Promotion of tracheal cartilage growth by intra-tracheal injection of basic fibroblast growth factor (b-FGF)

Makoto Komura, Hiroko Komura, Kenichirou Konishi, Tetsuya Ishimaru, Kazuto Hoshi, Tsuyoshi Takato, Yasuhiro Tabata, Tadashi Iwanaka

Abstract

Purpose: Basic fibroblast growth factor (b-FGF) is a very effective growth factor that induces the proliferation of chondrocytes. This study aimed to investigate whether intra-tracheally injected b-FGF solution promotes the growth of tracheal cartilage.

Methods: Group 1: 500 μl of distilled water was injected at the posterior wall of the cervical trachea (n = 5). Group 2: 100 μg/500 μl of b-FGF solution was injected at the posterior wall of the cervical trachea (n = 5). Group 3: Biodegradable gelatin hydrogel microspheres incorporating 100 μg/500 μl of b-FGF solution were injected at the posterior wall of the cervical trachea (n = 5). All animals were sacrificed 4 weeks later, and the outer diameter and luminal area of the cervical trachea at the site of b-FGF injection were measured.

Results: The cervical tracheas in the two b-FGF injection groups were spindleshaped and had a maximum diameter at the injection site. The median outer diameter of the cervical trachea in Groups 1, 2, and 3 was 7.3, 8.0, and 8.0 mm, respectively, showing a significant difference among Groups 1, 2, and 3 (P = 0.04). The median luminal area in Groups 1, 2, and 3 was 27.4, 29.4, and 32.1 mm², respectively. The ad hoc test showed a marginally significant difference only between Groups 1 and 3 (p = 0.056).

Conclusion: Intra-tracheal injection of slowly released b-FGF enlarged the tracheal lumen.

The treatment for severe tracheomalacia is controversial [1,2]. Traditionally, the symptoms of patients with moderate to mild tracheomalacia are thought to improve as the patients grow older [3–6]. We have also experienced some severe cases in which the patients’ tracheotomy tubes could be extubated in childhood or later. This phenomenon is believed to be caused by an increase in the size of the trachea.

Basic fibroblast growth factor (b-FGF) is a very effective growth factor that induces angiogenesis and wound healing as a result of its action on smooth muscle cells, endothelial cells, fibroblasts, and epithelial cells [7,8]. b-FGF is known as a chondrocyte growth factor [9,10]. Several studies have reported that b-FGF enhances cartilage regeneration, and suggested the possibility that a tracheal defect might be repaired using tracheal cartilage engineered by this technique [11–13]. We thought that rapid growth of tracheal cartilage induced by slowly released b-FGF might act as a novel treatment for tracheomalacia. We reported that slow-release b-FGF gelatin sheets placed outside of the trachea enlarged the tracheal lumen and thickness of the cartilage in rats [14]. Biodegradable hydrogel sheets should be inserted between the trachea and esophagus by an invasive surgical procedure. A less invasive surgical procedure is necessary for tracheomalacia patients. The aim of this study was to investigate whether injection of b-FGF solution from the inner side of the trachea promotes growth of the trachea.

1. Material 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 b-FGF solution

Trafermin (Fiblast Spray, Kaken Pharmaceutical Co. Ltd., Tokyo, Japan) is a commercially available human recombinant b-FGF. It has been authorized for use in patients by the Ministry of Health, Labor and Welfare in Japan. This solution was adjusted to a concentration of 0.2 μg/μl.
1.2. Preparation of slow-release forms of b-FGF

Gelatin microspheres were prepared by glutaraldehyde cross-linking as reported previously [15]. Briefly, gelatin with an isoelectric point of 4.9 was isolated from bovine bone collagen by an alkaline process (Nitta Gelatin Inc., Osaka, Japan). An aqueous solution of human recombinant b-FGF with an isoelectric point of 9.6 was obtained from Kaken Pharmaceutical Co. Ltd. (Tokyo, Japan). Ten microliters of a 1% aqueous solution of gelatin preheated to 40 °C was dribbled into 350 ml of olive oil with stirring at 420 rpm at 40 °C for 10 min. The emulsion was then cooled in crushed ice and stirred at 420 rpm for 30 min. Acetone (100 ml, 4 °C) was then added to the emulsion and stirring was continued for 60 min. The resulting microspheres were washed three times with acetone and recovered by centrifugation (5000 rpm, at 4 °C, 5 min). The microspheres were filtered through sieves with a pore size of 125 and 75 μm, and dried in air at 4 °C to obtain non-cross-link gelatin microspheres of 75–125 μm in diameter. The non-cross-linked gelatin microspheres (20 mg) were placed in 0.1% Tween 80 aqueous solution containing 0.13% glutaraldehyde, and cross-linking was allowed to proceed at 4 °C for 24 h. After collection by centrifugation (5000 rpm, 5 min), the microspheres were stirred in 20 ml of 100 mM glycine solution for 1 h. The resulting microspheres were washed with double distilled water, recovered by centrifugation (5000 rpm, 4 °C, 5 min) and freeze-dried. b-FGF was incorporated into the gelatin microspheres by dropping 1 mg/ml b-FGF solution (100 μl) on 10 mg of freeze-dried gelatin microspheres. The b-FGF-incorporated gelatin microspheres were suspended in saline (5 ml) before injection.

1.3. Tracheoscope and injection system

An ultra-thin endoscope (TELASA™, AVS Co. Ltd., Tokyo, Japan) was used as the tracheoscope in our study, according to a previous report [16]. This system consisted of a camera unit, handpiece and endoscopic fiber probe (Fig. 1a). The endoscopic fiber probe is 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 of 2 mm in diameter was used to protect the endoscopic fiber probe. Varixer (Top Co. Ltd., Tokyo, Japan) was used as the injection system. This needle was attached to the introducer outside of the endoscopic fiber probe by Steri-Strip (3M Co. Ltd., Tokyo, Japan) (Fig. 1b).

1.4. Surgical procedure

A total of 15 8-week-old, female New Zealand white rabbits were divided into 3 groups. The New Zealand white rabbits were anesthetized with propofol in a bolus of 20 mg/kg (Maruiishi Pharmaceutical Co. Ltd., Osaka, Japan) and halothane (Takeda Pharmaceutical Co. Ltd., Osaka, Japan). With the rabbit in a prone position, propofol was given as an intravenous drip at 60 mg/kg/h.

A tracheoscope was inserted into the trachea under deep sedation with spontaneous respiration. A needle was punctured into the membranous wall of trachea in the posterior tracheal wall, under visual observation. A 250 μl solution was injected into the submucosal space of the trachea two times, and submucosal elevation was confirmed under endoscopic view. Group 1 underwent injection of distilled water into the posterior tracheal wall, group 2 underwent injection of 100 μg b-FGF solution, and group 3 underwent injection of slow-release 100 μg b-FGF (n = 5 in each group).

1.5. Morphological and histological examinations

Four weeks after the surgical procedure, all rabbits were sacrificed for morphological and histological examinations. The outer diameter of the cervical trachea at the site of injection was measured using Super Caliper (Mitutoyo Co. Ltd., Kanagawa, Japan). The cervical tracheas were harvested and the specimens were embedded in Tissue-Tek OCT compound 4583 (Sakura Finetechnical Co. Ltd., Tokyo, Japan) and frozen. They were subsequently sliced into 7-μm sections and stained with hematoxylin and eosin (H & E), toluidine blue, and safranin O.

1.6. Measurement of luminal area

Images were obtained with a fluorescence microscope (BZ-9000, Keyence, Osaka, Japan). The luminal area of the cartilage was measured on cross-sections of each trachea using a commercially available image processing software (Medical Image Analyzer, Inotech Co. Ltd., Hiroshima, Japan).

1.7. Statistical analysis

Statistical analysis was performed by commercially available software IBM SPSS Statistics 21 (IBM, Armonk, NY) and JMP 9 (SAS Institute Inc. Cary, NC). Comparison among groups was performed by the Kruskal–Wallis test, and then non-parametric multiple comparison was performed by the Steel–Dwass test. A P value of < 0.05 was considered to be statistically significant. A P value of < 0.1 was considered to be marginally statistically significant.

2. Results

All rabbits in each group survived until the time of sacrifice and there was no change in breathing condition in any of the rabbits. Upon gross examination of the cervical tracheas, there was no difference in inflammation signs among the Distilled water group and the two b-FGF injection groups. In the Distilled water group, the
cervical trachea had a tapered shape from the thyroid cartilage to the carina tracheae (Fig. 2a). The cervical tracheas in the two b-FGF injection groups were spindle-shaped, and had a maximum diameter at the injection site (Fig. 2b and c). The height of each trachea cartilage ring in groups 2 and 3 with injected b-FGF, was more extensive than that in group 1. The trachea at the injection site of b-FGF was as stiff as it was in the non-injected parts, rather than stiffer, by palpation. The median outer diameter of the cervical trachea at the injection site in groups 1, 2 and 3 was 7.3, 8.0 and 8.0 mm, respectively, showing a significant difference among the three groups (P = 0.04; KW test) (Fig. 3). Marginally significant differences were observed between groups 1 and 2 (P = 0.09) and between groups 1 and 3 (P = 0.09) (Fig. 3).

Cross-sections of the tracheas in each group are shown in Fig. 4. The presence of cartilage was confirmed by H&E, toluidine blue, and safranin O staining. Upon histological examination of cross-sections of the tracheas, the thickest point of the trachea cartilage in groups 2 (Fig. 4b) and 3 (Fig. 4c) was thicker than that in group 1 (Fig. 4a).

The median luminal area in groups 1, 2 and 3 was 27.4, 29.4 and 32.1 mm², respectively (Fig. 5). A marginally significant difference was seen among groups 1, 2 and 3 (P = 0.06). A marginally significant difference was observed only between groups 1 and 3 (P = 0.056) using the Steel–Dwass test.

3. Discussion

The present results showed that 100 μg b-FGF incorporated in biodegradable hydrogel microspheres that were injected at the inner side of the trachea wall promoted growth of the trachea, albeit with only marginal statistical significance. The same volume of b-FGF solution injected as a single dose at the inner side of the trachea promoted growth of the trachea to an even lesser extent. This means that slowly released b-FGF in biodegradable gelatin hydrogel microspheres that were injected at the inner trachea had a greater effect on tracheal cartilage growth in the short term.

b-FGF is a chondrocyte growth factor [9,10]. Several studies have shown that slowly released b-FGF stimulated reproduction of tracheal cartilage [11–13]. There has been no study that aimed to promote growth of the tracheal cartilage using this agent. Recently, we reported that growth of the tracheal cartilage was promoted using b-FGF incorporated in biodegradable gelatin sheets that were placed between the esophagus and trachea by a surgical procedure [14]. In the present study, we achieved the same results of promoting growth of the trachea cartilage by injection of b-FGF incorporated in biodegradable gelatin hydrogel microspheres into the submucosal space of the trachea. The injected b-FGF migrated from the submucosal space to the perichondrium of trachea cartilage. Perichondrium is known to have chondrogenic potential [13] and to be a possible source of regenerative cartilage [13]. Cartilage tissue is an avascular tissue, and allows the exchange and transport of nutrients, gases, and metabolites by continuous diffusion instead of through the vasculature [17]. Therefore, we speculated that the enhanced growth of trachea cartilage observed in our study originated from perichondrium regeneration and chondrocyte proliferation in trachea cartilage induced by b-FGF.

In our study, group 3 which had been injected with the slow-release form of b-FGF, showed rapid growth of the trachea compared with group 2 which had been injected with simple b-FGF solution. The
reasons for the different result are that the half-life of b-FGF is very short [18–20] and b-FGF incorporated in gelatin hydrogel microspheres is released gradually for approximately 2 weeks as the gel degrades [21]. There was less enhancement of growth following injection of the simple b-FGF solution of the trachea than injection of the slow-release form of b-FGF at 4 weeks after administration. The current study only investigated short-term results. Growth enhancement by the slow-release form of b-FGF with statistical significance might have been observed with a longer observation period. Furthermore, we will investigate the optimal shot dose, frequency and interval for growth enhancement in future experiments.

We did not investigate the mechanical property of the trachea at the site of b-FGF injection. However, the respiratory condition of the rabbits did not change and there was no stridor at the expiratory phase. Also, the trachea at the injection site of b-FGF was as stiff as it was in the non-injected parts by palpation, rather than stiffer. Therefore, the trachea cartilage at the injected site was as stiff as normal trachea. The pathophysiologic mechanism in patients with tracheomalacia has not been investigated sufficiently. There are no data on the response of chondrocytes to b-FGF injection in patients with tracheomalacia. There is no tracheomalacia animal model. Therefore, we have to investigate whether b-FGF solution injection from the inner side of the trachea promotes growth of the trachea in tracheomalacia patients.

Patients with mild tracheomalacia do not require any specific treatment. The management of severe tracheomalacia is controversial. This is because tracheomalacia improves with growth and surgery may not completely abolish the cyanotic spells. Continuous positive airway pressure can be used to maintain a patent airway. However, this is not a good long-term solution. Tracheostomy is occasionally used for long-term airway support. However, the risk of iatrogenic problems is high. Surgical options for severe tracheomalacia include aortopexy, segmental trachea resection and external splinting of the trachea. However, the surgical procedures are invasive in patients with tracheomalacia with congenital malformation [22].

Our current study showed that injection of 100 μg of b-FGF to the submucosal space in the trachea increased the luminal area of the trachea. Therefore, administration of slowly released b-FGF to the tracheal cartilage from the submucosal space might make airway collapse more difficult and become an alternative to invasive surgical interventions for patients with severe tracheomalacia. Tracheomalacia is often detected in patients with esophageal atresia and in those who have undergone slide tracheoplasty [23–26]. Injection of slowly released b-FGF concurrently with diagnostic endoscopy for tracheomalacia may also improve symptoms of tracheomalacia. This procedure can be performed in an iterative fashion after checking the response to b-FGF injection.

Our results showed the feasibility of growth promotion of the trachea by injection of b-FGF from inside of the trachea using

---

**Fig. 4.** Histology of Cross-sections of the tracheas. The presence of trachea cartilage tissue was confirmed by H&E, toluidine blue, and safranin O staining. a. Group 1 underwent distilled water injection. b. Group 2 underwent injection of b-FGF solution. c. Group 3 underwent injection of slow-release 100 μg b-FGF. The thickest point in the trachea cartilage in groups 2 and 3 was thicker than that in group 1.

**Fig. 5.** Luminal area of the cervical trachea at the injection site. The median luminal area in groups 1, 2 and 3 was 27.4, 29.4 and 32.1 mm², respectively. A marginally significant difference was seen among groups 1, 2 and 3 (P = 0.06). The Steel–Dwass test showed a marginally significant difference only between groups 1 and 3 (P = 0.056).
bronchoscopically. Slowly released b-FGF is more effective in the short term in the growth phase. Our current procedure is a minimally invasive administration method to enhance growth of normal trachea.

Acknowledgments

This study was supported by grants from Kawano Masanori Memorial Foundation for Promotion of Pediatrics 2009 and the Ministry of Education, Culture, Sports, Science and Technology of Japan (No. 22591977). We appreciate the technical support provided by Nobuyuki Kikuchi, technician in the Department of Tissue Engineering in the Graduate School of Medicine, University of Tokyo.

References