Pulmonary effects of keratinocyte growth factor in newborn rats exposed to hyperoxia


1Institut National de la Santé et de la Recherche Médicale, Unité 955, Institut Mondor de Recherche Biomédicale, Équipe 13, Créteil; 2Faculté de Médecine, Université Paris-Val-de-Marne, IFR10, Créteil; 3PremUP, Paris; 4Centre Hospitalier Intercommunal, Créteil; 5Service de Médecine Néonatale de Port-Royal, AP-HP, Hôpital Cochin, Paris; and 6Faculté de Médecine, Université Paris Descartes, Paris, France

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Franco-Montoya ML, Bourbon JR, Durrmeyer X, Lorotte S, Jarreau PH, Delacourt C. Pulmonary effects of keratinocyte growth factor in newborn rats exposed to hyperoxia. Am J Physiol Lung Cell Mol Physiol 297: L965–L976, 2009. First published August 21, 2009; doi:10.1152/ajplung.00136.2009.—Acute lung injury and compromised alveolar development characterize bronchopulmonary dysplasia (BPD) of the premature neonate. High levels of keratinocyte growth factor (KGF), a cell-cell mediator with pleiotropic lung effects, are associated with low BPD risk. KGF decreases mortality in hyperoxia-exposed newborn rodents, a classic model of injury-induced impaired alveolarization, although the pulmonary mechanisms of this protection are poorly defined. These were explored through in vitro and in vivo approaches in the rat. Hyperoxia decreased by 30% the rate of wound closure of a monolayer of fetal alveolar epithelial cells, due to cell death, which was overcome by recombinant human KGF (100 ng/ml). In rat pups exposed to >95% O2 from birth, increased viability induced by intraperitoneal injection of KGF (2 µg/kg body wt) every other day was associated with prevention of neutrophil influx in bronchoalveolar lavage (BAL), prevention of decreases in whole lung DNA content and cell proliferation rate, partial prevention of apoptosis increase, and a markedly increased number of surfactant protein B-immunoreactive cells in lung parenchyma. Increased lung antioxidant capacity is likely to be due in part to enhanced CAAT/enhancer binding protein α expression. By contrast, KGF neither corrected changes induced by hyperoxia in parameters of lung morphometry that clearly indicated impaired alveolarization nor had any significant effect on tissue or BAL surfactant phospholipids. These findings evidence KGF alveolar epithelial cell protection, enhancing effects on alveolar repair capacity, and anti-inflammatory effects in the injured neonatal lung that may account, at least in part, for its ability to reduce mortality. They argue in favor of a therapeutic potential of KGF in the injured neonatal lung.

DESPITE CONSIDERABLE OBSTETRIC and neonatal advances in the care of very low birth weight (VLBW) neonates, bronchopulmonary dysplasia (BPD) continues to occur among 20–40% of survivors, and new ways for combating this disease must be found. Initially described as a fibrotic pulmonary end point following severe respiratory distress syndrome, BPD has considerably evolved with changes in the care of VLBW infants and because of survival of lower gestational age infants than in the original description. It is now usually considered to result from interrupted alveolar development exacerbated by life-sustaining but detrimental effects of invasive neonatal practice (4, 15). Newborn animals exposed to hyperoxia, mechanical ventilation, or airway lipopolysaccharide represent models reproducing the impaired alveolarization observed in premature infants with BPD (1, 41, 52).

Keratinocyte growth factor (KGF), also known as fibroblast growth factor (FGF)-7, is a critical growth factor in lung development (10, 47) and has been demonstrated as a protective agent after oxidant-induced lung injury in both adults and neonates (3, 6, 22, 38). This has potential clinical implication for BPD, since oxidative stress is a component in lung injuries leading to the occurrence of this disease in premature neonates (15). Moreover, previous investigation from our laboratory showed that in premature human neonates, high concentrations of KGF in airways were associated with low risk for BPD (17), whereas mechanical ventilation, another precipitating factor of BPD (15), was reported to downregulate KGF expression in premature rabbits (19). Last, it was shown that KGF protected premature newborn rats from hyperoxic lethality but not from hyperoxic inhibition of postnatal alveolar formation and early pulmonary fibrosis (22). The exact mechanisms of its protective effect have not yet been elucidated, however. Although a number of data about pulmonary effects of KGF have been gained from adult studies, they cannot be directly extended to the growing lung, since injuries interfere with developmental events, especially formation of definitive alveoli. Specific effects could therefore be expected from studies in developing animals.

Known effects of KGF on alveolar type II epithelial cells may account for the beneficial effects of this growth factor in hyperoxic exposure. Actually, inducible expression of KGF in mice exposed to hyperoxia protected the lung epithelium, but not the endothelium, from cell death, which is in keeping with the selective expression of KGF receptors on epithelial cells and not on endothelial cells (43). The well-established stimulatory effect of KGF on type II cell proliferation (14, 39, 53) may be insufficient, however, to explain its ability to restore lung tissue integrity in injured lungs (42). Indeed, KGF inhibited the alveolar damage induced by oxygen breathing in mice, despite the fact that its proliferative effect observed in normoxia was abolished under hyperoxia (6). The KGF protective effect may be mediated by the enhancement of surfactant synthesis evidenced in adult (48, 50, 61) as well as fetal (14) alveolar type II cells. Thus far, however, consequences of KGF treatment for surfactant synthesis in the injured neonatal lung remain unknown. In the adult rat lung in vivo, the KGF-
induced increase in surfactant protein mRNA per lung was shown to result from type II cell hyperplasia, whereas the mRNA content per cell was slightly diminished (63). Moreover, surfactant homeostasis was unchanged in type II cell hyperplasia (20). Finally, the contribution of an antiapoptotic effect of KGF to its protective effect is another likely hypothesis (12, 32). Reactive oxygen species (ROS) are released into the alveolar space and contribute to alveolar epithelial damage in patients with acute lung injury. It was shown that \( \text{H}_2\text{O}_2 \) inhibits alveolar epithelial wound repair in large part by induction of apoptosis and that apoptosis inhibition can maintain wound repair and cell viability in the face of ROS (24).

Consistent with this assumption, KGF inhibited \( \text{H}_2\text{O}_2 \)-induced cleavage of both procaspase-3 and the substrate of caspase-3, the poly(ADP-ribose) polymerase protein, in murine lung cells (42).

To further explore the mechanisms through which the protective effect of KGF is achieved in neonates, we used both in vitro and in vivo approaches for the protection of fetal/neonatal rat alveolar epithelial cells against disorders induced by hyperoxia.

**METHODS**

**Animals**

Pregnant Sprague-Dawley rats were purchased from Charles River Laboratories (Saint Germain sur l’Arbresle, France). Animal studies were conducted according to criteria established by the Institut National de la Santé et de la Recherche Médicale Animal Ethical Committee and performed with authorization of the French Ministry of Agriculture.

**In Vitro Experiments**

Cell isolation and culture. Alveolar epithelial cells were obtained from enzymatically dispersed fetal rat lung cells as previously described (14). Briefly, lungs from 20-day-old fetuses were dissected out under aseptic conditions. Cells were dissociated by incubation in trypsin-collagenase-DNase in MEM and then seeded on plastic to allow fibroblasts to adhere for three successive 45-min steps. Finally, six differential centrifugations were performed at 120 g for 3 min to eliminate remnant fibroblasts. Preliminary studies using modified Papanicolaou stain (lamellar body labeling) showed that alveolar type II cell purity of the final cell suspension was >90%; cell viability (trypan blue exclusion) was >95%. Isolated alveolar epithelial cells were counted, resuspended in 10% FBS-MEM supplemented with penicillin-streptomycin, plated at 4.5 x 10⁵ cells/well in 24-well polystyrene plates (1.9-cm diameter), and allowed to adhere overnight under 95% air-5% CO₂. The medium was then replaced by 0.5 ml of pure MEM and the mix was incubated for 2.5 h at 37°C to perform MTT quantification (34). Briefly, on day 2 of hyperoxia exposure, 62 µl of a 0.2% MTT solution were added to each well containing 0.5 ml of MEM, and the mix was incubated for 2.5 h at 37°C to perform MTT metabolization. The medium was then replaced by 0.5 ml of pure DMSO for 10 min. Next, 200 µl of supernatant were recovered and transferred to a 96-well plate to measure absorbance at 520 nm.

**Evaluation of in vitro cell proliferation by 5-bromo-2’-deoxyuridine labeling.** Proliferation was evaluated using the cell proliferation ELISA 5-bromo-2’-deoxyuridine (BrdU) kit (Roche Diagnostics, Meylan, France). Briefly, cells were seeded in 96-well plates (200 µl/well of a cell suspension adjusted to 450,000 cells/ml) and cultured for 24 h while exposed to hyperoxia or air, in the presence or absence of rhKGF. The DNA was then denatured by adding Fix Denat solution as usual. On day 2 of culture, cells were labeled with BrdU for 24 h and nuclei were fixed for morphometric/morphological analysis, or dropped in liquid nitrogen and kept frozen at −80°C until further assays.

**In Vivo Experiments**

Animal treatments. Rat pups born in the laboratory were divided into groups of equal numbers and body weights between each experimental group (i.e., room air or O₂ exposure) and kept on a 12:12-h light-dark cycle. Food pellets and water were given ad libitum to the dams.

Litters of randomly divided rat pups and their dams were placed in Plexiglas exposure chambers (Charles River Laboratories) and run in parallel with either >95% or 21% (room air) fraction of inspired oxygen, as previously reported (26) from day 0 to day 10. O₂ concentrations were monitored regularly. Because adult rats have limited resistance to high O₂, the dams were exchanged daily between O₂-exposed and room air-exposed litters. Chambers were opened for 20 min every day to switch dams, treat rat pups, and clean cages. Recombinant human KGF (2 µg/g body wt) or vehicle (controls) was injected intraperitoneally at birth and on days 1, 3, 5, and 7. Lung availability of rhKGF after intraperitoneal injection at 2 µg/g body wt was evaluated preliminary using a specific human kit ELISA (R&D Systems, Lille, France). Lung concentration was 20 pg/mg lung tissue 6 h after injection and decreased to 4 pg/mg after 24 h.

On days 5, 7, or 10, rat pups were killed by an intraperitoneal overdose of pentobarbital sodium (70 µg/g body wt) and exsanguinated by aortic transection. Lungs were either immediately lavaged or fixed for morphometric/morphological analysis, or dropped in liquid nitrogen and kept frozen at −80°C until further assays.
Bronchialveolar lavage and cell count in lung fluid. On day 7, rat pups were placed in supine position, and a cannula was inserted in the trachea. Isotonic saline was gently instilled with a syringe and then withdrawn. BAL was performed 12 times with 0.33 ml of sterile saline, and the 12 lavage samples were pooled. Total cell counts were performed on an aliquot fraction with a hemocytometer, and then samples were centrifuged at 300 g for 7 min. Cell pellets were resuspended in an adequate volume to obtain 10^6 cells/ml, and differential cell counts were performed on Cytospin preparations stained with Diff-Quik (Dade Behring, Paris-La Défense, France). A blinded observer counted a minimum of 300 cells to establish the differential cell count. Lavaged lung tissues were discarded.

Lung morphometry analyses. Ten-day-old pups were used. Methods used in this study have been described in detail previously (58). Briefly, lung fixation was performed by tracheal infusion of neutral buffered paraformaldehyde at 20 cmH2O pressure, and fixed lung volume was measured by fluid displacement. After routine processing and paraffin embedding, 4-μm-thick mediofrontal sections through lungs from 10-day-old pups. The assay was performed with ApopTag peroxidase in situ detection kit according to the recommendations of the manufacturer (Quiogen, Illkirch, France). Sections were counterstained with methyl green, dehydrated, and observed with light microscopy. As negative control, nonimmune rabbit serum was used. Five samples of four fields were counted for each tissue section. Three neonates were included for each condition. The mean total number of cells per field and percentage of SP-B-positive cells are presented.

Determination of the proportion of apoptotic lung cells. The terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL) assay was used to monitor the extent of DNA fragmentation as a measure of apoptosis in paraffin-embedded sections of lungs from 10-day-old pups. The assay was performed with ApopTag peroxidase in situ detection kit according to the recommendations of the manufacturer (Quiogen, Illkirch, France). Sections were counterstained with methyl green, dehydrated, and observed with light microscopy. Quantification of TUNEL-positive cells was performed similarly to BrdU labeling.

Western blot analysis of caspase-3. Lung tissues collected on days 5 and 10 were homogenized in RIPA buffer containing protease inhibitors cocktail (Roche Molecular Biochemicals, Meylan, France). Homogenates were centrifuged 10 min at 10,000 g, and protein concentration was determined in supernatants (Bradford assay method). Western blot analysis was performed to evaluate the activated caspase-3 level in lung homogenates at 5 and 10 days. One hundred micromolars of proteins were electrophoresed on a 12% SDS-polyacrylamide gel and transferred onto polyvinylidene difluoride membrane (Millipore, Saint-Quentin-en-Yvelines, France); transfer was checked by staining with Ponceau S dye (Sigma, L’Isle-d’Abeau, France) that also served as loading control. Membranes were exposed overnight at 4°C to a rabbit anti-caspase-3 antibody (Cell Signaling Technology, Danvers, MA) diluted 1:1,000, incubated for 1 h with horseradish peroxidase-conjugated goat anti-rabbit IgG antibody (Santa Cruz Biotechnology, Santa Cruz, USA) diluted 1:5,000, and then enhanced chemiluminescence detection reagent (Amersham Bioscience) for 1 min, and finally exposed to Kodak BioMax MS film for 2 min. Densitometry analysis of blots was performed using the VersaDoc imaging system (Bio-Rad) with the Quantity One program.

RNA extraction and quantitative RT-PCR analysis. Total RNA was extracted using the guanidinium isothiocyanate method (Trizol reagent; Invitrogen, Cergy-Pontoise, France) followed by purification with RNeasy columns (Qiagen, Courtaboeuf, France). RNAs were reverse-transcribed using 2 μg of total RNA, SuperScript II reverse transcriptase, and random hexamer primers (Invitrogen) according to the supplier’s protocol. Real-time PCR was performed on ABI Prism 7000 device (Applied Biosystems, Courtaboeuf, France) using initial denaturation for 10 min at 95°C and a two-step amplification program (15 s at 95°C followed by 1 min at 60°C) repeated 40 times. Melt curve analysis was used to check amplification of a single specific product. Reaction mixtures consisted of 25 ng of cDNA, SYBR green 2× PCR Master Mix (Applied Biosystems), and forward and reverse primers for the examined transcripts (Table 1) in a reaction volume of 25 μl. Primers were designed using Primer Express software (Applied Biosystems). Real-time quantification was monitored by measuring the increase in fluorescence caused by binding of SYBR green dye to double-stranded DNA at the end of each amplification cycle. Relative expression was determined using the ΔΔCt (threshold cycle) method of normalized samples (ΔCt) according to the manufacturer’s protocol in relation to the expression of a calibrator sample and of 18S rRNA.
used as a reference. Each PCR run included a no-template control and a sample without reverse transcriptase. All measurements were performed in triplicate.

**Statistical Analysis**

Data are means ± SE. Differences among three or more groups were evaluated using ANOVA or the Kruskal-Wallis test, as appropriate. Differences between two groups were evaluated using Student’s t-test, Fisher’s post hoc test, or the Mann-Whitney test, as appropriate and as stated. Overall rat pup survival in relation to treatment was evaluated using the Kaplan-Meier survival function, and the log-rank test was used for comparisons between treatment groups. All calculations were performed with StatView software version 5.0 (SAS Institute, Cary, NC). A *P* value <0.05 was considered to be statistically significant.

**RESULTS**

**In Vitro Experiments**

**Effects of hyperoxia on wound healing of alveolar type II cell monolayers.** Hyperoxia markedly impaired wound healing, decreasing the rate of wound closure by ~30% (Fig. 1A). This impairment was mainly due to cell death, presumably mainly because of necrosis, as observed by numbering of trypan blue-labeled cells that were increased about eightfold (Fig. 1B). Cell death was confirmed by an increase of LDH release in conditioned medium (Fig. 1C). Cell viability assessed by MTT test was decreased by 40% under hyperoxia (Fig. 1D), whereas cell proliferation rate was not different (Fig. 1E).

**Effects of KGF on alveolar type II cells exposed to hyperoxia.** Under control conditions (95% air-5% CO₂), KGF alone changed neither the speed of wound healing or cell death, as evaluated by either trypan blue exclusion or LDH determination, nor cell viability and proliferation (data not shown). Under 95% O₂, KGF at the concentration of 10 ng/ml had no effect on all the studied parameters, but when elevated to 100 ng/ml, KGF increased the closure speed of the wound about twofold (*P* <0.01, Fig. 2A), markedly reduced the dead cell surface area (*P* <0.001, Fig. 2B), and slightly decreased the level of LDH released in culture medium (*P* =0.03, Fig. 2C). KGF at 100 ng/ml also increased cell viability ~75% as evaluated by MTT assay (*P* =0.03, Fig. 2D) but did not change the rate of cell proliferation (Fig. 2E). Interestingly, addition of 5% FBS to control medium, which increased wound closure rate about threefold, failed by contrast with KGF to diminish cell death area extension and LDH release (not shown).

**In Vivo Experiments**

**Mortality rate.** KGF significantly improved the survival of neonates (Fig. 3). On day 10, 14 of 19 neonates exposed to
hyperoxia that had received intraperitoneal KGF were still
alive (73.7%), whereas only 6 of 18 neonates exposed to
hyperoxia that received only the vehicle solution were alive
(33.3%; log rank: \( P < 0.004 \)).

Lung morphometry. On day 10, survivors were evaluated for
lung morphometry (Table 2). Lung volumes did not differ be-
tween subgroups. Hyperoxia significantly altered lung growth.
Decreased alveolarization was evidenced by reduced alveolar
surface density (\( P < 0.001 \)), reduced total alveolar surface
(\( P < 0.001 \)), reduced interstitial volume density (\( P < 0.001 \)),
and increased alveolar airspace volume density (\( P < 0.001 \)).
Vascular volume density also was significantly reduced by
hyperoxia (\( P = 0.03 \)). No change was observed in airspace
volume density of airways. KGF did not attenuate hyperoxia-
induced lung development alterations and decreased alveolar
surface density.

Cells in BAL. Cell number and differential count in BAL
were determined on day 7, when the hyperoxia-induced mor-
tality rate began differing significantly between KGF-treated
rat pups and controls. Recovery of instilled saline was similar
in all groups. No significant increase in total BAL cell count
was observed in newborn rats exposed to hyperoxia (Fig. 4).
However, the distribution of cells was significantly changed,
with a decrease in alveolar macrophage proportion (\( P = 0.01 \))
and a significant neutrophil influx into alveoli (\( P < 0.005 \)); the
latter were extremely rare in basal conditions. KGF prevented
neutrophil influx (\( P < 0.004 \)).

Lung DNA content and proportion of SP-B-immunoreactive
cells. Hyperoxia induced a 40% decrease in whole lung DNA
content on day 5, which was totally prevented by KGF injec-
tions (Fig. 5). To focus on this KGF-induced cell protection in
distal lung, we evaluated on lung sections both the total
number of nuclei in alveolar walls and the proportion of
SP-B-positive cells, considered as representing al-
veolar type II cells. Hyperoxia decreased by \( \sim 40\% \) the total
number of cells in alveolar walls in newborns having received
the vehicle only (\( P < 0.001 \), Table 3). This decrease was not
observed for SP-B-positive cells, which means that the propor-
tion of SP-B-positive cells was significantly enhanced under
hyperoxia. KGF had a significant effect on SP-B-positive cells
(Table 3), with both an increase in their absolute number in
room air (\( P < 0.05 \) compared with vehicle-injected controls)
and an increase in their percentage under hyperoxia (\( P < 0.001 \)
compared with vehicle-injected controls exposed to \( O_2 \)).

Lung parenchymal cell proliferation. BrdU incorporation
into lung cells was evaluated at day 7. The percentage of cells
exhibiting BrdU incorporation was significantly reduced by
hyperoxia (\( P < 0.002 \)) but was not significantly changed by
KGF (Fig. 6). Mean values were \( 9.6 \pm 1.1, 6.4 \pm 1.9, 2.7 \pm \)
Caspase-3 that was activated significantly increased from cant difference was associated with KGF treatment. Significantly decreased activated caspase-3 (day 10).

Hyperoxia markedly decreased lung caspase-3 content, which was significantly more pronounced on day 10 (P < 0.05). KGF treatment tended to prevent this increase, since the value, intermediate between that of controls in air and hyperoxia, was no longer different from that of the former but not significantly different from that of the latter. Caspase-3 was evaluated by semiquantitative Western blot analysis (Fig. 7).

Apoptosis. Level of apoptosis evaluated by TUNEL assay was ~1% of cells in newborns exposed to room air (Table 3). Hyperoxia induced a 2.3-fold increase in this proportion (P < 0.05). KGF treatment tended to prevent this increase, since the value, intermediate between that of controls in air and hyperoxia, was no longer different from that of the former but not significantly different from that of the latter. Caspase-3 was more pronounced on day 5 (P < 0.005 between stages). KGF induced a significant decrease in caspase-3 content on day 5 independently of air or oxygen breathing (P < 0.001), but not on day 10. The fraction of caspase-3 that was activated significantly increased from day 5 to day 10 in room air neonates (P < 0.03). Hyperoxia significantly decreased activated caspase-3 (P < 0.02). No significant difference was associated with KGF treatment.

Surfactant phospholipids. DSPC and PG, the major phospholipid components of surfactant, were determined in whole lung tissue on day 5 (Table 4). Hyperoxia did not significantly change DSPC concentration, whereas it significantly decreased PG concentration by ~25%. KGF treatment had no significant effect on these changes. DSPC was also determined in BAL on day 7 and was found to be increased by hyperoxia; KGF did not induce changes in the proportion of DSPC either in room air or under hyperoxia (Table 4).

Gene expression of EGF receptor, transforming growth factor-α, CAAT enhancer binding protein α, and vanin 1. To further explore the possible underlying molecular mechanisms of KGF effects, we used quantitative PCR used to quantify the steady-state levels of the transcripts of I transforming growth factor (TGF)-α and its receptor, epidermal growth factor receptor (EGFR), previously shown to be involved in the mediation of KGF-enhanced spreading and migration of adult alveolar type II cells (2), and 2) of the transcription factor CAAT enhancer binding protein α (C/EBPα) and the ectoenzyme with pantetheinase activity, vanin-1 (Vnn1), known to be involved in pulmonary cytoprotection during hyperoxia exposure in mice (40, 62). These expression levels were determined in 5-day-old rat pups. Hyperoxia increased the transcripts of EGFR and TGF-α about 2.3- and 1.8-fold, respectively, in

**Table 2. Lung morphometry analysis in rat pups treated with rhKGF in room air or under hyperoxia**

<table>
<thead>
<tr>
<th></th>
<th>Room Air</th>
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<th>Hyreoxia</th>
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<tr>
<td></td>
<td>Controls</td>
<td>rhKGF</td>
<td>Controls</td>
<td>rhKGF</td>
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<tr>
<td>Body weight, g</td>
<td>20.6 ± 0.5</td>
<td>20.1 ± 0.4</td>
<td>21.1 ± 0.7</td>
<td>20.8 ± 1.0</td>
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<tr>
<td>Lung volume, ml</td>
<td>1.05 ± 0.05</td>
<td>1.05 ± 0.05</td>
<td>1.10 ± 0.08</td>
<td>1.00 ± 0.05</td>
</tr>
<tr>
<td>Sv(a,p), cm³/cm³</td>
<td>305 ± 29</td>
<td>290 ± 6</td>
<td>220 ± 19†</td>
<td>189 ± 4‡</td>
</tr>
<tr>
<td>Sa, cm³</td>
<td>263 ± 18</td>
<td>252 ± 6</td>
<td>207 ± 34</td>
<td>160 ± 11‡</td>
</tr>
<tr>
<td>Vva, cm³/cm³</td>
<td>0.658 ± 0.009</td>
<td>0.664 ± 0.009</td>
<td>0.739 ± 0.019†</td>
<td>0.748 ± 0.016‡</td>
</tr>
<tr>
<td>Vvi, cm³/cm³</td>
<td>0.247 ± 0.007</td>
<td>0.228 ± 0.007</td>
<td>0.173 ± 0.008†</td>
<td>0.163 ± 0.009‡</td>
</tr>
<tr>
<td>Vvv, cm³/cm³</td>
<td>0.048 ± 0.005</td>
<td>0.055 ± 0.006</td>
<td>0.038 ± 0.014*</td>
<td>0.035 ± 0.005*</td>
</tr>
<tr>
<td>VvA, cm³/cm³</td>
<td>0.047 ± 0.004</td>
<td>0.054 ± 0.006</td>
<td>0.051 ± 0.009</td>
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Values are means ± SE for 6 individuals in each group. *P < 0.05; †P < 0.001 compared with room air controls. ‡P < 0.05 compared with hyperoxia controls (multiple comparisons by ANOVA and 2-group comparisons by Fisher’s post hoc test).

Fig. 4. Bronchoalveolar lavage (BAL) cell contents in 7-day-old rats exposed to room air or hyperoxia from birth. Total cell (shaded bars), alveolar macrophage (open bars), and neutrophil counts (solid bars) are presented. Values are means ± SE for 6 individuals in each group. **P < 0.01 between groups (multiple comparisons by Kruskal-Wallis test and 2-group comparisons by Mann-Whitney test).

Fig. 5. Whole lung DNA content determined in 5-day-old rats exposed to room air or hyperoxia from birth. For each condition, newborns received either KGF (shaded bars) or vehicle (open bars). Values are means ± SE for 6 and 7 individuals per group in room air and hyperoxia, respectively. Hyperoxia diminished DNA content, i.e., total lung cell number, which was prevented by KGF treatment. ***P < 0.001 between groups (multiple comparisons by ANOVA and 2-group comparisons by Fisher’s post hoc test).
either the absence or presence of KGF (Fig. 8). The latter did not significantly change these expression levels, although mean values were slightly higher. C/EBPα transcript was not significantly changed by hyperoxia; KGF did not enhance its expression in the lung of pups exposed to room air, but the transcript, by contrast, was enhanced 88% (P < 0.05) in animals simultaneously exposed to O2 and KGF (Fig. 8). The steady-state level of Vnn1 transcript was increased in both O2-vehicle and O2-KGF groups compared with groups in room air, but the increase was statistically significant only for the O2-KGF group due to the wide dispersion of individual data in the O2-vehicle group (Fig. 8).

**DISCUSSION**

The present study was undertaken to explore at the cellular level possible mechanisms of KGF action susceptible to account for its protective effects toward the exposure of the developing lung to hyperoxia, used as a model of alveolar injury. We report an enhanced rate of alveolar cell wound closure in vitro and maintenance of lung cell content in vivo, likely due to enhanced survival of alveolar epithelial type II cells.

Most studies demonstrating a protective effect of KGF against lung injury have used the intratracheal route (38, 54), rather than the systemic route (6), whereas we administered KGF intraperitoneally and during oxygen exposure. Possible mechanisms to explain the protective effects of KGF in acute lung injury were recently reviewed (57) and are mainly based on effects on alveolar and airway epithelial cells, including increased proliferation (33, 39, 53, 63), increased surfactant production (14, 27, 50, 61), enhanced DNA repair (12, 51, 60), and decreased apoptosis (12, 43). These effects, however, were mainly studied in adult animals or cells and have scarcely been examined in the neonatal period.

In this study, we have demonstrated the protective effect of exogenous KGF on alveolar epithelial cells in neonatal rats exposed to hyperoxia, but we could not relate this effect to known effects of KGF observed in adult animals, except for the increase of C/EBPα expression. Giving KGF to newborn rats was not significantly associated with increased lung cell proliferation rate, decreased lung cell apoptosis, or increased production of surfactant but was shown to have a significant protective effect on alveolar type II cells and to significantly reduce alveolar neutrophil influx. Nevertheless, KGF treat-
Caspase-3 protein

Fig. 7. Lung caspase-3 content evaluated on days 5 and 10 by Western blot analysis. Top: representative blots with 35-kDa band of nonactivated caspase-3 and 17- and 19-kDa bands of activated caspase-3 at day 5 (left) and day 10 (right). Lanes from left to right: vehicle injections in air, KGF injections in air, vehicle injections in O2, and KGF injections in O2. Ponceau S labeling was used as loading control. Bottom: histogram represents means ± SE of densitometric analysis of blots (n = 4 individuals per group). Open bars indicate newborns having received the vehicle, shaded bars indicate newborns treated with KGF, and the hatched portion of bars indicate the activated fraction of caspase-3. Hyperoxia significantly decreased lung caspase-3 content. KGF induced a significant decrease in caspase-3 content on day 5, independently of air or oxygen breathing, but not on day 10. The activated fraction of caspase-3 significantly increased from day 5 to day 10 in room air neonates. Hyperoxia significantly decreased the activated caspase-3 fraction (P < 0.02), but no significant restoration was associated with KGF treatment. **P < 0.001 compared with room air. §P < 0.001 compared with vehicle-injected pups (multiple comparisons by Kruskal-Wallis test and 2-group comparisons by Mann-Whitney test).

For instance, although wet-to-dry lung weight ratio was not changed by KGF in rat neonates (22), an antiedematous effect might have contributed to lung protection, because KGF has been reported to enhance sodium and fluid transport and to prevent alveolar edema in a variety of experimental models of lung injury (3, 25, 44, 56, 65). An antiedematous effect, however, could hardly be explained by maintenance of the type II cell pool, since these cells account for 3–5% of alveolar surface only, or by restoration of damaged alveolar barrier by transdifferentiation of type II into type I cells, a process that is likely to occur principally during recovery after arrest of hyperoxic exposure. A direct effect of KGF on fluid transport by type I cells, which cover the major part of the alveolar surface, appears possible, since evidence has shown that type I cells not only reabsorb water but also contain functional sodium channels and appear responsible for the bulk of transepithelial Na⁺ transport in the lung (28). Last, FGF receptor inhibition has evidenced the key role of the FGF system in regulating vascular integrity (35). KGF therefore might also have prevented fluid leakage and/or favored fluid reabsorption through protection of the pulmonary microvascular network.

We cannot rule out the possibility that these mechanisms, together with those reported in this study, have contributed to enhanced survival in KGF-treated rat pups exposed to hyperoxia.

DNA determination used to evaluate lung cell number indicated that oxygen damage was associated with cell loss and that KGF treatment prevented this effect. Oxygen exposure is known to markedly reduce the turnover of alveolar cells. A previous study in adult mice showed no significant restoration by KGF (6). In the present study, KGF was able to prevent oxygen-induced mortality in neonatal rats despite a low lung-cell proliferation rate, arguing for a lung protective effect that is not necessarily associated with its capacity to sustain epithelial cell proliferation, since BrdU intake increased neither in vivo nor in vitro.

In fact, the main mechanism indicated by our results is the protective effect of KGF toward hyperoxia-induced cell toxicity. Exposure to oxygen is known to result in death of alveolar epithelial and endothelial cells (7, 16). It also was suggested that KGF exerted a protective effect on epithelial cells in adult mice (6). Consistently, we report that KGF protected fetal alveolar epithelial cells exposed to hyperoxia in vitro, as evaluated by the extent of cell death area, the level of released LDH, and increased cell viability. In vivo, prevention of lung DNA decrease and an elevated percentage of SP-B-positive cells also were consistent with protection and maintenance of alveolar type II epithelial cells. A reduced number of type II cells was similarly observed previously in neonatal mice recovering from hyperoxia (64), and the KGF-induced increase in type II cell number is consistent with previous findings in adult animals subjected to oleic acid-induced lung injury (54). KGF overexpression using adenoviral vector was recently shown to induce proliferation of surfactant protein C-positive cuboidal cells, i.e., alveolar type II cells, and also to prevent lung injury (3), which is in keeping with present findings. Type II cell preservation is crucial for the repair process, since alveolar type I cells, which are the most sensitive to injury, reconstitute from type II cells. A higher recovery potential may result from a higher percentage of surviving type II cells postinjury, which confers therapeutic value to KGF.
A recent investigation showed that the transcription factor C/EBPα is required for cytoprotection of type II cells during hyperoxia and that this effect is likely to be mediated through the transcriptional regulation of the vanin-1 gene (Vnn1) to trigger a variety of cellular redox-sensitive signaling processes (62). Vanin-1 is a membrane-bound protein with pantetheinase activity that produces cysteamine, a potent anti-oxidant, and indeed, tissues of Vnn1-deficient mice lack cysteamine (40). We therefore hypothesized that cytoprotection of alveolar cells by KGF could be mediated by enhanced C/EBPα and/or Vnn1 expression. The transcript level of C/EBPα was effectively increased in the lung of rat pups exposed to hyperoxia and treated by KGF compared with both air-exposed controls and hyperoxia-exposed pups injected with the KGF vehicle. Consistent with the present finding, we had previously evidenced enhanced C/EBPβ expression by KGF in isolated type II cells (5), but C/EBPα expression was rapidly lost in vitro, which prevented from determining KGF effects. KGF-increased expression of C/EBPα, which had not been reported formerly, provides a mechanistic support to type II cytoprotection against hyperoxia, likely to have contributed at least for a part to the decreased mortality of rat pups. By contrast, the Vnn1 transcript was augmented as a consequence of the sole exposure to hyperoxia and was not further enhanced by KGF. The effects of enhanced C/EBPα could therefore be mediated by the expression control of other genes, the products of which are involved in antioxidant defense mechanisms. Moreover, the status of Vnn1 appears to be rather complex, since Vnn1-deficient mice have been paradoxically reported to exhibit resistance to oxidative injury, despite the absence of cysteamine, due to elevated stores of glutathione (9).

Despite increased type II cell survival, and although KGF has been reported to enhance the synthesis of surfactant components in vitro (14, 50, 61) and in vivo (27), we found no effect of KGF on lung surfactant content in hyperoxia-exposed rat neonates. Indeed, the effects of hyperoxia on surfactant synthesis seem to be complex and may have interfered with those of KGF. Thus the expression of surfactant protein genes is known to be upregulated by injury, including hyperoxia or lipopolysaccharide exposure, in adult animals (37, 49) as well as in neonatal rat pups.

Table 4. Surfactant phospholipids in rat pups treated with rhKGF in room air or under hyperoxia

<table>
<thead>
<tr>
<th></th>
<th>Controls Room Air</th>
<th>rhKGF</th>
<th>Controls Hyperoxia</th>
<th>rhKGF</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lung-tissue phospholipids</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>DSPC, nmol/mg protein</td>
<td>45.3±2.0</td>
<td>40.7±0.5†</td>
<td>53.3±5.4</td>
<td>42.2±3.6</td>
</tr>
<tr>
<td>PG, nmol/mg protein</td>
<td>11.2±0.7</td>
<td>9.9±0.4</td>
<td>7.2±0.8†</td>
<td>7.1±0.3†</td>
</tr>
<tr>
<td>Lung-lavage phospholipids</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>TPL, nmol/ml</td>
<td>31.3±3.1</td>
<td>21.0±1.4*</td>
<td>35.2±0.1</td>
<td>31.1±2.5</td>
</tr>
<tr>
<td>DSPC, nmol/ml</td>
<td>19.0±1.8</td>
<td>13.8±0.8*</td>
<td>24.6±1.2*</td>
<td>22.3±1.1</td>
</tr>
<tr>
<td>DSPC/TPL, %</td>
<td>60.6±1.0</td>
<td>65.6±2.1</td>
<td>75.7±3.6*</td>
<td>71.7±3.1*</td>
</tr>
</tbody>
</table>

Values are means ± SE of determinations made in 5-day-old pups for tissue phospholipids (n = 6 or 7) and in 7-day-old pups for lung-lavage phospholipids (n = 4 or 5). Controls received the vehicle of rhKGF. DSPC, disaturated phosphatidylcholine. TPL, total phospholipids. In lung tissue, only phosphatidylglycerol (PG) was changed by hyperoxia, and this was unaffected by KGF. The proportion of DSPC in total phospholipids (DSPC/TPL) in lung lavage fluid was increased by hyperoxia but unchanged by KGF. *P < 0.05; †P < 0.01 compared with room air controls. ‡P < 0.05 compared with hyperoxia controls. (multiple comparisons by ANOVA and 2-group comparisons by Fisher’s post hoc test).

Fig. 8. Steady-state level of the transcripts for epithelial growth factor receptor (EGFR), transforming growth factor-α (TGF-α), CAAT/enhancer binding protein α (C/EBPα), and vanin-1 in the lung of 5-day-old rat pups. Hyperoxia increased EGFR and TGF-α transcripts; KGF increased the C/EBPα transcript in the presence of hyperoxia. *P < 0.05 compared with controls in room air. ‡P < 0.05 compared with vehicle-injected pups under hyperoxia (multiple comparisons by Kruskal-Wallis test and 2-group comparisons by Mann-Whitney test).
as in newborns (18). Phospholipid compartment had not been investigated in depth formerly in this condition. Alterations in individual phospholipid components may differ from those of surfactant proteins, since we found a decrease in lung tissue PG but an increase in BAL DSPC. On the other hand, surfactant homeostasis was maintained in rats in vivo following tracheal instillation of KGF despite type II cell hyperplasia (20). Although we did not evaluate the pulmonary content in surfactant proteins in the present study, it seems unlikely that beneficial effects of KGF on survival may relate to surfactant.

We also evaluated in vitro the effect of KGF on wound repair. By contrast with adult type II cells (23), KGF did not enhance wound closure in fetal cells under normoxic conditions. Nonetheless, KGF restored wound closure ability under hyperoxia, which appeared to be due mainly to prevention of cell death. This suggests increased repair potential of type II cells in the presence of KGF. From a mechanistic point of view, previous investigation (2) had evidenced that intratracheal KGF in vivo enhanced the repair potential of rat type II cells by nonmitogenic mechanisms through increased spreading and migration, as assessed in vitro on isolated cells. This was shown to require the mediation of TGF-α through binding to EGFR (2). We therefore hypothesized that the effects of KGF reported in this study could be mediated, at least in part, by control of either TGF-α or EGFR expression. In fact, the transcripts of both these proteins were markedly elevated by hyperoxic exposure in vivo, consistent in the instance of TGF-α with previous observations in neonatal rabbits (55). KGF, however, induced significant change under neither normoxia nor hyperoxia. The mechanism evidenced in adult rat cells therefore does not seem to account for the protective effects of KGF in rat neonates.

Cell death resulting from oxygen-induced adult lung injury presents features of both necrosis and apoptosis (7). Our data suggest that nonapoptotic cell death is the predominant effect of hyperoxia in newborns rat lungs, as indicated by a lack of increase in activated caspase-3 and the slight increase in TUNEL-positive cell number. This is also clearly supported by trypan blue exclusion study in cultured cells, although we did not evaluate apoptosis directly in this instance. Nonapoptotic cell death was previously recorded in cultured human lung epithelium exposed to O₂, with swelling of nuclei, increase in cell size, and no evidence of any augmentation in the levels of caspase-3 activity (21). The dissociation we observed between TUNEL and caspase-3 findings is in agreement with the involvement of multiple apoptotic pathways in hyperoxia (7). Thus, in adult mice, despite the evidence of changes specific for apoptosis, including internucleosomal DNA degradation, the marked increase in lung RNA or protein levels of p53, Bax, Bcl-x, and Fas, which are known to be expressed in certain types of apoptosis, contrasted with the absence of increase in proteases of the apoptosis “executioner” machinery, such as caspase-1 or -3 (7). Furthermore, it has not been possible so far to attenuate oxygen-induced injury by using antiapoptotic strategies (7). In fact, the main inducer of activated caspase-3 in the present study was postnatal age. Apoptosis is known to be involved in lung remodeling during normal pre- and postnatal development in humans and rats (45, 46). Postnately, the role of apoptosis appears to be to rid the lung of excess fibroblasts and epithelial cells to increase the gas exchange surface area (31, 46). Thus apoptosis levels were shown to abruptly increase at the time of spontaneous birth, up to 9% of cells, and to then rapidly decrease to ~1% of cells by day 2 of life (31). It remained low during alveolar septation, a stage that is conversely characterized by a high index of proliferation, but from day 13 (i.e., after completed septation), the number of lung fibroblasts undergoing apoptosis increased four- to fivefold (11).

Last, we found that KGF prevented alveolar neutrophil influx in hyperoxia-exposed newborns. A similar effect of KGF was previously shown in adult mice subjected to intratracheal acid instillation, with a decrease in macrophage inflammatory protein-2α gradients between BAL fluid and plasma suggested as the underlying mechanism (36). Presumably, cytoprotection of alveolar epithelial cells diminished inflammation signals involved in neutrophil recruitment, including expression of a variety of cytokines. Decreased neutrophil influx, in turn, might have diminished secondary injury to the epithelium. Moreover, KGF also was shown to decrease ICAM-1 and VCAM-1 expression and neutrophil adherence in bronchial epithelial cells, suggesting its involvement in the resolution of the inflammatory reaction (29).

In conclusion, we report evidence for KGF protection of alveolar epithelial cells, probably through enhanced antioxidant defense mechanisms, enhancing effects on alveolar repair capacity, and anti-inflammatory effects in the injured neonatal lung that may account, at least in part, for the ability of the factor to reduce pup mortality. Although no effects on impaired alveolarization were observed, this argues in favor of a therapeutic potential of KGF in neonatal chronic lung disease.

ACKNOWLEDGMENTS

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