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In situ tissue engineering for tracheal reconstruction using a luminar remodeling type of artificial trachea

Tatsuo Nakamura, MD,[a] Toshihiko Sato,[a] Masato Araki, MD,[a] Satoshi Ichihara, MD,[a] Akira Nakada, MD,[a] Makoto Yoshitani, MD,[a] Shin-ichi Itoi, MD,[a] Masaru Yamashita, MD,[b] Shin-ichi Kanemaru, MD,[b] Kouichi Omori, MD,[c] Yoshio Hori, MD,[a] Katsuaki Endo, MD,[d] Yuji Inada, MD,[a] and Katsumi Hayakawa, MD[a]

Background: After successful trials of tracheal reconstruction using mesh-type prostheses in canine models, the technique has been applied clinically to human patients since 2002. To enhance tissue regeneration, we have applied a new tissue engineering approach to this mesh-type prosthesis.

Methods: The prosthesis consists of a polypropylene mesh tube reinforced with a polypropylene spiral and atelocollagen layer. The cervical tracheas of 18 beagle dogs were replaced with the prosthesis. The collagen layer was soaked with peripheral blood in 6 of the dogs, with bone marrow aspirate in another 6, and with autologous multipotential bone marrow–derived cells (mesenchymal stem cells) in another 6. The dogs were humanely killed at 1 to 12 months after the operation.

Results: All 18 dogs survived the postoperative period. Bronchoscopically, 3 of 4 dogs in the peripheral blood group showed stenosis, whereas no stenosis was evident in all 8 of the dogs in the bone marrow and mesenchymal stem cell groups 6 months after the operation. Faster epithelialization and fewer complications, such as mesh exposure and luminal stenosis, were observed in these two groups than in the peripheral blood group. Histologically, the cells from autologous bone marrow were found to proliferate into the tracheal tissue during the first month. Cilial movement in these two groups was faster than that in the peripheral blood group and recovered to 80% to 90% of the normal level.

Conclusions: Bone marrow aspirate and mesenchymal stem cells enhance the regeneration of the tracheal mucosa on this prosthesis. This in situ tissue engineering approach may facilitate tracheal reconstruction in the clinical setting.

End-to-end anastomosis has been the standard clinical method for tracheal reconstruction since the 1970s.[1] However, in cases in which the resected segment is more than 6 cm long, high tension at the anastomosis site can sometimes cause severe and fatal postoperative complications such as dehiscence.[2] A variety of artificial tracheas have been designed for such cases and assessed, but so far none has proved satisfactory for clinical use.

We have developed a new mesh-type tracheal prosthesis on which autologous tracheal tissue is encouraged to regenerate on a collagen scaffold. We have already demonstrated that this prosthesis can be applied safely for circumferential replacement of the cervical trachea,[3] thoracic trachea,[2,4] or carinal bifurcation[5] in canine models. On the basis of the success of these experimental animal studies, clinical application of the tracheal prosthesis was begun for human patients with tracheal defects in 2002.[6]

In animal experiments, the initial type of prosthesis that was simply coated with amorphous collagen often developed mesh exposure or ulcers on the inner mucosa.[7] This indicated that early epithelial regeneration was a key factor required for successful use of this mesh-type tracheal prosthesis. In other words, enhancement of tissue recovery on the prosthesis was a crucial requirement for tracheal reconstruction.

In clinical trials, the prosthesis was moistened with autologous peripheral blood before anastomosis.[6] If tissue regeneration can be facilitated with an improved method, it would certainly contribute to the clinical feasibility of a tracheal prosthesis. Therefore, in the present study, we also used bone marrow aspirate and a suspension of bone marrow–derived mesenchymal stem cells (MSCs) as alternatives to peripheral blood. We also determined whether the seeded autologous bone marrow cells proliferated into the tracheal tissue in situ.

MATERIALS AND METHODS

Collagen

The collagen used was extracted from skin of young pigs (6 months old weighing about 120 kg) (supplied by Nippon Meatpackers Inc, Ibaraki, Japan). The extracted collagen was then digested with pepsin at 4°C for

From the Department of Bioartificial Organs,[a] Institute for Frontier Medical Sciences, Kyoto University, the Department of Otolaryngology,[b] Kyoto University, Kyoto, Japan; the Department of Otolaryngology,[c] Fukushima Medical University, Fukushima, Japan; and the Department of Physiology,[d] Kyoto Nerve Regeneration Research Center, Kyoto, Japan.

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Address for reprints: Tatsuo Nakamura, MD, Department of Bioartificial Organs, Institute for Frontier Medical Sciences, Kyoto University, 53 Kawahara cho, Sakyo-ku, Kyoto 606-8507, Japan (E-mail: nakamura@frontier.kyoto-u.ac.jp).

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Abbreviations and Acronyms
CBF = cilial beat frequency
MRI = magnetic resonance imaging
MSC = mesenchymal stem cell

48 hours. The supernatant consisted of type I collagen (70%–80%), and the rest was type 3 collagen, as confirmed by sodium dodecyl sulfate–polyacrylamide gel electrophoresis. During the pepsin treatment, the telopeptide of collagen, which is thought to have antigenicity, was removed, leaving only the atelocollagen.

Prosthesis

A straight tube 16 mm in diameter was manufactured from a sheet of polypropylene mesh with a pore size of 260×10^{-6} m (Bard mesh; Davol Inc, Cranston, RI) by melting the edges together. The tube was then reinforced with a polypropylene monofilament yarn (0.8 mm in diameter) (Figure 1). The yarn was spirally attached to the external surface of the mesh tube by thermal melt-bonding at 5-mm intervals and further fixed with 7-0 Prolene polypropylene suture (Ethicon, Inc, Somerville, NJ). The polypropylene spiral provided the tube with stiffness against compression. The interval of the yarn was determined so that the mechanical resistance against compression was the same as that of the native trachea.[8]

This polypropylene tube was then exposed to a coronal discharge at 9 kV for 10 minutes to activate the surface. The prosthesis was subsequently coated 10 times with the 1% collagen solution. The activated prosthesis was covalently immobilized with collagen molecules and then further physically coated with collagen to promote host tissue incorporation and render the prosthesis airtight during the initial stage of implantation. This coating process was repeated 20 times.

The collagen-coated prosthesis was placed in a cylindrical Teflon mold with an inner diameter of 37 mm and a depth of 6 cm. At the same time, a 5-mm–diameter Teflon tube was inserted into the center of the prosthesis. The 1% collagen solution, which had been stirred at 8000 rpm for 15 minutes, was poured into the space between the outer mold and the inner tube and then freeze-dried. In this freeze-drying process, the cast collagen became a porous structure with a pore size range of 100 to 500×10^{-6} m. Finally, the prosthesis was subjected to dehydrothermal treatment 140°C in vacuo for 24 hours to induce cross-links between the collagen molecules to prevent early breakdown of the collagen in vivo.

Animal Experiments

Eighteen beagle dogs weighing between 8 and 14 kg were divided at random into three groups. The cervical trachea was cut transversely 20 mm caudal to the thyroid cartilage, and a tracheal segment (40–50 mm) including seven tracheal cartilages was removed with the animal under general anesthesia. During the operation, the prosthesis was preclotted with 5 mL of autologous peripheral blood in 8 of the dogs (peripheral blood group). In another 8 dogs, the prosthesis was soaked with 5 mL of bone marrow aspirate obtained from the femoral bone during the operation (bone marrow group). In the remaining 8 dogs, from which bone marrow aspirate had been taken and incubated in advance, the prosthesis was soaked with an incubated bone marrow cell suspension (MSC group). In these 8 dogs of the MSC group, 2 mL of bone marrow aspirate was taken from the femur 4 weeks before the operation. The bone marrow aspirate was incubated with Dulbecco modified Eagle medium containing 10% fetal bovine serum. The incubation medium was changed every 3 days from the fifth day. Floating cells were removed when the medium was changed. Some cells became attached to the bottom of the incubation bottle and proliferated. These proliferated cells were detached with trypsin, and a suspension of these cells (10^7) was used in the operating room.

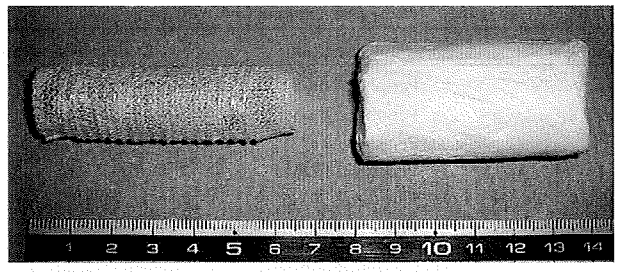

FIGURE 1. Mesh-type tracheal prosthesis. The framework is a polypropylene mesh tube reinforced with a spiral stent *(right)*. Freeze-dried collagen layers were made both inside and outside the framework as a scaffold of tissue regeneration *(left)*.

The porous collagen layer inside and outside the lumen shrank in volume on soaking. The prosthesis and the cut ends of the trachea were anastomosed with 3-0 Vicryl (Ethicon) interrupted sutures, the cut ends of the tracheal stumps being inserted into the prosthesis lumen (Figure 2). Neither omentopexy nor fibrin glue was used.

A 1000-mg dose of ampicillin was injected intramuscularly on the day of the operation, and a 500-mg dose was administered orally every day for 1 month thereafter. Bronchial cleaning with a fiberscope was only performed immediately after the operation on the same day. No additional care other than routine management was provided for the animals.

Bronchoscopic Examinations and Cilial Beat Frequency (CBF) Examination

A bronchofiberoscope (model BF1T20; Olympus Optical Co Ltd, Tokyo, Japan) examination was performed periodically under general anesthesia with an intramuscular injection of ketamine hydrochloride (10 mg/kg)

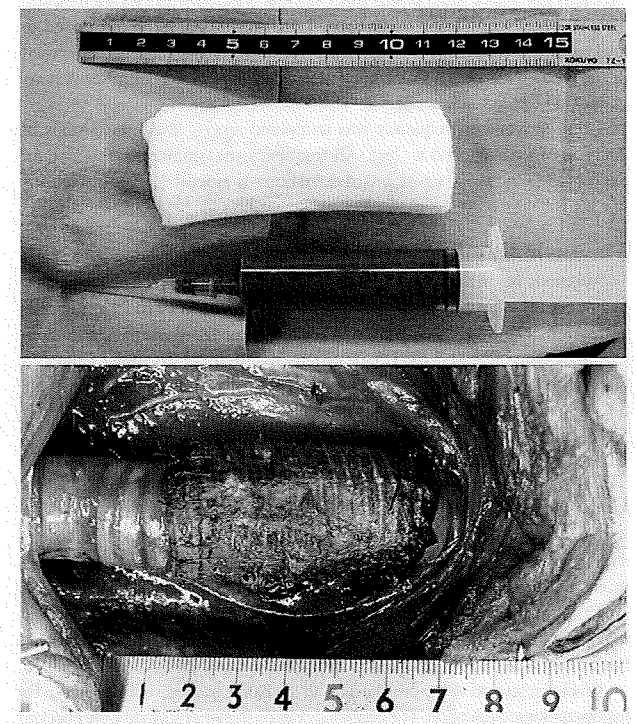

FIGURE 2. Intraoperative view of the reconstruction of a canine trachea with the prosthesis. The prosthesis was soaked with each medium before anastomosis. The prosthetic wall became airtight with this process.

TABLE 1. Results of tracheal reconstruction with prosthesis

Dog no.	Soak medium	Observation (mo)	Incorporation to host	Stenosis	Erosion
1	Peripheral blood	1	+	−	−
2	Peripheral blood	3	+	−	−
3	Peripheral blood	6	+	−	−
4	Peripheral blood	6	+	+	−
5	Peripheral blood	12	+	+	+
6	Peripheral blood	12	+	+	−
7	Bone marrow aspirate	1	+	−	−
8	Bone marrow aspirate	3	+	−	−
9	Bone marrow aspirate	6	+	−	−
10	Bone marrow aspirate	6	+	−	−
11	Bone marrow aspirate	12	+	−	−
12	Bone marrow aspirate	12	+	+	−
13	MSC suspension*	1	+	−	−
14	MSC suspension	3	+	−	−
15	MSC suspension	6	+	−	−
16	MSC suspension	6	+	−	−
17	MSC suspension	12	+	−	−
18	MSC suspension	12	+	+	−

Mesenchymal stem cells *(MSC)* were labeled with fluorescent marker DiI. *Stenosis is defined as a reduction of the tracheal lumen by more than one third.

and xylazine hydrochloride (4 mg/kg) to examine the luminal surface of the prosthesis. The replaced segments were examined carefully. Mesh exposure exceeding one third of the circumference was define as "++" and less than one third as "+." Stenosis was defined as a reduction of more than one third of inner luminal area.

At 6 months and 12 months, samples of the tracheal epithelium were removed with a brush inserted through the channel of the bronchoscope to evaluate cilial movement. The brush, which scrubbed the target (reconstructed) portion of the tracheal mucosa, was washed with culture medium. This medium (containing the scrubbed tracheal mucosa cells) dropped on a glass slide was then observed with a light microscope. The movement of the cilia on the slide was counted at 37°C with a photomultiplier system[9] connected to the microscope.

Cilial movement frequency was compared among the three groups by analysis of variance and a *t* test. All frequency measurements obtained are expressed as the mean (± standard deviation) of at least 10 determinations.

Magnetic Resonance Imaging

Magnetic resonance imaging (MRI) of the reconstructed trachea and adjacent organs was conducted on 1 dog from each group 12 months after implantation. The MRI system used was a Sonata MRI System with a static magnetic flux density of 1.5 T (Siemens Medical System, Erlangen, Germany) using a phase-array body coil with four active segments. The dog was anesthetized with an intravenous injection of propofol (Diprivan; 5 mg · min^{-1} · kg^{-1}) during the imaging.

Histologic Examination

One dog in each group was humanely killed with an injected overdose of sodium pentobarbital 1 and 3 months after tracheal reconstruction. Two dogs in each group were humanely killed 6 months after tracheal reconstruction. At 12 months, 2 dogs in the peripheral blood group and 1 each in the bone marrow and MSC groups were humanely killed. The remaining dog in each of the bone marrow and MSC groups was allowed to survive for further observation.

En bloc resection of the prosthesis with the native trachea was carried out for histopathologic evaluation. The epithelial cells on the inner surface were checked with a scanning electron microscope (S-450; Hitachi Ltd, Tokyo, Japan).

All of the surgical and euthanasia procedures were performed in accordance with the "Guide for the Care and Use of Laboratory Animals" published by the National Institutes of Health (NIH Publication No. 85-23, revised 1985). The experimental protocol was approved by Animal Experimental Committee of Kyoto University.

RESULTS

All animals in the three groups had uneventful postoperative courses, and no symptoms that necessitated unscheduled humane killing of an animal occurred (Table 1).

Bronchoscopic Examination

Bronchoscopic examinations at 1 month revealed that the luminal surface of the implanted prosthesis appeared glossy and whitish, suggesting complete coverage of the lumen by regenerated tissue (Figure 3). No mesh exposure was recognized even in the middle of the prosthesis, except in 1 dog (Table 1, dog 5), in which an area of exposed mesh measuring about 5 × 2 mm was observed at the center of the prosthesis at 1 month. Despite this mesh exposure, the dog was asymptomatic and its general condition was good, and no retention of sputum was observed in the lumen of the prosthesis up to 12 months. Stenosis of the prosthetic lumen was observed in 3 of the dogs in the peripheral blood group and in 1 dog in each of the other two groups. This stenosis occurred in the middle of the prosthesis and not at the sites of anastomosis. The definition of stenosis used in this study was reduction of the tracheal luminal diameter by more than one third. Whereas both dogs in the bone marrow group and the MSC group had only mild stenosis, severe stenosis that reduced the diameter of the tracheal lumen by more than half occurred in 3 of the 4 dogs in the peripheral blood group. The narrowest luminal diameter was 9 mm in both the bone marrow group and the MSC group, whereas that in the peripheral blood group was 6 mm.

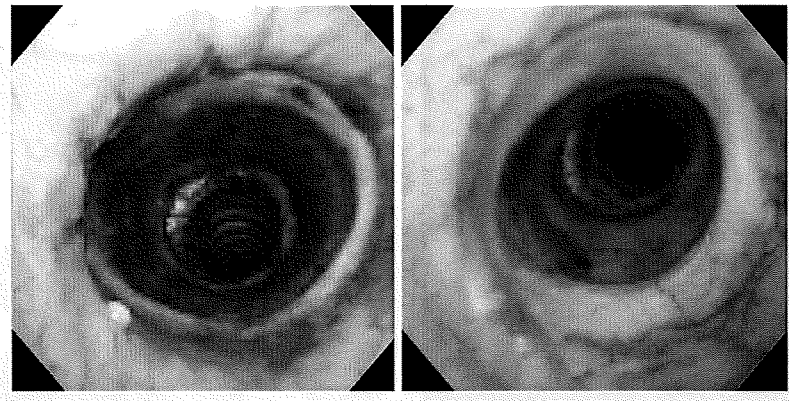

FIGURE 3. Bronchoscopic views of the reconstructed site of the bone marrow group (dog 11) 1 month *(left)* and 12 months *(right)* after the operation. Neither stenosis nor granulation is evident at the site of anastomosis with the trachea. Complete coverage of the mesh by regenerated tissue is evident.

Macroscopic Examination

At 1 month the inner surface of the prosthesis was covered with glossy tissue in all three groups (Figure 4). The prostheses were incorporated into the native tracheas, and there were no major complications such as erosion of the surrounding organs, abscess formation, pneumothorax, anastomotic dehiscence, or prosthetic dislocation in any of the 18 dogs that were humanely killed on schedule (Table 1).

MRI

MRI at 12 months indicated no scar formation around the prosthesis, and the inner lumen of the tube was maintained in the cervical space in all three groups (Figure 5). The implanted prostheses were well incorporated with the native trachea at the interfaces, and there was no morbid tissue reaction between adjacent organs including major blood vessels.

Histologic Examinations

Examination with a light microscope at 1 month revealed that the polypropylene mesh and polypropylene spiral had become buried in newly regenerated connective tissue and that the implanted collagen had disappeared. Capillary ingrowth was observed in the regenerated tracheal tissue in all dogs of the three groups. After 6 months, formation of respiratory epithelium was confirmed histologically from the upper to the lower anastomotic site of the prosthesis (Figure 6).

Although the extent of the epithelial lining of the regenerated mucosa on the prosthesis varied, 9 of the 12 dogs in the bone marrow and MSC groups had an epithelial lining covering more than 50% of the length of the prosthesis. In contrast, after 3 and 6 months, the peripheral blood group had poor epithelial lining, in which the squamous epithelium was thicker than that of the columnar bronchial epithelium. After 12 months, the extent of the epithelial lining in the peripheral blood group was similar to that in the other two groups. In each animal, ciliated columnar epithelium was observed near the anastomoses, the proportion of nonciliated cuboidal epithelium increased with distance from the anastomoses, and squamous epithelium was observed in the center of the prosthesis (Figure 7).

An area of exposed mesh measuring 5 × 2 mm without any sign of local infection was observed in dog 5 of the peripheral blood group. No granulation tissue formation was detected in the lumen.

Histologic examination of the stenotic part of the prosthesis in 5 dogs showed that the connective tissue, including vessels, had invaded the mesh pores, covering the inner surface of the prosthesis. Also in these animals, the neoepithelium appeared stretched from the cut end of the tracheal stump over the connective tissue layer.

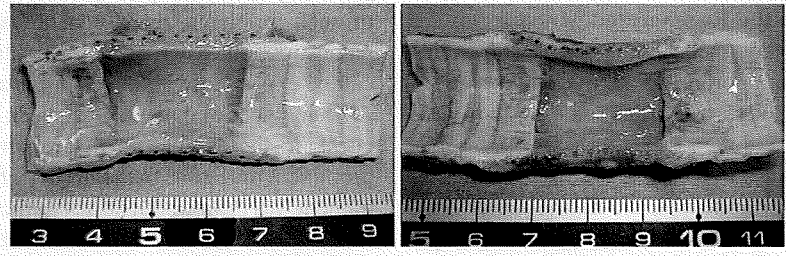

FIGURE 4. Luminal surface of the prosthesis (bone marrow group, dog 8). Three months after reconstruction, the prosthesis was incorporated into the native tracheas and inner surface was covered with glossy and whitish tissue. Polypropylene stents were seen in the cross-section of the reconstructed tracheal wall.

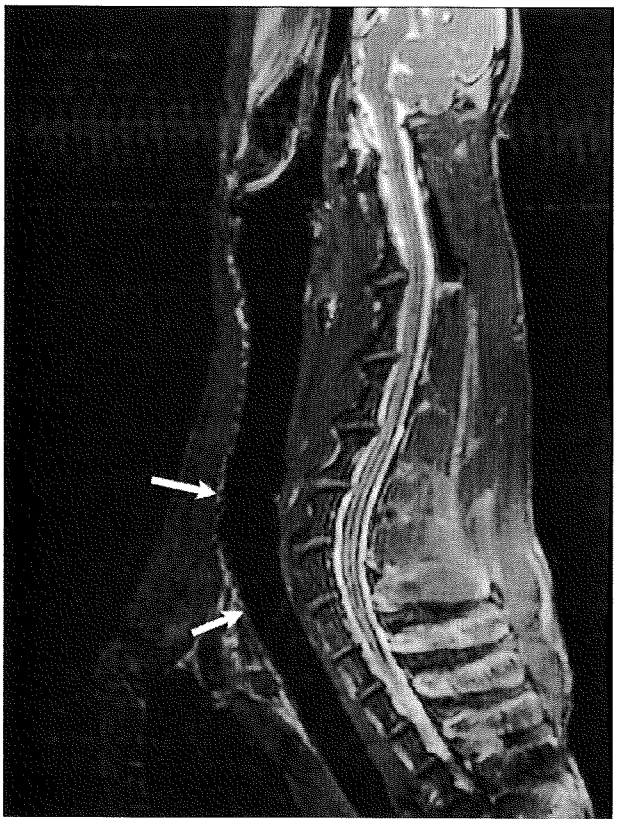

FIGURE 5. MRI appearance of the prosthesis implanted into the cervical trachea of a dog 12 months after the operation (MSC group). Replaced area is indicated by the *arrows*.

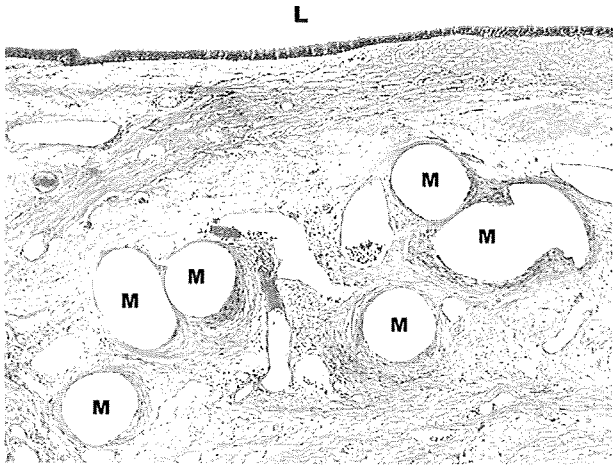

FIGURE 6. Regenerated mucosa with epithelial lining on the mesh of the prosthesis from a dog in the bone marrow group after 12 months. The mucosa is thicker than the normal tracheal mucosa. *L*, Tracheal lumen; *M*, mesh of the prosthesis (hematoxylin and eosin staining; original magnification, ×400).

Scanning electron microscopy examination revealed that the regenerated epithelial cells near the anastomoses possessed cilia similar to those of the normal tracheal epithelium in all groups. Moreover, a few ciliated epithelial cells were observed in areas where only a squamous cell lining was demonstrated by light microscopy (Figure 7).

Observation by transmission electron microscopy showed long and uniform cilia on the luminal surface over a large area, gathering closely together (Figure 8) on the regenerated part of the trachea in all three groups. In the area where ciliated cells were absent, the luminal surface was covered with the squamous cells.

Comparison of CBF in the Regenerated Mucosa

CBF in the regenerated tracheal mucosa is shown in Figure 9. CBF of the same portion of the trachea measured in intact normal beagle dogs using the same system was 14.6 ± 3.5.

At 6 months, CBF values in the bone marrow group and MSC group had recovered to almost the normal range and were significantly higher than that in the control group. There were no significant differences in CBF among the three groups at 12 months ($P > .05$).

Proliferation of Bone Marrow–Derived Cells

To determine the fate of the MSCs, we used MSC labeled with Di-I (Cell Tracer CM-DiI; Molecular Probes, Leiden, The Netherlands) in 1 dog (no. 13) of the MSC group, which was killed 1 month after the operation. At this time, the Di-I–labeled MSCs were recognized in the regenerated tracheal tissue. They remained in the submucosal tissue around the polypropylene mesh and were not present in the mucosa (Figure 10).

DISCUSSION

Direct end-to-end anastomosis has been the "gold standard" for airway reconstruction.[10] However, the range of possible reconstruction with this method is limited, and even within the feasible range (less than 6 cm), tension-reducing procedures are required during the operation. In the case of resections approaching the theoretical maximum, patients must endure a stressful drawn-in chin position with neck fixation for at least 2 weeks after the operation to avoid concentrating tension on the sites of anastomosis.[2] Thus, direct apposition and suturing of the tracheal stumps tends to place a heavy burden on the patient. For resolution of such problems, the use of a tracheal substitute makes reconstruction easier, and as a result, the surgical approach can be expanded with the use of a tracheal prosthesis.

Many types of material have been examined for use in tracheal prostheses since the first report by Daniel[11] in 1948. A prominent milestone in the history of artificial trachea development was the introduction of a silicone rubber prosthesis in the 1960s, which became commercially available in the 1970s[12] and was used clinically for a short time. However, because re-epithelialization could not be expected on the

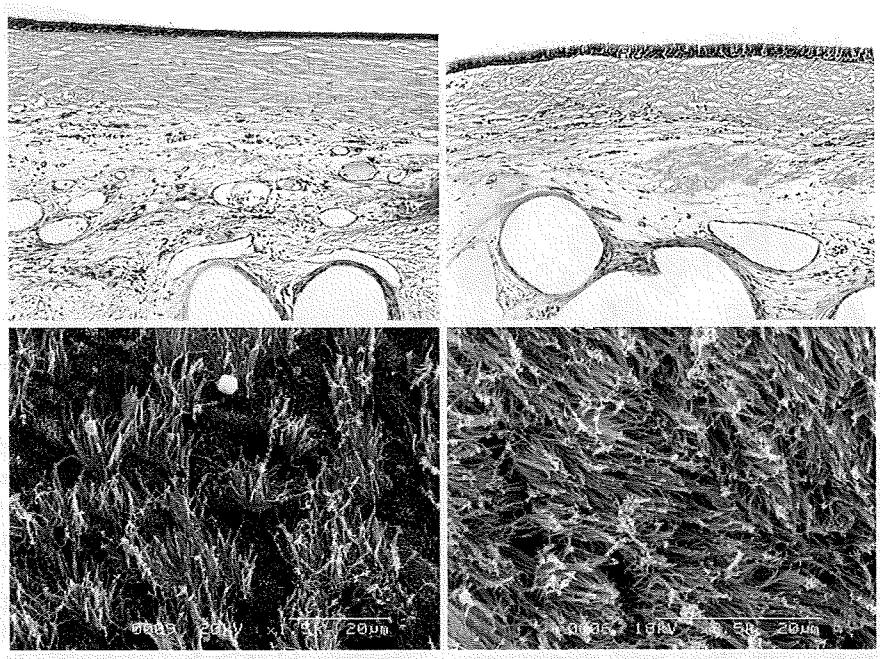

FIGURE 7. Ciliated epithelium is present on the regenerated mucosa near the anastomosis *(upper left)*, but only squamous epithelium is present in the middle of the prosthesis *(upper right)* in a dog in the bone marrow group after 12 months (hematoxylin and eosin staining; original magnification, ×200). *Bottom,* Scanning electron microscopy (SEM) views of the luminal surface. Dense cilia like those of the normal tracheal epithelium are present on the prosthesis near the anastomosis *(lower left)* and a few ciliated epithelial cells are present in areas where only a squamous cell lining was demonstrated by light microscopy *(lower right).* Although these findings were evident in all three groups, there were more ciliated cells in the bone marrow and MSC groups than in the peripheral blood group.

inner lumen, formation of granulation tissue or dehiscence occurred at the interface between the prosthesis and the native trachea, usually within several months.[13] For this reason, the nonporous tracheal prosthesis is now seldom used clinically.

One of the reasons that prosthetic tracheal reconstruction is more difficult than use of a vascular prosthesis may be that the trachea is in contact with the external environment (ie, it is an airway). At the interface of an implanted foreign body and host tissue, the wound healing process continues until the implanted foreign body is completely encapsulated or is rejected from the implant site. Such phenomena are often observed clinically by surgeons in relation to suture materials used on skin incisions. In this respect, an artificial trachea contrasts conspicuously with an artificial vascular graft, which has no external interface after implantation.

With this in mind, we[3,4,7,8,14] have designed a series of mesh-type tracheal prostheses that eliminate the interface facing the airway. In such prostheses, autologous tissue invades the pore spaces, and the interface of the foreign material with the airway finally disappears with re-epithelialization of the inner lumen.[15]

However, one problem with the mesh-type prosthesis that needs to be overcome is insufficient air sealing of the mesh structure, especially just after implantation. Indeed, to seal the porous tracheal prosthesis, a 2-stage operation was once designed for ensuring that the prosthesis was airtight.[16,17] However such a 2-stage operation places a heavy burden on the patient and seems too complicated for clinical use.

Therefore, to seal the mesh, we[14] began to apply collagen in the early 1980s in the expectation that the collagen might act not only as a palliative sealant but also as a scaffold for tissue regeneration. Collagen, the main element of the extracellular matrix, has been reported to play several important roles in tissues, such as cell adhesion, detachment, transformation, and proliferation.[18] For example, type 1 collagen is reported to promote the differentiation of tracheal ciliated epithelium.[19]

In this connection, tissue engineering, whereby a variety of tissues can be fabricated in incubation rooms using cell culture techniques, has been a focus of attention since 1990.[20] Tissue engineering has also been applied for tracheal reconstruction. In 1994, epithelial tissue was first induced on a tissue-engineered trachea.[21] In that study, tracheal epithelial cells were isolated from newborn lambs in advance to induce an epithelial lining on the reconstructed site. These epithelial cells were injected into a previously manufactured cylindrical cartilage tube made from cells harvested from calves. These in vitro–formed tubes were then implanted into subcutaneous pockets of 10 nude mice, aged 6 weeks. Four of the 10 implanted tubes were found to develop an epithelial lining on the inner surface, whereas

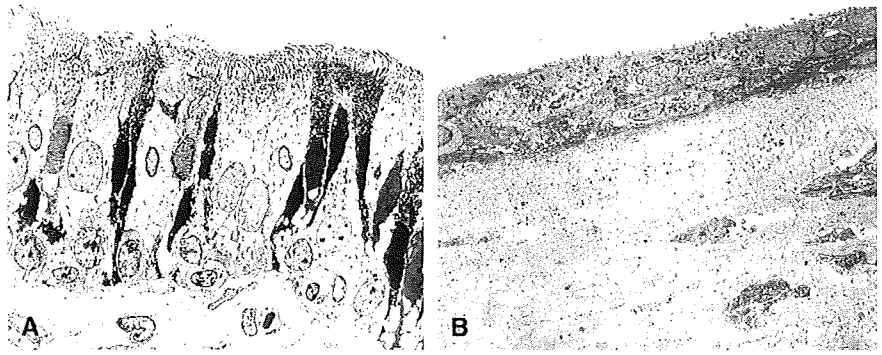

FIGURE 8. Transmission electron microscopic view of the regenerated mucosa of the reconstructed segment (center) 12 months after reconstruction in the MSC group (A). In the area where ciliated cells were absent, the luminal surface was covered with the squamous cells (B).

the other 6 tubes became infected. In 2001, in vitro–cultured tracheal epithelial cells were successfully transplanted onto a prefabricated capsule pouch surface with fibrin glue for possible use in tracheal reconstruction.[22]

Development of tracheal cartilage has also been studied in rats by means of tissue engineering.[23] Kojima and coworkers[24] have successfully constructed an autologous tissue–engineered trachea with sheep nasal chondrocytes and used it to reconstruct the trachea of 6 sheep. However, because of malacia and stenosis, their animals survived only 2 to 7 days after surgery. Okamoto and coworkers[25] have made tracheal cartilage using bone morphogenetic protein 2 in dogs. In their canine model, a 1 × 5-cm slit defect of the tracheal cartilage was created in the trachea in advance, while carefully preserving the tracheal mucosa. Subsequently, regeneration of the cartilage was recognized around the stumps of the resected cartilages. Although these tissue engineering trials have met with some limited success, achievement of a long-term durable tracheal cartilage with mechanical properties similar to those of native tracheal cartilage still seems a long way off.

In situ tissue engineering is a novel method wherein tissue is formed not in vitro previously, but in the patient's body.[26] That is to say, in conventional tissue engineering, a required tissue has to be made previously on an incubation dish in the laboratory room. By contrast, in in situ tissue engineering, a scaffold of tissue is placed directly at the site of a tissue defect in the body where regeneration of the tissue is required.

In our early experiment, in which a prototype Y-shaped tracheal prosthesis was simply coated with amorphous collagen alone, 13 of 20 dogs died within 11 days after tracheal reconstruction.[7] The main reason for these early postoperative deaths was air leakage from the trunk of the prosthesis. To address this problem, we[27] improved the structure of the tracheal wall by increasing the amount of collagen through introduction of a porous collagen layer that was laid over the amorphous collagen layer. This improvement made the surgical procedure easier, and neither fibrin glue nor pledget sealing was necessary at the anastomosis sites during the operation.

For evaluation of functional recovery of the reconstructed tracheal wall, we measured the CBF of the regenerated

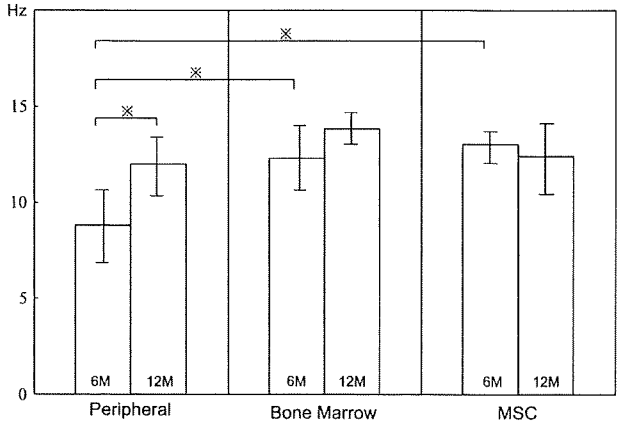

FIGURE 9. Cilial beat frequency of the regenerated cilia. *MSC*, Mesenchymal blood cell.

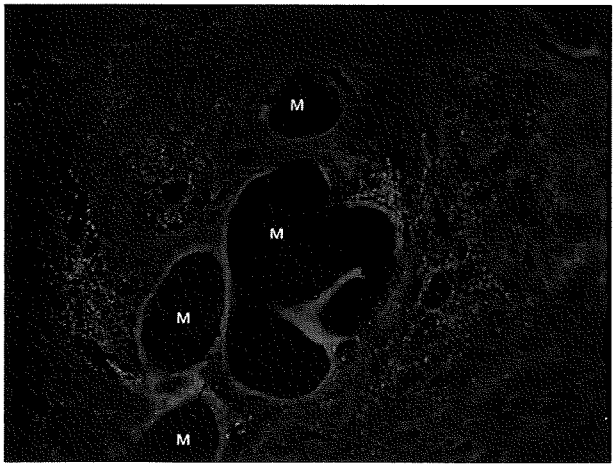

FIGURE 10. Microscopic appearance of the regenerated part of the tracheal wall after 1 month, showing numerous seeding MSCs, which are labeled with FM-DiI fluorescent tracer (original magnification, ×100). The labeled cells remained in the submucosa tissue around the polypropylene mesh and did not exist in the mucosa. *M*, Mesh of the prosthesis).

tracheal epithelium. Cilia play a major role in preserving the functional integrity of the airway, and CBF has been used as a marker of tracheal function in the field of pulmonology.[28] The CBF value of the regenerated cilial area was 80% to 90% of the normal value in the bone marrow and MSC groups at 6 months and significantly higher than that in the peripheral blood group, although by 12 months the difference among the three groups was not significant. This may indicate that the reconstructed area had recovered not only morphologically but also functionally.

In our previous series of experiments, we[2-6] had soaked the collagen sponge of the prosthesis with peripheral blood during the operation. Because collagen has high affinity for platelets,[29] we expected that the platelets attached to the collagen might provide platelet-derived growth factor or other active molecules that would help to enhance tissue regeneration. In the present study, in addition to peripheral blood used previously, we applied two other types of soaking medium: a bone marrow aspirate and a suspension of incubated bone marrow–derived cells. The latter cells have a tendency to attach to the bottom of the incubation bottle. Inasmuch as these cells have the potential to differentiate into several types of mesenchymal tissue, such as muscle, fat, and bone, they have been called multipotential bone marrow–derived cells or MSCs.[30] These cells have already been applied clinically as a cell source for tissue engineering.[31]

The results obtained in the bone marrow aspirate and MSC groups were better than those in the peripheral blood group. Mesh exposure and luminal stenosis occurred in only 2 dogs of the former two groups. These complications were milder in degree than those in the peripheral blood group. There was no significant difference in performance between the bone marrow aspirate group and the MSC group. In contrast, in the peripheral blood group, small areas of either mesh exposure or luminal stenosis occurred in all of the dogs but did not threaten the general condition of the animals or cause any significant problems. Evaluation of the functional recovery of the reconstructed site in terms of cilial movement revealed a similar tendency. Therefore, the application of bone marrow and MSCs to our prosthesis seemed to facilitate healing of the reconstructed tracheal region.

In the present study, we found that the implanted MSCs proliferated into the tracheal tissue. However it still remains unclear whether the cells in the soaked bone marrow aspirate also proliferated into the tracheal tissue. It will also be necessary to carry out further studies of the mechanism whereby soaked cells assist in tracheal remodeling on the collagen scaffold.

From a clinical viewpoint, use of a patient's own bone marrow aspirate seems to have many potential advantages. Aspiration of 2 mL of bone marrow from an anesthetized patient is relatively easy in the operating room and requires no incubation process to encourage cell proliferation. Eliminating the use of fetal bovine serum also obviates the risk of other unknown viral infections. In this regard, the application of MSCs in this experiment or iPS (induced pleuripotent) stem cells drawing current interest of the world have the same disadvantage; their clinical safety in terms of virus infection or tumorigenicity has not been established, inasmuch as fetal bovine serum or other nonautologous substances are now indispensable for preparation of such MSCs as well as iPS (induced pleuripotent) stem cells.[32]

In conclusion, although further long-term observation is required, our tracheal prosthesis appears promising for the repair of tracheal defects, and the application of autologous bone marrow may be clinically most feasible to assist the regeneration of tracheal tissue.

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The Laryngoscope

Chronic Vocal Fold Scar Restoration With Hepatocyte Growth Factor Hydrogel

Yo Kishimoto, MD; Shigeru Hirano, MD, PhD; Yoshiharu Kitani, MD; Atsushi Suehiro, MD; Hiroo Umeda, MD, PhD; Ichiro Tateya, MD, PhD; Shin-ichi Kanemaru, MD, PhD; Yasuhiko Tabata, PhD, DMedSci, DPharm; Juichi Ito, MD, PhD

Objectives/Hypothesis: Therapeutic challenges exist in the management of vocal fold scarring. We have previously demonstrated the therapeutic potential of hepatocyte growth factor (HGF) in the management of acute phase vocal fold scarring using a novel hydrogel-based HGF drug delivery system (DDS). However, the effect of HGF on matured vocal fold scarring remains unclear. The current study aims to investigate the effect of HGF-DDS on chronic vocal fold scarring using a canine model.

Study Design: Animal model.

Methods: Vocal folds from eight beagles were unilaterally scarred by stripping the entire layer of the lamina propria; contralateral vocal folds were kept intact as normal controls. Six months after the procedures, hydrogels (0.5 mL) containing 1 μg of HGF were injected into the scarred vocal folds of four dogs (HGF-treated group). Hydrogels containing saline solution were injected into the other four dogs (sham group). Histological and vibratory examinations on excised larynges were completed for each group 9 months after the initial surgery.

Results: Experiments conducted on excised larynges demonstrated significantly better vibrations in the HGF-treated group in terms of mucosal wave amplitude. Although phonation threshold pressure was significantly lower in the HGF-treated group compared with the sham group, no significant differences were observed in the normalized glottal gap between HGF-treated and sham groups. Histological examinations of the HGF-treated vocal folds showed reduced collagen deposition and less tissue contraction with favorable restoration of hyaluronic acid.

Conclusions: Results suggest that administration of HGF may have therapeutic potential in the treatment of chronic vocal fold scarring.

Key Words: Chronic vocal fold scarring, drug delivery system, hepatocyte growth factor.

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INTRODUCTION

There continue to be therapeutic challenges in the management of vocal fold scarring.[1] Vocal fold scarring occurs following injury, inflammation, or phonosurgery and disrupts the layered structure of the lamina propria altering the biomechanical properties of the vocal fold. Vocal fold scarring often causes glottal insufficiency and severe intractable dysphonia.

Previous histologic studies[2–5] on vocal fold scarring have revealed changes in the organization and distribution of extracellular matrix components (ECM), such as dense and/or disorganized type I collagen deposition, decreased elastin and decorin, increased fibronectin, and occasional decreases in hyaluronic acid (HA). These results confirmed the aberrant synthetic phenotype of vocal fold scar fibroblasts.[6] Given that these histological changes stiffen the properties of the vocal fold, phenotypic changes of vocal fold fibroblasts and a correction of the distribution of ECM components is needed to restore the vocal fold after scarring.

Hepatocyte growth factor (HGF) is a multifunctional polypeptide that plays a significant role in embryogenesis, angiogenesis, organ regeneration, and wound healing.[7] HGF has strong antifibrotic potency and has been shown to contribute to the prevention or complete resolution of fibrosis in the liver, kidney, and lung in animal models.[7] Another study has shown the therapeutic potential of HGF in the management of vocal fold scarring by demonstrating that HGF can increase HA production and decrease collagen production in vocal fold fibroblasts.[8]

From the Department of Otolaryngology–Head and Neck Surgery, Graduate School of Medicine, Kyoto University, Kyoto, Japan (Y.KISHIMOTO, S.H., Y.KITANI, H.U., I.T., J.I.); the Department of Otolaryngology, Vanderbilt University Bill Wilkerson Center for Otolaryngology and Communication Sciences, Nashville, Tennessee, U.S.A. (A.S.); the Department of Otolaryngology, Head and Neck Surgery, Kitano Hospital, Tazuke Kofukai Medical Research Institute, Osaka, Japan (S.-I.K.); and the Department of Biomaterials, Field of Tissue Engineering, Institute for Frontier Medical Sciences, Kyoto University, Kyoto, Japan (Y.T.).

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Send correspondence to Shigeru Hirano, Department of Otolaryngology–Head and Neck Surgery, Kyoto University Graduate School of Medicine, 54 Kawahara-cho, Shogoin, Sakyo-ku, Kyoto 606-8507, Japan. E-mail: hirano@ent.kuhp.kyoto-u.ac.jp

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We have previously shown the therapeutic potential of HGF in the management of vocal fold injury at acute phase.[9–11] Although those studies have shown the therapeutic potential of HGF, incomplete restoration of scarred tissue and individual variability of these effects were also reported.[11,12] These effects were attributed to insufficient retention time of HGF in the injected site, as the biological activity of HGF may be limited due to rapid dispersal by diffusion. To overcome this limitation and enhance the effect of HGF, we have developed a novel drug delivery system (DDS) for HGF using a gelatin hydrogel.[12] The previous study, however, revealed only the inhibiting effect on scar formation at the acute phase, and the effect of HGF on matured, chronic vocal fold scarring remains unclear. Given the clinical use of HGF it is important to determine if it has a restorative remodeling effect on chronic vocal fold scarring. The current study aims to investigate the effect of HGF-DDS on chronic vocal fold scarring using a canine model.

MATERIALS AND METHODS

Animals

Eight beagles weighing 10 to 17 kg were used in this study. All experimental protocols were approved by the Animal Committee of the Graduate School of Medicine, Kyoto University. Animal care was provided under the supervision of the Institute of Laboratory Animals of the Graduate School of Medicine, Kyoto University.

Preparation of HGF Hydrogel

Biodegradable hydrogels were developed by the Department of Biomaterials, Field of Tissue Engineering, Institute for Frontier Medical Sciences, Kyoto University.[13,14] The hydrogel was constituted by chemically cross-linking acidic gelatin with glutaraldehyde. A 50-mL quantity of acidic gelatin aqueous solution (5% w/w) was mixed with 50 μL of glutaraldehyde aqueous solution (25% w/w) to give a final concentration of 6.25 mM. The water content of the hydrogel was 94.8%. A solution of 1 μg of HGF (Human recombinant HGF; PeproTech Inc., Rocky Hill, NJ) in 20 μL of phosphate buffered saline (PBS) was dripped onto the gelatin hydrogels and left overnight at 4°C to create the HGF hydrogel.

Surgical Procedure

The surgical procedures that were used for generating the vocal fold injury models had been established in previous studies.[11,12] All animals were sedated under general anesthesia with intramuscular injections of ketamine hydrochloride (15 mg/kg) and xylazine hydrochloride (6 mg/kg). The glottis was visualized using a direct laryngoscope, and the vocal folds were unilaterally scarred by stripping the entire layer of the lamina propria down to the muscle. The contralateral vocal folds were kept intact as normal controls. The sides for scarring were randomly selected.

After stripping the vocal fold lamina propria of test animals, vocal fold scars were allowed to mature for 6 months. This period of vocal fold scar maturation was based on data from Rousseau et al., who proposed that it takes 6 months for vocal fold scarring to mature in canine and rat models.[3,4] Six months after the procedure, 0.5 mL of hydrogel solution containing 1 μg of HGF was injected into the scarred vocal folds of four dogs (HGF-treated group) using a transoral intracordal injector, and 0.5 mL of hydrogel solution containing 1 μg of PBS was injected into the scarred vocal folds of the four dogs in the sham group. Because HGF was expected to act on the fibroblast in the lamina propria, hydrogel was carefully injected into the subepithelial layer of the vocal fold. To enhance the effect of injection, the injection was performed twice at an interval of 1 month. It is reported that HGF acts on some kinds of cells in an autocrine manner,[15–17] and vocal fold fibroblasts may be similar to those cells. Thus, the effect of HGF might continue for some time after the administration and release period. For this reason, we set the interval to 1 month, which is longer than the 2-week release period.

All animals were euthanized 9 months after the surgery by intracardiac injection of Nembutal. The larynges were harvested and used for vibratory examinations then subjected to histological examination.

Setup for Vibratory Examination of Excised Larynges

Vocal fold vibration was examined with an excised larynx setup developed in previous studies.[10,12] For better visualization of the vocal folds supraglottic structures, including the epiglottis, false vocal folds and aryepiglottic folds were removed after resection of the superior portion of the thyroid cartilage. The arytenoid cartilages were sutured together, and an arytenoid adduction procedure was bilaterally performed using a 3-0 Prolene suture to close the glottis. The larynx was mounted on a table and an intubation tube was inserted into the trachea and tightly clamped. Air was pumped through the tube to generate vocal fold vibrations. During the vibratory examination, saline was dripped onto the vocal folds to prevent dehydration. A pressure sensor (PG-100; Nidec Copal Electronics Corp., Tokyo, Japan) was inserted into the tube to monitor subglottic pressure, and a high-speed digital imaging system (MEMRECAMci; NAC Image Technology, Osaka, Japan) was used to record vocal fold vibrations from the superior view. The camera was mounted 50 cm above the larynx, and the image was displayed on a monitor. The images were recorded at a frame rate of 1,000 frames per second, which is the maximum rate to give an acceptable resolution level in our equipment, and the images were then scanned into a computer.

As an indirect measurement, we used phonation threshold pressure (PTP) to evaluate the mucosal vibration. PTP, which is regulated by factors such as vocal fold thickness, property, and glottal width, is defined as the minimum pressure required to initiate phonation.[18,19] Further, the amplitude of the mucosal wave and glottal gap were measured using image analysis software (Scion Image beta4; Scion Corp., Frederick, MD). The distance (d1) from the midline of the glottis to the free edge of the vocal fold was measured at the anteroposterior middle portion of the vocal fold during the closed phase. Closed phase was recognized by the motion of the upper and lower lips of the vocal folds. The same distance (d2) was measured at the maximum open phase. The mucosal wave amplitude was defined by subtracting d1 from d2 and the amplitude ratio was derived by dividing the amplitude in the HGF-treated side by the amplitude in the normal side. The following formula was used: amplitude ratio (AR) = (d2−d1 in the HGF-treated side)/(d2−d1 in the normal side). The glottal gap was examined from the images during the closed phase. The length (L) from the anterior commissure to the vocal process and the glottal area (a) were measured, and the glottal area was normalized by dividing it by L^2. The following formula was used: normalized glottal gap (NGG) = $a/L^2 \times 100$ unit (u).

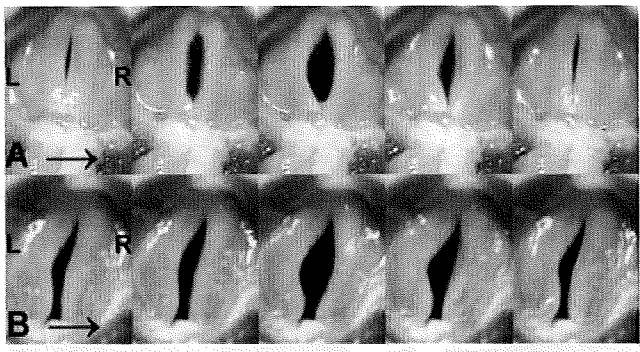

Fig. 1. Vibratory patterns experimentally generated from treated vocal folds of excised larynges. In both cases the left vocal fold was scarred. The hepatocyte growth factor-treated vocal fold showed almost normal mucosal vibration (A), however the sham-treated vocal fold was bowed and mucosal vibration was limited (B).

Histological Examination

Collagen, elastin, and HA in the lamina propria of each vocal fold were examined using light microscopy. The thickness of the lamina propria was also assessed to determine the degree of scar contraction. The thickness of the lamina propria was determined by measuring the distance from the free edge of the vocal fold down to the thyroarytenoid muscle and normalized by dividing the distance on the treated side (t1) by that of the normal side (t2). The following formula was used: normalized thickness of lamina propria (NTLP) = t1/t2.

Immediately following the vibratory examinations, the larynges were fixed in 10% formaldehyde for later tissue examination. Larynges were subsequently embedded in paraffin, and 5-μm-thick serial sections were prepared in the coronal plane from the anteroposterior middle portion of the vocal folds.

Elastica van Gieson staining was performed to identify collagen and elastin. Alcian blue staining was used to identify HA. A hyaluronidase digestion technique was used to detect HA. Images were captured with a BIOREVO BZ-9000 microscope (Keyence Corp., Osaka, Japan).

These assessments were performed in a blinded fashion, in which the examiners were not informed which slide belonged to each group.

Statistical Analysis

An unpaired t test was used to ascertain differences in PTP, AR, NGG, and NTLP between treatment groups. A P value $< .05$ was considered statistically significant.

RESULTS

Vibratory Examinations

The experiments on excised larynges showed better mucosal vibration in the HGF-treated group, as compared with the sham group. Figure 1 shows representative cases in the HGF-treated group (Fig. 1A) and in the sham group (Fig. 1B). Injured vocal folds were bowed and the mucosal vibration was limited in the sham group; however, their vibration was comparable to the uninjured side in the HGF-treated group.

An unpaired t test revealed significantly lower PTP in the HGF-treated group, compared with the sham group (Fig. 2A, $P = .015$). Although no significant differences were observed for NGG between the two groups (Fig. 2B), AR was significantly higher in the HGF-treated group compared to the sham group (Fig. 2C, $P = .012$).

Histological Examinations

Histological examinations revealed better restoration and less tissue contraction in the HGF-treated vocal fold compared with the sham-treated vocal fold.

Disorganized collagen deposition was found to be minimal in the HGF-treated vocal fold (Fig. 3A, 3B), whereas there was excessive collagen deposition in the sham-treated vocal fold (Fig. 4A, 4B). Elastin and HA in the HGF-treated vocal fold appeared to be well

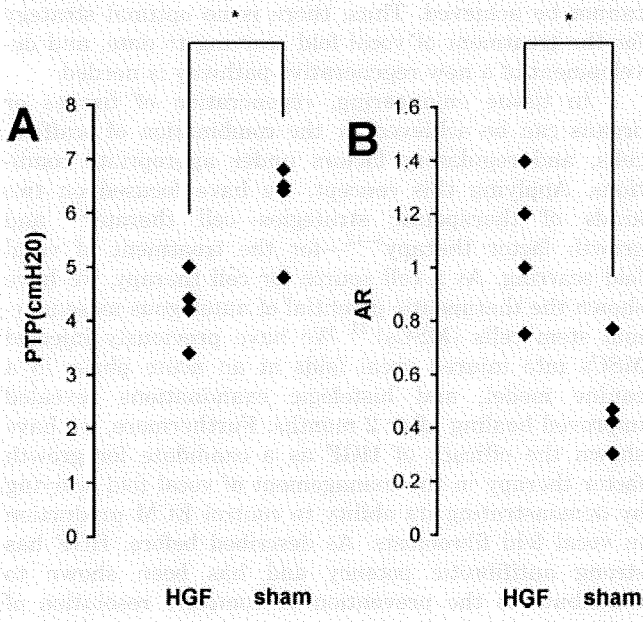

Fig. 2. Results of vibratory examinations. The hepatocyte growth factor (HGF)-treated group demonstrated significantly lower phonation threshold pressure (PTP) (A) and higher amplitude ratio (AR) (B) compared with the sham-treated group. No differences were observed for normalized glottal gap (NGG) between the two groups (C). * $P < .05$.

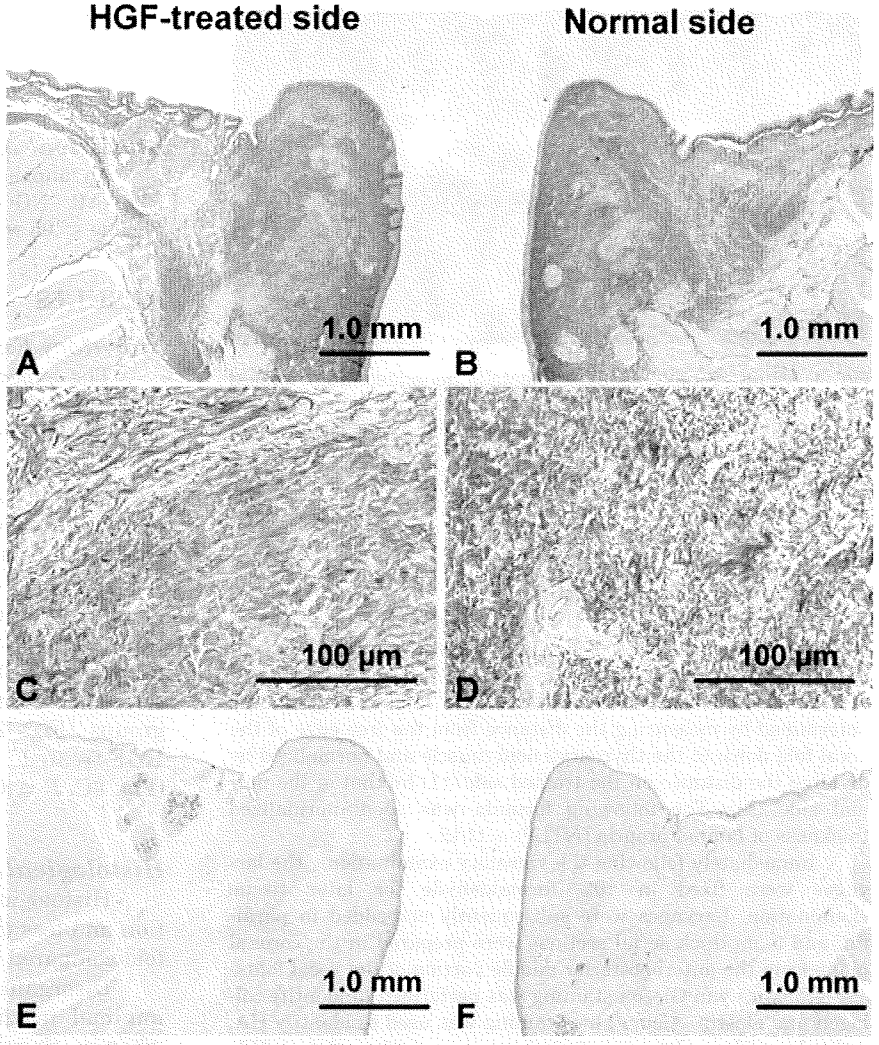

Fig. 3. Histologic findings in the hepatocyte growth factor (HGF)-treated group. (A–D) Elastica van Gieson stain. (E–F) Alcian blue stain. Tissue contraction and collagen deposition were found to be minimal (A, B), and elastin (C, D) and hyaluronic acid (E, F) were favorably restored.

organized, similar to that of the unscarred contralateral side (Fig. 3C–3F), whereas both were decreased in the sham-treated vocal fold (Fig. 4C–4F). There were no remarkable findings in terms of the underlying muscle.

NTLP was close to normal in the HGF-treated vocal folds, whereas NTLP was significantly thinner in the sham group as compared with the HGF-treated group (Fig. 5, $P = .03$).

DISCUSSION

The restoration of normal vocal fold properties is essential to the treatment of vocal fold scarring. With the advancement of phonosurgery, most voice disorders have been overcome, and many therapeutic strategies, including medialization thyroplasty, fat/collagen injection, and scar dissection have been tried in an attempt to restore normal properties to scarred vocal folds.[20] Medialization thyroplasty and fat/collagen injection result in augmentation effects that improve glottal insufficiency and facilitate entrainment of vocal fold vibrations. However, the restoration of normal vocal fold properties is not achieved by these treatments. The effect of scar dissection depends on the individual's healing ability, and stable outcomes with this approach cannot be achieved. Thus, there is no optimal strategy for the treatment of vocal fold scarring to date, and development of a new regenerative pathway is needed.

In tissue engineering, regeneration of tissues or organs can be achieved by the combination of scaffold, cells, and regulatory factors under appropriate conditions. Applying this concept, we have focused on two kinds of therapeutic strategies—cell therapy[21] and growth factor therapy[8–12]—for the treatment of vocal fold scarring. As a cell source for cell therapy, we have shown the therapeutic potential of autologous mesenchymal stem cells (MSCs).[21] We have previously injected MSCs into injured vocal folds at an acute phase in a canine model, and histologic examinations revealed improved healing after 2 months. Furthermore, we have shown the efficacy of HGF as a candidate for growth factor therapy in the management of vocal fold scarring by demonstrating its ability to control ECM production in vocal fold fibroblasts. As described before, HGF has strong antifibrotic potency and has been shown to contribute to the prevention or complete resolution of

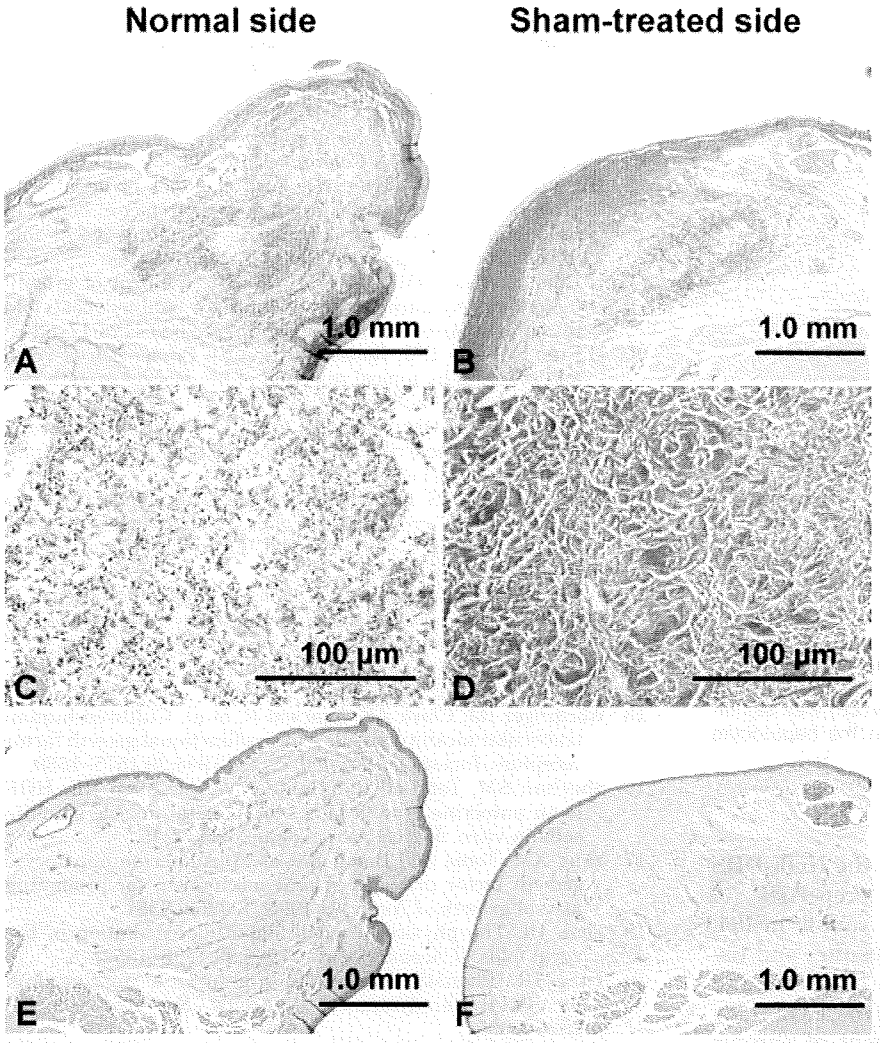

Fig. 4. Histologic findings in the sham-treated group. (A–D) Elastica van Gieson stain. (E–F) Alcian blue stain. Severe tissue contraction and excessive collagen deposition were observed in sham-treated vocal folds (A, B). Elastin (C, D) and hyaluronic acid (E, F) were decreased in the superior portion of the treated vocal fold.

fibrosis in some organs.[7] Hirano et al. reported the effects of HGF for the treatment of acute vocal fold injury using canine[11] and rabbit[10] models. In these studies, HGF was injected into injured vocal folds, and histological examination revealed reduced collagen deposition and decreased tissue contraction of the lamina propria in HGF-injected vocal folds as compared with saline-injected controls. However, these previous studies revealed only the inhibiting effect on scar formation at the acute phase of wound healing, and it is not clear whether these approaches have a restorative effect on the aberrant synthetic phenotype of vocal fold fibroblasts. In the current study we have investigated the effect of HGF administration on chronic matured vocal fold scarring.

A biodegradable hydrogel developed to enhance the in vivo regenerative effects of growth factors, such as HGF, basic fibroblast growth factor, platelet-derived growth factor, and epidermal growth factor, has been shown to be successful in the controlled release of biologically active growth factors in other parts of body.[13,14,22–24] In this system, HGF was embedded in gelatin hydrogel and gradually released in a continuous fashion over a 2-week period in vivo.

This study represents the first investigation of HGF for the treatment of matured, chronic vocal fold scarring in vivo. The vibratory experiments in the present study showed significant improvement of mucosal vibration in terms of PTP and AR in the HGF-treated group compared with the sham group. As mentioned above, PTP is regulated by the vocal fold property and glottal gap. Improvement in PTP without smaller NGG indicates that administration of HGF restored the scarred vocal fold in terms of stiffness and tissue contraction. Histological examination also showed positive restorative effects with the administration of HGF, including reduced collagen deposition, less tissue contraction, and improved restoration of elastin and HA. These results suggest that HGF-DDS has restorative remodeling effects on chronic vocal fold scarring; however, there was still individual variability and complete restoration could not be achieved. Particularly, a possible reason for incomplete improvement of glottal gap may be insufficient volume obtained in the treated vocal folds. Here may be some limitations in growth factor therapy, which warrant a combined use of cells and/or appropriate scaffoldings to obtain adequate tissue volume and function.

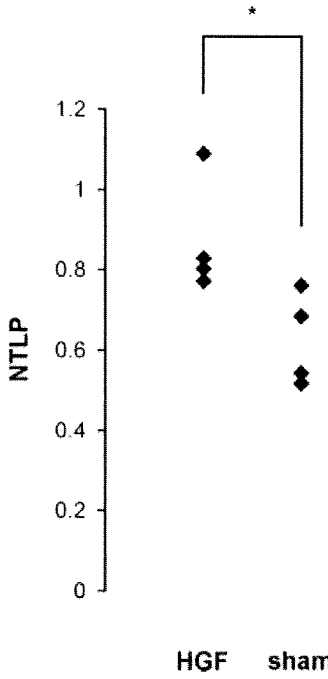

Fig. 5. Normalized thickness of lamina propria (NTLP) was significantly thinner in the sham-treated group than in the hepatocyte growth factor (HGF)-treated group. *$P < .05$.

CONCLUSION

The present study demonstrated that the HGF-DDS significantly improved the vibratory properties of matured, chronic vocal fold scarring in a canine model. HGF-DDS reduced excessive collagen deposition and tissue contraction with favorable restoration of elastin and HA. Results suggest that administration of HGF may have therapeutic potential in the treatment of chronic vocal fold scarring.

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