Original Article

Therapeutic effects of recombinant human keratinocyte growth factor-2 on hyperoxia-induced bronchopulmonary dysplasia in neonatal rats

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Abstract: Background: Bronchopulmonary dysplasia (BPD) is one of the most devastating conditions in premature babies; meanwhile, keratinocyte growth factor-2 (KGF-2) plays a key role in lung development. The aim of this study was to examine the therapeutic effects of recombinant human KGF-2 (rhKGF-2) in a hyperoxia-induced BPD rat model and explore the potential underlying mechanisms. Methods: A total of 75 newborn Sprague-Dawley (SD) rats were assigned to 5 groups, including normoxia control, normoxia+rhKGF2, BPD (no treatment), BPD+saline (NS) and BPD+rhKGF2 groups (n=15/group). For treatment, rats were intratracheally administered rhKGF2 (5 mg/kg) or equal volume of saline, and sacrificed 2 weeks later. Weights and wet-to-dry weight ratio (W/D), an indicator of lung edema) values of lung specimens were measured. Then, IL-1β, IL-6, TNF-α, and macrophage inflammatory protein-2 (MIP-2) levels were quantitated by enzyme-linked immunosorbent assay (ELISA) in bronchoalveolar fluid (BALF) samples. Next, lung tissue specimens were assessed by H&E staining, immunohistochemistry (VEGFa and NF-κB p65), quantitative real-time reverse transcription polymerase chain reaction (qRT-PCR; surfactant protein C or SPC) and immunoblotting (VEGFa, NF-κB p65 and p-p65). Results: In the hyperoxia-induced BPD rat model, rhKGF-2 promoted lung growth and development, and reduced pulmonary edema. Treatment with rhKGF-2 resulted in reduced BALF levels of inflammatory cytokines, increased SPC mRNA levels and VEGF protein expression in the lung, and slightly decreased lung NF-κB p65 nuclear expression and phosphorylation. Conclusion: rhKGF-2 alleviates hyperoxia-induced BPD, likely through reduced inflammation and induction of SPC.

Keywords: Bronchopulmonary dysplasia, keratinocyte growth factor-2, neonatal rats, inflammation, hyperoxia

Introduction

Bronchopulmonary dysplasia (BPD) is one of the major causes of adverse health outcome in premature infants [1]. As a result, long-standing consequences of BPD involve multiple organ systems, inducing adverse effects on pulmonary function [2]. Despite important advances in neonatology in the past two decades, BPD incidence remains above 30% in neonates with a gestational age below 30 weeks [3].

Currently, ventilation and oxygen therapy are often employed for respiratory failure in preterm neonates in order to improve survival [4]. However, the immature lung of neonates has inadequate antioxidant and anti-inflammatory capacities [5]. Therefore, oxygen treatment may cause oxygen toxicity and inflammation, resulting in hyperoxic lung injury, alveolar simplification and reduced-gas exchange, which is the hallmark of BPD [6]. Thus, it is imperative to develop novel therapeutic strategies that can improve alveolar growth and development. Indeed, treatment should go beyond current clinical options, to address various stages of the disease process.

Keratinocyte growth factor-2 (KGF-2), also referred to as fibroblast growth factor-10 (FGF-10), is a member of the fibroblast growth factor family that was first reported in 1996 [7]. KGF-2 is a 20-kD heparin-binding protein, predominantly expressed by mesenchymal cells [8]. KGF-2 binds to a spliced variant of fibroblast growth factor receptor 2-IIIb (FGFR-2IIIb) with
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high affinity on epithelial cells, and reduced affinity for FGFR-1IIIb on epithelial/endothelial cells [9, 10]. It mediates epithelial-mesenchymal interactions in a paracrine manner, and is essential for lung development [11]. The protective effects of KGF-2 have been reported in various disease conditions, including bleomycin-induced pulmonary fibrosis, high altitude pulmonary edema, lipopolysaccharide (LPS) toxicity, mechanical ventilation and ischemia/reperfusion-induced lung injury [12-16]. However, little is known about the therapeutic effects of KGF-2 on hyperoxia-induced lung injury in vivo.

Based on the above-mentioned properties of KGF-2, we hypothesized that it could alleviate BPD. Thus, this study aimed to assess the protective effects of rhKGF-2 on hyperoxia-induced BPD in neonatal rats, exploring the potential underlying mechanisms.

Materials and methods

Animals

Sprague-Dawley (SD) rats were purchased from the Academy of Military Medical Sciences, and bred in a specific pathogen free (SPF) environment, with rodent chow and water available ad libitum. All experimental protocols were approved by the Institutional Animal Care and Use Committee of the PLA Army General Hospital. All procedures were performed according to the guidelines of the National Institutes of Health concerning the care and use of laboratory animals.

Rat model of BPD and experimental groups

The experimental BPD rat model was induced as described by O’Reilly M, et al [17]. Newborn rats (n=75) were assigned to normoxia (21% O₂, n=30) and hyperoxia (85% O₂, n=45) groups, and placed in plastic chambers with continuous O₂ supply from postnatal day 1 to day 14. The neonates were housed with their mothers, and normoxia exposure in mother rats was performed with air break to prevent maternal O₂ toxicity. The 75 neonates were assigned to 5 groups, including (1) normoxia control, (2) normoxia+rhKGF2, (3) BPD (no treatment), (4) BPD+NS and (5) BPD+rhKGF2 groups (n=15/group). The success of model establishment was verified by randomly selecting 2 rats for pathological examination.

Animal treatments

The animals were treated twice, at 1-week interval [18]. Briefly, after model establishment, rats were intraperitoneally anesthetized with chloralhydrate (300 mg/kg). Then, rhKGF2 (Newsummit Pharmaceutical Company, China) (diluted with saline to a concentration of 1 mg/ml; 5 mg/kg) was injected intratracheally in the normoxia+rhKGF2 and BPD+rhKGF2 groups, with the same volume of saline administered to the BPD+NS group, using an 18 G catheter attached to a 1-ml syringe. Two weeks after the last intratracheal injection, the animals were sacrificed following anesthesia, and both lungs in each animal were collected and weighed. This was followed by lung lavage, for bronchoalveolar lavage fluid (BALF) collection. Lung tissues were subsequently stored at -80°C or fixed with 10% paraformaldehyde for further use.

Lung wet-to-dry weight ratio (W/D)

The right main bronchus was ligated, and the right lung was collected. After wet weight measurement, the middle lobe was placed in an oven at 60°C for 72 h, for determination of wet-to-dry weight ratio.

Bronchoalveolar lavage

The left lung was lavaged for BALF collection. In brief, 2 ml of PBS (4°C) was slowly infused for 1-2 min, and the fluid was slowly withdrawn, for 3 cycles. The resultant fluid was collected and stored at -80°C for further analysis.

H&E staining and immunohistochemistry

Lung tissue samples were fixed with 4% paraformaldehyde, dehydrated in a series of graded ethanol, paraffin embedded and sectioned (4 μm). For H&E staining, the sections were treated with xylene, a series of graded ethanol, and washed with distilled water. Hematoxylin and eosin were used to stain the nucleus and the cytoplasm, respectively. After dehydration and mounting, the sections were observed under a light microscope.

Immunohistochemistry was used to detect expression of VEGF and nuclear expression of NF-κB p65. For immunohistochemistry, endogenous peroxidase was blocked with 3% bovine serum albumin (BSA), and the sections were incubated with primary antibodies, including...
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anti-VEGF 
 anti-NF-κB p65, overnight at 4°C, followed by incubation with secondary antibody for 50 min at room temperature. DAB kit (DAKO, Denmark) was used for visualization, according to the manufacturer’s instructions. The sections were observed under a light microscope.

Enzyme-linked immunosorbent assay (ELISA)

Two weeks after the last rhKGF-2 intratracheal administration, BALF samples were collected and centrifuged at 2000-3000 rpm/min for 20 min at 4°C. The levels of cytokines, including IL-1β, IL-6, TNF-α and macrophage inflammatory protein-2 (MIP-2), were measured in the resulting supernatants by specific ELISA kits, according to the manufacturer’s instructions.

Quantitative real-time reverse transcription polymerase chain reaction (qRT-PCR)

Lung tissues were homogenized in liquid nitrogen, and total RNA was extracted with TRIzol reagent (Thermo Fisher, USA), according to the manufacturer’s instructions. Total RNA was subjected to reverse transcription into cDNA with a reverse transcription kit (Promega, USA). The primers of SPC used for qRT-PCR were 5′-ATGGACATGGGTAGCAAAGAGG-3′ and 5′-AC-TACCACCAACCCACGATGA-3′. And the primers of conference gene GAPDH were 5′-CTGGGAGATGCTTGTGC-3′. qRT-PCR was performed in triplicate using a detection system (Bio-Rad Laboratories, Inc., USA). A DyNAmo™ Flash SYBR® Green qPCR Kit (Thermo, USA) and primers were used with the following program: hot start at 95°C for 10 min, 40 cycles of annealing at various temperatures for 60 s and extension at 60°C for 5 min, followed by the final single-peak melting curve program. The comparative threshold cycle method (2-ΔΔCt) was used for data analysis. The relative expression of SPC gene mRNA was calculated and normalized based on the GAPDH levels using SDS software, version 1.4.

Western blotting

Statistical analysis

Statistical analyses were performed with SPSS, version 22.0 (SPSS, USA). Data are expressed as mean ± standard deviation (SD) and were assessed by an independent samples t-test (group pairs) or one-way analysis variance (ANOVA; multiple groups) followed by a post hoc LSD t test. P<0.05 was considered statistically significant.

Results

Hyperoxia-induced BPD model in neonatal rats

In this study, we first established a hyperoxia-induced BPD rat model. In the hyperoxia group, rats showed rough and disheveled hair seven days after birth, as well as reduced activity and anxiety. At 14 days, these animals were further deteriorated, with significantly reduced body weights, rough and disheveled hair, as well as
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anxiety, shortness of breath, and cyanosis of the lips and toes immediately after discontinuation of hyperoxia exposure. Meanwhile, the normoxia group was active and had normal body weight gain, with lustered hair and good response to stimulation. Body weights in the hyperoxia and normoxia groups were 34.92±4.23 g and 42.11±5.27 g, respectively, indicating a statistically significant difference (t=-6.529, P<0.001).

H&E staining revealed even alveolar septa in the normoxia group (Figure 1A); meanwhile, the lung structure showed typical BPD features, including thickened alveolar septa, scattered areas of hemorrhage and inflammatory cell infiltration, reduced alveoli, and inflammatory atelectasis in the hyperoxia group (Figure 1B). These findings indicate that the hyperoxia-induced BPD model was successfully established. No unexpected death occurred in experimental animals.

KGF-2 promotes lung development in hyperoxia treated rats

Next, the effects of KGF-2 on lung development in BPD rats were assessed. A total of 2 weeks after modeling, reduced activity and body weight gain were observed in untreated BPD rats (BPD and BPD+NS groups). Indeed, body weights in the BPD and BPD+NS groups were 129.15±8.92 g and 134.79±4.39 g, respectively, which were significantly lower than those of the normoxia group (t=-5.125, P<0.001; t=-4.299, P<0.001). Meanwhile, the normoxia+rhKGF2 and BPD+rhKGF2 groups showed normal activity and body weights of 147.14±5.14 g and 139.21±11.48 g, respectively, which were similar to those of the normoxia group (t=0.372, P=0.06; t=-1.818, P=0.305). Next, H&E staining was performed for histopathological analysis; in the normoxia (Figure 2A) and normoxia+rhKGF2 (Figure 2B) groups, alveolar septa appeared evenly, with no exudation; C and D. In the BPD and BPD+NS groups, respectively, lung tissues showed irregular alveolar structure, enlarged alveolar space and reduced number of alveoli. E. The alveolar structure was improved in the BPD+rhKGF2 group compared with the BPD and BPD+NS groups, with increased number of alveoli, less hemorrhage and reduced infiltration of inflammatory. Scale bar: 200 μm.

KGF-2 reduces hyperoxia-mediated edema and inflammation

To further evaluate the effects of rhKGF-2 in the rat model of BPD, lung edema and BALF levels...
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Significantly increased W/D values were observed in the BPD and BPD+NS groups compared with the normoxia group (P<0.05, BPD group and BPD+NS group vs. Normoxia group) (Figure 3A). Interestingly, intratracheal administration of rhKGF-2 resulted in decreased lung water content in BPD model animals (P<0.05, BPD+rhKGF2 group vs. BPD and BPD+NS groups) (Figure 3A). No significant difference in W/D between the normoxia+rhKGF2 and normoxia groups was found.

ELISA showed significantly increased levels of inflammatory cytokines, including IL-1β, IL-6, TNF-α and MIP-2, in BALF samples from the BPD and BPD+NS groups compared with the normoxia group (P<0.05, BPD group and BPD+NS groups) (Figure 3B-E). Interestingly, intratracheal administration of rhKGF-2 resulted in decreased lung water content in BPD model animals (P<0.05, BPD+rhKGF2 group vs. BPD and BPD+NS groups) (Figure 3A). No significant difference in W/D between the normoxia+rhKGF2 and normoxia groups was found.

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Figure 5. Effect of KGF-2 on VEGF protein expression in rat lung samples. High VEGF amounts were found in the normoxia (A) and normoxia+rhKGF-2 (B) groups, decreasing in the BPD (C) and BPD+NS (D) groups in comparison; the BPD+rhKGF-2 group (E) showed higher levels compared with the BPD group (DAB-based immunohistochemistry; 400×). Similar results were obtained by Western blotting (F). The expression of VEGF protein detected by immunohistochemistry was quantified as (G), and that detected by Western blotting was quantified as (H) (*P<0.05 vs. normoxia group; #P<0.05 vs. BPD and BPD+NS groups). Scale bar: 50 μm.
Figure 6. Effect of KGF-2 on NF-κb p65 protein nuclear expression and phosphorylation in rat lung samples. Low NF-κB p65 nuclear amounts were observed in the normoxia (A) and normoxia+rhKGF-2 (B) groups, increasing in the BPD (C) and BPD+NS (D) groups in comparison; the BPD+rhKGF-2 group (E) showed slightly reduced levels compared with the BPD group (DAB-based immunohistochemistry; 400×). Similar results were obtained by Western blotting (F). The nuclear expression of NF-κb p65 protein detected by immunohistochemistry was quantified as (G)
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BPD+NS group vs. Normoxia group) (Figure 3B-E). Meanwhile, intratracheal administration of rhKGF-2 significantly reduced the amounts of the above cytokines in the BALF (P<0.05; BPD+rhKGF2 group vs. BPD and BPD+NS groups) (Figure 3B-E). No significant differences were found in the expression levels of these cytokines between the normoxia+rhKGF2 and normoxia groups. These findings indicate that KGF-2 alleviated hyperoxia-mediated edema and decreased inflammation in BPD rats.

Effects of rhKGF2 on lung surfactant protein C (SPC) mRNA expression and vascular endothelial growth factor (VEGF) levels

Next, we determined the lung contents of SPC and VEGFa after treatment with rhKGF2 in BPD models. SPC mRNA expression levels significantly decreased in the BPD and BPD+NS groups compared with control values (P<0.05) (Figure 4). However, these values markedly increased in the BPD+rhKGF2 group (P<0.05; BPD+rhKGF2 group vs. BPD and BPD+NS groups) (Figure 4). The BPD+rhKGF2 and normoxia groups showed similar values (P>0.05, Figure 4).

In addition, immunohistochemistry, Western blotting and VEGF quantification showed that VEGF expression was reduced in the BPD (Figure 5C and 5G) and BPD+NS (Figure 5D and 5G) groups compared with control values. This effect was alleviated by treatment with rhKGF2 (BPD+rhKGF2 group; Figure 5E and 5G). No significant difference between the BPD+rhKGF2 and normoxia groups was observed (Figure 5E and 5G). Changes in VEGF protein expression were similar in Western blotting and immunohistochemistry (Figure 5F and 5H).

KGF-2 suppresses NF-κB p65 nuclear expression and phosphorylation

The NF-κB signaling pathway plays an important role in inflammation, and NF-κB is known to suppress KGF-2 expression [19]. As shown above, rhKGF-2 treatment reduced the expression levels of inflammatory cytokines. Thus, whether KGF-2 could inhibit the NF-κB expression was assessed. Since p65 is a key component of NF-κB, NF-κB p65 nuclear expression and phosphorylation were detected in this study. Immunohistochemistry showed that NF-κB p65 nuclear expression was lower in the normoxia and normoxia+rhKGF2 groups (Figure 6A, 6B and 6G), but significantly higher in the BPD and BPD+NS groups, compared with control values. Interestingly, the BPD+rhKGF2 group showed slightly decreased NF-κB p65 nuclear expression compared with the BPD and BPD+NS groups (Figure 6E and 6G). Western blotting for NF-κB p65 phosphorylation yielded similar results (Figure 6F and 6H).

Discussion

Approximately 15 million premature babies are born each year around the world, prematurity complications kill more newborns than any other disease [20]. BPD is one of the most devastating conditions in premature infants, with long lasting consequences, and involves multiple organ systems [2]. Classic BPD was first described by Northway and colleagues in 1967, as lung injury in preterm infants, resulting from oxygen and mechanical ventilation [21]. BPD is characterized by arrested alveolarization and vascularization due to extracellular matrix remodeling, inflammation, and impaired growth factor signaling in the immature lung during the early canalicular and saccular periods of lung development [22]. Infants who develop BPD often suffer from respiratory and neurological sequelae [23]. Currently, there is no effective treatment for BPD, and therefore, the development of effective treatments has been a focus in studies assessing BPD, for which a good animal model is essential. In this study, neonatal SD rats were subjected to hyperoxia exposure and a BPD model was successfully established.

KGF-2 mediates epithelial-mesenchymal interactions, which are essential for branch morphogenesis in lung [18]. Recently, it was shown that KGF-2 prevents lung injury after various stresses, including acute lung injury [24]. Injectable KGF-2 shows favorable safety in phase II trials in ulcerative colitis and mucositis [25]. Thus,
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we hypothesized that exogenous KGF-2 may protect against hyperoxia-induced BPD in neonatal rats. Indeed, we found that intratracheal administration of rhKGF2 promoted alveolar growth in BPD rats, improved clinical symptoms and lung histology, suggesting that KGF-2 prevents lung injury in hyperoxia-induced BPD.

Previously, Abman and Matthay described KGF-2 as a potent alveolar type II cell mitogen which plays an important role in preventing alveolar epithelial cells from DNA damage and apoptosis via the MAPK/ERK pathway [26]. In addition, pre-treatment with KGF-2 significantly improves high-altitude pulmonary edema in rats, most likely by reducing apoptosis and inducing proliferation in type II alveolar cells [13]. In this study, we wanted to explore the potential mechanisms underlying the therapeutic effects of KGF-2 in hyperoxia mediated BPD. As shown above, intratracheal administration of KGF-2 significantly decreased lung W/D ratios, indicating the therapeutic effect of KGF-2 on pulmonary edema. In addition, KGF-2 markedly reduced the secretion levels of the inflammatory cytokines IL-1β, IL-6, TNF-α and MIP-2 in BALF of BPD rats, indicating that it prevents lung injury in these animals. These findings suggested that KGF-2 may exert its preventive effects by combining alleviation of pulmonary edema, reduction of inflammatory cytokine secretion, and prevention of macrophage and neutrophil accumulation. These effects reduced lung damage and improved lung development in newborn rats. These findings corroborated previous studies showing that BPD development is associated with increased amounts of inflammatory cells and pro-inflammatory cytokines in the lung [27]. It is also known that decreased alveologenesis with interstitial thickness is associated with increased neutrophil cytokines in hyperoxia exposed rats [28].

SPC is a key marker of type II alveolar epithelial cells [29]. Therefore, we further evaluated SPC expression in lung epithelial cells in hyperoxia-induced BPD rats. Because of the low expression of SPC in alveoli, it is difficult to detect it by Western blotting. As the content of SPC in lung lavage fluid is also low, we detect its mRNA by qRT-PCR. As expected, SPC mRNA levels were reduced in the BPD group, and markedly increased after KGF-2 treatment (BPD+KGF-2 group). This strongly indicated that KGF-2 promotes growth in type II alveolar epithelial cells. The pathophysiological roles of VEGF have been intensively studied. For example, VEGF is considered a potent endothelial cell-specific mitogen that promotes vascular growth and remodeling [30]. In addition, VEGF is involved in the formation and maintenance of pulmonary and alveolar structures in infants and adults [31], and immature lungs are sensible to VEGF down-regulation [32]. Furthermore, reduced VEGF is associated with the pathology of BPD [33, 34]. In the current study, KGF-2 upregulated VEGF expression in BPD animals, subsequently preventing the pathological changes of hyperoxia mediated BPD. It is reported that KGF-2 is a major player in alveologenesis and regeneration of the lung after injury, by upregulating VEGF in the distal epithelium and directing the differentiation of the bipotent progenitor cells towards the AEC II lineage, but no report has shown that KGF-2 has any effect on normal lung tissue. The increased levels of SPC and VEGF were closely associated with the therapeutic effects of KGF-2 in BPD rats, but the levels of SPC and VEGF were not affected by KGF-2 in normal rats.

As a core transcription factor, NF-κB regulates cellular inflammatory, participates in immune responses, and regulates cell differentiation and apoptosis [35]. It is activated by a variety of stimuli that include cytokines, growth factors and lymphokines. The various stimuli that activate NF-κB cause phosphorylation of IκB, which is crucial to its role. Nuclear expression and phosphorylation of NF-κB p65 (a key component of NF-κB) are significantly increased in the lung tissues of BPD animals, whereas KGF-2 expression is markedly reduced [19]. Meanwhile, interactions between NF-κB p65 and SP3 inhibit KGF-2 expression [36]. In the current study, KGF-2 reduced the secretion levels of inflammatory cytokines. Thus, we hypothesized that KGF-2 may downregulate NF-κB. Indeed, as demonstrated above by immunohistochemistry and Western blotting, NF-κB p65 nuclear expression and phosphorylation were slightly reduced after KGF-2 administration in BPD rats. These findings suggested that KGF-2 may also, by inhibiting NF-κB p65 nuclear expression and phosphorylation, attenuate inflammation, thereby promoting lung repair in neonatal rats after hyperoxia-induced BPD.
Collectively, the current study demonstrated that rhKGF-2 prevents lung damage in hyperoxia-induced BPD rats via multiple routes; however, in terms of clinical application, further investigation is required to comprehensively determine the therapeutic effects of KGF-2 and the underlying mechanisms.

This study had several limitations. First, we did not use a comprehensive approach to assess various molecules and pathways involved in BPD pathology. In addition, we have only tentatively discussed the interactions of rhKGF2 and NF-κB, but have not explored the mechanisms of inhibiting inflammation in depth. Finally, whether results generated in SD rats would reflect the clinical situation remains unknown. Therefore, further investigation is required to confirm our findings.

Conclusion

In summary, KGF-2 promotes lung development in hyperoxia-mediated BPD in neonatal rats. These protective effects may combine alleviation of pulmonary edema, reduction of inflammatory cytokine secretion, and prevention of macrophage and neutrophil accumulation. These findings provide potential options for future clinical studies aiming at treating BPD patients to prevent long-term sequelae.

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Disclosure of conflict of interest

None.

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