Keratinocyte Growth Factor Gene Electroporation into Skeletal Muscle as a Novel Gene Therapeutic Approach for Elastase-Induced Pulmonary Emphysema in Mice

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Pulmonary emphysema is a progressive disease with airspace destruction and an effective therapy is needed. Keratinocyte growth factor (KGF) promotes pulmonary epithelial proliferation and has the potential to induce lung regeneration. The aim of this study was to determine the possibility of using KGF gene therapy for treatment of a mouse emphysema model induced by porcine pancreatic elastase (PPE). Eight-week-old BALB/c male mice treated with intra-tracheal PPE administration were transfected with 80 μg of a recombinant human KGF (rhKGF)-expressing FLAG-CMV14 plasmid (pKGF-FLAG gene), or with the pFLAG gene expressing plasmid as a control, into the quadriceps muscle by electroporation. In the lung, the expression of proliferating cell nuclear antigen (PCNA) was augmented, and surfactant protein A (SP-A) and KGF receptor (KGFR) were co-expressed in PCNA-positive cells. Moreover, endogenous KGF and KGFR gene expression increased significantly by pKGF-FLAG gene transfection. Arterial blood gas analysis revealed that the PaO_2 level was not significantly reduced on day 14 after PPE instillation with pKGF-FLAG gene transfection compared to that of normal mice. These results indicated that KGF gene therapy with electroporation stimulated lung epithelial proliferation and protected depression of pulmonary function in a mouse emphysema model, suggesting a possible method of treating pulmonary emphysema.

Key words: keratinocyte growth factor, emphysema, elastase, electroporation, gene therapy

I.   Introduction

Chronic obstructive pulmonary disease (COPD) is an increasing major public health problem that is characterized by air flow limitation. COPD is projected to rank as the third leading cause of death worldwide in 2020 and the prevalence of this disease for adults over age 40 in Japan is 10.9% [11, 33]. The mechanism of COPD has not been elucidated, and treatment efficacy is poor except for lung transplantation [15]. Regeneration medicine is a promising therapy for COPD, and several COPD animal models including an elastase-induced emphysema model have been examined [13, 23, 42]. Since Massaro *et al.* suggested the use of retinoic acid for elastase-induced pulmonary emphysema in 1997 [23], various regenerative studies using retinoic acid [18, 39], adrenomedullin [29], hepatocyte growth factor (HGF) [14, 36], granulocyte-colony stimulating factor (G-CSF) [17], basic fibroblast growth factor (bFGF) [27], and simvastatin [37] have been reported.

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A strong alternative candidate for regeneration therapy is keratinocyte growth factor (KGF) purified from the conditioned medium of human embryonic lung fibroblasts [9, 34]. KGF is a heparin-binding growth factor and a fibroblast-derived mitogen. KGF selectively stimulates epithelial cells through the keratinocyte growth factor receptor (KGFR) that is expressed specifically on epithelial cells [25]. KGF promotes the proliferation of alveolar type II cells *in vitro* [31] and *in vivo* [40], and stimulates their surfactant protein gene expression [6, 44]. Furthermore, Morikawa *et al.* showed that adenoviral vector-mediated KGF gene transfer stimulated alveolar type II cell proliferation [26]. In terms of therapeutic purposes, pre-treatment with KGF before lung injury had protective effects on bleomycin-induced lung injury [45], oleic acid-induced lung injury [41], hyperoxia-induced lung injury [4], and elastase-induced lung injury [32]. However, it is still unclear whether KGF has a beneficial effect for post-treated lung injury.

We have already reported that KGF augments lung epithelial proliferation in the remaining lung after trilobectomy following transfection by electroporation of a KGF-expressing plasmid DNA in rats [24]. Kaza *et al.* were the first to show that KGF enhanced compensatory lung growth after pneumonectomy in rats [19]. We also previously showed that administration of a KGF-expressing plasmid using electroporation significantly enhanced compensatory lung growth [24]. Moreover, exogenous KGF was shown to augment lung epithelial cell proliferation and to decrease the average airspace distance [12]. In terms of COPD treatment, we therefore considered that KGF, which is synthesized in different tissues far from the lung, may play a beneficial role in lung repair or preventing progression of emphysema.

In the present study, we investigated whether KGF gene therapy, through transfection of the KGF gene into muscles by means of electroporation, has a beneficial effect on lung repair in an elastase-induced lung emphysema mouse model.

II.   Materials and Methods

Animals

Eight-week-old BALB/c male mice weighing 23–29 g were purchased from SLC Japan (Shizuoka, Japan). The mice were housed in an air-conditioned and light-controlled room in the Biomedical Research Center, Center for Frontier Life Sciences, Nagasaki University. The principles of laboratory animal care (NIH publication No. 85-23, revised 1985) were followed. All experimental protocols were approved by the Animal Research Committee of Nagasaki University (No. 0601260487).

Pulmonary emphysema

Mice were anesthetized with 25 mg/kg sodium pentobarbital (Dainippon Seiyaku Co., Tokyo, Japan) adminis-

tered intraperitoneally, and were intubated with a 24-gauge intravenous catheter. Two units of porcine pancreas elastase (PPE, Sigma Chemical Co., St. Louis, MO, USA) in 60 μl saline were instilled intra-tracheally.

Plasmid DNA

Recombinant human KGF cDNA (kindly provided by Jeffrey Rubin, National Cancer Institute, Bethesda, MD, USA) was inserted between the BglII and XbaI restriction enzyme sites of the p3×FLAG-CMV14 vector (Sigma) to produce the pKGF-FLAG plasmid as reported previously [24]. pFLAG was generated as a control vector. Plasmid DNA was amplified in DH5α Escherichia coli, extracted using the Plasmid Mega kit (QIAGEN, Venlo, Netherlands), and dissolved in TE buffer (10 mM Tris-HCl buffer (pH 8.0) including 1 mM ethylenediaminetetraacetic acid (EDTA)). DNA concentration was determined at a wavelength of 260 nm using a UV photometer. KGF-FLAG protein expression from this plasmid was confirmed in vitro as described below in enzyme-linked immunosorbent assays using anti-KGF antibody and by Western blotting using anti-FLAG antibodies [24].

Plasmid administration, electroporation, and tissue preparation

For gene transfer, mice were electroporated at the plasmid injection site using an Electro Square Porator (T820; BTX Inc., San Diego, CA, USA). Immediately after instillation of PPE, the bilateral quadriceps muscles were denuded, and 40 μg of the pKGF-FLAG or the pFLAG vector were injected once into each bilateral muscle. The muscles were held by a pair of electrode disks (10 mm in diameter and 5 mm apart) attached to tweezers (449-10PRG; Meiwa Shoji, Tokyo, Japan), and electric pulses were delivered. Six square wave pulses of 75 V were administered for a duration of 20 ms at a rate of one pulse per second. The mice were sacrificed at various time-points after PPE administration with/without plasmid electroporation and the lungs were dissected out. As an additional test, plasmid was administered 4 days before PPE instillation to establish the prophylactic effect of KGF. Each experimental group consisted of 3 mice. The time scale of mouse treatments and sacrifice is shown in Figure 1. Following sacrifice, the lungs were fixed in 4% paraformaldehyde in phosphate-buffered saline (PBS; pH 7.4) overnight and embedded in paraffin. Serial sections 4-μm thick were cut and stained with hematoxylin and eosin.

Antibodies

Polyclonal antibodies against KGF and KGFR were prepared by immunizing rabbits with synthetic peptides that were produced in cooperation with Nichirei Co. (Tokyo, Japan) as previously described [43]. Anti-KGF IgG (5 μg/ml) and anti-KGFR anti-serum (1:800) were used for immunohistochemical analysis. Monoclonal mouse anti-proliferating cell nuclear antigen (PCNA; 1.2 μg/ml) was
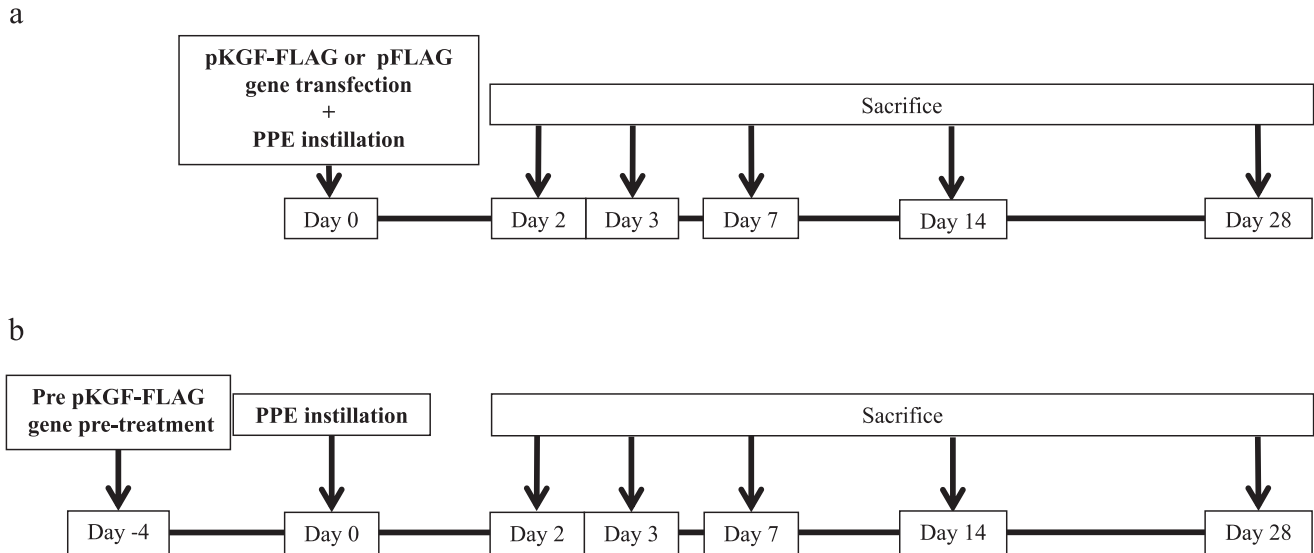
a

b

Fig. 1. Protocol for mouse emphysema induction, gene transfection and mouse sacrifice. Emphysema was induced in mice by instillation of porcine pancreas elastase (PPE). A plasmid encoding the FLAG-tagged keratinocyte growth factor (pKGF-FLAG), or control FLAG was electroporated into quadriceps muscle either immediately post-PPE treatment (**a**), or pre-PPE treatment (**b**). Mice were sacrificed on the indicated days for analysis.

purchased from DAKO (Glostrup, Denmark). Rabbit anti-surfactant protein A (SP-A) polyclonal antibody (1:5000) was purchased from Chemicon international (Temecula, CA, USA). Horseradish peroxidase (HRP) conjugated mouse monoclonal anti-FLAG antibody was purchased from Invitrogen (Tokyo, Japan). Normal goat IgG, normal rabbit IgG, and normal mouse IgG were purchased from Sigma. Normal goat serum and normal rabbit serum were purchased from DAKO. HRP conjugated goat anti-mouse IgG, and HRP conjugated goat anti-rabbit IgG were purchased from Chemicon International (Temecula, CA, USA).

Biochemicals and chemicals

Bovine serum albumin (BSA), Triton X-100, Brij 35, 3-amino-9-ethylcarbazole (AEC), proteinase K, yeast tRNA, salmon testis DNA, and dextran sulfate were purchased from Sigma. 3,3'-diaminobenzidine/4HCl (DAB) was purchased from Dojin Chemical Co. (Kumamoto, Japan). Paraformaldehyde (PFA) was purchased from Merck (Darmstadt, Germany). Biotin-16-dUTP and terminal deoxynucleotidyl transferase (TdT) were purchased from Roche (Mannheim, Germany). Blocking One was purchased from Nacalai Tesque (Kyoto, Japan). ECL Plus was purchased from Amersham Biosciences (Piscataway, NJ, USA). All other reagents used in this study were purchased from Wako Pure Chemicals (Osaka, Japan) and were of analytical grade.

Measurement of KGF concentration and Western blotting

The concentration of human KGF in mouse lungs and quadriceps muscle was measured using a KGF enzyme-linked immunosorbent assay (ELISA) (DKG00; R&D

Systems, Minneapolis, MN, USA). The tissues were homogenized (SK-200; Funakoshi Co., Tokyo, Japan) and diluted with a lysis buffer containing 50 mM Tris-HCl, 5 mM EDTA, 150 mM NaCl, 0.1 mM phenylmethylsulfonyl fluoride (PMSF), and lima bean trypsin inhibitor. After centrifugation, the supernatant was measured using an ELISA kit. This supernatant was also used for Western blotting of KGF. Samples were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis in a 4%–20% gradient gel (Daiichi Pure Chemical, Tokyo, Japan). The membranes were blocked with Blocking One for 0.5 hr and were then reacted with HRP-conjugated anti-FLAG antibody (1:6000) for 1 hr. After washing, the membranes were visualized with the ECL Plus reagent [24].

Blood gas analysis

To assess pulmonary function, arterial blood gas was analyzed using the i-STAT system (Brain Science Idea Co., Osaka, Japan). Blood samples were drawn from the abdominal aorta under ventilation.

Immunohistochemistry

Immunohistochemical analysis of the expression of KGF, KGFR, PCNA, and SP-A in lung tissues was performed as previously described [1, 3, 38, 43]. Paraffin sections were dewaxed with toluene and rehydrated in serial graded ethanol solutions. For KGFR detection, the sections were pretreated with 0.2% Triton X-100 in PBS for 10 min at room temperature. For KGFR and PCNA, the sections were autoclaved at 120°C for 10 min in 10 mM citrate buffer (pH 6.0). After inactivation of endogenous peroxidase activity with 0.3% H_2O_2 in methanol for 30 min, the sections were preincubated with 500 µg/ml normal goat IgG

and 1% BSA in PBS (PCNA, SP-A), or 10% normal goat IgG and 1% BSA in PBS (KGF, KGFR) for 1 hr to block nonspecific reactions of the primary antibody. The sections were then reacted with the primary antibodies for 2 hr (KGF, KGFR) or overnight (PCNA, SP-A). After washing with 0.075% Brij in PBS, the sections were incubated with HRP-labeled goat anti-mouse or anti-rabbit IgG for 1 hr. After washing with Brij, the HRP sites were visualized with DAB and H_2O_2 solution, or with DAB, Ni, Co, and H_2O_2 solution. As a negative control, normal mouse IgG, normal rabbit IgG, or normal rabbit serum was used instead of the primary antibody. For colocalization of PCNA with KGFR or SP-A, the sections were first reacted with anti-KGFR or anti-SP-A antibody, and HRP sites were visualized with DAB, Ni, Co, and H_2O_2 solution. The sections were then immersed in 0.1 M glycine-HCl buffer (pH 2.2) for 90 min and were then reacted with anti-PCNA antibody. HRP sites were subsequently stained with AEC.

Terminal deoxynucleotidyltransferase-mediated dUTP nick-end labeling (TUNEL) staining

TUNEL was performed to evaluate the presence of apoptotic cells. Briefly, the sections were dewaxed with toluene and rehydrated in serial graded ethanol solutions. After washing with PBS, the sections were immersed in proteinase K (1 μg/ml) for 15 min at RT, were rinsed with distilled water, and were then reacted with 1× TdT buffer (25 mM Tris-HCl buffer (pH 6.6) containing 0.2 M potassium cacodylate and 0.25 mg/ml BSA) for 30 min at RT. The slides were then reacted with 100 U/ml TdT dissolved in a TdT buffer supplemented with 5 μM biotin-16-dUTP, 20 μM dATP, 1.5 mM $CoCl_2$, and 0.1 mM dithiothreitol for 90 min at 37°C. Subsequently, the slides were washed with 0.05 M Tris-HCl buffer (pH 7.5) and immersed in 0.3% H_2O_2 in methanol for 15 min. After incubation with 500 μg/ml normal goat IgG in 5% BSA in PBS for 1 hr, the slides were reacted with HRP-goat anti-biotin antibody (1:100) in 5% BSA in PBS overnight. The signals were visualized with DAB, Ni, Co, and H_2O_2 solution [2, 22, 38].

In situ hybridization

In situ hybridization was performed as described previously [21, 43]. Briefly, paraffin sections (6 μm in thickness) were deparaffinized with toluene and rehydrated using serially graded ethanol solutions. The slides were immersed in 0.2 N HCl for 20 min and treated with pro-teinase K (20 μg/ml) for 15 min at 37°C. After post fixation with 4% PFA in PBS for 5 min, the slides were immersed twice in 2 mg/ml glycine PBS for 15 min and then kept in 40% deionized formamide in 4× standard saline citrate (SSC) until used for hybridization. Hybridization was carried out at 37°C with T-T dimerized antisense KGF oligo-DNA (1 μg/ml) or KGFR oligo-DNA (1 μg/ml) overnight in a medium containing 1 M Tris/HCl (pH 7.4), 5 M NaCl, 0.2 M EDTA, 100× Denhart's solution, 10 mg/ml yeast tRNA, 10 mg/ml salmon testis DNA, 50% dextran sul-

fate, 40% deionized formamide and 10 mM Tris/HCl-1mM EDTA (pH 7.4). Preparation of oligo-DNA probes for KGF and KGFR was described previously [21, 43]. After hybrid-ization, the sections were washed 3 times with 50% form-amide/2× SSC, then washed twice with 50% formamide/ 0.5× SSC at 37°C for 1 hr per wash, and finally washed twice with 2× SSC for 15 min per wash. The slides were then incubated with blocking solution (500 μg/ml mouse IgG, 5 M NaCl, 5% BSA, 100 μg/ml salmon testis DNA, and 100 μg/ml yeast tRNA) for 1 hr at room temperature and were then reacted with HRP-mouse anti-T-T antibody (1:80) at RT overnight. After washing 4 times with 0.075% Brij35 in PBS for 15 min, HRP sites were visualized with DAB, Ni, Co, and H_2O_2 solution. Positive cells were deter-mined using an image analyzer (DAB system; Carl Zeiss, Thornwood, NY, USA), and were evaluated based on the staining density divided by that obtained using the sense probe (negative control). 28S rRNA was used as a positive control to evaluate the hybridizable RNAs [43]. As control experiments, the sections were hybridized with an antisense probe in the presence of an excess amount of unlabeled antisense probe to check on the diminished signals [20].

Quantitative analysis

For the quantitative analysis of PCNA expression, at least 1,000 cells were counted in randomly selected fields. The number of positive cells was expressed as a percentage of positive cells per total number of counted cells (PCNA labeling index (LI)) [24]. The signal densities of KGF, KGFR, and SP-A were measured using an image analyzer (DAB system) and were calculated as the sum of gray values per area.

Statistical analysis

Data are expressed as mean standard deviation (SD). Statistical differences between treatment groups were examined by an unpaired t-test or a multiple comparison test (Bonferroni/Dunn test, or Steel Dwass test). P values of <0.05 were considered significant.

III.   Results

KGF expression by rhKGF gene transfection into quadriceps muscles

Expression of the KGF-FLAG protein in the quadri-ceps muscle of electroporated mice was examined by West-ern blot analysis using an anti-FLAG antibody. A specific band corresponding to KGF-FLAG (28 kDa) was detected on day 2 after pKGF-FLAG gene transfection (Fig. 2A). The human KGF protein levels in the mouse quadriceps and lung after pKGF-FLAG gene transfection were next measured using an ELISA. The levels of KGF protein were 12200±125 pg/mg of tissue weight on day 3 and 1475±327 pg/mg of tissue weight on day 7 in the quadriceps muscles (Fig. 2B), and 287±41.6 pg/mg of tissue weight on day 3 and 137±11.5 pg/mg of tissue weight on day 7 in the lung
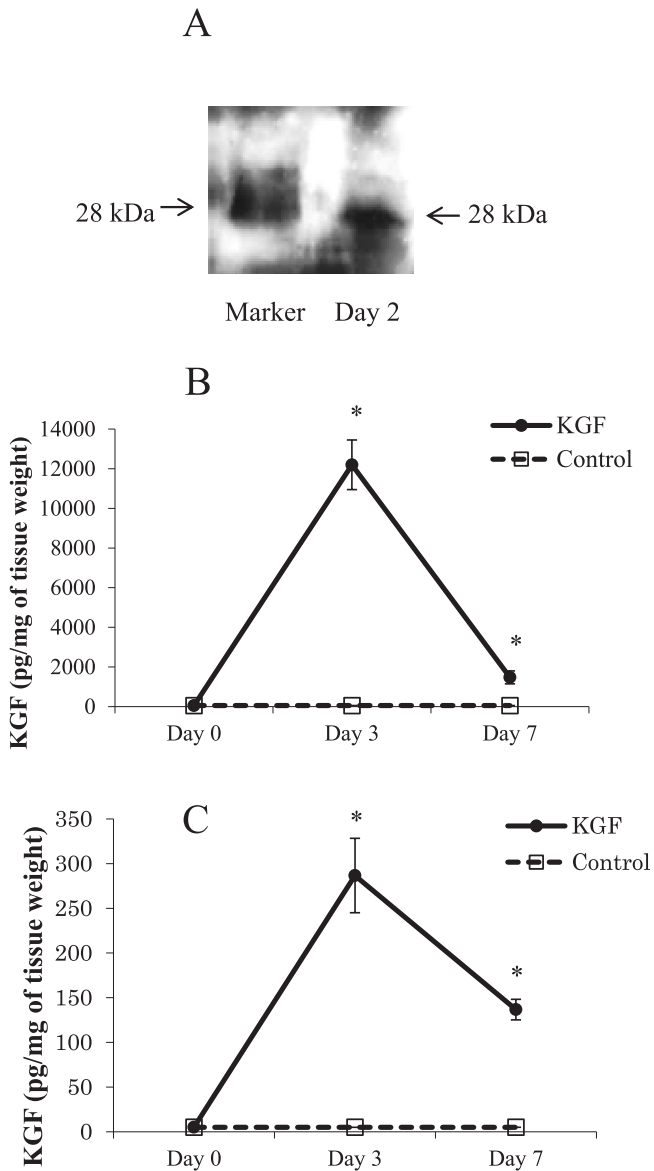
A

28 kDa → ← 28 kDa

Marker   Day 2

B

C

Fig. 2. Expression of KGF protein in mouse quadriceps muscle and lungs. KGF-FLAG expression in mouse quadriceps muscle was analyzed by Western blotting using an anti-FLAG antibody on day 2 after gene transfection of mice (**A**). The concentration of KGF in mouse quadriceps muscle (**B**) and lungs (**C**) on day 3 and day 7 after gene transfection was measured using an ELISA. * represents $p<0.05$ compared to pFLAG control.

(Fig. 2C). KGF protein levels in the mice transfected with the pFLAG gene were below measurable limits (<15 pg/ml) and endogenous KGF was not detected in the quadriceps and lung by ELISA. Therefore, changes in the KGF protein level detected by ELISA definitely reflect changes in the levels of the rhKGF protein. rhKGF expression in the quadriceps and the lung had decreased by day 7 after pKGF-FLAG gene transfection compared to day 3 after transfection. These results may indicate that systemic spreading of rhKGF was induced by pKGF-FLAG transfection into the leg muscles of mice.

Histological changes in lung emphysema after KGF gene transfection

Initially, we characterized histological changes in the lung resulting from KGF gene transfection by using a PPE-induced emphysema model. In this model, as described previously, after intra-tracheal instillation of PPE, lung injury with progressive alveolar wall destruction and enlargement of air space was caused in a time-dependent manner. Infiltration of inflammatory cells diminished gradually after PPE instillation, and, by day 28 after PPE instillation, emphysema was almost established and alveolar destruction was completed (Fig. 3A, B and C). As shown in Fig. 3D and E, KGF-FLAG gene transfection immediately after PPE administration did not induce any changes in lung morphological details compared to FLAG gene transfection when analyzed three days after transfection.

KGF and KGFR expression in the emphysematous lung after KGF gene transfection

We then immunohistochemically analyzed KGF and KGFR levels in the lungs of non-treated mice and PPE-treated mice. Endogenous KGF protein was detected in alveolar and stromal cells in the lungs of non-treated mice (Fig. 4A). In the 3-day PPE-treated lungs of mice whose quadriceps muscles were transfected with pFLAG gene, KGF expression in the lungs was almost equal to that of non-treated mice (Fig. 4B). However, in the 3-day PPE-treated lungs of mice whose quadriceps muscles were transfected with the pKGF-FLAG gene, the KGF signal was greatly increased after transfection compared to that of the FLAG vector control (Fig. 4C and D). Quantitative analysis of KGF signal density using an image-analyzer revealed that the density of KGF in the lung of these pKGF-FLAG gene-transfected mice was significantly increased to twice the level of that in the pFLAG gene-transfected control (pFLAG gene vs. pKGF-FLAG gene; 7.78%±3.37% vs. 15.09%±4.84% (pixel value/mm^2), p=0.001) (Fig. 4G). In contrast, expression of the KGFR in the PPE-treated lungs, which was expressed in epithelial cells, did not change significantly between the pFLAG gene- and pKGF-FLAG gene-transfected mice (Fig. 4E, F and H).

Expression of KGF and KGFR mRNAs in mouse lung after PPE instillation

We next performed in situ hybridization to examine changes in the expression of KGF and KGFR mRNA in the PPE-treated mouse lung. As shown in Fig. 5A and D, using an anti-sense labeled probe, KGF mRNA was detected in lung epithelial cells and macrophages on day 3 after PPE instillation with FLAG gene transfection. This signal was markedly increased on day 3 after PPE instillation with pKGF-FLAG gene transfection (Fig. 5B and E). As a negative control, serial lung sections were hybridized with sense probes, which resulted in no staining (data not shown). When serial sections of the lungs of PPE-instilled, pKGF-FLAG gene-transfected mice were hybridized with KGF
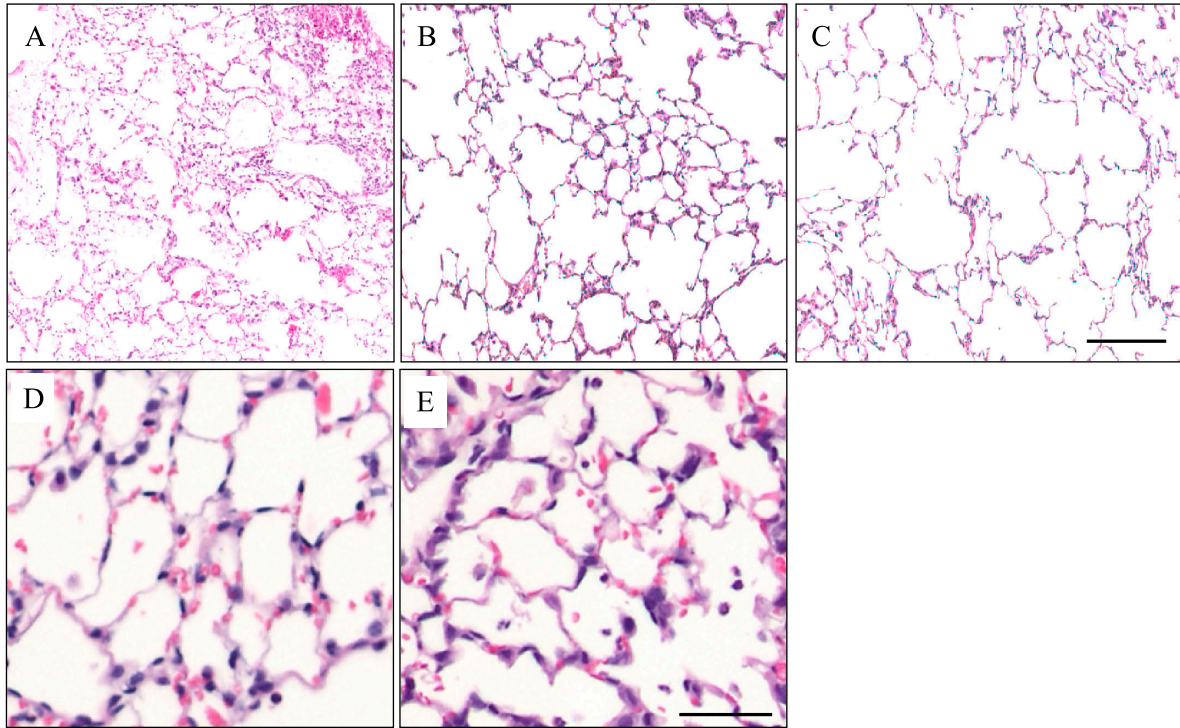
Fig. 3. Histological changes in mouse lung after instillation of PPE with or without KGF gene transfection. Lung tissues of mice treated PPE were fixed on day 2 (**A**), day 7 (**B**), and day 28 (**C**) after instillation of PPE and were stained with hematoxylin and eosin. Alveolar wall destruction and air space enlargement were seen in a time-dependent manner. Lungs of mice on day 3 after PPE instillation and transfection with the pKGF-FLAG gene (**D**) or with the pFLAG gene (**E**) were similarly analyzed. Infiltration of inflammatory cells and hemorrhage were also seen in these tissues. Data are representative of 3 mice per group. Magnification: **A, B** and **C** (×40), Bar=200 μm; **D** and **E** (×200), Bar=100 μm.
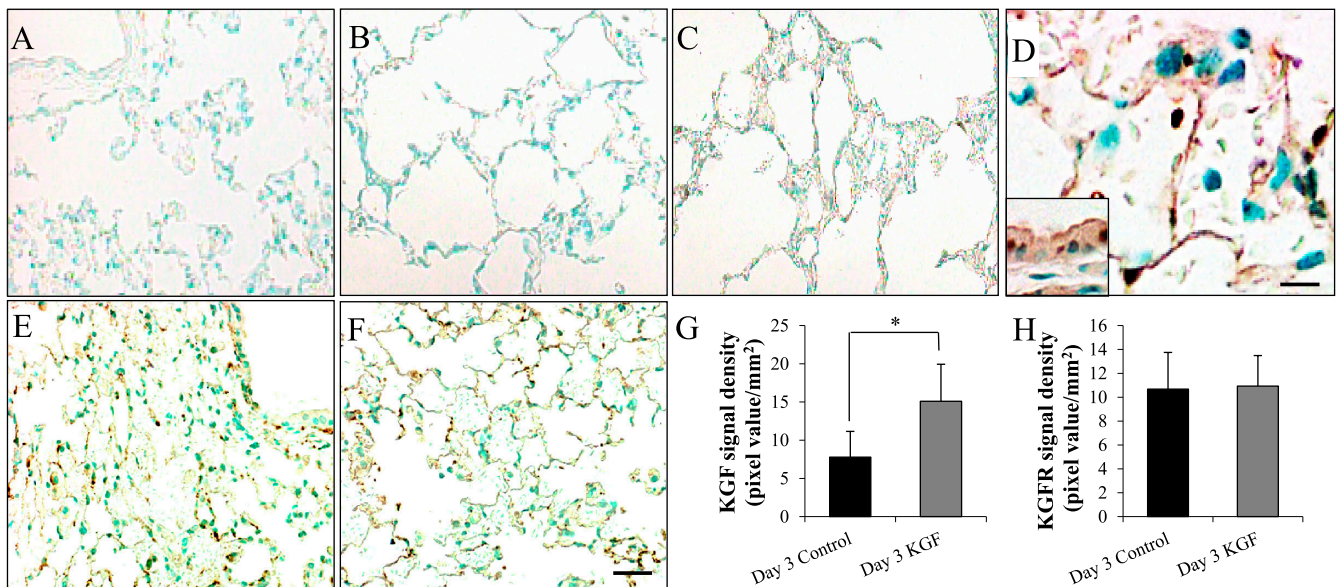

Fig. 4. Immunohistochemical localization of KGF and KGFR proteins in mouse lung after PPE instillation with or without KGF gene transfection. KGF protein expression in the mouse lung was immunohistochemically examined in a normal mouse (**A**), or on day 3 after PPE instillation into a mouse transfected with the pFLAG gene (**B**) or the pKGF-FLAG gene (**C** and **D**). KGF was detected in alveolar cells and bronchial cells. The signal density of KGF in the lungs of PPE-treated mice with KGF-FLAG gene transfection was significantly greater than that of PPE-treated mice with pFLAG gene transfection (**G**). KGFR protein expression in the mouse lung was immunohistochemically examined on day 3 after PPE instillation with pFLAG gene transfection (**E**) or with pKGF-FLAG gene transfection (**F**). KGFR signal density (**H**) was similar between (**E**) and (**F**). Data are representative of 3 mice per group. Magnification: **A, B, C, E** and **F** (×100), Bar=100 μm; D (×400), Bar=50 μm. * represents $p<0.05$.
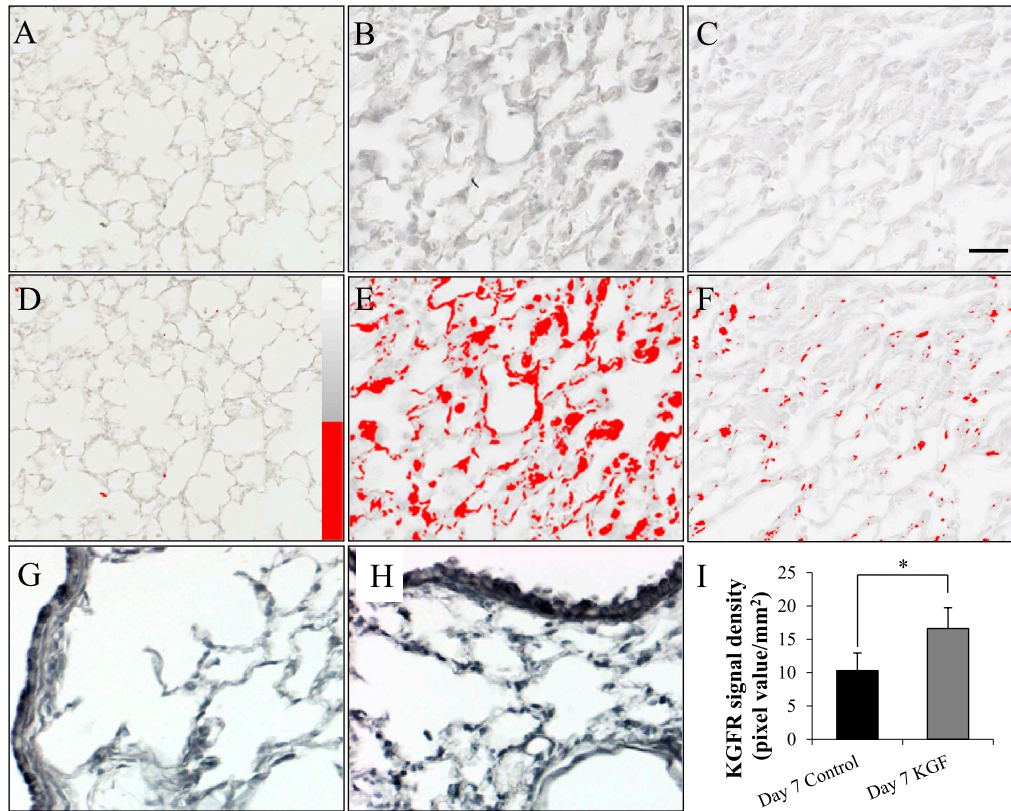
Fig. 5. *In situ* localization of KGF and KGFR mRNA in the mouse lung after PPE instillation with or without KGF gene transfection. KGF and KGFR mRNAs were analyzed in mouse lung sections by in situ hybridization on day 3 and day 7, respectively after PPE instillation with pFLAG or pKGF-FLAG gene transfection. (**A–F**): Sections of lung from mice 3 days after PPE instillation and transfection with the pFLAG gene (**A**) or the pKGF-FLAG gene (**B**) were hybridized with a KGF antisense probe. These slides were further analyzed using an image analyzer that applied pseudo color to the specific signal (**D** and **E**, respectively). A section from mice PPE-treated and transfected with the pKGF-FLAG gene was hybridized with a KGF antisense probe in the presence of an excess amount of unlabeled antisense probe (**C**) and the same slide was subjected to image-analysis (**F**). (**G–I**): Lung sections from mice 7 days after PPE instillation and transfection with the pFLAG gene (**G**) or the pKGF-FLAG gene (**H**) were hybridized with a KGFR antisense probe and the signal density was analyzed (**I**). The signal density of the KGFR in the lung section with pKGF-FLAG gene transfection was significantly greater than that with pFLAG gene transfection (**I**). Magnification: ×100, Bar=100 μm. * represents p<0.05.

antisense probes in the presence of a 100-fold excess amount of unlabeled antisense oligo DNA, little signal was detected, which confirmed the KGF specificity of the hybridization reaction (Fig. 5C and F). Furthermore, a signal for KGFR mRNA was detected in the lungs of mice 7 days after PPE instillation with pFLAG gene transfection, and this expression was significantly increased in the lungs of mice 7 days after PPE instillation with pKGF-FLAG gene transfection (Fig. 5G, H and I). These results indicated that rhKGF-FLAG gene administration could enhance the expression of both endogenous KGF and KGFR mRNA in the injured lung. Therefore, the KGF protein that is produced in a remote site could be systemically delivered to the lung.

Effect of KGF expression in quadriceps muscle on cell proliferation, SP-A production, and apoptosis in mouse lung instilled with PPE

We performed immunohistochemistry for PCNA to assess the effect of pKGF-FLAG gene transfection on the pro-

liferation of lung cells in the mouse emphysema model. The number of PCNA-positive cells in the lung increased on day 2 (LI=11.95%±3.94%) and day 3 (LI=14.60%±4.38%) after PPE instillation in control mice transfected with the pFLAG gene (Fig. 6A and C). When PCNA levels in the lungs of PPE-instilled mice with pKGF-FLAG gene transfection were compared to those of pFLAG gene-transfected PPE-treated mice, the number of PCNA-positive cells was significantly increased on day 2 (LI=13.90%±4.84%), and further increased on day 3 (LI=19.50%±4.52%) (Fig. 6B, D and E). Subsequently, the number of PCNA-positive cells had decreased by day 7 (LI=7.95%±2.19% with pFLAG gene transfection, LI= 8.95%±2.80% with pKGF-FLAG gene transfection). PCNA-positive cells were identified as alveolar epithelial cells, bronchial epithelial cells, endothelial cells and macrophages. To evaluate the effect of the pKGF-FLAG gene in more detail, we performed immunohistochemistry for SP-A, which was used as a marker of alveolar type II epithelial cells. Staining for SP-A was significantly increased on day 3 in the lungs of PPE-
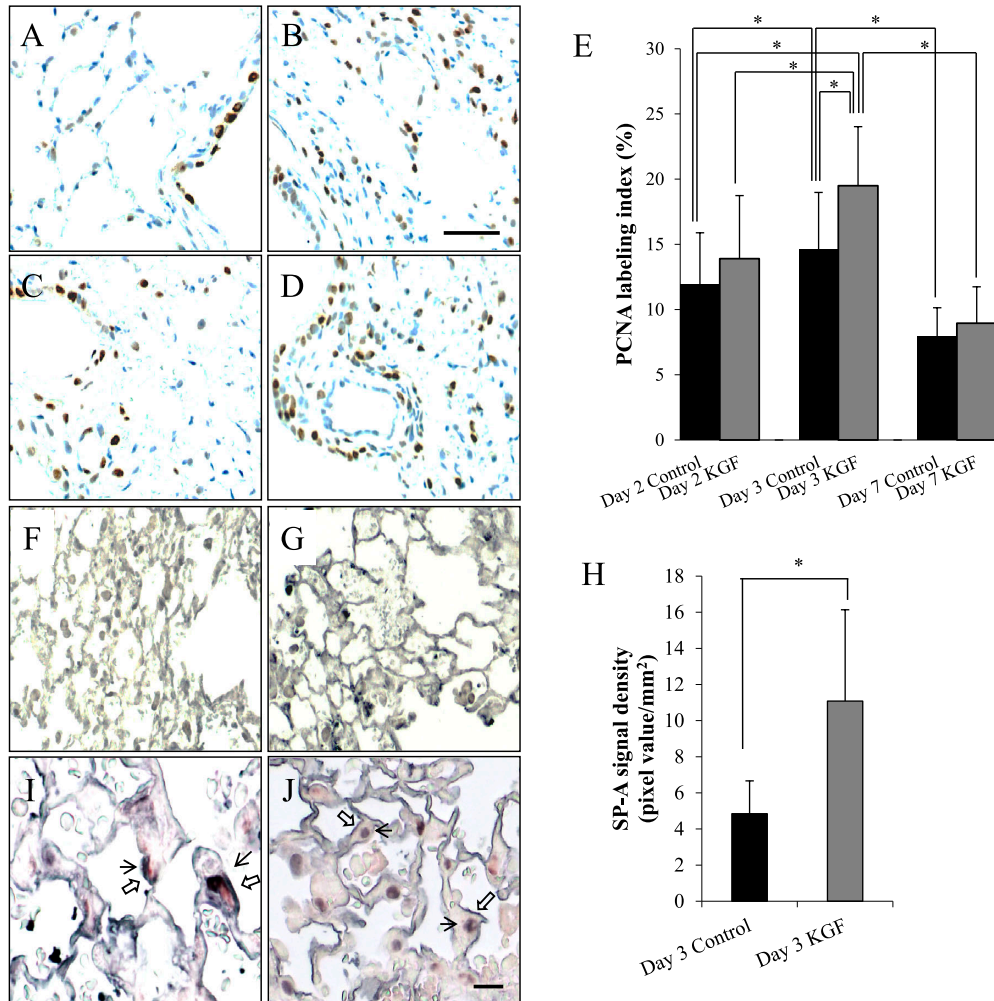
Fig. 6. Immunohistochemical staining of proliferation cell nuclear antigen (PCNA) and surfactant protein A (SP-A) in mouse lung after PPE instillation with or without KGF gene transfection, and double immunohistochemical staining for PCNA and SP-A or KGFR in mouse lung on day 3 after PPE instillation with KGF gene transfection. (**A–E**): PCNA-positive cells were detected immunohistochemically in the lungs of mice on day 2 (**A**) and day 3 (**C**) after PPE instillation with pFLAG gene transfection, and on day 2 (**B**) and day 3 (**D**) after PPE instillation with pKGF-FLAG gene transfection. The PCNA labeling index (LI) for day 2, 3 and 7 was calculated (**E**). (**F–H**): SP-A expression in the lungs of mice was detected immunohistochemically on day 3 after PPE administration with pFLAG gene transfection (**F**) or with pKGF-FLAG gene transfection (**G**). SP-A signal density was measured with an image-analyzer (**H**). (**I, J**): Co-localization of PCNA with SP-A (**I**) or KGFR (**J**) was immunohistochemically analyzed. PCNA (red) was detected in the nucleus and SP-A (black) was detected in the cytoplasm of alveolar epithelial cells (**I**). PCNA (red) was detected in the nucleus and KGFR (black) was detected in the cytoplasmic membrane of alveolar epithelial cells (**J**). Black arrows (PCNA) and open white arrows (SP-A or KGFR) point to cells with co-localized proteins. Magnification: **A, B, C, D, F** and **G** (×100), Bar=200 μm; **I** and **J** (×200), Bar=200 μm. * represents p<0.05.

instilled mice with pKGF-FLAG gene transfection, compared to that of PPE-instilled mice with pFLAG gene transfection (p=0.002) (Fig. 6F, G and H). When we performed double-staining for PCNA and SP-A or KGFR on day 3 after PPE instillation with pKGF-FLAG gene transfection, colocalization of PCNA and SP-A was detected in alveolar type II cells (Fig. 6I), and colocalization of PCNA and KGFR was detected in alveolar epithelial cells (Fig. 6J). TUNEL staining was also performed to assess apoptosis due to lung injury. As shown in Fig. 7A, B and C, there was no significant difference in the number of apoptotic cells in the emphysematous lung between mice with or without KGF gene at day 3.

KGF gene transfection protected gas exchange function in the emphysematous lung

To confirm the effectiveness of KGF gene transfection as a gene therapy, we analyzed gas exchange function in the PPE-treated emphysema lung with FLAG gene or with KGF-FLAG gene transfection and compared it to that of normal non-treated mice. As shown above (Fig. 3D and E), there was essentially no difference in lung morphological details between PPE-instilled mice transfected with pFLAG and those transfected with pKGF-FLAG on day 3 after transfection. However, when we analyzed arterial blood gas as an indicator of pulmonary function, the PaO_2 level on day 14 after PPE administration and KGF-FLAG gene
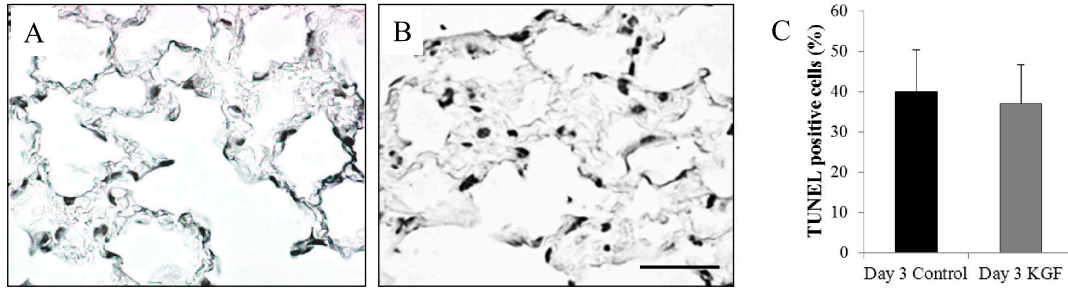
Fig. 7. TUNEL staining in mouse lung after PPE instillation with or without KGF gene transfection. (**A–C**): Sections of mouse lung on day 3 after PPE instillation and gene transfection with the pFLAG gene (**A**) or with the pKGF-FLAG gene (**B**) were analyzed for apoptosis by TUNEL staining. The number of TUNEL-positive cells was similar between the two groups (**C**). Magnification: ×200, Bar=100 μm.
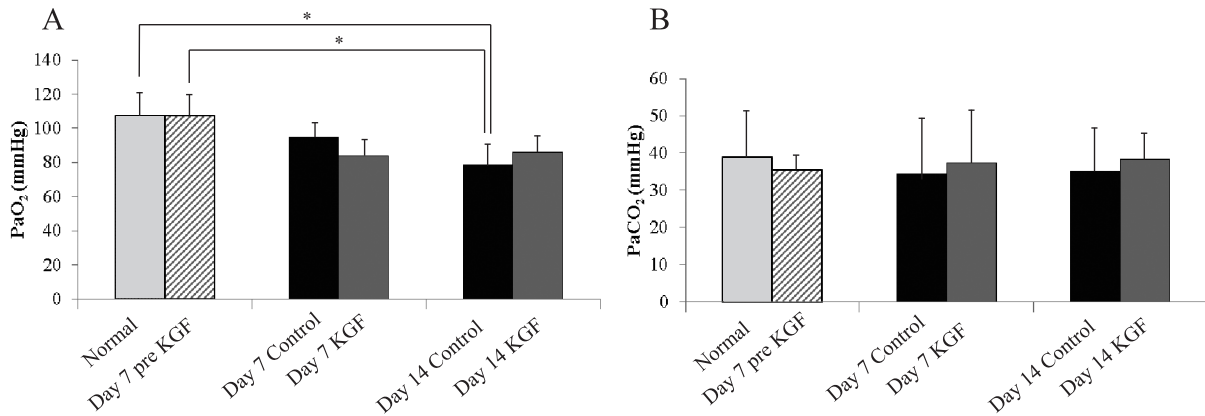

Fig. 8. Arterial blood gas analyses in mouse emphysema lung with or without KGF gene transfection. Mice were not transfected and not treated (normal) or were transfected with the pKGF-FLAG gene 4 days prior to PPE instillation (Pre-KGF), or were transfected with the pFLAG gene (control) or the pKGF-FLAG gene immediately after PPE instillation and the values of arterial PaO_2 and arterial $PaCO_2$ (**A** and **B**, respectively) were assessed on the subsequent indicated days. The PaO_2 level of mice on day 14 after PPE instillation with the pFLAG gene was decreased compared to normal mice. In contrast, the PaO_2 level of mice on day 14 after PPE instillation with the pKGF-FLAG gene and that of mice pretreated with KGF before PPE instillation for 7 days was comparable to that of normal lung. * represents $p < 0.05$.

treatment was nearly comparable to that of normal lung, while the PaO_2 level in the emphysema mice with the transfected pFLAG gene was significantly lower than that of normal lung (Fig. 8A). Of note, the PaO_2 level on day 7 after PPE instillation of mice that were pre-treated with KGF gene transfection 4 days prior to PPE instillation was almost the same as that of normal mice, demonstrating the protective effect of KGF gene therapy. There were no significant differences in $PaCO_2$ levels between the PPE-instilled mice treated with and without KGF gene transfection (Fig. 8B).

IV. Discussion

In the present study, we investigated whether KGF gene transfection into the skeletal muscles by means of electroporation could be a therapeutic tool in terms of preventing the progression for the emphysematous lung. Although there have been a number of reports regarding the protective effect of KGF pre-treatment in lung injury models, there have only been a few reports describing beneficial KGF post-treatment for lung injury. Sakamoto *et al.* showed that intratracheal administration of a KGF-

expressing adenoviral vector improved respiratory function and mortality in bleomycin-induced pulmonary fibrosis [35]. Yildirim *et al.* reported that oropharyngeal aspiration of the KGF protein promoted lung regeneration in elastase-induced emphysema [46]. However, the most appropriate route of administration of KGF for lung repair and regeneration remains unknown in these lung injury models. Here, we have shown that KGF produced in remote muscle tissue by transfection of the muscle with the pKGF-FLAG gene successfully promoted alveolar cell proliferation. Moreover, even post-injury treatment with pKGF-FLAG gene transfection resulted in the improvement of lung function in a PPE instillation mouse emphysema model.

We demonstrated that the expression of KGF and the number of PCNA-positive cells were significantly increased in the lung on day 3 after PPE instillation with pKGF-FLAG gene transfection compared to those in the lung after PPE instillation and transfection with pFLAG gene. SP-A and KGFR were co-expressed in some proliferating cells, indicating that exogenous rhKGF protein as well as endogenous KGF stimulated cell proliferation, in particular, of pulmonary type II cells through the KGFR. Moreover, we examined the expression of KGF mRNA in

the lung by *in situ* hybridization, and we detected expression of KGF mRNA on day 2 after pKGF-FLAG gene transfection (data not shown); it was markedly increased by day 3. It is not known whether exogenous KGF induces up-regulation of endogenous KGF, however it is indicated the potential for a KGF-dependent autocrine loop in pancreatic cancer [16]. Chedid *et al.* reported that proinflammatory cytokines up-regulated KGF gene expression and protein levels in fibroblasts from multiple sources [5], and Yildirim *et al.* reported that rhKGF induced proliferation of fibroblasts in emphysematous mice [46]. Furthermore, Niu *et al.* reported that KGF induced NF-kappaB activation, and stimulated the expression of NF-kappaB regulated genes such as IL-1 [30]. The mechanism of KGF up-regulation in this animal model is unknown, however these results strongly indicate that pKGF-FLAG gene transfection of skeletal muscle by electroporation provided rhKGF protein into the lung, and that this rhKGF might up-regulate the endogenous synthesis of KGF in the lung.

Emphysema is recognized as a progressive disease with a combination of long-term inflammation, oxidative stress, and protease-anti protease imbalance with alveolar wall destruction [33]. In the elastase-induced emphysema model that was used here, intra-tracheal elastase instillation was previously shown to induce hemorrhage and an early inflammatory exudate with neutrophils and macrophages, resulting in the destruction of the alveolar wall due to apoptosis [10]. Mouded *et al.* reported that airspace enlargement progressively continued in this animal model throughout the 21 days monitored after elastase instillation [28]. Thus, the behavior of this model seems to have some similarity with human emphysema. In the present study, arterial blood gas examination indicated that the PaO_2 level was on the rise again by day 14 in the lungs of mice transfected with the pKGF-FLAG gene, although no improvement in anti-apoptotic effect or inhibition of airspace destruction was observed on day 3 after PPE instillation and gene transfection. Plantier *et al.* reported that KGF pre-treatment in this model reduced the number of TUNEL-positive cells [32]. They administered KGF on day 3 before PPE instillation, and TUNEL staining was performed on day 7 after PPE instillation. Although it is difficult to make a direct comparison between their results and our results because of the different times of KGF administration, the different times of evaluation may contribute to the different results obtained.

Regarding the method of KGF administration, KGF gene transfection appears to be a useful alternative method to direct injection of recombinant human KGF protein. Although single instillation of the KGF protein should, in theory, be the simplest method of administering KGF, the effect of such instillation was reported to diminish within only a few days [8]. Gene therapy has advantages over intra-tracheal administration of the KGF protein in terms of prolonged protein expression and low cost [41]. For these reasons, we utilized gene transfer by electroporation to efficiently transfect the gene at a specific site and to make repetitive administration easy. In addition, plasmid DNA was injected into bilateral quadriceps muscle to avoid lung damage by gene transfection without general anesthesia. Gene transfection by electroporation is considered to be a safe and simple method that requires only a low dose of DNA without virus-associated adverse effects. However, there is some controversy surrounding the use of this method because of the lower levels of protein expression obtained compared to the use of viral vectors or of direct intra-tracheal administration of protein. Yildirim *et al.* reported that intra-tracheal multicycle application of 10 mg KGF protein/kg body weight was necessary to achieve a maximal proliferative response in alveolar type II epithelial cells in the elastase-induced emphysema model [46]. On the other hand, Furukawa *et al.* reported that the repeated administration of a low dose of KGF (0.4 mg/kg) gave the same effect as that of administration of a high dose (4 mg/kg) [12]. Currently, the optimal treatment threshold for the gene transfer method using electroporation in this animal model is unclear, because of differences in the route of administration and the absorption efficiency in the different studies. In our previous study, we showed enhanced alveolar septation and an increased number of alveoli on day 7 after pKGF-FLAG gene transfection in the trilobectomy rat model, in spite of the short duration of rhKGF expression, which had returned to the control level on day 7 after gene transfection [24]. Based on these reports, it seems likely that repeated gene transfection into skeletal muscle by electroporation has the potential to provide sufficiently effective treatment, even though KGF production by a single gene transfection is low.

In the present study, rhKGF expression in the quadriceps and lungs was detected on day 3 and, although it had decreased by day 7, it was still detectable and at a higher level than KGF protein expression in control mice transfected with the pFLAG gene. On the other hand, KGFR expression in the lung was not significantly enhanced on day 3 following KGF gene transfection, although co-expression of the KGFR and PCNA in alveolar type II epithelial cells was detected in these cells. Interestingly, the expression of endogenous KGFR mRNA in the lung had increased by day 7 after KGF gene transfection and PPE instillation and was higher than that in pFLAG gene-transfected cells. The mechanism of increasing KGFR expression is still unknown. D'Amici *et al.* reported that proinflammatory cytokines up-regulated FGFR2mRNA expression and gene transcription in epithelial or mesenchymal cells [7], however there have been very few reports about regulation of KGFR expression. Thus, expression of the KGFR as well as that of KGF might be up-regulated by transfection of the pKGF-FLAG gene. Therefore, we would like to propose that KGF gene therapy that is accompanied by lung epithelial cell proliferation has potential as a therapeutic tool for lung emphysema.

In conclusion, our results indicated that KGF gene

administration into skeletal muscles by electroporation in a mouse emphysema model enhanced the proliferation of lung epithelial cells, and relieved prolonged hypoxia. Although further work is clearly needed to apply this approach for the treatment of COPD, we believe that these findings support a future approach aimed at establishing gene therapy as a method for induction of lung regeneration.

V. References

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