Keratinocyte growth factor protects against elastase-induced pulmonary emphysema in mice

Laurent Plantier, Sylvain Marchand-Adam, Valeria G. Antico, Laurent Boyer, Cécile De Coster, Joëlle Marchal, Rafik Bachoual, Arnaud Mailleux, Jorge Boczkowski, and Bruno Crestani

1 Institut National de la Santé et de la Recherche Médicale, U700, Faculté Xavier Bichat, 2 Assistance Publique-Hôpitaux de Paris, Service de Pneumologie, Hôpital Bichat, 3 Université Paris 7, Unité de Formation et de Recherche Médicale Denis Diderot, Faculté Bichat, Paris, France; 4 Laboratory of Oxygen Metabolism, University Hospital, University of Buenos Aires, Buenos Aires, Argentina; 5 Department of Cell Biology, Harvard Medical School, Boston, Massachusetts; and 6 Assistance Publique-Hôpitaux de Paris, CIC 07, Hôpital Bichat, Paris, France

Submitted 21 November 2006; accepted in final form 24 August 2007

Keratinocyte growth factor protects against elastase-induced pulmonary emphysema in mice. Am J Physiol Lung Cell Mol Physiol 293: L1230–L1239, 2007. First published August 31, 2007; doi:10.1152/ajplung.00460.2006—Pulmonary emphysema is characterized by persistent inflammation and progressive alveolar destruction. The keratinocyte growth factor (KGF) favorably influences alveolar maintenance and repair and possesses anti-inflammatory properties. We aimed to determine whether exogenous KGF prevented or corrected elastase-induced pulmonary emphysema in vivo. Treatment with 5 mg·kg⁻¹·day⁻¹ KGF before elastase instillation prevented pulmonary emphysema. This effect was associated with 1) a sharp reduction in bronchoalveolar lavage fluid total protein and inflammatory cell recruitment, 2) a reduction in the pulmonary expression of the chemokines CCL2 (or monocyte chemoattractant protein-1) and CXCL2 (or macrophage inflammatory protein-2α) and of the adhesion molecules ICAM-1 and VCAM-1, 3) a reduction in matrix metalloproteinase (MMP)-2 and MMP-9 activity at day 3 and 4) a major reduction in DNA damage detected by terminal deoxynucleotidyltransferase-mediated dUTP nick end labeling (TUNEL) in alveolar cells at day 7. Treatment with KGF after elastase instillation had no effect on elastase-induced emphysema despite the conserved expression of the KGF receptor in the lungs of elastase-instilled animals as determined by immunohistochemistry. In vitro, KGF abolished the elastase-induced increase in CCL2, CXCL2, and ICAM-1 mRNA in the MLE-12 murine alveolar epithelial cell line. We conclude that KGF pretreatment protected against elastase-induced pulmonary inflammation, activation of MMPs, alveolar cell DNA damage, and subsequent emphysema in mice. We conclude that KGF pretreatment protected against elastase-induced pulmonary inflammation, activation of MMPs, alveolar cell DNA damage, and subsequent emphysema in mice. Therefore, the aim of this study was to determine whether the administration of exogenous KGF would prevent or reverse elastase-induced emphysema in mice and to explore the molecular mechanisms involved in its effect.

MATERIALS AND METHODS

Animal model. Studies were conducted in eight-week-old male C57BL/6 mice (Janvier, Le Genest Saint-Isle, France), in compliance with Institut National de la Santé et de la Recherche Médicale (INSERM) guidelines regarding the fair treatment of animals, under a license from the French administration to conduct animal research as described in the protocol. Mice were anesthetized with ketamine (1.6 mg; Merial, Lyon, France) and xylazine (300 μg; Bayer, Puteaux, France).

In a first set of experiments, we evaluated whether KGF prevented elastase-induced emphysema. Naïve mice did not receive any instillation. Saline mice received a tracheal instillation of 50 μl of saline.
Elastase mice received 100 μl/day saline subcutaneously for 3 days before the instillation of 5 U of porcine pancreatic elastase (Elastin Products, Owensville, MO) in 50 μl of saline into the surgically exposed trachea. KGf+Elastase mice received 5 mg·kg⁻¹·day⁻¹ recombinant human KGF (Amgen, Thousand Oaks, CA) in 100 μl of saline subcutaneously for 3 days before elastase instillation. For the determination of emphysema by computerized morphometry, 20 days after tracheal instillation mice were anesthetized with 50 μg of intraperitoneal halothane (Sigma, St-Quentin-Fallavier, France), and their lungs were removed. For determination of the mechanisms involved in the effect of KGF, other mice were anesthetized in a similar way, and their lungs were removed for further analysis at the 6th hour and 1, 3, 7, and 20 days after instillation.

In a second set of experiments, a curative effect of KGF was sought in mice treated with KGF or its vehicle from day 0 to day 7 after elastase instillation and killed at day 20 and in mice treated with KGF or its vehicle from day 20 to day 27 after elastase instillation and killed at day 27. Five mice were used in each group unless otherwise described.

The concentration of bacterial endotoxin in the elastase preparation determined with the Lonza-Biowhittaker KQCL endotoxin test (Lonza France, Levallois-Perret, France) was 0.21 U/ml below the 0.25 U/ml limit accepted under European Pharmacopeia rules.

**Morphological analysis.** The lungs were fixed with 2.5% glutaraldehyde at a transpleural pressure of 25 cmH₂O for 3 h and held in 4% paraformaldehyde (Sigma). Great-axis sagittal sections (5 μm) of the left lung were cut in a systematic fashion and were stained with hematoxylin and eosin. Five black-and-white digital photomicrographs were acquired from the cranial, medial, and caudal regions of lungs and expressed as a percentage.

**Zymography.** For gelatin zymography, 5 μl (6th hour time point) or 20 μl (other time points) of BALF was electrophoresed onto 8% sodium dodecyl sulfate-polyacrylamide gels containing 1 mg/ml bovine gelatin (Sigma). Sodium dodecyl sulfate was removed from the gels by shaking in 2.5% Triton X-100 (Sigma) twice for 1 h. The gels were then incubated for 2 days at 37°C in pH 7.5 50 mM Tris-HCl with 5 mM CaCl₂, 1 μM ZnCl₂, and 150 μM NaN₃. For elastin zymography, samples were electrophoresed onto 12% sodium dodecyl sulfate-polyacrylamide gels containing 1 mg/ml bovine soluble elastin (Elastin Products) and were incubated likewise for 5 days. Proteolysis bands were visualized by Coomassie staining and were quantified with the Bio-Vision system (Fisher Bioblock Scientific, Illkirch, France).

**RNA extraction and quantitative RT-PCR.** RNA was extracted from lung homogenate with TRizol reagent and was retrotranscribed with Moloney murine leukemia virus reverse transcriptase (Invitrogen, Cergy-Pontoise, France) according to the manufacturer’s instructions. Complementary DNAs (cDNAs) were quantified by quantitative PCR (94°C for 30 s, 60°C for 1 min, 40 cycles) with SYBR Green Jumpstart Taq Ready Mix (Sigma). The quantity of CCL2, CXCL2, elastin, collagen-1α1, intercellular adhesion molecule (ICAM)-1, vascular cell adhesion molecule (VCAM)-1, and tissue inhibitor of metalloproteinase (TIMP)-1, -2, -3, and -4 cDNAs was compared with that of a standard prepared from pooled mouse lung RNA. Likewise, the amount of the endogenous control ribosomal protein (RP)L13 cDNA was measured in samples and compared with the standard. The expression of a specific gene in a given sample was then determined by the following formula: gene expression = (gene of interest in sample/gene of interest in standard)/(RP)L13 in sample/(RP)L13 in standard). Primer sequences are listed in Table 1.

**TUNEL assay.** The lungs were insufflated ex vivo with 1 ml of a 1:1 mix of 0.9% saline with Tissue-Tek ornmith carbamoyltransferase fluid (Sakura, Zoetervoude, The Netherlands) and were snap-frozen in liquid nitrogen. Terminal deoxynucleotidyltransferase-mediated dUTP nick end labeling (TUNEL) was performed on 5-μm acetone-fixed slides of frozen lung with the DeadEnd System (Promega, Madison, WI). The number of TUNEL-positive nuclei was counted by a blinded observer (L. Plantier), compared with the total number of nuclei on five random fields taken at ×400 magnification, and expressed as a percentage.

**Immunohistochemistry for activated caspase-3.** The expression of activated caspase-3, a marker of apoptosis, was determined by immunohistochemistry on frozen and acetone-fixed lung slides. The rabbit primary antibody (ref. 9664, Cell Signaling Technology, Danvers, MA) was diluted 1:100 and incubated overnight at 4°C. Staining was revealed with a biotinylated anti-rabbit antibody (BA-1000, Vector, Burlingame, CA), Vectastain complex (Vector), and fast red substrate (Dako Cytomation).

**Table 1. Sequences of primers used for quantitative PCR experiments**

<table>
<thead>
<tr>
<th>Gene</th>
<th>Forward Primer</th>
<th>Reverse Primer</th>
</tr>
</thead>
<tbody>
<tr>
<td>RPL13</td>
<td>GCGTCGTGGTTTATTGAGC</td>
<td>CAGAAGGTACCCCAAA</td>
</tr>
<tr>
<td>CCL2</td>
<td>CCGCTGGTTGAAAGGCAAG</td>
<td>ATGCTGGAAGTTTCAGGG</td>
</tr>
<tr>
<td>Collagen-1α1</td>
<td>CACGTTTGGAACACACATC</td>
<td>CTGTTCAGTTACCTGAG</td>
</tr>
<tr>
<td>Elastin</td>
<td>TTTTCCTTCTTCCTTCCTT</td>
<td>CCGTGGAGGTCGAGCTG</td>
</tr>
<tr>
<td>TIMP-1</td>
<td>CGGAGGCTGTAGTACCTG</td>
<td>GGTGTCGAGGCCAGCCAG</td>
</tr>
<tr>
<td>TIMP-2</td>
<td>GCTGGGGTACCTGACTT</td>
<td>GAGCTGACCCACAGCAG</td>
</tr>
<tr>
<td>TIMP-3</td>
<td>TCCATGAGGTCGCTGACCT</td>
<td>CTTGTTGAGAGTTGAG</td>
</tr>
<tr>
<td>TIMP-4</td>
<td>AGTATCGAGGAGGAAGGTG</td>
<td>CTTGACGAGGACCCCTG</td>
</tr>
<tr>
<td>ICAM-1</td>
<td>CCGGGAATGACCCGATG</td>
<td>CTGGCAAGAAGAGAAG</td>
</tr>
<tr>
<td>VCAM-1</td>
<td>CGAGTGGGCTGCTGCTG</td>
<td>GGGCTGCTGACCTGCC</td>
</tr>
</tbody>
</table>

RPL13, ribosomal protein L13; TIMP, tissue inhibitor of metalloproteinase; ICAM-1, intercellular adhesion molecule 1; VCAM-1, vascular cell adhesion molecule 1.
**RESULTS**

*KGF pretreatment prevented elastase-induced emphysema.* Intratracheal instillation of elastase induced pulmonary emphysema (Fig. 1). Mice from the Elastase group had diffuse emphysematous lesions, and their mean chord length (63.8 ± 5.9 μm, n = 8) was markedly increased compared with the Saline group (46.1 ± 0.6 μm, n = 5; P = 0.02). By contrast, the lungs of most mice from the KGF+Elastase group showed only minimal and focal emphysematous changes. The mean chord length of KGF+Elastase mice (48.5 ± 4 μm, n = 9) was lower than that of Elastase mice (P = 0.009) and was not different from that of Saline animals.

*KGF prevented elastase-induced elevation in BALF proteins.* The total protein concentration in BALF was determined to assess the degree of elastase-induced injury in the lung. A sharp increase in BALF proteins was observed in the Elastase group from the 6th hour to day 3 (Fig. 2A), reaching 742.5 ± 241.5 μg/ml at day 3. In the KGF+Elastase group, BALF proteins were lower than in Elastase animals at the 6th hour (P = 0.008) and at day 3 (P = 0.02) after instillation, with the reduction reaching 75% at day 3.

*KGF prevented elastase-induced influx of inflammatory cells in BALF.* A sharp increase in BALF cellularity was observed in the Elastase group compared with the Saline group from the 6th hour to day 3 and at day 20 (Fig. 2B), with a maximum at the 6th hour (7-fold increase; P = 0.009). BALF cellularity in the KGF+Elastase group was markedly reduced compared with Elastase mice at the 6th hour (65% reduction; P = 0.014) and at day 3 (58% reduction; P = 0.014).

The composition of the inflammatory cell influx in BALF varied with time after elastase instillation. While neutrophils were very rare in BALF of animals from the Saline group at all time points, they formed the main fraction of BALF cells at the 6th hour after elastase instillation, with their numbers decreasing thereafter (Fig. 2C). KGF prevented the elastase-induced influx of neutrophils in BALF. Neutrophil numbers in BALF of mice from the KGF+Elastase group were reduced by 75% at the 6th hour (P = 0.014), 47% at day 1 (P = 0.021), and 83% at day 3 (P = 0.014) compared with mice from the Elastase group. Macrophage numbers increased in BALF of Elastase mice from the 6th hour to day 3 and at day 20 and were the

---

**Fig. 1.** Keratinocyte growth factor (KGF) pretreatment prevented elastase-induced pulmonary emphysema. Representative photomicrographs (×100 original magnification) of the lungs of mice treated with intratracheal saline (A), intratracheal elastase (B), and subcutaneous KGF (C) before intratracheal elastase. Mice were killed at day 20. D: mean chord length of alveoli in the lung of Saline (n = 5), Elastase (n = 8), and KGF (n = 9) mice. Data are means ± SE. *P < 0.05 vs. Saline; †P < 0.05 vs. Elastase.
KGF reduced elastase-induced expression of CCL2 and CXCL2 in the lung. To determine the mechanisms involved in the inhibition of the inflammatory cell influx observed in KGF-treated mice, we studied the pulmonary expression of CCL2 and CXCL2, two chemokines that are the main stimuli for the recruitment of monocytes/macrophages and neutrophils, respectively, to the site of inflammation (14).

CCL2 mRNA was increased 15-fold in mice from the Elastase group compared with mice from the Saline group ($P = 0.009$) at the 6th hour and remained elevated from the 6th hour to day 3 (Fig. 3A). KGF pretreatment completely prevented the elastase-induced elevation in lung CCL2 mRNA at the 6th hour and day 3. CCL2 mRNA levels were similar in all groups of animals past day 3 after instillation. CCL2 protein was undetectable in BALF of Saline as well as Naive mice (Fig. 3B). In the Elastase group, CCL2 protein was detected in BALF at the 6th hour ($66.6 \pm 15.7$ pg/ml), day 1 ($97.1 \pm 24.6$ pg/ml), and day 3 ($158.1 \pm 85$ pg/ml). BALF CCL2 levels in the KGF+Elastase group were markedly lower than in the Elastase group at the 6th hour ($16.6 \pm 7.8$ pg/ml; $P = 0.011$) and were not detectable from day 3 onward.

CXCL2 mRNA was increased 19-fold in the Elastase group compared with the Saline group at the 6th hour after instillation ($P = 0.009$) and decreased thereafter (Fig. 3C). In the KGF+Elastase group, the increase in CXCL2 mRNA at the 6th hour was reduced by 64% compared with the Elastase group ($P = 0.027$). CXCL2 mRNA levels were similar in all groups of animals past day 3 after instillation. CXCL2 protein was not detected in BALF of Saline as well as Naive mice (Fig. 3D). CXCL2 protein was detectable in BALF of Elastase and KGF+Elastase animals at the 6th hour and day 1. At the 6th hour after instillation, BALF CXCL2 levels were reduced by 23% in the KGF+Elastase group ($44.4 \pm 7.2$ pg/ml) compared with the Elastase group ($80.5 \pm 17.2$ pg/ml; $P = 0.024$).

KGF reduced elastase-induced expression of ICAM-1 and VCAM-1 in the lung. The coordinated expression of cell adhesion molecules, particularly ICAM-1 and VCAM-1, is implicated in the recruitment of macrophages and neutrophils to the site of injury in the lung (1). At the 6th hour after instillation, lung ICAM-1 mRNA was increased 6.5-fold in the Elastase group compared with the Saline group ($P = 0.009$; Fig. 4). This increase was blunted by 47% in the KGF+Elastase group compared with Elastase mice ($P = 0.027$). Likewise, the lung content of VCAM-1 mRNA was increased 3.8-fold in the Elastase group compared with the Saline group ($P = 0.009$), while the lung content of VCAM-1 mRNA was reduced by 39% in the KGF+Elastase group compared with Elastase mice ($P = 0.05$).

KGF prevented elastase-induced increase in CCL2, CXCL2, and ICAM-1 mRNA in MLE-12 cells in vitro. To determine whether elastase and KGF respectively induced and repressed CCL2 and CXCL2 expression in the lung through direct effects on alveolar epithelial cells, we exposed the MLE-12 murine alveolar epithelial cell line to both agents in vitro. Elastase induced a 3.6-fold increase in CCL2 mRNA ($P = 0.016$; $n = 6$), a 6.4-fold increase in CXCL2 mRNA ($P = 0.010$; $n = 6$), and a slight (25%) increase in ICAM-1 mRNA ($P = 0.049$, $n = 3$) compared with the control condition (Fig. 5). KGF completely prevented the elastase-induced increase in CCL2 ($P = 0.024$; $n = 6$), CXCL2 ($P = 0.01$; $n = 6$), and ICAM-1

dominant cell type from day onward (Fig. 2D), reaching a maximum at day 3 (4.3-fold increase compared with Saline group; $P = 0.034$). KGF pretreatment completely inhibited the elastase-induced increase in alveolar macrophages at all time points.

**Fig. 2.** KGF pretreatment prevented elastase-induced pulmonary inflammation. Total protein levels (A), total cellularity (B), polymorphonuclear neutrophil numbers (C), and macrophage numbers (D) in bronchoalveolar lavage fluid (BALF) of naive mice and mice treated with intratracheal saline (open bars), mice treated with intratracheal elastase (filled bars), and mice treated with subcutaneous KGF before intratracheal elastase (dotted bars) are shown. Data are means ± SE. *$P < 0.05$ vs. Saline. †$P < 0.05$ vs. Elastase. $n \geq 5$ in each group.
Elastase did not modulate the levels of VCAM-1 mRNA in MLE-12 cells (data not shown). Altogether, these results point to a strong anti-inflammatory effect of KGF pretreatment mediated by a reduction in the pulmonary expression of chemokines and adhesion molecules. This effect seems to be exerted at least partly through direct effects on alveolar epithelial cells.

**KGF did not modulate elastolytic activity in BALF.** We asked whether the protective effect of KGF pretreatment may have been due to a reduction in the diffusion of the instilled elastase from the proximal airway to the distal lung. At the 6th hour after instillation, elastin zymography showed the presence of two elastolytic bands located at 25 and 29 kDa in BALF of mice from the Elastase and KGF+Elastase groups, corresponding to endogenous neutrophil elastase and exogenous porcine pancreatic elastase, respectively (Fig. 6A). These bands were detected neither in the Saline group at all time points nor in the Elastase and KGF+Elastase groups past the 6th hour after instillation. The intensity of both bands was identical in Elastase and KGF+Elastase mice, demonstrating that the elastase instilled in the trachea of KGF-treated mice did reach the distal lung. This result also demonstrated that the preventive effect of KGF was not secondary to an increase in the antielastase shield before elastase instillation.

**KGF decreased matrix metalloproteinase-2 and -9 activity in BALF.** We determined whether the anti-inflammatory effect of KGF translated into the modulation of proteolytic activity in BALF. Gelatin zymography allowed the detection of a 73-kDa and a 97-kDa gelatinase, corresponding to MMP-2 and MMP-9, respectively (Fig. 6B). MMP-9 activity (Fig. 6C) was detected in BALF of mice from the Saline group at the 6th hour and at day 1. It was markedly increased in BALF of mice from the Elastase group compared with Saline mice from the 6th hour (13-fold increase; \( P = 0.021 \)) to day 3 (6-fold increase; \( P = 0.034 \)). BALF MMP-9 activity in the KGF+Elastase group was reduced compared with the Elastase group at day 3 (91% reduction; \( P = 0.05 \)).
MMP-2 activity was detected in all samples (Fig. 6D). Compared with the Saline group, MMP-2 activity was elevated in BALF of mice from the Elastase group at the 6th hour (59% increase; \( P = 0.043 \)), day 1 (6.7-fold increase; \( P = 0.05 \)), and day 3 (2.9-fold increase; \( P = 0.049 \)) but was reduced at day 20 (49% reduction; \( P = 0.021 \)). BALF MMP-2 activity in mice from the KGF+Elastase group was markedly lower that in animals from the Elastase group at day 3 (83% reduction; \( P = 0.025 \)).

Therefore, the potent early anti-inflammatory effect of KGF was associated with the inhibition of MMP-2 and MMP-9 activity in BALF on the third day after elastase instillation.

**KGF did not increase pulmonary expression of TIMPs.** Since KGF pretreatment decreased the activity of MMP-2 and MMP-9 in the lung, we determined whether KGF modulated the pulmonary expression of their specific inhibitors, the TIMPs. TIMP-1 mRNA was sharply increased in the lungs of Elastase compared with Saline mice at all time points studied, reaching a maximum on day 1 (6-fold increase, \( P = 0.014 \)); day 7 (16-fold increase, \( P < 0.001 \)); day 10 (6.7-fold increase, \( P = 0.021 \)); day 14 (6-fold increase, \( P = 0.025 \)); day 17 (6.2-fold increase, \( P = 0.021 \)); and day 20 (6-fold increase, \( P = 0.045 \)). TIMP-2 mRNA was not secondary to an increased anti-MMP shield.

**KGF reduced pulmonary content in elastin and collagen-Iα1 mRNA.** To determine whether KGF pretreatment prevented the degradation of alveolar structures through an elevation in the synthesis of extracellular matrix components, we determined the content of elastin and collagen-Iα1 mRNA in the lungs of mice from the Saline, Elastase, and KGF+Elastase groups. Elastase instillation was followed by a persistent increase in lung elastin mRNA peaking at day 7 (Fig. 7B). KGF pretreatment prevented that increase. Indeed, pulmonary elastin mRNA in mice from the KGF+Elastase group was reduced by 62% at day 3 and 68% at day 7 compared with mice from the Elastase group (\( P = 0.014 \)). The lung content of collagen-Iα1 mRNA was elevated in Elastase mice compared with Saline mice at the 6th hour, day 3, and day 7 (Fig. 7C). KGF pretreatment completely prevented the elastase-induced increase in lung collagen mRNA.

**KGF prevented elastase-induced DNA damage in alveolar septal cells.** We determined by the TUNEL method whether the protective effect of KGF was related to a reduction in DNA damage in alveolar cells on the 7th day after elastase instillation (Fig. 8). In contrast to animals from the Saline group, in which pulmonary cell DNA damage was very rare (1.2 ± 0.8% TUNEL-positive cells, \( n = 3 \)), large numbers of alveolar cell nuclei were stained by TUNEL in Elastase animals on day 7 (67.5 ± 12.4%; \( P = 0.045 \), \( n = 3 \)). KGF greatly reduced the number of alveolar septal cells showing DNA damage (9.5 ± 5.6%; \( n = 3 \)) after elastase instillation (\( P = 0.045 \)).

**Activated caspase-3 was not detected in the lung of elastase-treated animals.** While activated caspase-3 could be detected in thymus cells and sporadically in alveolar macrophages, activated caspase-3 was not detected in alveolar septal cells in any group of animals at 6 h and 1, 3, 7 and 20 days after elastase or saline instillation (data not shown).

**KGF posttreatment had no effect on elastase-induced emphysema.** In view of the strong protective effect of KGF pretreatment on elastase-induced emphysema, we asked whether KGF could have a curative effect in this model. In a first set of experiments, KGF was administered early after elastase instillation, from day 0 to day 7, and mice were killed at day 20. Early KGF treatment did not prevent the development of emphysema since the mean chord length of mice treated with KGF was 68.6 ± 4 μm compared with 66.7 ± 10.9 μm for mice treated with subcutaneous saline (\( P = 0.99 \), \( n = 4 \); Fig. 9). In a second set of experiments, we investigated whether KGF could induce alveolar regeneration after emphysema had been allowed to develop. KGF was administered later after elastase instillation, from day 20 to day 27, and mice were killed at day 27. Late KGF treatment did not induce alveolar regeneration. The mean chord length of mice treated with KGF was 76.3 ± 4.3 μm compared with 76.1 ± 5.5 μm for mice treated with subcutaneous saline (\( n = 4 \); \( P = 0.99 \)).

We asked whether the lack of a curative effect of KGF was due to a reduced expression of the KGF receptor in the lung of elastase-treated animals. We determined the expression of the...
Fig. 6. KGF pretreatment did not modulate elastolytic activity in the lung after elastase instillation but prevented the elastase-induced increase in matrix metalloproteinase (MMP)-9 and MMP-2 activity at day 3. A: representative elastin zymography at the 6th hour after instillation. Lanes 1 and 2, mice treated with intratracheal saline; lanes 3 and 4, mice treated with intratracheal elastase; lanes 5 and 6, mice treated with subcutaneous KGF before intratracheal elastase. Elastase activity was not detected in BALF after the 6th hour time point in any group. B: representative gelatin zymography at day 3 after instillation. Lane 1, Saline mouse; lanes 2 and 3, Elastase mice; lanes 4 and 5, KGF+Elastase mice. C: MMP-9 activity in BALF of Saline (open bars), Elastase (filled bars), and KGF+Elastase mice (dotted bars). MMP-9 activity was not detected in BALF after the day 3 time point in any group. D: MMP-2 activity in BALF of Saline, Elastase, and KGF+Elastase mice. Data are means ± SE. *P < 0.05 vs. Saline, †P < 0.05 vs. Elastase. n = 5 in each group. AU, arbitrary units.

KGF receptor in the lungs of elastase-instilled animals (n = 3) compared with saline-instilled mice (n = 3) at day 20 after instillation. The KGF receptor was detected in alveolar type 2 cells at the membrane level (Fig. 10). The percentage of alveolar cells expressing the KGF receptor was not different in saline (3.4 ± 0.8%) and elastase (4.4 ± 0.8%; P = 0.38)-instilled mice.

DISCUSSION

The main findings of this study are that KGF protected against elastase-induced pulmonary inflammation, activation of MMP-2 and MMP-9, alveolar cell DNA damage, and the subsequent development of emphysema in mice. The anti-inflammatory effect of KGF was related to a reduction in the...
pulmonary expression of the proinflammatory chemokines CCL2 and CXCL2 and of the adhesion molecules ICAM-1 and VCAM-1. In addition to its protective effect in vivo, KGF abolished the elastase-induced increase in CCL2, CXCL2, and ICAM-1 mRNA in a murine alveolar epithelial cell line in vitro.

The elastase-induced emphysema model has been used in research for more than 30 years (10) and has brought considerable insight into the pathogenesis of human disease. Previous data indicate a strong relationship between inflammation and emphysematous changes in this model (13). In support of a crucial role of inflammation in the development of emphysema, we observed that one of the main effects of KGF in this model was a marked reduction in the inflammatory reaction following the instillation of elastase as determined by total protein levels and the number of neutrophils and macrophages in BALF. An anti-inflammatory effect of KGF in the lung was previously reported in the acid aspiration-induced lung injury model (18).

To explore the mechanisms involved in the anti-inflammatory effect of KGF, we determined the pulmonary expression of the chemokines CCL2 and CXCL2, which are the main signals involved in the recruitment of macrophages and neutrophils, respectively, as well as that of the adhesion molecules ICAM-1 and VCAM-1, which play an essential role in leukocyte adherence and migration. In accordance with a major role of CCL2, CXCL2, ICAM-1, and VCAM-1 in the recruitment of inflammatory cells, these four factors were markedly upregulated in the lung after elastase instillation, while the levels observed in KGF-treated mice were markedly lower. It must be noted that a direct cause-and-effect relationship between the elevation of CXCL2 and CCL2 and the recruitment of neutrophils and macrophages in BALF of elastase-instilled animals was not demonstrated in this study.
Gain-of-function polymorphism in the promoter of MMP-9 is linked to the occurrence of pulmonary emphysema in humans (9), and the pharmacological inhibition of MMPs protects against emphysema in hamsters exposed to elastase (15). It was therefore interesting that while an elevation in MMP-2 and MMP-9 activity was initially detected at similar levels in BALF of mice early after elastase instillation (6th hour and day 1), MMP activation resolved much faster in KGF-treated mice. We suspect that the protective effect of KGF was mediated at least in part by this reduction in MMP-2 and MMP-9 activity in BALF. While alveolar epithelial cells have the capacity to secrete MMPs (19), alveolar macrophages are a main source of MMPs in the inflamed lung (6). The reduction in the numbers of BALF macrophages in KGF-treated mice may have contributed to the reduction in BALF MMP-2 and MMP-9 activity in these animals.

In addition to a reduction in elastase-induced pulmonary inflammation and protease excess, we observed that KGF pretreatment prevented elastase-induced DNA damage in alveolar septal cells as detected by the TUNEL method. Meanwhile, active caspase-3 was not detected in those cells at any time point, indicating that in our experiments DNA damage in the lung of elastase-exposed mice could not be attributed to caspase-dependent apoptosis. Indeed, apart from apoptosis, TUNEL staining has been shown to indicate other cell death processes such as necrosis or autolytic cell death (7). Nonetheless, the cytoprotective effect of KGF, which has been well described in vitro as well as in vivo and seems to be mediated principally by the Akt signaling axis (21), may have contributed to the prevention of emphysema in our experiments.

KGF favorably influences tissular repair in various organs, including the skin (23), cornea (22), and digestive tract (28), and we hypothesized that the beneficial effect of KGF in this model could have been related to the enhancement of lung repair. After elastase instillation in rodents, the initial acute inflammatory reaction is followed by a reparative phase during which several components of the extracellular matrix are up-regulated in the lung, including collagen and elastin (12). While this process fails to reestablish the normal pulmonary architecture ad integrum, it somehow manages to limit the extent of emphysematous changes since the pharmacological inhibition of elastin cross-linking leads to worsened emphysema (11). Unexpectedly, the increase in lung elastin and collagen-1a1 mRNA following elastase instillation was blunted in KGF-treated animals, suggesting that KGF exerted its protective effect by limiting the extent of the initial inflammatory injury rather than by enhancing alveolar repair systems. This conclusion is supported by the fact that in the present work, as in previous studies, KGF exerted a beneficial effect only when administered before the insult, although we verified that the expression of the KGF receptor was preserved in the lungs of elastase-treated animals.

While we were able to show only a protective and not a curative effect of KGF, the peculiarities of the elastase-induced emphysema model should be kept in mind when evaluating the therapeutic implications of this result. In particular, while in this model lung injury is unique and limited in time, in the case of human patients injurious events are repeated and span decades. For this reason, we think that therapeutic strategies aimed at protecting the alveolar epithelium from injury may prove useful for slowing the presently inexorable progression of pulmonary emphysema.

Alveolar type 2 cells have been shown to express CCL2, CXCL2, ICAM-1, and VCAM-1 (17, 26). Since the KGF receptor is predominantly expressed by alveolar type 2 cells, the fact that KGF could efficiently reduce the expression of CCL2, CXCL2, ICAM-1, and VCAM-1 indicated a probable role of those cells. This hypothesis is supported by the fact that elastase induced an increase in CCL2, CXCL2, and ICAM-1 mRNA in the MLE-12 murine alveolar epithelial cell line and that this effect was completely prevented by KGF. However, this last result should be considered with caution because it was obtained in a transformed cell line. The demonstration that alveolar epithelial cells play an important role in elastase-induced pulmonary inflammation would require the repetition of those experiments in primary alveolar epithelial cells. Moreover, it is unclear how the elastase concentration used for the in vitro experiments relates to those obtained in the lungs of mice.

While our results highlight the essential role of inflammation in the mechanisms leading to pulmonary emphysema, they also bring some insight into the role of MMPs in this model. MMPs are considered to be crucial for tissular remodeling processes in most organs, including the lung, and a role of MMPs in the development of pulmonary emphysema has been suspected. A gain-of-function polymorphism in the promoter of MMP-9 is...
ACKNOWLEDGMENTS

The authors thank Olivier Thibadeau (Plate-Forme de Morphologie, IFR 02, Hôpital Bichat) for his assistance with preparing anatomic specimens and Dr. Cyril Vadrot (Laboratoire de Microbiologie, Etablissement Pharmaceutique des Hôpitaux de Paris) for his help with endotoxin quantification in elastase samples.

GRANTS

L. Plantier was supported by a research fellowship from INSERM (Poste d’Accueil) and a grant from the Société de Pneumologie de Langue Française (SPLF). S. Marchand-Adam is the recipient of a grant from the Chancellerie des Universités (Legs Poix). J. Boczkowski was supported by a grant from the Fondation pour la Recherche Médicale (Prix Mariane Josso). S. Marchand-Adam is the recipient of a grant from the Fondation de la Recherche Médicale (Prix Mariane Josso). J. Boczkowski was supported by a grant from the Société de Pneumologie de Langue Française (SPLF). S. Marchand-Adam is the recipient of a grant from the Fondation de la Recherche Médicale (Prix Mariane Josso). J. Boczkowski was supported by a grant from the Société de Pneumologie de Langue Française (SPLF). S. Marchand-Adam is the recipient of a grant from the Fondation de la Recherche Médicale (Prix Mariane Josso).

REFERENCES


