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Regeneration of Aged Vocal Fold: First Human Case Treated With Fibroblast Growth Factor

Shigeru Hirano, MD, PhD; Yo Kishimoto, MD; Atsushi Suehiro, MD; Shin-ichi Kanemaru, MD, PhD; Juichi Ito, MD, PhD

Background/Objectives: Aged vocal folds are characterized by atrophy of the mucosa, which causes dysphonia and is difficult to treat. We have revealed a therapeutic potential of basic fibroblast growth factor (bFGF) for tissue regeneration of the aged vocal fold. We report here the first human case that has been treated with bFGF.

Study Design: Institutional review board approved clinical human trial.

Methods: A 63-year-old man with atrophied vocal folds was treated by local injection of 10 μg of bFGF into the left vocal fold under topical anesthesia. The effects of the injection were examined after 1 to 3 months by videostroboscopy, acoustic and aerodynamic measurements.

Results: The atrophy of the vocal fold was improved at 1 week after the injection and glottic gap disappeared. Aerodynamic and acoustic parameters also showed remarkable improvement. These positive effects were maintained up to 3 months.

Conclusion: The first case with aged vocal folds treated with bFGF administration is presented. The results are encouraging, suggesting therapeutic effects of bFGF for atrophied vocal folds in human.

Key Words: Aged vocal fold, basic fibroblast growth factor, regeneration.

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INTRODUCTION

Voice changes with age. It tends to become weak, harsh and breathy, especially in men.[1] This voice change is caused by the atrophy of the vocal fold which induces bowing, glottal insufficiency, and reduction of the mucosal traveling wave.[2,3] The atrophy occurs because of histological alterations of the lamina propria of the vocal fold, including excessive collagen deposition with formation of thick bundles, reduction of elastin and hyaluronic acid (HA).[4–7] It is required to address these histological changes to restore the aged vocal fold.

Fibroblasts in the lamina propria are the main provider of extracellular matrix (ECM). It has been revealed that fibroblasts decrease in number with age, and intracellular presence of golgi apparatus and rough endoplasmic reticulum also decrease, which suggests reduction of activity to synthesize ECM.[8,9] Therefore, it may be important to activate fibroblasts and control their function of ECM production to produce sufficient HA and suppress excessive collagen synthesis.

Basic fibroblast growth factor (bFGF) is a stimulant of growth of fibroblasts, and also induces modification of ECM production from the cells. In our previous in vitro study using aged rats has indicated that bFGF stimulates cell growth of fibroblasts in the aged vocal folds, and also induces a significant increase of HA production from those cells and suppresses production of collagen type I.[10] These effects seemed to be favorable for restoration of aged vocal fold lamina propria to younger state.

The effects of local administration of bFGF into aged vocal folds were also examined in our subsequent in vivo study using aged rats.[11] The results showed a recovery of HA content in the lamina propria of aged vocal folds in the bFGF treated group, which suggested a therapeutic potential of bFGF to restore an appropriate viscoelasticity of the vocal folds.

We then set up a protocol of use of bFGF for human cases with atrophied vocal folds caused by aging, which was approved by the institutional review board of Kyoto University. We report here the first human case treated by bFGF.

MATERIALS AND METHODS

Preparation of bFGF

Product of bFGF solution usable for human (Fibrast) was developed by Kaken Pharmaceutical (Tokyo, Japan) using artificial recombination of genes of human bFGF. Fibrast was

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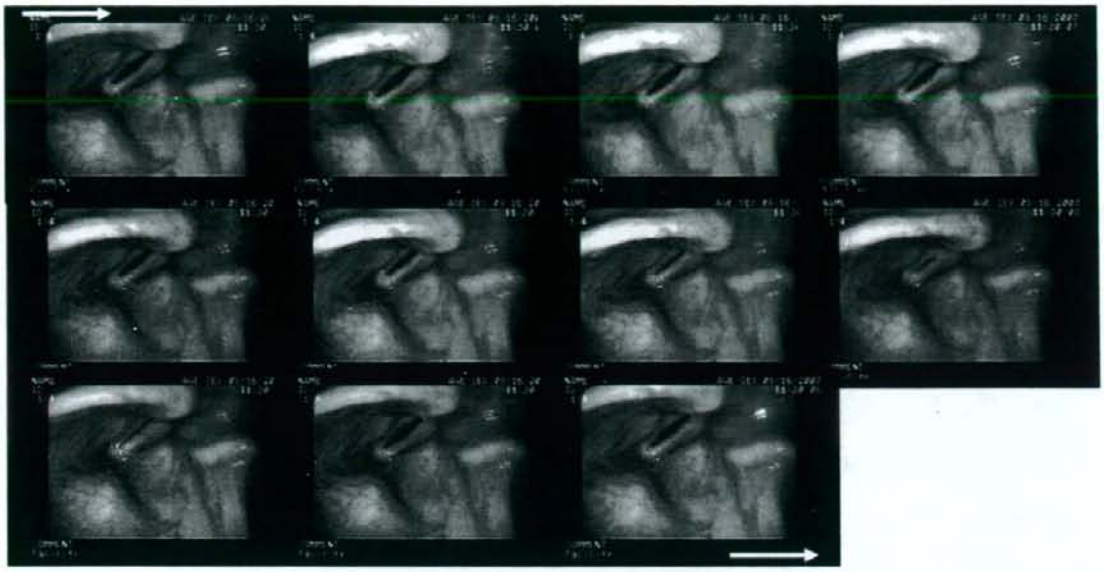

Fig. 1. Pretreatment videostroboscopic findings. Mucosal wave was reduced and glottic closure was incomplete.

approved for the treatment of skin ulcer in Japan and other limited countries, but not in the United States. No major safety issue has been reported so far. The only concern is that bFGF stimulates tumor growth if the patient has neoplasm on the applied site. Therefore, it is prohibited to use bFGF on the site of cancer.

Protocol

Unilateral transoral injection of bFGF (Fibrast, Kaken, Tokyo) is planned for patients, older than 50 years old, with vocal fold atrophy which causes hoarseness. Exclusion criteria include patients with organic or neurogenic voice disorders, a history of malignant tumors, hepatic diseases, kidney dysfunction, or other severe systemic disorders. Ten microgram of bFGF in 0.5 mL saline is prepared for local injection into the vocal fold. The dose was determined after one time dose of 10 to 20 μg used for skin. bFGF is injected into the superficial layer of the lamina propria under topical anesthesia using a curved injection needle. The injection is performed on one side because of safety issue. It is always required to minimize any unexpected adverse effects in a clinical trial.

The effects of the injection are assessed after 1 to 3 months by videostroboscopy, acoustic and aerodynamic examinations.

Patient

A 63-year-old man patient with aged vocal folds was recruited for the current trial. He had complained of a hoarse, breathy voice. Stroboscopic examination showed bilateral vocal fold atrophy with glottic insufficiency and reduction of mucosal wave amplitude (Fig. 1).

Ten microgram of bFGF in 0.5 mL saline was transorally injected into the left vocal fold under videolaryngoscopic monitoring after thorough topical anesthesia by 4% lidocaine spray (Fig. 2). The injection needle was placed at lateral portion of the vocal fold, and the bFGF solution was gradually infiltrated into the whole lamina propria.

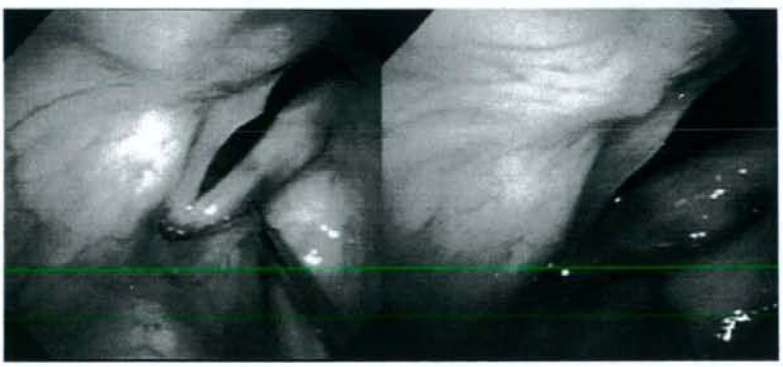

Fig. 2. Injection of bFGF solution under topical anesthesia. Left, injection needle was placed at lateral portion of the vocal fold. Right, Solution was injected inside the lamina propria of the fold.

Assessment

Stroboscopic examinations were performed using Digital Video Stroboscopy System Model 9295 (KayPentax, Lincoln Park, NJ) at 1 week, 1 month, and 3 months after injection to assess mucosal wave and glottic closure. The amplitude of vibration and glottal gap were examined using image analysis software (Scion Image beta3b, Frederick, MA). The distance (d1) from midline of the glottis to the free edge of the vocal fold was measured at

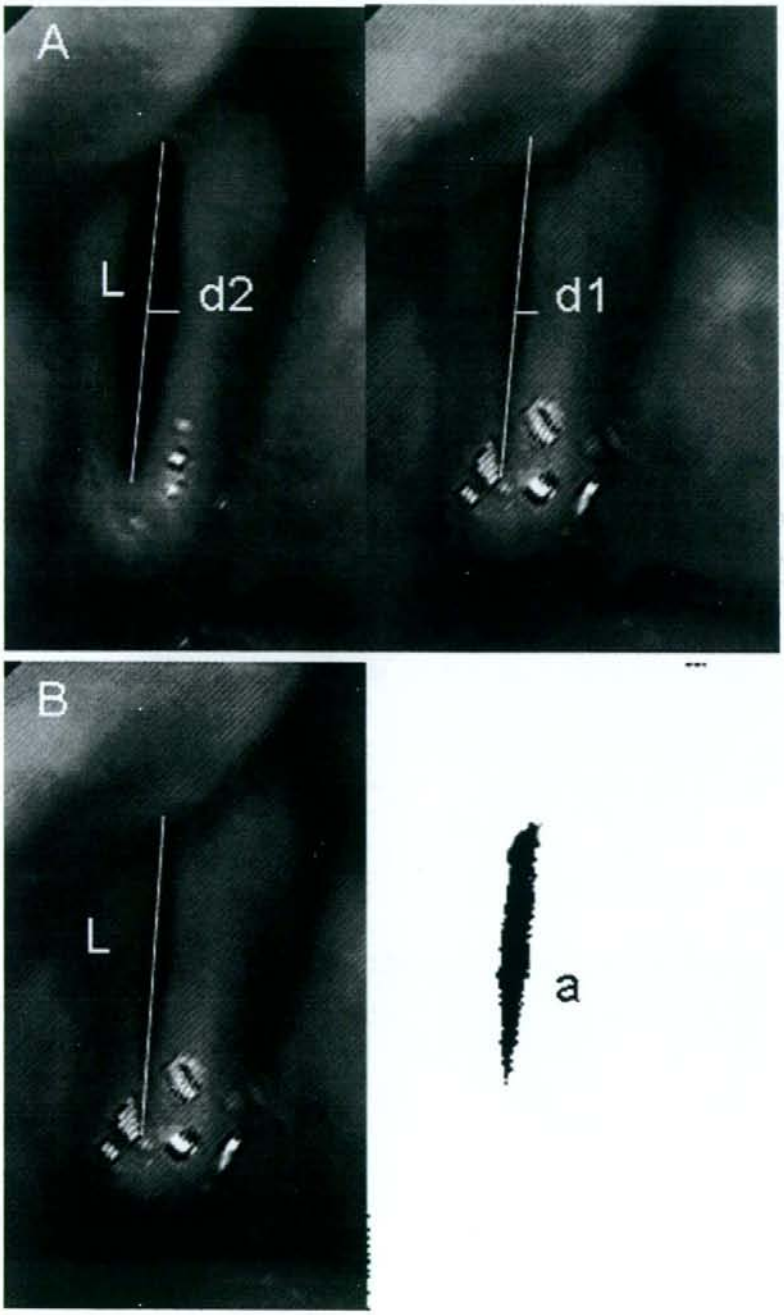

Fig. 3. Measurement of the amplitude of vibration and glottic gap. (A) Normalized mucosal wave amplitude (NMWA) = (d2 − d1)/L. (B) Normalized glottic gap (NGG) = a/L^2. (a: glottic area).

anteroposterior middle portion of the vocal fold at closed phase, and then the same distance (d2) was measured at maximum open phase (Fig. 3A). Mucosal wave amplitude was defined by subtracting d1 from d2, and normalized by dividing them by the length (L) from the anterior commissure to the vocal process: normalized mucosal wave amplitude = (d2 − d1)/L. This measurement was done on the left vocal fold (injected side). Glottal gap was examined on the images at closed phase. Glottal area (a) was measured and then normalized by dividing it by L^2 (Fig. 3B): normalized glottal gap = a/L^2.

Aerodynamic and acoustic measures were also completed at the same time points. Aerodynamic examinations included maximum phonation time and mean flow rate. Acoustic analyses evaluated amplitude perturbation quotient, pitch perturbation quotient, and noise-to-harmonic ratio.

RESULTS

Laryngoscopic examination found complete absorption of the injected solution in the vocal fold on the next day. Videostroboscopic examination at 1 week after the injection revealed an improvement of mucosal wave with complete glottic closure, and these positive effects were maintained up to 3 months (Fig. 4). Mucosal wave amplitude (normalized mucosal wave amplitude) and glottic closure (normalized glottal gap) also showed improvements from 1 week through 3 months after the administration of bFGF (Fig. 5).

The voice became stronger with less hoarseness. Maximum phonation time and mean flow rate improved at 1 week to 3 months. Acoustic parameters also showed improvements during the period from 1 week to 3 months (Fig. 6).

DISCUSSION

Vocal fold atrophy is characterized with alteration of histology of the lamina propria of the vocal fold mucosa. The superficial lamina propria is the most important portion for mucosal vibration, and is required to have an ideal viscoelasticity. With age, however, histological studies have revealed thinning of superficial lamina propria. Sato et al.[5,6] reported an increase of collagen, especially in men, forming thick bundles with high density, accompanied with reduction of elastin. A representative case indicated a massive collagen deposition in the lamina propria, showing no layer structure in the vocal fold mucosa. Excessive collagen deposition is thought to increase tissue strength,[12] which is not a favorable event for vibratory properties.

HA is one of the glycosaminoglycans distributed in the lamina propria. Several studies have indicated that HA is the key ECM molecule that maintains an optimal viscoelasticity of the vocal fold mucosa.[13,14] However,

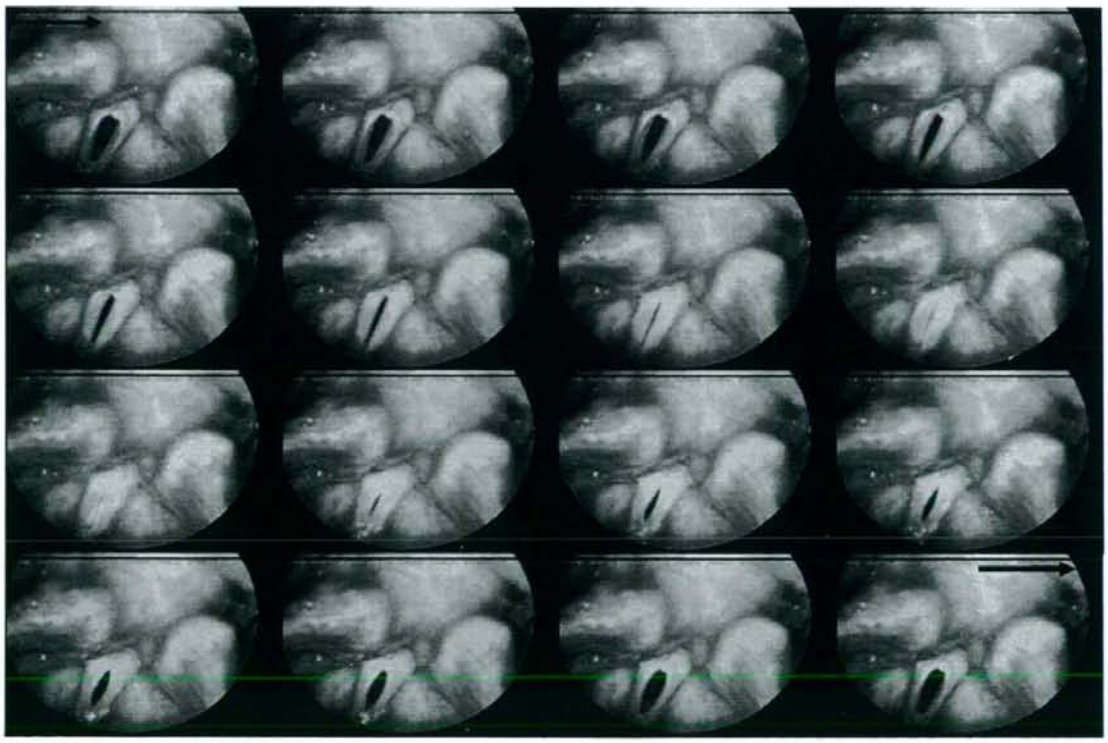

Fig. 4. Videostroboscopic findings at 3 months after bFGF injection. Mucosal wave improved with complete glottic closure.

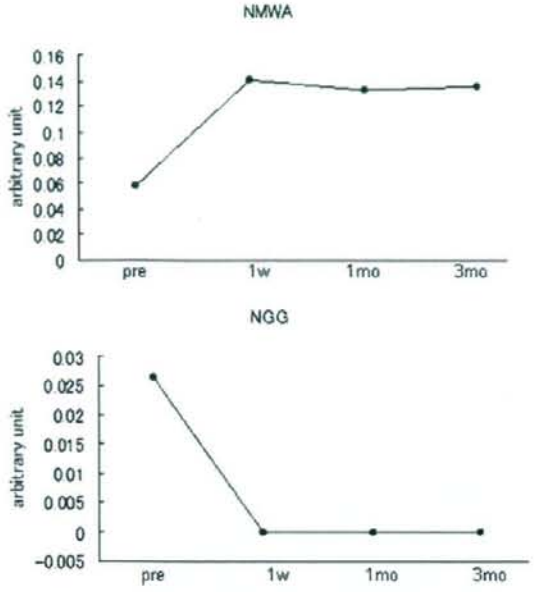

Fig. 5. Normalized mucosal wave amplitude (NMWA) and normalized glottic gap (NGG). Both values showed remarkable improvement after 1 week through 3 months after bFGF administration.

Butler et al.[7] reported a trend of decrease of HA in the whole lamina propria in human with age.

Above mentioned histological changes are attributed mainly to dysfunction of fibroblasts in the vocal fold with age. Sato et al.[9] demonstrated a decrease of number of the cells, decreases of intracellular organelle responsible for protein synthesis, and decreases of production of collagen or elastin from the cells, by histological examinations on aged human vocal folds. The macula flava is regarded as a "bank" of fibroblasts which is located at the anterior and posterior ends of the vocal ligament. These cells present with stellate shape and a high potential of ECM production. With age, however, Hirano et al.[8] showed drastic histological changes of the cells including changes of shape, poor development of intracellular organelle, increases of glycogen particles and lipofusion granules, suggesting a decrease in activities of protein production. Ding and Gray[15] examined gene expression of ECM in aged rat vocal fold tissues, and found decrease of gene expression of collagen type I, V, and proteinase, which suggested slow-down collagen turnover.

Based on the previously reported histological alterations of aged vocal folds, we have hypothesized that it should be essential and useful to increase HA deposition and decrease collagen to improve the tissue properties of aged vocal folds. We have also acclaimed that such regeneration of vocal fold atrophy requires tissue engineering strategy. Growth factor therapy is one of the most important elements in tissue engineering. Growth factors affect various cells and their function, and in that sense it is important to use them at appropriate places with appropriate dosage, timing, and route.

We have paid extensive attention on bFGF for the treatment of vocal fold atrophy. bFGF stimulates proliferation and migration of fibroblast and keratinocyte,[16] and thus has been used for reepithelialization of intractable skin ulcer. Hom et al.[17] indicated that exogenous bFGF also worked well for an improvement of wound healing of postirradiated skin in a pig study. As a clinical use in otolaryngology, Hakuba et al.[18] reported that topical administration of bFGF prompted regeneration of tympanic membrane in patients with a large perforation of the ear drum.

The effects of bFGF on ECM synthesis have also been well-described. Hong and Trackman[19] reported that bFGF reduced mRNA expression of collagen type I in gingival fibroblasts, while bFGF was reported to stimulate HA synthesis from skin fibroblasts.[20] We have also found that bFGF consistently stimulates ingrowths of fibroblasts of aged rat vocal folds, and that bFGF stimulates HA production and suppressed collagen type I production from the aged rat fibroblasts.[10] Based on these results, we were motivated to use bFGF for human patients with vocal fold atrophy caused by aging.

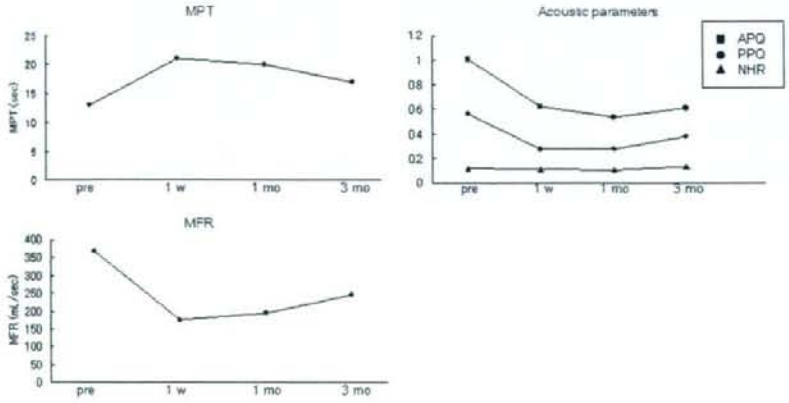

Fig. 6. Aerodynamic and acoustic measurements. MPT, MFR, PPQ, and APQ were improved at 1 week through 3 months after bFGF administration. MPT, maximum phonation time; MFR, mean flow rate; APQ, amplitude perturbation quotient; PPQ, pitch perturbation quotient; NHR, noise to harmonic ratio.

The present case study showed an improvement of tissue property of the aged human vocal fold after injection of bFGF in terms of mucosal vibration, glottic closure, aerodynamic and acoustic parameters. Such effects were already observed at 1 week after the injection. Because the injection material was solution, its volume inside the vocal fold disappeared on the next day, which means there were no effects of augmentation. Therefore biological activity of bFGF was thought to be so strong and rapid to improve the tissue properties of the vocal fold within a week, although it was impossible to confirm actual histological changes in the human study.

The present results have indicated that a single injection of bFGF has an ability to restore and maintain better mucosal vibration of aged vocal folds at least for 3 months, which seems to be quite useful for clinical treatment of human subjects. Growth factor therapy is considered as a "trigger" to jump start biological mechanisms,[21] which may be the reason why only a single injection of bFGF solution made such drastic effects as seen in the present study. Further studies are necessary to clarify how long the effects last and how consistently it works for different patients. It is at least suggested that the application of bFGF may be useful for regeneration of human aged vocal folds.

CONCLUSION

We report preliminary data of a human clinical trial of bFGF administration for an aged vocal fold. During a short-period observation, sufficient regenerative effects on the tissue properties of the treated vocal fold were noted without any adverse effect or toxicity. It is necessary to carry out further study with more cases and long-term follow up to confirm the regenerative effects of bFGF. It is at least suggested that local administration of bFGF may become a useful tool for the treatment of vocal fold atrophy.

BIBLIOGRAPHY

1. Ramig LO, Gray S, Baker K, et al. The aging voice: a review, treatment data and familial and genetic perspectives. *Folia Phoniatr Logop* 2001;53:252–265.
2. Woo P, Casper J, Colton R, Brewer D. Dysphonia in the aging: physiology versus disease. *Laryngoscope* 1992;102: 139–144.
3. Biever D, Bless D. Vibratory characteristics of the vocal folds in young adult and geriatric women. *J Voice* 1989;3: 120–131.
4. Hirano M, Kurita S, Sakaguchi S. Aging of the vibratory tissue of human vocal folds. *Acta Otolaryngol (Stockh)* 1989;107:428–433.
5. Sato K, Hirano M, Nakashima T. Age-related changes of collagenous fibers in the human vocal fold mucosa. *Ann Otol Rhinol Laryngol* 2002;111:15–20.
6. Sato K, Hirano M. Age-related changes of elastic fibers in the superficial layer of the lamina propria of vocal folds. *Ann Otol Rhinol Laryngol* 1997;106:44–48.
7. Butler JE, Hammond TH, Gray SD. Gender-related differences of hyaluronic acid distribution in the human vocal fold. *Laryngoscope* 2001;111:907–911.
8. Hirano M, Sato K, Nakashima T. Fibroblasts in geriatric vocal fold mucosa. *Acta Otolaryngol* 2000;120:336–340.
9. Sato K, Hirano M. Age-related changes of the macula flava of the human vocal fold. *Ann Otol Rhinol Laryngol* 1995;104: 839–844.
10. Hirano S, Bless DM, del Río A, Connor NP, Ford CN. Therapeutic potential of growth factors for aging voice. *Laryngoscope* 2004;114:2161–2167.
11. Hirano S, Nagai H, Tateya I, Tateya T, Ford CN, Bless DM. Regeneration of aged vocal folds with basic fibroblast growth factor in a rat model: a preliminary report. *Ann Otol Rhinol Laryngol* 2005;114:304–308.
12. Ehrlich HP. Collagen considerations in scarring and regenerative repair. In: Garg HG, Longaker MT, eds. *Scarless Wound Healing*. New York, NY: Marcel Dekker; 2000: 99–113.
13. Gray SD, Titze IR, Chan R, Hammond TH. Vocal fold proteoglycans and their influence on biomechanics. *Laryngoscope* 1999;109:845–854.
14. Chan RW, Titze IR. Hyaluronic acid (with fibronectin) as a bioimplant for the vocal fold mucosa. *Laryngoscope* 1999; 109:1142–1149.
15. Ding H, Gray SD. Senescent expression of genes coding collagens, collagen-degrading metalloproteinases, and tissue inhibitors of metalloproteinases in rat vocal folds: comparison with skin and lungs. *J Gerontol A Biol Sci Med Sci* 2001;56:B145–B152.
16. Rumalla VK, Borah GL. Cytokines, growth factors, and plastic surgery. *Plast Reconstr Surg* 2001;108:719–733.
17. Hom DB, Unger GM, Pernell KJ, Manivel JC. Improving surgical wound healing with basic fibroblast growth factor after radiation. *Laryngoscope* 2005;115:412–422.
18. Hakuba N, Taniguchi M, Shimizu Y, Sugimoto A, Shinomori Y, Gyo K. A new method for closing tympanic membrane perforations using basic fibroblast growth factor. *Laryngoscope* 2003;113:1352–1355.
19. Hong HH, Trackman PC. Cytokine regulation of gingival fibroblast lysyl oxidase, collagen, and elastin. *J Periodontol* 2002;73:145–152.
20. Heldin P, Laurent TC, Heldin CH. Effect of growth factors on hyaluronan synthesis in cultured human fibroblasts. *Biochem J* 1989;258:919–922.
21. Hom DB, Thatcher G, Tibesar R. Growth factor therapy to improve soft tissue healing. *Facial Plast Surg* 2002;18:41–52.

Replacement of the Left Main Bronchus With a Tissue-Engineered Prosthesis in a Canine Model

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Background. Stenosis of the left main bronchus caused by inflammatory diseases and neoplasms is a serious clinical problem because it can cause obstructive pneumonia and may require pneumonectomy. As an alternative to various treatments currently available, including balloon dilatation, stenting, and bronchoplasty, we propose the use of a prosthesis developed based on the concept of in situ tissue engineering for replacement of the left main bronchus.

Methods. The main frame of the tissue-engineered prosthesis is a polypropylene mesh tube, 12 to 15 mm in inner diameter and 30 mm in length, with reinforcing rings. Collagen extracted from porcine skin is conjugated to this frame. A consecutive series of 8 beagle dogs underwent replacement of the left main bronchus with this tissue-engineered prosthesis.

Results. All dogs survived the postoperative period with no morbidity except 1, which required intravenous administration of antibiotic for a week for pneumonia and recovered. Three dogs were euthanized for examination at 3 and 4 months after bronchus replacement, and the other five were monitored for more than 1 year. In two dogs, histologic examination revealed that the luminal surface was completely covered with ciliated columnar epithelium or nonciliated squamous epithelium. Exposure of the polypropylene mesh to various degrees was observed in 6 dogs, but the prosthesis remained stable and no adverse effects such as infection, sputum retention, or dehiscence were observed.

Conclusions. These long-term results suggest that our tissue-engineered prosthesis is applicable for replacement of the left main bronchus.

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Various therapeutic approaches for bronchial stenosis and obstruction have been reported, including bronchoscopic dilatation, laser ablation, airway stenting, and surgical bronchoplasty [1–3]. The objective of the present study was to test a possible alternative for the treatment of bronchial stenosis and obstruction. We have been developing a porous type of airway prosthesis conjugated with a collagen layer based on the concept of in situ tissue engineering. The intent of n situ tissue engineering is to create a local environment with a prepared scaffold suitable for tissue or organ restoration [4]. We have applied a collagen layer as a three-dimensional scaffold, which provides an appropriate environment into which surrounding host cells can migrate and proliferate, and applied it for regeneration of the trachea [5–8], esophagus [9, 10], stomach [11, 12], intestine [13], and peripheral nerves [14–16]. Collagen is a major component of the extracellular matrix and is known to promote cellular proliferation and tissue healing.

With this type of prosthesis, we have previously reported replacement of the intrathoracic trachea [5–7] and carinal part of the trachea [8] in animal experiments and the cervical trachea in humans [17, 18]. On the basis of these experiences, we designed a new prosthesis and adopted it for experimental replacement of the left main bronchus in dogs.

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Material and Methods

Bronchial Prosthesis

The collagen-conjugated bronchial prosthesis (20 mm long with an internal diameter of 12 to 15 mm) was made by a method similar to that described in our previous report concerning tracheal and carinal reconstruction [5]. The prosthesis consists of a fine polypropylene mesh cylinder with a pore size of 260 μm (Marlex mesh, CR Bard Inc, Billerica, MA), reinforced with 5 rings of polypropylene monofilament string (1 mm in diameter) wrapped around it. These rings were attached to the cylinder by thermal melt bonding to maintain the proper cylindrical form. This mesh cylindrical frame was exposed to a corona discharge at 9 kV for 5 minutes to make its surface hydrophilic.

The prosthesis was placed into a mold. A 2% collagen solution (supplied by Nippon Meat Packers Inc, Osaka, Japan) was poured into the mold, followed by freeze-drying, to form a collagen layer 5 mm thick around the

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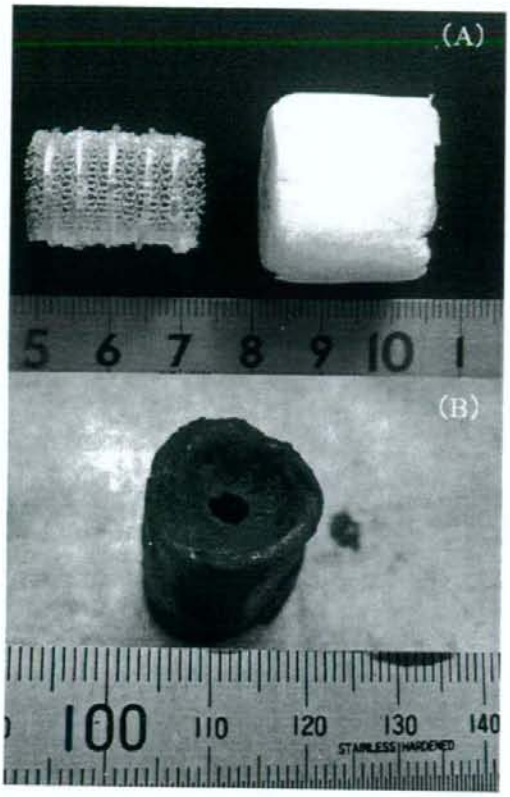

Fig 1. (A) The bulk structure of the prosthesis consists of a mesh cylinder reinforced with 5 rings of polypropylene string (left) and a covering of conjugated collagen (right). (B) This outer view shows the outer prosthesis conjugated with a collagen layer after it has been soaked with blood, just before replacement.

frame. In this process, the collagen became an amorphous layer with a pore size of 100 to 500 μm. The prosthesis was heated at 140°C under vacuum for a single or double 24-hour session of dehydrothermal treatment to induce moderate cross-linkage in the collagen molecules. Finally, the prosthesis was sterilized with ethylene oxide gas and stored dry until use. Figure 1A shows the polypropylene frame, and Figure 1B shows the collagen-conjugated prosthesis, which appears swollen after being soaked in blood.

Animal Experiment

Eight adult beagle dogs, weighing 8 kg to 15 kg, were anesthetized by the intramuscular administration of ketamine hydrochloride (15 mg/kg) and xylazine (7 mg/kg) and then intubated with an endotracheal tube. Respiration was maintained by a mechanical ventilator with halothane and nitrous oxide gas. A left thoracotomy was made through the 4th or 5th intercostal space according to the physique of each dog. The left main bronchus was exposed from the spur of the left upper bronchus to the carinal edge through the pulmonary aorta window. After the main bronchus had been transected at the spur of the left upper bronchus, the distal end was anastomosed with interrupted 3-0 Vicryl suture (Ethicon Inc, Somerville, NJ) to the prosthesis (Fig 2), which had been previously soaked with blood.

A 10-mm segment of the left main bronchus was resected and the carinal stump was anastomosed to the proximal end of the prosthesis. Both ends of the prosthesis were intended to be anastomosed in a telescopic manner, the bronchial end lying inside the prosthesis. A pedicled pericardial fat pad was fixed to the surrounding structure to cover the prosthesis.

The dogs were appropriately hydrated with extracellular fluid, and hydrocortisone (125 mg) was administered during the operation. Ampicillin was given intravenously at a dose of 1 g on the day of the operation and at 0.5 g daily for 3 days thereafter. Hydrocortisone (125 mg) was administered intravenously for 3 days after the procedure. All the animals received humane care in compliance with the *Guide for the Care and Use of Laboratory Animals* (Institute of Laboratory Animal Resources, Na-

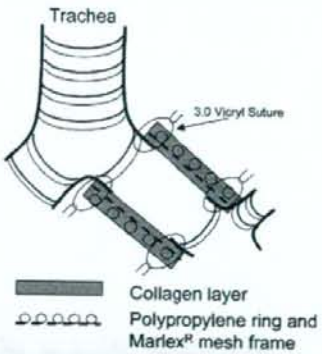

Fig 2. (Top) This drawing shows the schematics of the bronchial reconstruction. (Bottom) This photograph was taken during bronchial replacement with the artificial prosthesis (arrowhead). (Ao = aorta; PA = pulmonary artery.)

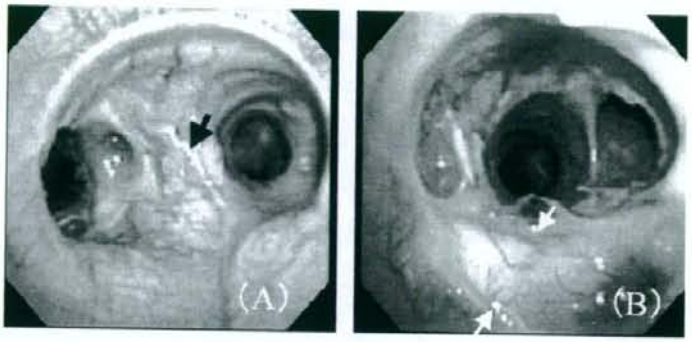

Fig 3. (A) Bronchoscopic views of dog 8 taken 18 months after tracheal replacement. Neither stenosis nor granulation is evident at the site of anastomosis. Black arrow shows the carina. The prosthesis was fully covered with regenerated tissue. (B) White arrows show the regenerated portion of the bronchus.

tional Research Councils, National Academy Press, revised 1996.)

Bronchoscopic Observation

Bronchoscopic observation was done periodically after induction of general anesthesia by an intramuscular injection of ketamine hydrochloride and xylazine hydrochloride. The luminal surface was observed with a bronchofiberscope (Model BF1T20, Olympus Optical Company, Ltd, Tokyo, Japan) to evaluate the coverage by host tissue and complications such as stenosis and dislocation of the prosthesis (Fig 3).

Evaluation of the Prosthesis

The replaced portion was examined bronchoscopically and macroscopically (Fig 4) after necropsy. Mesh exposure exceeding one-third of the circumference was defined as "exposed," and less than one-third as "spot exposed." Stenosis was defined as a reduction of more than one-third of the tracheal lumen cross-sectional area. Euthanasia was performed by intravenous injection of ketamine hydrochloride at a dose of 100 mg/kg, followed by necropsy.

Fig 4. Macroscopic view shows of the luminal surface of the prosthesis of dog 8 at 18 months after replacement. Arrows show the regenerated part. The luminal surface is covered with apparently normal mucosa, which is continuous with the native bronchus and appears whitish.

Histologic Examination

Immediately after macroscopic evaluation, specimens were placed in 10% formaldehyde solution, followed by paraffin embedding. Sections 5 μm thick were stained with hematoxylin and eosin for light microscopy. Another part of each fresh specimen was fixed with 2% glutaraldehyde and critical-point-dried by Au-Pd sputtering for scanning electron microscopic examination.

Results

The outcome of the experiments is reported in Table 1. All the dogs survived postoperative period without measurable morbidity, but severe pneumonia developed in 1 dog. Bronchoscopic and radiologic findings showed that this pneumonia was caused by postoperative atelectasis. This dog (No. 1 Table 1) recovered after the intravenous administration of antibiotic for a week and showed no dehiscence of anastomosis, or infection to the prosthesis during postoperative period.

Table 1. Results of Replacement of the Left Main Bronchus

Dog	Prosthesis Inner Diameter, mm	Dehydrothermal Treatment Time, hours	Stenosis	Mesh Exposure	Remarks
1	12	48	(−)	Spot	Sacrificed, 4 mon
2	12	48	(+)	(+)	Sacrificed, 3 mon
3	13	48	(+)	Spot	Sacrificed, 18 mon
4	15	48	(−)	(+)	Sacrificed, 4 mon
5	15	48	(−)	(+)	Sacrificed, 18 mon
6	15	24	(−)	(−)	Sacrificed, 18 mon
7	15	24	(−)	(−)	Sacrificed, 18 mon
8	15	24	(−)	(+)	Alive after 18 mon

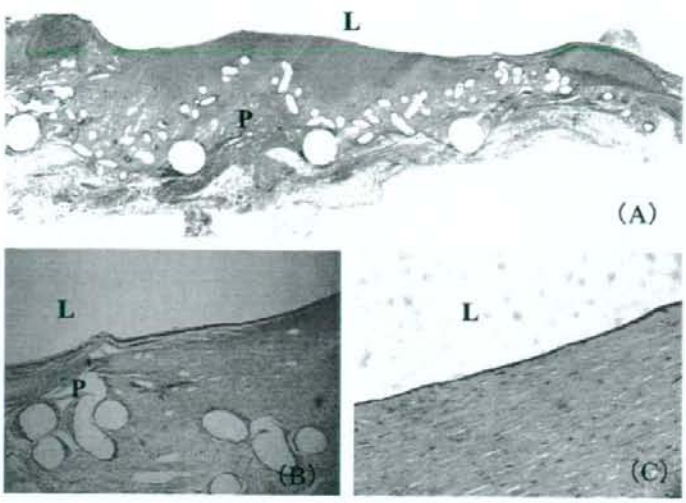

Fig 5. Microscopic examination of a longitudinal section of the regenerated tissue 18 months after replacement (hematoxylin and eosin staining). (A) Regenerated neomucosa with epithelial lining is observed on the prosthesis. (B) Ciliated epithelial cells have grown over the prosthesis showing continuity with the native bronchial epithelia, whereas (C) the center of the prosthesis is covered with a monolayer of squamous epithelium. (L = bronchial lumen, P = polypropylene mesh.) (Original magnification: ×10 for A, ×40 for B, ×100 for C.)

Bronchoscopic and Macroscopic Examination

Stenosis and excessive granulation were observed in dogs 2 and 3. One possible reason for the stenosis might have been mismatching of the prosthesis size in these 2 dogs, the prosthesis diameters being 12 and 13 mm, respectively, which were considered too small for the purpose. No stenosis or excessive granulation developed in the other 5 dogs, whose left main bronchi were replaced with a 15-mm-diameter prosthesis. Thus, the stenosis might have been caused by the small size of the prosthesis.

The evaluation for mesh exposure showed 4 dogs were classified as "exposed," 2 as "spot exposed," and 2 as fully covered. Dogs 2 and 3, which developed excessive granulation and stenosis, also showed concomitant mesh exposure.

Because mesh exposure was prominent in the first 4 dogs in this series, we reduced the dehydrothermal treatment time to 24 hours, with the expectation that this would enhance cell immigration to the prosthesis wall in the last 3 dogs. Complete epithelization was achieved in 2 dogs of this group, but the epithelization remained partial in 1 dog.

The host tissue incorporated into the framework of the prosthesis, and neither erosion nor nonfavorable interaction was observed between the framework and the adjacent vessels. No complications such as hemoptysis or intrathoracic hemorrhage were observed.

Histologic Findings

Ciliated columnar epithelium was microscopically observed near the anastomosis, but the proportion of squamous epithelium became larger than that of ciliated columnar epithelium with increasing distance; near the center of the prosthesis, only squamous epithelium was recognized (Fig 5, Fig 6). No immigration of inflammatory cells under the regenerated epithelium was evident, indicating that the prosthesis was well incorporated without an excessive foreign body reaction, which may lead to formation of granulation tissue and stenosis.

Comment

Reconstruction of the airway with an artificial prosthesis has been studied for decades. However, the degree of success has been limited because of problems such as local infection, stenosis, or rupture of the adjacent vessels [19–21]. The risk of such fatal complications currently precludes the clinical use of artificial prostheses in this situation.

Most recently, tissue-engineering techniques have been applied to regenerate a biologic prosthesis for implantation. Using ex vivo tissue engineering techniques, Kojima and colleagues [22] successfully created a tissue-engineered trachea with regenerated cartilage from bone marrow cells. However, the transplantation of such ex vivo tissue-engineered tissues has been associated with poor functional outcome [23, 24], and so their application is still problematic.

Another biologic approach for creation of prostheses has been reported by Martinod and colleagues [25]. They applied allogenic aorta for tracheal replacement, and the transplant was proven to be functional after removal of the inner stent [25, 26]. It is noteworthy that the allotransplant was replaced by a completely regenerated trachea provided with cartilage rings derived from the recipient. There is a similarity between their approach and ours, because the basic idea of in situ tissue engineering is to produce an environment suitable for host tissue regeneration at the required site.

The aortic graft played the role of a biologic incubator to allow host cells to migrate into it and form the regenerated trachea. One dissimilarity was that we used a polypropylene permanent framework as a substitute

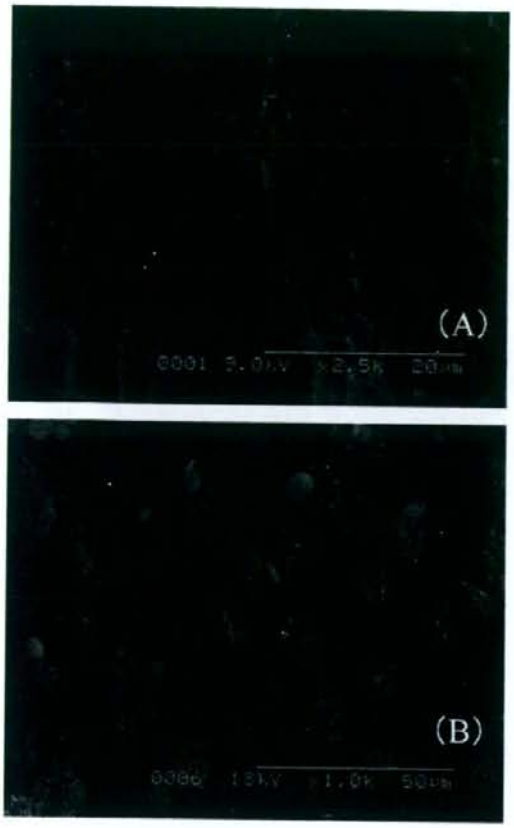

Fig 6. Scanning electron microscopy findings of the prosthesis 18 months after replacement. (A) The proximal portion of the anastomosis is covered with ciliated epithelial cells (×2500; bar, 20 μm), whereas (B) fewer such cells are observed in the middle of the prosthesis (×1000; bar, 50 μm).

for cartilage, whereas they applied an internal stent temporarily. Wurts and colleagues [27] have reported clinical application of this type of approach, and we await their long-term results, which may indicate whether cartilage would be generated in adult humans.

The requirements for an ideal prosthesis are (1) adequate durability that can be maintained long enough to restore the functional airway, (2) high biocompatibility, enabling incorporation into the host tissue, and (3) relatively low cost. We have been investigating a tissue-engineered collagen-conjugated prosthesis that potentially meets these requirements. Okumura and colleagues [5] designed a tracheal prosthesis consisting of a rigid polypropylene frame and a collagen monolayer. Animal experiments showed that this type of prosthesis has high biocompatibility with host tissue and overcomes serious major complications such as anastomotic leakage or postanastomotic stenosis. [7] With regard to mechanical properties, this type of tissue-engineered tracheal prosthesis has appropriate mechanical properties similar to those of the native trachea [28], and its feasibility has been proven by observation for more than 5 years in a canine tracheal replacement model [29]. For the cervical trachea, Omori and colleagues [17, 18; unpublished data] have applied this tissue-engineered tracheal prosthesis for 8 patients to date, with a maximum follow-up period of 4 years.

In terms of biocompatibility, we have developed a prosthesis conjugated with a thick collagen layer after a trial with a collagen monolayer type. Using this type of prosthesis, Teramachi and colleagues [6, 7] have achieved intrathoracic tracheal replacement, and Sekine and colleagues [8] have reported that a Y-shaped prosthesis for carinal reconstruction in a canine model showed good incorporation into the host tissue [6]. We consider that the collagen layer, which has a three-dimensional structure and provides an appropriate extracellular matrix in which migrating cells can proliferate, potentially enhances tissue regeneration.

As for affordability, this type of prosthesis is suitable for commercial production in various sizes and lengths at a low cost compared with biologic transplants and is free from any risk of donor infection. We used ethylene oxide gas for sterilization, because gamma-ray irradiation may have a degradative effect on collagen fibers. Gorham and colleagues [30] reported that neither method of sterilization of collagen-based wound repair materials produced any cytotoxic effect, although gamma-ray sterilization, which is more convenient, did lead to accelerated absorption.

On the basis of our previous experiences, we designed a prosthesis for replacement of smaller portions of the airway and applied it for repair of left main bronchial defects in a canine model. The aim of the present study was to clarify whether our previously developed tissue-engineered prosthesis would be applicable for smaller airway defects and to determine the optimum conditions for its use.

Severe stenosis developed in 2 of 3 dogs in which the left main bronchus was replaced with a 12-mm-diameter prosthesis. In contrast, no stenosis developed in the 5 dogs in which the bronchus was replaced with a 15-mm prosthesis. These results show that the inner diameter of the prosthesis should be 15 mm for replacement of the canine left main bronchus, which has a caliber of 10 to 12 mm, as measured at the site during the operation.

For the preparation of the collagen solution and the conditions for cross-linkage of the collagen molecules, we started with 1% collagen solution and 24 hours of dehydrothermal treatment. However, in the 2 dogs that received this type of prosthesis, the disruption of the prosthesis wall caused tension pneumothorax that proved to be too fragile for sealing the peripheral airway without an inner lining. These initial 2 dogs that were fitted with the 1% collagen prosthesis were therefore eliminated from the study. A prosthesis made with 2% collagen and cross-linked for 48 hours was applied in consecutive experiments, and the wall of this prosthesis

proved sufficiently durable to withstand the airway pressure. However, mesh exposure was prominent with this type of prosthesis, and epithelization of the lumen was poor compared with our previous study, in which 90% of dogs showed no mesh exposure.

Unlike our previous procedures for replacement of the intrathoracic trachea and carina [6, 8], we did not insert a silicone tube inside the prosthesis for extraction 8 weeks after implantation. This silicone tube had played a role in protecting the collagen layer from early degradation before tissue formation. However, because the canine left bronchus was considered too small for application of a silicone tube, we intended to try a simpler procedure. To improve epithelization, we reduced the dehydrothermal treatment time. Dehydrothermal treatment for 48 hours gives the collagen layer mechanical strength but may denature the collagen and thus decrease its favorable characteristics. Although we have not performed a sufficient number of trials to obtain precise data, it appears that collagen loses its ability to promote cell proliferation after 48 hours of dehydrothermal treatment because of excessive cross-linkage between the collagen molecules. Prostheses coated with 2% collagen that had been exposed to dehydrothermal treatment for 24 hours were transplanted with the expectation that this reduction in dehydrothermal treatment time would improve tissue regeneration. Two of the three dogs showed complete epithelization and no mesh exposure, and none showed dehiscence, disruption of the prosthesis wall, or stenosis. We therefore concluded that coating with 2% collagen solution and 24 hours of dehydrothermal treatment are the optimum conditions for preparation of the prosthesis.

In this canine model using the left main bronchus, the time for tissue regeneration was estimated to be 3 to 4 weeks by bronchoscopic observation. In patients where the cervical trachea was reconstructed, tissue regeneration was considered to take rather longer; Omori and colleagues [18] reported that this period ranged from 2 to 11 months in patients undergoing cervical tracheal replacement. Because no infection or dehiscence before complete epithelization occurred in these patients, mesh exposure might not be a fatal problem. However, to accomplish better epithelization, we are now investigating an improved prosthesis in which the luminal surface is lined with biodegradable polymer. In this new trial, we are observing better epithelization, with 90% of candidates accomplishing complete epithelization, although the observation period is not yet long enough.

We designed a new prosthesis for peripheral airway reconstruction. In a canine left main bronchus replacement model, this collagen-conjugated polypropylene prosthesis showed sufficient mechanical strength to support the airway without causing stenosis, and good biocompatibility. Although further assessment is essential before clinical application, our designed prosthesis may be a promising alternative for the treatment of left main bronchial stenosis and obstruction.

References

1. Kato R, Kakizaki T, Hangai N, et al. Bronchoplastic procedures for tuberculous bronchial stenosis. J Thorac Cardiovasc Surg 1993;106:1118–21.
2. Iwamoto Y, Miyazawa T, Kurimoto N, et al. Interventional bronchoscopy in the management of airway stenosis due to tracheobronchial tuberculosis. Chest 2004;126:1344–52.
3. Seijo LM, Sterman DH. Interventional pulmonology. N Engl J Med 2001;344:740–9.
4. Ikada Y. In situ tissue engineering: tissue engineering /fundamentals and applications. Challenges in tissue engineering. Burlington, MA: Academic Press (Elsevier); 2006:428.
5. Okumura N, Nakamura T, Natsume T, Tomihata K, Ikada Y, Shimizu Y. Experimental study on a new tracheal prosthesis made from collagen-conjugated mesh. J Thorac Cardiovasc Surg 1994;108:337–45.
6. Teramachi M, Nakamura T, Yamamoto Y, Kiyotani T, Takimoto Y, Shimizu Y. Porous-type tracheal prosthesis sealed with collagen sponge. Ann Thorac Surg 1997;64:965–9.
7. Teramachi M, Okumura N, Nakamura T, et al. Intrathoracic tracheal reconstruction with a collagen-conjugated prosthesis: evaluation of the efficacy of omental wrapping. J Thorac Cardiovasc Surg 1997;113:701–11.
8. Sekine T, Nakamura T, Matsumoto K, Ueda H, Matsumoto K, Shimizu Y. Carinal reconstruction with a Y-shaped collagen-conjugated prosthesis. J Thorac Cardiovasc Surg 2000; 119:1162–8.
9. Takimoto Y, Nakamura T, Yamamoto Y, Kiyotani T, Teramachi M, Shimizu Y. The experimental replacement of a cervical esophageal segment with an artificial prosthesis with the use of collagen matrix and a silicone stent. J Thorac Cardiovasc Surg 1998;116:98–106.
10. Yamamoto Y, Nakamura T, Shimizu Y, et al. Intrathoracic esophageal replacement in the dog with the use of an artificial esophagus composed of a collagen sponge with a double-layered silicone tube. J Thorac Cardiovasc Surg 1999;118:276–86.
11. Hori Y, Nakamura T, Matsumoto K, Kurokawa Y, Satomi S, Shimizu Y. Experimental study on in situ tissue engineering of the stomach by an acellular collagen sponge scaffold graft. Asaio J 2001;47:206–10.
12. Hori Y, Nakamura T, Kimura D, et al. Functional analysis of the tissue-engineered stomach wall. Artif Organs 2002;26:868–72.
13. Hori Y, Nakamura T, Kimura D, et al. Experimental study on tissue engineering of the small intestine by mesenchymal stem cell seeding. J Surg Res 2002;102:156–60.
14. Nakamura T, Inada Y, Fukuda S, et al. Experimental study on the regeneration of peripheral nerve gaps through a polyglycolic acid-collagen (PGA-collagen) tube. Brain Res 2004;1027:18–29.
15. Inada Y, Morimoto S, Moroi K, Endo K, Nakamura T. Surgical relief of causalgia with an artificial nerve guide tube: Successful surgical treatment of causalgia (Complex Regional Pain Syndrome Type II) by in situ tissue engineering with a polyglycolic acid-collagen tube. Pain 2005;117:251–8.
16. Yoshitani M, Fukuda S, Itoi S, et al. Experimental repair of phrenic nerve using a polyglycolic acid and collagen tube. J Thorac Cardiovasc Surg 2007;133:726–32.
17. Omori K, Nakamura T, Kanemaru S, et al. Regenerative medicine of the trachea: the first human case. Ann Otol Rhinol Laryngol 2005;114:429–33.
18. Omori K, Suzuki T, Nomoto Y, et al. Clinical Application of In situ Engineerin Using a Scaffolding Technique for Reconstruction of THe Larinx and Trachea. Annals of Otology, Rhinology & Laryngology (in press) 2008.
19. Neville WE, Bolanowski JP, Kotia GG. Clinical experience with the silicone tracheal prosthesis. J Thorac Cardiovasc Surg 1990;99:604–12;discussion 612–3.
20. Toomes H, Mickisch G, Vogt-Moykopf I. Experiences with prosthetic reconstruction of the trachea and bifurcation. Thorax 1985;40:32–7.

21. Deslauriers J, Ginsberg RJ, Nelems JM, Pearson FG. Innominate artery rupture. A major complication of tracheal surgery. Ann Thorac Surg 1975;20:671–7.
22. Kojima K, Ignotz RA, Kushibiki T, Tinsley KW, Tabata Y, Vacanti CA. Tissue-engineered trachea from sheep marrow stromal cells with transforming growth factor beta2 released from biodegradable microspheres in a nude rat recipient. J Thorac Cardiovasc Surg 2004;128:147–53.
23. Kojima K, Bonassar LJ, Roy AK, Vacanti CA, Cortiella J. Autologous tissue-engineered trachea with sheep nasal chondrocytes. J Thorac Cardiovasc Surg 2002;123:1177–84.
24. Grillo HC. Tracheal replacement. J Thorac Cardiovasc Surg 2003;125:975.
25. Martinod E, Seguin A, Holder-Espinasse M, et al. Tracheal regeneration following tracheal replacement with an allogenic aorta. Ann Thorac Surg 2005;79:942–8; discussion 949.
26. Seguin A, Martinod E, Kambouchner M, et al. Carinal replacement with an aortic allograft. Ann Thorac Surg 2006; 81:1068–74.
27. Wurtz A, Porte H, Conti M, et al. Tracheal replacement with aortic allografts. N Engl J Med 2006;355:1938–40.
28. Kawaguchi S, Nakamura T, Shimizu Y, et al. Mechanical properties of artificial tracheas composed of a mesh cylinder and a spiral stent. Biomaterials 2001;22: 3085–90.
29. Nakamura T, Teramachi M, Sekine T, et al. Artificial trachea and long term follow-up in carinal reconstruction in dogs. Int J Artif Organs 2000;23:718–24.
30. Gorham SS, S. French DA, Scott R. The effect of gamma-ray sterilization and ethylene oxide sterilization on collagen-based wound-repair materials. Journal of Materials Sciences: Materials in Medicine 1993;4:40–49.

Notice From the American Board of Thoracic Surgery Regarding Trainees and Candidates for Certification Who Are Called to Military Service Related to the War on Terrorism

The Board appreciates the concern of those who have received emergency calls to military service. They may be assured that the Board will exercise the same sympathetic consideration as was given to candidates in recognition of their special contributions to their country during the Vietnam conflict and the Persian Gulf conflict with regard to applications, examinations, and interruption of training.

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Richard H. Feins, MD
Chair
The American Board of Thoracic Surgery

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Endoscopic KTP Laser Photocoagulation Therapy for Pharyngolaryngeal Venous Malformations in Adults

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Objectives: Venous malformations are benign lesions with thin, fragile mucosa overlying a vascular stroma. Vascular anomalies often manifest as subglottic lesions in infants, but venous malformations in adults are rare in the pharyngolaryngeal region. The treatments include open and endoscopic surgery; intraoperative bleeding is often troublesome. Angiolytic lasers such as the potassium titanyl phosphate (KTP) laser enable photocoagulation for such hemorrhagic lesions without bleeding; we report findings from a series of adult patients.

Methods: Seven adults were treated with a KTP laser set at a low power of 1.5 W in continuous mode. Photocoagulation was easily performed for shallow lesions; however, laser irradiation of bulky venous malformations resulted only in surface photocoagulation. In such cases, the crust remaining after photocoagulation was removed, and laser energy was repeatedly delivered until no remnant lesion was seen. An office procedure using flexible endoscopy was performed under topical anesthesia for 1 patient with a limited lesion.

Results: The lesions were well controlled in all cases without major complications. A patient with a large obstructing lesion had a relapse. Because the recurrent lesion is small and the patient does not desire additional treatment at this time, she is being observed carefully.

Conclusions: Photocoagulation using the KTP laser is a feasible and relatively safe treatment for pharyngolaryngeal venous malformations in adults.

Key Words: adult pharyngolaryngeal venous malformation, KTP laser, photocoagulation therapy, potassium titanyl phosphate laser.

INTRODUCTION

Vascular anomalies are common in the head and neck region. They usually manifest as subglottic lesions in infants, whereas venous malformations in adults are rare in the pharyngolaryngeal region. Most venous malformations in adults do not progress, and small malformations can be observed without the need for aggressive treatments. Aggressive treatments are needed in case of bleeding, airway obstruction, or involvement of the digestive tract.

Many types of treatments have been reported, including systemic or local steroids, radiotherapy, and surgery.[1] Although all therapies have had some success, in the end, only surgery has become popularly used because of its certainty and relative safety.

The surgical treatments include both open and endoscopic surgery. In open surgery, lesions are accessed through pharyngotomy or laryngofissure. Considering intraoperative and postoperative morbidity, this approach is recommended only for extended pharyngolaryngeal venous malformations. On the other hand, endoscopic surgery is the more popular and preferred method because of its minimal invasiveness. However, because of the limited surgical field, intraoperative bleeding is often troublesome.

Recent advances in technology have allowed the use of lasers as an option in endoscopic surgery, and various lasers have been reported as part of the treatment of pharyngolaryngeal venous malformations. The potassium titanyl phosphate (KTP) laser has recently attracted the most attention because of its coagulation ability.

Since 1992, we have treated adults with pharyngolaryngeal venous malformations by use of photocoagulation therapy with the KTP laser. The KTP laser is preferentially absorbed by hemoglobin and enables photocoagulation for such hemorrhagic le-

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PATIENT DATA

Sex	Age (y)	Site	Initial Symptom	Anesthesia	Inpatient or Outpatient	Blood Loss	Duration of Procedure (min)	Preoperative Tracheotomy	Remnant or Relapse	Follow-Up Period (mo)
F	27	FVF, PWPG, PS	Discomfort	General	Inpatient	Little	100	+	–	3
F	45	Arytenoid	Asymptomatic	General	Inpatient	Little	145	+	–	12
M	46	Arytenoid, PS	Asymptomatic	Topical	Outpatient	Little	10	–	–	15
M	55	Arytenoid, PS	Discomfort	General	Inpatient	Little	22	–	–	12
M	71	PS, aryepiglottic fold	Asymptomatic	General	Inpatient	Little	44	–	–	12
F	77	Arytenoids, PS	Discomfort	General	Inpatient	Little	30	–	+	4
F	55	PS	Discomfort	General	Inpatient	Little	5	–	–	6

FVF = false vocal fold; PWPG = posterior wall of posterior glottis; PS = pyriform sinus.

sions without bleeding. Here, we report the findings for a series of 7 adults with pharyngolaryngeal venous malformations treated with photocoagulation therapy using the KTP laser. We examine the method's usefulness and safety and discuss how the laser should be used in the management of pharyngolaryngeal venous malformations.

MATERIALS AND METHODS

Patients. Clinical information for all 7 patients is summarized in the Table. The 7 adults (3 men, 4 women) with pharyngolaryngeal venous malformations were treated with endoscopic surgery using the KTP laser in 1992 to 2007 at Kyoto Medical Center and Kyoto University Hospital. Their ages ranged from 27 to 77 years (average, 53.7 years). The patients, except for case 3, were observed as inpatients, and the postoperative follow-up ranged from 3 to 15 months. The lesion involved the pyriform sinus in 6 of the 7 patients and the arytenoid cartilages in 4 of the 7 patients. Three of the 7 patients were asymptomatic; however, photocoagulation was performed to prevent possible bleeding.

Surgical Procedure. In 6 of the 7 patients, photocoagulation of the pharyngolaryngeal lesions was performed under general anesthesia. Tracheotomy was performed before surgery in 2 patients (cases 1 and 2) to prevent respiratory trouble due to postoperative edema. The glottis was exposed with a direct laryngoscope: either a Zeitels Universal Modular Glottiscope (Endocraft Co, Boston, Massachusetts) or a Kleinsasser laryngoscope (Karl Storz GmbH and Co, Tuttlingen, Germany). Pharyngolaryngeal lesions were photocoagulated with a KTP laser (Laserscope, San Jose, California) set at low power (1.5 W) with a defocused beam in continuous mode. During photocoagulation, the laser was delivered to the lesion through a flexible optical fiber attached to a curved handpiece with a suction channel for evacuating smoke. The beam was delivered from just above the surface of the lesion, and the tip of the fiber never contacted the lesion. For 1 patient (case 3) with a limited lesion, the procedure was performed via flexible endoscopy under topical anesthesia in an office setting.

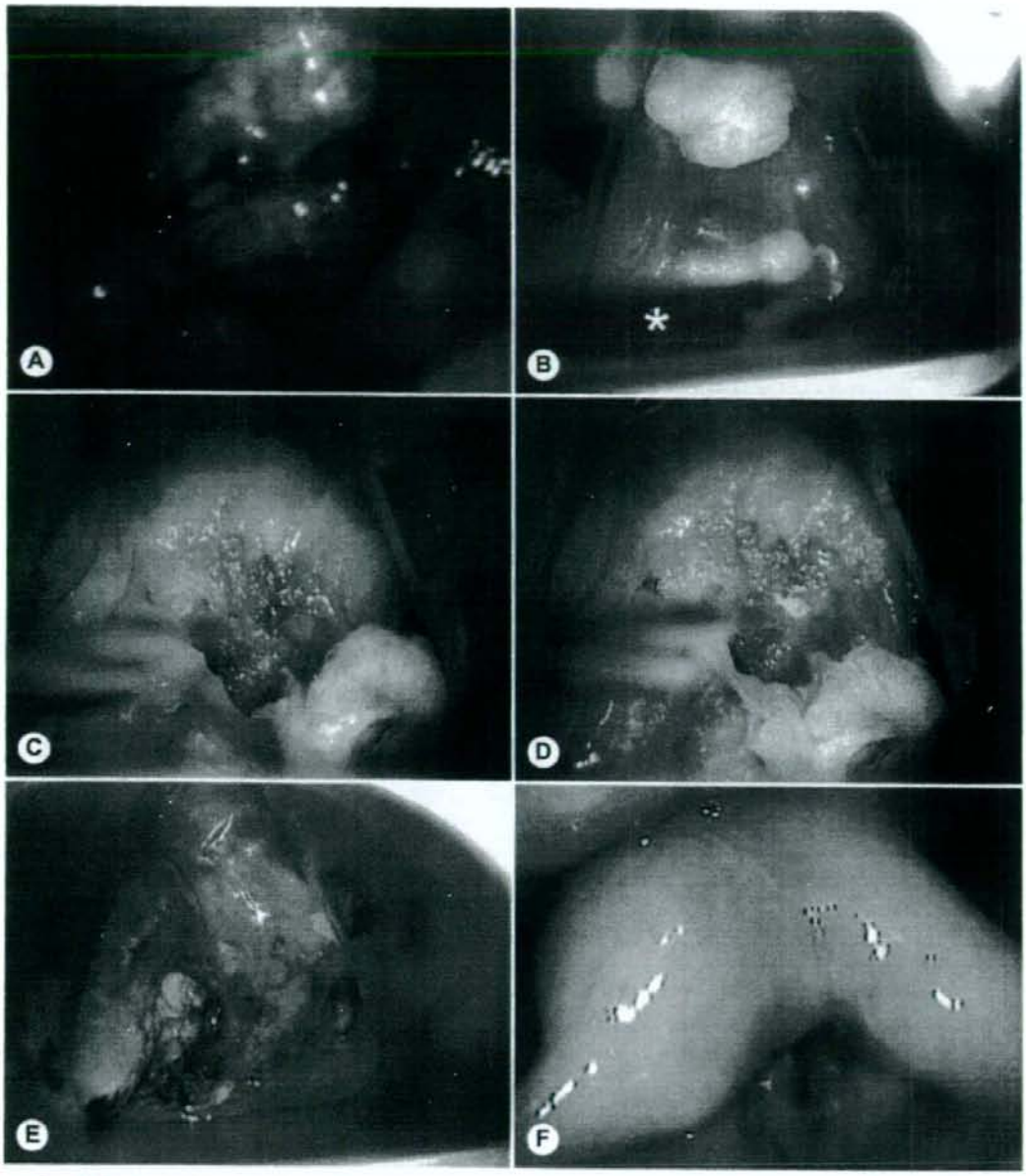
RESULTS

Intraoperative Findings During Photocoagulation. The laser irradiation was simple and relatively safe; photocoagulation was easily performed for all patients without any complication. At a low power level of 1.5 W, the laser beam is well absorbed by hemoglobin in the underlying vascular lesion, resulting in minimal damage to surrounding tissues.[2]

The Figure shows a typical photocoagulation of a bulky venous malformation with the KTP laser (case 6). In this case, the lesion involved the arytenoid cartilages and pyriform sinuses. At first, the laser irradiation resulted in photocoagulation of the surface only. As the surface was photocoagulated, the lesion shrank and the surface became covered with crust. The crusts were debrided, after which the laser energy was repeatedly delivered to the exposed lesion until the remnant lesion disappeared. Repeated laser irradiation was easily performed without complications. Also, when there was a network of vessels around the venous malformation, they were photocoagulated to prevent relapse. This step caused shrinkage of the vessels; no refilling was observed after photocoagulation.

As shown in the Table, the amount of intraoperative bleeding was very small in all cases. In the initial 2 cases, because we were not used to the procedure, the photocoagulation took much more time. From the third case, the duration of the procedure became significantly shorter.

Postoperative Course. There were neither remnants nor relapses, except in 1 patient (case 6). This patient, who had a bulky pharyngolaryngeal venous malformation, presented with a small remnant lesion at the pharyngoesophageal junction 3 months after surgery. The patient, however, has been observed



(Case 6) Photocoagulation of bulky venous malformation under direct microlaryngoscopy. **A)** Preoperative findings of venous malformation on arytenoid cartilages and pyriform sinuses. **B)** Laser irradiation resulted in photocoagulation of surface and shrinkage of lesion. Asterisk — handpiece of laser. **C)** Post-photocoagulated crusts were scraped out. **D)** Laser energy was repeatedly delivered to underlying lesion. **E)** Photocoagulation was completed with no remnant lesion. **F)** Postoperative findings at 1 month after surgery indicated that lesion mostly disappeared.

without further treatments to date, because she has no symptoms and does not want further intervention. All patients became asymptomatic. No postoperative complications, such as bleeding, respiratory troubles, or scar formation, occurred.

DISCUSSION

Vascular anomalies are common in the head and

neck. Especially in the pharyngolaryngeal region, they often manifest as subglottic mucosal-covered lesions in infants. Lesions in this region are rarely encountered in adults. Histologically, anomalies in infants are of the capillary type with tangled nests of small vessels. Anomalies in adults, on the other hand, are of the cavernous or mixed type with thin, fragile mucosa overlying the vascular stroma.[3]

In the pharyngolaryngeal region, the clinical signs relate to the size and location of the lesion. Patients with small pharyngolaryngeal venous malformations usually have no symptoms, and they can be managed conservatively without aggressive treatments. However, large venous malformations cause various symptoms, such as hoarseness, cough, foreign body sensation, and dysphagia. Further treatments are needed in cases with bleeding, airway obstruction, or involvement of the digestive tract.

Treatment of venous malformations is challenging, and results have been reported for various kinds of treatments. As mentioned above, endoscopic laser surgery has been generally accepted and has become a popular therapeutic modality. Many reports describe the use of various kinds of lasers, such as the carbon dioxide (CO_2) laser,[4,5] the neodymium:yttrium aluminum-garnet (Nd:YAG) laser,[6] and the KTP laser.[7] However, the appropriate use of lasers for pharyngolaryngeal venous malformations in adults is still controversial, because only case reports or limited case series are currently available.

Since Simpson et al[4] first reported CO_2 laser therapy for subglottic vascular anomalies in 1979, the CO_2 laser has been used frequently in the management of pharyngolaryngeal vascular anomalies. It has a wavelength of 10,600 nm in the invisible light range and requires an aiming beam during operation. The beam of this laser is well absorbed by tissues with a high water content without tissue penetration, and therefore CO_2 laser cauterization is less effective in hemostasis.[7] Lucioni et al[5] treated 6 cases of supraglottic adult vascular anomalies using a CO_2 laser and reported intraoperative bleeding complications in 3 cases. Postoperative scarring caused by CO_2 laser therapy often becomes a severe problem, especially when the scarring involves the subglottic region.

The Nd:YAG laser has a wavelength of 1,064 nm in the invisible light range and also requires an aiming beam.[3] This laser can be delivered through optic fibers and is easy to handle. This wavelength is more readily absorbed in blood than in surrounding tissue.[8] The emitted laser therefore has a deep coagulation effect, whereas the damage to the overlying watery mucosa is minimal. However, there is significant risk of transmural injury and a risk of scar formation. Nicolai et al[9] compared CO_2 laser therapy with Nd:YAG laser therapy for subglottic vascular anomalies and concluded that the former is superior to the latter in terms of postoperative complications.

The KTP laser has a wavelength of 532 nm in the visible light range and emits bright-green laser light.[10] This laser is produced by a crystal of KTP (potassium titanyl phosphate), doubling the frequency of the Nd:YAG laser. It does not require an aiming beam and can be delivered through optic fibers. Because the wavelength is near the absorption peak of hemoglobin, the laser energy is preferentially absorbed by hemoglobin molecules and therefore has deeper penetration of tissue and a deeper coagulation effect than those of the CO_2 laser. Irradiation with this laser is thought to be effective in treatment of hemorrhagic lesions such as vascular anomalies. Although excessive irradiation with this laser causes transmural injury, it has a lower risk of thermal damage to surrounding tissue than does the Nd:YAG laser. Kacker et al[11] treated 8 patients with subglottic vascular anomalies using the KTP laser, and because of its characteristics and system, they concluded that the KTP laser represents an improvement over the CO_2 laser in treating subglottic vascular anomalies. In addition, to prevent postoperative deterioration of the mucosal wave due to scarring, it is important to preserve as much of the normal tissue as possible when treating microvascular lesions of the vocal fold. Hirano et al[10] treated 12 cases of microvascular lesions of the vocal fold using the KTP laser (set at a low power) without adverse effects and concluded that KTP laser photocoagulation therapy is a relatively safe method for treating microvascular lesions of the vocal fold.

In our case series, there were no problems associated with KTP laser therapy in terms of bleeding or scar formation. As shown in the Figure, even a bulky venous malformation was photocoagulated with minimum bleeding; the surgical view was never hampered. In 2 patients with a bulky venous malformation, we performed tracheotomy before surgery; however, the postoperative edematous changes were minimal. Thus, tracheotomy is not always necessary. In addition, we were able to treat 1 patient with a small venous malformation with photocoagulation therapy using flexible endoscopy in the office under topical anesthesia. For patients with small venous malformations, this method seems to be ideal in terms of the burden on the patients. Although 1 patient with a bulky venous malformation presented later with a small remnant lesion, she has been observed without further treatment, because no

symptoms or lesion progression have been observed so far. In conclusion, this study suggests that photocoagulation therapy using the KTP laser is a simple and relatively safe method that has great potential in the treatment of venous malformations.

CONCLUSIONS

We performed endoscopic photocoagulation therapy using the KTP laser for 7 adult patients with pharyngolaryngeal venous malformations. One small venous malformation was successfully photocoagulated in the office under topical anesthesia with flexible endoscopy. Even bulky venous malformations were successfully photocoagulated by repeated delivery of the laser beam, without any complications such as bleeding. Photocoagulation therapy with the KTP laser is a feasible and relatively safe method for treatment of pharyngolaryngeal venous malformations in adults.

REFERENCES

1. Rahbar R, Nicollas R, Roger G, et al. The biology and management of subglottic hemangioma: past, present, future. Laryngoscope 2004;114:1880-91.

2. Hsiung MW, Kang BH, Su WF, Pai L, Wang HW. Clearing microvascular lesions of the true vocal fold with the KTP/532 laser. Ann Otol Rhinol Laryngol 2003;112:534-9.

3. Yellin SA, LaBruna A, Anand VK. Nd:YAG laser treatment for laryngeal and hypopharyngeal hemangiomas: a new technique. Ann Otol Rhinol Laryngol 1996;105:510-5.

4. Simpson GT, Healy GB, McGill T, Strong MS. Benign tumors and lesions of the larynx in children. Surgical excision by CO_2 laser. Ann Otol Rhinol Laryngol 1979;88:479-85.

5. Lucioni M, Marioni G, Della Libera D, Rizzotto G. Adult laryngeal hemangioma CO_2 laser excision. A single institution 3-year experience (Vittorio Veneto 2001-2003). Acta Otolaryngol 2006;126:621-6.

6. Cholewa D, Waldschmidt J. Laser treatment of hemangiomas of the larynx and trachea. Lasers Surg Med 1998;23: 221-32.

7. Madgy D, Ahsan SF, Kest D, Stein I. The application of the potassium-titanyl-phosphate (KTP) laser in the management of subglottic hemangioma. Arch Otolaryngol Head Neck Surg 2001;127:47-50.

8. Poetke M, Berlien HP. Laser treatment in hemangiomas and vascular malformations. Med Laser Appl 2005;20:95-102.

9. Nicolai T, Fischer-Truestedt C, Reiter K, Grantzow R. Subglottic hemangioma: a comparison of CO_2 laser, Neodym-Yag laser, and tracheostomy. Pediatr Pulmonol 2005;39:233-7.

10. Hirano S, Yamashita M, Kitamura M, Takagita S. Photocoagulation of microvascular and hemorrhagic lesions of the vocal fold with the KTP laser. Ann Otol Rhinol Laryngol 2006; 115:253-9.

11. Kacker A, April M, Ward RF. Use of potassium titanyl phosphate (KTP) laser in management of subglottic hemangiomas. Int J Pediatr Otorhinolaryngol 2001;59:15-21.

Regeneration of Skeletal Muscle Using *In Situ* Tissue Engineering on an Acellular Collagen Sponge Scaffold in a Rabbit Model

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Because of the limited ability of skeletal muscle to regenerate, resection of a large amount of muscle mass often results in incomplete recovery due to nonfunctional scar tissue. The aim of this study was to regenerate skeletal muscle using *in situ* tissue engineering in a rabbit model. In 18 male rabbits, a muscle defect (1.0 × ~1.0 × ~0.5 cm) was created in the vastus lateralis of both legs. A piece of cross-linked atelocollagen sponge was then inserted into the defect in one leg, whereas the defect in the other leg was left untreated. Both defects were finally covered with fascia. Twenty-four weeks after surgery, the defect that had been filled with the cross-linked atelocollagen sponge scaffold showed mild concavity and slight adhesion to the fascia, while the control side showed severe scar formation and shrinkage. Histologically, the regenerating myofibers at the site containing the collagen sponge were greater in number, diameter, and length than those at the control site. These results indicate that cross-linked atelocollagen sponge has the potential to act as a scaffold for muscle tissue regeneration. *ASAIO Journal* 2007; 53:506–513.

Skeletal muscle is composed of bundles of myofibers that are contracted by motor nerve stimulation. Skeletal muscle controls movement, posture, and respiration, and has the potential to regenerate to a certain degree. However, loss of a large amount of muscle mass often results in incomplete recovery, with the development of nonfunctional scar tissue.[1–3] Such muscle loss has been conventionally managed by muscle transplantation or transposition techniques.[4,5] However, such methods have limited applicability, and often fail to restore complete muscle function. One approach to address such difficulty may be *in situ* tissue engineering, which allows tissue regeneration at the site of a defect at its intended position in the patient's body, rather than *in vitro*.[6] Collagen sponge has the potential to act as a scaffold to promote tissue regeneration, and has been used successfully for repairing tissue defects in the trachea, esophagus, stomach, small intestine, and peripheral nerves.[7–14]

In the present experimental study, we tested the use of collagen sponge as a scaffold for regeneration of skeletal muscle by *in situ* tissue engineering in a rabbit model.

Materials and Methods

Collagen Sponge Scaffold

Collagen was extracted from porcine skin, chopped into small pieces, and suspended in cold 0.01 M HCl for 24 hours. The pieces were then homogenized with a Polytron homogenizer (Kinematika, Lucerne, Switzerland), followed by treatment with pepsin (substrate/enzyme ratio by weight, 100:1) at 4°C for 48 hours. The homogenate was centrifuged at 7000*g* for 20 minutes and the pellet was discarded. The supernatant was collected and found to consist predominantly of type I collagen (70–80%); the remainder was type III collagen (confirmed by sodium dodecylsulfate-polyacrylamide gel electrophoresis [SDS-PAGE]).[8,15] In this initial processing of the skin, the telopeptide of collagen, which is considered antigenic, was removed. The extracted atelocollagen was dissolved in HCl at pH 3 to give a final concentration of 1.0% by weight. The collagen solution was agitated together with air in a homogenizer (Nissei AM3, Nihon Seiki Kaisha Ltd., Japan) at 8000 rpm for 15 minutes on ice. This whipped collagen solution was poured into a plastic mold, frozen at −80°C to produce a porous sponge, and immediately freeze-dried for 48 h (Ulvac GCD135XA, Shinkukiko, Tokyo, Japan). The freeze-dried collagen sponge was then heated at 140° under vacuum for 24 hours to introduce cross-linking between the collagen molecules, resulting in a microstructure. The pore diameters of the collagen sponge were controlled to within the range of 50 to 100 μm.[9] The final collagen sponge pieces were cut to a size of 1.0 × ~1.0 × ~0.5 cm and sterilized with ethylene oxide gas.

Animals and Surgical Procedure

Eighteen male rabbits weighing 2.0 to 2.5 kg were used. Surgical procedures were performed in accordance with the Rules and Regulations for Animal Research at Kyoto Prefectural University of Medicine, 2002.

The animals were anesthetized with intravenously administered sodium pentobarbital (Nembutal, 30 mg/kg; Dainippon Pharmaceutical, Japan) and then given an intramuscular injec-

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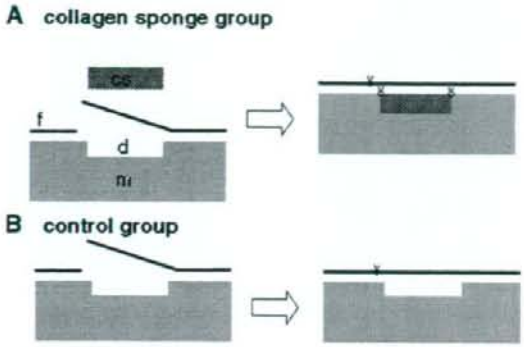

Figure 1. Schema of the operations performed in this study. (**A**) Collagen sponge implantation. (**B**) Control. cs, collagen sponge scaffold; d, defect; f, fascia; m, native muscle tissue.

tion of 2% xylazine hydrochloride (Celactal, 4 mg/kg; Bayer, Tokyo, Japan). A 5-cm skin incision was made in the lateral thigh of each leg, and the vastus lateralis muscle was exposed beneath the fenestrated fascia. After retraction of the muscle, a defect measuring 1.0 × ~1.0 × ~0.5 cm was created in the middle of the vastus lateralis muscle on both sides. In one thigh, the defect was filled with a piece of collagen sponge that had been soaked in Dulbecco's modified Eagle medium (Gibco, Tokyo, Japan). The collagen sponge was fixed in place with 4-0 polypropylene suture (Prolene; Ethicon, Tokyo, Japan) at four corners, and the fenestrated fascia was closed with 4-0 polyglactin suture (Vicryl, Ethicon) (**Figure 1**). In the opposite thigh, the fenestrated fascia was closed with 4-0 polyglactin suture without inserting a piece of collagen sponge. After the operation, cefazolin sodium (Cefamezin, 500 mg/body IM; Astellas Pharmaceutical, Tokyo, Japan) was administered. All rabbits were housed in separate cages with free access to food and water without immobilization.

Macroscopic observation

At 1, 2, 3, 4, 12, or 24 weeks after the operation, three rabbits were killed and sites were opened. The operation sites were observed for color, hardness, and the extent of the adhesion to fascia. To investigate the influence of scar contraction to muscle regeneration, the scar contraction was assessed by measuring surface area of the operation sites. At the initial operation, the four corners of the surface area of the muscle defect were marked with 4-0 polypropylene sutures. The surface area surrounded by markers was measured at each time point. **Table 1** shows the sequence comparison of the area of the operation sites with the initial value (%).

Histologic Evaluation

After macroscopic observation, the implants and surrounding tissues were removed, fixed in 20% neutral buffered formalin, and embedded in paraffin. All muscle specimens were cut longitudinally into 5 μm sections and stained with hematoxylin and eosin for routine structural analysis.

Table 1. Sequence Comparison of the Area of the Operation Sites with the Initial Value (%)

Operation Duration	Control Group	Collagen Sponge Group
1 wk	84.5 ± 3.1	96.5 ± 3.4*
2 wk	61.3 ± 1.4	72.8 ± 2.9*
3 wk	66.7 ± 4.4	77.2 ± 2.6*
4 wk	56.4 ± 3.4	80.4 ± 2.0*
12 wk	84.8 ± 5.1	88.9 ± 1.0
24 wk	100.8 ± 1.6	105.7 ± 6.3

Values are mean ± SD, n = 3 in each group.
*Significantly different from control sites, ≤0.01.

Immunohistochemistry

Mouse monoclonal anti-desmin antibody (D33; Dako Japan, Kyoto, Japan) was used as a primary antibody for detection of intermediate filament proteins and actively regenerating muscle fibers. After sections had been deparaffinized and their endogenous peroxidase activity blocked by application of peroxidase blocking reagent (Dako) for 5 min, incubation with the primary antibody was carried out for 60 min at room temperature. The sections were then incubated with peroxidase-labeled polymer conjugated to goat anti-mouse immunoglobulin for 30 minutes at room temperature, incubated with diaminobenzidine chromogen for 5 minutes, counterstained with hematoxylin, and finally mounted and coverslipped. Between individual steps in antibody staining, sections were rinsed gently with phosphate-buffered saline including 5% skim milk to inhibit nonspecific binding. Control sections were incubated in the absence of primary antibody.

Results

All the animals survived uneventfully until 24 weeks after the operation. No wound infections or other major complications occurred.

Macroscopic Findings

In the thigh bearing the collagen sponge, the sponge had a smooth white surface and a distinct margin 1 week after the operation. At 2 weeks, the space between the ends of the collagen sponge and the cut ends of the muscle fibers had narrowed, and was covered with a thin collagen layer free of adhesions, with an underlying hematoma. Overall, the border between the native muscle and the sponge had become indistinct. At 3 weeks after the operation, this area was slightly reddish and had become more indistinct. At 4 weeks, the junction of the sponge and muscle was soft and similar in color to the nearby normal muscle, but its surface had become slightly concave. Even at 4 weeks after the operation, no scar, contracture, or adhesion to the fascia was observed.

In the control thigh at 1 week after the operation, the surface of the muscle defect had collapsed, resembling an ulcer in appearance. From 2 to 3 weeks, the defect gradually began to shrink due to scar formation, and adhered tightly to the fascia. At 4 weeks, the defect was completely filled with whitish connective tissue. The surface of the defect was concave and firm, and adhesions between the surface of the muscle defect and the fascia were particularly dense.