

Intratracheal Administration of Recombinant Human Keratinocyte Growth Factor Promotes Alveolar Epithelial Cell Proliferation during Compensatory Lung Growth in Rat

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Keratinocyte growth factor (KGF) is considered to be one of the most important mitogens for lung epithelial cells. The objectives of this study were to confirm the effectiveness of intratracheal injection of recombinant human KGF (rhKGF) during compensatory lung growth and to optimize the instillation protocol. Here, trilobectomy in adult rat was performed, followed by intratracheal rhKGF instillation with low (0.4 mg/kg) and high (4 mg/kg) doses at various time-points. The proliferation of alveolar cells was assessed by the immunostaining for proliferating cell nuclear antigen (PCNA) in the residual lung. We also investigated other immunohistochemical parameters such as KGF, KGF receptor and surfactant protein A as well as terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling. Consequently, intratracheal single injection of rhKGF in high dose group significantly increased PCNA labeling index (LI) of alveolar cells in the remaining lung. Surprisingly, there was no difference in PCNA LI between low and high doses of rhKGF with daily injection, and PCNA LI reached a plateau level with 2 days-consecutive administration (about 60%). Our results indicate that even at low dose, daily intratracheal injection is effective to maintain high proliferative states during the early phase of compensatory lung growth.

Key words: recombinant human KGF, intratracheal injection, compensatory growth, trilobectomy, PCNA

I. Introduction

Chronic obstructive pulmonary disease (COPD), including emphysema, is increasing, and by 2020 will be the third leading cause of death worldwide. COPD patients tend to merge lung cancer. Especially, patients of combined pulmonary fibrosis and emphysema syndrome have a high prevalence of lung cancer [11]. However, the high rates of morbidity and mortality are actually associated with their

lower forced expiration volume in one second. Therefore, development of effective therapies to promote lung regeneration is critically needed.

Because of the structural complexities, lung regeneration has been known to be difficult, compared to that of liver, neuron or skin. On the other hand, compensatory lung growth after lung injury or surgical resection was reported in the previous papers [5, 9, 24]. Although the compensatory lung growth is a transient event and cannot be continued for a long time, it should have a possibility to prevent the decline of lung function or maintain the function after lung resection. The growth process in compensatory lung growth involves increasing lung volume, weight, cell proliferation, and pulmonary function. In fact, it has been

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reported previously that many pathways are involved in the compensatory lung growth [8, 10, 19], and they seem to be interacted mutually making the situations complicated. Eventually, there remain many hurdles to be considered for the application to clinical therapy.

Rubin and colleagues discovered keratinocyte growth factor (KGF) as a mesenchymal cell-derived epithelial cell mitogen [18], and subsequently KGF receptor (KGFR; *bek IIIb*) was identified in various epithelial cells [7]. KGF has been reported as a pleiotropic growth factor with morphogenic, motogenic, tumorigenesis and anti-apoptotic properties in the lung [16, 17, 20, 23, 26]. KGF mainly stimulated proliferation and function of lung alveolar type II cells [6], but also promoted the regeneration of tracheal cartilages in rodents [1].

For recovery of pulmonary function in damaged lung, the increase of surface area of alveoli should be essential to maintain the abilities of gas exchange. Alveolar surface area is covered by alveolar type I cells predominantly, which are terminally differentiated from alveolar type II cells. Hyperplasia of alveolar epithelial type II after instillation of recombinant human KGF (rhKGF) leads to terminal differentiation to alveolar type I cells [6]. KGF expression is upregulated in rodent models of lung injury and has been implicated in the induction of alveolar epithelial cell proliferation [4]. Ulich *et al.* reported the progression of alveolar cell type II hyperplasia in the normal adult rat lung at 72 hr after intratracheal KGF administration [23]. Kaza and associates demonstrated that intraperitoneal administration of KGF enhanced compensatory lung growth after postpneumonectomy through alveolar proliferation until 21 days after operation [10]. This means that alveolar cell proliferation by KGF instillation would be logically important for pulmonary function. But the effect of the direct administration of rhKGF at early phase of compensatory lung growth was still unclear. Later, we confirmed the involvement of KGF and KGFR in the compensatory lung growth and then the usefulness of gene therapy using KGF expression vector to accelerate it after lung resection [12]. The gene therapy seemed to be suitable for short-term acceleration of lung compensatory growth, but could cause some complication including pneumothorax and pulmonary bleeding.

In the present study, we examined a direct and short-term effect of intratracheal administration of rhKGF on alveolar cell proliferation as well as on the expression of KGFR during lung compensatory growth after trilobectomy in rats. As a result, we found a dramatic increase in the number of proliferating alveolar cells and pleural cells, and defined the optimal protocol to administer rhKGF to accelerate lung compensatory growth.

II. Materials and Methods

Animals, surgical procedure and tissue preparation

Pathogen-free 8-weeks-old male Lewis rats weighing 280–320 g were purchased from Charles River Japan

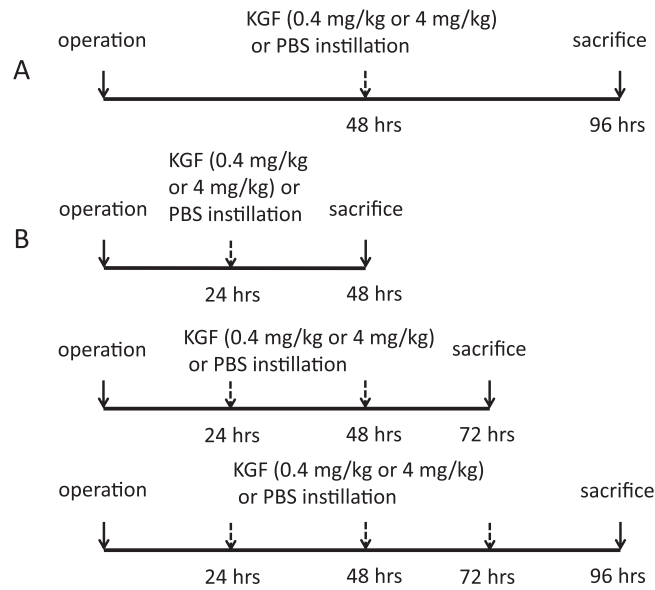
(Kanagawa, Japan). Each experimental group consisted of three or four rats at each given time-points. Rats were anesthetized with intraperitoneal injection of 25 mg/kg sodium pentobarbital (Dainippon Sumitomo Pharma, Osaka, Japan), and then underwent a right posterolateral thoracotomy. The right lung of rats consisted of 4 lobes; the cranial, middle, accessory and caudal lobes were freed from the inferior pulmonary ligament. Three lobes (cranial, middle and accessory lobe) were excised as previously described [12]. The remaining caudal lobe after trilobectomy was used for the following investigation after fixed in 4% paraformaldehyde in PBS (pH 7.4) (Merck, Darmstadt, Germany) and embedded in paraffin. Serial 5- μ m sections were cut, and each specimen was stained with hematoxylin and eosin. All animal experiments were performed in accordance with National Institutes of Health guidelines dictated by the Animal Care Facility at Nagasaki University Graduate School of Medicine (approval code 0708100614).

Administration of KGF protein

Recombinant human KGF protein (Amgen, Thousand Oaks, CA) was administered intratracheally after the operation. According to our previous results, the proliferating cell nuclear antigen labeling index (PCNA-LI) was $1.74\% \pm 0.75\%$ at day 0 and increased significantly at day 2 and reached a maximum ($8.49 \pm 1.83\%$) at day 4 after trilobectomy [12]. And then the PCNA-LI returned to the control level at day 14. For titration of rhKGF, rats were divided into three groups (PBS alone, 0.4 mg KGF/kg body weight (BW) and 4 mg KGF/kg BW), and each group was administered with PBS alone or KGF at 48 hr after operation (Table 1). They were sacrificed 96 hr after operation and analyzed. For the experiment with daily injections of rhKGF, rats were divided into three groups as described above and each group was further subdivided into three groups by times of injection (one day, two days and three days injection) (Table 1).

Immunohistochemistry of KGF, KGFR and surfactant protein A (SP-A) in the lung

Immunohistochemistry was performed to determine the expression of KGF and KGFR in tissue sections using anti-KGF and anti-KGFR antibodies as previously described [2, 15, 22, 26]. Sections of rat lung tissue were deparaffinized and those for KGFR expression were pre-treated with 0.2% Triton X-100 in PBS for 15 min at RT. Endogenous peroxidase activity was inactivated with 0.3% H_2O_2 in methanol, and the sections were preincubated with 500 μ g/ml normal goat IgG in 1% BSA in PBS to block nonspecific reaction with the first antibody. The sections were then incubated for 2 hr with primary antibody at 1.0 μ g/ml for the anti-KGF antibody, 1 : 600 for anti-KGFR antiserum and 1 : 30 for the anti-SP-A antibody (Santa Cruz Biotechnology, Santa Cruz, CA), and then incubated with horseradish peroxidase (HRP)-conjugated goat anti-rabbit IgG (1 : 100) (MBL, Nagoya, Japan) for KGF, KGFR and SP-A for 1 hr as a secondary antibody. HRP sites were

Table 1. Protocol for single injection (A) and for daily injections (B)

visualized by treatment with 3,3'-diaminobenzidine-4HCl (DAB; Dojindo, Kumamoto, Japan) and H₂O₂.

Terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL) Staining

To analyze internucleosomal DNA fragmentation as a hallmark of apoptosis, TUNEL was performed as previously described [2, 3]. The sections were reacted with 200 U/ml TdT (Roche, Munich, Germany) dissolved in TdT buffer (25 mM Tris/HCl buffer, pH 6.6, containing 0.2 M potassium cacodylate and 0.25 mg/ml BSA) (Roche, Munich, Germany) supplemented with 0.5 μM biotin-16-dUTP, 20 μM dATP, 1.5 mM CoCl₂, and 0.1 mM dithiothreitol for 90 min at 37°C. The reaction was terminated by washing with 50 mM Tris-HCl buffer (pH 7.4) and the sections were reacted with HRP-goat anti-biotin antibody (1 : 100, diluted with 5% BSA in PBS) (Vector, Burlingame, CA). The HRP sites were visualized with DAB and H₂O₂ in the presence of nickel and cobalt ions [2].

Evaluation of proliferating activity of lung cells

Lung cell proliferation was analyzed immunohistochemically using an anti-PCNA antibody (DAKO, Glostrup, Denmark) according to the previous paper [1, 2, 12]. Briefly, the sections were autoclaved and incubated with anti-PCNA antibody (1 : 100) as the primary antibody and HRP-goat anti-mouse IgG (1 : 100) (Chemicon International Temecula, CA) as the secondary antibody. Negative control sections were incubated with normal mouse IgG in place of primary antibody.

Quantitative analysis of PCNA positive cells

PCNA LI was measured according to the previous paper [1, 12, 21]. Briefly, PCNA staining was graded as positive or negative, compared to the negative control. For

each section, more than 2,000 cell nuclei were counted over randomly selected fields at 400× magnification. The number of positive cells was expressed as the percentage of cells with positive nuclei per total number of counted nuclei. This method avoided counting error due to cell size.

Statistical analysis

Data were expressed as mean±SEM. Values of different groups were compared using one-way analysis of variance. For statistical analysis, we used the unpaired Student's *t* test and a *P* value of <0.05 was considered statistically. All statistical analyses were performed using JMP software (SAS, Cary, NC).

III. Results

Effect of rhKGF administration on histological and histochemical characters in the remnant lung during compensatory growth after trilobectomy

First, we examined the effect of intratracheal administration of rhKGF at a dose of 0.4 mg/kg BW on histology of the growing lung at 48 hr after trilobectomy. As shown in Fig. 1A and 1E, the rhKGF administration markedly increased the thickness of pleura and alveolar septum of the remnant lung, compared to the control group with PBS alone. In accordance with the histological finding, the expression of KGFR was much more widely induced in both alveolar and pleural cells (Fig. 1F), while a few significant increase in KGFR positive cells was detected in the control lung with PBS alone (Fig. 1B). The number of KGF positive cells was also clearly increased in alveolar and pleural parts although the origin of KGF, i.e., rat KGF or rhKGF was not discriminated in the present study (Fig. 1C and 1G). Similarly, PCNA positive cells were more abundant in both parts of the remnant lung with

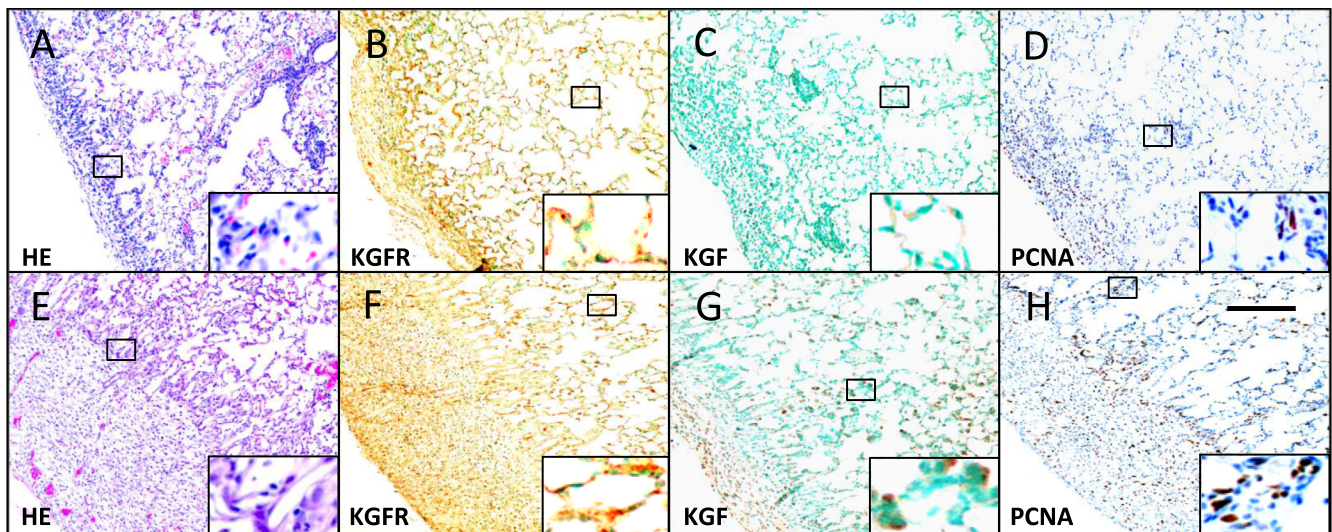


Fig. 1. Effect of rhKGF administration on the compensatory growth of remnant lung after trilobectomy. The rats were treated with PBS (A–D) or rhKGF (E–H) at 48 hr after trilobectomy and sacrificed 48 hr later. Histological examination was done with hematoxylin-eosin staining (A, E). The expression of KGF (B, F), KGFR (C, G) and PCNA (D, H) was examined immunohistochemically. Magnification; $\times 40$ (Bar=500 μm).

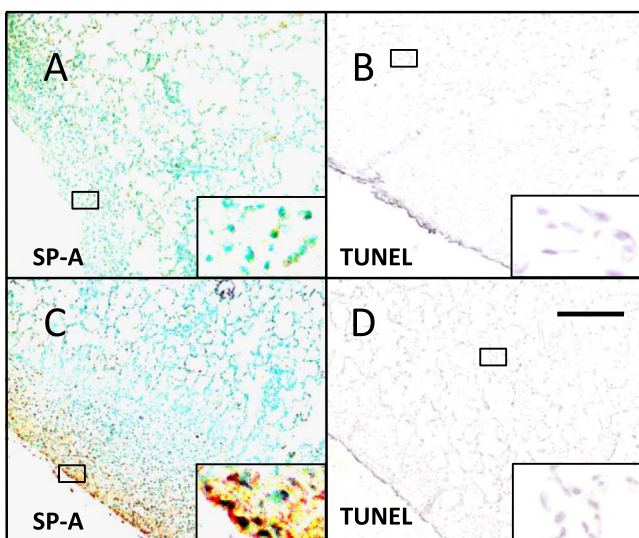


Fig. 2. Effect to SP-A expression and apoptosis by rhKGF administration on the compensatory growth of remnant lung after trilobectomy. The rats were treated with PBS (A, B) or rhKGF (C, D) at 48 hr after trilobectomy and sacrificed 48 hr later. The remaining lungs were analyzed for the expression of SP-A (A, C) and TUNEL (B, D). Magnification; $\times 40$ (Bar=500 μm).

rhKGF administration (Fig. 1H), compared to the control (Fig. 1D).

Moreover, SP-A expression was increased by rhKGF, especially in the pleural area (Fig. 2A and 2C), indicating the promotion of the function of type II alveolar cells. In addition, there was no change in TUNEL positive cell population in the presence or absence of the extraneous rhKGF (Fig. 2B and 2D).

Effect of single administration with different doses of exogenous rhKGF on alveolar cell proliferation

To explore an optimal condition of rhKGF administration, the effect of low and high doses of rhKGF on alveolar cell proliferation was investigated by PCNA LI. As shown in Fig. 3A, PCNA positive cells were found in the remnant lung after trilobectomy without rhKGF administration, where the PCNA LI was $14.9 \pm 6.47\%$ for alveolar epithelial cells. On the other hand, single intratracheal administration of rhKGF after trilobectomy markedly increased PCNA positive alveolar epithelial cells, as shown in Fig. 3B and 3C. Very interestingly, the size of alveolar cells was markedly enlarged at the same time. To analyze quantitatively, PCNA LI was measured at 48 hr after single administration of PBS alone or rhKGF (Fig. 4). In PBS group, PCNA LI was not significantly changed, compared to the sham control (without injection $14.9 \pm 6.4\%$, PBS $21.1 \pm 5.9\%$, $p=0.27$). In rhKGF injected groups, PCNA LI was significantly increased dose-dependently; the LI in 4 mg/kg KGF group was significantly higher than that of 0.4 mg/kg KGF group ($61.9 \pm 6.31\%$ vs. $46.3 \pm 6.8\%$ respectively, $p=0.017$).

Effect of consecutive daily administrations with rhKGF on alveolar cell proliferation

Finally, we examined the effectiveness of daily administrations with rhKGF on alveolar epithelial cell proliferation with different timings and doses, as described in Table 1. Histological examination revealed that the daily application of rhKGF enhanced thickening of alveolus and pleural region more effectively (Fig. 3D). Surprisingly, there was no significant difference in PCNA LI between low and high doses of rhKGF at any time-tables, as shown in Fig. 5. Furthermore, the maximal level of PCNA LI

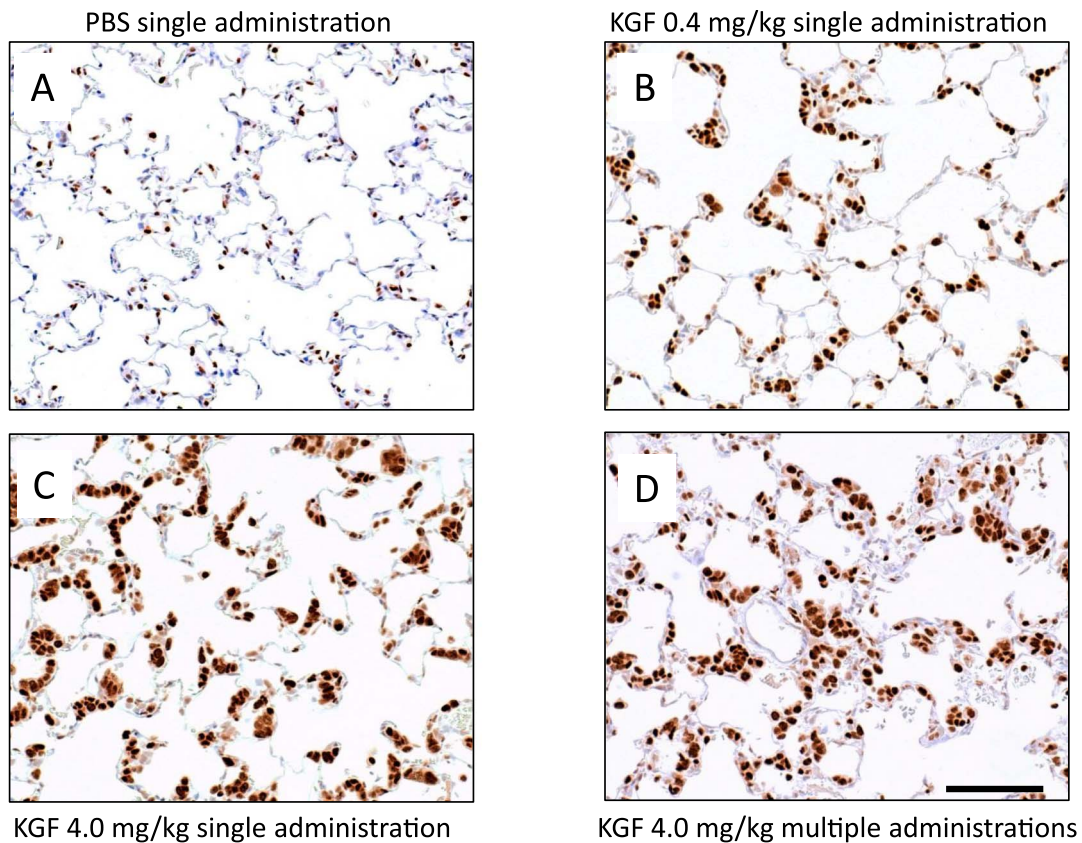


Fig. 3. Immunostaining for PCNA in the remnant lung administrated with rhKGF in various experimental conditions. The remnant lungs treated with PBS (A), 0.4 mg/kg KGF with a single injection (B), 4 mg/kg KGF with a single injection (C), and 4 mg/kg KGF with consecutive 3 days injection (D) were analyzed for the expression of PCNA immunohistochemically. Magnification; $\times 200$ (Bar=100 μm).

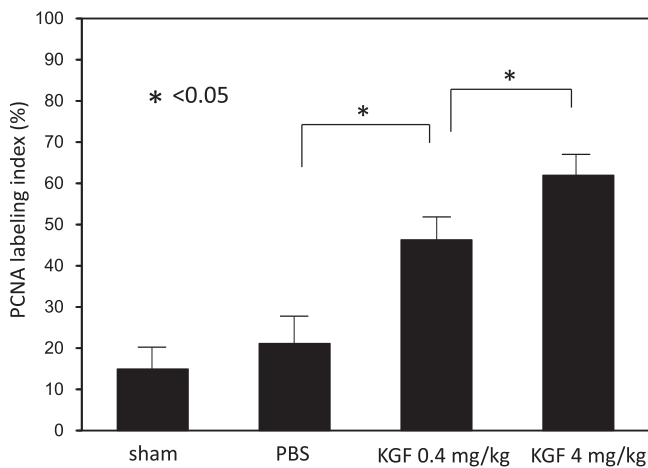


Fig. 4. Effect of single injection with different doses of rhKGF on alveolar cell proliferation after trilobectomy. Dose-dependent changes in PCNA LI were analyzed in the remnant lung with a single intratracheal injection of PBS or rhKGF. There was a significant difference between 0.4 mg/kg KGF and 4 mg/kg KGF (46.3 \pm 6.8% vs. 61.9 \pm 6.31% respectively, $p=0.017$). * $P<0.05$.

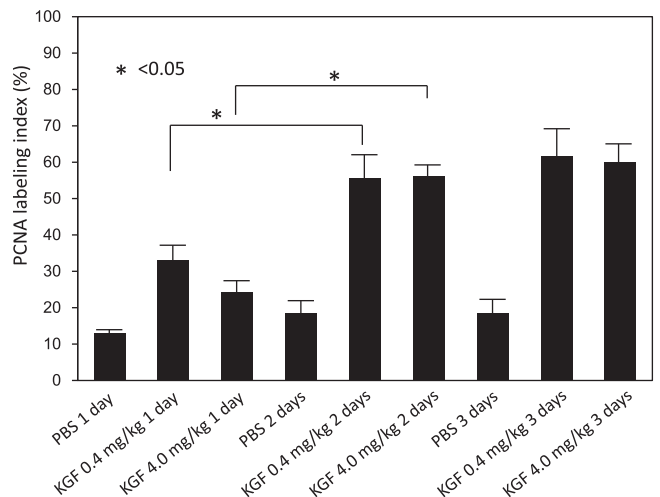


Fig. 5. Effect of daily injections with different doses of rhKGF on alveolar cell proliferation after trilobectomy. Dose-dependent changes in PCNA LI were analyzed in the remnant lung with daily intratracheal injection of PBS or rhKGF. There was a significant change between day 1 and day 2 in the groups of 0.4 mg/kg KGF and 4 mg/kg KGF (0.4 mg/kg; 33.2 \pm 5.6, 55.6 \pm 9.38% respectively, $p=0.003$, 4 mg/kg; 24.2 \pm 3.77, 56.3 \pm 6.6%, respectively, $p=0.002$). PCNA LI reached a plateau level with two days-injection. There was no significant difference between 0.4 mg/kg KGF and 4 mg/kg KGF at any time-points. * $P<0.05$.

seemed to be about 60% in any administration protocols used here. Therefore, 2–3 days consecutive administrations with the low dose of rhKGF were enough to attain the maximum value of PCNA LI even at 72 hr after operation.

IV. Discussion

In the present study, we investigated a possibility of intratracheal injection of rhKGF to promote the compensatory lung growth after trilobectomy. Acceleration in alveolar cell proliferation without any effect on apoptosis indicated that rhKGF may be a novel drug to be used for intratracheal therapy for improvement of pulmonary function after lung resection. Importantly, we found that even small amount of KGF was enough to support a maximal proliferation of alveolar epithelial cells during compensatory lung growth with daily administration protocol. Furthermore, the increase in SP-A expression by KGF strongly suggested that rhKGF affected alveolar type II cells effectively. Interestingly, intratracheally injected KGF seemed to exert its effect throughout a whole remnant lung since the increase in pleural cell proliferation and pleural thickness was occurred.

Compensatory lung growth after lung resection has been actively investigated and it should be noted that many factors were implicated in the events. For example, retinoid acid enhanced lung growth after pneumonectomy in rats [9]. Basic fibroblast growth factor induced an increase in pulmonary blood flow in the damaged canine lung [13]. Hepatocyte growth factor was reported to be a potent mitogen for rat alveolar type II cells and might work simultaneously on various types of cells [19]. Moreover, it was reported that epidermal growth factor stimulates the differentiation of alveolar type II cells and biosynthesis of surfactant precursor proteins [8], and the expression of vascular endothelial growth factor was up-regulated by the stimulus for alveolarization [14]. However, it is still too complicated to explain the total story. Now KGF is considered as one of the most important growth factors in this situation [10, 12].

Kaza *et al.* reported that weekly intraperitoneal administrations of 21 mg KGF/kg BW enhanced compensatory lung growth after postpneumonectomy through alveolar proliferation until 21 days after operation, while no induction of alveolar cell proliferation, even though with KGF administration, was found in sham-operated rats [10]. On the other hand, Ulich and Fehrenbach reported that proliferation of lung epithelial cells was induced 2 or 3 days after intratracheal injection of 5 mg/kg KGF in normal adult rat lung [6, 23]. The major differences between these experiments are routes and doses for the drug administration. The former administrated 21 mg/kg KGF intraperitoneally, but the latter gave only 5 mg/kg KGF intratracheally. Nevertheless, the latter protocol seemed more effective for alveolar cell proliferation. Recently, we have shown the usefulness of gene therapy using KGF expression vector for lung compensatory growth after lung resection [12]. However,

this gene therapy method has some difficulty to use especially in direct transfection into lung. In clinical practice, complications including pneumothorax or hemothorax should arise remarkably by the method [25]. Therefore, in this study, we chose intratracheal injection of rhKGF to evaluate short term protocols for acceleration of alveolar cell proliferation after trilobectomy.

Ulich *et al.* reported that a single intratracheal injection of KGF caused a prominent dose-dependent proliferation of alveolar cells; a substantial increase of alveolar cells was found at 5.0 and 10.0 mg/kg KGF and a mild increase was found at 1.0 mg/kg KGF in normal adult rat lung [23]. With rat compensatory lung growth model, we confirmed that a single intratracheal administration of rhKGF induced alveolar cell proliferation significantly and dose-dependently. To our surprise, however, daily administrations of KGF could induce the maximal level of increase of alveolar epithelial cells even at the low dose (0.4 mg KGF/kg BW) after trilobectomy. Moreover, our previous study revealed that PCNA LI reached a maximum at day 4 after trilobectomy [12], indicating that the daily administration of rhKGF accelerated to reach the maximal PCNA LI from day 4 to day 3 after trilobectomy. Based upon these results, we concluded that two days of intratracheal injection with rhKGF are enough to promote compensatory lung growth effectively. Considering that KGF acts to prevent cells from apoptosis, these results indicate that daily administration of rhKGF during compensatory lung growth after lung resection could lead to continuous proliferation of alveolar cells and pleural cells, and there is a possibility of further administration to give rise quick recovery of pulmonary function after lung resection.

Intratracheal drug administration by using nebulizer is often used as a simple and convenient medical examination for expectorant clinically. Therefore, we believe that rhKGF has a potential as a key drug that can promote compensatory lung growth after lung resection, although further studies are needed to clarify the effect of intratracheal rhKGF administration on a variety of respiratory functions in compensatory lung growth.

In conclusion, our results clearly demonstrated that the protocol with daily intratracheal administration of rhKGF can promote the compensatory lung growth significantly even at a low dose. We believe that the findings in this study would be useful to develop a new instillation therapy with KGF and other growth factors for the promotion of lung compensatory growth.

V. Acknowledgments

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VI. References

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