学位論文

Regeneration of emphysematous lungs using gelatin sheets that release basic fibroblast growth factor

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ORIGINAL ARTICLE

Regeneration of emphysematous lungs using gelatin sheets that release basic fibroblast growth factor

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Abstract

Purpose Basic fibroblast growth factor (bFGF) induces regeneration and neovascularization of the lungs. We conducted this study to demonstrate the regeneration of emphysematous lungs achieved by gelatin sheets that slowly release bFGF into the visceral pleura in a canine model.

Methods Porcine pancreatic elastase was used to induce bilateral lower lobe pulmonary emphysema in dogs. Slow-release bFGF gelatin sheets were attached to the visceral pleura of the left lower lobe via thoracotomy. The subjects were divided into two groups: one treated with gelatin sheets containing slow-release bFGF ($bFGF^+$ group, $n=5$), and the other, treated with only gelatin sheets ($bFGF^-$ group, $n=5$). The subjects were euthanized after 28 days and histologic lung assessment was performed. The results were evaluated in terms of the mean linear intercept (MLI) and microvessel count.

Results The MLI was significantly shorter in the $bFGF^+$ group than in the $bFGF^-$ group; (110.0 ± 24.38 vs. 208.9 ± 33.08 μm; $P=0.0006$). The microvessel count was not significantly different between the $bFGF^+$ and $bFGF^-$ groups (12.20 ± 3.007 vs. 5.35 ± 2.3425; $P=0.075$); however, it was significantly higher in the bFGF-attached lungs than in the emphysema group (12.20 ± 3.007 vs. 4.57 ± 0.8896; $P=0.012$).

Conclusions Attaching gelatin sheets with slow-release bFGF to the visceral pleura induced lung regeneration and vascularization in a canine pulmonary emphysema model.

Keywords Lung regeneration · Basic fibroblast growth factor · Emphysema · Chronic obstructive lung disease · Canine model

Introduction

Emphysema is defined as irreversible collapse of the alveolar structures in the lung, caused mainly by smoking. In 2020, it was recognized as the third most common cause of death worldwide [1]. Lung transplantation is the only known curative treatment for emphysema; however, donor shortage means only a limited number of these patients will receive a lung transplant [2]. Volume reduction surgery has been used as a viable surgical treatment option in the past, but this procedure does not confer any survival advantage [3] and lung function often declines postoperatively [4, 5]. To date, two clinical trials have been conducted on the regeneration of emphysematous lungs [6, 7]. One of these was a randomized controlled trial conducted on 262 patients using γ-selective retinoic acid, which demonstrated no improvement in respiratory function [6]. The other trial used allogeneic mesenchymal stem cells, which had a satisfactory safety profile, but no pulmonary regeneration was observed [7]. To our knowledge, there are no drugs with proven efficacy for the regeneration of emphysematous lungs.

Basic fibroblast growth factor (bFGF) is a member of the FGF family, which comprises 23 members [8]. During the fetal stage, bFGF activates the differentiation of various cells that originate from the mesoderm, ectoderm, and endoderm. Furthermore, bFGF immunoreactivity has been observed in the cells of the respiratory epithelium, basement membrane, and extracellular matrix during infant development [9]. Interestingly, recent studies have reported cases of alveolar regeneration achieved by bFGF in rat and mouse

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emphysematous models [10, 11]. In our previous experiments, we succeeded in regenerating tracheal cartilage, intrathoracic fibrous materials, and alveoli, by slowly releasing various growth factors in vivo [12–15]. In particular, we reported reconstructing emphysematous lung tissue histologically with improved oxygenation via the slow release of bFGF from bioabsorbable gelatin microspheres injected into the pulmonary artery in a canine model [16, 17]. No adverse events were observed in the animal subjects of these experiments; however, because the administration of intravascular gelatin microspheres can lead to respiratory failure, this study was not approved by the Ethics Review Board. Thus, it is necessary to develop a rational method that can be applied safely in clinical practice.

The visceral pleura has a rich lymphatic network that drains lymph from the interlobular region into the alveoli and bronchioles [18, 19]. Furthermore, it has been reported that multipotent mesenchymal cells can be distributed in a wide variety of adult tissues [20]. Therefore, we predicted that lung regeneration is achievable through slow bFGF release around the destroyed alveoli via a bioabsorbable gelatin sheet attached to the visceral pleura. This method can be implemented easily in clinical practice using video-assisted thoracoscopic surgery. The application of sheets that release biological substances to the visceral pleura has been performed clinically, and its safety is established [21].

Methods

The protocol of this study was approved by the Animal Care and Use Committee of Kagawa University (approval number: 2020-20615), and performed in accordance with the ethical standards of the 1964 Declaration of Helsinki and its later amendments. Hybrid female beagle dogs (age 1–2 years; weight 9–12 kg) were prepared from the Kitayama Shizuoka Laboratory Animal Center (Shizuoka, Japan).

Study design

Fifteen hybrid beagle dogs were used in this study. Five were subjected to left lower lobectomy and assigned to the normal group ($n=5$). In the other 10 dogs (emphysema group, $n=10$) porcine pancreatic elastase (PPE) was administered into the lower lobes of both lungs with bronchoscopy to induce experimental emphysema of the lower lobes. The emphysematous right lower lobes of the emphysema group were evaluated and compared between the normal and emphysema groups. Thereafter, in five dogs from the emphysema group, gelatin sheets with bFGF were attached to the visceral pleura ($bFGF^+$ group, $n=5$); in the other five, gelatin sheets without bFGF ($bFGF^-$ group, $n=5$) were attached to the emphysematous left lower lobe (Fig. 1). Then, the data

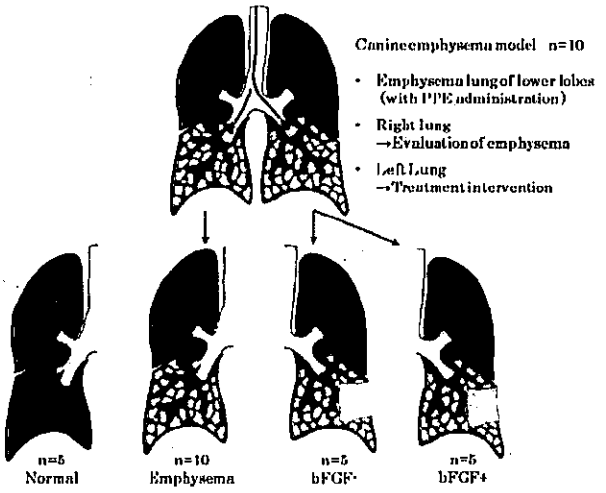

Fig. 1 Study protocol. Comparison between the normal and emphysema groups indicated induced emphysema. We compared the basic fibroblast growth factor ($bFGF^+$) and $bFGF^-$ groups to evaluate the mean liner intercept. Vascularization was assessed by counting microvessels and comparing the results among all the groups

of dogs in the $bFGF^+$ and $bFGF^-$ groups were compared pathologically.

Development of the canine emphysema model

In a previous report, we described the first successful canine emphysema model in large animals [22], which we applied in this study. First, PPE (700 U/kg body weight; Elastin Products Co., Owensville, MO) was dissolved in an isotonic sodium chloride solution (1 mL/kg body weight). The 10 animals in the experimental group were anesthetized using intramuscular injections of xylazine (Selactal, 5.0 mg/kg; Byer Ltd., Tokyo, Japan) and ketamine (Ketalar, 10 mg/kg; Sankyo Co. Ltd., Tokyo, Japan). After introducing a bronchoscope (Olympus: 1T40, Tokyo, Japan), under mechanical ventilation, we inserted a catheter into both sides of the lower bronchus. The PPE solution was injected selectively into both lower lobes in sequence. This procedure was performed once a week for 3 consecutive weeks. Completion of induced emphysema was achieved by 4 weeks after the last administration of the PPE solution.

Preparation of Gelatin sheets incorporating bFGF

Gelatin, as a 5% aqueous solution with an isoelectric point of 5.0 (Nitta Gelatin Co, Osaka, Japan) containing 0.1% glutaraldehyde (Wako Pure Chemical Industries, Osaka, Japan), was cast into a mold and stored at 4 °C for 12 h to induce complete chemical cross-linking. The resulting material was soaked in a glycine solution (Nacalai Tesque Inc., Kyoto, Japan) at 37 °C for 1 h to reduce any glutaraldehyde,

then rinsed in distilled water and freeze-dried. The gelatin sheet was obtained and cut into uniform pieces of approximately 30×50×2 mm. Immediately before implantation of the sponge, 100 μg of aqueous bFGF solution was dissolved in 3 mL of isotonic sodium chloride solution, and the resulting solution was added to the empty dry gelatin sheets. This sheet was left for 15 min to allow the bFGF solution to completely soak in. Previous works have shown that bFGF, ionically immobilized on the gelatin sheet by this method, was released from the gelatin sheet slowly for approximately 2 weeks as it gradually degraded [23, 24].

Intrapleural administration of bFGF gelatin sheet

By 4 weeks after the administration of PPE through bronchoscopy, thoracotomy was performed under general anesthesia. Thoracotomy was performed on the left side of the chest through a 10 cm incision, and gelatin hydrogel particles were attached to the visceral pleura of the left lower lobe. Five animals had gelatin hydrogel sheets infiltrated with bFGF implanted ($bFGF^+$ group), and the other five had gelatin hydrogel sheets infiltrated with isotonic sodium chloride solution instead of bFGF implanted ($bFGF^-$ group).

Evaluation

The study subjects were euthanized after 28 days for evaluation. S6 (upper lobe and superior segment) and the basal segment to which a gelatin sheet was attached were sliced and hematoxylin–eosin (HE) staining was performed in both the normal and emphysema groups. Mean linear intercept (MLI), which is the most usual technique for measuring alveolar diameter, was calculated in the following four groups: normal, emphysematous, bFGF-treated, and bFGF-untreated lungs. Our aim was to assess the degree of emphysematous changes [25]. Random evaluation was performed starting from just below the pleura to the deep part of the lungs. The MLI was evaluated by drawing a line to approximately 100,000 μm for each animal.

Arterial blood gas analysis was performed at three points: before the administration of the elastase injection, before gelatin sheet attachment, and before lung resection under the same ventilation conditions (FiO_2: 1.0, respiratory rate: 10 breaths/min, tidal volume: 10 mL/kg). Vascularity was evaluated by counting each animal's microvessels, identified by immunohistochemical staining for factor VIII. We counted an average of 10 areas under a light microscope at ×200 magnification.

Statistical analysis

Statistical analyses were performed using EZR (Saitama Medical Center, Jichi Medical University, Saitama, Japan), a graphical user interface for R (The R Foundation for Statistical Computing, Vienna, Austria). We conducted an unpaired *t* test to compare the MLI for the two groups. We used the Kruskal–Wallis test to compare the microvessel counts among all the groups. The differences in arterial blood gas analysis results before and after FGF+/− sheet attachment were analyzed using the paired *t* test. Statistical significance was set at $P < 0.05$.

Results

The study animals weighed 9–12 kg (mean weight 10.2 kg). No animals died after PPE injection or the gelatin sheet attachment procedure. By 4 weeks after the last PPE administration, bullae or blebs were observed macroscopically in the bilateral lower lobes of all the dogs (Fig. 2a). The gelatin sheet was integrated with the visceral pleura 4 weeks post-application (Fig. 2b, c). Figure 3 shows the microscopic findings of each lung section. Centrilobular emphysema was observed in the PPE-administered emphysema group (Fig. 3b).

The mean MLI was significantly higher in the emphysema group than in the normal group (177.7 ± 36.76 vs. 61.96 ± 7.346 μm; $P = 0.00001$) (Fig. 4). The MLI was significantly lower in the $bFGF^+$ group than in the bFGF- group (110.0 ± 24.38, vs. 208.9 ± 33.08 μm; $P = 0.0006$) (Fig. 5). Blood gas analysis showed no significant differences in pO_2 before and after the attachment of $FGF^{+/-}$ sheets (FGF^+: 513.4 ± 50.47 vs. 497.8 ± 56.64 mmHg, $P = 0.488$) (FGF^-: 452.4 ± 161.3 vs. 431.4 ± 175.4 mmHg; $P = 0.219$) (Fig. 6). Vascularization was assessed by counting each animal's individual microvessels, which were identified using immunohistochemical staining for factor VIII (Fig. 7). The microvessel count was significantly higher in the bFGF group than in the emphysema group (12.20 ± 3.007 vs. 4.57 ± 0.8896; $P = 0.012$), but there was no significant difference between the $bFGF^+$ and $bFGF^-$ groups (12.20 ± 3.007 vs. 5.35 ± 2.3425; $P = 0.075$) (Fig. 8).

Discussion

Transbronchial bFGF administration has been reported to increase blood flow to the lungs and improve blood gas analysis in a canine emphysema model [26, 27]. These studies have revolutionized the treatment of emphysema through regeneration using bFGF. As bFGF has a short half-life [28], frequent and repeated administration is required to achieve optimal results. Thus, we developed a slow-release mechanism to overcome this and to secure a stable method of administering bFGF. Two of our previous studies demonstrated successful regeneration of emphysematous lungs

Fig. 2 Macroscopic changes in the visceral pleura. **a** A macrograph of minute bullae or blebs in the lower lobes. **b** The gelatin sheet was sutured to the visceral pleura. **c** By 4 weeks after applying the gelatin sheet, it was integrated with the visceral pleura

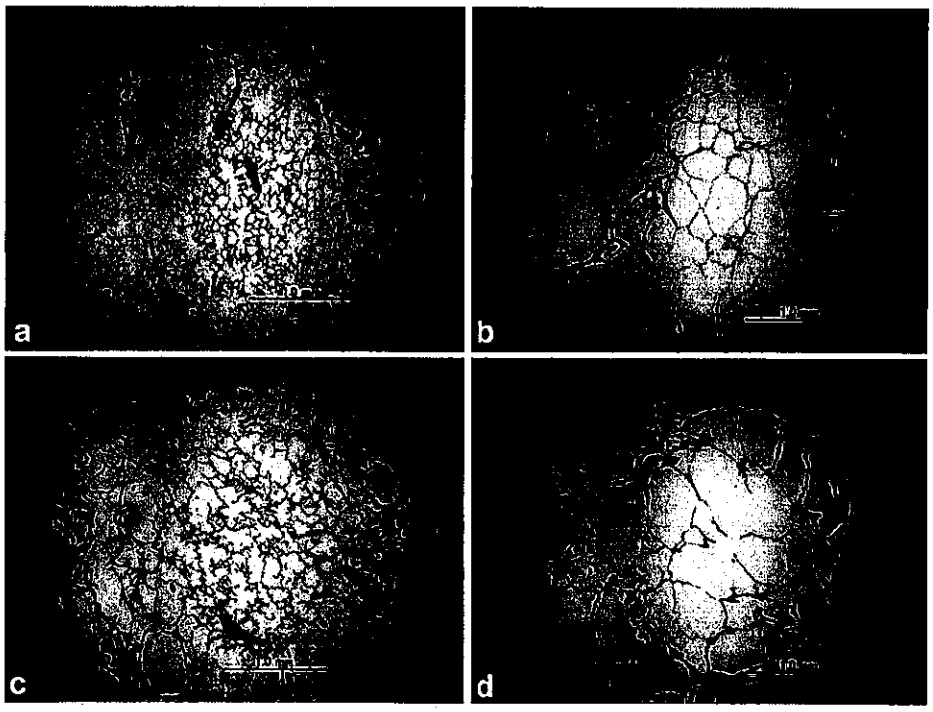

Fig. 3 Representative microscopic views of the lungs from each dog. **a** normal group. **b** emphysema group: centrilobular emphysema was observed. **c** $bFGF^{+}$ group. **d**: $bFGF^{-}$ group. (Hematoxylin and eosin staining, × 100)

by embolizing gelatin beads and slowly releasing bFGF into the pulmonary artery in a canine model [16, 17]. Both studies demonstrated improved oxygenation and respiratory function in addition to regenerating pulmonary structures; however, the clinical application of the method was difficult because of the possibility of respiratory failure following the embolism of gelatin beads. There is a rich lymphatic flow in the visceral pleura, and it has been proven that various substances are transported through lymphatic vessels [18, 19, 29]. In this study, the microscopic findings of the rich lymphatic ducts in the parenchyma after attaching the bFGF gelatin sheets were informative (Fig. 9). Notably, alveolar regeneration and vascularization were seen after the application of bFGF to the visceral pleura; then, the bFGF was distributed in the lung parenchyma via the lymphatic flow. The bFGF can also be carried by lymphatic vessels from the

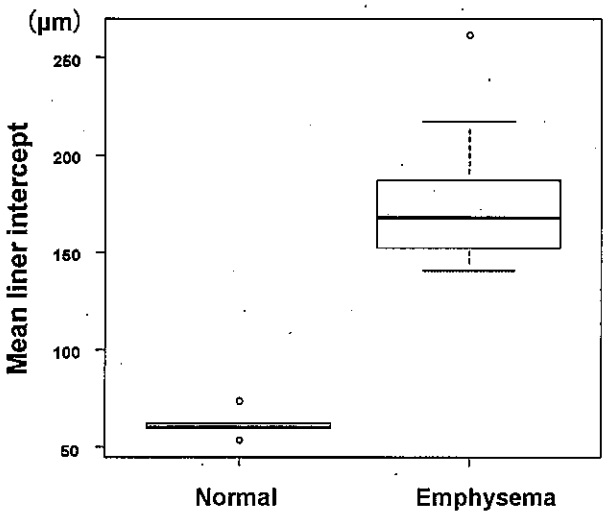

Fig. 4 Comparison of the average mean linear intercept (MLI) between the normal and emphysema groups. The MLI was significantly higher in the emphysema than in the normal group (177.7±36.76 vs. 61.96±7.346 μm, $P=0.00001$)

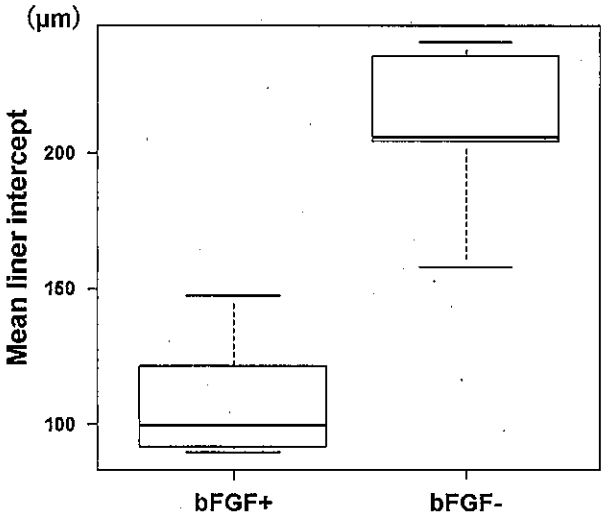

Fig. 5 Comparison of the mean linear intercept (MLI) between the $bFGF^+$ and $bFGF^-$ groups. The MLI was significantly lower in the $bFGF^+$ than in the bFGF- group (110.0±24.38 vs. 208.9±33.08 μm, $P=0.0006$)

visceral pleura to the deep lung parenchyma and the alveoli are regenerated through angiogenesis.

We succeeded in regenerating the structure of the emphysematous lung and increasing the microvessel count, but improvement in oxygenation was not demonstrated. In our previous study, we created a PPE-induced total emphysema model and tried to improve the condition of one lung through the therapeutic effect of bFGF; however, half of the animals died after PPE administration [17]. Therefore,

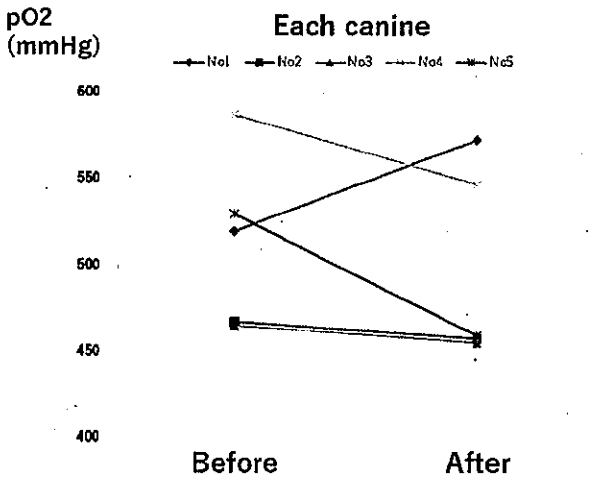

Fig. 6 Blood gas analysis revealed no significant differences in the pO_2 levels before and after the attachment of $bFGF^+$ gelatin sheets (FGF^+: 513.4±50.47 vs 497.8±56.64 mm Hg; $P=0.488$)

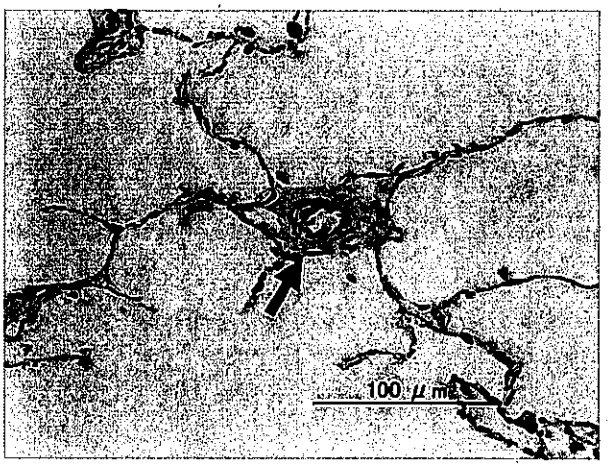

Fig. 7 Representative microscopic findings of factor VIII immunochemical staining-positive vessels for evaluating vascularity

in this study, we changed the approach to inducing emphysema only in the lower lobes of both sides. As healthy lung structure was retained in the anterior and middle lobes, there was no remarkable improvement in oxygenation. Moreover, as the sheet was applied to only part of the visceral pleura instead of the entire lower lobe, the effect did not cover a wide area, and increasing blood flow/perfusion was shown by the presence of abundant microvessels. Although some microvessels may indicate an increase in matching alveolus, successful angiogenesis would not necessarily improve pulmonary function because of the perfusion/ventilation mismatch or shunting effects; therefore, oxygenation was not improved. There was no perioperative death in either of our $bFGF^+$ or $bFGF^-$ groups, and the safety of treatment with

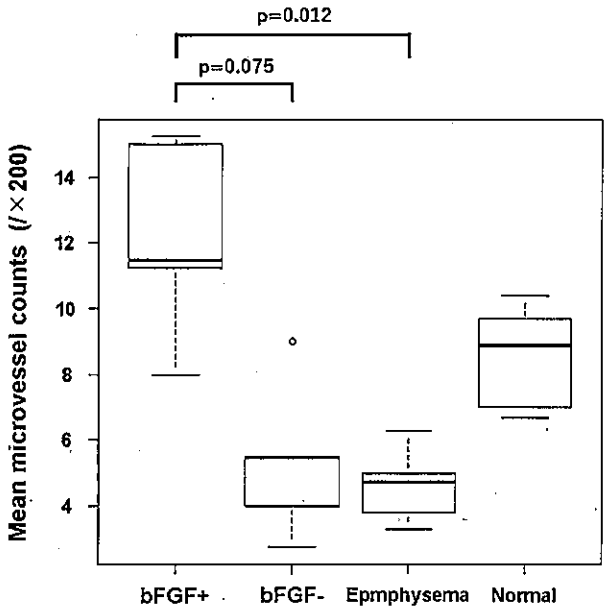

Fig. 8 The microvessel count was significantly greater in the FGF+ than in the emphysema group (12.20±3.007 vs. 4.57±0.8896; $P=0.012$)

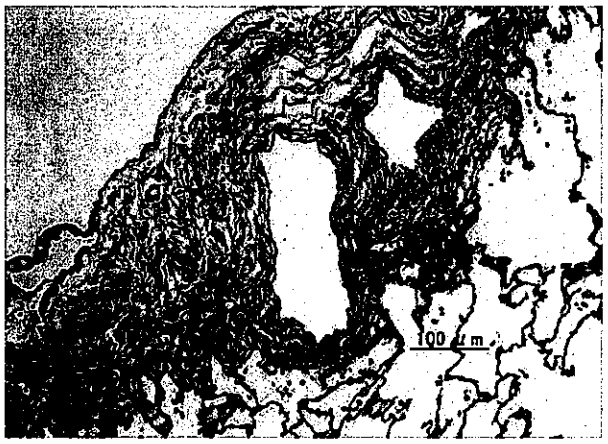

Fig. 9 Microscopic findings of the rich lymphatic ducts in the parenchyma after attaching the bFGF gelatin sheets (Hematoxylin and eosin staining, ×200)

slow bFGF release gelatin sheets attached to the visceral pleura was also conclusive.

This study had some limitations. First, the dogs were monitored for only 4 weeks after attaching the gelatin sheets; however, a longer observational period would be needed to evaluate the long-term effects and safety of this method. Second, as the multiple dose regimen of bFGF was evaluated, it was necessary to study the changes in the therapeutic effect and safety profile of bFGF administration by increasing the dosage. Third, the only outcome evaluated was emphysematous lung regeneration. The speed and mechanism of alveolar epithelial cell regeneration remain unknown, as this study was not designed to identify this. A variety of growth factors other than bFGF are involved in lung development. Although bFGF is a proven important agonist of alveolar proliferation, the exact mechanism of action has not been elucidated. In this study, regeneration might have been attributed to enhanced differentiation of multipotent mesenchymal cells alone, self-repair of damaged alveolar epithelium, enhanced cell division of damaged alveoli, or a combination of these factors. Future studies are necessary to understand the mechanism of alveolar regeneration by bFGF and to identify other factors that may have similar therapeutic benefits.

We conclude that it is possible to regenerate the canine emphysematous lung using gelatin sheets that slowly release bFGF into the visceral pleura. Although the clinical application of this technique is still limited, it may emerge as a new surgical option for the treatment of emphysema with continuous improvements.

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Declarations

Conflict of interest We have no conflicts of interest to declare.

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