Keratinocyte Growth Factor Improves Repair in the Injured Tracheal Epithelium

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Keratinocyte growth factor (KGF) is a critical growth factor in lung development and is a protective agent after lung injury, although the exact mechanisms of this protective effect have not yet been elucidated. Our laboratory has shown that circulating epithelial progenitor cells can traffic to the airway and that they appear to be derived from the bone marrow. On this basis, we hypothesized that KGF and its putative receptor (KGFR) would be important to these cells. We showed that the KGFR, which is found almost exclusively on epithelial cells, was present on cells in the bone marrow and circulation of mice that identified a subpopulation of cytokeratin 5+ circulating epithelial progenitor cells (CEPC). In addition, the KGFR co-localized with a population of cytokeratin 5+ basal cells in the repairing proximal airway. Systemic administration of KGF resulted in a significant increase in mobilization of cytokeratin 5+ CEPC at 6 h after injection. Administration of KGF to mouse recipients of heterotopic syngeneic tracheal transplants resulted in protection and more rapid repair of the tracheal epithelium, with an increase in the number of CEPC in the epithelium of the airway, and this effect was abrogated by blocking CEPC with anti-CXCL12 antibodies. KGF therefore appears to be an important growth factor for local resident progenitor epithelial cell repair and for mobilization and enhanced engraftment of CEPC to the injured proximal airway epithelium.

Keywords: stem cells; mobilization; proximal airway repair; tracheal transplantation

Keratinocyte growth factor (KGF) is a member of the fibroblast growth factor family and is also known as FGF-7 (1, 2). The receptor for keratinocyte growth factor (KGFR or FGFR2-IIIb) is the sole known high-affinity receptor for KGF (3). The unique, almost exclusive, localization of the KGFR on epithelial cells, and the production of the KGF ligand by stromal cells, suggests that this growth factor acts in a paracrine manner to exert its important effects on epithelial-mesenchymal interactions (4, 5). As would therefore be predicted, KGF plays a critical role in lung development, and overexpression of KGF in the lung results in papillary cystadenomas with enlargement of bronchial airspaces (6). Inhibiting the effects of KGF with a dominant-negative mutant of the KGFR results in an absence of branching morphogenesis within the developing lung (7).

KGF is an important growth factor for a number of organs. It has been shown to be involved in proliferation, migration,

CLINICAL RELEVANCE

These findings are important for our understanding of proximal airway repair mechanisms and provide a potentially useful new clinical application for keratinocyte growth factor.

and differentiation of epithelial cells in the skin, cornea, intestines, and distal airway (8–16). KGF plays a unique role in wound healing and has been best studied in skin wounding, where it is a potent and highly specific mitogen for keratinocytes (17). KGF therefore has important functions in repair and regeneration.

KGF administration has also been shown to protect the lung from a number of toxins, including hyperoxia (18), radiation, bleomycin exposure (19, 20), acid instillation (21), and graft-versus-host disease after allogeneic bone marrow transplantation (22), in both mouse and rat model systems. Cytokines, such as IL-1 β , TNF- α , and IL-6 have been shown to increase KGF mRNA expression in cultured fibroblasts (23). To date, investigators have shown a number of effects of KGF on the airway epithelium including cell proliferation (9, 15, 16, 24), migration, differentiation (9, 16), survival, DNA repair (25), and induction of enzymes involved in the detoxification of reactive oxygen species (26). The Akt signaling pathway appears to be an important mediator of the anti-apoptotic effects of KGF (27). However, the beneficial effects of KGF *in vivo* appear to be multifactorial, and all the mechanisms have not yet been elucidated.

We have identified a circulating epithelial progenitor cell (CEPC) that is present in the bone marrow and circulation that expresses the basal cell immature cytokeratin, cytokeratin 5 (CK5) (28). These CEPCs contribute to repair of the proximal airway epithelium in a mouse model of syngeneic tracheal transplantation (28). We hypothesized that mobilization of these CEPCs to the circulation would facilitate more rapid airway epithelial repair. We further postulated that an epithelial-specific growth factor could be important in mobilizing CEPC and enhancing engraftment of CEPC to injured airway epithelium. The epithelial growth factor, KGF, is expressed by fibroblasts as well as endothelial cells (29) and activated $\gamma\delta$ T cells (30), which could be involved in the secretion of KGF in the circulation to mobilize KGFR-expressing cells after injury. We therefore examined KGF as a candidate growth factor for CEPC mobilization and potential improvement of airway repair in the murine heterotopic tracheal transplant model.

MATERIALS AND METHODS

Mouse Tracheal Transplant Model

We used a well-established, reproducible murine model of tracheal epithelial regeneration using syngeneic subcutaneous tracheal transplants from wild-type C57Bl/6 mice into C57Bl/6 GFP+ mice (Jackson

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Labs, Bar Harbor, ME) (10, 11). Mice that received tracheal transplants were treated with recombinant human KGF (10 μ g/mouse) intraperitoneally on Days -3, -2, and -1 before tracheal transplantation. Control animals received 10 μ g of mouse serum albumin by intraperitoneal injection on the same dosing schedule. The mice that donated their tracheas for transplantation did not receive human KGF or control vehicle. Animal use for these studies was approved by the Department of Laboratory Animal Medicine, David Geffen School of Medicine at UCLA. We transplanted four tracheas heterotopically into each recipient mouse. A total of three mice were used per group, and 12 tracheal transplants examined and scored per group. The experiment was performed in duplicate.

Epithelial Repair Score

Histopathologic sections from the tracheal transplants were stained with hematoxylin and eosin and examined for epithelial cell repair. A scoring system was devised and applied by a pathologist who was blinded as to whether the tracheal transplant sections were from KGF or control vehicle-exposed mice. A score of 1 was given for a few cells to 1 layer of epithelial cells on the basement membrane. A score of 2 reflected 2 layers of epithelial cells, a score of 3 was for 3 layers of more columnar epithelial cells, 4 was for pseudostratified epithelium without ciliated and mucus cells, and a score of 5 represented a fully repaired pseudostratified columnar epithelium with mucus and ciliated cells.

Mobilization of CEPC

Recombinant human KGF (10 μ g/mouse; Sigma, St. Louis, MO) was injected intraperitoneally ($n = 5$ mice), and bone marrow and buffy coat were harvested for FACS analysis of the CK5+ cells at two time points, namely 6 and 24 h after the administration of KGF. Negative control animals ($n = 5$ mice) were treated with 10 μ g of mouse serum albumin by intraperitoneal injection on the same dosing schedule and their bone marrow and buffy coat were also harvested for FACS analysis of CK5+ cells at the same time points. The experiment was performed in duplicate. We also injected mice with KGF or vehicle control and compared CK5+ and Collagen1+ cell populations in the buffy coat 6 h after the injections ($n = 5$). The recombinant human KGF, which has the native KGF sequence, has a half life of about half a day (31).

Depletion of CXCL12

F(Ab')₂ fragments of neutralizing goat anti-mouse CXCL12 antibody were injected intraperitoneally 1 h before tracheal transplant (28).

Immunohistochemistry

Tracheal tissue was fixed in 4% paraformaldehyde overnight and then embedded in paraffin and sectioned. Sections (4 μ m) were deparaffinized in xylene and rehydrated in graded ethanols. The sections were boiled in glyca-based antigen retrieval buffer (Biogenex Laboratories Inc., San Ramon, CA) for 20 min. Blocking was performed with Universal Blocking Reagent (Biogenex). The primary antibodies used were goat-anti-GFP (Rockland anti-GFP antibody with minimum crossreactivity to mouse) (dilution 1:200; Gilbertsville, PA), monoclonal-anti-mouse FGFR2 (IIIb) (KGFR) (dilution 0.3 μ g/ μ l; R&D, Minneapolis, MN), rabbit-anti-mouse CK5 (dilution 1:200; Abcam, Cambridge, MA), rabbit-anti-human/mouse activated caspase-3 antibody (dilution 1:500; R&D) and anti-PCNA (dilution 1:1,000; Abcam). Appropriate biotinylated secondary antibodies were used and the signal amplified with the ABC kit (Vector Labs, Burlingame, CA) per the manufacturer's protocol. Peroxidase staining was detected with DAB substrate (Vector Labs) and hematoxylin was used as a counterstain.

The commercially available anti-KGFR antibody that we used reacts with the IIIb isoforms of the mouse fgfr2 receptor. It has no crossreactivity with the IIIc isoform of the mouse fgfr2 receptor or with the mouse fgfr3 receptor. The antibody is therefore specific for the mouse KGFR.

Immunofluorescence

Tissue sections were deparaffinized, and antigen retrieval and blocking were performed as for immunohistochemistry above. The primary antibodies used were as for immunohistochemistry above: anti-mouse CK5 (dilution 1:200; Abcam), anti-GFP (dilution 1:200; Rockland), and anti-

KGFR (dilution 1:200; R&D). Biotinylated secondary antibody (Jackson Immuno Research Labs, West Grove, PA) followed by Streptavidin-Cy3 (Jackson Immuno Research Labs) was used to amplify the signal. For dual immunostaining, avidin and biotin (Vector) blocking steps were performed before repeating the above protocol with the second primary antibody. Microscopy was performed with a Zeiss Axioskop 2 Plus fluorescent microscope and Axiocam HRC camera (Carl Zeiss, Oberkochen, Germany) and imaged with an Axiovision scanner and LE Real 4.2 software.

FACS Analysis of Buffy Coat and Bone Marrow Cells

FACS staining was performed in 96-well disposable plates. Bone marrow was isolated from mouse femurs by flushing with PBS through a 26-gauge needle into RPMI-1640 medium. Collagenase A (Roche Biosciences, Palo Alto, CA) and DNase (Sigma) were added to the bone marrow, thoroughly mixed, and incubated at room temperature for 20 min to create a single-cell suspension. The red blood cells were lysed and 1×10^6 cells were plated in each well of the 96-well plate for immediate FACS analysis. Peripheral blood was obtained from mice by retro-orbital bleeding. Five hundred microliters of mouse whole blood was collected from a single mouse in an Eppendorf tube with 50 μ l of heparin to prevent clotting. Single mouse samples were not combined. The whole blood was centrifuged at 3,000 rpm for 10 min to separate out the fractions. The layer of nucleated cells between the plasma layer and the red blood cell layer forms the buffy coat. The buffy coat was aspirated and red blood cells lysed using Ammonium Chloride Potassium lysis buffer (ACK). A quantity of 1×10^5 cells was placed into each well of a 96-well plate for immediate FACS analysis. We used the purified rat anti-mouse CD16/CD32 (Fc γ III/II receptor) monoclonal antibody (Mouse BD Fc block, 0.5 μ g/well) in all of our conditions to reduce nonspecific antibody binding to the Fc γ III/II receptor. Antibodies used were anti-mouse-CK5 (Abcam), PerCP-anti-mouse-CD45 (BD Biosciences, San Jose, CA), anti-mouse-KGFR (R&D), and anti-mouse-Collagen1 (Chemicon, Temecula, CA). Cells were permeabilized with Cytotfix/Cytoperm (BD Biosciences) after surface staining but before CK5 and KGFR staining, as CK5 is an intracellular protein and the KGFR antibody is to the intracellular portion of the receptor. Isotype antibody controls were performed for all antibodies used and were added either before or after cell permeabilization, depending on whether the antigen was intra- or extracellular. Fluorescent cell staining was analyzed with a BD Flow Cytometer and Cellquest software (BD Biosciences).

RESULTS

Characterization of KGFR in the Bone Marrow, Circulation, and during the Repair of the Airway Epithelium

The KGFR is expressed specifically on epithelial cells. We used FACS analysis of uninjured mouse buffy coat (BC) and bone marrow cells and demonstrated that there were nucleated cells in the circulation and bone marrow that expressed KGFR (Figure 1). The KGFR-expressing cells in the circulation represented 2.9% of nucleated cells (Figure 1ii), with $1.9 \pm 0.2\%$ of nucleated cells expressing both KGFR and CD45 (Figure 1iii). The percentage of CD45+, KGFR+ nucleated cells (R1) that also expressed CK5 was $74.4 \pm 7.4\%$ (Figure 1iv). The percentage of CD45+, KGFR+ cells (R1) that also expressed the chemokine receptor CXCR4 was $16.6 \pm 2.6\%$ (Figure 1v). In the bone marrow, 1.2% of nucleated cells (Figure 1vii) expressed the KGFR, and $1.5 \pm 0.4\%$ of the nucleated cells co-expressed CD45 and the KGFR (Figure 1viii). Of these CD45+, KGFR+ bone marrow cells (R2), $22 \pm 3.4\%$ also expressed CK5 (Figure 1ix). The difference between CK5 expression on CD45+, KGFR+ cells from the bone marrow and BC was statistically significant ($P < 0.001$). Of the CD45+, CK5+ cells, $12 \pm 5.5\%$ (R2) also expressed CXCR4 (Figure 1x). There was no statistically significant difference between the expression of CXCR4 in the CD45+, KGFR+ cells from the bone marrow or BC ($P = 0.09$).

Figure 1. Representative FACS analysis of KGFR expression on cells from the BM and buffy coat. (i–v) FACS analysis on buffy coat ($n = 5$). (i) Isotype antibody control of nucleated cells from the buffy coat. (ii) The KGFR-expressing cells in the circulation represented 2.9% of nucleated cells. (iii) Of the KGFR-expressing cells, 1.5% also expressed CD45 (R1). (iv) The percentage of CD45+, KGFR+ nucleated cells (R1) that also expressed CK5 was 92%. (v) The percentage of CD45+, KGFR+ cells (R1) that also expressed the chemokine receptor CXCR4 was 17.2%. (vi–x) FACS analysis on bone marrow ($n = 5$). (vi) Isotype antibody control of nucleated cells from the buffy coat. (vii) 1.2% of nucleated cells expressed the KGFR. (viii) 1.4% of the nucleated cells co-expressed CD45 and the KGFR. (ix) Of these CD45+, KGFR+ bone marrow cells (R2), 16.4% also expressed CK5. (x) Of the CD45+, CK5+ cells, 10.8% (R2) also expressed CXCR4.

To determine the expression pattern of the KGFR on airway epithelial cells in our tracheal transplant model, we performed IHC of KGFR on sections of transplanted tracheas at Days 1, 3, 14, and 21 after transplant. The KGFR was not expressed on the submucosal gland ducts but was expressed on the sloughed epithelium in the lumen of the trachea at 1 d after transplant (Figure 2Ai). The isotype control for Figure 2Ai is shown in Figure 2Aii. At Day 3 after transplant there was robust expression of the KGFR in the repairing airway epithelium and submucosal glands (Figure 2Aiii). By Day 14 after transplant the KGFR was present on the apical epithelial cell surface of the airway (Figure 2Aiv), and remained present at Day 21 after transplant in the fully repaired airway epithelium (Figure 2Av). Isotype control immunostaining at Day 21 after transplant is shown in Figure 2Avi. To determine whether the KGFR is expressed on basal resident CK5+ cells in the repairing airway epithelial tracheal transplant model, we performed dual immunofluorescence (IF) with primary antibodies for CK5 and the KGFR. We found a subpopulation of the repairing proximal airway epithelium that expressed both the KGFR and CK5 as seen by the *yellow-stained cells* in Figure 2B at Day 7 aftertransplant (Figure 2B). Many of the basal resident CK5+ cells (*red cells*), however, did not express the KGFR (Figure 2B). There were also some cells that expressed KGFR but did not also express CK5 (*green cells*, Figure 2B).

KGFR Mobilizes Circulating Epithelial Progenitor Cells

We have identified circulating epithelial progenitor cells (CEPC) that are CK5+ and are involved in repair of the airway epithelium (28). We hypothesized that since we saw KGFR+ cells in the circulation and BC, one mechanism of action for the protective effect of KGF after airway injury could be by mobilization of CEPC from bone marrow into the circulation. We therefore injected mice intraperitoneally with KGF ($n = 5$) or vehicle control ($n = 5$) and examined the number of CK5+ cells in the circulation and bone marrow. At 6 h after injection we detected a 1.7-fold increase in the number of CK5+ cells in the circulation (i.e., BC) (Figures 3A and 3B) ($P < 0.05$), but no change in the

number of CK5+ cells in the bone marrow (Figure 3B). By comparison, intraperitoneal CXCL12 injection resulted in a 3-fold increase in the number of CK5+ cells in the circulation 6 h after injection (Figure 3A) ($P = 0.001$). At 24 h after injection of KGF, we did not detect any further increase in the number of CK5+ cells in the circulation (BC) or bone marrow (Figure 3B). There was no statistically significant difference between mobilization of CEPC with KGF or CXCL12 ($P = 0.1$).

To examine whether KGF specifically mobilizes only CEPC or other progenitor cells too, we injected uninjured mice ($n = 5$) with recombinant human KGF or vehicle control ($n = 5$) and examined the buffy coat from the mice 6 h later by FACS analysis. We found an increase of 2.9×10^5 CK5+ cells to 6.1×10^5 CK5+ cells after KGF ($P < 0.05$). We also found an increase of 2.7×10^5 Collagen1+ cells to 5.2×10^5 Collagen1+ cells after KGF ($P < 0.05$).

KGFR Improves Epithelial Repair in the Tracheal Transplant Model

KGF is used clinically in patients receiving bone marrow transplants as it has been shown to prevent mucositis and improve patient outcomes. We therefore used the same dose and timing of KGF as is used clinically, and injected mice intraperitoneally with KGF (10 μ g per dose) ($n = 5$) or control vehicle ($n = 5$) on Days -3, -2, and -1 before tracheal transplantation (Figure 4A). Mice were killed 24 h after injection and tracheal transplants were examined. An epithelial repair score was devised by a pathologist (M.C.F.), who scored the epithelial repair of the tracheal transplant tissue in a blinded fashion. The scoring system was graded by the best appearing area of epithelium (Figure 4B).

Using this scoring system, recipient mice that received KGF before tracheal transplantation had a mean repair score of 3.5 and a median repair score of 5, at 24 h after transplantation (Figure 4C). This was statistically significantly different ($P = 0.001$) when compared with control vehicle-injected mice, which had a mean repair score of 1.5 and a median of 1 (Figure 4C). At the same time point after transplantation, we also graded

Figure 2. (A) Immunostaining of KGFR expression in the repairing syngeneic tracheal transplants. (i) The KGFR was not expressed on the submucosal gland ducts but was expressed on the sloughed epithelium in the lumen of the trachea at 1 d after transplant. (ii) Isotype control immunostaining at Day 1 after transplant. (iii) At 3 d after transplant there was robust expression of the KGFR in the repairing airway epithelium and submucosal glands. (iv) By 14 d after transplant the KGFR was present on the apical epithelial cell surface of the proximal airway. (v) The KGFR was still present at 21 d after transplant in the fully repaired airway epithelium. (vi) Isotype control immunostaining at Day 21 after transplant (vi). (B) Dual immunofluorescence of KGFR and CK5 expression in the repairing airway epithelium at 7 d after tracheal transplantation. CK5 is stained with Cy3 (red) and KGFR is stained with FITC (green). Dual staining of CK5 and KGFR is seen in yellow cells. A subpopulation of the repairing proximal airway epithelial cells expressed both the KGFR and CK5 (yellow cells) at 7 d after transplant. Many of the basal resident CK5+ cells (red cells) did not express the KGFR. There were also some cells that expressed the KGFR but did not co-express CK5 (green cells).

the epithelial injury by examining the percentage of tracheal epithelium that was sloughed in the tracheal transplants. In the tracheal transplants from mice that were transplanted into KGF-exposed mice, $43.3 \pm 7.0\%$ of the tracheal epithelial circumference was sloughed compared with control vehicle-exposed recipient mice, in which the tracheal epithelium had a percentage of sloughed epithelium of $74.2 \pm 6.6\%$ ($P = 0.012$) at 24 h after transplantation (Figure 4D).

To determine whether KGF effected the presence of CEPC in the airway epithelium during tracheal injury, we next transplanted wild-type tracheas into GFP+ mice to evaluate the number of CK5+ cells in the repairing airway epithelium 24 h after transplant that were derived from the circulation and were therefore also GFP+ (Figure 5). The absolute number of CK5+GFP+ cells per high-power field (PHF) was determined and found to be 11.2 ± 2.1 cells PHF in the recipient mice that received KGF, as compared with 3.4 ± 0.6 cells PHF in the control vehicle-treated mice ($P = 0.01$) (Figures 5A and 5B).

To determine whether there was a connection between KGF/KGFR and CXCL12/CXCR4 on CEPC, we examined the effect of anti-CXCL12 antibodies on the CXCR4-expressing CEPC by performing tracheal transplants in recipient mice that received

either KGF alone intraperitoneally or KGF and anti-CXCL12 F(Ab')₂ fragments intraperitoneally (Figure 5C). The epithelium of the tracheal transplants was scored 24 h after transplant, as described in Figure 4. We found that compared with the group of recipient mice that received KGF alone and had a mean tracheal repair score of 4 and a median repair score of 5, the KGF and anti-CXCL12 F(Ab')₂ antibodies injected group had a mean repair score of 2.7 and a median of 2 ($P < 0.001$) (Figure 5C).

KGF Prevents Apoptosis and Promotes Proliferation of the Submucosal Cells in the Tracheal Transplants

KGF has been shown to have anti-apoptotic and proliferative effects in the injured lung parenchyma. We therefore considered whether these may also be playing a role in the airway of the tracheal transplants. Immunohistochemical staining for activated caspase-3 revealed that the proximal airway epithelium of the tracheal transplants was protected by treating recipient mice with KGF (Figure 6i) with almost no staining for activated caspase-3, when compared with the control vehicle-treated mice where the sloughed off epithelium, as expected, showed some cells that

Figure 3. (A) Mobilization of circulating epithelial progenitor cells by CXCL12 and KGF. At 6 h after intraperitoneal injection of CXCL12, we detected a 3-fold increase in the number of CK5+ cells in the circulation ($P < 0.001$) ($n = 5$). At 6 h after intraperitoneal injection of KGF we detected a 1.7-fold increase in the number of CK5+ cells in the circulation ($P < 0.05$) ($n = 5$). There was no statistically significant difference between mobilization of CK5-expressing cells with KGF or CXCL12 ($P = 0.1$). (B) Fold changes in CK5+ cells at two time points in the BM and buffy coat after KGF administration. At 6 h after intraperitoneal injection of KGF, we detected a 1.7-fold increase in the number of CK5+ cells in the circulation but no change in the number of CK5+ cells in the bone marrow ($n = 5$). At 24 h after injection we did not detect any increase in the number of CK5+ cells in the circulation or bone marrow. (C) Mobilization of circulating epithelial progenitor cells and fibrocytes by KGF. At 6 h after intraperitoneal injection of KGF we found a 1.8-fold increase in the number of CK5+ cells compared with injection of vehicle control ($P < 0.001$). We also found a 1.4-fold increase in the number of collagen1+ cells in the buffy coat ($P < 0.05$) ($n = 5$).

positively stained for activated caspase-3 (Figure 6iii). The isotype controls are shown in Figures 6ii and 6iv.

To assess proliferation, we performed immunohistochemistry for the proliferative nuclear marker, PCNA, which revealed no difference in the number of PCNA⁺ proliferating epithelial cells PHF in the KGF-exposed mice compared with the control vehicle-exposed mice (1.12 ± 0.46 versus 1.59 ± 0.84 cells PHF; $P = 0.19$) (Figures 7A and 7B). However, there was a significant difference in the number of proliferating cells PHF in the submucosal area of the tracheal transplants in the KGF-exposed recipient mice compared with the control vehicle-exposed mice (1.32 ± 0.34 versus 2.36 ± 0.94 cells PHF; $P = 0.049$) (Figure 7A). Figure 7Bi demonstrates PCNA nuclear staining in the epithelium of a tracheal transplant section from a control vehicle-treated recipient. Figure 7Bii demonstrates PCNA nuclear staining from a KGF-treated tracheal transplant. Positive nuclei are seen in the epithelium and submucosal areas. The isotype antibody controls are shown in Figures 7Bii and 7Biv.

DISCUSSION

Our data demonstrated that KGFR was present on cells in the bone marrow and circulation and that a subpopulation of these cells also expressed CK5. We found differences between the KGFR⁺ cells in the bone marrow and circulation, in that more than 90% of the KGFR-expressing cells in the bone marrow also expressed CD45, while $\sim 66\%$ of the KGFR-expressing cells in the circulation co-expressed CD45. This was analogous to our previous studies in which the CK5+ population of cells lost CD45 expression in circulation (28). In addition, CD45+ KGFR⁺ cells demonstrated a significant increase in CK5 expression in the circulation compared with the bone marrow.

We showed that KGF mobilized CK5+ cells into the circulation at 6 h after injection, and this effect did not persist out to 24 h after injection. This would suggest that mobilization of CEPC is a fairly rapid event, which is analogous to the mobilization of hematopoietic cells. In addition, no expansion in CK5+ cells after KGF was seen at 6 or 24 h after injection in the bone marrow. We chose a dose of KGF that approximates the dose of KGF currently used in patients to relieve mucositis after bone marrow transplantation (~ 1 mg/kg). In addition, our data demonstrate that KGF does not selectively mobilize CEPC and that Collagen1+ fibrocytes are also mobilized, suggesting that KGF may be important as an initial response for wound healing.

Many investigators have shown the mitogenic and proliferative effects of KGF in the lung parenchyma and bronchial airways (9, 10, 15, 16, 18–22, 24–27, 32, 33). We have now demonstrated that KGF is also important for tracheal airway repair and that the KGFR is present on some of the CK5+ basal progenitor airway epithelial cells in the trachea. The KGFR has been shown to be present on other epithelial progenitor cell populations and has been associated with the transiently amplifying pool of CD133-negative basal cells in the prostate (34) and with the basal cells of the limbus of the cornea (35). The KGFR may therefore represent a novel marker that defines the transiently amplifying pool of basal progenitor epithelial cells.

KGF has been shown to improve distal airway repair by a number of local mechanisms including mitogenic effects, anti-apoptotic effects, and inhibition of oxygen free radicals (9, 10, 15, 16, 18–22, 24–27, 32, 33). Previous work showed that terminal airway epithelial cell proliferation peaked at Days 1–2 after endobronchial administration of recombinant human KGF (36). Our findings support the potential of another mechanism of

Figure 4. (A) Schema of KGF dosing. The KGF dosing schedule was based on the dose and timing of KGF that is currently used clinically in patients undergoing bone marrow transplantation. The recipient mice received KGF (10 μ g) or mouse serum albumin intraperitoneally on three consecutive days, 24 h apart (i.e., days -3, -2, and -1). The tracheal transplants were performed 24 h after the last dose of KGF on Day 0. The recipient mice were killed 24 h after receiving tracheal transplants on Day +1, and the tracheal transplants were harvested for studies. The donor mice did not receive KGF. (B) Epithelial repair score. The best area of epithelium on the tracheal transplants was graded as follows. Score 1: few epithelial cells to one layer of epithelial cells on the basement membrane; Score 2: two layers of cells; Score 3: three layers of epithelial cells and a more columnar appearance of the cells; Score 4: multiple layers of epithelial cells but no ciliated or mucus cells; Score 5: fully repaired pseudostratified columnar epithelial cells with ciliated, mucus, and secretory cells. (C) Epithelial repair scores of tracheal transplants from vehicle control and KGF-injected mice. Recipient mice that received KGF before tracheal transplantation had a mean repair score of 3.5 and a median repair score of 5 ($n = 12$ tracheal transplants). This was statistically significantly different ($P = 0.001$) when compared with control vehicle injected mice who had a mean repair score of 1.5 and a median of 1 ($n = 12$ tracheal transplants). (D) Examination of the percentage of tracheal epithelium that was sloughed in the tracheal transplants. In the tracheal transplants from mice that were transplanted into mice that received KGF, $43.3 \pm 7.0\%$ of the tracheal epithelial circumference was sloughed compared with control vehicle-exposed recipient mice, in which the tracheal epithelium had a percentage of sloughed epithelium of $74.2 \pm 6.6\%$ ($P = 0.012$).

action for KGF in mobilizing CEPC from the bone marrow into the circulation. We found that KGF decreased the percentage of tracheal epithelial circumference that sloughed after the ischemic injury of tracheal transplantation. In the areas of the proximal airway epithelium that were preserved 24 h after transplantation, KGF appeared to have an anti-apoptotic effect, and most of these cells were derived from the donor mouse epithelium and not from the circulation. In contrast, repairing areas of tracheal epithelium contained numerous CK5+GFP+ cells (CEPC) at 24 h after transplantation, which were statistically significantly increased in the mice that received KGF compared with control vehicle-exposed mice. In addition, the CXCR4/CXCL12 biological axis appeared to play a role in the recruitment of the CEPC, as neutralizing antibodies to CXCL12 abrogated the effect on enhanced repair that resulted from the mobilization of CEPC by KGF. Taken together, these results suggest that the ability of KGF to strengthen epithelial integrity includes

recruitment of CEPC to aid in airway repair and that this occurs in addition to the other described mechanisms of action of KGF.

In the airway, KGF expression has been found in cultured bovine tracheal explants and the timing of KGF expression varied on the basis of the type of injury to the airway and whether the basement membrane was intact. In this model, KGF expression was first seen at 16–24 h after injury in the submucosal area after chemical injury with an intact basement membrane (37). In contrast injury to the suprabasal layer of proximal airway epithelium did not result in KGF expression (37). In our studies, we saw by immunohistochemistry very little KGFR expression in the submucosal glands at 24 h after injury. However, we saw a robust increase in KGFR expression at Day 3 after transplant in the tracheal epithelium and submucosal glands, which correlated with the increase in KGF expression at 16–24 h after injury.

Other mechanisms of action of KGF were also apparent in the tracheal transplants. In the recipient mice that were exposed

Figure 5. (A) Determination of the number of CK5+GFP+ cells in the repairing airway epithelium at 24 h after transplant. The absolute number of CK5+, GFP+ cells PHF was determined by dual immunofluorescence and found to be 11.2 ± 2.1 in the recipient mice that received KGF as compared with 3.4 ± 0.6 in the control vehicle-treated mice ($P = 0.01$) ($n = 12$ tracheal transplants per group). (B) Representative dual immunofluorescence of tracheal transplant tissue at 24 h after transplant. (i) CK5+GFP+ cells in the repairing airway of a tracheal transplant from a recipient mouse that received KGF. (ii) CK5+GFP+ cells in the repairing airway of a tracheal transplant from a recipient mouse that received control vehicle. (C) Tracheal repair scores 24 h after transplantation from recipient mice that received KGF alone or KGF and anti-CXCL12 F(Ab')₂ antibodies. The tracheal transplants from recipient mice that received KGF alone had a mean repair score of 4 and a median epithelial repair score of 5. The tracheal transplants from recipient mice that received KGF and anti-CXCL12 F(Ab')₂ antibodies had a mean repair score of 2.7 and a median repair score of 2 ($P < 0.001$).

to KGF, an anti-apoptotic effect was seen in the tracheal epithelium. However, we did not see at 24 h after transplant an increase in proliferation of tracheal epithelial cells using PCNA. We did, however, note an increase in PCNA-positive cells in the submucosal area in cells with a mesenchymal phenotype. The submucosal glands and intercartilaginous regions are considered the source of resident progenitor cells in the airway. It is therefore possible that the initial effects of KGF may be found in the submucosal area of the airway, rather than in the epithelium. KGF is a potent mitogen, so it is possible that proliferation of epithelial cells occurs at later time points in the repair process.

In conclusion, we have demonstrated that KGF exerts a protective effect on the airway after ischemic injury. This is an important finding because the integrity of the airway epithelium is critical for host defense, and many toxins, viruses, and bacteria cause loss of this epithelium, resulting in respiratory diseases. We have also shown that the bone marrow is a potential reservoir of KGFR-expressing cells that can be recruited to the injured airway under appropriate conditions. While fibrocytes were also mobilized by KGF, we saw an improvement in epithelial repair at 24 h after transplant, which suggests that there are likely other factors that determine engraftment of CEPC or fibrocytes after injury.

Figure 6. Immunohistochemistry for activated caspase-3 on representative tracheal transplant tissue. (i) Almost no nuclear staining is seen in the preserved epithelium in the tracheal transplant from a recipient mouse that received KGF. (ii) Isotype control antibody staining for i. (iii) Activated caspase-3-positive cells are seen with nuclear staining in some of the sloughed cells in the lumen of the tracheal transplant from a recipient mouse that received vehicle control (arrows). (iv) Isotype control antibody staining for iii.

Figure 7. (A) Proliferating cells in the tracheal transplants at 24 h after transplant. Immunohistochemistry for the proliferative nuclear marker, PCNA, revealed no difference in the number of PCNA⁺ proliferating epithelial cells PHF in the KGF-exposed mice compared with the control vehicle-exposed mice (1.12 ± 0.46 cells versus 1.59 ± 0.84 cells; $P = 0.19$). However, there was a significant difference in the number of proliferating cells PHF in the submucosal area of the tracheal transplants in the KGF-exposed recipient mice compared with the control vehicle-exposed mice (1.32 ± 0.34 cells versus 2.36 ± 0.94 cells; $P = 0.049$). (B) Immunohistochemistry with a PCNA antibody in representative tracheal transplants. (i) Nuclear staining for PCNA in a tracheal transplant from a recipient mouse exposed to control vehicle (arrow). (ii) Isotype antibody control for i. (iii) Nuclear staining for PCNA in the epithelium and submucosal area of a tracheal transplant from a recipient mouse exposed to KGF. (iv) Isotype antibody control for ii.

There are a number of clinical conditions in which patients are exposed to viruses or pollutants that cause sloughing of the airway and reduce host defense. KGF may prove to be a valuable tool for improving airway epithelial repair in these situations and improving patient outcome. KGF may therefore have a therapeutic benefit to help maintain the epithelial integrity of the airway epithelium, both by local effects on the resident cells, as well as through mobilization of CEPC to help with the airway repair process.

Conflict of Interest Statement: R.M.S. has lectured as a visiting scientist at Biogen, Novartis, and InterMune and has received an honorarium for talks. None of the authors has a financial relationship with a commercial entity that has an interest in the subject of this manuscript.

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