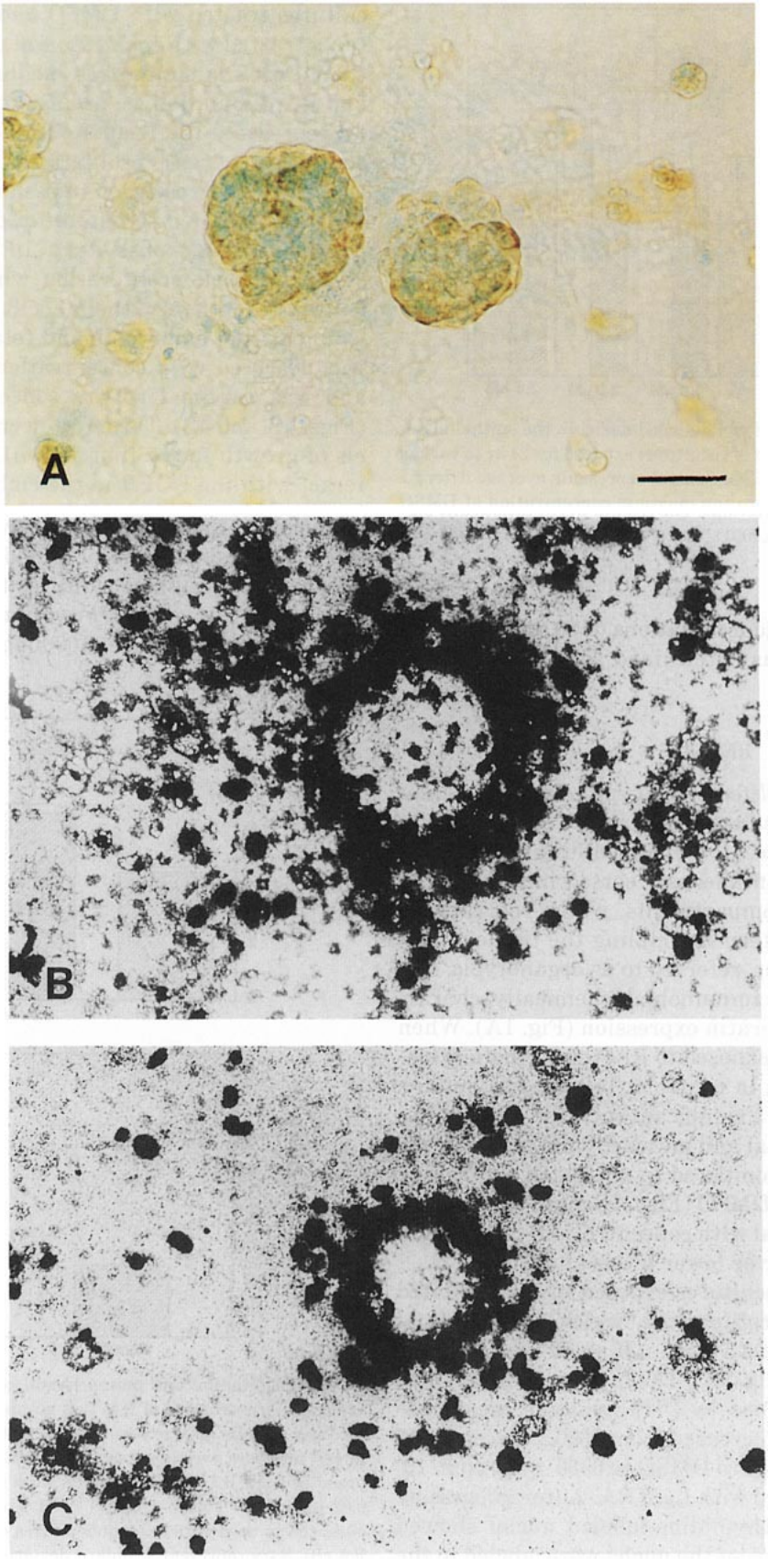
Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Lungs from Days 12 and 13 of gestation were incubated overnight in methionine-free medium supplemented with 100 μCi/ml of [^{35}S]methionine (1.0–1.4 Ci/mmol; NEN-Dupont, Boston, MA). After incubation, the tissues were lysed and centrifugated at 10,000*g* for 10 min. The desired protein was precipitated from the cell lysate with a 1:200 dilution of the corresponding antibody (anti-EGFR, anti-EGF, and anti-laminin) and protein A–Sepharose (Sigma) according to the protocol of Ruddon *et al.* (1979). The immunoprecipitates were eluted and fractionated in a polyacrylamide gel according to Laemmli (1970). Radioactive bands were visualized by exposing the dried gels to X-ray film (Kodak XAR-2).

Enzyme-linked immunosorbent assay (ELISA). Epithelial and mesenchymal monocultures were incubated for 3 hr in serum-free MEM containing 0.02% BSA (MEM-BSA). The culture fluids were then collected and added to wells of a 96-well plate (Falcon Plastics, Oxnard, CA) in aliquots of 0.1 ml/well. MEM-BSA served as a negative control. Serial concentrations of EGF were added to the assay plates to serve as standard. After the 4-hr incubation, the fluids were removed and ELISA was performed as described (Varani *et al.*, 1983) using an anti-EGF antibody. Laminin and fibronectin production were determined in the same way. Purified laminin and fibronectin (Collaborative Biomedical) served as standards.

Branching activity assays and blocking experiments. Lung explants were treated with either RA, at concentrations ranging from 0.1 to 3 μM, or equivalent volumes of DMSO for periods of 24 to 48 hr and occasionally for up to 1 week. In additional studies, lung explants were cultured for 24 to 48 hr in the presence of EGF or TGF-α at concentrations ranging from 2 to 100 ng/ml. The extent of branching activity was determined by direct counting of the peripheral terminal buds.

Blocking experiments were carried out in an attempt to determine whether EGF endogenously synthesized by the lung was a mediator for the RA effects in the developing organ. Lung organ cultures were simultaneously treated with 1 μM of RA or 50 ng/ml of EGF and

FIG. 1. Epithelial–mesenchymal cocultures of cells obtained from fetal lungs. Organotypic rearrangement takes place in these cultures. The mesenchymal cells form a monolayer and the epithelial cells rearrange into polarized clusters which immunoreact positively (light brown color) with antibodies to cytokeratins (A). B and C: Tritiated thymidine incorporation and autoradiography of a coculture exposed to 1 μM RA (B) or an equivalent concentration of DMSO (C). Note the larger size of the epithelial cluster containing numerous radiolabeled nuclei in the RA-treated coculture (B) compared to the epithelial cluster in the DMSO-treated coculture (C) which is smaller and contains few labeled nuclei. Scale bar, 20 μm.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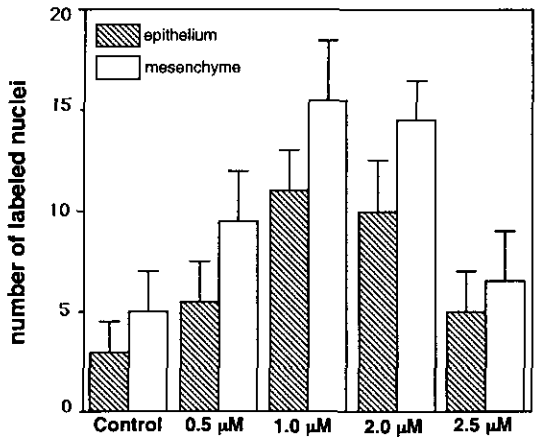


FIG. 2. Average number of radiolabeled nuclei in the epithelial and mesenchymal compartments of cocultures exposed for 24 hr to various concentrations of RA or DMSO. Control represents average determinations in cultures exposed to the maximal concentration of DMSO. Bars represent standard deviations. The results were statistically significant for 0.5–2 μM ($P < 0.0001$).

antibodies to EGF at concentrations of 10 and 25 μg/ml. Branching activity was determined after 48 hr in culture and compared.

RESULTS

Effects of RA on proliferation of epithelial and mesenchymal cells in monoculture and coculture. As previously reported (Schuger *et al.*, 1992), mixed lung cell populations in coculture spontaneously sorted into epithelial and mesenchymal compartments while rearranging themselves into a pattern resembling the tissue of origin. In these cocultures, referred to as organotypic, epithelial clusters were immunohistochemically characterized by their cytokeratin expression (Fig. 1A). When these cocultures were exposed to RA, the epithelial clusters grew larger than in controls. Tritiated thymidine incorporation showed a significant increase in the number of labeled epithelial and mesenchymal nuclei in the RA-treated cultures compared to untreated controls or controls treated with DMSO (Figs. 1B and 1C). Cell proliferation was maximal with concentrations of 1 μM and decreased with higher or lower levels of RA (Fig. 2).

Mesenchymal monocultures exposed to RA exhibited an increase in cell proliferation, which was maximal with concentrations of 0.5 and 1 μM and decreased with higher levels of RA (Fig. 3A). The increment in cell proliferation was confirmed by [^{3}H]thymidine incorporation into DNA which increased from 2850 ± 256 cpm/10^6 cells in cultures exposed to DMSO to 6580 ± 310 cpm/10^6 cells in cultures treated with 1 μM RA. Autoradiography and counting of [^{3}H]thymidine-labeled nuclei showed similar results (24 ± 8% of the nuclei were labeled in the cultures treated with DMSO and 54 ± 14% in the cultures treated with 1 μM RA; ± standard deviation). Epithelial cells in serum-free medium did not proliferate and RA did not induce proliferation of these cells (Fig. 3B represents the results obtained with 1 μM RA and control). Absence of proliferation was confirmed by [^{3}H]-thymidine incorporation (not shown).

Effects of EGF on proliferation of epithelial and mesenchymal cells in monoculture. In contrast to RA, EGF stimulated cell proliferation when added to primary cultures of either epithelial cells or mesenchymal cells isolated from embryonic and fetal lungs. Proliferation was observed with concentrations as low as 10 ng/ml and was maximal with a concentration of 50 ng/ml (Figs. 4A and 4B). The effect decreased with higher levels of growth factor (not shown). Since TGF-α may interact with the EGFR in the embryo and play a role in lung morphogenesis, additional studies were conducted to elucidate this possibility. However, no statistically significant response was obtained upon exposure of lung mesenchymal cell monocultures to TGF-α.

Effects of RA and EGF on branching activity in cultured lung explants. Over 100 lung explants from Day 12

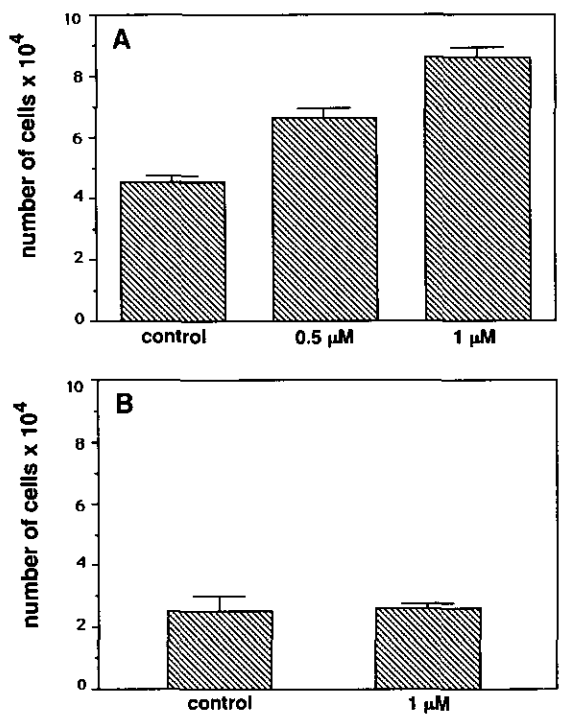


FIG. 3. Epithelial and mesenchymal cell monocultures exposed to various concentrations of RA. Cell numbers were determined at time zero and after 24 hr of exposure to RA or to DMSO. A twofold increase in cell numbers was observed in the mesenchymal monocultures exposed to 1 μM RA (A). The results were statistically significant ($P < 0.02$ for 0.5 μM and $P < 0.0001$ for 1 μM). No cell proliferation was observed in the epithelial monocultures exposed to a similar doses of RA (B). Bars represent standard deviations.

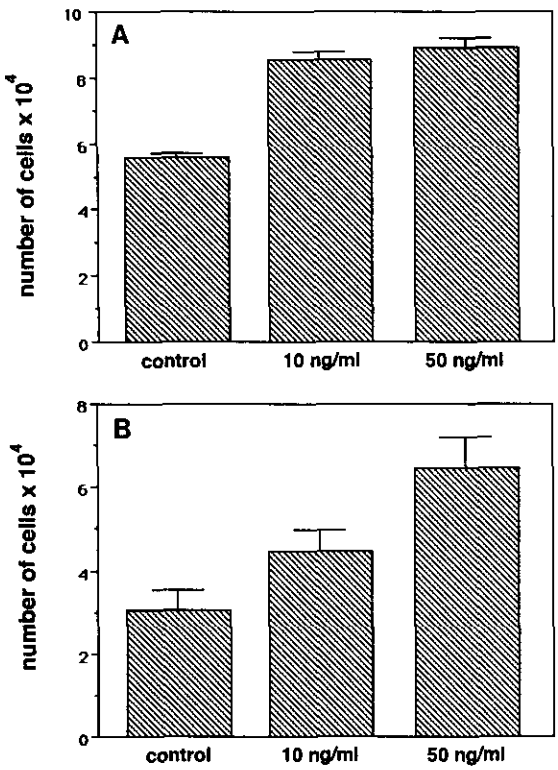


FIG. 4. Epithelial and mesenchymal cell monocultures exposed to various concentrations of EGF. Cell numbers were determined at time zero and after 24 hr of exposure. An increase in cell numbers was observed in both mesenchymal (A) and epithelial (B) cell monocultures in response to EGF. Bars represent standard deviations. These results were statistically significant ($P < 0.02$ for mesenchymal cells and $P < 0.02$ and 0.01 for epithelial cells exposed to 10 and 50 ng/ml, respectively).

and more than 80 from Day 13 were exposed to RA. A gradual increase in branching activity was observed as the RA concentration in the medium was increased from 0.1 to 1 μM, which was the maximal stimulatory dose (Fig. 5). Higher concentrations of RA were inversely correlated with their ability to stimulate branching. The stimulatory effect was statistically significant for doses of 0.5 to 2.5 μM (Fig. 6A). Finally, concentrations of 3 μM and higher increased ^{51}Cr release in a cytotoxicity assay (more than 10% of the total ^{51}Cr incorporated into the explants was released during an 18-hr incubation period) and were considered toxic to the explants.

Tritiated thymidine incorporation followed by autoradiography confirmed that the increased branching activity seen in the RA-treated lungs was associated with an increase in cell proliferation in both the epithelial and mesenchymal compartments. The average percentage of labeled epithelial cell nuclei was 50 ± 12% in six lungs treated with 1 μM of RA compared to 14 ± 5% in the six lungs exposed to DMSO. Labeled mesenchymal nuclei were 56 ± 10% in presence of 1 μM RA and 15 ± 7% in presence of DMSO.

In addition to the RA-treated lungs. Over 80 lungs were exposed to EGF at various concentrations and examined for branching activity. Like RA, EGF also stimulated branching activity. The effect was dose-dependent and statistically significant for EGF concentrations ranging from 10 to 50 ng/ml (Fig. 6B). Lower and higher concentrations were less effective in stimulating branching. Over 30 additional lungs were exposed to TGF-α at various concentrations and examined for branching activity. TGF-α failed to stimulate branching, suggesting that TGF-α may not play a major role in this aspect of lung organogenesis.

Effects of RA on synthesis of EGFRs in cocultures and monocultures of lung cells. Immunohistochemical studies were conducted to confirm the presence of EGFR in the lung during the developmental period studied. On Day 12 the lungs showed a diffuse positive immunoreaction for the receptor in the epithelial and mesenchymal cells, seen as a light brown color (Fig. 7A). A gradual increase in EFGR immunoreactivity (darker brown tone) was observed in the bronchial smooth muscle and blood vessels and in a minor degree in the bronchial epithelium (Fig. 7B). This pattern remained constant up to Day 17, which was the latest day of gestation studied. EGFR was identified by immunoprecipitation followed by SDS-PAGE in lungs at Days 12 and 13 of gestation (Fig. 7D).

Since RA is known to stimulate the expression of EGFR in many cell types (Jetten, 1980; Komura *et al.*, 1986), efforts were made to determine whether RA also upregulates EGFR synthesis in the developing lung. [^{125}I]EGF binding assays demonstrated a significant increase in the number of EGFR in primary cultures of mixed lung cells exposed to 0.5 to 1.5 μM RA (Fig. 8). This increase in the number of receptors upon RA exposure was detected in both the embryonic and fetal periods. Receptor expression was also increased in mesenchymal and epithelial cell monocultures exposed to 0.5 to 1.5 μM RA (not shown). Higher concentrations of RA resulted in a gradual decrease in the levels of EGFR expression.

The mesenchyme as the main source of endogenous lung EGF. ELISAs were used to assess EGF production by lung cells in monoculture. These studies showed that mesenchymal cell monocultures synthesize EGF in increasing quantities as the lung develops. The values ranged from 0.1 ± 0.1 ng/10^5 cells with cells isolated from Day 13 lungs to 5 ± 0.2 ng/10^5 cells in cells isolated from Day 17 lungs. Epithelial cell monocultures produced only traces of EGF (less than 0.001 ng/10^5 cells). Immunoprecipitation and SDS-PAGE showed that most of the EGF was present in the lung as EGF precur-

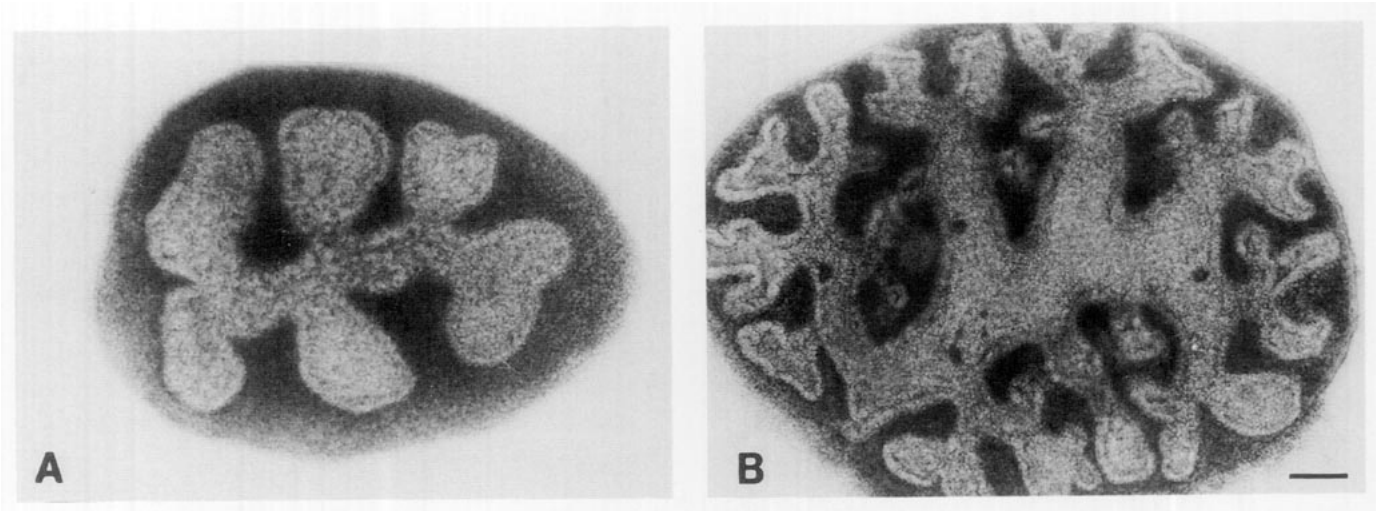


FIG. 5. Microphotographs of two lung explants from Day 12 mouse embryos exposed for 48 hr to DMSO (A) or to 1 μM RA (B). Significant branching activity occurred only in the explant exposed to RA. Scale bar, 100 μm.

sor. The EGF precursor is a 128-kDa protein which includes several EGF-like amino acid sequences (Gray *et al.*, 1983). A band of M_r 6000 (mature EGF) was observed by exposing the gels for longer periods of time (Fig. 9).

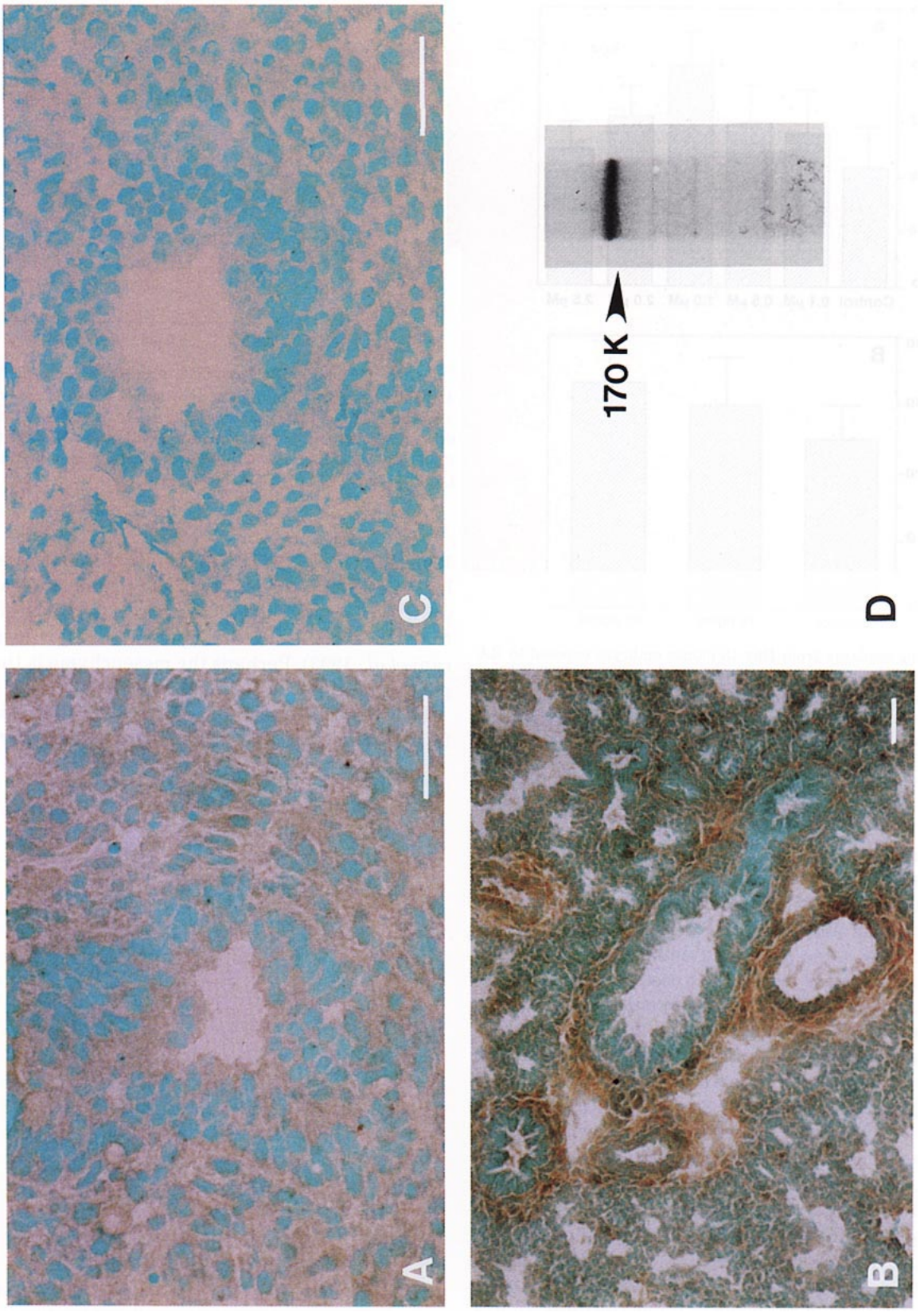
Previous studies indicated a role for the extracellular matrix, particularly laminin and fibronectin, in the process of branching morphogenesis (Roman *et al.*, 1989; Schuger *et al.*, 1990a,b, 1991). Although RA is a modulator of laminin production by many cell types, ELISA performed on supernatants from epithelial and mesenchymal cell monocultures showed no changes in the levels of secreted laminin or fibronectin after RA stimulation. In addition, SDS-PAGE showed no alterations in laminin chain composition in the RA-exposed lung explants.

Antibodies to EGF blocked EGF-induced but not RA-induced branching. The stimulation of branching activity produced by 1 μM of RA was not inhibited by antibodies to EGF at any of the concentrations used (10 or 25 ng/ml). However, the same concentrations of anti-EGF antibodies were effective in blocking the branching stimulation produced by 50 ng/ml of EGF. The control lung explants, which received mouse IgG, exhibited an average number of terminal buds of 23 ± 2, the lung explants exposed to 50 ng/ml of EGF exhibited an average number of 39 ± 5 terminal buds and those explants that were concomitantly treated with 50 ng/ml of EGF and 10 and 50 ng/ml of antibody showed a number of terminal buds of 20 ± 1 and 21 ± 6, respectively. The experiment was repeated three times with similar results.

DISCUSSION

Lung morphogenesis is a complex process requiring coordinated activity in the epithelial and mesenchymal compartments. In both organ cultures of embryonic mouse lung and organotypic cultures derived from embryonic lung cells, epithelial proliferation and development into branched airway tubules required the presence of a functional mesenchyme (Grobstein, 1954; Schuger *et al.*, 1990b). How the mesenchyme contributes to epithelial development is not fully understood. On the one hand, we (Schuger *et al.*, 1990a, 1991) and others (Spooner and Faubion, 1980, Roman *et al.*, 1989) have shown that the extracellular matrix is critical. The mesenchyme is thought to provide essential components of the matrix. Alternatively, studies of epithelial and mesenchymal cells in culture have shown that mesenchymal cells synthesize a number of epithelial growth factors, including insulin-like growth factors (Barreca *et al.*, 1992; Ristow and Messmen, 1988), fibroblast-pneumonocyte factor (Smith, 1979) fibroblast-derived keratinocyte growth factor (Finch *et al.*, 1989; Yaeger *et al.*, 1991), and, perhaps, interleukin-1 (Ristow, 1987). Among these, fibroblast-pneumonocyte factor and insulin-like growth factors have already been shown to play a role in lung development (Smith, 1979; Stiles and D'Ercole, 1990; and articles therein). Obviously, these two activities are not mutually exclusive and epithelial development may require both an intact matrix and mesenchymal growth factors for development.

FIG. 7. Immunolocalization of the EGFR in the developing lung. (A) On Day 12 of embryogenesis a weak positive staining (seen as light brown) is diffusely present in the epithelial and mesenchymal compartments of the lung. (B) On Day 17 of gestation EGFR positivity intensifies in the smooth muscle surrounding air ways and vessels (dark brown). (C) Immunohistochemical control in which the primary antibody has been omitted. Scale bar, 20 μm. (D) EGF receptor (170 kDa) immunoprecipitated from lung lysates at Day 12 of embryogenesis.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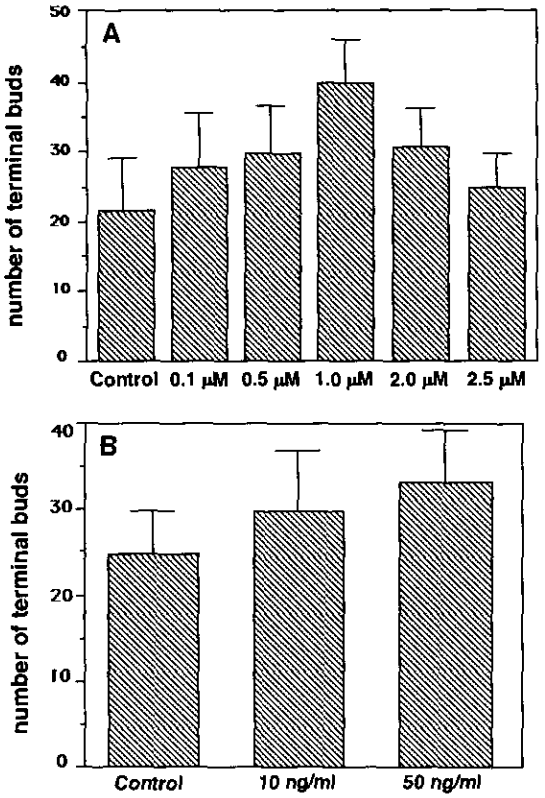

FIG. 6. Lung explants from Day 13 mouse embryos exposed to RA (A) or EGF (B). The extent of branching activity was determined after 48 hr by direct counting of terminal buds in each explant. Both RA and EGF produced an statistically significant stimulation in branching activity ($P < 0.0001$ for 1 μM and $P < 0.02$ for 0.5–2.5 μM RA, and $P < 0.06$ for 10 ng/ml and $P < 0.002$ for 50 ng/ml EGF). Bars represent standard deviations.

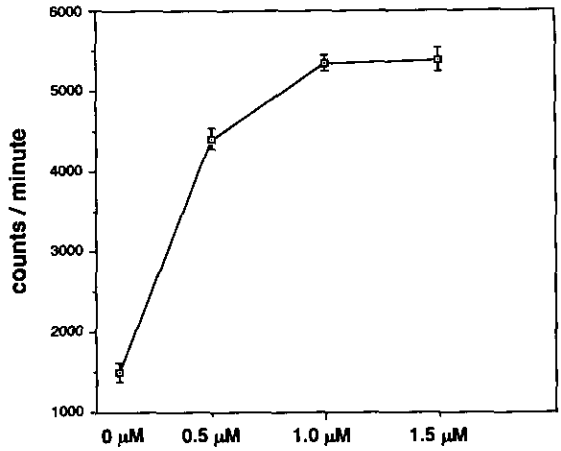

FIG. 8. EGFR binding assays. Cells were incubated with [^{125}I]EGF at 4°C and radioactive binding was determined 1 hr later. The results are expressed as counts/minute per 10^5 cells. Nonspecific binding was subtracted from all points. Bars represent standard deviations ($P < 0.0001$ for 1 μM RA). Higher doses of RA resulted in gradually lower binding levels (not included in the graphic).

The present study extends our efforts to understand the process of embryonic lung morphogenesis. Here we have used a number of culture systems, including monocultures of lung epithelial and mesenchymal cells, cocultures of the same cells, and embryonic lung organ cultures, to elucidate events that occur during lung development induced by RA. In organ culture, RA stimulated terminal branch formation and this was accompanied by increased mitotic activity in both epithelial and mesenchymal compartments. Increased proliferation of both cell populations was also seen in cocultures treated with RA. Interestingly, however, when epithelial and mesenchymal cells were treated with RA in monoculture, only the mesenchymal cells responded. This suggests that the stimulatory activity of RA on the epithelial component of the developing lung is either an indirect effect or that induction of epithelial proliferation requires an additional signal along with RA. It is interesting that RA has been shown to induce proliferation of some types of epithelial cells in monoculture, including normal human and mouse keratinocytes (Varani *et al.*, 1989, Tong *et al.*, 1988). Yet despite this, our recent studies suggest that the ability of RA to induce keratinocyte growth in organ-cultured skin is an indirect effect and that the dermal fibroblasts are the major target (Varani *et al.*, 1993). Perhaps the mesenchyme is the principal target of RA in the lung as well.

How RA acts to stimulate lung cell proliferation is not fully understood. The mechanisms underlying this ef-

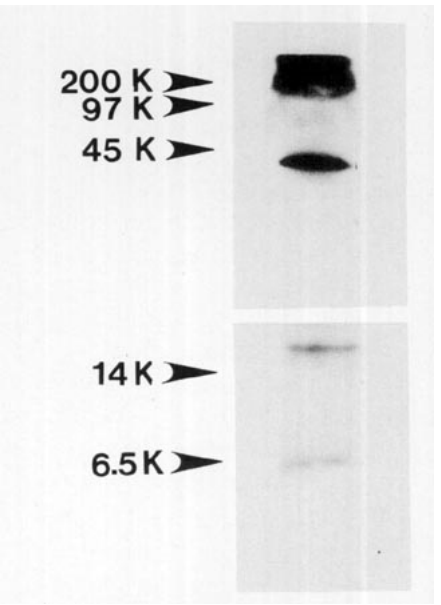

FIG. 9. EGF precursor (≅ 130 kDa) and an additional band (≅ 42 kDa) were immunoprecipitated by antibodies against murine EGF. Two additional bands, including one compatible with mature EGF (6 kDa), were identified by exposing the gels for longer periods of time.

fect are likely to be complex as is suggested by the multiple biological activities of vitamin A. One of the mechanisms thereby RA may stimulate cell proliferation is by the induction of EGFR expression with the resulting amplification of the cellular responses mediated through this receptor (Jetten, 1980, 1982; Thompson and Rosner, 1989). EGFRs have been previously detected in mouse embryos (Nexo *et al.*, 1980; Adamson *et al.*, 1981; Hortsch *et al.*, 1983; Partanen and Thesleff, 1987). Their presence in the developing lung was confirmed by our studies. We found EGFRs throughout the embryonic and fetal stages in the epithelial and mesenchymal compartments of the lung. However, expression was particularly high in the smooth muscle surrounding the air ways and blood vessels. That increased EGFR expression might be functionally linked to RA-induced proliferation was suggested by the finding that there was a direct relationship between RA-induced EGFR expression in lung cocultures and RA-induced proliferation.

Further evidence for a role for EGFR in lung development comes from studies with EGF itself. When EGF was added to lung cultures, it stimulated branching activity under the same conditions as RA. A mitogenic effect of EGF in the developing lung had been shown earlier (Goldin and Opperman, 1980; Warburton *et al.*, 1992). In addition, EGF stimulated proliferation of both epithelial cells and mesenchymal cells in either coculture or monoculture. Based on these observations, it can be suggested that EGFR expression is important for lung cell proliferation during the developmental period and that EGF, itself, may play an important role. The actions of RA appear to be mediated, at least in part, through upregulation of its receptor. Whether RA has additional effects, such as stimulating EGF synthesis is not known. The mesenchyme appears to be the principal source of EGF in the developing lung (this report and Snead *et al.*, 1989). If EGF synthesis is a critical intermediary in RA-induced proliferation, the lack of EGF synthesis by the epithelial cells in the embryonic stage could explain their inability to respond to RA in monoculture despite an increase in EGFR expression. The increase in EGF production by epithelial cells that occurs during later development (Fisher and Lakshmanan, 1990; and articles therein) as well as synthesis of other ligands for the EGFR (e.g., TGF-α and amphiregulin) (Elder *et al.*, 1989; Cook *et al.*, 1991) may also explain why fetal and adult epithelial cells are successfully cultured in the absence of mesenchyme and why they can be directly stimulated to proliferate by RA (Lechner *et al.*, 1982).

We used an antibody to mouse EGF in an effort to provide direct evidence for the involvement of EGF in RA-induced lung organogenesis. However, we were unsuccessful in blocking RA-induced branch formation while the same antibody inhibited branching activity produced by exogenous EGF. There could be a number of explanations for the failure of the antibody to block branch formation. First, EGF may not be the critical ligand. A series of other proteins work through the EGF receptor to stimulate epithelial cell growth (Elder *et al.*, 1989; Cook *et al.*, 1991). Another possibility is that the antibody raised against mature mouse EGF may not block activity of the embryonic form of the ligand. Further, the antibody may not penetrate the tissue in high enough concentration to effectively block all of the endogenous ligand. Simply blocking the activity of the exogenously provided EGF is not sufficient to indicate similar effectiveness with the endogenous molecule. Antibodies to the EGFR may help to sort through some of these possibilities. It should be noted that RA may not be playing a role during early lung organogenesis since it has been shown that Day 11 lungs can develop *in vitro* without the addition of RA (Warburton *et al.*, 1992). It may be possible, however, that during the first stages of lung organogenesis, the lung has larger quantities of retinyl esters, the stored form of RA, so that *in vitro* development may still take place in the absence of exogenous RA.

In conclusion, we found that RA stimulates branching activity in embryonic lung explants. RA, likewise, stimulates proliferation of lung epithelial cells and mesenchymal cells in coculture. Stimulatory activity appears to be direct for mesenchymal cells but mesenchymal cells are required for an epithelial cell response. RA effect may be accomplished in part through upregulation of EGFR expression but whether mesenchymal-derived EGF is the critical ligand remains to be determined.

This work has been supported by NIH Grant HL48730-01, American Lung Association Grant RG/058N, and a grant from The Council for Tobacco Research.

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