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.

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LARYNGOLOGY

Expression of extracellular matrix proteins in the vocal folds and bone marrow derived stromal cells of rats

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Abstract Vocal fold scarring remains a therapeutic challenge. Our research group has indicated that bone marrow-derived stromal cells (BSCs) may have therapeutic potential in restoration of injured vocal folds. However, it is still unclear how BSCs restore the viscoelasticity of vocal fold mucosa. Since a feature of vocal fold scarring is the disorganization of the extracellular matrix (ECM), it is important to understand how BSCs produce ECM. The present study aimed to clarify ECM gene expression in BSCs, and also examined the effects of hepatocyte growth factor (HGF) on this expression. BSCs obtained from the femurs of four Sprague–Dawley rats were cultured with or without HGF. The mRNA expression of ECM components (type I procollagen, decorin, Has2, CD44, MMP-1, and GAPDH) were examined in cultured BSCs and the vocal fold mucosa by the reverse transcription-polymerase chain reaction (RT-PCR). The mRNA expression of Has2 and MMP-1 was significantly stronger in BSCs than in the vocal folds ($P < 0.05$). Expression of Has2 in BSCs was significantly increased by the administration of HGF ($P < 0.05$). There was no significant difference in the gene expression of other ECM molecules between BSCs and vocal fold mucosa. Increased expression of Has2 and MMP-1 genes from BSCs may have a positive potential in the treatment of vocal fold scarring.

Keywords Vocal fold scarring · Hepatocyte growth factor · Bone marrow-derived stromal cells · Extracellular matrix

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Introduction

Vocal fold scarring occurs following injury or inflammation to the vocal fold. Scarring results in a disruption of the structure of the lamina propria, which is essential for optimal vibration. It also changes the viscoelasticity of the vocal fold mucosa and often results in severe and intractable dysphonia. Although various injectable materials such as bovine autologous/homologous collagen, autologous fat and mitomycin-C have been used in an attempt to treat scarring [1–5], treatment remains difficult.

Previous animal studies on vocal fold scarring have reported deterioration in the organization and distribution of various extracellular matrix (ECM) components, including dense and/or disorganized type I collagen deposition, decreased elastin and decorin, increased fibronectin, and occasional decreases of hyaluronic acid (HA) [6, 7]. Restoration of ECM distribution is essential for achieving adequate regeneration of scarred vocal folds. Of all the ECM components, HA has been regarded as a key molecule for the maintenance of optimal viscoelasticity of the vocal fold. This has led to the concept that administration or increase of HA may be useful for softening scarred vocal folds [8, 9].

Hepatocyte growth factor (HGF) has strong antifibrotic activity that has been shown to contribute to the prevention or the complete resolution of fibrosis in liver, kidney and

lung in animal models [10]. The senior author in this study has suggested that HGF may also have a therapeutic potential in restoring scarred vocal folds since HGF increased HA production and decreased collagen production in vocal fold fibroblasts [11, 12].

Mesenchymal stem cells (MSCs) are pluripotent cells with the potential to differentiate into chondrocytes, osteoblasts, adipocytes and other tissues of mesenchymal origin [13, 14]. Bone marrow-derived stromal cells (BSCs) are easily accessible from bone marrow aspirates and are enriched sources of MSCs. Using a canine model, we examined the effects of local injection of cultured BSCs into injured vocal folds on tissue regeneration of the mucosa. Our results showed that the group injected with BSCs showed better wound healing and regeneration of the injured mucosa both morphologically and histologically [15]. However, it is still unclear how injected BSCs work in regeneration of the vocal fold. Our previous study revealed that injected BSCs differentiated into epithelial and mesenchymal cells in the vocal fold, suggesting that this multipotency may contribute to regeneration of vocal fold tissue [16]. Another important aspect may be the effect of BSCs on ECM reorganization, since ECM organization determines the tissue properties of the vocal fold; however, it is still unknown how BSCs influence ECM deposition. The present study aimed to clarify gene expression of the ECM in BSCs and also addressed how HGF influences gene expression in BSCs.

Materials and methods

Animals

Four Sprague–Dawley rats, aged 8 weeks, were used in this study (Japan SLC Inc., Hamamatsu, Japan). All experimental protocols were approved by the Animal Research Committee of the Graduate School of Medicine, Kyoto University, Japan. Animal care was under the supervision of the Institute of Laboratory Animals of the Graduate School of Medicine, Kyoto University, Japan.

Isolation and culture of rat bone marrow-derived stromal cells

Animals were anesthetized by intramuscular injection of ketamine hydrochloride (75 mg/kg) and xylazine hydrochloride (9 mg/kg). The femurs of each animal were exposed and a 1 mm diameter hole was drilled using a diamond bur. The medullary cavity was aspirated and 0.1 ml of bone marrow was collected. Marrow cells were cultured in a 10 cm dish with 10 ml Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and antibiotics, at 37°C under 5% CO_2. The medium was changed twice a week until the cells were 80% confluent. Non-adherent cells, such as hematogenic cells, were removed during the medium change procedure and adherent cells were collected. We investigated the expression of ECM proteins in primary culture cells with no passaging (P0), secondary culture cells after one passage (P1), and secondary culture cells with HGF (100 ng/ml) for 24 h (P1 + HGF).

Harvest of rat vocal fold mucosa

Animals were euthanized by intracardiac injection of Nembutal. The larynx was immediately dissected out and the lamina propria and epithelium of the vocal fold were removed using a microscope.

Gene expression analysis

Extraction of total RNA

Rat vocal folds and BSCs were homogenized using a Mixer Mill MM 301 (F. Kurt Retsch GmbH & Co. KG, Haan, Germany), and total RNA was isolated using an RNeasy Mini Kit (Qiagen, Valencia, CA, USA). The quantity of total RNA was determined using the A260/A280 ratio, and the quality was evaluated based on the appearance of the 18S and 28S ribosomal RNA bands on electrophoresis.

Complementary DNA (cDNA) synthesis

To eliminate genomic DNA contamination, total RNA was treated with DNase I (Ambion, Austin, TX, USA). The cDNA was prepared by reverse transcription (RT) using TaqMan® Reverse Transcription Reagents (Applied Biosystems, Foster City, CA, USA). The total volume of the RT reaction was 50 μl and the final concentrations of the reagents in the reaction mixture were as follows: RT buffer (1×), $MgCl_2$ (5 mM), deoxynucleoside triphosphate (dNTP) mixture (0.5 mM for each), random hexamer mix (2.5 μM), RNase inhibitor (0.4 units/μl), MultiScribe™ Reverse Transcriptase (1.25 units/μl), and 2 μL of total RNA. The reactions were performed using a GeneAmp PCR System 9700 (Applied Biosystems, Foster City, CA, USA) with the following parameters: 25°C for 10 min, 48°C for 30 min, 95°C for 5 min and 4°C for 5 min.

Primer design

The primers for type I procollagen, decorin, CD44, hyaluronic acid synthase 2 (Has 2), matrix metalloprotease-1 (MMP-1), and glyceraldehyde-3-phosphate dehydrogenase (GAPDH, a constitutively expressed gene included as a

control) were initially designed and tested elsewhere [17–19].

Primers were synthesized by Hokkaido System Science Co. Ltd, Hokkaido, Japan. All primers generated a single PCR band of the expected size and PCR products were verified by DNA sequencing. The sequences and optimal conditions of the primer sets as well as the expected sizes of the resulting PCR products are shown in Table 1.

Amplification of cDNA with polymerase chain reaction (PCR)

In order to avoid saturation effects resulting from excess cDNA template, the amount of cDNA used for each primer was first determined by an initial optimization step. Two-fold serial dilutions of cDNA were amplified simultaneously for 35 cycles. The densities of the PCR products were plotted against the amount of starting cDNA. The cDNA concentration chosen produced a PCR product that fell within the linear range before it reached a maximum plateau. GAPDH was coamplified with each experimental group to confirm the use of equal amounts of cDNA. PCR amplification was performed in a reaction volume of 50 μl, using the GeneAmp PCR System 9700. Five micro liter of each cDNA sample was amplified with 2.5 μl (0.5 μM) of target specific primers (Table 1), 5 μl of dNTPs (0.2 mM for each) and 25 U/ml Ex Taq™ polymerase (TaKaRa Bio Inc., Shiga, Japan) in PCR buffer (5 μl, Tris–HCl, pH 8.3; 50 mm KCl; 1.5 mM $MgCl_2$). The cycling conditions for all primer combinations were as follows: denaturation at 94°C for 30 s, annealing at the optimal primer temperature (Table 1) for 30 s and extension at 72°C for 1 min. All reactions were performed in duplicate. PCR products were electrophoresed through a 2% agarose gel (Wako, Osaka, Japan) containing 0.5 mg/ml ethidium bromide (Wako, Osaka, Japan). Images were captured by printgraph (Atto Bioinstrument, Tokyo, Japan) and analyzed using Scion Image (Scion Corporation, Frederick, MD, USA) on a Windows computer. The rate of gene expression was analyzed by comparing the mean band intensity normalized to the mean band intensity of GAPDH.

Statistical analysis

Statistical analysis was performed using a one-way non-parametric analysis of variance, followed by the Mann–Whitney U test. $P < 0.05$ was considered to be statistically significant.

Results

The results of the RT-PCR analysis are shown in Fig. 1. Despite differences in intensity, mRNA of type I procollagen, decorin, CD44, Has2 and MMP-1 were detected in the BSCs and vocal folds of each rat.

The mRNA expression of Has2 in the BSCs was found to be significantly stronger than that in the vocal folds, regardless of the passage of time ($P < 0.05$; Fig. 2). The mRNA expression of Has2 in the P1 BSCs with HGF (P1 + HGF) was significantly stronger than that in the P1 BSCs without HGF (P1 alone) ($P < 0.05$; Fig. 3).

The mRNA expression of MMP-1 in the BSCs was significantly stronger than that in the vocal folds regardless of the passage time ($P < 0.05$; Fig. 4). No significant difference was noted in mRNA expression of MMP-1 between P1 BSCs with or without HGF (Fig. 5).

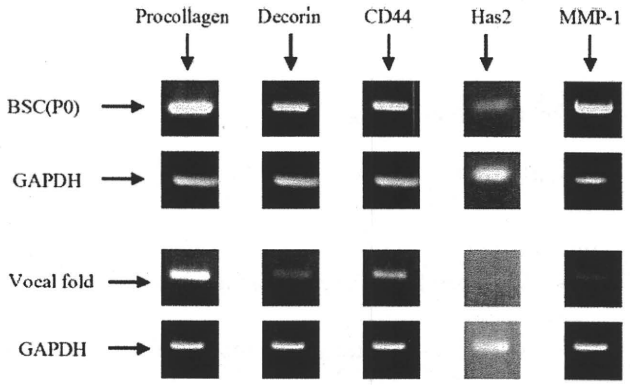

Fig. 1 The mRNA expression of type I procollagen, decorin, CD44, Has2, MMP-1 and GAPDH in BSCs and vocal folds. mRNA expression was detected in all of the samples

Table 1 Primer sequences and optimal PCR conditions

	Primers (5′–3′)		Product (bp)	$MgCl_2$ (mM)	AT (°C)
	Forward	Reverse			
Type I procollagen	cctgctggtccttctggcc	ccactctgggtggctgagtc	356	1.5	56
Decorin	tgggtctggacaaagtaccc	tcccagagtttttcagtggg	363	1.5	56
CD44	tcatgttagagcatccgtgc	gggttgtacatcatgcctcc	373	1.5	56
Has2	catgatggacatcttcagtgaag	gtgtctgagtcacacacctg	257	1.5	56
MMP-1	ttgttgctgcccatgagctt	actttgtcgccaattccagg	639	1.5	56
GAPDH	acccccaatgtatccgttgt	tactccttggaggccatgta	299	1.5	56

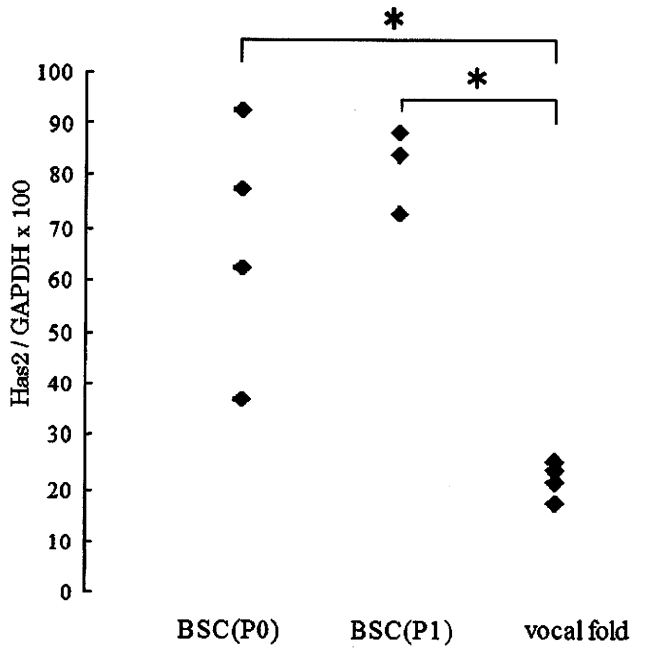

Fig. 2 Analysis of normalized mRNA expression of Has2 in BSCs and vocal folds. Expression was found to be significantly stronger in BSCs than in vocal folds regardless of the passage of time ($^*P < 0.05$)

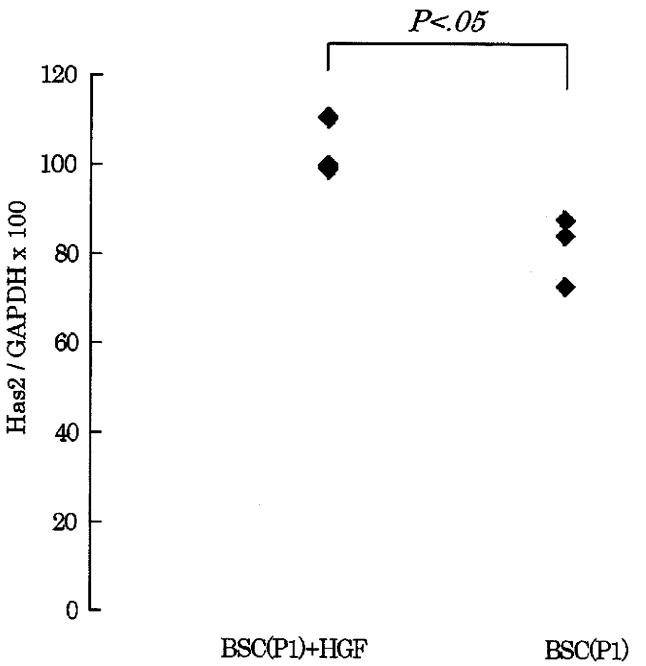

Fig. 3 Analysis of normalized mRNA expression of Has2 in P1 BSCs with or without HGF. The expression in BSCs with HGF (P1 + HGF) was found to be significantly stronger than in BSCs without HGF (P1) ($P < 0.05$)

No significant difference was found in the mRNA expression of type I procollagen, decorin, or CD44 between BSCs and vocal folds.

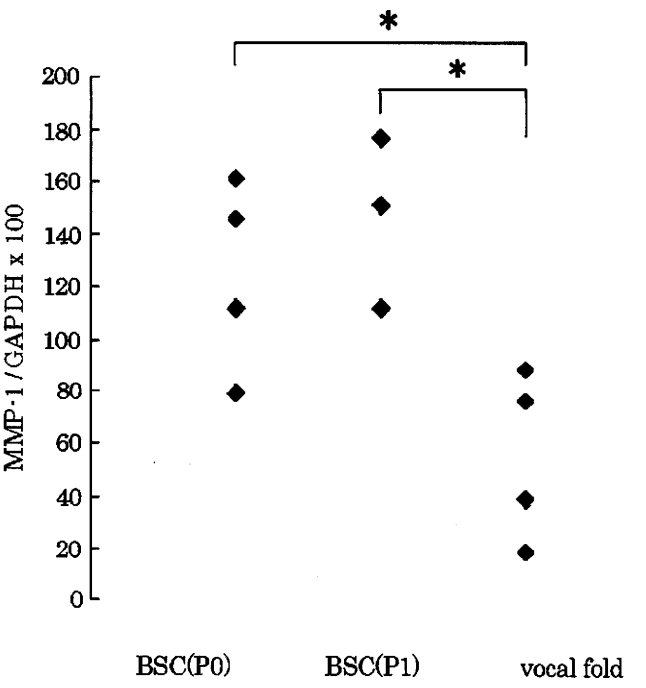

Fig. 4 Analysis of normalized mRNA expression of MMP-1 in the vocal folds and BSCs. Compared to the expression in the vocal folds, the expression in BSCs was found to be significantly stronger regardless of the passage of time ($^*P < 0.05$)

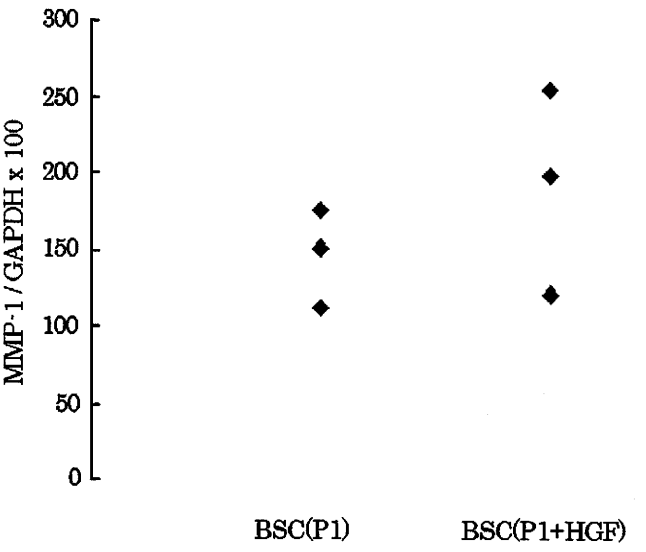

Fig. 5 Analysis of normalized mRNA expression of MMP-1 in P1 BSCs with or without HGF. No difference was detected with or without HGF ($P < 0.05$)

Discussion

Vocal fold scarring remains a therapeutic challenge. At the Chevalier Jackson Lecture at the annual meeting of the American Bronchoesophagological Association in 1995,

Hirano stated that treatment of vocal fold scarring was one of the problems that awaited improvement in the future, and that future directions would include developing techniques to replace scar tissue with normal pliable mucosa, or to soften scar tissue with medicine or physical energy [20]. Tissue engineering and regenerative medicine has helped to shed light on this field. A tissue engineering technique was proposed by Langer et al. [21] to regenerate organs or tissues. This technique was composed of three fundamental elements: cells, scaffold and growth-regulation factors. Our research group has focused on the use of cells and growth factors for regeneration of the vocal fold mucosa. Previously, we used BSCs for regeneration of injured vocal folds and found that BSCs have great potential for treatment of vocal fold scarring [15, 16].

Since the ECM is the key to determining tissue properties of the vocal fold, it is important to take into account the relevance of BSCs to ECM synthesis in order to better understand and implement cell therapy using BSCs for vocal fold scarring. This study investigated gene expression of several ECM components that have been regarded as important for the vocal fold architecture. Type I collagen is known to be responsible for the tensile strength of tissue, and dense and/or disorganized type I collagen deposition has been found in scarred vocal folds [6]. Excessive deposition of disorganized collagen may have a negative effect on vibratory properties due to increased tensile strength. Matrix metalloproteinases have various subtypes and are the major factors that degrade ECM [19]. MMP-1 (interstitial collagenase) degrades extracellular fibers comprised of types I and III collagen. It is well known that HGF enhances the activity of MMP-1 in several cells, which in turn may dissolve the fibrous matrix [22]. Decorin, a small proteoglycan, is abundant in the superficial layer of the lamina propria [8]. It binds to the surface of collagen fibrils through its core protein and affects the rate of fibril formation [23]. Decorin has been found to be decreased in scarred vocal folds of rabbits [7]. Hyaluronic acid (HA) is an important ECM component that is thought to play a major role in maintaining the viscoelasticity of the lamina propria in the vocal fold [8]. CD44 is a family of plasma membrane glycoproteins encoded by a single gene, and is known as the most abundant receptor for HA [9]. In this study, mRNA expression of Has2 and MMP-1 was significantly stronger in BSCs than in the vocal folds, whereas there was no significant difference in gene expression of the other molecules examined. These characteristics of BSCs are thought to be useful in the treatment of scarring since an increased level of HA would be expected to reduce scarring, and enhanced MMP-1 would work to dissolve deposited collagen in the lamina propria of the vocal fold. HGF has also been proven to increase the level of gene expression of Has2 in BSCs. These results suggest that the combination of BSCs and HGF could provide powerful effects for improving vocal fold scarring.

It is not certain whether implanted BSCs produce more HA and MMP-1 in vivo, due to the alteration of function in producing ECM from phenotypic changes after differentiation or mitosis. However, the genetic features of BSCs are at least in part regarded as favorable for restoration of scarred vocal folds.

Conclusion

The current study has shown that expression of Has2 and MMP-1 was significantly stronger in BSCs than in vocal folds. We also found that expression of Has2 in BSCs was significantly enhanced by administration of HGF. The genetic features of BSCs in terms of ECM production may have positive effects on treatment of vocal fold scarring.

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One Year Outcome of Damaged Lingual Nerve Repair Using a PGA-Collagen Tube: A Case Report

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Traumatic injury of the lingual nerve is in many cases iatrogenic, extraction of the third molar being the most frequent cause.[1-4] Deficits in taste, thermal and touch sensations, and abnormal sensations (eg, paresthesia and dysesthesia) are frequently caused by peripheral nerve damage, leading to difficulties in eating and/or speech. In contrast to natural improvement after slight or moderate sensory impairment in the orofacial region, several studies have reported the use of surgical intervention with either direct anastomosis or autograft to reconstruct nerve gap and/or remove neuromas.[5-10] Gap repairs often result in unsatisfactory results and donor site morbidity.

A new and effective treatment method for complete sensory loss is thus desirable. We report here a patient with lingual nerve damage and a complete deficit in lingual sensation who received surgical repair using a new artificial nerve of a polyglycolic acid (PGA) tube containing collagen (PGA-collagen tube) (Kyoto University, Kyoto, Japan). The result 1 year after operation was an excellent improvement of sensory and behavioral difficulties despite a long nerve gap, indicating that such a treatment method could demonstrate efficacy.

Report of a Case

A 30-year-old woman underwent extraction of an impacted third molar of the right mandible. Tooth extraction seemed to have been conducted without difficulty, although the patient remembered an electrical shock running in the lower jaw when local anesthesia was performed in the retromolar area. Almost 1 week later, she noticed prolonged numbness in her right lingual area. Over a period of several weeks, she began to have trouble in talking and eating because of difficult tongue movements and a slight but gradually increasing chronic lingual pain. Four months after the extraction, she was admitted to the dental hospital of our university and diagnosed with complete sensory loss in the right lingual nerve. The surface of her tongue looked flat and white and the taste buds were completely lost on the right side of the tongue (Fig 1). Tongue movements were less controlled, resulting in difficulties in talking and eating, but not dysphasia. She also noticed the loss of the taste sensation on the right side of her tongue that contrasted to a normal feeling on the other side. She suffered from chronic pain in the tongue.

A quantitative sensory test was conducted according to previously described methods.[11] Briefly, brush stroke perception was evaluated as follows: the patient was blindfolded and asked to respond to a light touch brush stroke direction (eg, anterior, posterior, rightward, or leftward). Perception was assessed and calculated from successful rates of 15 trials. The mechanical touch threshold was measured using Semmes-Weinstein monofilaments. The pa-

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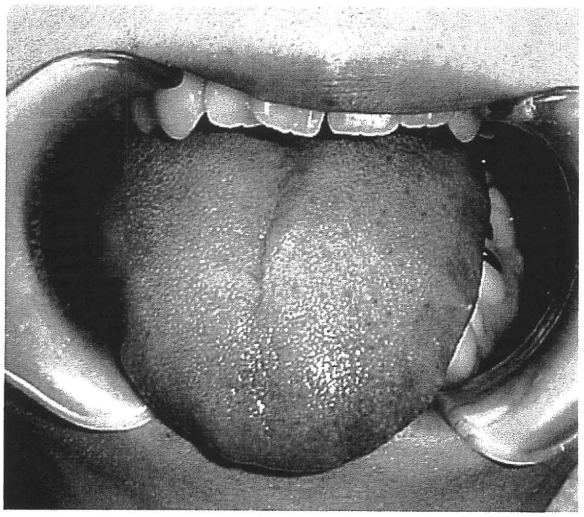

FIGURE 1. Photograph of the patient's tongue at first admission. Taste buds on the right side are completely diminished.

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tient was also blindfolded and asked to declare when she felt the filament attachments on her tongue. The value for each tongue site was determined by calculating the average of 2 or 3 descending and ascending series trials applied to the same site. Application of a thermal applicator to the tongue for just a few seconds only, to prevent tissue damage, was performed to estimate ability to perceive 60°C. Two-point discrimination was estimated by the minimum perception length required for a 2-touch test as 2 separate points when 2 filaments were applied to the tongue surface in different parts or directions, and the average of minimum lengths from 5 trials was calculated. Taste sensitivity was evaluated by an electrogustometer (TR-06, Rion Co Ltd, Tokyo, Japan) and was compared with the corresponding part on the opposite side of the tongue.

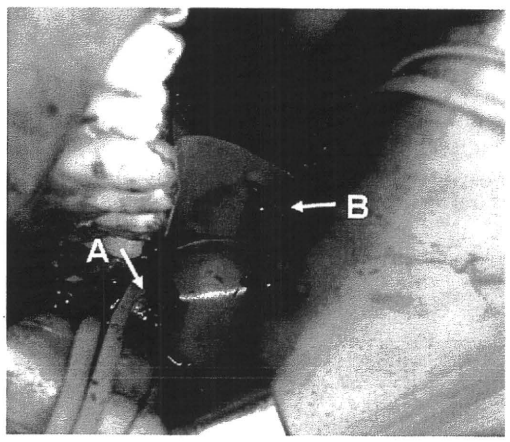

FIGURE 2. Intraoral view of the patient just before repair. The left side represents the maxilla and the right side the mandibular region. Two stump-like enlargements of the cut nerve end are observed. Arrows *A* and *B* indicate the neuroma on the proximal and distal sites, respectively, of the damaged lingual nerve.

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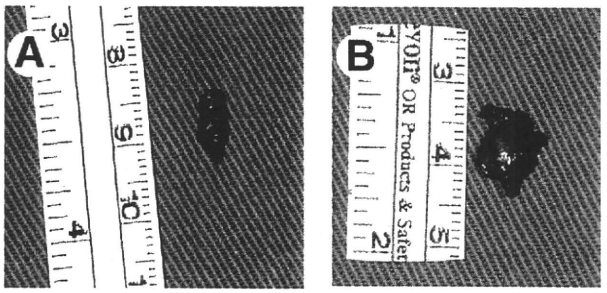

FIGURE 3. Neuromas resected from the distal (*A*) or proximal (*B*) end of the injured lingual nerve.

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At first admission, all tests showed complete sensory loss in the anterior part of the right side of the tongue. The patient could not correctly detect any moving direction in any part on the right side of the tongue, or recognize between 2 points using the 2-different touch application test. As well, mechanical touch threshold was out of scale and the patient could not detect heat even at 60°C. These results strongly suggested the complete loss of continuity of the lingual nerve; therefore, surgical intervention was recommended. Informed consent was obtained from the patient to use a new PGA-collagen tube on the damaged part of the lingual nerve.

The operation was conducted under general anesthesia 9 months after the extraction. The anterior lingual ridge of ramus was slightly reduced for a wide view of the lingual space (Fig 2). The medial end of the lingual nerve was found in the inside lower part of ramus. This was shaped like a sphere, forming a neuroma of 7 mm in diameter (Fig 3B). By contrast, the distal end was attached to the periosteum of the lingual mandible close to a canine region and its shape resembled a stump, also forming a neuroma of 7 × 3 × 4 mm (Fig 3A). These resected nerve ends were located with an over 40 mm interval between them.

Neuromas were removed from each end of the lingual nerve, and the freshly cut ends inserted and fixed to the insides of both ends of a PGA-collagen tube (length, 50 mm; outer diameter, 5 mm) under microsurgery (Fig 4). No complication occurred during or after the operation.

Chronic pain observed before the operation diminished soon afterward. Difficulties in talking and eating improved rapidly, and the patient's quality of daily life activities sig-

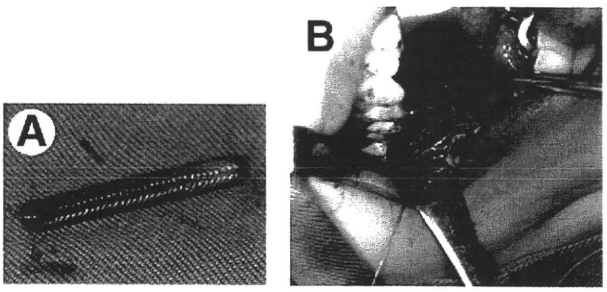

FIGURE 4. *A*, PGA collagen (polyglycolic acid-collagen) tube. Outer diameter 5 mm and length 5 cm. *B*, Intraoral view of the patient just after finishing reconstruction using the tube.

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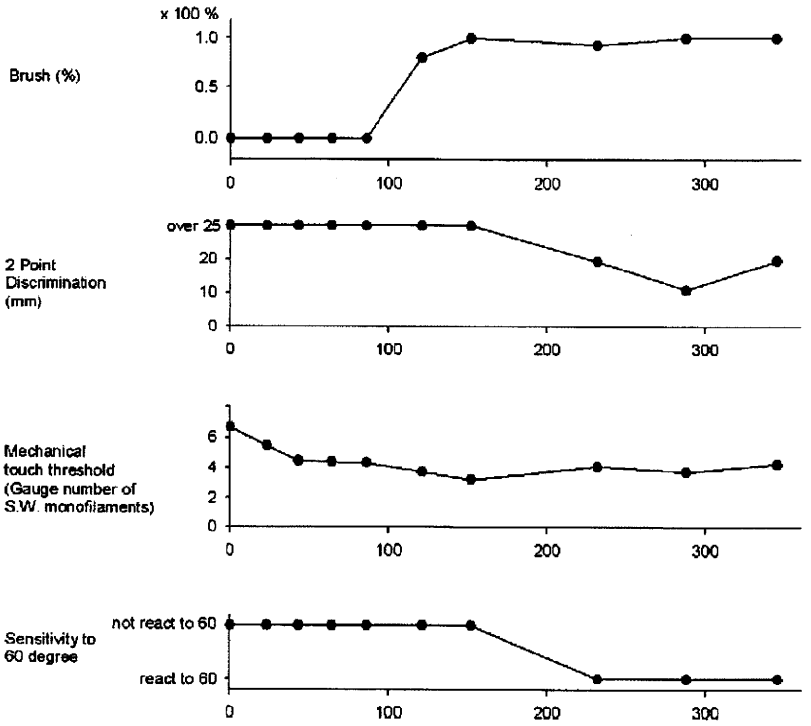

FIGURE 5. Changes in sensory parameters evaluated after operation. Brush stroke perception rate, 2-point discrimination, and mechanical touch threshold measured by Semmes-Weinstein monofilaments; and thermal perception are presented. Abscissa represents days after operation.

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nificantly improved because there was no more dysesthesia in the tongue during movement. For several weeks after the operation, jaw opening movements induced pain, resulting in mild trismus (approximately 20 mm between the upper and lower incisors). Daily administration of anti-inflammatory agents resolved this problem, resulting in no pain while eating or speaking. Changes in quantitative sensory tests were followed postoperatively (Fig 5). Recovery of brush stroke sensation began at 90 days after operation; and 150 days later had reached a normal level. Mechanical touch threshold started to improve very early after the operation, but just reached a low grade level compared with the reported normal value and did not improve any further. Two-point discrimination and the ability of heat perception improved very slowly, taking approximately 200 days from the operation. Taste sensation did not change subjectively, but slight changes in recordings from the electrogustometer began to increase to 20 dB at the anterior part, and to 30 dB at the lateral part of the damaged side at 1 year and later after the operation. Further observations will be needed to confirm the longer term prognosis.

Discussion

The case presented here exhibited neurotmesis or Sunderland's' classification class 5.[12] No papilla on the injured site of the tongue was detected, implicating loss of continuity in the lingual nerve caused by lack of supply of neurotrophin. This also supported a complete disruption of the lingual nerve trunk. Symptoms of spontaneous and/or elicited type dysesthesia suggested inclusion of neuroma formation.

Surgical intervention was thus appropriate for the complete peripheral nerve disruption in this case. This treatment consists of a direct suture, an autograft, and an indirect suture of the separated nerve gap using some other tissue or biomaterial. Autogenous nerve graft, as for a sural nerve, has been used for lingual nerve injury.[6,10] Based on previous criteria,[5,10] our case indicated surgical intervention was called for.

The PGA-collagen tube used in this case is a bioabsorbable material. Its natural absorption is also beneficial for foreign body implantation. Collagen applied in the PGA tube can act as a growth medium within a conduit, and can facilitate regeneration of myelinated fibers.[13] Collagen-filled PGA tubes also allow functional recovery from sensory deficits and are desirable materials for treatment of complete nerve deficits. Several reports have shown that PGA tubes filled with collagen result in excellent outcomes both experimentally[14,15] and clinically.[16-18] Our study using a 50-mm PGA-collagen tube in the trigeminal area is the first to report the successful use of such a long tube in this particular area.

As well, a PGA-collagen tube is effective for treatment of neuropathic pain. Inada[17] successfully used

PGA-collagen tubes to treat causalgia. This could be explained by the surgical resection of the neuroma that sends ectopic afferent discharges relating to dysesthesia.[19-21] However, the mechanism of pain relief and the prolonged maintenance of pain-free status after operation remain unclear.

With regard to the timing of surgical reconstruction, the patient in our case underwent surgery 9 months after the original injury. Electrogustometric examinations suggested taste function recovery took over 1 year from the operation. Therefore, while PGA-collagen tubes for nerve repair might allow the recovery of taste sensation even after a long period following injury, recovery might require longer than expected. Taste buds in the human tongue are connected to unmyelinated nerve fibers,[22] and while several reports have described recovery of myelinated fibers, there are none reported for unmyelinated fibers.[13-15] This indicates that nerve recovery has different features, and prognosis might depend on the type of the damaged peripheral nerve fibers (eg, myelinated or unmyelinated).

In conclusion, we reported the successful recovery of sensory loss in the tongue through the use of PGA-collagen tubes sutured to nerve ends with a 50-mm nerve gap. Functional recovery was partial according to a neurosensory test. Neuromas were detected on both sides of the injured nerve; dysesthesia disappeared soon after their resection. Difficulties in eating and speaking also rapidly resolved. The present case represented a pioneering trial to regenerate oral sensory function in chronic nerve injury with a long nerve gap.

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OTOLOGY

Efficiency of a transtympanic approach to the round window membrane using a microendoscope

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Abstract There has been increasing interest in cochlear drug delivery through the round window membrane (RWM). However, placing drugs on the RWM is difficult because of anatomical barriers. We examined the efficacy of a microendoscope for a transtympanic approach to the RWM. We evaluated the visibility of the RWM using four approaches: transtympanic microendoscopic, transtympanic microscopic, transmastoid microendoscopic, and transmastoid microscopic in ten human temporal bones. For the transtympanic approach, we made a fenestration (2 × 1 mm) in the postero-inferior quadrant of the tympanic membrane. For the transmastoid approach, conventional posterior hypotympanotomy was performed. The transtympanic microendoscopic approach enabled visualization of the RWM in all specimens, whereas the transtympanic microscopic approach only permitted visualization in three specimens. Through the transmastoid approach, the RWM was visible in all specimens using either a microendoscope or a microscope. The transtympanic microendoscopic approach can be utilized for cochlear drug delivery through the RWM.

Keywords Microendoscope · Round window membrane · Cochlea · Drug delivery

Introduction

Sensorineural hearing loss (SNHL) is one of the most common disabilities in industrial countries. Systemic administration of steroids has been widely used for the treatment of acute profound hearing loss [1]; however there are limitations in their clinical efficacy [2]. At present, therapeutic strategies are limited to hearing aids and cochlear implants for patients with chronic SNHL. Based on this background, basic investigations have elucidated several agents that are effective for the treatment of SNHL. However, the problem of how to deliver drugs to the inner ear has been a considerable obstacle to the development of treatments for SNHL. The blood-inner ear barrier prevents the transportation of serum drugs to the inner ear, and the blood flow to the inner ear is very limited.

Drug transduction through the round window membrane (RWM) is one option for delivering drugs into the inner ear. Continuous infusion of RWM with an osmotic pump and microcatheter has been reported as an effective and safe approach [3]. However, it requires surgery and the invasion cannot be overlooked. Recently, new local drug application procedures using biodegradable substances are gaining interest [4, 5]. The inner ear is one of the targets for local drug administration using biodegradable gelatin hydrogels [6, 7]. In this drug delivery system, positively charged proteins or peptides are electrostatically trapped in negatively charged gelatin polymer chains. As the gelatin polymer chains degrade, proteins or peptides are released from the hydrogel. The released protein is conveyed through the RWM into the inner ear via a concentration gradient. Therefore, close contact of biodegradable hydrogels with the RWM is critical for efficient drug delivery to inner ear fluids.

The RWM is situated perpendicular to the tympanic membrane and deep in the round window niche. In some cases, a false membrane covers the RWM. For safe and certain drug administration, hydrogels containing drugs should be placed on the RWM under direct visualization. Use of a

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microendoscope is an effective method for visualization of the RWM [8]. It is equipped with a working channel, which can be used in drug administration. However, the potential of microendoscopes for placing substrates on the RWM has not been evaluated, and it is important to clarify the prevalence of subjects in whom the RWM is microendoscopically visible. In the present study, we examined the potential of a specially modified microendoscope for a transtympanic approach to the RWM using human temporal bones.

Materials and methods

Ten formalin-fixed temporal bones with no middle or inner ear diseases were obtained from six individuals (aged from 68 to 76 years at death, five male, and one female). A microendoscope (0.9 mm in outer diameter, 50 mm in length; FiberTech, Tokyo, Japan) was specially modified in the fit angle for observation of the RWM through the tympanic membrane. The tip is curved 15° (Fig. 1). The view angle is 70°. It is equipped with a working channel (0.3 mm in diameter).

We used four different approaches to observe the RWM as follows: (1) transtympanic microendoscopic, (2) transtympanic microscopic, (3) transmastoid microendoscopic, and (4) transmastoid microscopic. For the transtympanic approach, a small fenestration (2 × 1 mm) was made in the posterior inferior quadrant of the tympanic membrane using a knife (Fig. 2). The microendoscope was inserted into the middle ear through this fenestration and set to provide the best view of the RWM. For observation with a microscope, the fenestration edge in the tympanic membrane was gently pushed with a curved needle to obtain the best access to the

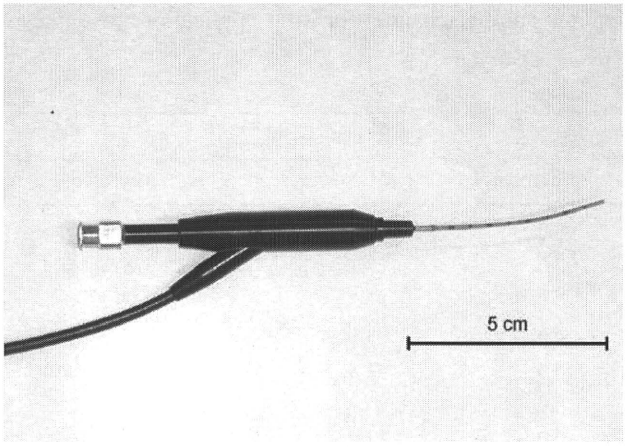

Fig. 1 A microendoscope specially modified for better visualization of the RWM. The outer diameter is 0.9 mm and the length is 50 mm. The view angle is 70°. It is equipped with a working channel (0.3 mm in diameter)

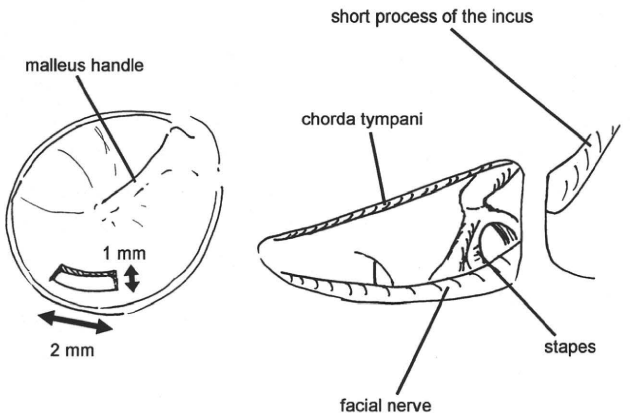

Fig. 2 A small fenestration (2 × 1 mm) was made in the posterior inferior quadrant of the tympanic membrane using a knife. Posterior hypotympanotomy was made as large as possible. In all specimens, the facial nerve and chorda tympani were skeletonized

RWM. For transmastoid approaches, canal-wall up complete mastoidectomy and posterior hypotympanotomy were performed under conventional microscopy (Leica M300, Leica Microsystems, Wetzlar, Germany). The bones covering the middle cranial fossa dura, the posterior fossa dura, and the sigmoid sinus were drilled to be as thin as possible. The bony wall of the external auditory canal was preserved. The facial nerve and chorda tympani nerve were skeletonized and the facial recess was opened as large as possible (Fig. 2).

The RWM was observed through a posterior hypotympanotomy with a microendoscope or a microscope. Surgical procedures were performed by one author (Harukazu Hiraumi). The view of the RWM and surrounding structures using the four approaches was video-captured. Frames showing best view of the RWM were converted into still images, and the area of the RWM was measured using image-processing program, ImageJ. An angled hook (1.0 mm sharp tip) was used as a reference. Total area of the RWM was measured after drilling the round window niche. The visibility of the RWM was calculated and graded into three classes: Grade I as no or little visualization of the RWM (<20%), Grade II as defined by >20%, and Grade III as defined by >70%. In three samples, the round window niche was covered with false membranes. In these cases, the false membranes were removed with a curved needle under microscopic view via posterior hypotympanotomy.

Results

A microendoscope was smoothly inserted into the middle ear cavity and the incuidostapedial joint was observed easily in all the specimens. The percentage of the area of the

RWM under direct vision was shown in the Table 1. The transtympanic microendoscopic approach enabled visualization of the RWM in all the specimens (Fig. 3). In three specimens, the RWM was totally observed (Fig. 4a). We used the incuidostapedial joint as a landmark to identify the location of the round window niche and the tip of the microendoscope was safely oriented to the RWM. No hazardous events such as ossicular dislocation or disruption of the tympanic membrane occurred. In contrast to the transtympanic microendoscopic approach, a transtympanic approach using a microscope provided visualization of the RWM in only three specimens (Fig. 3). Even in those three specimens, the view of the RWM was very limited (Fig. 4c). In the other seven specimens, the RWM was not observed, as the overhang of the round window niche was an obstacle for visualization. The visibility of the RWM through the transtympanic microendoscopic approach was significantly superior to that through transtympanic microscopic approach (Fig. 3, $P < 0.01$, Wilcoxon matched-pair signed-rank test).

In all the specimens, the transmastoid approach provided an excellent view of the RWM using either microendoscope (Fig. 4b) or microscope (Fig. 4d). The transmastoid microendoscopic approach provided a wide view of the middle ear cavity; for instance more than 70% of the tympanic membrane was visible in nine (microendoscopic), and seven (microscopic) specimens.

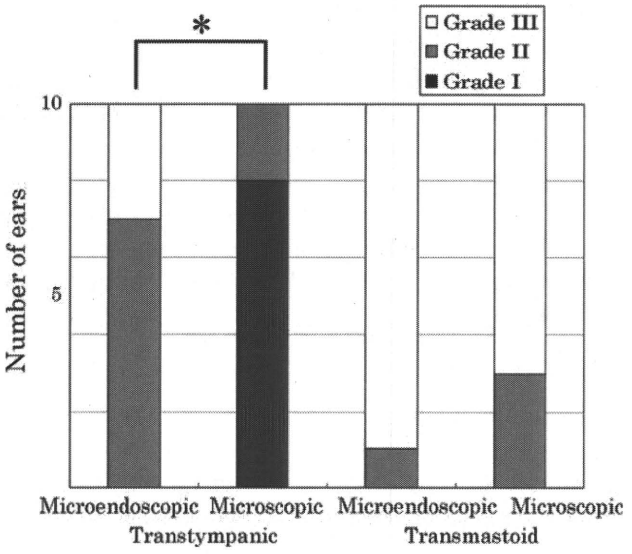

Fig. 3 The visibility of the RWM for four approaches. Grade I as no or little visualization of the RWM (<20%), Grade II as defined by >20%, and Grade III as defined by >70%. The visibility through the transtympanic microendoscopic approach was better than that with transtympanic microscopic approach

Discussion

The present results demonstrate that a microendoscope provided a satisfactory view of the RWM through a transtympanic approach with only a 2-mm incision on the tympanic membrane. Although the transmastoid microscopic approach provides an excellent view and favorable access to the RWM, this approach requires mastoidectomy and is not adequate for local drug application for treatment of SNHL. In contrast, the transtympanic microendoscopic approach requires only a small fenestration in the tympanic membrane. Therefore, the transtympanic microendoscopic approach may be applicable for office-based treatment.

Conventional endoscopes with 30° provide good visualization of the RWM [9, 10]. However, endoscopes with attached CCD cameras are not easy to handle. In office-based usage, the endoscope is usually placed just outside of the tympanic membrane [11], and tools used for drug application can hinder the view. The outer diameter is 1.7 mm or larger, requiring larger myringotomy. In addition, use of a conventional endoscope for drug delivery onto the RWM requires another channel for drug application, resulting in

Table 1 The percentage of the visible area of the round window membrane using four approaches

No	Side	Transtympanic		Transmastoid	
		Microendoscope (%)	Microscope (%)	Microendoscope (%)	Microscope (%)
1	Left	80.2	0.0	91.6	70.1
2	Left	54.5	0.0	78.1	72.0
3	Left	78.8	23.0	87.3	79.6
4	Left	59.1	0.0	73.3	84.8
5	Left	48.2	14.6	94.8	71.6
6	Right	49.7	0.0	80.7	61.3
7	Right	79.9	0.0	87.6	75.7
8	Right	39.5	0.0	66.2	42.3
9	Right	62.0	20.1	84.9	83.2
10	Right	56.9	0.0	82.8	65.4

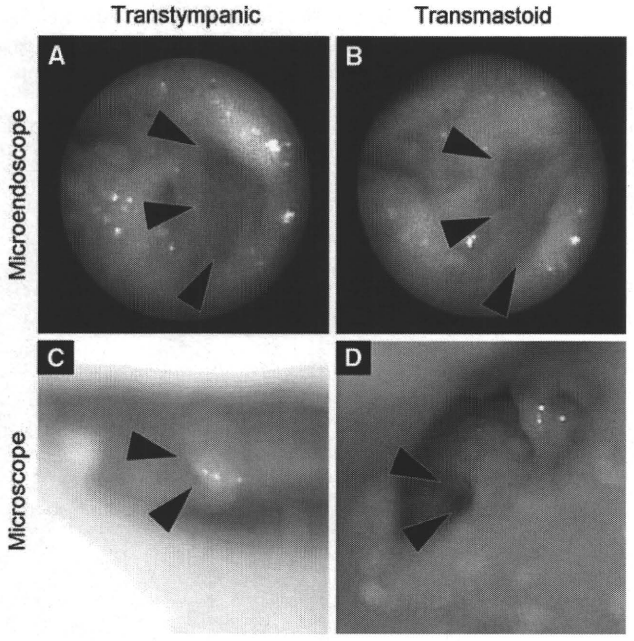

Fig. 4 The RWM of bone three observed through four approaches (*arrow heads*). The transtympanic microendoscopic approach (**a**), transmastoid microendoscopic approach (**b**), and transmastoid microscopic approach (**d**) provided good views. In the transtympanic microscopic approach (**c**), only a small part of the RWM was observed with the aid of a curved needle

increase of surgical invasion on the tympanic membrane. This means that enlargement of the size of tympanotomy or making additional tympanotomy site is necessary. Conventional microendoscopes are made for the inspection of the nasolacrymal ducts, and their tips are straight. The external auditory canal is S-shaped [12], and it is difficult to direct straight microendoscope to the RWM. The modified microendoscope used in the current study is quite smaller than conventional ones, and is connected to a CCD camera system via a cable. The curved tip fitted the external auditory canal. This configuration provides excellent handling of equipment for drug delivery. In addition, the microendoscope used in this study has a working channel that can be utilized for application of substrates onto the RWM.

The aim of the current study was to evaluate the accurate RWM drug application efficacy of a microendoscope with angles modified to ease RWM access. For clinical use of previously developed local drug delivery systems [3, 8], safe and stable visualization of the RWM through the tympanic membrane is necessary. In this manuscript, we compared the transtympanic microendoscopic approach with the transmastoid microscopic approach, since it is the most common procedure to access the RWM. The transmastoid microscopic approach is the most reliable approach for observation of the RWM, and additional removal of the round window niche enabled measurement of the total area of the RWM, which was indispensable for quantitative analysis in the present study. The view provided by a microendoscope is enough to deliver drugs or biomaterials incorporating drugs onto the RWM, although it is not satisfactory for precise surgical procedures. Previous studies have demonstrated the efficacy of biodegradable gelatin hydrogels for local application of brain-derived neurotrophic factor [6] and insulin-like growth factor 1 [7, 13]. The present findings resolve the problem of how to place a hydrogel onto the RWM in the clinic.

This study also found some drawbacks for this instrument. The resolution of the microendoscope is not as high as that of conventional microscopes, which may impede the differentiation of the false membrane from the RWM [14]. Sufficient understanding of the surgical anatomy of the middle ear is necessary for appropriate use of the microendoscope in drug delivery onto the RWM. However, we consider that refinement of the quality of view provided by microendoscopes may resolve this problem.

Conclusion

The transtympanic microendoscopic approach provided satisfactory visualization of the RWM through the tympanic membrane, indicating that the microendoscope is a useful tool for placing drugs or drug-containing materials onto the RWM.

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Conflict of interest We do not have a financial relationship with the organization that sponsored the research.

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ティッシュエンジニアリング治療 4
気道の再生治療

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● 気道の再生治療と *in situ* ティッシュエンジニアリング

従来の組織工学（ティッシュエンジニアリング）では培養室のシャーレの中で細胞を培養増殖させて目的とする組織を作り，それを体内に移植してきた。これに対し，欠損した組織を体内のその場所（*in situ*）で再生させる方法は *in situ* ティッシュエンジニアリングとよばれる。現在，臨床研究が進んでいる自己組織再生型人工気管は，この *in situ* ティッシュエンジニアリングを応用したものである。

● 人工気管開発の歴史

気道は中枢の気管と末梢の気管支・細気管支によって構成されるが，外科的な再生（再建）治療は気管支より中枢が対象になる。気管支よりさらに末梢の気道や肺胞レベルの再生は内科治療の対象である。

外科的な気道再建の対象となる疾患は，腫瘍の気道浸潤や外傷や気管チューブのカフによる瘢痕狭窄などである。病変部気管を切除して，ゴム管などの人工チューブで欠損部を再建しようとする研究が1940年代より始まった[1]。しかしながら，人工血管とは対照的に，人工気管開発は遅れ，60年以上経った現在でも市販されている人工気管はないのが現状である。

一方，合成吸収性縫合糸をはじめとするバイオマテリアルの進歩に伴い，気管外科学は1970年代から大きく発展した。米国ハーバード大学のGrillo博士のグループは，6 cm以下の気管切除に際しては端々吻合で気管再建が可能であることを明らかにした。以来，気管の端々吻合は気管外科の標準術式となっている。しかし，広範囲の切除や，さまざまな理由で気管の端々吻合ができない症例も多い。また，端々吻合は術後1か月間の頸部前屈位の保持が必要で，患者の負担がきわめて大きい。しかも臨床で症例の多い左主気管支狭窄に対しては，端々吻合では再建できない。安全に使用できる人工気管の開発が待たれるゆえんである。

縫合輪付きのシリコンチューブ型のノンポーラス人工気管は，1970年代に一度米国で商品化されたものの，その後吻合部が離解してしまうことが判明し，使われなくなった。一方，日本では内腔面に上皮が再生する新しいタイプの人工気管研究が進められた。

● 人工気管の構造とその生体親和性の向上

自己組織再生型の人工気管は，円筒状にしたファインマーレックスメッシュ（現 BIRD® mesh）にポリプロピレン製のモノフィラメントステントを外側に巻き付けた支持構造（フレーム）にコラーゲンを複合化させてある（**図1**）。コラーゲン部分には自己の細胞が入り込み自己組

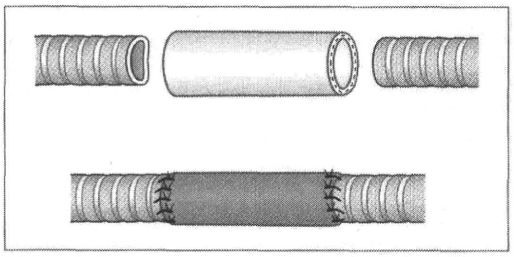

図 1　自己組織再生型の人工気管手術のシェーマ
人工気管はポリプロピレン製のフレームの周囲に豚皮由来のアテロコラーゲンが複合化されている。切除した気管を人工気管で置換して，そこに自己組織を再生させる。埋入手術時に気管壁のコラーゲンを自己血液，穿刺骨髄液，間葉系幹細胞で浸潤させることにより，人工気管は気密になる。生体気管端を人工気管に内挿するかたちで，吸収性縫合糸を用いて縫合固定する。

織になるため，結果としてフレームは生体に取り込まれる。

フレームのプラスチックは，表面を分子修飾することにより，生体親和性が飛躍的に向上する。この新しい技法は 1977 年に開発された。プラスチックの表面にプラズマ処理をして反応基を作り，その反応基にコラーゲン分子を共有結合させる方法である[2]。

● 気道の力学強度と人工気管の構造設計

内部が中空である気管は，解剖学的にみると気管壁内の軟骨構造に支えられている。この軟骨輪が，外部からの圧迫や咳嗽時の陰圧に抗して内腔を保持している。

1970 年代に硬いポリエチレン製のヘビーマーレックスメッシュ（heavy Marlex Mesh）が気管再建用材料として米国で発売されたことがある。この気管再建用ヘビーメッシュは筒状に巻いて気管再建に用いられた。しかしながら，この気管再建用ハードメッシュは硬すぎて隣接する血管からの大出血を起こす事故が発生し[3]，患者が死亡したため使われなくなった。そこで，新しい自己組織再生型人工気管では力学強度と剛性を考慮した設計が行われた。

人工気管に用いたファインメッシュの力学強度はそれだけではきわめて弱く，内腔保持はできない。そこで，気管軟骨に相当する支持材として弾性のあるステントを巻き付ける必要があった。この気管軟骨の代わりのステントとして，細く弾性のあるモノフィラメント繊維をメッシュチューブに一体化した。用いられた繊維は医療用のメディカルグレードポリプロピレン製で，メッシュと繊維は熱融合で固定し，さらに補強のために 7-0 プロリン（外科用縫合糸）で 2 mm 間隔に結節固定した。

● ビーグル犬を用いた動物実験による安全性の確認と機能評価

体重 10 kg 程度のビーグル犬は人間と気管の太さが近いため，気管の再生実験にはビーグル犬が用いられる。ビーグル犬の頸部気管で 4〜6 気管軟骨輪分を切除した後にこのメッシュ状人工気管で置換すると，再建部の人工気管内腔面は気管上皮に覆われることがまず確かめられた[4]。次に広範気管切除時の臨床応用を想定して 10〜16 軟骨輪を切除して，吻合部に抗張力が 900〜1000 g 重加わった状態を作り，この欠損を人工気管で再建した。その過酷な条件下でも人工気管は生体に取り込まれ，抗張力下でも吻合不全は生じなかった[5]。

さらに，7〜9 気管軟骨輪に相当する長い人工気管を用いた置換実験が行われた。この実験では，置換手術後，内腔保持のために一時的にメッシュ管の内側にシリコンチューブを挿入した。この管状置換でも人工気管は宿主気管と術後速やかに一体化して，メッシュ内に生体組織が再生し内腔の上皮化が進んだ。22 か月後に病理組織学的検討を行うと，連続した上皮再生が口側から尾側端まで認められた[6]。

● コラーゲンの足場と自己組織の再生

体内に埋入されたアテロコラーゲンにはまず線維芽細胞が侵入し，そこでコラーゲンを分泌する。人工気管のアテロコラーゲンは，自己のコラーゲンと置き換わる。そのコラーゲンを足場に自己組織が再生し，最終的には密集した線毛に覆われた上皮が再生部を覆うが，この過程は 1〜6 か月で起こる。

● 人工気管に用いる新しい工夫

頸部気管から縦隔内気管へと再建実験が進められた。合計24頭のビーグル犬縦隔内気管に5 cm長の人工気管を埋め込んだ。この実験では，内腔狭窄やメッシュの露出は大網被覆を行うと減少することが判明した[7]。

組織再生の足場となるコラーゲンの改良も続けられた。それまで人工食道に応用されて良好な成績を上げていたコラーゲンスポンジを人工気管に用いると，6〜24か月に屠殺するまで全頭経過良好に生存した。手術時にもスポンジに自己の血液を含潤させることにより，針穴からのリークが起こらず気密となる[8]。現在，臨床で用いられている自己組織再生型の人工気管では，薄フィルム多房構造という微細構造のコラーゲンを用いることにより，組織再生を促進させている。

分岐部用のY字型人工気管[9]や左側主気管支再建[10]においても，再建部気道内面は完全に上皮が再生し，安全に使用可能であることが確かめられた。

● 気管再建における軟骨再生の試み

整形外科領域では近年，軟骨の再生治療が始まっている。気管再建においても軟骨再生の研究が進められている[11]。しかし，気管軟骨の場合は力学的強度が低下すると，気道が虚脱し，ただちに生命に関わる。このため，再生軟骨を気管で臨床応用する前に，少なくとも10年間は再生軟骨の強度低下がないことを確認する必要があろう。しかし，大型動物を使ったこのような長期観察はなかなか困難なのが実情である。その点，長期に安定であるポリプロピレンメッシュを芯にした自己組織再生型人工気管は，力学強度の低下を起こさない。5年にわたる長期観察でも逸脱することなく安全であることが，動物実験で確認されている[12]。

● 組織再生型人工気管の日本での臨床応用

自己組織再生型の人工気管は京都大学医学部附属病院，福島県立医科大学において倫理委員会の審査を経て，2002年より臨床使用が世界に先駆けて始まっている[13]。2009年3月現在で13例の頸部気管壁欠損（甲状腺腫瘍の気管浸潤）や瘢痕性気管狭窄症例に使用され，良好な組織再生と内腔面の上皮化をみている。

● 現状と今後の展望

さらに，コラーゲン上における組織再生を促進させるために穿刺骨髄液や，骨髄から分離培養した間葉系幹細胞（mesenchymal stem cell：MSC）を併用する手法も検討された[14]。ビーグル犬を用いた検討では，自己のMSCは，再建部位のコラーゲン上に散布すると気管壁の組織に分化することが判明した。

また2008年には，allograftを用いた気道再建の臨床報告がされ，注目を集めた。これはスペインのMacchiarini らが死体気管の軟骨構造を用いたもので，そこに組織工学的手法で患者由来の上皮細胞と軟骨細胞を播種し，それを30歳女性患者に移植した。その報告では4か月後にも内腔を保持している[15]。しかしながら，長期に気道を支えるに足る軟骨の再生は検証されておらず，安全性の評価には慎重を期す必要があると考えられる[16]。

● おわりに

iPS（induced pluripotent stem）細胞を代表とする再生医学研究は急速に進められているが，実際に臨床に応用されているものはまだ少ない。そのなかで，本稿で紹介した *in situ* ティッシュエンジニアリングを用いた気道の再生治療はすでに臨床応用が始まっており，今後さらに一般医療として普及する期待が高まっている。

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