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

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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). KGF and 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 costs of publication of this article were defrayed in part by the payment of page charges. The article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.
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 both in whole lung and in individual airway epithelial cells. All of these changes are accompanied by an up-regulation of TTF-1 mRNA expression in whole lung.

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 BALB/MK 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). 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 purified 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% (wt/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 SP-D antibody-conjugated horseradish 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 (Fierce 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.
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 (GITC) 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 centrifugation at 500 g for 10 min, washed, and recentrifuged twice in PBS, and then lysed in GITC. Total cellular RNA from whole lung tissue or from isolated type II cells was isolated by ultracentrifugation through a 5.7 M CCl₃ 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 membrane (Nytran; Schleicher & Schuell, Keene, NH) by capillary action. The isolation and characterization of cDNAs for rat SP-A, SP-B, SP-C, and SP-D have been described previously (35, 37). The cDNAs for rat CC10 and rat TTF-1 were provided by Dr. Arun Rishi (Boston University, Boston, MA) and Dr. Lyn Thet (University of Wisconsin, Madison, WI), respectively. cDNAs were labeled to high specific activity by random priming using [α-32P]dCTP and a commercially available kit (both from Amersham Life Sciences, Arlington Heights, IL). Membranes were prehybridized, hybridized, washed, and autoradiographed on Kodak XAR5 film (Eastman 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, version 3.3 (Molecular Dynamics, Sunnyvale, CA).

A 28S ribosomal RNA (rRNA) oligonucleotide probe was used for normalizing results of Northern blots for rat SP-A, SP-B, SP-C, SP-D, CC10, and TTF-1. The sequence of the oligonucleotide was 5′-ACGATCAGATGAGTTTGTGGAACAGCAC-3′, which is complementary to bases 4011–4036 of the human 28S rRNA. The oligonucleotide was synthesized on an Applied Biosystems model 381A DNA synthesizer (Foster City, CA) and was 5′-end labeled with T4 polynucleotide kinase (GIBCO BRL) and [γ-32P]dATP (Amersham Life Sciences). Previously hybridized nylon membranes were stripped of SP-A, SP-B, SP-C, SP-D, CC10, or TTF-1 probe by washing with 50% formamide and 6× sodium chloride-sodium citrate (SSC) at 65°C for 2 h. The membranes were prehybridized at 37°C for 2 h with 6× SSC, 0.1 M sodium phosphate (pH 6.8), 1 mM EDTA (pH 8.0), 0.1 mg/ml denatured salmon sperm DNA, 0.5% SDS, and 0.1% nonfat dry milk. Hybridization was performed at 37°C for 16 h in the same solution as the prehybridization buffer. The membranes were washed once for 10 min at room temperature and three times for 30 min at 55°C in 6× SSC with 0.1% SDS. After the membrane was washed, it was wrapped in a plastic bag, exposed to Kodak XAR5 film or Hyperfilm (Amersham) as described (34), and the signal intensity for the SP-A, SP-B, SP-C, SP-D, CC10, or TTF-1 mRNA band was divided by that for the 28S rRNA band.

RNAse protection assay. In some experiments, total RNA isolated from elutriated type II cells was analyzed for surfactant protein mRNA expression using a RNase protection assay (RPA) that allowed simultaneous measurement of mRNAs for SP-A, SP-B, SP-C, and SP-D. Fragments of different sizes for the four surfactant proteins were isolated by polymerase chain reaction using full-length cDNAs as templates. The forward primers included a BamH 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 directional cloning into pGEM-4Z (Promega). The primers for SP-A were 5′-CGGATCCGAGGAGGACGACGAC-3′, coding sense and corresponding to nucleotides 424–444, and 5′-GGAATTCCGGTTTCCTCTCTAGGACCCCGTC-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′-CCGATCCGAGGAGGACGACGAC-3′, coding sense and corresponding to nucleotides 997–1017, and 5′-GGAATTCCGAGGAGGACGACGAC-3′, coding antisense and corresponding to nucleotides 1152–1172. The probe transcribed from this clone identified a fragment of 176 bp. The primers for SP-D were 5′-CCGATCCGAGGAGGACGACGAC-3′, coding sense and corresponding to nucleotides 831–851, and 5′-GGAATTCCGAGGAGGACGACGAC-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 BamH I, and radiolabeled antisense probes were transcribed in vitro using a commercially available kit (Promega) and [α-32P]CTP (800 Ci/mmol; ICN). The RNA probe was purified on an 8% polyacrylamide-7 M urea gel and eluted from the most intense band detected by autoradiography. An 18S rRNA probe, which was synthesized using pT7 RNA 18S template, T7 MEGashort-scription Kit (Ambion, Austin, TX), and [α-32P]CTP, was used as an internal standard for RNA quantitation. Three to five micrograms of total cellular RNA were hybridized at 45°C for 24 h with 1 × 105 counts/min of antisense probe. Probe not protected by hybridization with target RNA was digested with a mixture of RNase A and T1 (RPA II kit; Ambion). The protected RNA duplex fragments were precipitated, resuspended, and separated on an 8% polyacrylamide-7 M urea gel. The gel was dried and exposed to Hyperfilm (Amersham Life Sciences).
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 33P-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 (gift 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 were significantly different. Post hoc comparisons were made using the Bonferroni test. A value of P < 0.05 was considered statistically significant.

Fig. 2. Temporal changes in surfactant protein (SP) mRNA levels in the lung after rhKGF instillation. Rats were instilled with rhKGF or normal saline intrabronchially into the left lung and were killed 1–7 days after treatment. The rats on day 0 were the uninstilled controls. Values are means ± SE of the ratio of signal intensity of the respective SP mRNA to that of 28S rRNA expressed as a percentage of the mean values for untreated rats; nos. in bars, no. of animals/experimental group. Statistical analysis was applied between the untreated control group and the treated groups at the same time point. A: SP-A. There was an increase in SP-A mRNA level on day 1, with a maximal response on day 2 followed by a decrease to the untreated control level on day 3 after rhKGF instillation. B: SP-B. There was an increase in SP-B mRNA level on day 1, with a maximal response on day 2 followed by a decrease to the untreated control level on day 3 after rhKGF instillation. C: SP-C. SP-C mRNA levels in rhKGF-treated animals increased on day 2 through day 5. D: SP-D. There was an increase in SP-D mRNA level on days 1 and 2 followed by a decrease to the untreated control level on day 3 after rhKGF instillation. *P < 0.05 vs. untreated group, †P < 0.05 between saline-treated and rhKGF-treated groups.
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).
A 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

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<th>Treatment Group</th>
<th>Days After Instillation</th>
<th>n</th>
<th>Total Protein, µg/ml</th>
<th>SP-A, µg/ml</th>
<th>SP-A/Total Protein, %</th>
<th>SP-D, µg/ml</th>
<th>SP-D/Total Protein, %</th>
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<td>Untreated</td>
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<td>1</td>
<td>3</td>
<td>215 ± 62</td>
<td>0.62 ± 0.02</td>
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<td>0.24 ± 0.07</td>
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<td>153 ± 58</td>
<td>0.34 ± 0.05</td>
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<td>84 ± 11</td>
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<td>66 ± 11†</td>
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<td>64 ± 17</td>
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<td>113 ± 16</td>
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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 ± 5.2 × 10⁶ cells from rhKGF-treated rats, 41.1 ± 2.2 × 10⁶ cells from saline-instilled rats, and 38.5 ± 2.7 × 10⁶ cells from unin- stalled 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 general 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 hyper- tropic 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.

![Fig. 6. Temporal changes in 10-kDa Clara cell protein (CC10) mRNA levels. Rats were instilled with rhKGF or normal saline intrabronchially and were killed 1–7 days after the treatment, and the RNA was processed as stated in legend to Fig. 2. A: representative Northern blots of CC10 mRNA and 28S rRNA. B: mean ± SE values for the ratio of signal intensity for CC10 mRNA to that for 28S rRNA.](http://ajplung.physiology.org/)
signal over the bronchiolar cells appeared less after KGF instillation than in the controls. The intensity of the signal over the bronchiolar walls in sections from the untreated lung appeared the same as the uninflamed 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.

DISCUSSION

KGF (FGF-7) is a well-known mitogen specific for epithelial cells. In terms of alveolar type II epithelial cells, Panos et al. 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. 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 increase 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.

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. In situ hybridization, there appears to be a slight increase in expression of TTF-1 in both bronchiolar and alveolar cells (Fig. 9).

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Fig. 7. In situ hybridization for CC10 mRNA. Rats were instilled with rhKGF or normal saline and killed 2 days later. The rats on day 0 were the uninflamed controls. Tissue sections were hybridized with antisense cRNA probe for CC10. Hybridization with CC10 probe resulted in labeling over cells only on bronchiolar walls in sections from the untreated lung (A). Saline-instilled lungs appeared the same as the uninflamed 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.
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 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 rhKGF-stimulated ternary 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 airway 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-

![Fig. 8. Temporal changes in thyroid transcription factor-1 (TTF-1) mRNA. Rats were instilled with rhKGF or normal saline intrabronchially and were killed up to 7 days after treatment. RNA was isolated and prepared as stated above. A: representative Northern blot of TTF-1 mRNA and 28S rRNA. B: mean ± SE values for the ratio of signal intensity for TTF-1 mRNA to that for 28S rRNA are expressed as a percentage of the mean values of untreated rats. The number of individual animals in each treatment group is listed in the bars. Statistical analysis was applied between the untreated control and the treated groups at the same time point. There was an increase in the mRNA levels for TTF-1 2–7 days after KGF instillation. Saline instillation had no effect on TTF-1 mRNA levels. *P < 0.05 vs. untreated group. †P < 0.05 between saline and rhKGF-treated groups.](http://ajplung.physiology.org/figure8.png)
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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