0031-3998/89/2505-0535$02.00/0
PEDIATRIC RESEARCH

Vol. 25, No. 5, 1989
Printed in U.S.A.

Ontogeny of Epidermal Growth Factor Receptor/ Kinase and of Lipocortin-1 in the Ovine Lung[1]

MAHLON D. JOHNSON, MARY E. GRAY, GRAHAM CARPENTER, R. BLAKE PEPINSKY, HAKAN SUNDELL, AND MILDRED T. STAHLMAN

Departments of Pathology [M.D.J., M.E.G.], Biochemistry [G.C.] and Pediatrics [H.S., M.T.S.], Vanderbilt University, School of Medcicine, Nashville, Tennessee 37232, and Biogen Research Corporation, Cambridge, Massachusetts 02142 [R.B.P.]

ABSTRACT. We have examined the ontogeny and distribution of the epidermal growth factor receptor/kinase (EGF receptor) and of lipocortin-1, a major cellular substrate of the EGF receptor, in a developmental series of 13 normal ovine fetal lungs (44–145 d of gestation) using the peroxidase anti-peroxidase technique with two extensively characterized polyclonal antibodies recognizing the EFG receptor and one polyclonal antibody recognizing lipocortin-1. Immunoreactive EGF receptor/kinase and lipocortin-1 were detected in conducting airway epithelium by the end of the first trimester of pregnancy before bronchial glands could be identified. This was followed at two-thirds of gestation by immunoreactivity in bronchial glands and large bronchioles adjacent to postive bronchi. By seven-eighths of gestation conducting airway epithelium no longer contained consistently detectable immunostaining for EGF receptor, although lipocortin-1 was identified until term in all levels of conducting airways. In contrast, neither EGF receptor nor lipocortin-1 immunoreactivity was detected in alveolar type I or type II epithelial cells, fibrocytes, chondrocytes, smooth muscle, or endothelial cells at any gestational age. These findings suggest that EGF receptor and lipocortin-1 may participate in early airway development. (*Pediatr Res* 25:535–541, 1989)

Abbreviations

EGF, epidermal growth factor
PAP, peroxidase-antiperoxidase

EGF is a 53-amino acid polypeptide found in many mammalian tissues including the lung (1–3). Administration of EGF stimulates proliferation of epithelial cells *in vivo* in the lungs of fetal and newborn lambs (4), accelerates lung maturation in fetal rabbits (5), and induces increased thymidine uptake in rat type II cells *in vitro* (6). EGF also enhances synthesis of surfactant associated-protein by type II cells (7) and phosphatidylcholine synthesis in fetal rat lung explants (8). These effects are thought to be initiated by growth factor binding to high affinity EGF receptors recently identified in cultured type II cells and in fetal rabbit lung homogenates (9); however, the localization of the EGF receptor in intact lung has not been demonstrated.

Received September 29, 1988; accepted December 27, 1988.
Correspondence and reprint requests: Dr. Mildred Stahlman, Professor of Pediatrics and Pathology, Vanderbilt University, School of Medicine, Nashville, TN 37232.
Supported by NIH Grants HL 37726, CA 24071, and SCOR HL 14214.
Presented in part at the meetings of the American Pediatric Society, Society for Pediatric Research, Washington, DC, May 1988.

The EGF receptor is a transmembrane protein containing an extracellular EGF binding site and an intracellular tyrosine kinase domain (10–12). Binding of EGF to the receptor activates the kinase activity which in turn catalyzes the phosphorylation of specific cytoplasmic proteins and of the receptor itself (10, 11). Recent work has identified lipocortin-1, a 35 kD protein (p35), as a major physiologic substrate for the EGF receptor/ kinase (13–15). In *in vitro* assays, lipocortin-1 has been shown to inhibit phospholipase A_2 and bind calcium, acidic phospholipids and actin filaments (16–18); however, the cellular function of lipocortin-1 is unknown. Lipocortin-1 has been found at high concentrations in the mouse and rat lung (17) and has also been isolated from bovine lung (19); however, its distribution in the lung has not been described.

The present study evaluates the ontogeny and distribution of EGF receptor immunoreactivity and lipocortin-1 immunoreactivity in a developmental series of fetal lamb lungs. In the developing ovine lung, both proteins showed a similar cellular distribution confined to bronchi, bronchioles and bronchial glands.

MATERIALS AND METHODS

The ontogeny and distribution of EGF receptor and lipocortin-1 immunoreactivity were analyzed in a series of 13 normal ovine fetuses from time-dated pregnancies (20) of 44–145 d of gestation (term gestation is 147 d). Lungs from Hamshire or Suffolk ovine fetuses were rapidly collected after delivery by cesarean section after the ewe and fetus were killed by secobarbital overdose. Gestational ages of the lambs are listed in Table 1. Right lungs of the tiny 44- and 51-d old fetuses were collected without separation into lobes for EGF receptor immunohistochemistry. At these times, the wt of these fetuses ranged from 9.5 to 19.2 g, and the crown-rump lengths ranged from 6.8 to 8.5 cm. In fetuses of 75 d or older, a time when fetal wt had increased to 235 g and the crown-rump length to 20.5 cm, sections from two or three lobes from each lamb lung were evaluated for EGF receptor. Blocks from one or two lobes from each lung, fixed both in 10% formalin and Gendre's fluid and embedded in paraffin, were evaluated for lipocortin-1 immunoreactivity. Each lung was sectioned, evaluated for gross abnormalities, and tissue for EGF receptor immunoanalysis was rapidly frozen in liquid nitrogen for storage at −80°C. Frozen sections 5 μm thick were cut from each lung at −20°C and thaw-mounted onto aminoalkylsilane-coated microscope slides (21). Sections were fixed in freshly prepared phosphate-buffered 4% paraformaldehyde pH 7.6 for 15 min, rinsed in 70% ethanol for 10 min, and stored at −20°C. Additional tissue for identification of lipocortin-1 immunoanalysis was fixed in 10% phosphate-buffered formalin and Gendre's fluid, paraffin embedded, sectioned at 5 μm and mounted on aminoalkylsilane-coated slides (21). A human epi-

Table 1. *Appearance of EGF receptor immunoreactivity in the developing lamb lung**

Lamb No.	Gestational age (d)	Bronchi: Lining epithelium	Bronchi: Glands	Bronchioles lining epithelium	Bronchiolo-alveolar portals
1	44	0	0	−	0
2	44	0	0	±§	0
3	51	+	0	−	0
4	75	++	0	+	0
5	75	++	0	+	0
6	90	+	±	−	−
7	108	+	++	+	−
8	117	++	++	+	−
9	129	+	++	−	−
10	141	−	+	+	−
11	142	−	−	±	−
12	144	−	++	+	+
13	145	+	++	+	±

* ++, heavy staining; +, faint staining; ±, questionable staining; −, no staining identified; 0, structure not in section; §, largest conducting airways but no intrapulmonary cartilage present.

dermal carcinoma cell line (A-431), previously shown to possess 2.5×10^6 EGF receptor/cell, was used as a positive control for EGF receptor immunoreactivity. A-431 cells (American Type Culture Collection, Rockville, MD), grown using previously outlined methods, were injected at concentrations of 1×10^6 cells (22) into irradiated nu/nu (nude) mice to produce tumors harvested 14 d later (23). These tumors were frozen in liquid nitrogen and stored at −80°C or fixed and sectioned as described above. A-431 cell tumors, sheep skin, and placenta containing high concentrations of lipocortin-1 were used as positive controls for lipocortin-1 immunoreactivity (13, 24).

Immunohistochemistry. Epidermal growth factor receptor immunoreactivity was localized utilizing two extensively characterized polyclonal antibodies produced after immunization with either a native form (#451) or a denatured form (#310) of the EGF receptor (24–26) and the PAP method (27). Both antisera exhibited optimal immunoreactivity only in frozen sections. Greater immunoreactivity in frozen control tissues denatured with paraformaldehyde was frequently seen with the #310 antibody raised against denatured EGF receptor. However, the pattern of distribution was similar with both antibodies. The specificity of these antibodies for EGF receptor has been established by ablation studies with EGF receptor-rich membranes of epidermoid carcinoma (A-431) cells (28). Specificity was also assessed by absorption of the antisera to cultures of A-431 cells.

Lipocortin-1 immunoreactivity was localized in tissue fixed in 10% formalin and in Gendre's fluid using a polyclonal rabbit antibody to recombinant human lipocortin-1 (Biogen Research Corp., Cambridge, MA). The specificity of this antibody was assessed by preabsorption of the minimum effective concentration of antiserum (1:5000 dilution) with 1.0 mg of recombinant lipocortin-1 bound to cyanogen-bromide-activated sepharose 4B

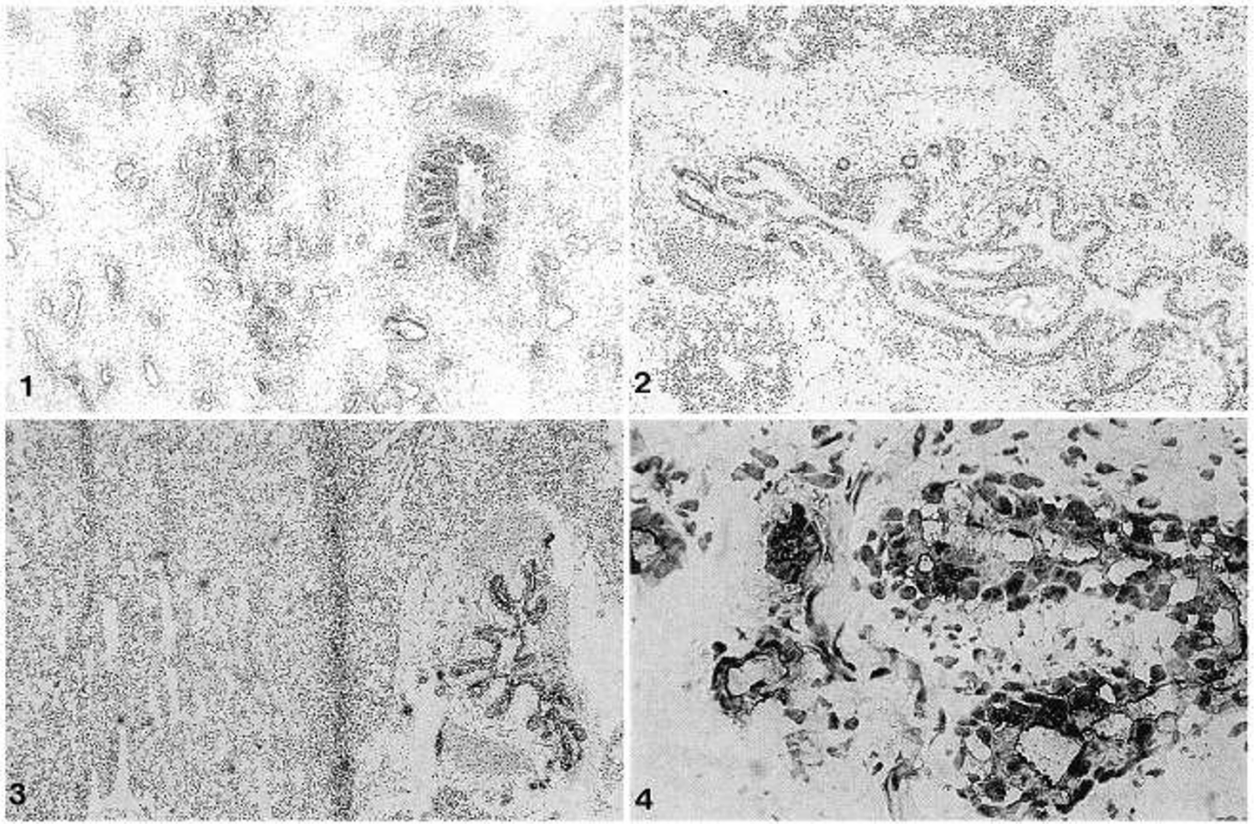

Fig. 1. Photomicrograph of the lung of a lamb of 75 d of gestation showing extensive immunostaining of the epithelium of a small bronchus for EGF receptor using antibody #310. No glands are identified. The lung parenchyma is not immunostained (frozen section, immunoperoxidase and hematoxylin × 48).

Fig. 2. Photomicrograph of the lung of a lamb of 108 days gestation. Bronchial submucosal glands are immunostained for EGF receptor using antibody #310 (frozen section, immunoperoxidase and hematoxylin × 60).

Fig. 3. Photomicrograph of the lung of a lamb of 117 d of gestation. Bronchial epithelial lining and submucosal glands are immunostained for EGF receptor using antibody #310 (frozen section, immunoperoxidase and hematoxylin × 48).

Fig. 4. Detail of Figure 3 showing immunostaining of cells lining crypts and submucosal glands of the bronchus seen in Figure 3. The most intense immunostaining for EGF receptor is at the luminal surface (frozen section, immunoperoxidase and hematoxylin × 480).

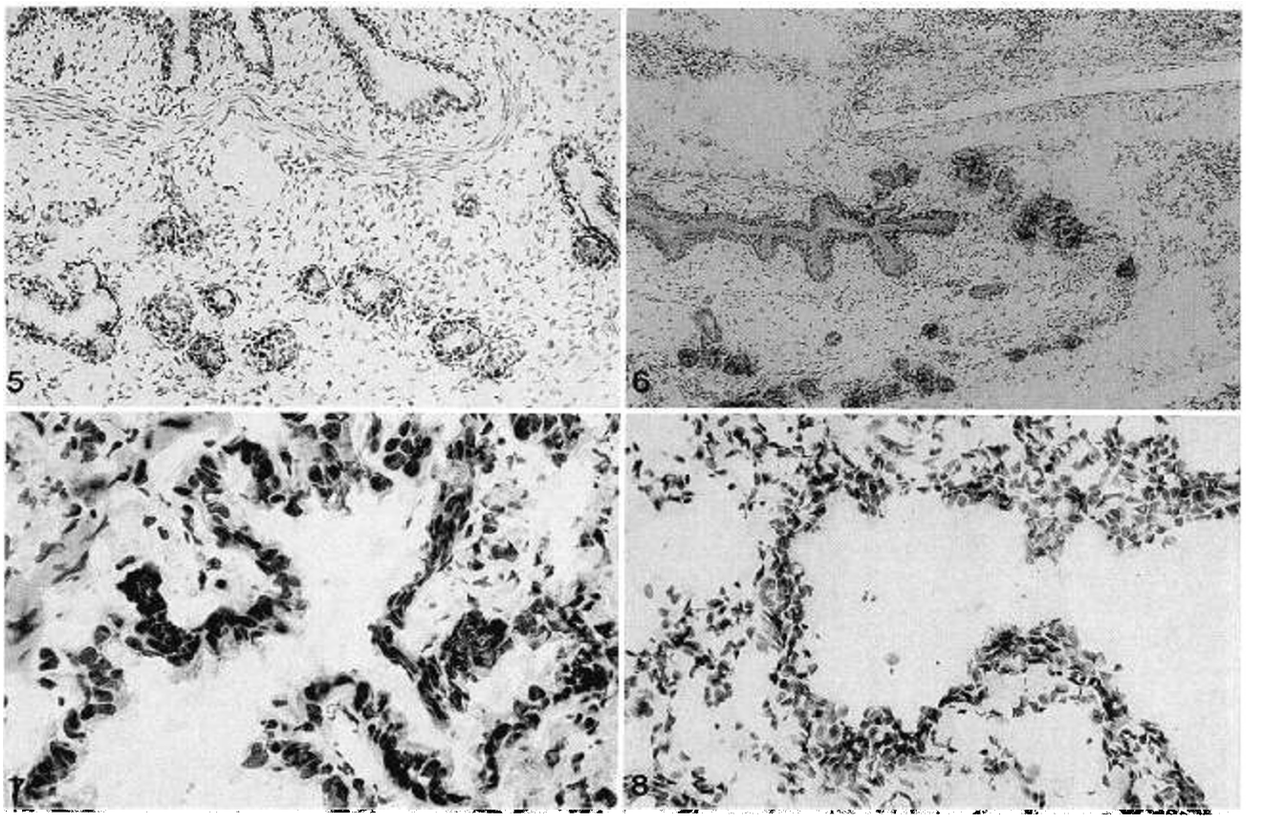

Fig. 5. Photomicrograph of the lung of a lamb of 129 d of gestation. Bronchial submucosal glands are extensively immunostained for EGF receptor using antibody #310 (frozen section, immunoperoxidase and hematoxylin × 120).

Fig. 6. Photomicrograph of the lung of a lamb of 144 d gestation. Submucosal bronchial glands are intensely immunostained for EGF receptor as well as is fluid filling the bronchial lumen using antibody #310 (frozen section, immunoperoxidase and hematoxylin × 78).

Fig. 7. Photomicrograph of the lung of a lamb of 141 d of gestation. Bronchiolar epithelium is lightly immunostained for lipocortin-1 in cells lining deep folds (immunoperoxidase and hematoxylin × 480).

Fig. 8. Photomicrograph of the lung of the same lamb as in Figure 6 showing immunostaining for EGF receptor at the bronchiolo-alveolar portal using antibody #310 (frozen section, immunoperoxidase and hematoxylin × 480).

(Sigma Chemical Co., St. Louis, MO) before use on control tissues and on four sections of lung previously found to contain extensive lipocortin-1 immunoreactivity (29). Lipocortin-1 antiserum does not react with lipocortin-2 (30).

Sections of lung as well as placenta were washed in a solution of 0.5% hydrogen peroxide in 100% methanol for 10 min, then preincubated with 10% normal swine serum in PBS (pH 7.6). After blotting, sections were incubated overnight at room temperature with either primary antisera against EGF receptor or lipocortin-1, then sequentially incubated with swine antirabbit immunoglobulin (dilution 1:20) and rabbit PAP (dilution 1:50) for 30 min each. All incubations were followed by rinsing and two 10-min washes in PBS. The tissues were subsequently exposed to the peroxidase substrate 3,3′diaminobenzidine (DAB; Sigma) 0.5 mg/mL in 0.05M Tris (pH 7.6) containing 0.05% hydrogen peroxide (28). Slides were counterstained with hematoxylin.

Extensive EGF receptor immunoreactivity was seen in all viable cells of the A-431 cell tumor. Immunoreactivity was not seen in endothelial cells lining vascular spaces or in fibroblasts making up the capsule surrounding the tumor. EGF receptor immunoreactivity was completely ablated by preabsorption on A-431 cells. EGF receptor immunoreactivity was also seen throughout the epidermis of ovine skin with greatest intensity in keratinocytes populating the basal and spinous layers.

Extensive lipocortin-1 immunoreactivity was also seen in sections of tumors from A-431 cells. Intravascular neutrophils also immunostained. Immunoreactivity was seen at dilutions of 1:250–1:5000 with an optimal signal to noise ratio at 1:1000–1:2000. Lipocortin-1 immunoreactivity was also demonstrated in the sheep epidermis and in human and ovine placenta where chorionic plate staining was especially intense. Preabsorption of lipocortin-1 antisera on Sepharose 4B containing a limited concentration of recombinant lipocortin-1 produced complete ablation of lipocortin-1 immunoreactivity in the sections of placenta and lung.

The distribution and degree of immunostaining was independently evaluated by three of the authors (MJ, MG, MS). Questionable areas of immunostaining were labeled as ±, distinct but faint immunostaining was labeled as +, and heavy staining was labeled as ++.

RESULTS

Knowledge of the times of appearance of bronchi, defined by the presence of cartilage, and of bronchial submucosal glands, is necessary for interpreting the observations we are reporting. In the fetal lamb, cartilage can be identified in large bronchi near the hilus between 35 and 40 d of gestation when fetal crown-rump length is approximately 3.6 cm (31, 32). If subsequent development proceeds in the orderly fashion described for the human fetus (33), cartilage will form progressively in more peripheral airways as gestation proceeds. From 75 d of gestation onward, bronchi were located easily in fetal lambs.

Faure-Fremiet and Dragoiu (31) reported that bronchial submucosal glands first appear in the fetal lamb of about 80 d of

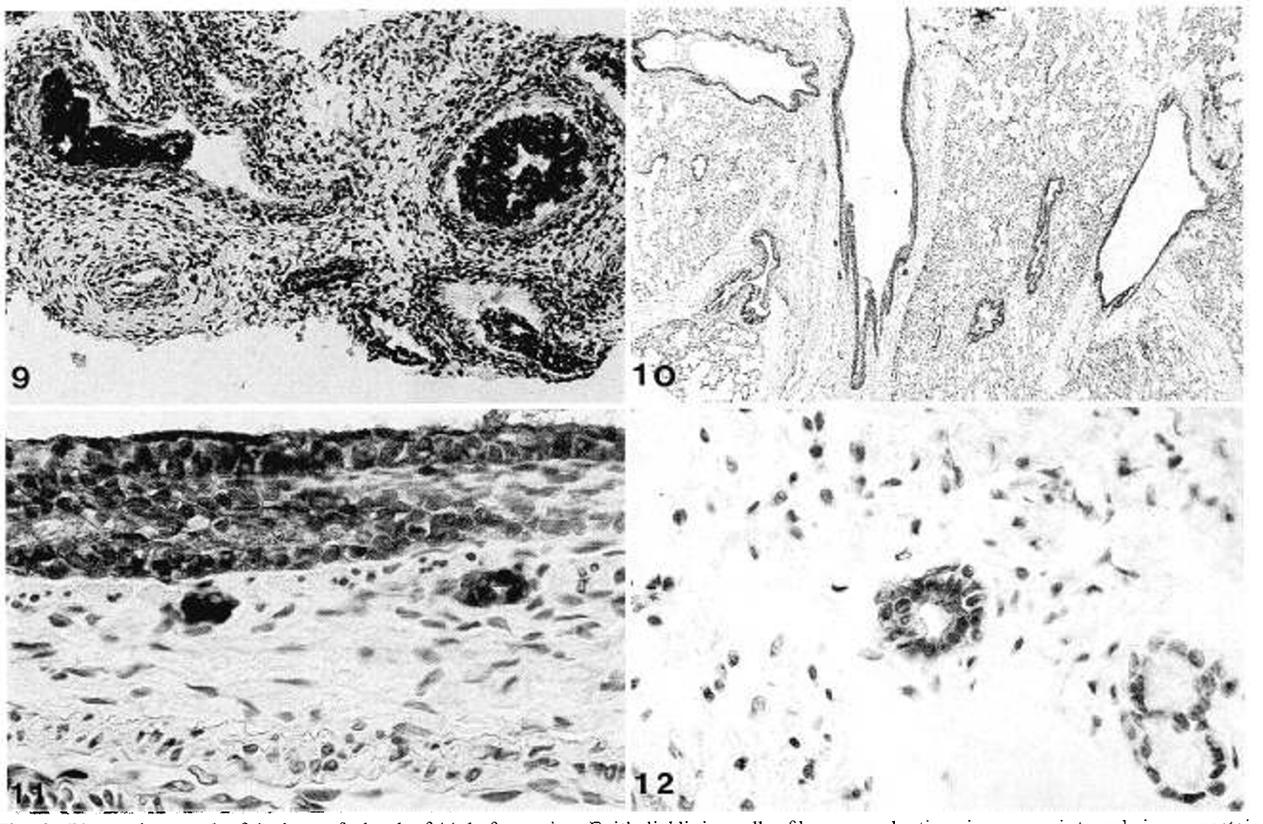

Fig. 9. Photomicrograph of the lung of a lamb of 44 d of gestation. Epithelial lining cells of larger conducting airways are intensely immunostained for lipocortin-1 (immunoperoxidase and hematoxylin × 150).

Fig. 10. Photomicrograph of the lung of a lamb of 129 d of gestation. Bronchial epithelium and epithelium of both large and small bronchioles are intensely immunostained for lipocortin-1 (immunoperoxidase and hematoxylin × 48).

Fig. 11. Detail of Figure 10 showing immunostaining for lipocortin-1 of bronchial epithelium and adjacent small tubular structures which appear to be necks of glands (immunoperoxidase and hematoxylin × 48).

Fig. 12. Photomicrograph of the lung of a lamb of 117 d of gestation. What appears to be a section through the neck of a submucosal gland is immunostained for lipocortin-1, whereas acini containing mucous cells are unstained (immunoperoxidase and hematoxylin × 480).

gestation. At this time, solid buds from epithelial basal cells penetrate the lamina propria and form ramifying tubular structures. Acinar cells remain undifferentiated until about 130 d of gestation (crown-rump, 40 cm) when mucous cells can be identified. Serous cells are probably differentiated slightly later.

EGF receptor-IR localization (Table I). In the lungs of 44-d fetal lambs, no cartilage was identified and only questionable immunostaining of larger conducting airway epithelium was seen. However, in the lung of the 51-d lamb, where an intrapulmonary bronchus, as defined by the presence of cartilage, was identified, immunostaining for EGF receptor appeared in the lining epithelium. By 75 d of gestation, bronchial lining epithelium contained extensive immunoreactivity in the frozen sections (Fig. 1). Bronchial lining epithelium continued to be immunostained for EGF receptor through 129 d of gestation, but was absent in the near-term lambs with one exception (case 13). When bronchial submucosal glands were first identified at 90 d of gestation, EGF receptor immunoreactivity was present in some of the cells of the glandular epithelium (Fig. 2). Staining of cells in the bronchial submucosal glands was present from this point on in gestation with one exception (case 11) (Figs. 3, 4, 5, 6). Bronchiolar epithelial immunostaining appeared at 75 days and, with one exception, the 90-d lamb, was present throughout gestation (Fig. 7). In both bronchi and bronchioles it was the deep folds in the epithelium that contained the most intense immunoreactivity. Bronchioloalveolar portals (34), defined as points of junction between cuboidal bronchiolar and attenuated alveolar epithelia, were identified from 90 d onward, but first appeared immunostained for the EGF receptor in the near term lambs of 144 and 145 d (Fig. 8).

Table 2. *Appearance of lipocortin-1 immunoreactivity in the developing lamb lung**

Lamb No.	Gestational age (d)	Bronchi		Bronchioles lining epithelium	Bronchiolo-alveolar portals
		Lining epithelium	Glands		
1	44	0	0	++§	0
2	44	0	0	++§	0
3	51	0	0	++§	0
4	75	++	+	++	0
5	75	++	−	++	0
6	90	++	+	++	++
7	108	++	+	++	+
8	117	++	+	++	++
9	129	++	+	+	+
10	141	++	+	++	+
11	142	++	+	++	++
12	144	++	+	+	+
13	145	++	+	+	−

* ++, heavy staining; +, faint staining; ±, questionable staining; −, no staining identified; 0, structure not in section; §, largest conducting airways but no intrapulmonary cartilage present.

Lipocortin-1 localization (Table 2). In the series of lamb lung sections immunostained for lipocortin-1, no bronchi were iden-

tified in the 44- and 51-d fetal lambs. However, two discrete size levels of conducting airway were apparent. The largest of these conducting airways were immunostained for lipocortin-1 at both these gestational ages (Fig. 9). Because of the absence of cartilage these airways were designated as bronchioles in Table 2. Bronchi, as defined by the presence of cartilage, were first identified at 75 d of gestation. At this time, bronchial lining epithelium immunostained intensely for lipocortin-1 and continued to immunostain throughout the remainder of gestation (Figs. 10, 11). At 75 d of gestation the bronchial submucosal glands were not well differentiated, and true acini were not present. However, the epithelium lining the necks of some of the glands was immunostained (Figs. 11, 12). From 75 d onward, necks of the bronchial submucosal glands and some of the nonmucous acinar cells immunostained throughout the remainder of gestation (Fig. 13). Smaller, more peripheral conducting airways were not immunostained for lipocortin-1 in early gestation, but larger bronchioles were stained throughout this series of lambs (Figs. 10, 14, 15, 16). Bronchioloalveolar portals, first identified at 90 d, were immunostained for lipocortin-1 throughout the remainder of gestation with one exception (case 13).

DISCUSSION

Recent findings suggest that EGF and TGF-α, another ligand for the EGF receptor, appear during the development of fetal tissues including lung (35–37), thus raising the possibility that EGF or EGF-like TGFs may influence development of the fetal lung. Fundamental to this hypothesis is the demonstration of the EGF receptor in developing lung tissue *in vivo*. Data presented here shows that EGF receptor immunoreactivity is present in the developing ovine lung from 51 d gestation onward. A similar ontogeny of EGF-binding sites has been demonstrated in ovine and mouse liver which exhibit EGF-binding sites by midgestation and an increase in EGF receptor before birth (36, 38). In the human fetus, bronchial epithelia initially exhibit EGF receptor immunoreactivity near the end of the first trimester; immunoreactivity in bronchial glands appears shortly thereafter (Johnson M, unpublished data). During fetal development, EGF receptor synthesis may, therefore, coincide with fetal production or transplacental passage of its ligands.

EGF receptor immunoreactivity was demonstrated only in epithelial cells lining conducting airways or peribronchial glands. In a previous study, we have shown that EGF administration stimulated proliferation of tracheal and bronchial epithelium in the ovine fetus (4). The apparent diminution or loss of EGF-receptor immunoreactivity in conducting airway epithelium in late gestation, despite continued EGF receptor immunoreactivity presence in the epithelium of peribronchial glands, may merely reflect reductions in EGF receptor concentrations during differentiation below the detection limits of our immunohistochemistry. Alternatively, it is conceivable that EGF receptor synthesis wanes as differentiation of respiratory epithelium proceeds.

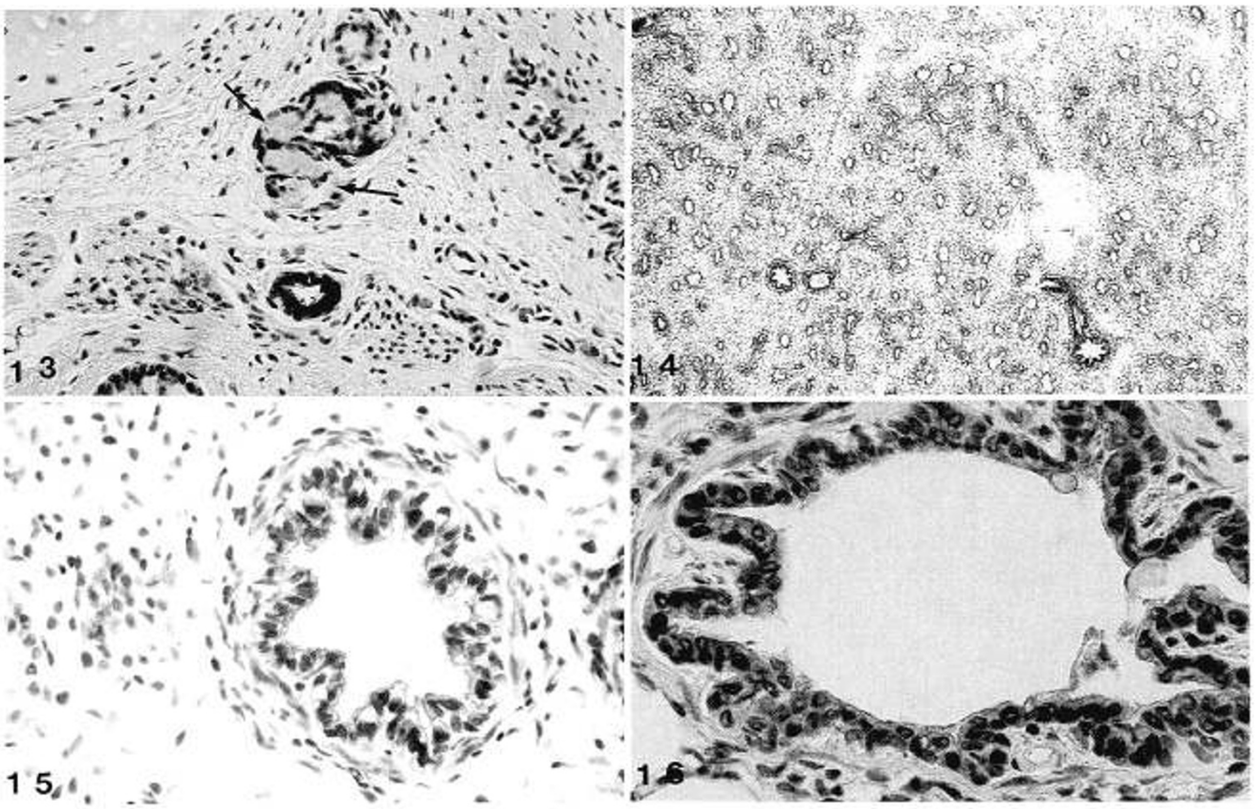

Fig. 13. Photomicrograph of the lung of a lamb of 144 d of gestation. Serous demilunes of submucosal glands show immunostaining for lipocortin-1. Acini primarily made up of mucous cells (*arrows*) are unstained. Cross-section of gland duct is intensely immunostained (immunoperoxidase and hematoxylin × 480).

Fig. 14. Photomicrograph of the lung of a lamb of 108 d of gestation. Epithelial lining cells of large bronchioles are intensely immunostained for lipocortin-1 (immunoperoxidase and hematoxylin × 48).

Fig. 15. Photomicrograph of the lung of a lamb of 117 d of gestation. Epithelial lining of bronchiole is immunostained for lipocortin-1 (immunoperoxidase and hematoxylin × 480).

Fig. 16. Photomicrograph of the lung of a lamb of 144 d of gestation. Bronchiolar epithelium is immunostained for lipocortin-1 (immunoperoxidase and hematoxylin × 480).

The immunostaining of cells at bronchioloalveolar portals suggests that these cells may remain as progenitor cells for more distal airways during late gestation and possibly in the fully developed lung. Alveolar type II cells, smooth muscle cells, and fibroblasts also possess EGF receptors *in vitro* (11). The absence of distinct immunoreactivity in these cell types *in vivo* may reflect differences in EGF-receptor-gene expression *in vitro* and *in vivo*. However, the absence of EGF-receptor immunostaining in fibrocytes *in vivo* also suggests that the lower concentrations of EGF receptor present on nonepithelial cells might also lie below the threshold of immunodetection for our antibodies.

The significance of immunostaining of bronchial fluid is not clear. It is conceivable that some of the immunostaining reflects the presence of antibody binding fragments of epithelium carried into the lumen during the frozen sectioning process. However, it is also possible that staining was produced by unquenchable endogenous peroxidase.

The physiologic effects initiated by EGF-receptor activation late in gestation are not known. Our previous work indicated that EGF stimulated epithelial proliferation in the fetal trachea and bronchi. Nonproliferative roles for EGF have also been reported including suppression of fetal lung fluid secretion (39), stimulation of surfactant-associated protein synthesis in the lung (7) and suppression of chloride secretion in the stomach (40). Current evidence suggests that stimulation of EGF receptor/kinase may result in phosphorylation and subsequent changes in activity in cytoplasmic or membrane bound regulatory proteins such as lipocortin-1 (13–17).

Previous studies have reported isolation of lipocortin-1 (35 kD) from rat, porcine and bovine lung (17, 19, 24). Immunoreactive 35 kD protein has also been identified in the columnar epithelia of the respiratory systems of the adult mouse and rat (41). However, the ontogeny and distribution of lipocortin-1 in the developing ovine lung have not been reported. Findings reported here suggest the lipocortin-1 immunoreactivity is distributed in epithelial cells lining conducting airways and in peribronchial glands, once they appear, throughout development. Preliminary studies suggest that lipocortin-1 has a similar distribution in a series of near-normal fetal and neonatal human lungs (Johnson M, unpublished data).

The distribution of lipocortin-1 immunoreactivity is of particular interest due to its potential role as a modulator of EGF effects. Several reports suggest that lipocortin-1 is an important regulatory peptide phosphorylated by the EGF receptor/kinase (13–17). Localization of lipocortin-1 immunoreactivity at sites bearing EGF receptor immunoreactivity raises the possibility that EGF may influence cellular proliferation and differentiation by altering the activity of intracellular regulatory proteins such as lipocortin-1. The lack of complete similarity of the timing of appearance and distribution of EGF receptor immunoreactivity and lipocortin-1 immunoreactivity in this study may represent real differences in ontogeny and location, or simply concentrations of EGF receptor below the level of immunodetection with our antibodies.

Lipocortin-1, initially isolated as a substrate for the tyrosine kinase of the EGF receptor, is a calcium and phospholipid-dependent membrane-binding protein that in *in vitro* assays inhibits phospholipase A_2 activity, binds actin, and causes lipid vesicles to aggregate (16–18, 42). Phosphorylation of lipocortin-1 appears to alter its activity (43, 44); however, the physiologic effects of lipocortin-1 and thus consequences of phosphorylation are not established. The inhibitory effects of lipocortin-1 on phsopholipase A_2 have been proposed as a mechanism through which prostaglandin synthesis by inflammatory cells is inhibited (16, 17). Inhibitory effects on pulmonary prostaglandin synthesis have also been described by Cirino *et al.* (45) who found that lipocortin-1 inhibits thromboxane release in perfused guinea pig lung. Recently, Glenney *et al.* (18) have also demonstrated a submembrane distribution for lipocortin-1 (which they refer to as Calpactin II) and suggest that this protein acts as a membrane to a cytoskeletal linkage molecule.

In summary, the pattern of appearance of the EGF receptor/kinase and its substrate, lipocortin-1, in ovine lung begins with immunostaining of conducting airway lining epithelium near the end of the first trimester of pregnancy before bronchial glands could be identified. The larger the airway, the earlier was the appearance of immunostaining. This was followed by staining of bronchial glands and of large bronchioles adjacent to stained bronchi at ⅔ of gestation. By ⅞ of gestation, conducting airway epithelium was no longer stained consistently for the EGF receptor except for the most distal cells lining bronchioloalveolar portals, but lipocortin-1 was still identified until near term in all levels of airways, including those of bronchioloalveolar portals. The most striking immunostaining for lipocortin-1 late in gestation was that in bronchial submucosal glands and the cells lining the necks of glands.

This pattern of development of the EGF receptor expression suggests early influence of EGF or EGF-like ligands on developing airway epithelium during a rapid proliferative stage. As intrapulmonary bronchial glands were identified at about ⅔ of gestation and stained heavily for EGF receptor and for lipocortin-1, we postulate that the EGF receptor might promote EGF action in an autocrine or paracrine fashion, as these glands also are sites of human EGF immunostaining from midgestation onward (46).

Acknowledgments. The authors wish to thank Dr. J. Kamso-Pratt, Mrs. Sandy Olson, Ms. Tracie Reynolds, Mr. Rao Gaddipati, Mr. Stanley Poole and Mrs. Patricia Minton for excellent technical assistance and Ms. Diana Nelson for manuscript preparation. We are also indebted to Dr. Roy Fava for helpful discussions.

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