KGF regulates pulmonary epithelial proliferation and surfactant protein gene expression in adult rat lung

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Yano, Toshiyuki, Robert J. Mason, Tianli Pan, Robin R. Deterding, Larry D. Nielsen, and John M. Shannon. KGF regulates pulmonary epithelial proliferation and surfactant protein gene expression in adult rat lung. Am J Physiol Lung Cell Mol Physiol 279: L1146–L1158, 2000.—Keratinocyte growth factor (KGF, FGF-7) is a potent mitogen for epithelial cells. We instilled recombinant human KGF to determine the effects of KGF on alveolar epithelial cells. Left lungs of adult rats were instilled intrabronchially with KGF (5 mg/kg) or normal saline. KGF instillation resulted in epithelial cell hyperplasia, and the alveolar bronchoepithelium (BrDU) labeling index peaked at 35% on day 2 after instillation. The mRNA levels for the surfactant proteins (SPs) SP-A, SP-B, and SP-D were increased in whole lung tissue on days 1 and 2 after KGF treatment and then returned to control levels on days 3–7. SP-C mRNA levels were increased on days 2–5 after KGF instillation. However, all surfactant protein mRNAs were reduced in type II cells isolated from rats instilled with KGF 2 or 3 days before isolation. These observations were confirmed by in situ hybridization. Instillation of KGF also increased the amount of SP-A and SP-D in lavage fluid. Transcripts for CC10, the 10-kDa Clara cell protein, were decreased. KGF increases the mRNA for the surfactant proteins per lung because of type II cell hyperplasia, but the mRNA per cell is slightly diminished as measured in isolated cells or estimated by in situ hybridization.

IN THE NORMAL LUNG, the alveolar epithelium comprises two principal cell types. Type I cells are flat, squamous cells devoid of secretory organelles and cover >90% of alveolar surface. Type II cells have a cuboidal shape, are located in the alveolar corners, and occupy <10% of alveolar surface area. Type II cells synthesize, store, and secrete pulmonary surfactant, which reduces surface tension and stabilizes alveolar units for efficient gas exchange. Type II cells are also the progenitor cells for type I cells, which are very sensitive to damage by a variety of agents. When type I cells are damaged, type II cells divide, migrate, and spread along the denuded basement membrane surface, reform the epithelium, and then finally differentiate into type I cells.

Type II cells also transport sodium from the transepithelial surface to the interstitium to minimize alveolar fluid. In addition, they secrete a variety of cytokines that can modify the inflammatory response to injury. Although type II cells are essential for pulmonary epithelial homeostasis and repair of the epithelium after lung injury, regulation of their proliferation and differentiated function is not completely understood.

Mesenchymal cells are critical to development of the lung epithelium during organogenesis and may be very important to repair of the epithelium after lung injury. Several factors that are produced by mesenchymal cells could serve as signaling molecules to regulate epithelial growth and differentiation. Proliferation and differentiation of glandular stage fetal rat lung epithelium can be sustained in vitro in the absence of mesenchyme by replacement with a combination of soluble factors (10). One of the critical factors for these cultures is keratinocyte growth factor (KGF) also referred to as fibroblast growth factor-7 (FGF-7). KGF is a heparin-binding growth factor that was originally isolated from adult and fetal lung fibroblast-conditioned media (13, 30, 33). The biological activity of KGF is restricted to epithelial cells (1, 33). This unique target cell specificity is derived from its binding to the KGF receptor (KGFR), a splice variant of FGF receptor 2 (FGFR2), which is expressed only in epithelial cells (5). KGFR transcripts are found in fetal and adult rat lung tissues (27). In vitro studies demonstrate that KGF is a potent mitogen for type II cells (30). KGF has also been shown to be a mitogen for type II cells and bronchiolar epithelial cells in vivo (29). Two and three days after a single tracheal instillation of KGF (5 mg/kg), the adult rat lung shows prominent type II cell hyperplasia (39, 44). These data suggest that endogenous KGF is involved in regulating both proliferation and differentiation of type II cells and bronchiolar epithelial cells.

Pulmonary surface-active material is composed predominantly of phospholipid and associated proteins. Currently there are four known surfactant proteins (SPs), designated SP-A, SP-B, SP-C, and SP-D. The
proteins SP-A, SP-B, and SP-D are synthesized and secreted from both type II cells and bronchiolar epithelial cells, whereas SP-C is produced only by type II cells. After lung injury due to silica instillation, hypoxia, or lipopolysaccharide, there is an increase in the total number of type II cells and an increased expression of SP-A, SP-B, or SP-C mRNAs in whole lung. Moreover, type II cell hyperplasia is a hallmark of pulmonary fibrosis. SP-A and SP-D concentrations in serum of patients with interstitial pulmonary fibrosis are higher than those of normal patients. Since KGF increases SP-A and SP-B mRNA levels of type II cells in vitro (37) and induces a hyperplasia of type II cells containing abundant immunoreactive SP-B and SP-C when instilled into rats (39, 44), we hypothesized that KGF regulates the expression of surfactant proteins associated with pulmonary epithelial hyperplasia in vivo.

We sought to determine the relationship between the response of pulmonary epithelium and expression of the surfactant proteins and their mRNAs after KGF instillation. Thyroid transcription factor-1 (TTF-1) has been found to activate mammalian SP-A, SP-B, and SP-C gene transcription (41). Additionally, TTF-1 binding sites are present in the genes for SP-A, SP-B, SP-C, and the 10-kDa Clara cell protein (CC10) (43). We hypothesized that KGF might interact with TTF-1 in regulating the expression of the surfactant proteins in type II cells and CC10 in nonciliated bronchiolar epithelial cells. We report here that exogenous KGF administered in the lung induces type II cell hyperplasia and increases mRNA levels for SP-A, SP-B, SP-C, and SP-D in whole lung, as well as SP-A and SP-D protein content in lavage fluid. However, the transcripts for the surfactant proteins per alveolar type II cell is slightly reduced. CC10 mRNA levels are reduced when instilled into rats (39, 44), we hypothesized that KGF treated group received normal saline (0.5 ml) as the vehicle for KGF. The KGF-treated group was given KGF (5 mg/kg in 0.5 ml of saline). The day of saline or KGF instillation was designated day 0. Animals were killed 1, 2, 3, 5, and 7 days after instillation. Only the left lung was harvested for analysis after intrabronchial administration. Type II cells isolated by centrifugal elutriation were obtained from both lungs, which had been instilled with KGF intratracheally to provide as many type II cells as possible.

Protein assays in bronchoalveolar lavage. SP-A, SP-D, and total protein were quantitated in lung lavage fluid. After tracheostomy, the thorax was opened and the right main stem bronchus was ligated. The left lung was lavaged five times with 4-ml aliquots of normal saline. A 2-ml aliquot of the pooled lavage fluid was immediately centrifuged at 300 g for 10 min at 4°C to remove the cellular constituents, and then the supernatant was gently aspirated and stored at −20°C for subsequent analyses.

SP-A and SP-D were measured by ELISA. Rat SP-A and SP-D, which were secreted from lavage fluid from rats 28 days after silica instillation, were used as a standard for the assay. Polyclonal anti-rat SP-A or anti-rat SP-D rabbit IgG (10 μg/ml in 0.1 M sodium bicarbonate) was bound to wells in microtiter plates (Immulon 1 plates; Dynatech Laboratories, Alexandria, VA) at room temperature overnight. The wells were then incubated with a 5%/vol) solution of nonfat dry milk in PBS (pH 7.2) to block nonspecific binding (blocking buffer). After a wash with the blocking buffer, 100 μl containing purified rat SP-A or SP-D (0–20 ng) for standards or appropriately diluted lavage samples were added to each well. Plates were incubated for 90 min at 37°C and then washed with 20% blocking buffer and 1% Triton X-100 (vol/vol) in PBS (antibody buffer). Two hundred microliters of anti-SP-A or anti-SP-D antibody-conjugated hors eradish peroxidase (2 and 30 μg/ml in antibody buffer for SP-A and SP-D, respectively) were added to the wells, and the plates were incubated for 90 min at 37°C. After further washing with 1% Triton X-100 in PBS, 200 μl of the color-developing agent (0.1% o-phenylenediamine and 0.015% hydrogen peroxide in 0.1 M citrate buffer, pH 4.6) were added. The reaction was carried out for 5 min at room temperature in a darkened room and was stopped by the addition of 100 μl of 1 M sulfuric acid. The absorbance at an optical density of 490 nm was recorded with a Microplate Autoreader EL-311s (BIO-TEK instruments, Winooski, VT). The total lavage protein concentration was determined colorimetrically using the BCA protein assay kit (Pierce Chemical, Rockford, IL) with BSA as the standard.

Isolation and elutriation of alveolar type II cells. Alveolar type II cells were isolated from both lungs of untreated rats and of rats 2 or 3 days after KGF instillation by elastase dissociation and purification on a discontinuous metrizamide density gradient (11). In one set of experiments, the type II cells were then further purified by centrifugal elutriation described previously (16). For elutriation, suspensions of isolated cells were loaded into the elutriator mixing chamber and pumped into the rotor separation chamber at a constant rotor speed of 2,000 rpm (Beckman model J2–21M centrifuge with a JE-6B elutriator rotor; Beckman, Palo Alto, CA). Cells were eluted by stepwise increases in flow rate at 9, 14, 18, 22, 28, and 34 ml/min. The cells in each fraction were centrifuged at 300 g for 10 min at 4°C, resuspended, and counted in a standard hemacytometer. The percentage of type II cells was determined from smears stained by the modified Papanicolaou stain. Cell viability was determined by exclusion of the vital dye erythrosin B.

MATERIALS AND METHODS

Recombinant human KGF. Recombinant human KGF (rhKGF) was produced in *Escherichia coli* and purified to homogeneity by conventional methods as previously described (39). Bioactivity of this KGF, which was free of endotoxin, was assayed in BAX/LM/KE keratinocytes.

Animals and instillation method. Pathogen-free Fischer 344 rats purchased from Harlan Sprague Dawley (Indianapolis, IN) and weighing 165–225 g were housed in a laminar airflow hood. Instillations were performed as previously described (44). Briefly, rats anesthetized with xylazine and ketamine were intubated with a 16-gauge intravascular Teflon catheter (Quick-Cath; Baxter, Deerfield, IL) in a supine position. Either KGF or saline was instilled intrabronchially into the left lung or intratracheally into both lungs (44). For intrabronchial administration, rats were placed in the left lateral position, and a small polyethylene tube was positioned and inserted through the tracheal catheter to the level of the left main stem bronchus.

Experimental design. There were three treatment groups of animals: 1) an untreated group of controls, 2) a vehicle-treated group, and 3) a KGF-treated group. The untreated controls were neither anesthetized nor instilled. The vehicle-treated group received normal saline (0.5 ml) as the vehicle for KGF. The KGF-treated group was given KGF (5 mg/kg in 0.5 ml of saline).
Isolation of RNA. Excised left lungs were immediately homogenized in 4 M guanidinium isothiocyanate, 0.5% laurylsarcosine, and 0.1 M β-mercaptoethanol in 25 mM sodium citrate buffer (GTC) with a Polytron tissue homogenizer (Brinkman Instruments, Westbury, NY) and stored at −70°C until used. Type II cells isolated by metrizamide density gradient centrifugation or elutriation were pelleted by cen-
trifugation at 500 g for 10 min, washed, and recentrifuged twice in PBS, and then lysed in GTC. Total cellular RNA from whole lung tissue or from isolated type II cells was isolated by ultracentrifugation through a 5.7 M CsCl cushion at 150,000 g for 18 h at 20°C.

Northern blot analysis. Northern blot analysis was performed as described previously (34). Briefly, total cellular RNA was electrophoresed through a 1% agarose gel under denaturing conditions and then blotted onto a nylon mem-
brane (Nytran; Schleicher & Schuell, Keene, NH) by capil-
lary action. The isolation and characterization of cDNAs for rat SP-A, SP-B, SP-C, and SP-D have been described previ-
ously (35, 37). The cDNAs for rat CC10 and rat TTF-1 were
previously described (34). Hybridized blots were washed once for 10 min at room temperature
with the same solution as the prehybridization buffer. The mem-
branes were prehybridized, hybridized, and washed, and autoradiographed on Kodak XAR5 film (East-
heights, IL). Membranes were prehybridized, hybridized,
and washed with 50% formamide and 6
3
ences). Previously hybridized nylon membranes were
isolated from elutriated type II cells was analyzed for surfac-
tant proteins using full-length cDNAs as tem-
plates. The forward primers included a BamHI I restriction site added to the 5′-end, and the backward primers included
an EcoR I restriction site added to the 5′-end to facilitate direction cloning into pGEM-4Z (Promega). The primers for SP-A were 5′-CGGATCCAGTCCTCAGCTTTGCAGAGC
TC-3′, coding sense and corresponding to nucleotides 424–
444, and 5′-GGAAATCCGTTCTCCTCGAGGGATCTCG-3′, coding antisense and corresponding to nucleotides 549–569. The probe transcribed from this clone identified a fragment of 146 bp. The primers for SP-B were 5′-CGGATCCAGGAGGTTTGTGGGACACGAC-3′, coding sense and corre-
sponding to nucleotides 997–1017, and 5′-GGAAATTCTG
GTCCTTTGTTGACAGGTTG-3′, coding antisense and corre-
sponding to nucleotides 1152–1172. The probe transcribed from this clone identified a fragment of 176 bp. The primers for SP-C were 5′-CGGATCCCCATCTAGAGATGTGCTCTTT
GAG-3′, coding sense and corresponding to nucleotides 202–
222, and 5′-GGAAATTCTTGGAGACCATCTTCTATGATG-3′, coding antisense and corresponding to nucleotides 381–401. The probe transcribed from this clone identified a fragment of 200 bp. The primers for SP-D were 5′-CGGATCCCGAGAA
GACCTTTGAGGAGT-3′, coding sense and corresponding
to nucleotides 831–851, and 5′-GGAAATTCCAGGTGC
GCCCCCTCATTTG-3′, coding antisense and corresponding to nucleotides 1054–1075. The probe transcribed from this clone identified a fragment of 245 bp.

The vectors were linearized with BamHI, and radiolabeled antisense probes were transcribed in vitro using a com-
ercially available kit (both from Amersham Life Sciences, Arlington Heights, IL). Membranes were prehybridized, hybridized, wash,
and autoradiographed on Kodak XAR5 film (East-
man Kodak, Rochester, NY) or on Hyperfilm (Amersham) as
previously described (34). Hybridized blots were placed on a phosphorus screen for a direct quantitation of radioactive counts using Molecular Dynamics ImageQuant software, ver-
sion 3.3 (Molecular Dynamics, Sunnyvale, CA).

A 28S ribosomal RNA (rRNA) oligonucleotide probe was used for normalizing results of Northern blot hybridiza-
tion (RPA) that allowed simultaneous measurement of
mRNAs for SP-A, SP-B, SP-C, and SP-D. Fragments of dif-
ferent sizes for the four surfactant proteins were isolated by polymerase chain reaction using full-length cDNAs as tem-
plates. The forward primers included a BamHI I restriction site added to the 5′-end, and the backward primers included
Life Sciences) at −70°C. Radioactive counts were obtained from protected fragments for SP-A, SP-B, SP-C, and SP-D by ImageQuant and normalized to 18S rRNA.

In situ hybridization and immunocytochemistry. Excised lungs were instilled with freshly prepared 4% paraformaldehyde and 2% low-melting-point agarose (GIBCO BRL) in RNase-free PBS (pH 7.4) (19). The instilled lungs were then immersed in 4% paraformaldehyde in PBS and fixed overnight at 4°C. The fixed lungs were cut into blocks, washed in PBS, and then stored in 70% ethanol until paraffin embedding.

In situ hybridization was performed as previously described (9). Tissue sections (4–6 µm) were mounted on Super Frost II glass slides (Fisher Scientific, St. Louis, MO) and hybridized with 32P-labeled sense or antisense RNA probes transcribed from full-length rat cDNAs for SP-A, SP-B, SP-C, SP-D, and CC10. After a series of high-stringency washes, the slides were dipped in Kodak NTB-2 nuclear track emulsion. Autoradiograms were exposed in light-tight boxes for 3–11 days at 4°C, developed, and then counterstained with hematoxylin.

The method for immunocytochemistry has been described previously (7, 42, 44). Deparaffinized 4-µm sections were rehydrated through graded ethanols and treated with methanol and hydrogen peroxide to remove any endogenous peroxidase. To enhance antigen detection, the sections were treated with guanidinium hydrochloride and then with trypsin (7, 44). The sections were subsequently incubated in PBS containing 3% goat serum (GIBCO BRL) for 20 min to block nonspecific binding sites and then overnight with a polyclonal rabbit IgG against rat SP-A, rat SP-D (gifts of Dennis Voelker, Denver, CO), or the propeptide of human SP-C (proSP-C, a gift from Jeffrey Whitsett, Cincinnati, OH) (40). Washing, secondary antibody treatment, color development, and counterstaining with hematoxylin were performed as previously described (7, 42, 44).

Labeling indexes. To determine the percentage of cells synthesizing in the S phase, animals were injected intraperitoneally with bromodeoxyuridine (BrdU; 200 mg/kg) 4 h before death. Left lungs were removed and fixed for immunocytochemistry, and BrdU incorporated into nuclei was detected by immunostaining using mouse monoclonal antibody M774 (DAKO, Carpinteria, CA). The labeling index of at least 1,000 epithelial cells was determined in randomly selected fields of sections through the hilum of the left lung. The sections were coded, randomized, and quantitated in a blinded manner. Measurements were made separately for alveolar walls and septa and for the bronchial epithelium.

Statistics. A one-way analysis of variance (ANOVA) was used to determine whether means of continuous outcomes...
differed by treatment. Fisher’s protected least significant difference multiple comparison procedure was then used to determine which pairs of means differed. When variances differed for the different treatment groups, Welch’s ANOVA was used followed by the Games-Howell multiple comparison procedure for unequal variances (38). Values are presented as the means ± SE. Statistical significance was defined as \( P < 0.05 \).

RESULTS

BrdU immunocytochemistry and labeling index. KGF has been reported to stimulate type II cell proliferation in vivo, and micrographs including immunocytochemistry have been published (7, 12, 29, 39, 44). In the uninstilled control lung, the labeling indexes for alveolar cells (0.7 ± 0.1%) and bronchial epithelial cells (0.3 ± 0.1) were low. With saline instillation, there was a transient increase in labeling at 1 day that returned to baseline values at 2 days. KGF produced a significant increase in labeling at 2 and 3 days in the alveolar compartment, which returned to baseline levels at 7 days (Fig. 1). The peak alveolar labeling index was 34.3 ± 3.6% at 2 days. Panos et al. (29) previously demonstrated by double-label immunocytochemistry that the cells incorporating BrdU are type II cells. In the airway epithelium, compared with the saline control, there was an increase in labeling after KGF administration at 2 days but not at 1 or 3 days. Saline instillation increased the labeling index in the bronchial epithelium on 1 day (data not shown).

Surfactant protein mRNA levels in whole lung. In the whole lung, KGF instillation increased surfactant protein mRNA levels for all the surfactant proteins. SP-A, SP-B, and SP-D mRNA levels increased on days 1 and 2 and then sharply decreased to the untreated control levels on day 3 (Fig. 2). Maximum levels of mRNA expression were attained on day 2 for SP-A and SP-B and on days 1 and 2 for SP-D. The expression of SP-C mRNA was increased in response to KGF instillation on day 2 (Fig. 2), but the extent of the increase was relatively less but more sustained than that seen for SP-A, SP-B, and SP-D mRNAs. There was an increase in mRNA for SP-C on days 2, 3, and 5. The lungs from saline-instilled animals had small, transient, but significant increases in SP-A and SP-D mRNA expression on day 1 after instillation compared with untreated controls. After KGF instillation, this increase in mRNA levels for the surfactant proteins in whole lung could be due to an increase in type II cells, alterations in expression per cell, or a combination of these possibilities.

Immunolocalization of SP-A and SP-D. To evaluate the protein expression within cells and to document that the proliferating cells were indeed alveolar type II cells, sections of lung were immunostained for SP-A, proSP-C, and SP-D. We have previously reported immunocytochemistry for proSP-C after instillation of KGF (7, 44). The hyperplastic alveolar epithelial cells were readily stained with antibodies to proSP-C. Immunostaining for SP-B was not done but has been reported previously (39). However, tissue localization is important for SP-A and SP-D, which are expressed in both alveolar and bronchiolar epithelial cells, and has not been reported previously. We evaluated sections on days 1, 2, 3, and 7. The peak response occurred at days 2 and 3, which appeared very similar, and by day 7, the lung had returned nearly to the normal state.
The hyperplastic epithelial cells were readily stained with antibodies to SP-A and SP-D (Fig. 3). To our surprise, the bronchiolar epithelium showed a slightly different response. In the bronchioles, Clara cells protrude into the lumen and are readily stained by immunocytochemistry for SP-A and SP-D. KGF produced a more uniform hyperplastic cuboidal epithelium, and the expression of SP-D appeared more widespread than that of SP-A. Nearly all the bronchiolar epithelial cells stained for SP-D, whereas only a few cells stained for SP-A.

In situ hybridization for surfactant protein mRNA. In situ hybridization provides a means of defining local expression and is complementary to the protein localization studies. In situ hybridization of the untreated lung demonstrated silver grains derived from SP-A, SP-B, and SP-D mRNAs over both cells along the alveolar septa and bronchiolar epithelium (Fig. 4), whereas silver grains for SP-C mRNA were confined to cells of the alveolar epithelium (Fig. 4, E and F) (21). Two days after rhKGF instillation, the number of cells expressing SP-A, SP-B, SP-C, and SP-D cRNA probes increased along the alveolar wall. However, in the bronchiolar epithelium, there was a decrease in the cells identified with the SP-A probe but an increase in cells labeled with the SP-B and SP-D probes. The number of silver grains for SP-C mRNA/cell seemed to be slightly decreased after rhKGF instillation (E and F). Saline instillation did not have any significant effect on all surfactant protein mRNAs at 2 or 3 days after instillation (data not shown). The results from animals instilled with KGF 3 days before were the same as seen here (data not shown).
batch of probes was used for the KGF-instilled and control sections, and the exposures were developed after the same length of time. The general impression was that there was a slight decrease in expression for SP-A, SP-B, and SP-C in the alveolar epithelial cells after instillation of KGF. SP-D was harder to evaluate because of a lower level of expression in alveolar epithelial cells in the normal lung. In bronchiolar cells on day 2, the density of silver grains for SP-A mRNAs per cell was clearly reduced in rhKGF-instilled lungs (Fig. 4) compared with saline-instilled lungs and uninstilled lung (Fig. 4, A and B), whereas the silver grains per cell for SP-D over the bronchiolar cells appeared to be increased (Fig. 4, G and H). Analysis for in situ hybridization of the surfactant proteins was also performed on day 3 after instillation, and the results were similar to those done on day 2 (data not shown).

Table 1. Effect of rhKGF on proteins in bronchoalveolar lavage fluid

<table>
<thead>
<tr>
<th>Treatment Group</th>
<th>Days After Instillation</th>
<th>( n )</th>
<th>Total Protein, ( \mu g/ml )</th>
<th>SP-A, ( \mu g/ml )</th>
<th>SP-A/Total Protein, %</th>
<th>SP-D, ( \mu g/ml )</th>
<th>SP-D/Total Protein, %</th>
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</thead>
<tbody>
<tr>
<td>Untreated</td>
<td>0</td>
<td>4</td>
<td>115 ± 6</td>
<td>0.49 ± 0.05</td>
<td>0.43 ± 0.06</td>
<td>0.13 ± 0.03</td>
<td>0.12 ± 0.03</td>
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<tr>
<td></td>
<td>1</td>
<td>3</td>
<td>215 ± 62</td>
<td>0.62 ± 0.02</td>
<td>0.33 ± 0.07</td>
<td>0.24 ± 0.07</td>
<td>0.11 ± 0.01</td>
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<tr>
<td></td>
<td>2</td>
<td>2</td>
<td>153 ± 58</td>
<td>0.34 ± 0.05</td>
<td>0.25 ± 0.06</td>
<td>0.30 ± 0.11</td>
<td>0.20 ± 0.01</td>
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<tr>
<td></td>
<td>3</td>
<td>3</td>
<td>84 ± 11</td>
<td>0.29 ± 0.03</td>
<td>0.35 ± 0.03</td>
<td>0.13 ± 0.01</td>
<td>0.15 ± 0.01</td>
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<tr>
<td></td>
<td>5</td>
<td>3</td>
<td>66 ± 11†</td>
<td>0.42 ± 0.02</td>
<td>0.72 ± 0.18</td>
<td>0.14 ± 0.01</td>
<td>0.22 ± 0.05</td>
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<td>7</td>
<td>3</td>
<td>64 ± 17</td>
<td>0.50 ± 0.12</td>
<td>0.80 ± 0.07*†</td>
<td>0.13 ± 0.01</td>
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<td>Saline</td>
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<td>4</td>
<td>173 ± 49</td>
<td>0.53 ± 0.08</td>
<td>0.36 ± 0.06</td>
<td>0.35 ± 0.04*</td>
<td>0.23 ± 0.04</td>
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<tr>
<td></td>
<td>2</td>
<td>4</td>
<td>133 ± 13</td>
<td>0.98 ± 0.13*:††</td>
<td>0.75 ± 0.10*:††</td>
<td>0.69 ± 0.06*:††</td>
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<td></td>
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<td>5</td>
<td>134 ± 21</td>
<td>3.11 ± 0.51*:††</td>
<td>2.60 ± 0.57*:††</td>
<td>0.39 ± 0.07*:††</td>
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<tr>
<td></td>
<td>5</td>
<td>5</td>
<td>145 ± 14††</td>
<td>0.51 ± 0.06</td>
<td>0.37 ± 0.07</td>
<td>0.19 ± 0.02</td>
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<tr>
<td></td>
<td>7</td>
<td>5</td>
<td>113 ± 16</td>
<td>0.40 ± 0.05</td>
<td>0.37 ± 0.39*††</td>
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<td>0.14 ± 0.02</td>
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<td>rhKGF</td>
<td>1</td>
<td>4</td>
<td>173 ± 49</td>
<td>0.53 ± 0.08</td>
<td>0.36 ± 0.06</td>
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<td></td>
<td>10</td>
<td>5</td>
<td>103 ± 13†</td>
<td>0.42 ± 0.05</td>
<td>0.67 ± 0.39*:††</td>
<td>0.21 ± 0.02</td>
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</table>

Values are means ± SE; \( n \), no. of animals. Instillation of saline and recombinant human keratinocyte growth factor (rhKGF) was intrabronchial into the left lung, and only the left lung was lavaged. Statistical analysis was applied among the untreated control and groups at the same time point after treatment. *P < 0.05 vs. untreated control. †P < 0.05 vs. saline-instilled group.
ical data were hard to evaluate in quantitative terms. In one set of experiments (Fig. 5), we isolated type II cells 2 days after the instillation of KGF or saline. In these experiments, there was no increase in the number of type II cells isolated (51.3 ± 2.2 × 10⁶ cells from rhKGF-treated rats, 41.1 ± 2.2 × 10⁶ cells from saline-instilled rats, and 38.5 ± 2.7 × 10⁶ cells from uninstilled rats) or alteration in their viability (rhKGF 92.3 ± 1.5%, saline control 94.4 ± 1.2%, and uninstilled control 93.3 ± 1.0%) or purity (rhKGF 86.9 ± 2.39%, type II cells, saline 84.2 ± 1.0%, and uninstilled controls 86.1 ± 1.5%). There was a relative decrease in the specific mRNA levels for all the surfactant proteins (Fig. 5). These results were confirmed by a second set of experiments in which type II cells were isolated by elutriations 3 days after instillation and the mRNA was quantitated by a dot blot analysis (data not shown). In this second set of experiments, there was a generalized decrease in the mRNAs for all the surfactant proteins relative to 28S ribosomal RNA to ~50% of the values for uninstilled controls, and there were no marked differences between normotrophic and hypertrophic type II cells as defined by elution at different flow rates (15, 31). Hence both studies with the isolated type II cells confirmed the observations made by in situ hybridization that the increase of total mRNA for the surfactant proteins in whole lung on days 2 or 3 was due to an increased number of type II cells and not due to increased expression per cell. The expression per cell was decreased.

SP-A and SP-D levels in lavage fluid. Although there was increased mRNA for SP-A and SP-D in whole lung, we next sought to determine the amount of SP-A and SP-D protein in alveolar fluid as estimated by lavage. SP-A concentration in lavage fluid increased slightly on day 2 and showed a sixfold increase at the peak on day 3 after rhKGF instillation when the values are compared with untreated and saline-instilled controls. On days 5 and 7, the levels of SP-A in the lavage fluid of rhKGF-treated animals returned to control levels. However, on day 7, there was a slight increase in those animals that received saline compared with those animals that received rhKGF and the uninstilled controls (Table 1). SP-D concentration in lavage fluid after rhKGF instillation increased on day 1 and reached a maximal increase of fourfold on day 2. On day 3, SP-D concentration decreased but remained at a higher value than untreated and saline-instilled controls. Saline instillation did not alter the SP-D concentration in lavage (Table 1).

Time course of CC10 mRNA levels. Expression of the Clara cell secretory protein CC10 was different from the surfactant proteins. Instillation of saline caused a slight but statistically significant decrease when compared with the untreated controls on days 2 and 3 and then gradually recovered to control values on days 5 and 7. KGF administration markedly reduced the expression of CC10 mRNA throughout the 7-day observation period. CC10 mRNA levels were ~35% of the untreated control levels on days 2 and 3 (Fig. 6).

The observations for CC10 mRNA in whole lung were confirmed by in situ hybridization (Fig. 7). In the untreated lung, silver grains for CC10 mRNA were indistinguishable from that in the lung from untreated control rats.
Signal over the bronchiolar cells appeared less after KGF instillation than in the controls. Two days after rhKGF instillation, number of silver grains for CC10 mRNA/cell decreased (B). The intensity of the signal over the bronchiolar cells appeared less after KGF instillation than in the controls. Saline-instilled lungs appeared the same as the uninstilled controls (data not shown). Two days after rhKGF instillation, number of silver grains for CC10 mRNA/cell decreased (B). The intensity of the signal over the bronchiolar cells appeared less after KGF instillation than in the controls.

Time course of TTF-1 mRNA in whole lung tissue. The levels of TTF-1 mRNA in the lung were not altered by saline instillation. In rhKGF-treated rats, TTF-1 mRNA increased on day 2, reached a maximum on day 3, and remained elevated on days 5 and 7 (Fig. 8). The time course for the increased expression of TTF-1 was delayed relative to the time for the increases in mRNAs for SP-A, SP-B, and SP-D and the decrease in expression in CC10. By in situ hybridization, there appears to be a slight increase in expression of TTF-1 in both bronchiolar and alveolar cells (Fig. 9).

DISCUSSION

KGF (FGF-7) is a well-known mitogen specific for epithelial cells (33). In terms of alveolar type II epithelial cells, Panos et al. (30) identified KGF as an important growth factor for type II cells in conditioned medium from human lung fibroblasts, and several studies have demonstrated rat alveolar type II cell proliferation in vivo after instillation of rhKGF (7, 12, 29, 39, 44). We found a time-dependent increase in BrdU incorporation in the alveolar cells. The magnitude of the response was similar to that reported by Panos et al. (29) and the time course was similar to that reported by Ulich et al. (39). KGF causes a transient wave of proliferation of alveolar type II cells followed by a return to normal at 7 days (12, 39). The change from focal type II cell hyperplasia to the normal alveolar epithelium is associated with apoptosis of the alveolar epithelial cells (12). Proliferation has been verified both by BrdU labeling and by expression of Ki67 (12). This study confirms the observation of type II cell proliferation in vivo and demonstrates that rhKGF increases the mRNAs for the surfactant proteins in whole lung and increases SP-A and SP-D protein in lavage fluid.

The increases in whole lung mRNA for the surfactant proteins were due to an increase in the number of cells expressing these proteins, but there was decreased expression per cell. This conclusion is based on both in situ data and direct measurement in isolated cells. By in situ hybridization, there appeared to be less specific mRNA. It could be argued that it is difficult to assess the level of expression by in situ hybridization. However, attention was given to using the same probes, the same length of time for exposure, and controls for each experiment. The results were reproducible and obvious to different observers. We quantified the mRNA relative to rRNA in isolated type II cells from control and KGF-instilled lungs. We evaluated on days 2 and 3 and isolated the cells both by the usual elastase digestion and metrizamide density centrifugation and by elutriation. In all these studies, there was a consistent decrease in mRNA for the surfactant proteins. These results indicate that the most likely reason for the increase in SP-A and SP-D protein in lavage fluid was due to an increase in the number of type II cells. We conclude that the type II cells stimulated by KGF in vivo have a slightly reduced differentiated function as determined by the mRNA levels of the surfactant proteins. The effects of KGF in vitro indicate that KGF can increase differentiation of alveolar type II cells. The results of the studies in vitro will depend on the exact culture conditions and when KGF is added. In general, KGF will increase differentiation compared with cultures without KGF in terms of expressing the surfactant proteins SP-A, SP-B, and SP-D and Na-K-ATPase (4, 37, 42). However, the cells have to be plated on a permissive substratum such as Engelbreth-Holm-Swarm mouse tumor extracellular matrix (Matrigel; Becton Dickinson, Franklin Lakes, NJ). There may be differences in the results if KGF is added throughout the culture or after the cells have dedifferentiated. KGF appears to increase SP-C if added throughout the culture time but decreases or has no effect if added late in the culture period (unpublished observations). KGF will also delay or prevent the spreading or flattening of type II cells in culture and their acquisition of markers associated with the type I cell phenotype such as aquaporin 5 and T1α (3, 4).

The regulation of SP-C expression by KGF appears to be different from that of SP-A, SP-B, and SP-D. The mRNAs for the later proteins increased concomitantly with the morphological proliferation of type II cells. The response of SP-C mRNA was slightly delayed and prolonged. In vitro, Sugahara et al. (37) found SP-C
mRNA unaffected or diminished 48 h after treatment of type II cells with KGF in vitro. The reason for this disparity in the changes in the mRNA for SP-C compared with the other surfactant proteins is unknown. In vitro, the expression of the surfactant proteins appears to be independently regulated in that conditions that appear to be important for one surfactant protein, e.g., corticosteroids or cAMP, may be unimportant or even inhibitory for the others (Shannon, unpublished observations). In vitro maintenance of SP-C expression appears to be the most difficult surfactant protein to retain at the level of expression observed in freshly isolated type II cells. The precise reason for the independent regulation of the surfactant proteins in vitro and by KGF in vivo is not known. Presumably this is due to transcriptional regulation, but the possibility of mRNA stability is not excluded.

One of the transcription factors thought to be important in regulating the mRNAs for SP-A, SP-B, and SP-C is TTF-1. There was increased mRNA for TTF-1 after instillation of KGF. However, the maximal increase occurred after the increase in the expression of SP-A, SP-B, and SP-D but was coincident for the increased expression of SP-C. TTF-1 is a 38-kDa nuclear protein, which was initially identified as a thyroid transcription factor that regulated thyroid-specific gene expression (17). In the lung, TTF-1 is confined to the bronchial and alveolar epithelia (24). Recent studies demonstrate that TTF-1 activates the transcription of SP-A, SP-B, and SP-C in vitro, and TTF-1 binding sites are recognized on 5'-flanking region of their and CC10 genes (6, 22, 25, 43). The temporal and spatial patterns of TTF-1 expression and SP-B expression are similar in the developing lung. This study did not address the specific importance of TTF-1 in the regulation of the mRNAs for the individual surfactant proteins but only demonstrated that the whole lung mRNA for TTF-1 increased after KGF instillation. There was decreased expression of CC10 despite an increase in TTF-1.

Although the focus of this study was on the alveolar epithelium, there were also changes in the bronchiolar epithelium. rhKGF stimulated DNA synthesis in rhesus monkey bronchial cells (33), and there is a proliferative effect in bronchiolar epithelial cells in rats instilled with KGF (39). There was a decrease in mRNA for CC10 observed in whole lung and in bronchiolar cells in sections examined by in situ hybridization. CC10 expression is diminished in a variety of acute conditions associated with airway inflammation and presumed epithelial proliferation such as inhalation of ozone and bleomycin (20). There was also a change in the relative immunostaining in the alveolar epithelium and in situ hybridization for SP-A and SP-D. In the normal lung, SP-A and SP-D are observed only in the easily identified protruding Clara cells. However, after instillation of KGF, there is diffuse staining for SP-D in the bronchiolar epithelium, whereas there is less and more focal staining for SP-A. By in situ hybridization, there also was no marked increase in the number of bronchiolar cells expressing SP-A. This is the first in vivo observation on the independent expression of SP-A and SP-D in bronchiolar cells to our knowledge.

The critical issue for KGF as a therapeutic agent is to stimulate the alveolar and bronchiolar epithelium in a time-dependent manner without adverse effects in the lung. In the current study as well as in previous studies, the focal epithelial cell proliferation returns to normal by 7 days. There is no appar-
ent fibrosis or residual tissue inflammation. Pretreatment with KGF has already been demonstrated to lessen the inflammatory and fibroproliferative effects of a variety of agents including 100% oxygen, bleomycin, irradiation, acid instillation, and administration of α-naphthol urea (7, 18, 26, 28, 36, 44, 45).

The increase in the expression of surfactant proteins by KGF could be part of the beneficial effect to KGF pretreatment in animal models of lung injury, but it is unlikely to be the sole or critical effect. KGF has pleiotropic effects on the alveolar epithelium. These effects include a reduction in the alveolar surface area composed of the very sensitive type I cells, generalized increased expression of antioxidant enzymes, and increased Na-K-ATPase (2, 8, 12, 29). Frank et al. (14) have suggested that the induction of a non-selenium glutathione peroxidase is important for the cytoprotective effect of KGF on keratinocytes. This same protein has been referred to as Clara cell protein 26 and is also thought to have phospholipase activity (23, 32). KGF likely exerts a generalized cytoprotective effect on epithelial cells, but the specific biochemical alterations remain to be defined.

In conclusion, KGF instillation produces a transient and reversible focal type II cell hyperplasia. This is associated with an increase in the mRNA for all the surfactant proteins. However, the specific mRNA for the surfactant proteins in individual type II cells is reduced. Nevertheless, presumably because of the increase in total numbers of type II cells, there is an increase in the amount of SP-A and SP-D recovered in lavage. Instillation of KGF also causes a decrease in CC10 mRNA levels in bronchiolar epithelial cells.

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