Keratinocyte growth factor induces Akt kinase activity and inhibits Fas-mediated apoptosis in A549 lung epithelial cells

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Keratinocyte growth factor (KGF), also known as fibroblast growth factor (FGF)-7, is a member of the FGF family and is produced exclusively by fibroblasts. Unlike most FGFs that have broad mitogenic activity, KGF is specific for the epithelium and inhibits Fas-mediated apoptosis in A549 lung epithelial cells. Acute respiratory distress syndrome (ARDS) is a syndrome characterized by the rapid influx of protein-rich edema fluid into the air spaces. The magnitude of alveolar epithelial cell injury is a key determinant of disease severity and an important predictor of patient outcome. The alveolar epithelium is positioned at the interface of the host response in the initiation, progression, and recovery phase of the disease. Keratinocyte growth factor (KGF) is a potent survival factor unique to the epithelium that promotes lung epithelial cell survival, accelerates wound closure, and reduces fibrosis. We therefore hypothesized that KGF preserves lung function by inhibiting apoptosis through activation of a signal transduction pathway responsible for cell survival. To test this hypothesis we determined that KGF inhibits death following Fas activation, a relevant apoptosis pathway, and then determined that cell survival is mediated through activation of the phosphatidylinositol 3'-kinase (PI3K)/Akt kinase signal transduction pathway. We found that KGF induces a dose- and time-dependent increase in Akt kinase activity and that, as expected, activation of Akt via KGF is PI3K dependent. KGF inhibited Fas-induced apoptosis as measured by a reduction in apoptotic cells and caspase-3 activity. This investigation supports our original hypothesis that KGF protects the lung epithelium by inhibiting apoptosis and that protection occurs through activation of PI3K/Akt-mediated cell survival pathway. Our results are in agreement with other reports that identify the PI3K/Akt axis as a key intracellular pathway in the lung epithelium that may serve as a therapeutic target to preserve epithelial integrity during inflammation.

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mulate fluid in the airway and alveolus, resulting in severely compromised ventilation. In addition, this injury can generate maladaptive restoration of the epithelial barrier resulting in fibrotic scarring (2, 3). The pathogenic mechanisms responsible for fluid accumulation and tissue remodeling remain unclear but are believed to involve overattenuation of the inflammatory response and accelerated apoptosis of the epithelium (29). Attenuation of the Fas/Fas ligand (Fas/FasL) pathway has been implicated as one of the primary apoptotic pathways activated in the alveolar epithelium at the onset of ARDS in humans (1, 7, 12). In support of this, Fas-mediated apoptosis of the lung epithelium causes acute lung injury and fibrosis in mice (11, 15, 17) and leads to the development of ARDS and fibrosis in humans (18, 22). Our laboratory has shown that Fas-mediated apoptosis of human lung epithelial cells is modulated by proinflammatory cytokines (5).

Based on these observations we predict that, during localized stress, PI3K and Akt are mobilized by external signals present in the tissue microenvironment, including growth factors, to internally protect the lung epithelium from external apoptotic stimuli that promote an untimely death. Cell preservation becomes a “tug-of-war” between exposure to both pro- and antiapoptotic factors. Specifically, we hypothesized that KGF preserves lung integrity by inhibiting alveolar epithelial cell apoptosis via activation of the Akt survival pathway. To test this we determined that KGF directly activates Akt kinase and that upregulation of kinase activity is linked to inhibition of Fas-mediated apoptosis in A549 cells, a human lung epithelial cell line. We also provide evidence that KGF prevents apoptosis via Akt activation. Based on this, we propose that the PI3K/Akt survival pathway represents a novel proximal therapeutic target that can be exploited to preserve or restore lung function in patients with ARDS.

MATERIALS AND METHODS

Cell culture. A549 cells, a human lung epithelial cell line, were purchased from the American Type Culture Collection (Rockville, MD) (19). Cells were cultured under standard conditions in F-12 medium supplemented with 10% fetal bovine serum (Hyclone, Logan, UT), 100 U/ml penicillin, and 100 μg/ml streptomycin (GIBCO-BRL) (complete growth medium). A549 cells were passaged at 80–90% confluence using 0.25% trypsin, 1 mM EDTA (GIBCO-BRL) (as previously described). At the onset of each experiment, cultures were serum-starved for a period of 48 h before addition of any factors to eliminate background signal noise contributed by serum containing medium.

Experimental conditions for apoptosis and caspase activity measurements. A549 cells were plated at 80–90% confluence in either 25-cm² tissue culture flasks (Falcon/Fisher, Pittsburgh, PA) or eight-well glass chamber slides (Fisher). Cells were incubated for 2 days postpassage before further manipulation to allow cells to reach full confluence. After serum starvation, KGF (10–100 ng/ml, R&D Systems) and/or recombinant human IFN-γ (250 units/ml; BioSource International, Camarillo, CA) were added to cultures. After 24 h, cells were given a second stimulus of either a Fas cross-linking antibody (FasAb, 10–100 ng/ml clone CH-11; Kamiya Biomedical, Seattle, WA) or an isotype control (10–100 ng/ml IgM; Sigma, St. Louis, MO). Cells were incubated an additional 24 h before analysis for the number of apoptotic cells or caspase activity. A549 cells were also stimulated as described above; however, 30 min before the initial stimulus, cells were given the PI3K inhibitor LY-294002 (10 μM; CalBiochem, San Diego, CA) or an equivalent dose of the solvent DMSO as the vehicle control. After 24 h, cells were given a second dose of the above inhibitors or solvent at half the original dose, and 30 min later the cells were stimulated with the FasAb as described above.

Cell extracts. After the treatment described above, adherent cells were trypsinized and pooled with nonadherent cells present in the original medium. Cells were then pelleted at 300 g for 10 min at room temperature, supernatants were discarded, and the cell pellet was resuspended in 200 μl of 1× KPM complete buffer (2× KPM = 100 mM PIPES, pH 7; 100 mM KCl; 20 mM EGTA; and 3.84 mM MgCl₂, 1 mM DTT, 0.1 mM PMSF, 10 μg/ml chymotatin B, 2 μg/ml leupeptin, 2 μg/ml antipain, and 2 μg/ml pepstatin A. Resuspended cells were transferred to 0.5-ml Eppendorf tubes and pelleted again (3,000 rpm for 10 min). Cell pellets were resuspended in 15 μl of 1× KPM complete buffer, frozen in liquid nitrogen, and stored at −70°C.

Enzymatic caspase activity measured with amino trifluoro methyl coumarin. For all amino trifluoro methyl coumarin (APC) preparations, cells (3 × 10⁶ cells) were collected by centrifugation and washed with KPM buffer and lysed by 4 cycles of freeze-thawing as previously described (5). The presence of active caspases was determined by APC assay using a specific fluorophore-substrate as previously described. Lysates were incubated with DEVD-APC in a cyto-buffer (10% glycerol, 50 mM PIPES pH 7.0, and 1 mM EDTA) containing 1 mM DTT and 20 μM DEVD-APC (Enzyme Systems Products). The release of free APC was determined using a Cytofluor 4000 fluorimeter (Perceptive, Framingham, MA; filters: 400 nm for excitation, 505 nm for emission).

Immunohistochemistry. A549 cells were stimulated as described above. Adherent cells were nonenzymatically disadhered and pooled with disadhered cells followed by cytospin preparation of cells onto silane-treated slides. Cells were then rinsed twice with 1× PBS and fixed in ice-cold pure methanol for 30 min at −20°C. After being washed twice with washing buffer (1× PBS and 0.1% Tween 20, buffered with 0.1% Triton X-100), frozen in liquid nitrogen, and stored at −70°C, cells were trypsinized and pooled with nonadherent cells present in the supernatant. Cells were then pelleted at 200 g for 20 min at 4°C. The resulting supernatant or cell extract was removed to a fresh 0.5-ml Eppendorf tube, and an aliquot was analyzed for protein concentration by the Bradford method (Bio-Rad, Hercules, CA), frozen in liquid nitrogen, and stored at −70°C.

Akt in vitro kinase assay. The dose and time response of Akt activation by KGF was determined. A549 cells were first treated with...
increasing doses of KGF (0–100 ng/ml). Next, the optimum dose was used to determine the time-dependent response of Akt activation. Initial studies were done with extended time points (0–12 h). From our initial observations, Akt activation occurred within minutes. On the basis of this, all further studies evaluated Akt with time points ranging between 1 and 20 min. Under all conditions, cells were lysed in 1 ml of ice-cold buffer (50 mM Tris-HCl pH 7.5, 0.1% Triton X-100, 1 mM EDTA, pH 8.3, 1 mM EGTA pH 8.0, 50 mM NaF, 10 mM β-glycerophosphate, 5 mM sodium pyrophosphate, 10 mg/ml aprotime, and 10 mg/ml leupeptin, 1 mM sodium vanadate, and 14.2 mM 2-mercaptoethanol) for 15 min. Nuclei were removed by centrifugation, and then cytoplasmic extracts were immunoprecipitated with an immobilized Akt antibody (Cell Signaling Technology, Beverly, MA). The immunoprecipitates were then removed from beads in cold lysis buffer and measured for Akt kinase activity using glycogen synthase kinase-3 (GSK-3, Cell Signaling Technology) as substrate. Proteins and peptides were separated on a 12% SDS-polyacrylamide gel and then transferred onto nitrocellulose membranes. The lower portion of the membrane was immunoblotted with phospho-GSK-3-specific antibody and then subject to autoradiography. The upper portion of the membrane was immunoblotted with an antibody that recognizes total Akt to confirm equal loading and standardize results.

We semiquantified densitometry of the phospho-GSK-3 by standardizing each sample to its own total Akt blot and presenting it as a complement to the autoradiography results.

**Detection of apoptotic vs. necrotic cells.** The Vybrant Apoptosis Assay Kit (V-13243, Molecular Probes) was used to distinguish apoptotic and necrotic cells under our culture conditions. Briefly, at the end of the treatment period cells were washed in cold PBS and then stained per the manufacturer’s recommendations with green fluorescent YO-PRO-1 dye, which enters and stains apoptotic cells only, and propidium iodide, which enters and stains necrotic cells. Cells were immediately prepared for viewing in a cytospin preparation and enumerated under fluorescent microscopy.

**Statistical analysis.** All data were expressed as means ± SE. Paired t-tests were used for single comparisons (Microsoft Excel; Microsoft, Redmond, WA). For comparisons that involved multiple variables and observations, two- and three-way ANOVA (JMP; SAS Institute, Cary, NC) was used. Having passed statistical significance by ANOVA, individual comparisons were made using the Tukey multiple-comparison test. Statistical significance was defined as a P value < 0.05.

**RESULTS**

**KGF prevents apoptosis.** Caspase processing of a key structural intermediate filament protein unique to epithelial cells, CK18, was used to quantify cellular apoptosis. CK-18 has been identified as a substrate for caspases-3, -6, and -7 during epithelial cell apoptosis (4). Activation of Fas with an anti-FasAb in conjunction with IFN-γ resulted in apoptosis in >20% (23.2 ± 8.8%) of the total population of cells (Fig. 1). An isotype control antibody matched to the FasAb did not induce apoptosis (data not shown). Pretreatment with KGF before the addition of IFN-γ and FasAb significantly decreased the incidence of apoptosis by approximately one-half (12.2 ± 4.9%). Addition of the PI3K inhibitor LY-249002, before KGF, IFN-γ, and FasAb, increased the number of apoptotic cells to >70% of the total population, even in the presence of KGF (74.5 ± 13.2%). Treatment with LY-249002 alone also resulted in apoptosis, without evidence of necrosis as measured by YO-PRO-1 staining, but approximately threefold less (26.5 ± 7.4%) than that measured in the LY-249002/KGF/IFN-γ/FasAb treatment group as well as the LY-249002/IFN-γ/FasAb treatment groups. On the basis of these findings we concluded that the PI3K/Akt survival axis contributes to KGF protection against Fas-mediated apoptosis.

**KGF induces Akt kinase activity.** KGF has previously been shown to promote lung epithelial cell survival, but the mechanisms by which this occurs are not fully understood. Conflicting reports indicate that KGF protects the lung epithelium via mitogen-dependent (28, 34) as well as -independent events (31). KGF did not induce proliferation in A549 cells as measured by a common cell proliferation assay (data not shown), thereby indicating that mitogenic activity is not responsible for cell survival. We reasoned that, to inhibit apoptosis, KGF preserves cell viability through activation of Akt, a key survival factor in mammalian cells. To establish this we first measured the Akt kinase activity in A549 lung epithelial cells in response to increasing doses of KGF. Confluent cultures of serum-starved A549 cells were treated with recombinant KGF for 15 min at increasing doses, and then total cell lysates were analyzed for Akt in vitro kinase activity. Our findings indicate that A549 cells cultured in 10% BSA have a relatively high constitutive level of Akt kinase activity that is reduced approximately twofold with serum starvation (Fig. 2). The high constitutive level of kinase activity can likely be attributed to growth factors present in serum and the fact that the cell line is of cancer origin. Importantly, in the absence of serum, KGF treatment alone restored and elevated Akt kinase activity to levels observed in BSA-positive conditions in a dose-depen-

![Fig. 1. Keratinocyte growth factor (KGF) inhibits Fas-mediated apoptosis.](http://ajplung.physiology.org/)
dent manner. Increasing concentrations of KGF proportionally induced the level of Akt kinase activity with maximum stimulation at 4.5-fold above serum-starved baseline levels at a concentration of 100 ng/ml. Next, we evaluated the time course of Akt kinase induction by KGF. Using a concentration of 100 ng/ml, we found that KGF reproducibly generated a peak in Akt kinase activity within 20 min of exposure (Fig. 2). We did not find additional modulation in kinase function following evaluation of extended time points (data not shown).

Akt induction by KGF requires PI3K. We expected that KGF-mediated RTK activity would result in the recruitment and activation of p85 and p110 PI3K, leading to activation of Akt. To confirm this we evaluated KGF’s ability to induce Akt kinase function in the presence of the PI3K inhibitor LY-249002. Inhibition of PI3K by LY-249002 resulted in a substantial reduction of Akt kinase activity following treatment with KGF in serum-starved A549 lung epithelial cells compared with DMSO treatment alone (Fig. 3). This effect was highly reproducible and observed under optimal conditions for growth factor stimulation following 20 min of exposure. We also consistently showed a moderate decrease in KGF-induced Akt kinase activity following treatment with the vehicle DMSO alone. This is not entirely unexpected since DMSO has antioxidant properties and Akt function is linked to oxidant activation (33).

KGF-induced Akt activation inhibits caspase-3. The intracellular apoptotic cascade that ensues following Fas receptor stimulation requires activation of the “initiator” caspase-8 followed by activation of “executioner” caspases including caspase-3. Our previous result demonstrated that CK-18, a caspase-3 substrate, is enzymatically processed following Fas receptor activation and that cleavage is inhibited by exposure to KGF. Based on this we reasoned that the preservation of lung epithelial cells by KGF is linked to downmodulation of caspase-3 activation by the PI3K/Akt signaling axis. Consistent with previous data, the level of caspase-3 activity increased in cells treated with the combination of IFN-γ and anti-Fas antibody compared with baseline levels (Fig. 4). Pretreatment with KGF significantly decreased the amount of caspase activity in cell lysates. Treatment with LY-249002 reversed the protective effect of KGF and significantly increased the amount of caspase-3 activity. This further supports our previous observation that KGF inhibits apoptosis. Consistent with our earlier findings, LY-249002 treatment alone induced caspase activity. Again, this observation is not surprising when considering that the A549 cell line is derived from human adenocarcinoma tissue and that many cancer cells have been found to possess a relatively high basal level of Akt kinase activity (8).

DISCUSSION

Previously, we reported that apoptosis of the lung epithelium is controlled by inflammatory cytokines and that caspase activation is inhibited by interleukin-1β, an activator of the PI3K/Akt signaling axis (5). To expand upon these observations we turned our attention to KGF, a growth factor unique to the
epithelium with known protective effects that we reasoned would inhibit alveolar epithelial cell apoptosis through activation of a well-known survival signal transduction pathway. To test this hypothesis we determined that KGF inhibits lung epithelial cell death following Fas activation, a relevant apoptosis pathway in ARDS, and then determined that cellular preservation was dependent on activation of the PI3K/Akt kinase signal transduction pathway. In particular, KGF inhibited apoptosis initiated by IFN-γ and FasAb as measured by a reduction in caspase-3 activity following exposure to IFN-γ/H9253. Addition of the PI3K inhibitor LY-294002 further enhanced caspase-3 activity and blocked the ability of KGF to inhibit caspase-3 activation. *P < 0.05.

We predict that PI3K/Akt provides an essential function in the alveolar epithelium as a central survival pathway during intervals of localized stress associated with inflammation and apoptosis. In this scenario, PI3K and Akt are mobilized by external signals present in the tissue microenvironment, including growth factors and cytokines, to internally protect the cell from external apoptotic stimuli that promote an untimely death. Cell preservation then becomes a tug-of-war between external stimuli and the relative timing of exposure to both pro- and antiapoptotic factors. If terminal caspase activation, such as caspase-3, precedes growth factor stimulation, a commitment to death is made that becomes very difficult to reverse. Therefore, therapeutic intervention is complicated by the fact that agents directed at the alveolar epithelium of “at risk” individuals susceptible to developing acute lung injury must be administered before the onset of damage. Our results obtained from A549 cells indicate that KGF provides protection but only when PI3K is active. In the presence of PI3K inhibition, KGF lost the ability to protect cells from apoptosis. Interestingly, a recent investigation conducted in primary rat alveolar type II cells reports that KGF activation was mediated through the extracellular signal-regulated kinase pathway in addition to the PI3K pathway (24). The implication from this as it relates to our findings is that the lung epithelium has redundant signaling mechanisms to convey RTK protection.

Fas, also known as CD95/Apo-1, is constitutively expressed by human alveolar epithelial cells (7, 12) and is believed to play a major role in controlling lung epithelial cell apoptosis during inflammation. Excessive Fas activation in the lung causes significant tissue pathology. Topical administration of FasL to the airway accelerates apoptosis of epithelial cells and causes pulmonary fibrosis in mice (10, 11). Aerosolization of pathogenic bacteria promotes Fas-FasL-dependent increases in permeability and tissue pathology (21). In humans, increased levels of soluble bioactive FasL in bronchoalveolar lavage fluid directly correlate with disease severity in adults with ARDS (1, 22). These observations suggest that Fas-mediated apoptosis of the lung epithelium is an important process in the development of acute lung injury at the onset of ARDS. For this reason, we investigated Fas-mediated apoptosis in A549 cells as a representative model to identify key cellular events that determine cell survival. We investigated external signals that converge upon a common, internal biochemical pathway that facilitates cell survival. We anticipated that identification of biochemical events involved with protection against inflammatory stimuli that promote cell death would reveal a potential treatment strategy to prevent acute lung injury. KGF is a potent survival factor for the lung epithelium and provides protection against many external stimuli. On the basis of our investigation and others, we believe that protection afforded by KGF is at least in part related to activation of the PI3K/Akt pathway and perhaps others. Activation of this survival pathway helps to protect tissue barrier function during inflammatory stress and facilitate wound repair when the barrier is compromised. Sev-
eral reports demonstrate that KGF is a mitogen that promotes repair by inducing cell proliferation (28, 34), whereas others indicate that KGF modifies the cell phenotype by reducing sensitivity to apoptosis without inducing cell division (31). We found that prior treatment with KGF prevented Fas-mediated death without inducing proliferation. Therefore, we predict that one mechanism by which KGF modifies lung epithelial behavior is through activation of survival pathways that arrest the Fas-induced apoptosis before caspase activation. This would also indicate that prophylactic therapeutic strategies can be directed toward viable, differentiated lung epithelium without a requisite for cell proliferation. This is supported by previous studies demonstrating that growth factor stimulation prevents or inhibits apoptosis via the PI3K/Akt pathway (9, 16, 25, 32).

Our results corroborate previous investigations and further establish a framework to dissect the role of KGF-induced inhibition of apoptosis in the human lung epithelium. Our findings indicate that KGF triggers the PI3K/Akt response and induces cell protection presumably through modulation of intermediate proteins located upstream from terminal caspases. We anticipate that this investigation will help to further identify biochemical events that can prevent acute lung injury as it pertains to the epithelium. This investigation supports the hypothesis that KGF can preserve epithelial cell integrity by inhibiting apoptosis and suggests that protection is mediated in part through activation of the PI3K survival pathway. Therefore, the PI3K/Akt axis may serve as a therapeutic target to preserve epithelial integrity and provide a useful strategy to block apoptosis thereby limiting acute lung injury in humans.

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