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Tracheal cartilage growth by intratracheal injection of basic fibroblast growth factor

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ABSTRACT

Background/Purpose: We have previously shown that intratracheal injection of slowly released (in gelatin) basic fibroblast growth factor (bFGF) significantly enlarged the tracheal lumen by a slight margin. This study aimed to investigate differences in tracheal cartilage growth by the intratracheal injection of bFGF doses in a rabbit model.

Methods: Water (group 1; n = 7; control) or 100 μg (group 2; n = 8) or 200 μg (group 3; n = 8) of bFGF dissolved in water was injected into the posterior wall of the cervical trachea of New Zealand white rabbits using a tracheoscope. All animals were sacrificed four weeks later.

Results: The mean circumferences of cervical tracheas for groups 1, 2, and 3 were 18.8 ± 0.83, 21.1 ± 2.0, and 22.1 ± 1.3 mm, respectively. A significant difference was found between groups 1 and 2 (P = 0.034) and groups 1 and 3 (P = 0.004). The mean luminal areas of cervical tracheas for groups 1, 2, and 3 were 27.0 ± 2.1, 32.2 ± 4.8, and 36.3 ± 4.6 mm^2, respectively. A significant difference was found between groups 1 and 3 (P = 0.001).

Conclusion: Intratracheal injection of bFGF in the dose range used significantly promoted the growth of tracheal cartilage in a rabbit model.

Levels of Evidence: Level II at treatment study (animal experiment).

Since 2006, we have been developing an engineered airway using patch tracheoplasty for tracheal stenosis [1–3]. An engineered airway construction, generated using a biodegradable scaffold and chondrocytes grown *in vitro*, was implanted into a tracheostomy to reconstruct an airway. However, engineered cartilage cannot be easily grown in an *in situ* implantation site [3]. Basic fibroblast growth factor (bFGF) is a very effective growth factor that induces the proliferation of chondrocytes, as well as angiogenesis and wound healing, by affecting smooth muscle cells, endothelial cells, fibroblasts, and epithelial cells [4–7]. The half-life of bFGF is very short [8–10]. Therefore, to grow engineered cartilage during patch tracheoplasty at an *in situ* implantation site, slow-release bFGF was administered into the site using a drug delivery system [3,11]; this comprised gelatin hydrogel microspheres that gradually released bFGF as the gel degraded over approximately two weeks [12]. The slow release of bFGF generated engineered cartilage constructed with biodegradable scaffold seeded with proliferating chondrocytes *in vitro*. The swallowed stump of native tracheal cartilage was also recognized in histological findings [3]. We speculated that slowly released bFGF could also promote the growth of native tracheal cartilage.

Traditionally, symptoms in children with moderate to mild tracheomalacia are thought to improve as they grow older [13–16]. We have case-based experience whereby a patient's tracheostomy tubes can be extubated later in childhood without ill effect. Speculation exists that this phenomenon is caused by a growth-related increase in the size and mechanical properties of the trachea. Therefore, the rapid growth of induced tracheal cartilage may act as a novel treatment for tracheomalacia.

We have previously reported that slow-release bFGF gelatin sheets placed on the outer surface of the rat trachea enlarged the tracheal lumen and thickness of the cartilage in a bell-shaped, dose-dependent fashion [17,18]. Also, a less invasive surgical procedure was necessary for tracheomalacia patients with premature and congenital malformations. In this respect, we noted that the intratracheal injection of slowly

Abbreviations: bFGF, basic fibroblast growth factor.

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released bFGF enlarged the tracheal lumen, but with a marginally significant difference [19]. In general, the sustained release formulation was administered to a spot with slowly increasing concentrations, and a consequent delayed effect noted. The concentration of bFGF by a sustained release formulation decreased slowly, and side effects occurred easily. In a rat model, we noted that the slow release of bFGF induced a bell-shaped, dose–response curve of tracheal cartilage thickening in the luminal area, with a peak observed using 5 μg of bFGF [18]. In this rat model, the luminal area of tracheal cartilage from rats administered 50-μg bFGF became thickened to three times that of the control group, subsequently developing into an airway stenosis. Therefore, it is necessary to investigate the efficacy of the injection of bFGF only, without gelatin, in the promotion of tracheal cartilage growth in order to avoid such stenosis. This study aimed to investigate differences in tracheal cartilage growth by the intratracheal injection of bFGF doses in a rabbit model.

1. Materials and Methods

The study protocol was approved by the Animal Care and Use Committee of the University of Tokyo (protocol No. P12–25), and all experiments were performed in accordance with the Guidelines for Proper Conduct of Animal Experiments of the University of Tokyo.

1.1. Preparation of bFGF solution

Trafermin (Fiblast Spray, Kaken Pharmaceutical Co. Ltd., Tokyo, Japan) is a commercially available, recombinant human bFGF. It has been authorized for use in patients by the Ministry of Health, Labor and Welfare of Japan. This solution was adjusted to a concentration of 0.2 and 0.4 μg/μL of bFGF.

1.2. Tracheoscope and injection system

An ultra-thin endoscope (TESALA™, AVS Co. Ltd., Tokyo, Japan) was used as a tracheoscope in our study as previously described [19,20]. This system consisted of a camera unit, hand piece and endoscopic fiber probe (1.6 mm in diameter, 150 mm in length, and 17,000 pixel counts). The endoscope was connected to a monitor for visualization. An introducer, which was 2 mm in diameter, was used to protect the endoscopic fiber probe. An injection needle (Varixer; Top Co. Ltd., Tokyo, Japan) was used as the injection system. This needle was attached to the introducer outside the endoscopic fiber probe by a 3 M Steri-Strip adhesive skin closure (3 M Co. Ltd., Tokyo, Japan).

1.3. Surgical procedures

New Zealand white rabbits were anesthetized with a propofol bolus of 20 mg/kg (Maruishi Pharmaceutical Co. Ltd., Osaka, Japan) and halothane (Takeda Pharmaceutical Co. Ltd., Osaka, Japan). With the rabbit in a prone position, propofol was given through an intravenous drip at 60 mg/kg/h. A tracheoscope was inserted into the trachea, with animals under deep sedation and with spontaneous respiration. A needle puncture was made into the membranous, posterior wall of the trachea under visual observation. A 250-μL solution of distilled water, with or without bFGF, was then injected twice into the submucosal space of the trachea, and a localized submucosal elevation was confirmed under endoscopic view. Eight-week-old, female New Zealand white rabbits were divided into three groups: Group 1 underwent an injection of distilled water into the posterior tracheal wall ($n = 7$), group 2 underwent injection of a 100-μg bFGF solution ($n = 8$), and group 3 underwent injection of a 200-μg bFGF solution ($n = 8$).

1.4. Morphological and histological examinations

Four weeks after the surgical procedure, all rabbits were sacrificed for morphological and histological examinations. Cervical tracheas were harvested and tissue specimens were embedded in Tissue-Tek OCT compound 4583 (Sakura Finetechnical Co. Ltd., Tokyo, Japan) and frozen. Embedded tissues were subsequently sliced into 7-μm sections and stained with hematoxylin and eosin (H & E), toluidine blue, or safranin O.

1.5. Measurement of circumference and luminal area

Images were obtained with an H & E stained section of trachea (BZ-9000, Keyence, Osaka, Japan). The circumference and luminal area of cartilage was measured in cross-sections of each trachea using an automatic measurement system in commercially available image processing software (Medical Image Analyzer, Inotech Co. Ltd., Hiroshima, Japan).

1.6. Statistical analysis

Statistical analysis was performed using commercially available software SAS 9.4 (SAS Institute Inc. Cary, NC, USA). At first, a test of normality was performed for each group data set using the Kolmogorov–Smirnov test. Parametric multiple comparisons were performed by Tukey's test. Results were expressed as the mean ± standard deviation (SD). A P value of <0.05 was considered to be statistically significant.

2. Results

All rabbits in each group survived until sacrificed; changes in breathing were not noted. Upon gross examination of the cervical tracheas, differences in inflammatory signs among the distilled water and two bFGF injection groups were not observed. For the distilled water group, cervical tracheas displayed a tapered shape, from the thyroid cartilage to the carina tracheae (Fig. 1). Cervical tracheas from the two bFGF injection groups were spindle shaped, and displayed a maximum diameter at the injection site (Fig. 1). The width of each tracheal cartilage ring for bFGF groups 2 and 3 was noticeably greater than that of control group 1, but was not measured.

Histological examination of cross-sections of tracheas in each group are shown in Fig. 2. The presence of cartilage was confirmed by H & E, toluidine blue, and safranin O staining. The thickest point of each tracheal cartilage in bFGF groups 2 and 3 appeared thicker than that in control group 1, but was not measured (Fig. 2).

Data for each group was found to be normal ($P > 0.15$). The mean circumferences ± SD of cervical tracheas for groups 1, 2 and 3 were 18.8 ± 0.83, 21.1 ± 2.0 and 22.1 ± 1.3 mm, respectively; a significant difference was noted between groups 1 and 2 ($P = 0.03$), and between groups 1 and 3 ($P = 0.004$; Fig. 3). The mean luminal areas ± SD for groups 1, 2 and 3 were 27.0 ± 2.1, 32.2 ± 4.8 and 36.3 ± 4.6 mm^2, respectively; a significant difference was noted between groups 1 and 3 ($P = 0.001$; Fig. 4).

3. Discussion

Biodegradable hydrogel sheets need to be inserted between the trachea and esophagus by an invasive surgical procedure in order to promote growth of the tracheal wall. A less invasive surgical procedure, the injection of bFGF solution from the inner side of the trachea can also promote growth, with a significant peak noted at 200 μg of bFGF for both circumference and luminal area. However, a 100-μg bFGF solution injected as a single dose in the inner side of the trachea did not significantly enlarge the trachea luminal area. This suggests that intratracheal injection of bFGF significantly promoted the growth of tracheal cartilage in a rabbit model in the dose range used.

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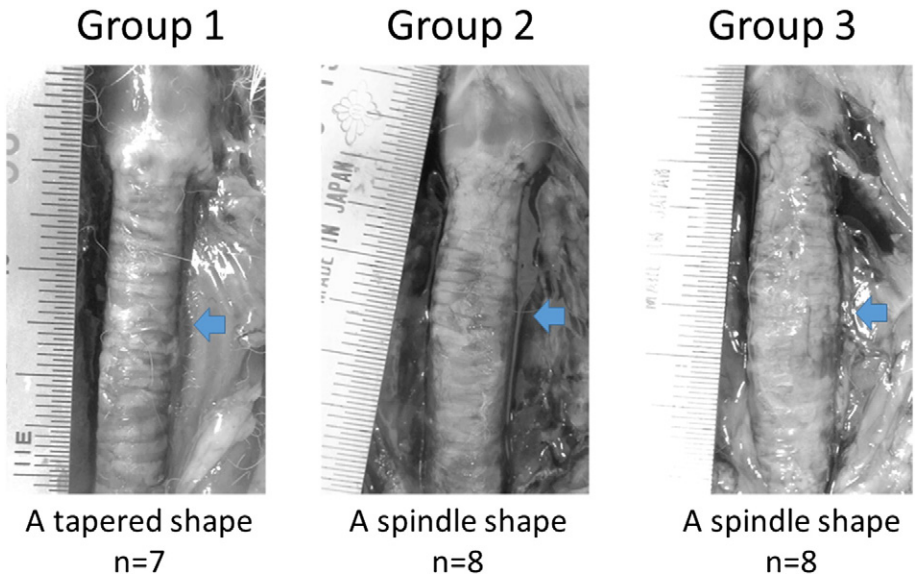

Fig. 1. Shape of cervical tracheas. New Zealand white rabbits were injected with bFGF into the posterior tracheal wall: group 1 (control group, water), group 2 (100-μg bFGF) and group 3 (200 μg bFGF). For group 1, cervical tracheas showed a tapered shape between the thyroid cartilage and the carina tracheae. For groups 2 and 3, cervical tracheas were spindle-shaped, and displayed their maximum diameter at the injection site. The width of each cartilage ring was greater for bFGF groups 2 and 3 than for control group 1.

We have previously shown in a rabbit model that 100 μg of slowly released bFGF injected into the trachea significantly enlarged the tracheal lumen, but in a marginal manner [19]. With regard to our knowledge of tracheal cartilage, several studies have shown that only slow-release bFGF stimulated tracheal growth [3,21,22]. In contrast, we showed that the injection of a 200-μg bFGF solution in the inner side of the trachea promoted tracheal cartilage growth, with a significant difference from control, four weeks after administration. This is the first report of chondrocyte proliferation and chondrogenesis in response to a bFGF solution (i.e. without gelatin), with remarkable cartilage generation and growth in part of the trachea's cartilage.

Exogenously administered bFGF can stably bind heparan sulfate within the extracellular matrix, and can then be subsequently released by enzyme activity. Injected bFGF has been shown to reach the perichondrium of tracheal cartilage, which is known to have chondrogenic potential [21], and to be a possible source of regenerative cartilage [21], to enhance the growth of tracheal cartilage. Indeed, a high enough dose of bFGF, with its short half-life, can induce the proliferation of

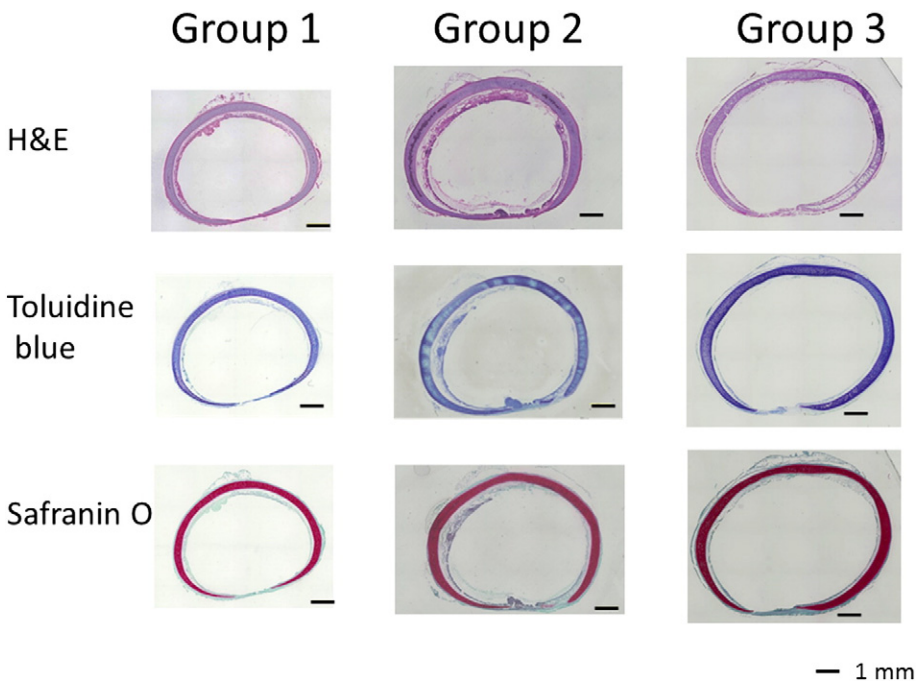

Fig. 2. Histology of tracheal cross-sections at the injection site. New Zealand white rabbits were injected into the posterior tracheal wall: group 1 (control group, water), group 2 (100-μg bFGF) and group 3 (200 μg bFGF). Frozen sections of tracheal cartilage were stained with H & E, toluidine blue, or safranin O. The thickest tracheal cartilage was found at injection sites for groups 2 and 3.

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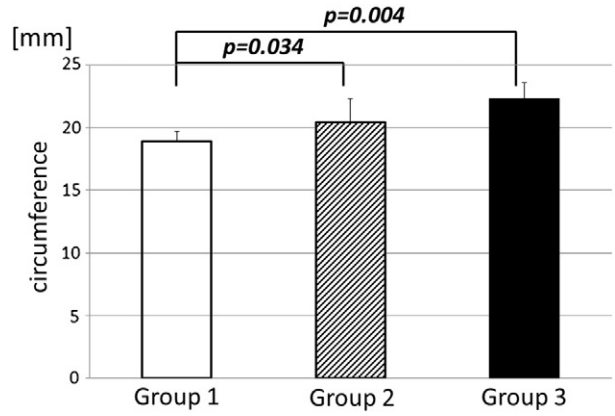

Fig. 3. Mean circumference of cervical tracheas at the injection site. New Zealand white rabbits were injected into the posterior tracheal wall: group 1 (control group, water), group 2 (100-μg bFGF) and group 3 (200 μg bFGF). The circumference of cartilage was measured in cross-sections of each trachea using a fluorescence microscope and imaging software.

cartilage stem cells to promote rapid trachea growth *in vivo*. Therefore, the administration of slow-release bFGF is not always necessary to promote significant and remarkable growth of tracheal cartilage.

In a rabbit model, we previously demonstrated that the bioavailability of 100 μg of a slow-release bFGF formulation was superior to a 100-μg bFGF solution [19]. However, in a rat model, slow-release bFGF administration displayed a bell-shaped dose–response curve for the luminal area, with a peak at 5 μg of bFGF [18]. In such a rat model, the tracheas of rats treated with 50-μg bFGF on the inner side showed thickening of the tracheal cartilage three times greater than that of control rats. A uniform direction of tracheal growth is necessary for the enlargement of the tracheal lumen by optimal bFGF stimulation. A sustained release formulation can support a high, local concentration of bFGF for a long time, and a long, effective concentration can induce cartilage growth. An optimal amount of bFGF-impregnated gelatin sponges, as a sustained release formulation, can control the size and shape of three-dimensionally engineered cartilage [23]. It follows, therefore, that the choice of a slow-release bFGF dosage requires great circumspection to promote optimal trachea cartilage. Furthermore, we still need to investigate optimal drug delivery systems, injection doses, frequencies and intervals for the promotion of trachea cartilage over a longer observation period.

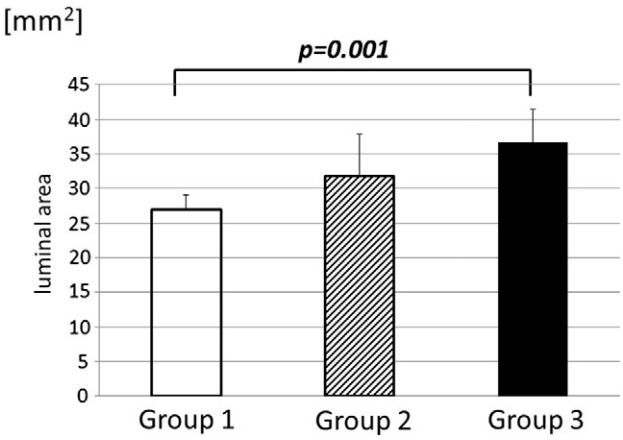

Fig. 4. Mean luminal areas of cervical tracheas at the injection site. New Zealand white rabbits were injected with bFGF into the posterior tracheal wall: group 1 (control group, water), group 2 (100-μg bFGF) and group 3 (200 μg bFGF). The luminal area of cartilage was measured in cross-sections of each trachea using a fluorescence microscope and imaging software.

In conclusion, intratracheal injection of a bFGF solution with a short half-life in the dose range used significantly promoted the growth of rabbit tracheal cartilage.

Acknowledgements

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Conflicts of interest: none.

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