Keratinocyte growth factor protects against 
*Pseudomonas aeruginosa*-induced lung injury

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Viget, Nathalie B., Benoit P. H. Guery, Florence Ader, Remi Nefiere, Serge Alfandari, Colette Creuzy, Micheline Roussel-Delvallez, Claude Foucher, Carol M. Mason, Gilles Beaucaire, and Jean-François Pittet. Keratinocyte growth factor protects against *Pseudomonas aeruginosa*-induced lung injury. *Am J Physiol Lung Cell Mol Physiol* 279: L1199–L1209, 2000.—We have previously reported that keratinocyte growth factor (KGF) attenuates α-naphthylthiourea-induced lung injury by upregulating alveolar fluid transport. The objective of this study was to determine the effect of KGF pretreatment in *Pseudomonas aeruginosa* pneumonia. A 5% bovine albumin solution with 1 μCi of 125I-labeled human albumin was instilled into the air spaces 4 or 24 h after intratracheal instillation of *P. aeruginosa*, and the concentration of unlabeled and labeled proteins in the distal air spaces over 1 h was used as an index of net alveolar fluid clearance. Alveolo-capillary barrier permeability was evaluated with an intravascular injection of 1 μCi of 131I-albumin. In early pneumonia, KGF increased liquid clearance (LLC) compared with that in nonpretreated animals. In late pneumonia, LLC was significantly reduced in the absence of KGF but increased above the control value with KGF. KGF pretreatment increased the number of polymorphonuclear cells recovered in the bronchoalveolar lavage fluid and decreased bacterial pulmonary translocation. In conclusion, KGF restores normal alveolar epithelial fluid transport during the acute phase of *P. aeruginosa* pneumonia and LLC in early and late pneumonia. Host response is also improved as shown by the increase in the alveolar cellular response and the decrease in pulmonary translocation of bacteria.

Pneumonia; lung liquid clearance

*Pseudomonas aeruginosa* is the most common pathogen involved in nosocomial pneumonia and is responsible for both a high mortality and morbidity in critically ill patients (7). Instillation of this pathogen into the air spaces increases the protein permeability of the lung epithelium to induce both interstitial and alveolar edema (38). Active sodium and chloride transport is the primary mechanism driving alveolar liquid clearance in the normal lung (11, 31). The maintenance of active ion transport is critical for the resolution of alveolar edema (20, 36). Alveolar liquid clearance is upregulated by cAMP-dependent or -independent mechanisms in pathological conditions such as septic shock (30) and endotoxin instillation (25).

Keratinocyte growth factor (KGF) is a specific mitogen for epithelial cells (8, 29). It has been shown to promote the proliferation and differentiation of type II pneumocytes in vitro and in vivo (24, 35). In cultured alveolar epithelial type II (ATII) cells, KGF stimulated vectorial ion transport by increasing Na-K-ATPase expression (2, 37). KGF also prevented the decrease in the number of functional sodium pumps per cell after exposure of ATII cell monolayers to oxygen for 48 h (3). Comparable results were obtained in vivo in a model of α-naphthylthiourea-induced lung injury (12, 19). Additional studies (4, 6, 32, 39, 42) have also shown that KGF may improve the lung response to injury by mechanisms other than improvement of liquid clearance. For example, Gillis et al. (9) showed that KGF stimulated angiogenesis and protected the endothelial barrier against hydrogen peroxide- and vascular endothelial growth factor-induced increases in permeability. Consistent with these findings, Barazzone et al. (1) found that KGF prevented not only the alveolar epithelium but also the lung endothelium against oxygen-induced lung injury. This effect was mediated by a decrease in the expression of protease inhibitor and restoration of the fibrinolytic activity in the lung. How-
ever, whether KGF would protect the alveolar epithelium against *P. aeruginosa*-induced injury is unknown. Therefore, the objective of this study was to determine the effect of KGF pretreatment on alveolar epithelial barrier function in *P. aeruginosa* pneumonia in rats. Using an experimental model that measures both the permeability of the alveolar epithelium to protein and its capacity to actively transport fluid from the air space, we tested the hypothesis that KGF may improve *P. aeruginosa*-induced lung injury. We studied KGF effects in early (4-h) and late (24-h) pneumonia after pathogen instillation. We found that KGF increased lung liquid clearance (LLC) in early and late *P. aeruginosa* pneumonia. Pretreatment with KGF was associated with an increase in alveolar inflammatory cell recruitment and a more rapid bacterial clearance from the air spaces.

**METHODS**

**Animals**

Specific pathogen-free Sprague-Dawley rats (280–300 g; Centre d’Élevage Dépré, St. Doulchard, France) were housed in the Lille University Animal Care Facility (Lille, France) and allowed food and water ad libitum. All experiments were performed with the approval of the Lille Institutional Animal Care and Use Committee. A total number of 248 rats were used for all experiments.

**KGF Injection**

As previously described (12), for the intratracheal injection, the animals were anesthetized with ether (Mallinkrodt, Paris, France), and a small midline incision was made in a sterile fashion on the neck ventral surface after the surface was swabbed with 70% ethanol. The trachea was exposed by blunt dissection. Either KGF (recombinant human KGF, 5 mg/kg in 0.5 ml; Amgen, Thousand Oaks, CA) or an equal volume of phosphate-buffered saline (PBS; pH 7.2) was injected directly into the trachea with a 28-gauge needle followed by an injection of 0.5 ml of air. The incision was then closed with surgical clips, and the animal was allowed to recover. All intratracheal injections of KGF were performed 48 h before the injury.

**P. aeruginosa-Induced Lung Injury**

A clinical isolate of *P. aeruginosa* was used for this study as previously described (5). *P. aeruginosa* was incubated in 125 ml of tryptic soy broth at 37°C in a rotating shaking water bath for 8 h. The culture was then centrifuged (3,000 g for 10 min), washed twice with PBS (pH 7.4), and resuspended in PBS. The resulting bacterial suspension contained 2 × 10⁸ colony-forming units (cfu)/ml. The instillate was constituted with 0.5 ml/kg of this suspension.

Pneumonia was produced according to the method described by Pennington and Ehrie (26). Two days after KGF or PBS injection, under ether anesthesia, the same incision was used to instill *P. aeruginosa* intratracheally. A 28-gauge needle was used for the injection, and 0.5 ml/kg of bacterial suspension was instilled in the trachea followed by an injection of 0.5 ml of air.

### Ex Vivo Measurement of the *P. aeruginosa*-Induced Lung Injury

**Isolated perfused lung preparation.** The isolated perfused lung model has been previously described (12, 13). Briefly, 2 h after pneumonia was induced, the animals were anesthetized with ketamine (50 mg/kg; Parke Davis, Courbevoie, France) and xylazine (5 mg/kg; Sanofi, Libourne, France) intramuscularly, and the trachea was cannulated and connected to a constant-volume pump (Harvard Apparatus, South Natick, MA). The lungs were ventilated with a constant tidal volume of 10 ml/kg of 95% oxygen and 5% carbon dioxide at 60 breaths/min, with 2 cmH₂O positive end-expiratory pressure. A catheter was inserted into the left carotid artery, and after administration of 1.0 ml of sodium heparin solution (5,000 U/ml), the animal was exsanguinated. The chest was then opened, and the main pulmonary artery was exposed. The pulmonary artery was cannulated through a right ventriculotomy, and the lungs were perfused with Krebs-Henseleit buffer. Next, the left atrial appendage was also cannulated through the left ventricle. The lungs and heart were removed en bloc from the chest and placed in a heated water jacket. The lungs were perfused with a Masterflex pump (Cole-Parmer Instrument, Barrington, IL) with a mixture of one part Krebs-Henseleit buffer to three parts heparinized blood at a rate of 4.5 ml/min. For the protocols studying the effect of the changes in the perfusate osmotic pressure on fluid movement, the lungs were perfused with Krebs-Henseleit solution with and without 50 mg/ml of serum albumin (Sigma, St. Quentin Fallavier, France). The perfusate was circulated through a 37°C heat exchanger. The effluent from the cannulas emerging from the left atrium was directed to the perfusate reservoir so that all drainage from the preparation returned to the reservoir, which was constantly stirred magnetically to provide adequate mixing. Airway pressure, respiratory flow, pulmonary arterial pressure, left atrial pressure, and pressure in the blood-buffer reservoir were recorded for a 30-s period every 10 min, and data were stored in a personal computer [RESDIAG analysis system (27), INSERM U279, Lille, France]. The isolated lungs were ventilated and perfused ex vivo for a study period of 180 min after a 15-min equilibration period. Protocols evaluating the influence of osmotic pressure on the preventive effect of KGF were shortened to 60 min because the preparation remained stable for 90 min.

**Wet-to-dry lung weight ratios.** To evaluate the amount of extravascular lung water, each group of animals had lung wet-to-dry weight ratios performed. For these experiments, the lungs were prepared as described in Isolated perfused lung preparation and set up in the isolated perfused lung model. At the end of the experiment, the lungs were removed, and the wet weight was recorded. The lungs were then placed in a 37°C incubator for 7 days, at which time the dry weight was recorded. For each pair of lungs, the wet-to-dry weight ratio was calculated. Measurements of lung homogenate supernatant hemoglobin contents allowed the calculation of bloodless lung wet-to-dry weight ratios.

**Quantitative perfusate cultures and pulmonary bacterial load.** One hundred microliters of perfusate were sampled and diluted in 0.9 ml of isotonic saline, and 0.1 ml of serial 10-fold dilutions of the solution was plated on agar plates. At the end of the ex vivo experiment, the lungs were removed and homogenized in 7 ml of Tris-buffered saline, and viable bacteria were enumerated by plating 0.1 ml of serial 10-fold dilutions of the homogenates on agar plates.
In Vivo Measurement of the Alveolar Liquid Clearance in P. aeruginosa-Induced Lung Injury

Lung barrier function studies. Surgical preparation and ventilation. Sprague-Dawley male rats were anesthetized with pentobarbital sodium (Sanofi, Libourne, France). A catheter (PE-50) was inserted into the left carotid artery to monitor systemic arterial pressure and to obtain blood samples. Pancuronium bromide (0.3 mg·kg⁻¹·h⁻¹ iv) was given to achieve neuromuscular blockade. An endotracheal tube (PE-220) was inserted through a tracheostomy. The rats were ventilated with a constant-volume pump (Harvard Apparatus) with an inspired oxygen fraction of 1.0, a peak airway pressure of 8–12 cmH₂O, and a positive end-expiratory pressure of 2 cmH₂O.

Preparation of instillate. The test solution that was used for alveolar instillation was prepared as follows. Briefly, a 5% bovine albumin solution was prepared using Ringer lactate and was adjusted with NaCl to be isosmolar with the rat circulating plasma, 3 ml/kg of the 5% bovine albumin solution was prepared using Ringer lactate (Gif sur Yvette, France) to the 5% albumin solution. Also, anhydrous Evans blue dye (0.5 mg) was added to confirm the location of the instillate at the end of the study. A sample of the instilled solution was saved for total protein measurement, radioactivity counts, and water-to-dry weight ratio measurements so that the dry weight of the protein solution could be subtracted from the final lung water calculation.

General protocol. For all experiments, the following general protocol was used. After the surgical preparation, heart rate and blood pressure were allowed to stabilize for 1 h. After that, the rat was placed in the left lateral decubitus position (to facilitate liquid deposition into the left lung).

To calculate the flux of plasma protein into the lung interstitium, a vascular tracer, 1 μCi of 131I-labeled HSA, was injected into the bloodstream (22, 28). 131I-HSA was prepared in our institution according to a standardized technique (16). To calculate the flux of protein from the air spaces into the circulating plasma, 3 ml/kg of the 5% bovine albumin solution with 1 μCi of 125I-albumin were instilled 30 min later into the left lung over a 10-min period with a 1-ml syringe and polypropylene tube (0.5-mm ID) (21).

One hour after the beginning of the alveolar instillation, the abdomen was opened, and the rat was exsanguinated. Urine was sampled for radioactivity counts. The lungs were removed through a sternotomy, and fluid from the distal air spaces was obtained by passing a polypropylene tube (0.5-mm ID) into a wedged position in the lower lobe. Total protein concentration and radioactivity of the liquid samples were measured. Right and left lungs were homogenized separately for water-to-dry weight ratio measurements and radioactivity counts.

Measurements. Hemodynamics, pulmonary gas exchange, and protein concentration. Systemic arterial and airway pressures were continuously measured. Arterial blood gases were measured at 1-h intervals. Arterial PO₂ was used to quantify the oxygenation deficit (21, 22, 28). Samples from the instilled protein solution, the final distal air space fluid, and the initial and final blood were collected to measure total protein concentration with an automated analyzer (Hitachi 917).

Albumin flux across endothelial and epithelial barriers. Two different methods were used to measure the flux of albumin across the lung endothelial and epithelial barriers. The first method measures residual 125I-albumin (the alveolar protein tracer) in the lungs and accumulation of 125I-albumin in the plasma. The second method measures 131I-albumin (the vascular protein tracer) in the extravascular spaces of the lungs.

The first method requires measurement of the total quantity of 125I-albumin (the air space protein tracer) instilled into the lung. This was determined by measuring duplicate samples of the instilled solution for total radioactivity counts (in counts·min⁻¹·g⁻¹) and multiplying this value by the total volume instilled. To calculate the residual 125I-albumin in the lungs at the end of the study, the average radioactivity counts of two 0.5-g samples obtained from the lung homogenate was multiplied by the total weight of lung homogenate. The 125I-albumin in the lung homogenate was added to the recovered counts in the final aspirated distal air space fluid to calculate the amount of instilled 125I-albumin that remained in the lungs at the end of the study. The 125I-albumin in the circulating plasma was measured in a sample of plasma obtained at the end of the experiment. The plasma fraction was accounted for by multiplying the counts per gram times the plasma volume [body weight × 0.071(1 – hematocrit)].

The second method requires measurement of the vascular protein tracer 131I-albumin in the alveolar and extravascular spaces of the lungs. We estimated the quantity of plasma that entered the instilled lungs by measuring the transfer of the vascular protein tracer 131I-albumin into the extravascular spaces of the instilled lung with the equation of plasma equivalents previously described (21, 22, 28). Briefly, the extravascular lung plasma equivalents were determined by the total counts of 131I-albumin in the lung, subtracting the fraction in the blood in the lung as measured by the gravimetric method and then dividing by the mean counts in the circulating plasma over 90 min. The 131I-albumin counts in the final air space samples are expressed as a ratio to the plasma counts as we have done previously (21, 22, 28).

LLC (Excess Lung Water Measurement). The extravascular water content of the lungs was measured by the gravimetric method (21, 22, 28). In addition, changes in the water-to-dry weight ratio of the noninstilled lung were used as an index of lung endothelial injury because the noninstilled lung did not have the confounding presence of the instilled protein solution in its air spaces.

Bronchoalveolar Lavage

Lungs from each experimental group were lavaged with a total of 30 ml in 5-ml aliquots of PBS with 3 mM EDTA. The returned fluid was pooled and centrifuged (200 g for 10 min), and the cellular pellet was washed two times in PBS. A cell count was performed directly with a hemacytometer, and the total number of cells was calculated. Cellular monolayers were prepared with a cytocentrifuge and stained with Wright-Giemsa stain. Cellular morphotype differential was obtained by counting 200 cells/sample and expressing each type of cell as a percentage of the total number counted.

Histology

Lung samples from each group were analyzed. Samples devoted to the histological study were fixed for 24 h in a 10% paraformaldehyde-buffered solution (pH 7.2, 133 mM NaCl), embedded in paraffin, and sectioned at 5 μm. Serial lung sections were made and stained with hematoxylin-eosin-safran, May-Grünwald-Giemsa to appreciate the nature of inflammatory cells (eosinophil and mastocyte), Masson stain (either with methylene blue or aniline blue), Sirius red stain and reticulin stain to reveal fibrosing evolution, orcein stain to appreciate elastic fibers, and Perls method for hemosiderin...
detection. Most of these staining methods were also performed on semithin sections.

For the ultrastructural study, small lung samples (1 \times 1 \times 1 \text{mm}) were fixed by immersion in a high osmolarity 2.5% phosphate-buffered glutaraldehyde solution (0.1 M, pH 7.5) for 3 h at room temperature. The osmolarity of the fixative solution was adjusted to 600 mosM by the addition of NaCl. Samples were postfixed in 1% OsO4 for 1 h, dehydrated in graded ethanol, and embedded in Epon. Ultrathin sections (80 nm) were stained with 2% uranyl acetate and lead citrate. Samples were observed and photographed at 60 kV with an electron microscope (Zeiss LEO 906).

Experimental Protocols

**Experimental groups.** The following experimental groups were studied: 1) intratracheal KGF and intratracheal PBS 48 h later (KGF-PBS); 2) intratracheal PBS and intratracheal *P. aeruginosa* 48 h later (PBS-Pa); 3) intratracheal KGF and intratracheal *P. aeruginosa* 48 h later (KGF-Pa); and 4) intratracheal PBS and intratracheal PBS 48 h later (PBS-PBS; control).

**Ex vivo studies. Baseline study.** Four hours after the pathogen instillation, animals of the four groups were studied as previously described in the ex vivo lung preparation to evaluate lung leak and water-to-dry weight ratios.

**Evaluation of the oncotic gradient consequences in KGF protective role.** Three groups, PBS-PBS, PBS-Pa, and KGF-Pa, were studied in the isolated lung setup with a blood-free perfusate with and without serum albumin added. Five animals were used in each group.

**Translocation across the alveolocapillary barrier.** Two groups of animals were studied, the PBS-Pa group (n = 5) and the KGF-Pa group (n = 6). In these animals, the lungs were isolated and perfused, and *P. aeruginosa* were added ex vivo into the trachea. The perfusate culture was added every 10 min for a 60-min period to monitor emergence of the pathogen from the alveolar side.

**In vivo studies.** In vivo studies to evaluate lung fluid transport and permeability of the alveolocapillary barrier to proteins were performed in each of the four groups. These measurements were obtained 4 (early) and 24 h (late) after pathogen instillation (53 animals were studied for these experiments).

Statistical Analysis

Results are presented as means \pm SD. Data were analyzed with the Kruskal-Wallis test and the Mann-Whitney test where appropriate. *P* values < 0.05 were regarded as significant.

RESULTS

**KGF Improves Survival of *P. aeruginosa* Pneumonia**

Four groups of twenty animals were constituted. We followed the spontaneous mortality over a 3-wk period. We observed 90% death in the PBS-Pa group and 50% in the KGF-Pa group at the end of the 3-wk study period (*P < 0.05*); death occurred within the first 48 h. No mortality was observed in KGF-PBS and PBS-PBS groups.

**Early *P. aeruginosa*-Induced Lung Injury**

**KGF restores normal LLC in early pneumonia.** In vivo experimental model of pneumonia. The PBS-Pa group had a significantly greater lung leak over the 3-h observation period (9.16 \pm 1.3 ml; n = 6 animals) than the PBS-PBS group (2.16 \pm 0.1 ml; *P < 0.001*; n = 6 animals) or KGF-Pa group (4.8 \pm 0.58 ml, *P = 0.0032*; n = 5 animals; Fig. 1). There was also a significantly higher lung wet-to-dry weight ratio for the PBS-Pa group (18.7 \pm 2.12) compared with that in the other groups (PBS-PBS, 8.22 \pm 0.29; KGF-Pa, 13.36 \pm 2.1; Fig. 1). The KGF-PBS group (n = 5 animals) was comparable to the PBS-PBS group. Lung dry weights were comparable between the KGF-Pa and PBS-Pa groups, and both were significantly different from the PBS-PBS and KGF-PBS groups. Throughout the experiment, airway pressure and pulmonary arterial pressure increased 0.85 \pm 0.3 and 2.35 \pm 0.8 cmH2O, respectively, in the PBS-PBS group. In the PBS-Pa group, the increase was much more important (13.1 \pm 2.9 and 6.9 \pm 1.7 cmH2O, respectively). Preadministration of KGF completely prevented both airway and pulmonary arterial pressure increases (3.5 \pm 1.5 and 3.7 \pm 1.2 cmH2O, respectively). From these experiments, we hypothesized that KGF could improve alveolar fluid transport in early pneumonia, and, therefore, we measured these parameters using an in vivo animal model of pneumonia.

**In vivo experimental model of pneumonia.** Pneumonia was associated with a significant decrease in LLC in the PBS-Pa group (n = 6 animals) compared with that in the PBS-PBS group (n = 7 animals). In contrast, pretreatment with KGF in pneumonic rats (n = 6) restored LLC to the control value (Table 1). Lung endothelial permeability to protein was, however, similarly increased as shown by the influx of the vascular...
The efflux of 125I-albumin from the lung was used as an alveolar tracer. *Significantly different from PBS-PBS, \( P < 0.05 \). KGF did not improve this parameter (PBS-PBS, 0.08 ± 0.01; KGF-PBS, 0.13 ± 0.06; PBS-Pa, 0.51 ± 0.07; KGF-Pa, 0.38 ± 0.11). The efflux of 125I-albumin from the lung was comparable between both pneumonic groups and significantly higher than in the control group (Table 2).

**Histological Findings after 4 H of Pneumonia.** We examined lung samples from infected rats with and without KGF pretreatment and compared them with samples from uninfected animals (\( n = 4/\)group). In the animals without infection pretreated with KGF for 48 h, type II pneumocytes were hyperplasic and hypertrophied, with a large multilobar nucleus as observed in KGF-pretreated animals. There was no exudate in the bronchiolar lumina, and no modification of the interstitial framework (Fig. 2). After KGF pretreatment, one-fourth to one-half of the lungs were injured, and lesions were less extensive. The exudate was less hemorrhagic, less edematous, and richer in inflammatory cells, with some macrophages and many neutrophils. Type II pneumocytes were hyperplasic and hypertrophied, with a large multilobar nucleus as observed in KGF-pretreated animals without pneumonia. Similar to the nonpretreated KGF groups, no fibrosis and no densification of the elastic framework was evident except for a slight densification of the reticular framework (Fig. 2).

The morphological aspect was similar on semithin sections. At the ultrastructural level, numerous microorganisms were observed in the alveolar lumen. In KGF groups, the alveolar basement membrane was not altered, and type II pneumocytes were enlarged and more hypertrophic than in normal lungs.

### Late *P. aeruginosa*-Induced Lung Injury

**KGF restores LLC in pneumonia.** LLC was decreased in rats with *P. aeruginosa* pneumonia (\( n = 7 \)) compared with that in the control group (\( n = 10 \)). KGF restored LLC to values comparable to control values in both pneumonic (\( n = 7 \)) and nonpneumonic (\( n = 5 \)) animals (Table 1). Consistent with these findings, lung wet-to-dry weight ratio was significantly increased in the pneumonic group compared with that in the control group, and KGF-pretreated animals (\( n = 7 \)) presented a wet-to-dry weight ratio comparable to control values (Table 1).

**Decrease in the oncotic gradient does not change LLC in KGF-pretreated pneumonia.** We observed that KGF increased LLC in pneumonia. Interstitial resorption depends on four variables; the vascular and lymphatic pathways are the most important compared with the pleural and mediastinal pathways. The histology showed an increase in the vascular wall on the arterial side as well as venular side of the KGF-Pa group; we hypothesized that this increase could be responsible for the preservation of an oncotic gradient after KGF pretreatment. To test this hypothesis, we studied the isolated perfused lung setup in the same groups but perfused the lungs with a buffer solution (Krebs-
Henseleit) with and without proteins (50 g/dl). A total number of 16 animals (4 animals/group) were used. Without protein in the perfusate, we observed in the control group a slight increase in the lung wet-to-dry weight ratio to 8.89 ± 0.6; pneumonic animals had a significantly higher ratio (14.22 ± 4.6), and KGF pre-treatment prevented a lung wet-to-dry weight ratio increase (6.69 ± 0.11).

**KGF does not affect protein permeability across the alveolocapillary barrier.** The evaluation of the epithelial permeability measuring the efflux of 125I-labeled albumin from the lung showed an increased leak in both pneumonic groups (pretreated or not pretreated with KGF) compared with that in the control groups (Table 2).

Bidirectional protein movement across the alveolocapillary barrier was also affected. We observed an
increase in the ratio of $^{131}$I-albumin in the aspirate to that in the plasma in the PBS-Pa group ($0.38 \pm 0.12$) and the KGF-Pa group ($0.38 \pm 0.16; P < 0.05$) compared with that in the PBS-PBS group ($0.08 \pm 0.01$) and the KGF-PBS group ($0.15 \pm 0.04$).

The slight increase in the ratio of $^{131}$I-albumin in the aspirate to that in the plasma observed in the KGF-PBS group was not related to a small degree of injury caused by the KGF instillation but more probably to an increase in alveolar fluid clearance. To confirm this hypothesis, we therefore measured the level of native protein in the bronchoalveolar lavage fluid, and we did not find any statistical difference ($0.15 \pm 0.02$ in the KGF-PBS group vs. $0.2 \pm 0.05$ in the PBS-PBS group).

Histological findings after 24 h of pneumonia. As we did at 4 h, we examined lung samples from pneumonic rats with and without KGF pretreatment and compared their lung morphology to that in control rats (2 animals/group). Seventy-two hours after KGF administration, we found a heterogeneous type II cell hyperplasia but no atypical nuclei and a densification of the reticulin framework. Small arteries had a thicker wall with a reduced lumen, an increase in the collagen framework, and smooth muscle cell proliferation. Venules presented hypertrophy of the vascular wall too but only collagen related. Similar to the type II cell hyperplasia, this vessel aspect was very heterogeneous in the lung (Fig. 3).

In pneumonic animals without KGF pretreatment, small vessels did not show any significant morphological changes except for a slight increase in collagen around them. When KGF was instilled before P. aeruginosa, small arteries were found to be hypertrophic with a reduced lumen, comparable to the features described in the KGF group but less pronounced.

Pneumonia without KGF was extensive (76% of total area) but less congestive than at 4 h. Most alveolar spaces contained inflammatory cells (mainly neutrophils and macrophages) that were more abundant around bronchovascular bundles. Localized type II pneumocyte hyperplasic foci were observed.

After KGF pretreatment, parenchymal injury was less extensive, mainly in the perihilar regions (70% of total area; the difference was not significant). There was less pulmonary edema and a more important infiltrate of inflammatory cells (macrophages and neutrophils) that was prominent around bronchovascular bundles. Type II cell hyperplasia was more important than in the PBS-Pa group. Results of a representative animal of each group are shown in Fig. 4.

Bacteriological and Cellular Responses

KGF decreases pulmonary translocation of the pathogen from the airway to the vascular space. We tested the hypothesis that KGF pretreatment may decrease translocation of P. aeruginosa across the alveolocapillary barrier. Animals were pretreated with KGF or its vehicle 48 h before the pneumonia ($n = 6$ and 5, respectively). The lungs were then removed from the chest and suspended in the organ chamber. P. aeruginosa was added in the trachea (0.5 ml/kg of the inoculum, $2 \times 10^9$ cfu/ml), and the appearance of the pathogen was monitored in the perfusate for 60 min to study lung translocation. We observed a progressive increase in the count of bacteria in the perfusate in both groups (Fig. 5). The perfusate bacterial load was significantly lower in the KGF-Pa group compared with that in the PBS-Pa group at the end of the 60-min period ($1.08 \times 10^8$ vs. $6.8 \times 10^7$ P. aeruginosa/g; $P = 0.04$). Similarly, the lung wet weight was significantly lower in the KGF-Pa group compared with that in the PBS-Pa group ($3.1 \pm 0.15$ vs. $5.8 \pm 0.79$ g; $P = 0.0034$).

KGF increases P. aeruginosa clearance at 24 h. We measured lung bacterial count per gram of parenchyma 24 h after tracheal instillation of P. aeruginosa in rats pretreated or not pretreated with KGF ($n = 22$). KGF reduced the bacterial load from $6.63 \times 10^3 \pm 1.96 \times 10^3$ cfu/g in the PBS-Pa group to $7.46 \times 10^2 \pm 3.44 \times 10^2$ P. aeruginosa/g in the KGF-Pa group ($P < 0.02$).

KGF increases lung inflammatory cell recruitment in pneumonia. The number of inflammatory cells recovered from the bronchoalveolar lavage fluid was significantly increased in KGF-pretreated pneumonic animals ($5.1 \times 10^7$ cells/ml) compared with that in the PBS-PBS (1.7 $\times 10^6$ cells/ml), KGF-PBS (3.0 $\times 10^6$ cells/ml), or PBS-Pa (2.2 $\times 10^7$ cells/ml) group ($P < 0.02; n = 16$). More than 90% of the cells were alveolar macrophages in nonpneumonic rats with or without KGF.

Figure 3. Histological sections prepared with hematoxylin and eosin stain from the KGF-PBS group (A) 72 h before the study. The wall of the small artery is thicker than in the PBS-PBS group (B), involving both endothelial and smooth cell proliferation. Original magnifications: $\times 40$ in A; $\times 25$ in B.
the pneumonic groups, neutrophils were predominant, representing 98% of the cells.

**DISCUSSION**

Preservation of active fluid reabsorption from the air spaces is one of the main determinants in acute lung injury-related survival. KGF has been shown to increase alveolar epithelial fluid transport in normal as well as in pathological lungs (12, 37). Thus the overall objective of this study was to determine whether pretreatment with KGF would attenuate *P. aeruginosa*-induced lung injury. We focused our work on factors involved in the resolution of the injury such as removal of fluid from the air spaces, capacity of the pathogen to translocate across of the alveolocapillary barrier, and bacterial clearance from the air spaces.

The first important finding of these studies was that KGF restored normal LLC 4 and 24 h after instillation of live *P. aeruginosa* into the air spaces of the lung. Wang et al. (37) recently reported that intratracheal administration of KGF induced a sustained upregulation of alveolar epithelial fluid transport in normal rats. In addition, these authors showed that catecholamines had an additive effect in upregulating alveolar liquid clearance in rats pretreated with KGF.

Several studies (10, 11, 31) have established active sodium and chloride transport as the primary mechanism driving alveolar liquid clearance in the normal lung. The maintenance of this active transport is critical for the resolution of alveolar edema in humans (20). In hydrostatic edema, preservation of alveolar fluid clearance is associated with a better oxygenation and a lower hospital mortality (36).

KGF has been shown to be protective when administered as a pretreatment in different types of acute lung injury such as hyperoxia (23), acid (41), bleomycin (43), radiation (34), and α-naphthylthiourea (19). One mechanism responsible for the benefit of KGF is the stimulation of ion transport. In cultured ATII cells,
KGF-dependent preservation of sodium pump expression prevented a hyperoxia-induced decrease in active ion transport (3). In bleomycin-induced lung injury, Yi et al. (42) showed a decrease in pulmonary edema with KGF. Similarly, in the α-naphthylthiourea-induced lung injury, Mason et al. and Guery et al. obtained comparable results, with a decrease in the extravascular lung water (19) related to an increase in the transalveolar sodium reabsorption (12). In our study, KGF pretreatment significantly increased the survival of pneumonic rats, probably because it stimulated removal of edema fluid from the air spaces.

There are several mechanisms that can explain why KGF pretreatment increased fluid removal from the air spaces. First, KGF is a heparin-binding growth factor, a member of the fibroblast growth factor family (8, 29). KGF stimulates epithelial cell proliferation (14) and particularly DNA synthesis in and proliferation of rat ATII cells in primary cultures (24). In vivo, Ulich et al. (35) observed a similar effect with a knob micropapillary overgrowth of alveolar septal epithelial cells within 48 h and diffuse hyperplasia of type II cells in 72 h. Second, Borok et al. (2) demonstrated an upregulation of active ion transport in cultured ATII cells exposed to KGF. This effect was mainly due to the increase in Na-K-ATPase α₁-subunit mRNA expression.

Recently, Gillis et al. (9) showed that KGF at sub-nanomolar concentrations induced in vivo neovascularization in the rat cornea. Moreover, KGF was not effective in vitro against endothelial cells cultured from large vessels but acted on those cultured from small vessels. KGF also protected against vascular endothelial growth factor or vascular permeability and hydrogen peroxide-induced increases in the permeability of capillary endothelial cell monolayers. Our histological work underlined a profound modification of the vascular wall, with an increase in the muscularis and a reduction in the lumen; this effect was predominant in small arteries and was heterogeneous (potentially related to KGF intratracheal instillation). This vascular effect was obvious in late pneumonia; however, some morphological changes of small arteries were observed after only 48 h of KGF pretreatment in the early pneumonia study. Even if Gillis et al. showed a direct effect of KGF on the endothelial cell, stimulation of the other components of the vascular wall were not shown, and we did not address this issue in our work. However, other members of the fibroblast growth factor family, basic and acidic fibroblast growth factors, are mitogenic for vascular smooth muscle and endothelial cells (15).

We therefore hypothesized that in KGF-pretreated animals, preservation of the interstitial oncotic pressure may be responsible, at least in part, for the restoration of LLC. We postulated that inhibition of this gradient between the vascular and interstitial compartments could inhibit the beneficial effect of KGF. The perfusion of an ex vivo preparation with a buffer solution without any protein failed to inhibit the protective effect of KGF, so even if the oncotic pressure contributes to LLC, this mechanism is not responsible by itself for KGF-associated protection. Consistent with this finding, the influx of the vascular tracer ¹²⁵I-albumin was significantly increased in the alveolar fluid in early and late pneumonia and caused a similar increase in extravascular lung water. We cannot show from these data that the protective effect of KGF was related to an endothelial effect.

The final important finding of our study is that KGF increased bacterial clearance from the air spaces at 24 h. The number of neutrophils in the bronchoalveolar lavage fluid was statistically higher in the KGF-Pa group compared with that in the PBS-Pa group. There was also a decrease in the pathogen translocation through the alveolocapillary barrier in the KGF-pretreated group. We did not study the mechanisms responsible for these effects on the pathogen, but KGF could act through surfactant protein A production. Mariencheck et al. (18) showed a major role for surfactant protein A in P. aeruginosa infection; they showed that surfactant protein A acted as an opsonin to stimulate macrophage phagocytosis of a live mucoid strain of P. aeruginosa (18). KGF demonstrated a potent stimulating effect on those collectins (40). KGF-related epithelial type II cell hyperplasia may also be responsible for the increased strength of the barrier. Independent of the mechanisms involved, increased pathogen clearance represents an important feature of KGF-associated effects, considering the pathogenesis of P. aeruginosa pneumonia and the major role of alveolar epithelium injury (17).

Histological findings showed an extensive injury independent of KGF pretreatment. In pneumonic animals, typical aspects were found; early studies evidenced mainly hemorrhagic and exudative features. At 4 h, KGF pretreatment decreased the extension not only of alveolar edema but also of alveolar hemorrhage. However, inflammatory cell infiltrate and type II cell hyperplasia were increased. At 24 h, pneumonic lesions were less congestive, consistent with the stimulation of fluid transport, with a more important infiltrate of inflammatory cells within the alveolar spaces and around bronchiovascular bundles. KGF-pretreated animals had less extensive lung injuries with more inflammatory cells.

In conclusion, KGF pretreatment improved survival in P. aeruginosa-induced lung injury in an experimental rat model of pneumonia. This beneficial effect was related to the improvement of fluid reabsorption from the air spaces in the injured lung. KGF also stimulated the host cell response to the pathogen by increasing recruitment of inflammatory cells and bacterial clearance from the air spaces. From these data, it seems interesting to further evaluate KGF in pneumonia, especially with a therapeutic administration.

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