Keratinocyte growth factor stimulates bronchial epithelial cell proliferation in vitro and in vivo

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Michelson, Peter H., Margaret Tigue, Ralph J. Panos, and Peter H. S. Sporn. Keratinocyte growth factor stimulates bronchial epithelial cell proliferation in vitro and in vivo. Am. J. Physiol. 277 (Lung Cell. Mol. Physiol. 21): L737–L742, 1999.—Airway epithelial cell (AEC) proliferation is crucial to the maintenance of an intact airway surface and the preservation of host defenses. The factors that regulate AEC proliferation are not known. Keratinocyte growth factor (KGF), also known as FGF-7, is a member of the fibroblast growth factor family and a known epithelial cell mitogen. We studied the influence of KGF on the growth of cultured human bronchial epithelial cells and on bronchial cells of rats treated with KGF in vivo. First, we demonstrated the mRNA for the KGF receptor (KGFR) in both normal human bronchial epithelial (NHBE) cells and BEAS-2B cells (a human bronchial epithelial cell line). KGF caused a dose-dependent increase in DNA synthesis, as assessed by thymidine incorporation, in both cell types, with a maximal twofold increase in NHBE cells after 50 ng/ml KGF (P < 0.001). KGF also induced a doubling in NHBE cell number at 10 ng/ml (P < 0.001). Finally, we determined the effect of intratracheal administration of KGF to rats on proliferation of AEC in vivo. Measuring bromodeoxyuridine (BrdU) incorporation in AEC nuclei, KGF increased BrdU labeling of rat AEC in both large and small airways by approximately threefold compared with PBS-treated controls (P < 0.001). Thus KGF induces proliferation of bronchial epithelial cells both in vitro and in vivo.

KGF is a heparin-binding growth factor that selectively stimulates proliferation of a broad range of epithelial cells including those in the skin, gastrointestinal tract, and mammary gland (23). This effect is transduced through the KGFR, a tyrosine kinase receptor (16), which appears to be uniquely expressed by epithelial cells. Signaling through KGFR is essential for normal murine lung growth and development (20). In addition, KGF enhances the differentiation of alveolar type II cells and/or their progenitors and promotes alveolarization during branching morphogenesis (25).

Furthermore, KGF has been shown to stimulate proliferation of alveolar type II cells in vitro (18) and in vivo (28) and to protect against diffuse alveolar damage and mortality caused by many different injurious agents (1). Treatment with KGF reduces lung injury caused by hyperoxia, bleomycin, naphthylthiourea, and hydrochloric acid in rats (6, 9, 17, 32, 33). Furthermore, KGF facilitates repair of radiation-induced DNA damage in alveolar epithelial cells in vitro (27). Finally, KGF protects against permeability defects in cultures of human airway epithelial cells exposed to H2O2 (29).

In this study, we examined the mitogenic effects of KGF on cultured human airway epithelial cells and on airway epithelium of rats treated with the growth factor intratracheally. Our results demonstrate that KGF induces proliferation of airway epithelial cells both in vitro and in vivo.

MATERIALS AND METHODS

Cell Culture Conditions

The transformed human bronchial epithelial cell line BEAS-2B (23) was obtained from ATCC (Manassas, VA). Normal human bronchial epithelial (NHBE) cells were obtained from Clonetics (Walkersville, MD). Cells were cultured at 37°C in humidified 5% CO2 in air. The BEAS-2B cells were grown in keratinocyte serum-free medium (K-SFM; Gibco BRL, Gaithersburg, MD) supplemented with recombinant human epidermal growth factor (rhEGF) and bovine pituitary extract (PE). NHBE cells were grown in bronchial epithelial growth medium (Clonetics). Bronchial epithelial growth medium consists of bronchial epithelial basal medium (BEBM) supplemented with rhEGF, PE, insulin, hydrocortisone, transferrin, retinoic acid, epinephrine, and triiodothyronine. To facilitate adherence, plasticware was precoated with 30 μg/ml Vitrogen 100 (Cetrix, Santa Clara, CA) and 10 μg/ml FCS for 2 h at 37°C. Cells were allowed to adhere for 12 h in the above supplemented medium before individual experiments.

In each experimental condition, before exposure to exogenous growth factor, cell growth was slowed by altering medium conditions. BEAS-2B cells were cultured in K-SFM without PE or rhEGF (basal K-SFM); the NHBE cells were cultured in BEBM containing the above-described additives except for PE and rhEGF (basal BEBM).
Additionally, the Ca\(^{2+}\) concentration ([Ca\(^{2+}\)]) of K-SFM and BEBM was supplemented to physiological levels to slow spontaneous proliferation of BEAS-2B and NHBE cells, respectively. Both K-SFM ([Ca\(^{2+}\)] = 0.15 mM) and BEBM ([Ca\(^{2+}\)] = 0.11 mM) were supplemented with tissue culture grade CaCl\(_2\) (Sigma, St. Louis, MO) to raise the [Ca\(^{2+}\)] to 1.0 mM. Cells were exposed to KGF at varying concentrations in both the absence (0.15/0.11 mM) and presence (1.0 mM) of supplemental Ca\(^{2+}\). Cell viability, as determined by trypan blue exclusion, remained >97% in all cell types under all culture conditions.

Northern Analysis

Total RNA was isolated from NHBE and BEAS-2B cells using the acid guanidium thiocyanate-phenol-chloroform method (24). RNA was size fractionated by electrophoresis through 1% agarose gels under denaturing conditions and transferred to Nytran by capillary action. Blots were then hybridized with human KGFR cDNA (the 178-bp fragment) labeled with \(^{32}\)PdCTP (Amersham, Arlington Heights, IL) by nick translation. Hybridization signals were quantitated using a Fuji Bio-Imaging Analyzer (Fuji, Elmsford, NY), and autoradiograms were prepared by exposure of blots to X-ray film.

\[^{3}H\]Thymidine Incorporation

To determine the effect of KGF on epithelial cell DNA synthesis, incorporation of thymidine was measured. Both BEAS-2B and NHBE cells were seeded onto 24-well plates at a density of 25,000 cells/well. The cells were allowed to adhere and were incubated in their respective growth medium for 12 h. The media were removed, and spontaneous proliferation was slowed by culturing in basal K-SFM for the BEAS-2B cells and basal BEBM for the NHBE cells, both with supplemental Ca\(^{2+}\). After quiescence, the cells were washed and exposed to 0, 1, 10, or 50 ng/ml recombinant human KGF (Amgen, Thousand Oaks, CA) in medium containing 0.1 \(\mu\)Ci/ml \[^{3}H\]thymidine (Amersham). After preliminary experiments demonstrated that maximal \[^{3}H\]thymidine incorporation occurred at 48 h, all subsequent experiments utilized this duration of KGF exposure. At 48 h, the cells were washed, and DNA was precipitated with cold 10% trichloroacetic acid. The precipitate was solubilized with 1.0 M sodium hydroxide, which was neutralized with 1.0 M sodium acetate. Aliquots were collected, and radioactivity was quantified by liquid scintillation spectrometry as described (18).

Determination of Cell Number

NHBE cells were plated in six-well plates at 50,000 cells/well. To slow spontaneous proliferation, cells were cultured in Ca\(^{2+}\)-supplemented basal BEBM ([Ca\(^{2+}\)] = 1.0 mM). After 48 h, the cells were washed, and exposed to 0, 1, 10, or 50 ng/ml of KGF in basal BEBM either with or without supplemental Ca\(^{2+}\) for 24 and 48 h. Cells were then detached by exposure to 0.05% trypsin (GIBCO), which was neutralized by the addition of trypsin neutralizing solution (Clonetix). Trypsinization was monitored under the microscope to ensure that all cells detached. Aliquots (0.2 ml) of cell suspensions were diluted 50-fold, and cells were counted using a Coulter counter (Coulter Electronics, Hialeah, FL). Each cell suspension was counted two times, and the results were averaged.

KGF Treatment of Rats and Bromodeoxyuridine Incorporation

Rats were treated with KGF, and tissue specimens were prepared as outlined previously (17). This protocol was approved by the Animal Care and Use Committee of the Veterans Affairs Chicago Health Care System, Lakeside Division. Briefly, adult male Sprague-Dawley rats were anesthetized, intubated, and given KGF (1 or 5 mg/kg in 0.6 ml of PBS) or vehicle (0.6 ml of PBS) intratracheally. All animals received 100 mg/kg bromodeoxyuridine (BrdU) by intraperitoneal injection 18 h before death. Forty-eight hours after instillation of intratracheal KGF (or PBS), the animals were euthanized, and the lungs were excised, fixed with 10% buffered formalin at 20 cm H\(_2\)O, and embedded in paraffin. Sections were deparaffinized, rehydrated, and incubated with 3% horse serum in PBS. They were then incubated in 1% H\(_2\)O\(_2\) for 10 min and washed in PBS. After incubation in 100 \(\mu\)g/ml Pronase E (Sigma) in Pronase buffer (0.02 M Tris·HCl and 20 mM CaCl\(_2\), pH 7.6), the sections were rinsed with buffer and then incubated in ice-cold 0.1 N HCl for 10 min to remove DNA binding proteins. DNA was denatured by incubating the slides in 2 N HCl for 30 min at 37°C, and then the acid was neutralized with 0.1 M borax, pH 8.5. The slides were next incubated for 1 h at room temperature with anti-BrdU monoclonal antibody, Bu20a (DAKO, Carpinteria, CA), diluted 1:50 in 3% horse serum. After being washed in PBS, horseradish peroxidase-conjugated goat anti-mouse IgG (Sigma), diluted 1:75 in 3% horse serum, was added for 1 h. Diaminobenzidine was added, and the peroxidase reaction was intensified using nickel-silver enhancement. Slides were counterstained with hematoxylin and were mounted in 90% glycerol. Bronchi were identified microscopically, and bronchial epithelial cells with black nuclear staining were counted as BrdU positive. Proliferation index was calculated as the number of BrdU-positive bronchial epithelial cells divided by the total number of bronchial epithelial cells, multiplied by 100. At least 200 cells were counted per specimen.

Statistics

For in vitro experiments with NHBE and BEAS-2B cells, duplicate or triplicate determinations were made for each experimental condition. The results of replicates were averaged and expressed as one data point. Each experiment was repeated three to six times. For experiments measuring the effects of KGF on \[^{3}H\]thymidine incorporation and cell proliferation, data were normalized and are expressed as a percentage of the control (no KGF) condition. The statistical significance of differences between groups was assessed by analysis of variance and the Bonferroni multiple comparisons test. P values < 0.05 were considered significant.

RESULTS

Expression of KGFR mRNA in Human Bronchial Epithelial Cells

To ascertain whether the bronchial epithelial cells BEAS-2B and NHBE express the receptor for KGF, Northern analysis was performed using a KGFR cDNA probe. As seen in Fig. 1, both BEAS-2B and NHBE cells demonstrated a hybridization band at ~180 bp, indicating the presence of the KGFR mRNA transcript. A housekeeping gene, glyceraldehyde-3-phosphate dehydrogenase, was also included to demonstrate equal loading of the mRNA for each cell type. Additionally, we found expression of KGFR mRNA in A549 and Calu-3 cells, epithelial cell lines derived from human bronchogenic carcinomas (data not shown).
Effect of KGF on Growth of Human Bronchial Epithelial Cells In Vitro

DNA synthesis. The effect of KGF on bronchial epithelial cell DNA synthesis was determined by assaying incorporation of [3H]thymidine. As shown in Fig. 2A, KGF failed to stimulate incorporation of [3H]thymidine in BEAS-2B cells cultured in basal K-SFM. Because these cells are SV40 transformed, they proliferate rapidly. This rapid spontaneous growth is promoted by culture in K-SFM, which contains much less Ca\(^{2+}\) (0.15 mM) than either standard tissue culture medium or physiological extracellular fluid. Thus, to reduce the rapid growth of these cells, K-SFM was supplemented with CaCl\(_2\) to raise the [Ca\(^{2+}\)] to 1.0 mM, close to the physiological range. Ca\(^{2+}\) supplementation reduced the basal [3H]thymidine incorporation by BEAS-2B cells two- to threefold. In the presence of 1.0 mM Ca\(^{2+}\), KGF caused a dose-dependent increase in DNA synthesis in BEAS-2B cells, achieving statistical significance at the 10 ng/ml dose (Fig. 2B).

DNA synthesis in primary bronchial epithelial cells exposed to KGF was measured under similar conditions. Of note, the basal level of [3H]thymidine incorporation in the NHBE cells was 10-fold less than in the BEAS-2B cells. In basal BEBM ([Ca\(^{2+}\)] = 0.11 mM), KGF increased [3H]thymidine incorporation by NHBE cells in a dose-dependent fashion, reaching a maximum, nearly twofold, in response to 50 ng/ml (Fig. 3A). KGF also enhanced [3H]thymidine incorporation in NHBE cells cultured in medium with supplemental Ca\(^{2+}\) ([Ca\(^{2+}\)] = 1.0 mM; Fig. 3B). Unlike BEAS-2B cells, NHBE cells exhibited a diminished response to KGF in Ca\(^{2+}\)-supplemented compared with unsupplemented medium.

Cell proliferation. To determine whether the increases in DNA synthesis caused by KGF were accompanied by increases in cell number, epithelial cells were enumerated using a Coulter counter. As shown in Fig. 4A, KGF increased NHBE cell numbers in a dose-dependent manner, with more than a doubling of control cell counts at a dose of 10 ng/ml KGF at 48 h. Like it did for DNA synthesis, Ca\(^{2+}\) supplementation of BEBM blunted the change in cell numbers in response to KGF, although significant increases were still seen (Fig. 4B). KGF did not induce significant increases in cell number in the more rapidly proliferating BEAS-2B line in either the absence or presence of supplemental Ca\(^{2+}\) (data not shown).

Effect of KGF on Growth of Rat Bronchial Epithelial Cells In Vivo

To determine if KGF stimulated bronchial epithelial cell proliferation in vivo, we measured BrdU incorporation by rat airway epithelial cells 48 h after intratracheal instillation of either KGF or PBS. As shown in...
Fig. 5, both 1 and 5 mg/kg KGF significantly increased rat bronchial epithelial cell labeling with BrdU. The bronchial epithelial cell BrdU labeling index in rats that received 5 mg/kg of KGF was three times that in rats that received PBS. Photomicrographs of representative BrdU-labeled airways from rats treated with PBS and 5 mg/kg KGF are presented in Fig. 6.

**DISCUSSION**

In this study, we have assessed the ability of bronchial epithelial cells to respond to the fibroblast-derived growth factor KGF. Our data show that primary and transformed human bronchial epithelial cells express mRNA for the KGFR. Exposure to KGF not only augmented the level of DNA synthesis in primary human bronchial epithelial cells but also stimulated proliferation, increasing epithelial cell numbers. In contrast, BEAS-2B cells, an SV40-transformed cell line, were less responsive to the proliferative effects of KGF. Finally, intratracheal instillation of KGF into the lungs of uninjured rats induced a dose-dependent increase in bronchial epithelial cell DNA synthesis compared with PBS-treated control animals. Thus KGF stimulates bronchial epithelial cell proliferation both in vitro and in vivo.

By Northern analysis, we demonstrated that bronchial epithelial cells express the KGFR and thus are capable of responding to its proliferative signaling. A splice variant of the FGF receptor-2, KGFR, has high affinity for both KGF and acidic FGF and has lower affinity binding for basic FGF (23). In both BEAS-2B cells and NHBE cells, the transcript for KGFR was detected.

Modulation of receptor expression in dominant-negative KGFR transgenics (20) or by altering [Ca²⁺] (15) has been shown to reduce the effects of KGF. This may be significant in activating KGFR and initiating postreceptor signaling (4, 14). Recent investigations of KGFR expression in human pancreatic cancer reveal overexpression in the neoplastic cells compared with normal cells (11). Additionally, there appears to be...
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Fig. 5. Effect of intratracheal KGF on bronchial epithelial cell bromodeoxyuridine (BrdU) incorporation in rats. KGF (1 or 5 mg/kg) or PBS was given intratracheally, and rats were killed 48 h later. BrdU was administered 18 h before death. Immunohistochemistry was performed, and BrdU labeling index was calculated for n = 3–4 experiments as described in MATERIALS AND METHODS. *P < 0.01 vs. KGF = 0 ng/ml; †P < 0.001 vs. KGF = 0 ng/ml.

Fig. 6. Photomicrographs of representative BrdU-labeled airway sections from rat treated intratracheally with KGF or PBS control. Bronchiolar epithelium shown in A (5 mg/kg KGF) and B (PBS); epithelium of central airways shown in C (5 mg/kg KGF) and D (PBS). Original magnification, ×250 for A and B and ×400 for C and D. Arrowheads represent BrdU-labeled cells. Arrows indicate airway cartilage.

colocalization of both KGF and KGFR in the neoplastic tissue, suggesting KGF acts in both an autocrine and paracrine fashion in enhancing pancreatic tumor cell growth. KGFR has been described in a number of nonrespiratory epithelial cells (14), but its expression has not been described in bronchial epithelium. Although it is not known whether KGFR overexpression occurs in bronchogenic carcinoma (lung neoplasms derived from bronchial epithelium), these findings may be significant in understanding the malignant potential of airway epithelia and the development of lung neoplasms.

A number of growth factors, including epidermal growth factor and hepatocyte growth factor, stimulate airway epithelial cell proliferation (7, 19, 26). Epidermal growth factor has also been shown to facilitate repair of tracheal epithelial cell wounds by enhancing cell spreading and migration (12). Utilizing primary human bronchial epithelial cells, our data demonstrate that KGF too has proliferative effects on airway epithelial cells. When compared with the proliferative response seen in alveolar type II cells, our results support the findings of previous investigators (18, 28), with a two- to threefold increase in cell number after exposure to KGF. Additionally, our data demonstrate a dose-dependent effect, with responses in the dosing range described for the alveolar type II cells.

BEAS-2B cells, an immortalized cell line that is phenotypically similar to primary bronchial epithelium (21), did not demonstrate a significant proliferative response to KGF. These cells, whose population doubling time is more rapid than primary bronchial epithelial cells (3, 21), continued to proliferate rapidly, even in unsupplemented medium. To slow proliferation and assay more effectively the response to KGF, comparative experiments were performed in physiological (1.0 mM) and usual media (0.15 mM) [Ca\(^{2+}\)]. As reported by Wu et al. (30), increased [Ca\(^{2+}\)] in culture medium may affect tracheal epithelial cell proliferation and differentiation without altering cell viability. In Ca\(^{2+}\)-supplemented culture medium, cellular proliferation was reduced without untoward effects on viability or cellular integrity. Although it is not clear whether the alteration in [Ca\(^{2+}\)] may have some impact on postreceptor signal transduction, one possible explanation for the altered response to KGF may be increased KGFR expression. This possibility is currently being explored.

To assess the effect of KGF on growth of airway epithelial cells in vivo, we administered KGF intratracheally to rats. KGF stimulated proliferation of epithelial cells in both large and small airways, similar to its effect on rat alveolar epithelium, as demonstrated previously (17). KGF has been shown to ameliorate lung injury caused by hyperoxia (17), bleomycin (33), and hydrochloric acid (32) as well as to facilitate repair after H\(_2\)O\(_2\) and radiation-induced DNA damage (27, 29). Besides promoting alveolar type II cell proliferation and stimulating the reepithelialization of the alveolar surface, KGF increases surfactant protein expression (31), which may further limit injury to the alveolus. Our current results suggest that an additional mechanism for the protective effect of KGF may be through the stimulation of bronchial epithelial cell proliferation and repair of the airway epithelial lining.

Understanding how these mesenchymal cell-derived growth factors reach the epithelial cell receptor and induce proliferation is an important area of further investigation. Epithelial-fibroblast cross talk and epithelial cell-extracellular matrix signaling may further modulate the proliferative potential of epithelial cells (2, 13). Insight into this interaction and the postreceptor signaling will be critical not only in providing understanding of the mechanism of signal transduction but also as potential therapies after injury or to increase incorporation of exogenous DNA vectors in epithelial cells in gene transfer protocols (8).

The factors that regulate the proliferation of the airway epithelium during development and repair after injury are not yet known. In this study, we demonstr-
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