Keratinocyte growth factor and its receptor are involved in regulating early lung branching

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SUMMARY

Lung branching morphogenesis depends on mesenchymal-epithelial tissue interactions. Keratinocyte growth factor (KGF) has been implicated to be a regulator of these tissue interactions. In the present study, we investigated the role of KGF in early rat lung organogenesis. Reverse transcriptase-polymerase chain reaction analysis revealed KGF mRNA expression in the mesenchymal component of the 13-day embryonic lung, while message for KGF receptor (KGFR) was expressed in the epithelium, confirming the paracrine nature of KGF/KGFR axis. Antisense KGF oligonucleotides inhibited DNA synthesis of embryonic lung explants. This inhibitory effect of antisense KGF was partially reversed by the addition of exogenous KGF. Recombinant KGF was mitogenic for 13-day isolated embryonic lung epithelial cells. Medium conditioned by 13-day lung mesenchymal cells also stimulated DNA synthesis of 13-day embryonic lung epithelial cells. This stimulatory effect was partially abrogated by a neutralizing KGF antibody. The number of terminal buds of lung explants cultured in the presence of antisense KGF oligonucleotides was significantly reduced compared to control explants. Exogenous KGF partially abrogated the inhibitory effect of antisense KGF on early lung branching. Sense or scrambled KGF oligonucleotides had no inhibitory effect on lung growth and branching. Addition of neutralizing KGF antibodies to the explants also reduced the degree of branching, while non-immune IgG and neutralizing acidic FGF antibodies had no effect. Explants incubated with antisense oligonucleotides targeted to the initiation site of translation of both the splice variants of the fibroblast growth factor receptor-2 (FGFR2) gene, KGFR and bek, exhibited a similar reduction in lung branching as observed with antisense KGF oligonucleotides. Antisense KGFR-specific oligonucleotides dramatically inhibited lung branching, while exposure of explants to antisense bek-specific oligonucleotides resulted in reduced branching albeit to a lesser degree than that observed with antisense KGFR-specific oligonucleotides. Neither sense nor scrambled KGFR-specific oligonucleotides had any effect on early lung branching. These results suggest that the KGF/KGFR system has a critical role in early lung organogenesis.

Key words: keratinocyte growth factor, epithelial-mesenchymal interactions, embryonic lung development, rat lung

INTRODUCTION

Many organs, including lung, are composed of two primary tissue layers, namely, epithelium and mesenchyme. Bidirectional interactions of these two tissues have been demonstrated to be essential for the sequential events of organogenesis – determination, growth, morphogenesis, and cytodifferentiation (Grobstein, 1953; Wessels, 1977). In embryonic organs such as lung, mammary gland and salivary gland, which are formed by a process of progressive branching of the epithelium, the mesenchyme plays a determining role in the formation of the organ's characteristic morphology (Kratchowill, 1969; Spooner and Wessels, 1970; Wessels, 1979). Dependence of lung epithelial branching on its surrounding mesenchyme was first demonstrated by Rudnick (1933). This observation has been confirmed by later studies (Dameron, 1961; Alescio and Cassini, 1962). The nature of the morphogenetic signals regulating lung epithelial branching remains, however, largely unknown. Recently,

several soluble factors such as growth factors, neuropeptides and vitamins have been implicated in lung branching morphogenesis (Warburton et al., 1992; Matrisian et al., 1992; Jaskoll et al., 1994; Peters et al., 1994; Serra et al., 1994; King et al., 1995; Souza et al., 1995). However, few growth factors have been identified with characteristics compatible with transferring information between mesenchyme and epithelium. Using an antisense strategy to block translation of endogenous platelet-derived growth factors (PDGF), we recently reported that PDGF-AA and its receptor represent an important epithelial-mesenchymal interaction, which plays a critical role in early lung branching morphogenesis (Souza et al., 1995). PDGF-AA is synthesized in the epithelium while its receptor, the α-receptor, is expressed in the mesenchyme of the rat lung primordia. Apositional expression is also observed for PDGF-BB and the β-receptor (Holmgren et al., 1991) but the BB isoform and β-receptor are not involved in the control of lung branching (Leveen et al., 1994; Soriano, 1994; Souza et al.,

1994). Thus, PDGF-AA appears to be a diffusible epithelium-derived mediator of epithelial-mesenchymal tissue interactions during early branching morphogenesis. In several organs, including lung, keratinocyte growth factor, a member of the fibroblast growth factor family (FGF-7, KGF), has been implicated as a diffusible mesenchymal mediator of epithelial-mesenchymal tissue interactions (Rubin et al., 1995). In embryonic lung, KGF mRNA is expressed in the mesenchyme (Mason et al., 1994), whereas the KGF receptor, a FGFR2-IIIb splice variant (Miki et al., 1992, Yayon et al., 1992; Dell and Williams,1992), which has an high affinity for KGF and acidic FGF (FGF1, aFGF), is expressed in airway epithelium (Orr-Urtreger et al., 1993). A targeted expression of a dominant negative KGFR in transgenic mice resulted in dramatically reduced bronchial branching (Peters et al., 1994). In addition, homozygous deletions of *breathless*, a FGF-receptor gene, caused abnormal tracheal branch migration in *Drosophila* (Glazer and Shilo, 1991). Although these data are consistent with KGF being an important mesenchyme-derived signal for branching of lung epithelium, other FGFs cannot be excluded. Recent studies suggest that acidic FGF (FGF1, aFGF), but not basic FGF (FGF2, bFGF), stimulates epithelium of lung rudiments to branch in Matrigel (Nogawa et al., 1995). To better understand the biological role of the KGF/KGFR complex during early lung organogenesis, we have conducted inhibition studies for KGF and KGFR, using a similar antisense strategy as previously reported for the PDGF ligand-receptor complexes (Souza et al., 1994, 1995). We report that an intact KGF/KGFR system is required for early lung organogenesis.

MATERIALS AND METHODS

Materials

Female (200-250 g) and male (250-300 g) Wistar rats were obtained from Charles River (St. Constant, Quebec) and were bred in our animal facilities. Rats were killed at days 12-14 of gestation (term = 22 days). Culture media, antibiotics, fetal bovine serum and ascorbic acid were purchased from Gibco (Grand Islands, NY). Four well culture dishes were from Nunc (Intermed, Denmark) and culture inserts from Millipore (Co, Bedford, MA). Dispase was from Becton Dickinson Labware (Bedford, MA) and [^{3}H]thymidine from Amersham (Arlington Heights, IL). Rabbit polyclonal IgG against an epitope corresponding to amino acids 805-821 at the carboxy terminus of bek was from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). The epitope is identical to corresponding KGFR sequence, indicating that the antibodies crossreact with KGFR (Dionne et al., 1990; Miki et al., 1992). Recombinant human KGF (rKGF), bovine aFGF and neutralizing antibodies to rKGF and aFGF were from R & D systems (Minneapolis, MN).

Sequence of antisense and sense oligonucleotides

Phosphorothioate oligonucleotides were synthesized on a PCR Mate EP 391 DNA synthesizer from Applied Biosystems (Foster City, CA). We synthesized oligonucleotides targeted against sequences adjacent to the AUG initiation codon of KGF (GenBank no. M60828) and KGFR mRNA (Miki et al., 1992). Previous studies have demonstrated that antisense oligonucleotides targeted to sequences adjacent to initiation codons are very efficient in inhibiting translation (Souza et al., 1994, 1995). Furthermore, it has been found that short 15-20 mer oligonucleotides are taken up in sufficient amount by lung explants and provide sufficient specificity for hybridization to corresponding target mRNA (Souza et al., 1994, 1995). The oligonucleotide sequences were.

(a) antisense KGF ON: 5′-TTTGTGCATTATAGT-3′; sense KGF ON: 5′-ACTATAATGCACAAA-3′; scrambled KGF ON: 5′-TTTCTGGATAATTGT-3′,

(b) antisense KGFR/bek ON: 5′-GCTGACCATGGTCAC-3′; sense KGFR/bek ON: 5′-GTGACCATGGTCAGC-3′.

As KGFR and bek are both splicing variants of the FGFR2 gene (Miki et al., 1992; Dell and Williams, 1992; Dionne et al., 1990) and have an identical initiation sequence, an antisense ON targeted against this region will not discriminate beween these two receptors. Therefore, we also synthesized antisense oligonucleotides to KGFR- and bek-specific exon sequences (Orr-Urtreger et al., 1993):

(c) antisense KGFR ON: 3′-AGGCAGACTGGTTGGCCTG-5′; sense KGFR ON: 3′-CAGGCCAACCAGTCTGCCT-5′ scrambled KGFR ON: 5′-AGGCTCAGTCGATCGCGTG-3′,

(d) antisense bek ON: 3′-CTGCAGAACTGTCAAC-5′; sense bek ON: 3′-GTTGACAG TTCTGCAG-5′.

All ONs were dissolved in double distilled water and quantitated by optical density at A_{260nm}.

Reverse-transcriptase polymerase chain reaction (RT-PCR)

Total RNA was isolated using RNeasy total RNA kit (Qiagen, Chatham, CA) and RT-PCR was performed as described previously (Wang et al., 1994). The primer set chosen for amplification of KGF was based on the mouse KGF mRNA sequence (GenBank no. M60828). KGFR-specific amplification was carried out as described by Orr-Urtreger et al. (1993). The primers chosen for amplification of β-actin (Nudel et al., 1983) and cytokeratin 18 (Singer et al., 1986) were based on mouse mRNA sequences. The primer set chosen for cytokeratin 8 (Hsieh et al., 1992) and vimentin (GenBank no. X62952) mRNA amplification were based on rat mRNA sequences. Primers used for amplification were:

(a) KGF cDNA: (forward primer): 5′-ATCCTGCCAACTCT-GCTCTACAGA-3′, (reversed primer): 5′-CTTCCCTTTGACAG-GAATCCCCTT-3 (predicted product size = 509 bp);

(b) KGFR cDNA: (forward primer): 5′-AACGGTCACCACAC-CGGC-3′, (reversed primer): 5′-AGGCAGACTGGTTGGCCTG-3′ (predicted product size = 312 bp);

(c) β-actin cDNA: (forward primer): 5′-TTGTAACCAACTG-GGACGATATGG-3′, (reversed primer): 5′-GATCTTGATCTTC-ATGGTGCTAGG-3 (predicted product size = 764 bp);

(d) Cytokeratin 8 cDNA: (forward primer): 5′-CTGGAGGTG-GACCCCAACAT-3′, (reversed primer): 5′-TTGATCTCGTCG-GTCAGTCC-3′ (predicted product size = 414 bp);

(e) Cytokeratin 18 cDNA: (forward primer): 5′-ACTCGCTCCAC CACCTTCTTCACCAACTAC-3′, (reversed primer): 3′-CCACA-GAATTCGCAAGGATC-3′ (predicted product size = 373 bp);

(f) Vimentin cDNA: (forward primer): 5′-CTTCGAAGCCAT-GTCCACCAGC-3′, (reversed primer): 5′-GATCCACCTGCCG-GCGCAA-3′ (predicted product size = 500 bp).

The identity of the PCR reaction products was confirmed by sequencing. Sequence analysis of the PCR products revealed a 96% sequence identity for the KGF cDNA product with the mouse KGF gene (Mason et al., 1994) and 97% for the KGFR cDNA product with the mouse KGFR gene (Miki et al., 1992). DNA contamination was excluded by performing PCR on each sample without first transcribing mRNA with reverse transcriptase.

Microdissection and organ culture

Pregnant dams were killed by diethylether excess on days 12-14 of pregnancy. Embryos were aseptically dissected from uterine decidua and staged by external features. Lung rudiments were microsurgically dissected from the embryos under a dissection microscope. If required, epithelial and mesenchymal components were separated with microscalpels and gentle trituration (Wang et al., 1994). For explant cultures, 12-day embryonic lung rudiments were placed on 0.4 μm porous culture inserts and incubated for 48 hours at 37°C as

a submersion culture in serum-free medium as previously described (Souza et al., 1994, 1995). The explants were incubated in the presence or absence of oligonucleotides. Each culture insert contained 4 lung explants.

Isolation and culture of embryonic epithelial cells

Pregnant dams were sacrified by diethylether excess on day 13 of pregnancy. Embryos were aseptically removed from uterine decidua and lung rudiments were microsurgically dissected from the embryos under a dissection microscope. The rudiments were incubated at 37°C with 10 caseinolytic units of dispase in HBSS. After 1 hour of incubation, fetal bovine serum was added to inactivate proteolytic enzyme activity. Epithelial and mesenchymal components were then separated with microscalpels and gentle trituration (Wang et al., 1994). Mesenchymal cells were grown to confluence in Ham's F12/DMEM plus 10% (v:v) FBS. Epithelial organoids (4 epithelial fragments/well) were seeded in separate wells of a 48-well culture plate containing Ham's F12/DMEM plus 10 % (v:v) FBS and left to grow for 48 hours.

Conditioned medium was obtained from confluent cultures of 13-day fetal lung mesenchymal cells. The cells were rinsed once with serum-free MEM and then incubated for 12 hours in serum-free MEM. At the end of the incubation, the medium was collected and centrifuged at 420 **g** for 5 minutes to remove any cell debris. The conditioned medium was then split in aliquots for storage at −20°C until mitogenic activities were measured.

DNA synthesis

Twelve-day embryonic lung explants, cultured for 48 hours with and without antisense and sense ONs, were incubated in the presence of 5 μCi of [^{3}H]thymidine/ml of medium. After a 5-hour incubation, explants were washed 3 times with PBS, homogenized and DNA extracted as described by Greenstein et al. (1984). Protein content was determined according to Bradford (1976). Thymidine incorporation into DNA was calculated as dpm/μg protein and expressed as a percentage of control.

Thirteen-day embryonic lung epithelial cells, cultured for 48 hours in Ham's F12/DMEM + 10 % (v:v) FBS, were incubated for 6 hours in serum-free MEM prior to a 24-hour incubation with either KGF, aFGF or mesenchymal conditioned medium in the presence of 2-10 μCi/ml [^{3}H]thymidine. At the end of the incubation period the media were aspirated and the cells were rinsed twice with ice-cold PBS. The amount of radioactive thymidine incorporated into DNA was then measured as described previously (Caniggia et al., 1991). Thymidine incorporation into DNA was calculated as dpm/well.

Quantification of branching morphogenesis

To assess branching morphogenesis, 12-day lung explants were monitored daily by phase contrast microscopy. Branching morphogenesis was quantitatively assessed by counting the number of terminal buds. Lung explants after 48 hours in culture, were randomly photographed and each image was printed on photographic paper at a final magnification of ×693.

Cytotoxicity

Cellular integrity was assessed by measuring lactate dehydrogenase (LDH) release into the medium. LDH activity was determined according to Fanestil and Barrows (1965). None of the oligonucleotides used in the present study increased the LDH release compared to control explants cultured in medium alone.

Immunohistochemistry

At days 12-15 of gestation, whole embryos were removed from the mothers and immersion fixed in 4% (w/v) paraformaldehyde in phosphate-buffered saline (PBS) overnight and washed in PBS, pH 7.4, for 24 hours before processing. The embryos were then embedded in paraplast (Oxford labware, St. Louis, MO), 5 μm sections were cut and mounted on α-aminopropyltriethoxysilane-coated slides. The avidin-biotin immunoperoxidase method was used to study the immunolocalization of KGF and KGFR/bek in embryonic rat lungs. Tissue sections, embedded in paraffin, were dewaxed in 100% ethanol and rehydrated in a graded series of 100% ethanol dilutions. The sections were transferred to a plastic container containing 10 mM sodium citrate pH 6.0 and antigens were retrieved by boiling the sections twice in a microwave (med-high for 6 minutes). Tissue sections were then transferred to PBS. Endogenous peroxidase enzyme activity was quenched with 1% (v:v) hydrogen peroxide in methanol. Non-specific binding sites were blocked by using 5% (v:v) normal goat serum (NGS) and 1% (w:v) bovine serum albumin (BSA) in PBS. The excess blocking solution was carefully removed and tissue sections were incubated overnight at 4°C with either a 1:1 dilution of mouse monoclonal anti-KGF or a 1:150 dilution of rabbit anti-KGF/bek. The tissue sections were washed three times in PBS, then incubated with a 1:300 dilution of biotinylated secondary sheep anti-mouse or goat anti-rabbit IgG. After washing with PBS, tissue sections were incubated with an avidin-biotin complex for 2 hours. The tissue were washed again in PBS and developed in 0.075%(w:v) 3,3 diaminobenzidine in Tris-HCl buffer, pH 7.6, containing 0.002% (v:v) hydrogen peroxide. All other procedures were conducted at room temperature. After lightly counterstaining with Carazzi's haematoxylin, the sections were dehydrated in an ascending ethanol series then in xylene, and mounted. In control experiments, antisera were replaced with blocking solution (5% NGS and 1% BSA). No immunostaining was observed under these conditions. Immunoabsorption studies were also conducted by incubating the primary antibodies with either excess recombinant KGF or KGFR peptide for 4 hours prior to immunostaining procedures. Negative immunoreactivity was also observed with these studies.

Western blot immunoanalysis

To analyze KGF and KGFR protein content, 12-day embryonic lung explants, cultured for 2 days in the presence of scrambled or antisense ODNs, were homogenized in ice-cold PBS (phosphate-buffered saline, pH 7.4) containing 1% (w/v) Triton X-100 and 1 mM phenylmethylsulfonyl fluoride (PMSF). The homogenates were centrifuged for 10 minutes at 10,000 **g**. Protein content was estimated according to Bradford (1976). Aliquots of lung homogenate, containing 25 μg of protein, were diluted with sample buffers and subjected to 5 and 15% (w/v) SDS-PAGE for KGFR and KGF, respectively. After electrophoresis, proteins were transferred to a nitrocellulose membrane. Nonspecific binding was blocked by incubation with 3% (w/v) nonfat milk powder in PBS at 4°C for 60 minutes. The membrane was then incubated with designated primary antibodies (2 μg/ml goat anti-KGF or 1 μg/ml rabbit anti-bek/KGFR). After overnight incubation at 4°C, the membrane was washed three times with PBS, followed by incubation with horseradish peroxidase-conjugated rabbit anti-goat IgG (1:20,000 in PBS containing 1% (w/v) BSA and 5% (v/v) normal rabbit serum) or goat anti-rabbit IgG (1:30,000 in PBS containing 1% (w/v) BSA plus 5% (v/v) normal goat serum). After washes with PBS, blots were developed with an enhanced chemiluminescence detection kit. The films were quantified with the use of an Ultrascan XL Laser densitometer.

Data presentation

All data are presented as means ± s.e.m. Statistical significance was determined by one way analysis of variance followed by assessment of differences using Student-Newman-Keuls test. Significance was defined as $P<0.05$.

RESULTS

Expression of KGF and KGFR in embryonic rat lung

KGF and KGFR gene expression was followed during the embryonic period of rat lung development. Expression of KGF
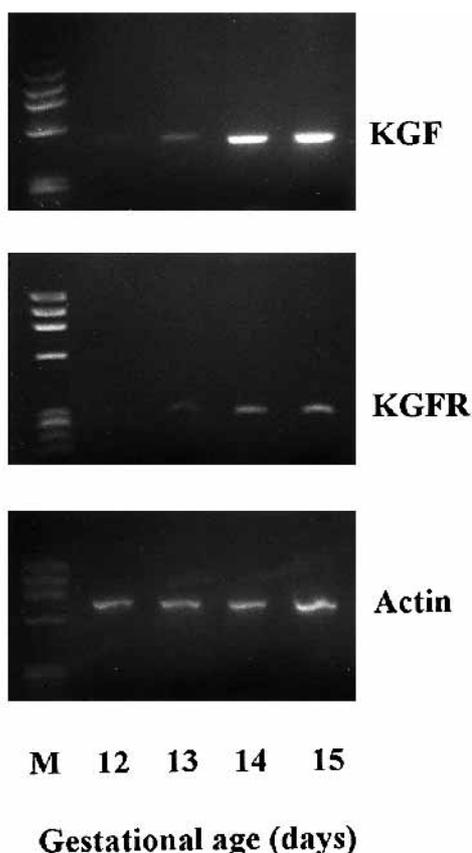
Fig. 1. Expression of KGF and KGFR mRNA in embryonic rat lung. RT-PCR analysis was carried out on total RNA (2 μg) from embryonic rat lung of 12-15 days of gestation. An ethidium bromide-stained agarose gel showing KGF (509 bp) and KGFR (312 bp) products in embryonic rat lung after 30 cycles of amplification. β-Actin (764 bp) amplification (20 cycles) is shown as a control. The number of KGF and KGFR transcripts in embryonic rat lung increased with advancing gestation. Similar results were obtained in a second experiment.

and KGFR was readily detectable by RT-PCR in rat lung as early as 12 day gestation (Fig. 1). Expression for both KGF and KGFR appeared to increase with advancing gestation, while that of β-actin remained constant (Fig. 1). In order to identify the source of KGF in the embryonic lung, mesenchymal and epithelial tissue components were separated as described in the Materials and Methods. The epithelial and mesenchymal purity of both separated tissue components were assessed by RT-PCR for cytokeratin (8 and 18) and vimentin, respectively. Messenger RNA integrity was also demonstrated by β-actin RT-PCR. The results show that KGF is expressed in the lung mesenchyme but not in the epithelium (Fig. 2). These data are in agreement with the existing body of evidence that KGF

mRNA expression is restricted to mesenchymal cells (Rubin et al.,1995). KGFR mRNA expression was detected in the airway epithelium, consistent with in situ hybridization studies (Orr-Urtreger et al., 1993). At 12-13 days gestation, KGF and KGFR/bek were predominantly immunolocalized to lung epithelium (Fig. 3). This localization pattern was similar for 14- and 15-day embryonic rat lung (not shown). A possible explanation for the discrepancy between KGF immunolocalization and mRNA expression may be that KGF secreted by mesenchymal cells is bound and internalized by epithelial cells. Alternatively, secreted KGF may be bound to the epithelial cell surface by heparan sulfate containing proteoglycans (Ruoslahti and Yamaguchi, 1991). Taken together, the data demonstrate that the embryonic rat lung express KGF and KGFR in adjacent but separate tissue layers at the initiation of lobar and segmental bronchial branching (day 12-13 in the rat, term = 22 days).

Mitogenic effects of KGF on embryonic rat lung

First, we evaluated whether antisense KGF oligonucleotide (ON) treatment affected KGF protein synthesis. Addition of antisense KGF ON, at a concentration of 10 μM, decreased the KGF protein content in the explants when compared to control explants (Fig. 4). We then assessed the effect of antisense KGF ON on lung cell proliferation by measuring DNA synthesis (Fig. 5). Antisense KGF ON (10 μM) significantly decreased the incorporation of [^{3}H]thymidine into DNA of embryonic rat lung explants. Control explants, cultured in the presence of an equivalent concentration of sense KGF ON, exhibited no such effect. The inhibitory effect of antisense KGF ON on DNA synthesis was partially overcome by the addition of exogenous rKGF, at a concentration of 10 ng/ml. Exogenous KGF (0-20 ng/ml) alone did not affect DNA synthesis (not shown), suggesting that sufficient KGF for a maximal response was already present in the explant culture system. Once separated from their mesenchymal component, however, embryonic lung epithelial cells responded mitogenically to rKGF in a concentration-dependent manner (Fig. 6A), consistent with these cells being target cells for endogenous KGF. Acidic FGF, which binds with high affinity to KGFR (Miki et al., 1992; Yayon et al., 1992), was also mitogenic for embryonic epithelial cells (Fig. 6B). Moreover, mesenchyme-conditioned medium stim-
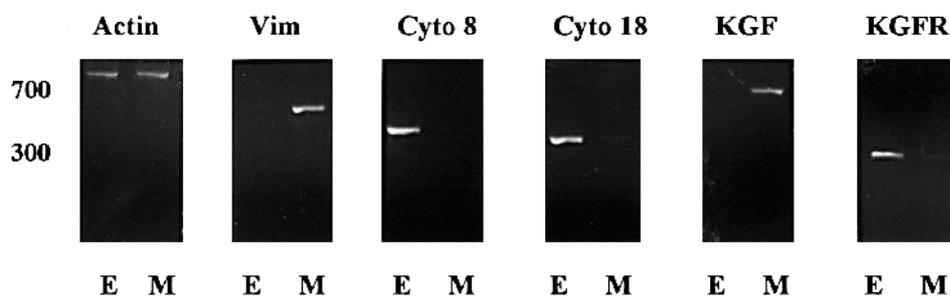

Fig. 2. Expression of KGF and KGFR mRNA in different tissue layers in embryonic rat lung at 13 days of gestation. RT-PCR analysis was carried out on total RNA (2 μg) from either embryonic rat lung epithelium (E) or mesenchyme (M). Epithelial and mesenchymal tissue purity was confirmed by RT-PCR for cytokeratin [Cyto 8 (25 cycles) and Cyto 18 (25 cycles)] and vimentin [Vim (20 cycles)], respectively. An ethidium bromide-stained agarose gel showing amplified KGF (509 bp) and KGFR (312 bp) PCR products in mesenchyme and epithelium, respectively, after 30 cycles of amplification. Similar results were obtained in a second experiment.

Fig. 3. Immunolocalization of KGF and KGFR/bek in rat embryonic lung. (A,C) KGF and (B,D) KGFR/bek. Strong positive immunoreactivity to KGF is present in airway epithelial cells of 12-day (A) and 13-day (C) embryonic lung but is weak in the surrounding mesenchyme. The airway epithelial lining of the 12-day (B) and 13-day (D) lung buds also show positive immunoreactivity to KGFR/bek but the surrounding mesenchyme is negative. Scale bar: 10 μm.
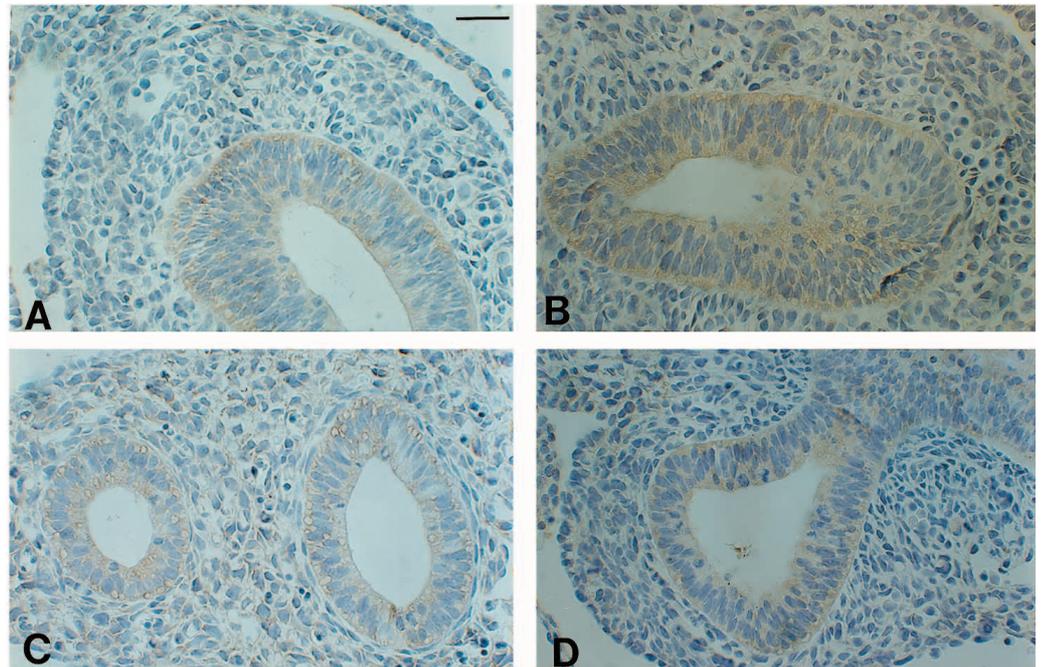
ulated DNA synthesis of 13-day fetal lung epithelial cells (Fig. 6B). This stimulatory effect was partially abrogated by neutralizing KGF antibodies, consistent with mesenchymal cells elaborating KGF.

Morphogenic effects of KGF on embryonic rat lung

In order to examine the morphogenic effects of KGF on early lung branching, 12-day embryonic lung explants were incubated in a serum-free system in the absence or presence of antisense KGF ON. In previous studies, we have demonstrated that the embryonic rat lung is a self-directed entity, because the lung rudiment, when cultured under serum-free conditions,

will continue to branch, albeit to a lesser degree than in vivo (Souza et al., 1995). Twelve-day lung explants cultured for 48 hours in the presence of antisense KGF ON showed significantly less branching than control explants cultured in medium alone (Fig. 7, Table 1). Neither sense- nor scrambled ON-treated lung rudiments affected branching morphogenesis (Table 1). Antisense KGF ON inhibited lung branching in a concentration-dependent manner with maximal inhibition at 10 μM (Fig. 8). Removal of antisense ON from medium after 24 hours restored the degree of branching to that of sense ON-treated explants or medium alone (not shown). Exogenous rKGF (0-20 ng/ml) alone had no effect on lung branching. Addition of rKGF, at a concentration of 10 ng/ml, to the antisense KGF ON-treated explants partially reversed the inhibitory effect on lung branching (Table 1). A neutralizing
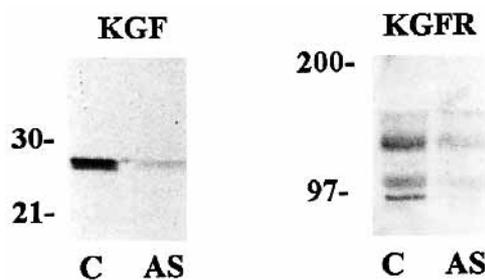

Fig. 4. Antisense inhibition of KGF and KGFR translation. Twelve-day embryonic rat lung rudiments were cultured for 48 hours in the presence or absence of either 10 μM antisense KGF or KGFR ON and then lysed. Aliquots of lung lysates, normalized for protein content, were electrophoresed and immunoblotted with antibodies to KGF and KGFR/bek. The polyclonal antibody against KGF recognized a 28×10^3 M_r protein, consistent with previous reported molecular mass for KGF (Rubin et al., 1989). Two distinct proteins with approximate molecular masses of 130×10^3 and 94×10^3 were detected with the polyclonal antibody to KGFR/bek, which is in agreement with the reported molecular mass of the mature and immature the KGFR, respectively (Dell and Williams, 1992; Miki et al., 1992). Molecular mass marker positions ($\times 10^{-3}$) are displayed on the right. C, control explants; AS, antisense treated explants.

Table 1. Inhibition of KGF blocks early lung branching.

Treatment	n	Culture time (hours)	Number of terminal buds
12-day fresh lung	3	–	2
Control	6	48	7.23±0.17
Antisense KGF	5	48	3.15±0.19*
Sense KGF	3	48	7.60±0.34
Scrambled KGF	3	48	6.93±0.32
Antisense KGF + KGF	3	48	5.83±0.31
KGF	3	48	7.83±0.48
Anti-KGF-IgG	3	48	3.17±0.15*
Anti-aFGF-IgG	3	48	6.85±0.21
Nonimmune IgG	3	48	7.00±0.10

  Day-12 embryonic rat lung rudiments were maintained for 48 hours in the presence of 10 μM antisense KGF ON, 10 μM sense KGF ON, 10 μM scrambled KGF ON, 10 ng/ml KGF, 10 μg/ml KGF or aFGF neutralizing antibody, 10 μg/ml nonimmune IgG or antisense ON with and without 10 ng/ml KGF. Branching morphogenesis was quantitatively assessed by counting the number of terminal buds. Values are mean ± s.e.m, n = separate experiments with 4 lungs per group. *P<0.05 compared to control.
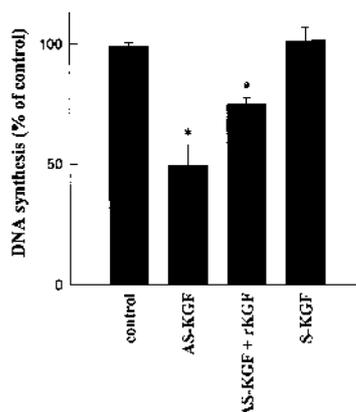
Fig. 5. Antisense KGF ON inhibition of embryonic lung growth. Antisense KGF ON treatment reduced DNA synthesis. The inhibitory effect of antisense KGF ON (AS-KGF) was partially overridden by the addition of exogenous rKGF. Sense KGF ON (S-KGF) treatment did not affect DNA synthesis, determined as a percentage of the control (3325±312 dpm of [^{3}H]thymidine incorporated into DNA per mg of total protein), n=3 separate experiments. *P<0.05 compared with control.

KGF antibody had a similar inhibitory effect on early lung branching as antisense KGF ON (Table 1). The inhibition was specific as incubation of explants with an equivalent amount of non-immune IgG or medium alone had no effect. Neutralizing antibodies to aFGF did not affect early lung branching morphogenesis (Table 1). Also, antisense KGFR/bek ON inhibited lung branching similar to that of antisense KGF ON-treated explants (Fig. 7, Table 2). Control explants, cultured with sense KGFR/bek ON, displayed no such effect. As the antisense KGFR/bek ON will not discriminate between KGFR and bek, we also incubated the explants with antisense ONs targeted to either the KGFR-specific or bek-specific exons (Miki et al., 1992; Orr-Urtreger et al., 1993; Dell and Williams, 1992). KGFR-specific antisense ON inhibited lung branching to the same degree as antisense KFGR/bek ON. Indeed, antisense KGFR ON significantly decreased the KGFR protein content in the explants (Fig. 4). Treatment of explants with an antisense ON to the bek-specific exon resulted also in reduced

Table 2. Inhibition of KGFR blocks early lung branching.

Treatment	n	Culture time (hours)	Number of terminal buds
12-day fresh lung	3	–	2
Control	4	48	7.29±0.29
Antisense KGFR/bek	7	48	3.59±0.14*,†
Sense KGF/bek	3	48	7.05±0.12
Antisense bek	4	48	5.00±0.24*
Sense bek	4	48	6.89±0.22
Antisense KGFR	5	48	3.65±0.02*,†
Sense KGFR	4	48	6.95±0.28
Scrambled KGFR	3	48	6.77±0.26

Day-12 embryonic rat lung rudiments were maintained for 48 hours in the presence or absence of 10 μM antisense, sense or scrambled ON. Branching morphogenesis was quantitatively assessed by counting the number of terminal buds. Values are mean ± s.e.m, n = separated experiments with 4 lungs per group. *P<0.05 compared to control. †P<0.05 compared to antisense bek.
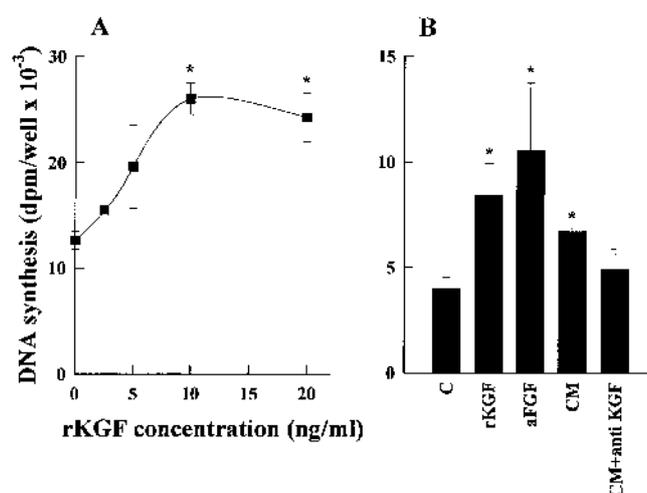

Fig. 6. Effect of KGF on DNA synthesis of day-13 embryonic rat lung epithelial cells. (A) A dose-dependent effect of rKGF on [^{3}H]thymidine incorporation into DNA. The maximal stimulation of DNA synthesis was observed with a concentration of 10 ng/ml. (B) The effect of rKGF (10 ng/ml), aFGF (10 ng/ml) and medium conditioned by day-13 mesenchymal cells on [^{3}H]thymidine incorporation into DNA. Both rKGF and aFGF equally stimulated DNA synthesis. Mesenchymal conditioned medium (CM) was also mitogenic for day-13 embryonic cells. This mitogenic effect of CM was partially abolished (approx. 50%) by a neutralizing KGF antibody (10 μg/ml). All data are expressed as mean ± s.e.m, n=3 separate experiments. *P<0.05 compared with control.

lung branching but to a lesser degree than that observed with antisense KGFR/bek ON and KGFR-specific ON (Table 2). Sense and scrambled ONs had no effect on lung branching morphogenesis.

DISCUSSION

In the present study, we demonstrate that treatment of embryonic rat lung explants from 12-day gestation with antibodies and antisense oligonucleotides to KGF and its receptor, KGFR, inhibited early lung organogenesis. This was manifested by reduced DNA synthesis and a lesser degree of branching compared to explants incubated with medium alone and nonimmune IgG or sense and scrambled oligonucleotides. Similar observations have been reported for PDGF-A and its α-receptor (Souza et al., 1995), in which PDGF-AA was identified as a soluble epithelium-derived mediator of mesenchymal-epithelial tissue interactions during early lung organogenesis. The appositional expression of KGF and KGFR during early lung development is in accord with previous studies (Mason et al., 1994; Orr-Urtreger et al., 1993; Finch et al., 1995) and implies a mesenchymal-to-epithelial direction of KGF action. Such a direction of KGF action in lung is supported by our observation that rKGF stimulated 13-day fetal lung epithelial cell growth in vitro. Further, the stimulatory effect of medium conditioned by embryonic mesenchymal cells on DNA synthesis by embryonic lung epithelial cells was partially abolished by a neutralizing KGF antibody. This is consistent with previous reports that 20-day fetal lung fibrob-
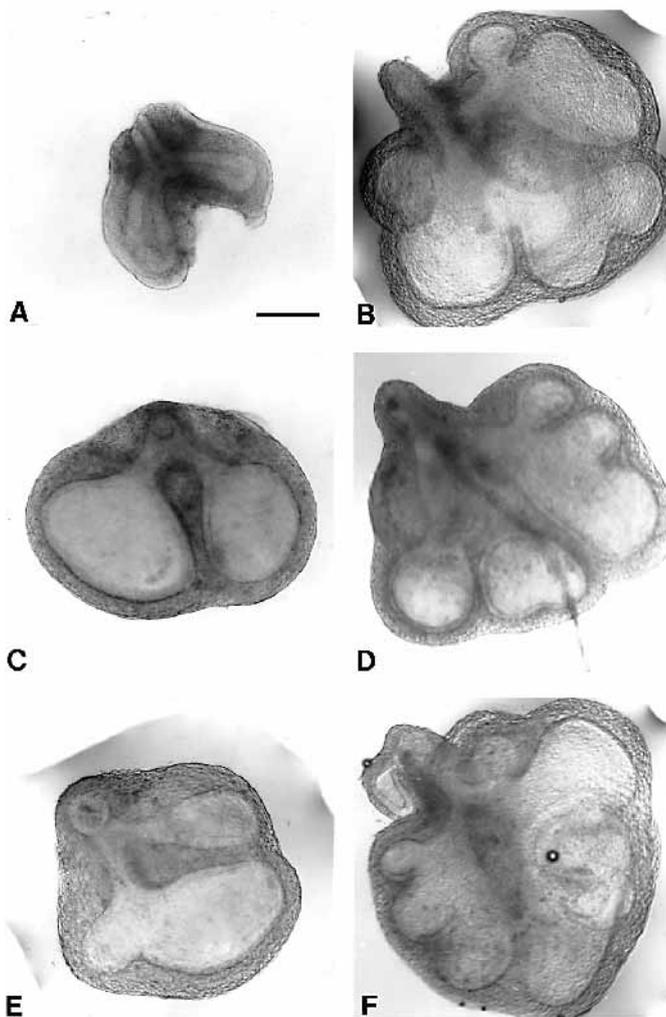
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Fig. 7. Efect of antisense KGF and KGFR ON on embryonic lung morphology. Day-12 embryonic rat lung rudiments were maintained for 48 hours in the presence of either antisense or scrambled ON. (A) Lung rudiment immediately after dissection. The trachea and main bronchi are present. (B) Lung explant after culture in serum-free medium. Increased branching is present compared with freshly isolated lung rudiment. Branching is significantly reduced in (C) antisense KGF and (E) KGFR ON treated lung explants when compared to control explants. (D) Scrambled KGF and (F) KGFR ON treated lung explants are not different from control explant. Each picture is representative of 3-7 separate experiments with 4 lungs per group. Scale bar, 100 μm.

recent study has shown that aFGF, which binds to KGFR and bek, is crucial for branching of embryonic mouse epithelium in mesenchyme-free culture (Nogawa and Ito, 1995). Basic FGF, which binds to bek, but not to KGFR (Miki et al., 1992; Yayon et al., 1992), did not affect epithelial branching in these cultures (Nogawa and Ito, 1995), suggesting that the effect of aFGF on epithelial branching is mediated via the KGFR. Our antisense KGFR and bek results are compatible with KGFR being more important in regulating epithelial branching mor-phogenesis than bek. KGFR has been shown to be the pre-dominant FGFR2 splice variant expressed in embryonic mouse lung (Orr-Urtreger et al., 1993). Moreover, transgenic mice with targeted expression of a dominant negative KGFR to the lung epithelium exhibited reduced branching morphogenesis (Peters et al., 1994). The studies with KGFR mutant mice and antisense KGFR oligonucleotides do not differentiate between either ligand, aFGF or KGF, regarding their importance in the process of early lung branching. Acidic FGF has been immunolocalized to the extracellular matrix of lung mes-enchyme, while the epithelium of embryonic rat lung was negative (Fu et al., 1991), suggesting that, like KGF, it is produced by the mesenchyme. Although we observed that aFGF was mitogenic for isolated embryonic epithelial cells, the almost complete blockage of branching with antisense oligonu-cleotides and antibodies to KGF but not with antibodies to aFGF, suggests that aFGF is either not expressed in sufficient amounts to overcome the KGF loss or it cannot biologically substitute for KGF in the explant system.

Several studies have reported antisense approaches to inhibit specific translation products during tooth (Kronmiller et al., 1992; Diekwisch et al., 1993), heart (Runyan, 1991), kidney (Sariola et al., 1991) and muscle (Florini et al., 1991) devel-opment. Using an antisense approach, we have dissected out the role of PDGF-A (Souza et al., 1995) and PDGF-B (Souza et al., 1994) in early lung development. In these previous studies, we reported that antisense oligonucleotides, targeted
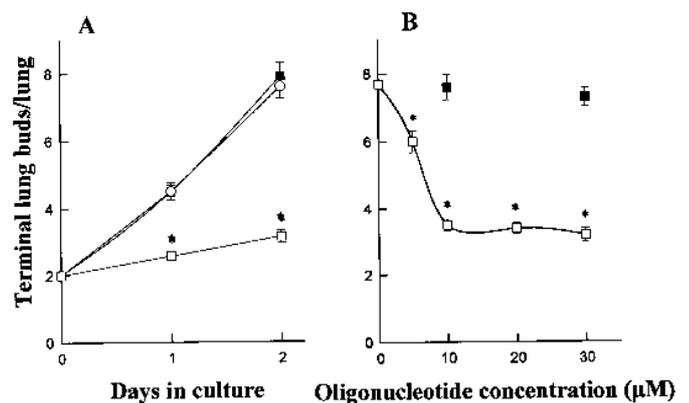
lasts secreted KGF into their medium (Panos et al., 1993), which stimulates adult type II pneumocyte proliferation in vitro (Panos et al., 1993) and in vivo (Ulich et al., 1994; Panos et al., 1995). The mesenchymal-epithelial distribution of KGF and KGFR expression during development has been docu-mented for other branched organs, such as salivary gland, kidney and mammary gland (Rubin et al., 1995). In addition, apositional expression of KGF and KGFR has also been reported for organs within the gastrointestinal and urogential systems, whose development is dependent upon mesenchymal-epithelial interactions (Finch et al., 1995).

To our knowledge, no previous studies have reported that KGF plays a critical role in embryonic lung development. A

Fig. 8. Effect of antisense KGF ON on lung branching morphogenesis. (A) Time-dependent effect of antisense KGF ON (10 μM) on early lung branching. Branching inhibition by antisense ON was already noticeble after a 24-hour culture. (B) Concentration-dependent effect of antisense ON on early lung branching. Branching inhibition was optimal when antisense ON was added at a concentration of 10 μM or higher. All data are expressed as mean ± s.e.m. of 3 separate experiments. *$P<0.05$ compared with control. (○) control explants; (□) antisense ON treated explants; (■) sense ON treated explants.

to sequences adjacent to the initiation site of translation, inhibited protein synthesis in an efficient and specific manner. Herein, we show that antisense treatment decreased KGF and KGFR protein synthesis. The specificity of the antisense approach was suggested by the absence of any effects in sense- and scrambled ON-treated explants and the partial reversal of inhibitory effects of antisense KGF on DNA synthesis and branching by exogenous rKGF. In addition, neutralizing KGF antibodies exhibited similar inhibitory effects on branching as antisense KGF oligonucleotides. The incomplete recovery with exogenous rKGF may be due to KGF-matrix interactions which may affect the penetration of exogenous rKGF into the explants (Ruoslahti and Yamaguchi, 1991).

The present and previous studies (Souza et al., 1995) identified KGF and PDGF-AA as soluble mediators of mesenchymal-epithelial tissue interactions during early lung branching. PDGF-AA is an epithelial-to-mesenchyme mediator (Souza et al., 1995), whereas KGF is a mesenchymally derived factor acting on lung epithelium. The mechanism whereby PDGF-AA affects epithelial cell growth and branching remains to be elucidated. Recent studies have demonstrated that KGF expression in lung and dermal fibroblasts can be modulated by a number of growth factors and cytokines, including interleukin 1(IL-1), interleukin 6, transforming growth factor alpha and PDGF (Chedid et al., 1994; Brauchle et al., 1994). Another mitogen with motogenic and morphogenic activities is hepatocyte growth factor/scatter factor (HGF/SF). During mouse development, HGF/SF and its receptor, c-met, are expressed in different but adjacent tissues, suggesting that they transduce mesenchymal-epithelial signaling (Sonnenberg et al., 1993; Rosen et al., 1994). HGF/SF gene expression has also been shown to be regulated by growth factors, such as PDGF (Gohda et al., 1994). Coculture experiments of human keratinocytes and dermal fibroblasts have suggested that the keratinocytes release factors such as IL-1, that induce KGF gene expression in fibroblasts, which consequently stimulates keratinocyte growth (Smola et al., 1993). It is plausible that an analogous mechanism involving PDGF-AA and KGF may be operative during embryonic lung development.

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