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Expression of Basic Fibroblast Growth Factor and Receptor: Immunolocalization Studies in Developing Rat Fetal Lung

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ABSTRACT. To study the role of basic fibroblast growth factor (bFGF) in fetal lung development, the distribution of bFGF peptide and FGF receptor (FGF-R) was examined by immunohistochemistry in embryonic and fetal rat lung [d 12 to term (term = 22 d)]. Throughout development bFGF was localized to airway epithelial cells, their basement membranes, and their extracellular matrix. FGF-R was also detected in airway epithelial cells, especially in the branching areas, and in interstitial cells as early as d 13. The number of FGF-R immunoreactive cells increased during the embryonic and pseudoglandular stages of lung development, followed by fluctuations in reactivity during the canalicular stage. No FGF-R was detected in tissue from the saccular stage of lung development. The presence of bFGF and FGF-R in developing airway epithelium and mesenchyme is compatible with a role for this growth factor during fetal lung development. In the developing lung, bFGF seems to be sequestered and stored in the extracellular matrix, and may be released at times of need. Furthermore, FGF-R up- and down-regulation offers another mechanism by which the growth of specific cell populations may be controlled during fetal lung development. (*Pediatr Res* 31: 435–440, 1992)

Abbreviations

bFGF, basic fibroblast growth factor
aFGF, acidic fibroblast growth factor
FGF-R, fibroblast growth factor receptor
HSPG, heparan sulfate proteoglycan

The mammalian lung develops as an invagination of the endodermal tube from the ventral wall of the foregut into the surrounding splanchnic mesoderm. This embryonic stage is followed by the pseudoglandular stage of sequential tubular bifurcations. Vasculogenesis of the embryonic and fetal lung occurs in parallel with branching morphogenesis of the airways. Microvascular formation is greatest during the subsequent canalicular stage, which is followed by the saccular stage of acinar development (1).

Polypeptide growth factors, in concert with extracellular matrix molecules, are believed to play a well-coordinated role in lung morphogenesis. Epidermal growth factor has been shown to influence growth of airway epithelium (2, 3), and IGF has been localized to the respiratory epithelium (4, 5). Transforming growth factor-β_1, which may modulate extracellular matrix composition, also appears to play a role in lung branching morphogenesis (6, 7). Recently, we described the distribution of immunoreactive platelet-derived growth factor in the developing fetal rat lung (8, 9).

bFGF is a pluripotential growth factor that is mitogenic toward a broad spectrum of mesoderm- and ectoderm-derived cells (10). *In vivo* studies have shown that it is also a potent angiogenic (11) and neurotrophic factor (10). It may play a role in embryonic (12) and fetal (13) development and seems also to be involved in tumorigenesis (10). bFGF is well conserved through evolution. The possession of two heparin-binding domains in its sequence is one of the main reasons why bFGF binds avidly to heparin and HSPG in the extracellular matrix. In addition, bFGF can also bind to low-affinity cell surface proteoglycans (13). Heparin and HSPG stabilize and protect bFGF from heat, acid, and proteolytic degradation (14–16). Recently, it has been shown that the binding of bFGF to its receptor requires binding either to heparan sulfate side chains of a membrane HSPG or to free heparan sulfate (heparin) chains (17). These observations suggest that proteoglycans may modulate the bioactivities of bFGF.

Growth factors induce their diverse functions by binding to specific high-affinity cell surface receptors. Two FGF-R with molecular weights of 125 and 145 kD, which recognize both aFGF and bFGF, have been identified. Although both aFGF and bFGF will compete for binding to those receptors, there is evidence that the 145-kD receptor has a stronger affinity for bFGF (18). Recently, two novel FGF-R have been added to the receptor family, FGF-R3, which is commonly shared by both aFGF and bFGF (19), and an aFGF-specific FGF-R4 (20).

A number of bFGF immunolocalization studies in various organs and tissues of chick embryo (21), cow (22), developing fetal and adult rat (23, 24), and adult human (25) have been reported. However, bFGF and FGF-R expression in the developing lung has not, to our knowledge, been specifically investigated. *In vitro* studies have demonstrated that microvascular endothelial cells (26) and epithelial cells (27) of fetal rat lung do respond to exogenous bFGF. In addition, it has been shown that bFGF is a potent mitogen for adult lung pneumocytes (28). To better define the expression of bFGF and FGF-R during lung development, we examined immunohistochemically the distribution and expression of bFGF and FGF-R in embryonic and fetal rat lung.

MATERIALS AND METHODS

Materials. Mouse monoclonal anti-bovine bFGF, polyclonal rabbit anti-human FGF-R, human recombinant bFGF and FGF-R peptide were from Upstate Biotechnology, Inc. (Lake Placid, NY). The rabbit peptide antiserum FGF-R was raised against the synthetic peptide (DALPSAEDDDEDDSSSEEKEADNTK)

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corresponding to the deduced human cDNA sequence of the external domain of the FGF receptor (FLG/BEK/CEK gene product). Goat anti-mouse peroxidase-conjugated IgG was from Calbiochem (San Diego, CA) and goat anti-rabbit peroxidase conjugated IgG was from Boehringer Mannheim Biochemica (Mannheim, Germany). Nitrocellulose membrane was from Schleicher and Schuell (Keene, NH) and Zysorbin was from Zymed (San Francisco, CA). The enhanced chemiluminescence detection kit was from Amersham (Ontario, Canada). An avidin-biotin complex (Vectastain) kit was from Vector Laboratories (Burlingame, CA), and embedding media (O.C.T. compound) was from Miles Research (Elkhart, IN).

Wistar rats were obtained from Charlest River (St. Constant, Quebec) and were bred in our animal facilities. Pregnant dams of known gestational age were killed by exposure to an excess of diethylether. The uterus was exposed, and the embryos and the fetuses were delivered. Whole embryos were studied on d 12 of gestation, and the lungs were removed for study from d 13 to 22 of gestation (term = 22 d).

Immunoprecipitation and Western blot immunoanalysis for FGF-R. Freshly frozen d-20 fetal lung was homogenized in PBS, followed by centrifugation at 10 000 × *g* for 15 min at 4°C. The supernatant was spun in a Beckman TLA-100 table-top ultracentrifuge for 60 min at 50 000 × *g* at 4°C. The membrane-enriched pellet was washed once with ice-cold PBS and dissolved in lysis buffer (0.15 M NaCl, 1% Triton X-100, 0.01 M Tris, pH 8.0). The sample was precleaned by incubation with nonimmune rabbit IgG for 30 min at 4°C, followed by incubation with 10% (vol/vol) formalin-fixed *Staphylococcus aureus* Cowan strain A (Zysorbin) in PBS for another 30 min at 4°C. Rabbit anti-human FGF-R (0.025 μg) was added to the sample and incubated overnight on an end-over-end rotator at 4°C. Zysorbin was used to precipitate the immune complexes (29). After 30 min of incubation at 4°C, the immune complexes were washed three times with lysis buffer, dissociated by boiling for 3 min in sample buffer (10% glycerol, 2% SDS, 5% β-mercaptoethanol, 0.0025% bromophenol blue, 0.06 M Tris, pH 8.0), and subjected to 5% SDS gel electrophoresis as described by Laemmli (30). Proteins were electrophoretically transferred to a nitrocellulose membrane (31). Nonspecific binding was blocked by incubation with 3% nonfat dry milk in PBS at 4°C for 1 h, followed by an overnight incubation with rabbit anti-human FGF-R antibody (1:300). The membrane was washed three times with PBS and incubated with peroxidase-conjugated goat anti-rabbit IgG (1:30 000) for 2 h. After several washes with PBS, the enhanced chemiluminescence detection kit was used to develop the membrane.

Western blot immunoanalysis of bFGF. Freshly frozen d-21 fetal lung was homogenized in lysis buffer (0.15 M NaCl, 1% Triton X-100, 0.01 M Tris, pH 8.0), followed by 10 000 × *g* centrifugation for 15 min at 4°C. Protein content of supernatant was determined according to Bradford (32). Sample buffer (10% glycerol, 0.0025% bromophenol blue, 0.06 M Tris, pH 8.0) was added to 15 μL of supernatant containing 60 μg of protein, and the proteins were separated by gel electrophoresis on a 15% (wt/vol) SDS-free polyacrylamide gel (30). Proteins were electrophoretically transferred to nitrocellulose membrane (31). Western blot immunoanalysis was performed as described above by using monoclonal mouse anti-bovine bFGF IgG (type I; 1:50 dilution) and peroxidase-conjugated goat anti-mouse IgG (1:15 000 dilution). The enhanced chemiluminescence detection kit was used to develop the membrane.

Immunohistochemistry of bFGF and FGF receptor. Tissues for cryosection were fixed as described by Simmons *et al.* (33). Sections (5 μm) were cut on a cryotome and mounted on α-aminopropyltriethoxysilane-coated slides. The avidin-biotin immunoperoxidase method (34) was used to study the immunolocalization of bFGF and FGF-R in fetal rat lungs. Embedding medium (O.C.T. compound) from the cryosections was dissolved in PBS. Endogenous peroxidases were quenched in 1% (vol/vol) hydrogen peroxide in methanol for 30 min. Nonspecific binding sites were blocked by using 5% (vol/vol) normal goat serum and 1% (wt/vol) BSA in PBS for 30 min. The excess of blocking solution was carefully blotted at the edges of the tissue sections, and the sections were incubated overnight at 4°C with a 1:15 dilution of monoclonal anti-bovine bFGF and a 1:300 dilution of polyclonal rabbit anti-human FGF-R. Subsequent procedures were conducted at room temperature. The tissue sections were washed three times in PBS, then incubated for 2 h with a 1:100 dilution of biotinylated anti-mouse IgG for bFGF immunolocalization studies and a 1:200 dilution of biotinylated anti-rabbit IgG for FGF-R immunolocalization studies. After washing three times in PBS, the tissue sections were incubated with an avidin-biotin complex for 1 h. Slides were washed again in PBS and developed in 0.075% (wt/vol) 3,3′-diaminobenzidine tetrahydrochloride in Tris-HCl buffer, pH 7.6, containing 0.002% (vol/vol) hydrogen peroxide. Tissue sections were lightly counterstained with Carazzi's hematoxylin, dehydrated in an ascending ethanol series, then in xylene, and mounted.

In control experiments, the anti-bFGF IgG and anti-FGF-R IgG were replaced with preimmune or nonimmune mouse or rabbit sera or with blocking solution [5% (vol/vol) normal goat serum and 1% (wt/vol) BSA]. No immunostaining was observed under these conditions. Immunoabsorption studies were also conducted by incubating the primary antibodies with excess (double the amount of antibody) bFGF peptide and recombinant human FGF-R peptide for 4 h at 4°C before immunostaining procedures. Negative immunoreactivity was observed with these studies, indicating that the antibodies are highly specific.

RESULTS

Immunoanalysis of FGF-R and bFGF. Initially, the specificity of the antibodies used in this study was validated by immunoprecipitation and Western blot immunoanalysis. The polyclonal antibody raised against a synthetic FGF-R peptide immunoprecipitated and recognized (Fig. 1) two distinct lung proteins with approximate molecular weights of 125 kD and 145 kD, consistent with previously reported molecular mass for FGF-R (18).

A 15-kD lung protein was detected (Fig. 1) with the MAb to bovine bFGF (type I), which is in agreement with the reported molecular mass of bFGF (37). These data indicate that the

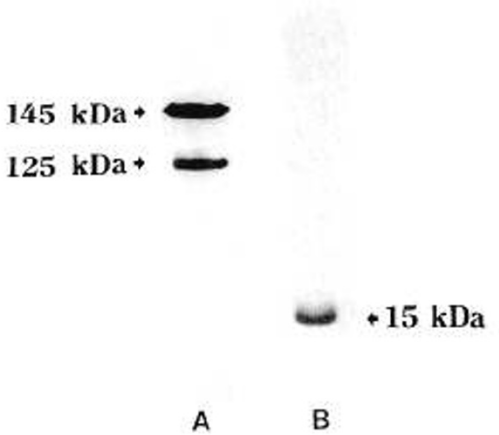

Fig. 1. *A*, Immunoprecipitation and Western blot immunoanalysis of FGF-R in rat fetal lung of 20 d gestation. Immunoprecipitated FGF-R-containing protein was loaded and separated on 5% polyacrylamide gel under reducing conditions. Protein was transferred to a nitrocellulose membrane and immunostained with FGF-R antibody. Protein bands were detected at ≈125 kD and ≈145 kD. *B*, Western blot immunoanalysis of bFGF in rat fetal lung of 21 d gestation. Protein (60 μg) from fetal lung homogenate was loaded and separated on a 15% polyacrylamide gel under nonreducing conditions. Protein was transferred to a nitrocellulose membrane and immunostained with bFGF antibody. A single protein band of ≈15 kD was detected.

antibodies recognize rat bFGF and FGF-R and, thus, can be used for immunolocalization studies in rat lung.

Immunoreactivity of bFGF and FGF-R. The lung bud from whole embryo sections of 12 d gestation showed positive immunoreactivity to bFGF in the epithelial lining of the primitive airway, in the mesenchymal cells, and in the extracellular matrix. A similar staining pattern of bFGF was observed for d-13 embryonic lung (Fig. 2*a*) with intense immunoreactivity to bFGF in airway epithelial cells, both intracellularly and in the basement membrane (Fig. 2*b*). Immunoreactivity to bFGF was also evident in mesenchymal and mesothelial cells and in the extracellular matrix. Negative staining in d-13 embryonic lung was seen with immunoadsorbed bFGF antisera (Fig. 2*c*). Weak positive immunoreactivity to FGF-R was seen in airway epithelial cells of d-13 embryonic lung. In addition, solitary cells in the mesenchyme and some mesothelial cells demonstrated positive immunoreactivity to FGF-R. Day-15 fetal lung showed intense bFGF immunoreactivity in airway epithelial cells, with homogenous and diffuse immunostaining in the extracellular matrix (Fig. 2*d*). The intensity of FGF-R immunoreactivity in airway epithelial cells and in mesothelial cells increased on d 15 (Fig. 2*e*) and d-17 (Fig 2*h*) through 19 of gestation. During the pseudoglandular stage (d 15–18) of lung development, FGF-R immunoreactive airway epithelial cells were localized primarily to the branching areas of the developing airway (Fig. 2*e*). The number of FGF-R immunoreactive solitary cells, which were present in close vicinity to internal vascular plexuses (Fig. 2*f*), also increased between d 15 and 18 of gestation. Intense bFGF immunoreactivity was also evident in epithelial cells of both bronchial and distal airways of d-17 fetal lung (Fig. 2*g*). Extracellular matrix and smooth muscle cells surrounding the large vessels were also bFGF immunopositive, but the endothelial cells of major vessels and bronchial smooth muscle were not (Fig. 2*g*). Immunoreactivity to bFGF in the matrix of d-19 fetal lung, which represents the early canalicular stage of lung maturation, was more localized to the periphery of the developing lung compared with the diffuse homogenous pattern seen during the pseudoglandular stage at d 17. Reactivity to bFGF was also seen in epithelial cells of both bronchial and distal airways (Fig. 3*a*). At d 19, a few bronchial and distal airway epithelial cells showed weak immunoreactivity for FGF-R, and a few patchy areas of FGF-R-positive cells were located in the interstitium, especially at the peripheral region (Fig. 3*b*). Most of the epithelial cells in the airways and in the interstitium showed negative immunoreactivity to FGF-R. Neither bFGF nor FGF-R were detected in mesothelial cells from d 19 of gestation onward. bFGF immunoreactivity similar to that seen at d 19 was observed in d-20 fetal lung, especially in the epithelial cells lining the developing air spaces (Fig. 3*c*). Under this high-power magnification, bFGF seemed to be localized not only intracellularly, but also on the apical cell membrane of the bronchial and distal airway epithelial cells. In contrast to d 19, a dramatic increase in the number of FGF-R immunoreactive cells, especially bronchial and distal airway epithelial cells (Fig. 3*d*), was noted in d-20 fetal lung. Solitary cells with FGF-R immunoreactivity that were evident at earlier gestations were not distinguishable from other FGF-R immunoreactive interstitial cells in d-20 fetal lung and were absent during the saccular stage of lung development. Day-22 fetal lung, representing the saccular stage of lung development, demonstrated intense bFGF immunoreactivity both intracellularly and extracellularly (Fig. 3*e*). Day-21 and -22 fetal lungs (Fig. 3*f*) showed negative immunoreactivity to FGF-R.

DISCUSSION

Previous bFGF immunolocalization studies in d-18 fetal rat (24) demonstrated that bFGF immunoreactivity was restricted to basement membranes of the digestive tract, skin, lung, kidney, müllerian and wolffian ducts, salivary glands, pancreas, sex cords, and sebaceous glands. Although intracellular bFGF staining was evident in endocrine cells of adrenal cortex, testis, and ovary, no cytoplasmic immunoreactivity of bFGF was observed in fetal airway epithelial cells. However, intracellular localization of bFGF has been demonstrated in tracheal and bronchial epithelial cells of adult human lung (25), and bFGF mRNA has been detected in rat pleural mesothelial cells (35). Despite *in vitro* responsivity to bFGF, the cytoplasmic bFGF immunoreactivity *in vivo* was patchy or absent in alveolar pneumocytes of adult human lung. In this study, bFGF immunoreactivity was localized both to extracellular matrix and to cytoplasm of fetal tracheal, bronchial, and distal airway epithelial cells. The intensity of bFGF immunoreactivity appeared to increase with gestation. This could be due to either increased reactivity of individual cells or an increase in the number of reactive cells. The staining of extracellular matrix for bFGF was homogenous during the embryonic and pseudoglandular stages of lung development, but was more intense peripherally during the canalicular stage when active vascularization of acini occurs.

High-power magnification revealed bFGF immunoreactivity not only on extracellular matrix, but also on the apical cell membranes of bronchial and distal airway epithelial cells, suggesting that bFGF binds to membrane-bound proteoglycans. Studies have demonstrated that bFGF binds to an HSPG and can be released as an active heparan sulfate-bFGF complex when extracellular matrix HSPG is degraded by its specific endoglycosidase heparinase or heparatinase (36). The heparan sulfate-bFGF complex, which is freely diffusible in extracellular matrix, is capable of binding to high-affinity cell surface receptors for bFGF (37). Moreover, heparin-like molecules that are present on the cell membrane have been shown to modulate the binding of bFGF to its receptors (17). Both cell- and extracellular matrix-associated HSPG or heparin may thus function as storage depots for bFGF and regulate bFGF bioavailability, whereas cell surface heparin-like molecules modulate the binding of bFGF to its receptors.

Although the exact physiologic role of bFGF for fetal airway epithelial cells is unknown, recent studies have demonstrated that epithelial cells (27) of fetal rat lung do respond to exogenous bFGF. In addition, it has been shown that bFGF is a potent mitogen for adult lung pneumocytes (28). These studies, and the observation that bFGF is present in fetal lung epithelium, suggest that bFGF may be involved in the control of lung epithelial cell proliferation. The finding of FGF-R immunoreactivity on epithelial airway cells supports the idea that these cells are target cells for bFGF during lung development. Weakly positive FGF-R immunoreactive epithelial cells were detected in embryonic lung, suggesting that bFGF is involved in airway epithelial cell proliferation from the very early stage of lung development. Further evidence for a role of bFGF in regulating airway epithelial proliferation is the observation that the decrease in number of FGF-R-positive airway epithelial cells at 19 d gestation, which is followed by a surge in FGF-R immunoreactive epithelial cells 1 d later, correlates with previously published epithelial labeling indices for both days (38). The finding of patchy areas of FGF-R immunoreactive cells in d-19 fetal lung indicates that there is uneven growth in different areas at the early canalicular stage of development. Strong FGF-R immunoreactivity present in the branching areas of d-15 airway epithelium suggests that bFGF may also play a role in lung-branching morphogenesis.

In addition to epithelial cells, vascular smooth muscle cells, microvascular endothelial cells, mesothelial cells, and chondrocytes of trachea rings also demonstrated intense bFGF immunoreactivity. The finding of FGF-R immunoreactive solitary cells in the mesenchyme close to the internal microvascular plexuses during the embryonic and pseudoglandular periods of lung development suggests that these positive cells could be the precursors of endothelial cells. *In vitro* studies (26) have shown that pulmonary microvascular endothelial cells do respond to exogenous bFGF, suggesting that bFGF, in concert with other angi-

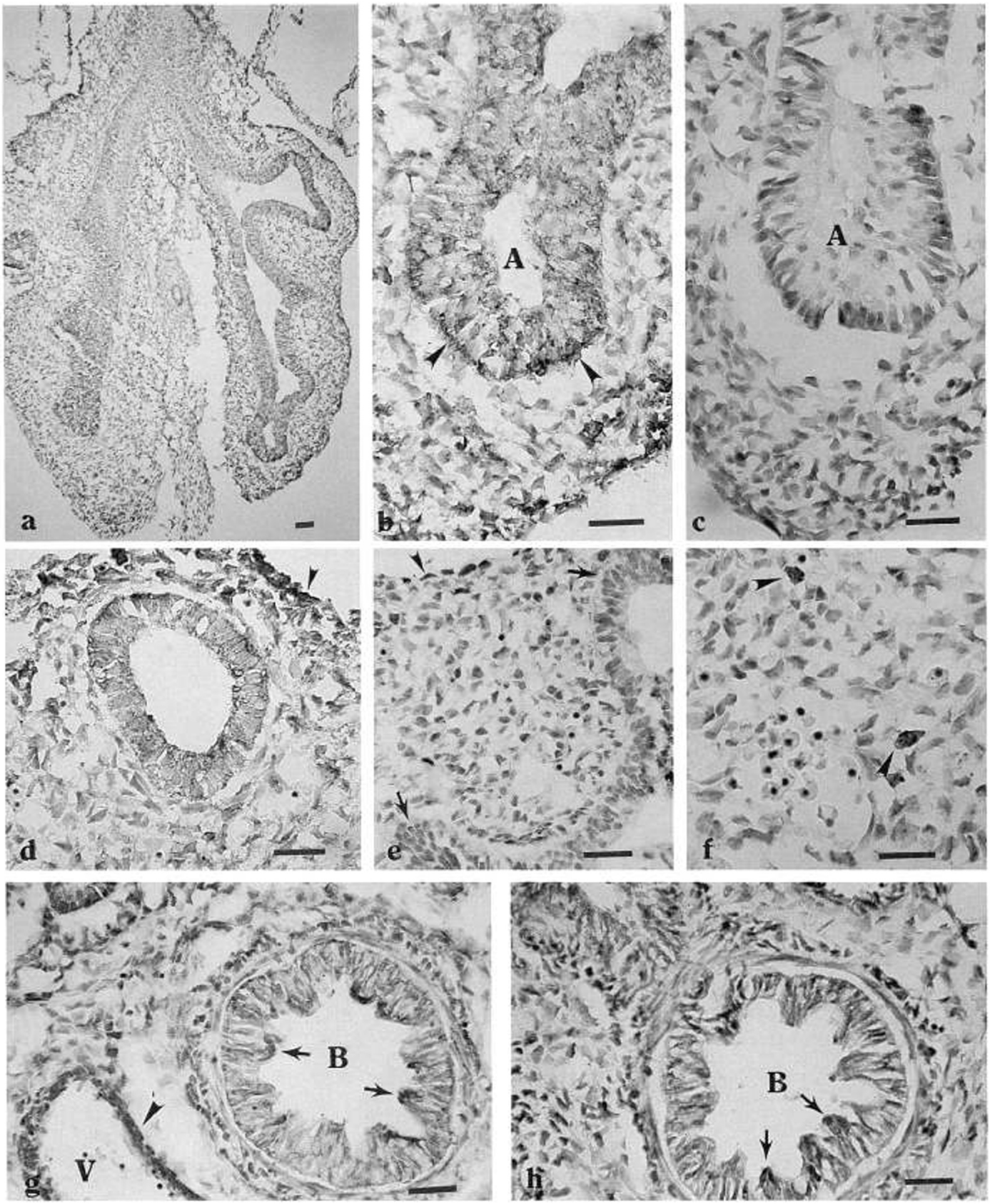

Fig. 2. Immunolocalization of bFGF and FGF-R in fetal rat lung at the embryonic and pseudoglandular stages of lung development. *Bars* = 10 μm. *a*, bFGF immunoreactivity in d-13 embryonic lung. *b*, Intense bFGF immunoreactivity in airway (*A*) epithelial lining and in basement membrane (*arrowheads*) shown by higher magnification of the lower right corner of *a*. *c*, Negative bFGF immunoreactivity in airway (*A*) and surrounding mesenchyme of d-13 embryonic lung after immunoabsorption. *d*, Fetal lung at d 15, showing positive immunoreactivity to bFGF in airway epithelial cells, interstitial cells, mesothelial cells (*arrowhead*), and extracellular matrix. *e*, Positive immunoreactivity to FGF-R in airway epithelial cells, especially in branching areas (*arrows*), interstitial cells, and mesothelial cells (*arrowheads*). *f*, FGF-R immunoreactive solitary cells, close to the internal vascular plexus in d-15 fetal lung. *g*, Fetal lung at 17 d gestation, demonstrating intense bFGF reactivity in bronchial (*B*) airway epithelial cells (*arrows*), vascular (*V*) smooth muscle cells (*arrowheads*), and the extracellular matrix. *h*, Positive FGF-R immunoreactivity in bronchial (*B*) epithelial cells (*arrows*) and interstitial cells.

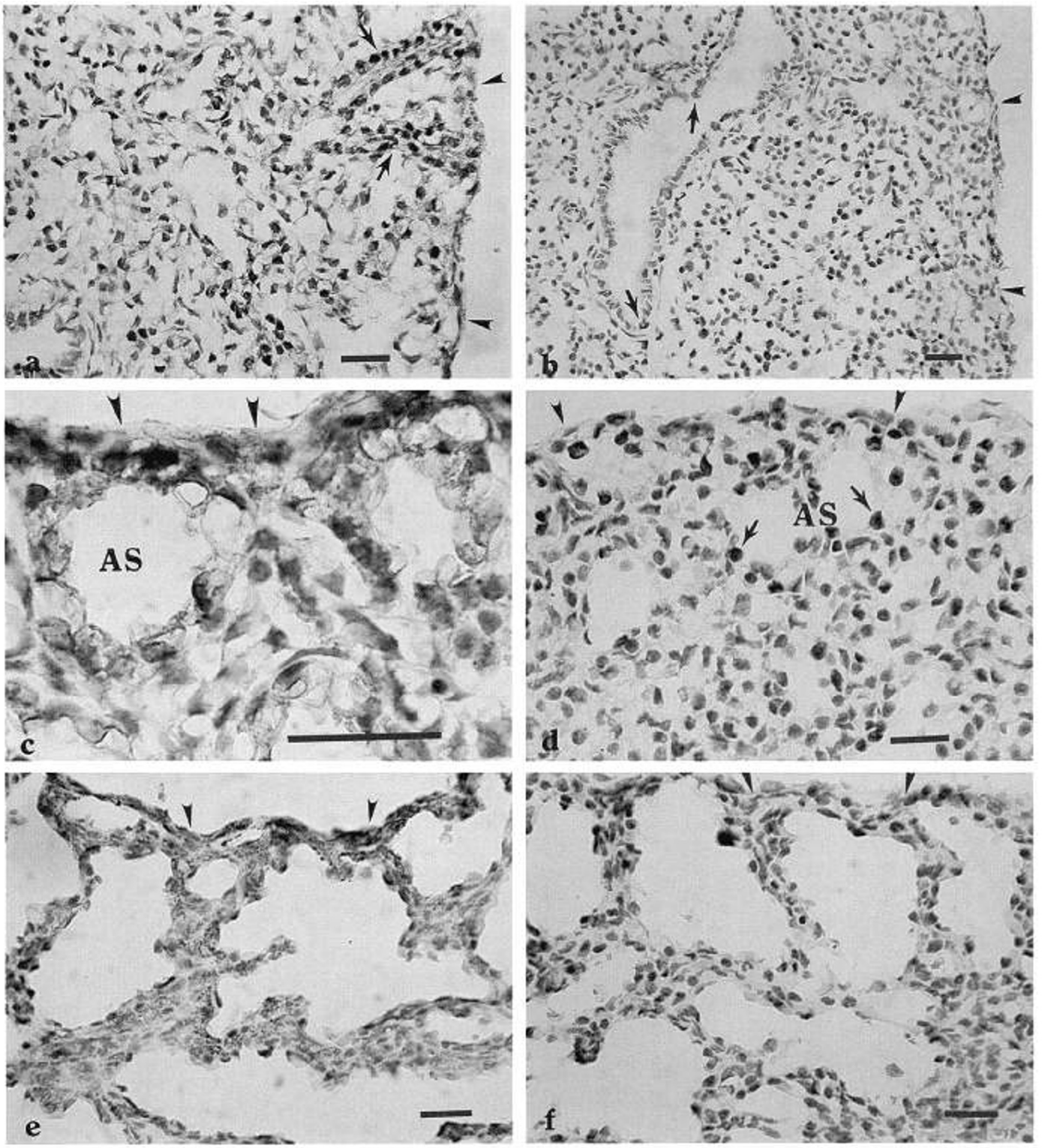

Fig. 3. Immunolocalization of bFGF and FGF-R in fetal rat lung at the canalicular and saccular stages of lung development. *Bars* = 10 μm; *arrowheads* denote mesothelial lining. *a*, Fetal lung at 19 d gestation, demonstrating bFGF reactivity in epithelial cells lining distal respiratory tubules (*arrows*) and in the extracellular matrix. *b*, Collection of FGF-R immunoreactive cells in the periphery of d-19 fetal lung with few FGF-R reactive airway epithelial cells (*arrows*). Most of the airway epithelial cells and interstitial cells showed negative immunoreactivity to FGF-R. *c*, Intense bFGF immunoreactivity in cells lining the developing air spaces (*AS*) and in the extracellular matrix during the canalicular stage of lung development at 20 d gestation. *d*, FGF-R immunoreactivity in cells (*arrows*) lining the air spaces (*AS*) and in interstitial cells of d-20 fetal lung. *e*, bFGF immunoreactivity in d-22 fetal lung, especially in the extracellular matrix. *f*, Negative FGF-R immunoreactivity in d-22 fetal lung.

ogenic factors, may also be involved in lung vasculogenesis and angiogenesis.

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