Keratinocyte Growth Factor and Autocrine Repair in Airway Epithelium

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**Background:** Delayed or nonreepithelialization of the large conducting airway (ie, trachea and bronchus) is a clinically recognized but poorly understood result of airway trauma. This delay results in granulation tissue formation and scarring, which impairs mucociliary transport and can critically compromise gas exchange. Keratinocyte growth factor (KGF) is a known epithelial cell mitogen that is derived from mesenchymal cells. We previously observed its expression in injured tracheal explants, and in the present study we investigated its origin.

**Design:** Freshly isolated porcine tracheal epithelial cells were cytopointed onto glass slides for immunohistochemical identification and localization of KGF and for in situ hybridization localization of its messenger RNA. Polymerase chain reaction analysis for KGF was also performed.

**Results:** Freshly isolated respiratory epithelial cells were identified as being of epithelial origin and uncontaminated by fibroblasts, as evidenced by stains that were positive for AE3 and negative for vimentin. Immunohistochemical analysis and in situ hybridization revealed a subset of cells that were positive for both the protein and the message for KGF.

**Conclusion:** This subset of KGF-expressing respiratory epithelial cells may participate in a hitherto undescribed autocrine loop for stimulating KGF production in response to injury.

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T R A U M A TO THE LARGE CONDUCTING AIRWAY (ie, trachea and bronchus) that disrupts the basement membrane often leads to aberrant repair manifested by excessive proliferation of granulation tissue and replacement of the respiratory epithelium with cicatricial tissue. A lack of well-defined in vitro model systems with which to study repair and an incomplete knowledge of the growth factors and cytokines involved have impeded understanding of the physiologic basis for respiratory epithelial cell (REC) migration and proliferation after injury.

Keratinocyte growth factor (KGF) is a member of the structurally related fibroblast growth factor family of mitogens. Unlike other fibroblast growth factor members with mitogenic activities for cells that are derived from all 3 germ layers, KGF is highly specific for epithelial cells. It has been widely regarded as a paracrine factor that is produced by mesenchymal cells to stimulate epithelial cell migration and proliferation. It is an early-response gene product and a key growth factor in wound healing. Its expression is increased after injury in many organ systems, which suggests that it has a potentially important role in the general response to injury. Also, exogenous application of KGF enhances wound healing. In the upper airway, KGF increases bronchial epithelial cell migration and spreading in response to wounding, overcoming the inhibitory effect of mechanical strain on wound healing, and induces proliferation both in vitro and in vivo.

We have previously shown that after an injury that violates the basement membrane occurs, KGF is up-regulated in tracheal organ explants, both in the underlying submucosa and in the epithelium adjacent to the wound. The rapid expression of KGF in the injured epithelium led us to hypothesize that this protein might be pivotal in an autocrine repair process. We observed that freshly isolated RECs expressed KGF, which belied the possibility of paracrine transport as the sole source of KGF in injured respiratory epithelium. Although reports in-
volving other organ systems also suggest that the messenger RNA for KGF can be produced by epithelium.\textsuperscript{12-14} These organ systems have mixed mesenchymal and epithelial elements.\textsuperscript{14} Respiratory epithelium is the only pure epithelium (vimentin negative) reported to produce KGF (messenger RNA and protein).

### METHODS

#### CELL ISOLATION

Porcine tracheas, which were obtained within 24 hours of necropsy from a supplier (Spear Products, Quakertown, Pa), were cleaned and the epithelium and submucosa were removed from the cartilage layer. The tissue was soaked overnight at 4°C in minimal essential medium containing antibiotics and antimycotics to reduce subsequent contamination. Respiratory epithelial cells were isolated using elastase digestion as previously described.\textsuperscript{15} The cells were washed twice in Dulbecco's phosphate-buffered saline instead of the primary antibody. Furthermore, during culture, there was no overgrowth of fibroblasts (not shown). Immunostaining for KGF protein revealed a positive subset of RECs (Figure 2B). Likewise, when tracheal organ explants were wounded, KGF expression was increased both in the submucosa beneath the injury and in the adjacent uninjured epithelium.\textsuperscript{13} These combined observations prompted us to investigate the source of the KGF within the epithelial cells. In situ hybridization for KGF messenger RNA in cytospin preparations of freshly isolated RECs localized the negative control in tubes with and without added primers. Probe-free and ribonuclease-digested controls were also included.

Polycarbonate chain reaction detection of KGF

Total RNA was obtained from freshly isolated cells using a nucleic acid and protein isolation reagent (Tri Reagent; Molecular Research Center Inc, Cincinnati, Ohio), according to the manufacturer’s directions. The RNA (5 µg) was reverse transcribed and used in subsequent polymerase chain reaction (PCR) analysis to detect the message for KGF. The KGF primers were as follows: 5' primer, 5'-TGA TCA AGC TGG ACT TGT GC-3'; and 3' primer, 5'-TGT CTT CTT CCA GGA TTT GC-3', producing a 995-base pair (bp) product corresponding to nucleotides 1163-2157 of the human KGF complementary DNA. Identity of the PCR product was confirmed by sequencing. The PCR conditions were as follows: The 20-µL reactions consisted of 2 µL of template complementary DNA, 1X PCR buffer, 2mM magnesium chloride, 300mM forward and reverse KGF primers, 200µM deoxynucleotide triphosphates, and 2.5 U Taq polymerase. Amplification was carried out in a thermocycler (M. J. Research, Boston, Mass) as follows: hot start at 94°C for 5 minutes, followed by 94°C for 1 minute; annealing at 60°C for 45 seconds; and elongating at 72°C for 45 seconds, for a total of 30 cycles. A final extension was carried out for 10 minutes at 72°C. The 995-bp PCR product was visualized in an ethidium bromide–stained 2% agarose gel. Adult human dermal fibroblasts (Cascade Biologics, Portland, Ore) were used as a positive control, and water (no DNA added to PCR) was the negative control in tubes with and without added primers.

#### IMMUNOHISTOCHEMICAL ANALYSIS

Freshly isolated RECs or a basal cell–enriched population were cytospun onto 3-aminopropyltriethoxy-silane–treated glass slides and air dried.\textsuperscript{16,17} Positive control cells for vimentin staining were adult human dermal fibroblasts. Endogenous peroxidase activity was quenched with 3% hydrogen peroxide in deionized water, and nonspecific binding was blocked using 0.05% casein in phosphate-buffered saline. Keratinocyte growth factor polyclonal antibody (Chemicon, Temecula, Calif) was used at 1:50; vimentin (mesenchymal cell marker) was used at 1:1200; and AE3 mouse monoclonal epithelial cell keratin antibody (ICN Biomedicals, Aurora, Ohio) was used at 1:50. After incubation in primary antibody, the slides were washed and peroxidase-labeled secondary antibodies (ICN Biomedicals) were added to the AE3 and vimentin slides. For KGF, preabsorbed biotinylated donkey anti–rabbit secondary antibody was applied, followed by a streptavidin peroxidase conjugate (Shandon Lippshaw, Pittsburgh, Pa). The brown color reaction product was then rehydrated using diaminobenzadine as the chromogen, and the cells were counterstained with hematoxylin, dehydrated and coverslipped. Isotype-matched IgG controls were used at the same concentrations as the relevant primary antibodies, and an additional control consisted of using phosphate-buffered saline instead of the primary antibody.

#### RESULTS

Freshly isolated RECs were not contaminated with fibroblasts, as the cells were positive for epithelial cell cytokeratin (AE3) and negative for vimentin (mesenchymal cell marker) (Figure 1A and B, respectively). Furthermore, during culture, there was no overgrowth of fibroblasts (not shown). Immunostaining for KGF protein revealed a positive subset of RECs (Figure 2B). Isotype-matched IgG control (Figure 2A) and sense-strand riboprobes in situ hybridization signals (Figure 2D) were negative, as were the probe-free and ribonuclease-digested PCR controls (not shown).
tion analysis detected KGF RNA in positive control fibroblast cells and in freshly isolated RECs (Figure 3, lanes 3 and 4, respectively). The bands were the predicted 995-bp size. No bands were observed in lane 1, where there was no input complementary DNA template or primers, or in lane 2, where the template was absent but the primers were present.

**COMMENT**

Our studies demonstrated the presence of both the message and the protein for KGF in a subset of RECs. The epithelial origin of the RECs was confirmed by the absence of the mesenchymal intermediate filament protein vimentin and by the presence of the epithelial cytokeratin marker AE3. The KGF was detected in freshly isolated cells, and the presence of both KGF message and protein is suggestive of a potential autocrine growth regulatory mechanism in these cells. This idea has been put forth by Parrott et al., who found that ovarian surface epithelium expressed KGF RNA and protein. Ovarian surface epithelium cells also responded to KGF both in an autocrine manner and in a paracrine manner through interactions with adjacent stromal cells. Ovarian surface epithelial cells differ from RECs in that they possess characteristics of both epithelial and mesenchymal cells and may be relatively immature uncommitted cells. Interestingly, ovarian surface epithelial cells, endometrial epithelial cells, and RECs are all embryologically derived from endoderm. The stem cell of the airway has not been definitively identified, and there is considerable debate about which of the major epithelial cell types (basal, mucous,
and that of other epithelial motogens and mitogens, such as KGF, promote keratinocyte cell attachment and migration to cover tracheal damage. Keratinocyte growth factor (KGF) promotes keratinocyte cell proliferation and migration in collagen and fibronectin. Cell Adhes Commun. 1999; 7:211-221.

One possible reason for autocrine growth regulation in tracheal epithelium is that unlike excessive granulation tissue formation in skin, in which interstitial tissue overgrowth (fibrosis) and scarring are generally not life threatening, excessive granulation tissue formation in the trachea may result in airway narrowing and compromise. If epithelial regeneration can in part be independent of connective tissue control, then intraepithelial wound repair could stimulate migration and proliferation to cover tracheal defects before fibroblasts and connective tissue overgrow the airway's lumen. Keratinocyte growth factor acting as a primary response protein would stimulate its own secretion and that of other epithelial motogens and mitogens, such as transforming growth factor α. Our observations of KGF message and protein in RECs suggest that this growth factor may play a central role in stimulating rapid restitution of these cells to restore barrier function of the trachea. Augmenting local levels of KGF and/or targeted delivery of KGF to areas of injury may prove to have important clinical applications to repair of the large conducting airway and other organ systems.

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