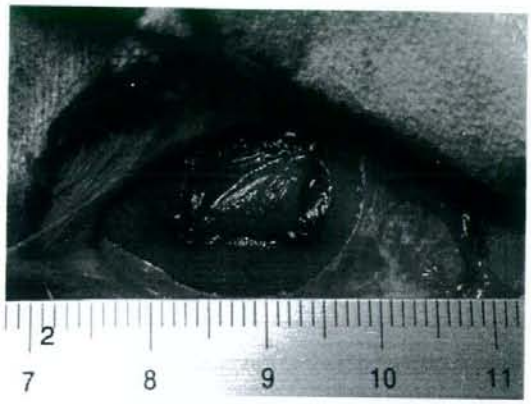

Figure 2. Microscopic findings in muscle defects treated with collagen sponge. (**A**) At 1 week after surgery, between the collagen sponge (asterisk) and the stumps of the muscle fibers (arrow), invasion of inflammatory cells, including phagocytes (arrowhead) ingesting the collagen sponge, is evident. Most of the collagen sponge remains in the muscle defect. (**B**) At 2 weeks after surgery, a small amount of the collagen sponge remains in the center of the muscle defect (asterisk). At the periphery of the muscle stumps (arrow), numerous regenerating myoblasts are recognized. (**C**) At 3 weeks after the operation, the collagen sponge has disappeared completely. Elongation of regenerating myofibers showing mutual fusion is evident adjacent to the stumps among the native collagen fibers (asterisk). (**D**) At 4 weeks after the operation, native collagen fibers are more extensive with numerous myofibers (asterisks). (**A–B**) the border between the muscle stumps. (**C–D**) the ends of regenerating myofibers. Hematoxylin and eosin. Bar 500 μm.

Four weeks after treatment, the surface area of operation site shrank to 80.4% in the collagen sponge group, as compared with 56.4% in control group. There was significant difference between two groups until 4 weeks after operation, but after 12 weeks no significant difference was observed (**Table 1**).

Histologic Observations

In the thigh bearing the implanted collagen sponge, the sponge was identifiable at 1 week after the operation. A large number of erythrocytes were recognized in the pores of the sponge, but other cells had not migrated into the pores. Between the muscle stumps and the collagen sponge, a number of inflammatory cells had invaded, with macrophages phagocytizing the sponge at the ends adjoining the muscle fiber stumps (**Figure 2A**). At 2 weeks after the operation, macrophages had phagocytized the collagen sponge to a greater extent, and consequently a small amount of collagen sponge remained at the center of the muscle defect. At the periphery of the muscle stumps, numerous regenerating myoblasts were recognized. A number of these had fused and begun to extend toward the middle of the defect (**Figure 2B**). At 3 weeks after the operation, the collagen sponge had disappeared completely. Elongation of regenerating myofibers due to their mutual fusion could be seen adjacent to the stumps among the native collagen fibers (**Figure 2C**). At 4 weeks after the operation, native collagen fibers were more extensive with an increase in the number of extending myofibers. However, regenerating myofibers remained small in diameter. They were not found in the center of the muscle defect, where native collagen and newly formed small vessels were observed (**Figure 2D**). The thickness of the defect gradually diminished to two thirds by 24 weeks, and the center of the defect had changed into reticular tissue before 12 weeks. This reticular tissue became reduced in size before 24 weeks, and the ends of the regenerating fibers from both stumps had partially connected together in the middle of the defect.

In the control thigh at 1 week after the operation, the defect was occupied by hematoma and numerous invading inflammatory cells. The edges of the native myofibers had degenerated (**Figure 3A**). At 2 weeks after the operation, numerous myoblasts were seen adjacent to the muscle stumps. The muscle defect gradually thinned (**Figure 3B**), and at 3 weeks after

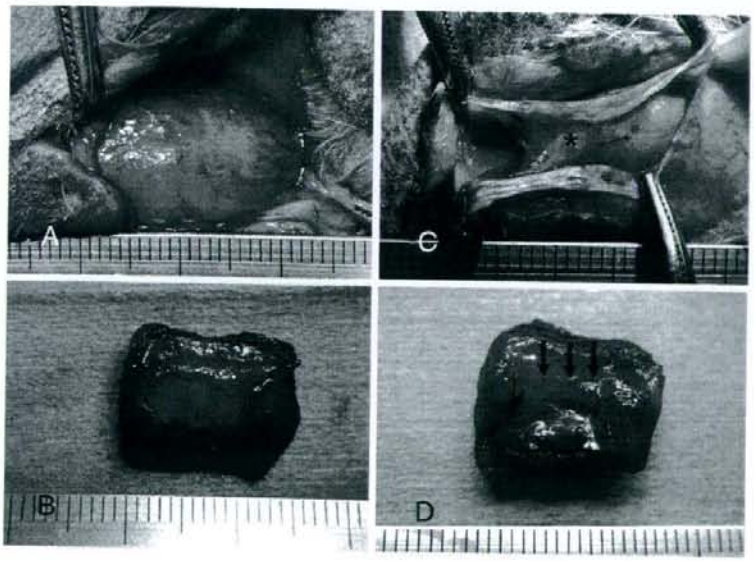

Figure 3. Microscopic findings at the border between the muscle stumps in the control (untreated) defect. (**A**) At 1 week after the operation, hematoma and numerous invading inflammatory cells are present in the defect. (**B**) At 2 weeks after the operation, the muscle defect has thinned. (**C**) At 3 weeks after the operation, the muscle defect has become filled with excessive collagen fibers. (**D**) At 4 weeks after the operation, myofibers adjacent to the stumps show fusion but have not extended. Hematoxylin and eosin. Bar 500 μm.

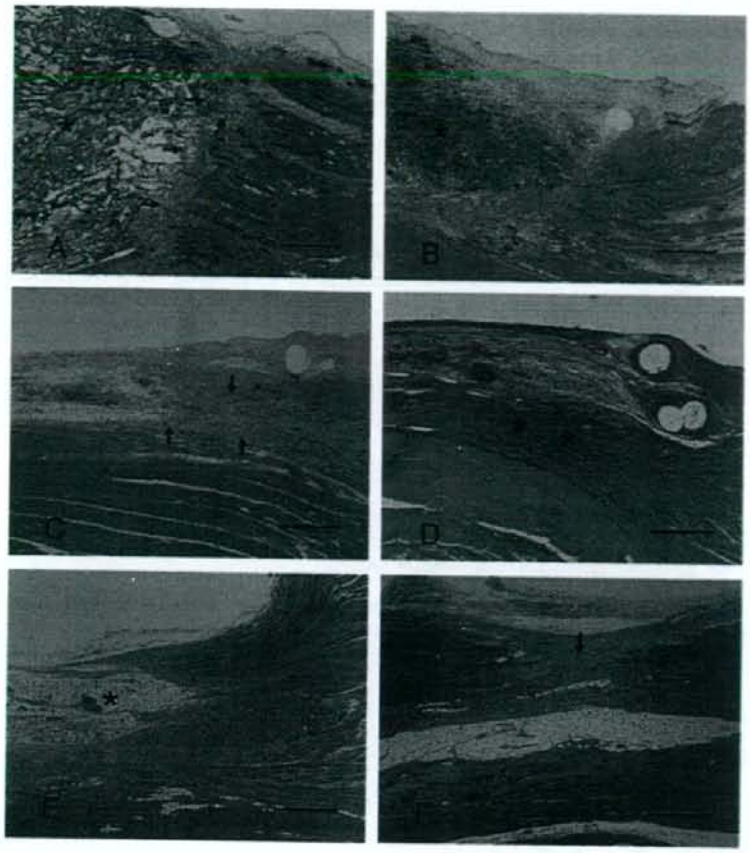

Figure 4. Microscopic findings in muscle defects treated with collagen sponge (**A, C, E**) and in untreated (control) muscle defects (**B, D, F**) after immunohistochemical staining for desmin at 4 (**A, B**), 12 (**C, D**), and 24 (**E, F**) weeks after the operation. (**A**) Desmin-positive fibers in the deep part of the defect are stratified toward the center (asterisk). Each fiber has a small diameter and length. (**B**) Regenerating fibers at the bottom of the muscle defect are small number, diameter, and length. (**C**) The ends of the fibers regenerating from both ends show partial interconnection inside the reticular tissue (asterisks). (**D**) The regenerating fibers have not extended into the reticular tissue. (**E**) The reticular tissue has been replaced partially by regenerating fibers with a mature diameter and length (asterisks), but the defect has not been completely filled with regenerated fibers. (**F**) The fibers regenerating from both ends have not connected together inside the reticular tissue. Bar 500 μm.

the operation, it was filled with thick scar tissue composed of excessive collagen fibers (**Figure 3C**). At 4 weeks after the operation, regenerating myofibers adjacent to the stumps showed no branching extension to the middle of the defect (**Figure 3D**). The center of the defect had changed into reticular tissue by 12 weeks. Even 24 weeks after the operation, regenerating myofibers adjacent to the stumps did not extend any further, and excessive scar tissue persisted in the muscle defect.

Immunohistochemistry

Desmin is one of the earliest structural proteins detected during myogenesis. Normal skeletal muscle fibers have been found to be slightly immunopositive for desmin, while regenerating fibers are strongly immunopositive.[16,17] The high level of desmin synthesis has been used as a marker to target neomyogenesis in regenerating muscle after experimental injury.

In the thighs containing collagen sponge, the head of regenerating myofibers were strongly positive for desmin beginning at 1 week after the operation. Desmin-positive cells generally had rounded ends and many clear, central nuclei with prominent nucleoli. Between the collagen sponge and native muscle fibers, some desmin-positive cells appeared to have fused at 1 week after the operation. Desmin-positive angiogenesis was also observed. At 2 weeks after the operation, desmin-positive muscle fibers appeared to extend as the collagen sponge was absorbed. These regenerating fibers from the two stumps with strongly desmin-positive ends showed elongation, but each individual fiber appeared disordered. The desmin-positive fibers in the deeper part of the defect were stratified toward the middle of the defect where the collagen sponge had been phagocytized. Regenerating myofibers had between one third and one half of the normal diameter at 4 weeks (**Figure 4A**). The ends of the regenerating fibers from the two stumps had partially connected together inside the reticular tissue by 12 weeks (**Figure 4C**). Regenerating myofibers had a diameter equivalent to that of normal myofibers by 24 weeks. The reticular tissue was partially replaced by the regenerating fibers, but the defect was not completely filled with regenerated fibers even after 24 weeks (**Figure 4E**).

In the control thigh at 1 week after the operation, several single or fused desmin-positive cells were present adjacent to the stumps. Even at 2 weeks after the operation, these desmin-positive cells did not extend, even though some fusion was evident. In the bottom of the muscle defect, muscle fibers strongly immunopositive for desmin were stratified toward the

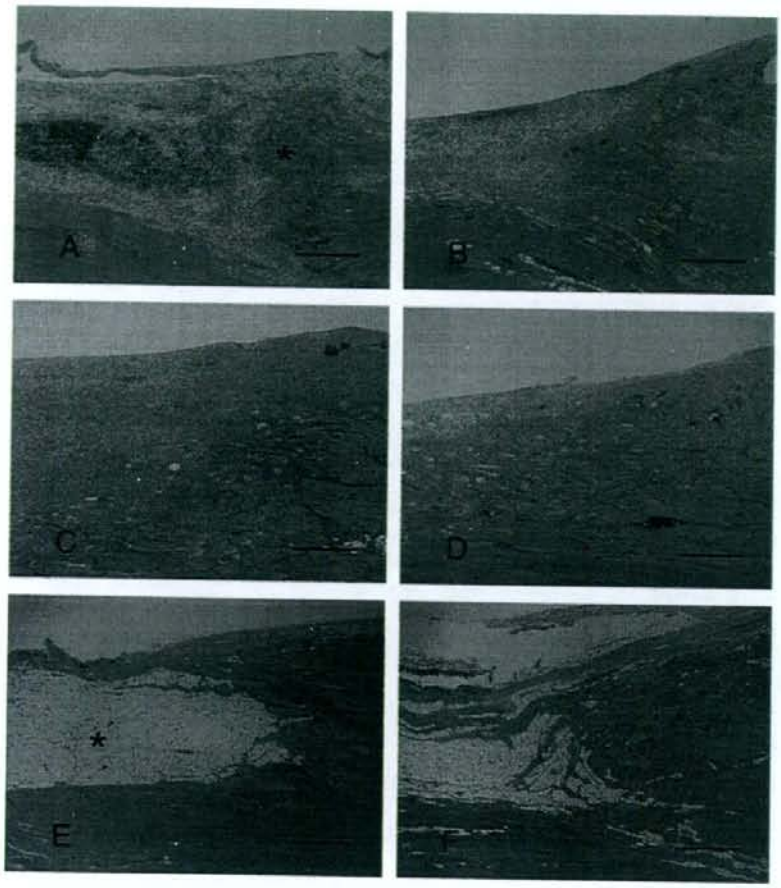

Figure 5.

middle of the defect. Their length, diameter, and number were less than in the defects treated with collagen sponge (**Figure 4B**). The regenerating fibers did not extend into the reticular tissue at 12 weeks (**Figure 4D**). Even after 24 weeks, the strongly immunopositive muscle fibers had not shown further growth at the bottom or sides, and the defect was filled with reticular tissue (**Figure 4F**).

Discussion

It was previously thought that injured skeletal muscle was replaced by fibrous scar tissue. However, over the last 40 years, accumulated evidence has suggested that skeletal muscle has the potential to regenerate to a certain degree, particularly following trauma and also in some forms of muscular dystrophy. Molecular biologic techniques have also revealed the molecular pathways of muscle regeneration.

The process of regeneration of injured skeletal muscle has been determined using a variety of experimental models in which skeletal muscle damage was created by laceration,[18–22] contusion,[23,24] crushing,[22,25] freezing,[26] or chemicals.[27,28] The generally accepted process of muscle regeneration after laceration injury is thought to involve initial retraction of the cut myofibers to both sides and the creation of a gap between them.[29] Because skeletal muscle has a rich vascular supply, the gap becomes filled with a hematoma, which is then invaded by inflammatory cells and fibroblasts. The fibroblasts synthesize connective tissue proteins such as collagen.[19] The cut myofibers become necrotized from the stumps over a distance of 1 or 2 mm inside their preserved basement membrane. Blood-derived inflammatory cells gain immediate access to the injury site and substances released from the necrotized muscle serve as chemoattractants for further inflammatory cells.[30] Polymorphonuclear leukocytes in the acute phase are soon followed by monocytes, which are transformed into macrophages. The latter then begin to actively phagocytose the necrotic muscle.[29,30] Satellite cells, which lie underneath the basement membrane,[31] become activated by growth factors secreted by macrophages.[32] The satellite cells proliferate, differentiate into myoblasts, and fuse with each other into multinucleated myotubes. Multinucleated myotubes fuse with the preserved myofibers at the stumps and extend through connective tissue at the center of the defect.[19,29]

Previous studies have already demonstrated that an injured muscle can regenerate successfully. However, to our knowl-

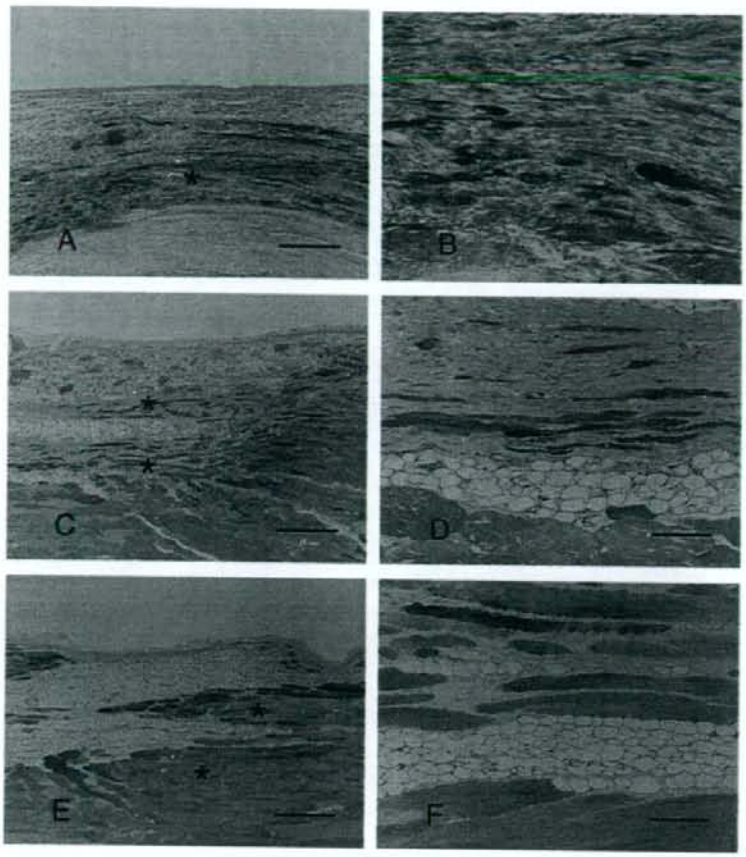

Figure 6.

edge, muscle regeneration across a large defect has never been demonstrated in mammals. In the present study, we attempted to regenerate muscle tissue using an atelocollagen sponge scaffold in muscle defects created in rabbits. Collagen has been widely applied for medical use in an artificial gel or sponge form because of its biocompatibility and absorbability. Collagen sponge has been used as a scaffold for repairing several types of tissue, such as nerves, trachea, and periodontium.[7-14] Attempts have also been made to use it to regenerate the esophagus, stomach and small intestine.[8-10] These studies showed that collagen sponge could induce regeneration of mucous membrane, glands and fibrous tissue of the gastrointestinal tract. However, the regenerated tissue lacked a muscle layer. There has been one previous report of a study in which a 5-cm-long gap in the canine cervical esophagus was replaced successfully using a collagen sponge scaffold, and complete host tissue regeneration, including the muscle layer, occurred.[8] Therefore in the present study, we applied an atelocollagen sponge scaffold to achieve muscle regeneration.

Macroscopically, in the defects containing the collagen sponge, soft tissue had filled the gap, and only mild concavity and slight adhesion to the fascia was observed. In the control defects, replacement by firm scar tissue and severe shrinkage were evident. Histologically, in the defects containing the collagen sponge, the sponge scaffold remained in place for 2 weeks where inflammatory reaction was minimal. The control defects, on the other hand, were filled with hematoma and numerous inflammatory cells, and excessive scar formation was observed between the stumps until 2 weeks.

Although there was no significant muscle regenerated, both contracted group and uncontracted group, this excessive scarring may impede the regeneration of myofibers and reinnervation.[19] The collagen sponge remained in place between the muscle stumps for 2 weeks and induced platelet aggregation around it to allow limited development of hematoma and the infiltration of inflammatory cells.[33] These properties of the collagen sponge might prevent the excessive development of scar tissue and allow elongation of regenerating myofibers into the center of the defect.

Collagen has also been reported to elicit cytokine release from platelets.[34] The effects of cytokines on muscle regeneration or scar tissue formation deserve further investigation.

In this study, the collagen sponge was heated at 140°C for 24 hours to introduce cross-linking between the collagen molecules. The degradation rate of collagen sponge depends on the degree of cross-linking that occurs during sponge preparation.[12] With this particular preparation, the collagen sponge gradually disappeared over a period of 3 weeks. Since myo-

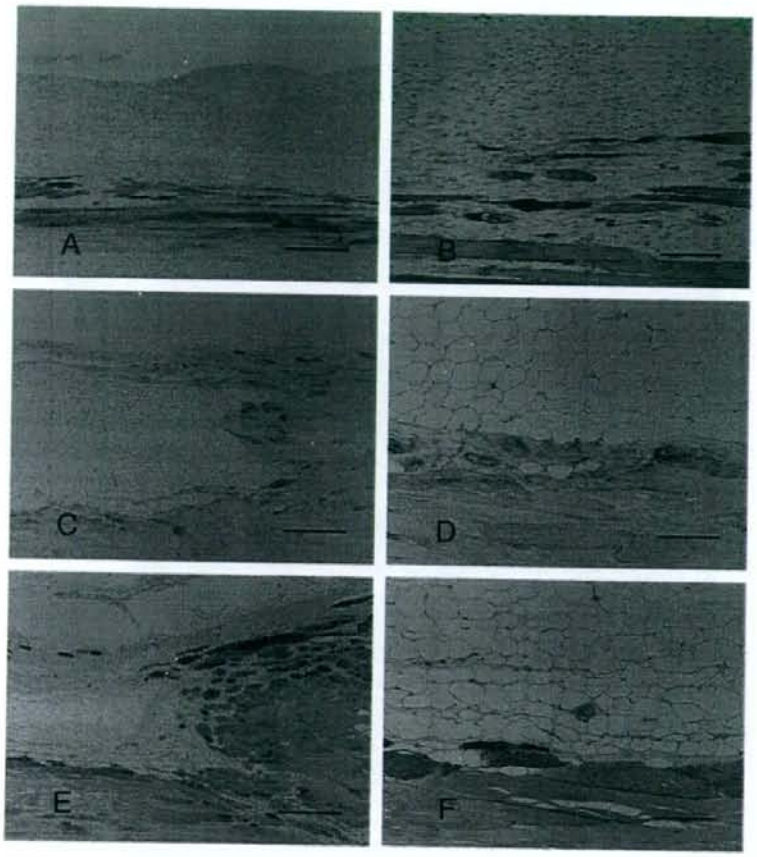

Figure 7.

blasts mature into myofibers at about 3 weeks after injury in laceration injury models,[18] this degradation rate of collagen sponge seems suitable for skeletal muscle regeneration.

In conclusion, regeneration of a large amount of skeletal muscle was not recognized 24 weeks after implantation in this laceration model using an atelocollagen scaffold. However, the implanted atelocollagen scaffold controlled the amount of bleeding and inhibited the infiltration of inflammatory cells, consequently preventing excessive scar formation in the muscle defect. These phenomena may indicate that atelocollagen provides a field for the regenerational extension of muscle filaments. In this preliminary experiment, neither stem cells nor cytokines (signal molecule) were applied to the collagen scaffold. If such elements were added, this system might be promising for regeneration of skeletal muscle.

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Eur Arch Otorhinolaryngol (2008) 265:669–674
DOI 10.1007/s00405-007-0522-7

LARYNGOLOGY

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

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Received: 26 June 2007 / Accepted: 25 October 2007 / Published online: 8 November 2007

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).

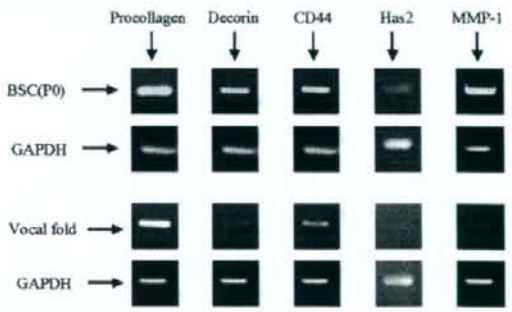

Fig. 1 The mRNA expression of type I procollagen, decorin, CD44, Has2, MMP-1 and GAPDH in BSCs and vocal folds. mRNA expression was detected in all of the samples

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	accccaatgtatccgttgt	tactccttggaggccatgta	299	1.5	56

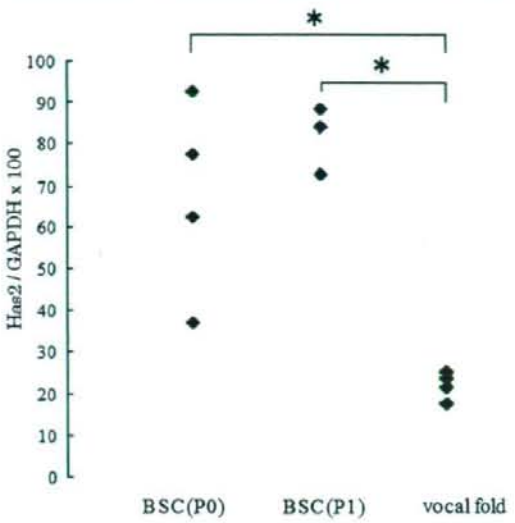

Fig. 2 Analysis of normalized mRNA expression of Has2 in BSCs and vocal folds. Expression was found to be significantly stronger in BSCs than in vocal folds regardless of the passage of time ($^*P < 0.05$)

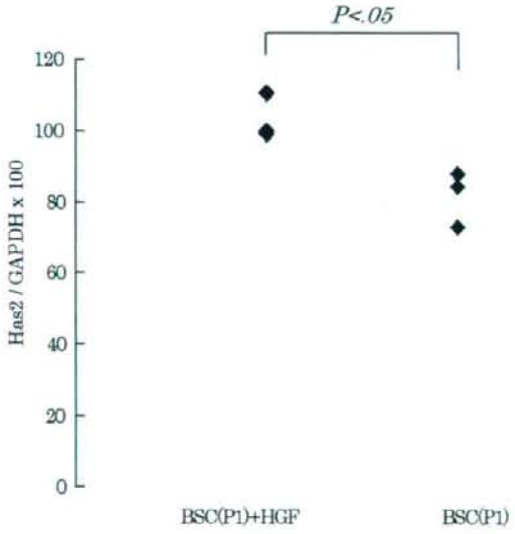

Fig. 3 Analysis of normalized mRNA expression of Has2 in P1 BSCs with or without HGF. The expression in BSCs with HGF (P1 + HGF) was found to be significantly stronger than in BSCs without HGF (P1) ($P < 0.05$)

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

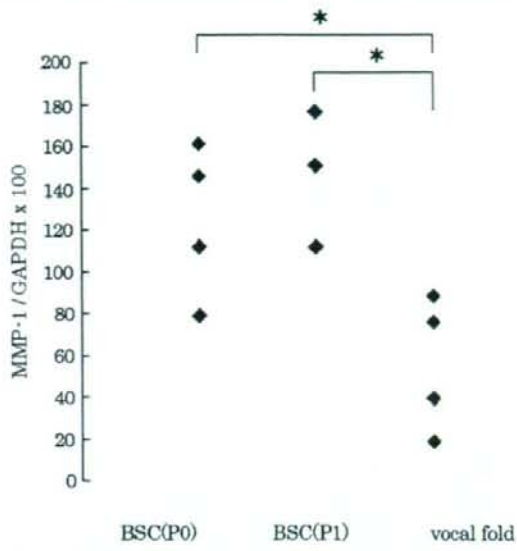

Fig. 4 Analysis of normalized mRNA expression of MMP-1 in the vocal folds and BSCs. Compared to the expression in the vocal folds, the expression in BSCs was found to be significantly stronger regardless of the passage of time ($^*P < 0.05$)

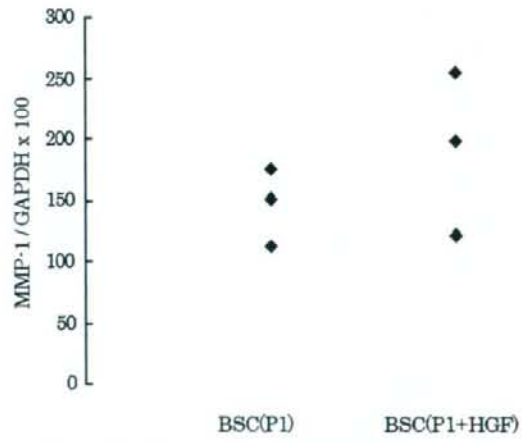

Fig. 5 Analysis of normalized mRNA expression of MMP-1 in P1 BSCs with or without HGF. No difference was detected with or without HGF ($P < 0.05$)

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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J Oral Maxillofac Surg
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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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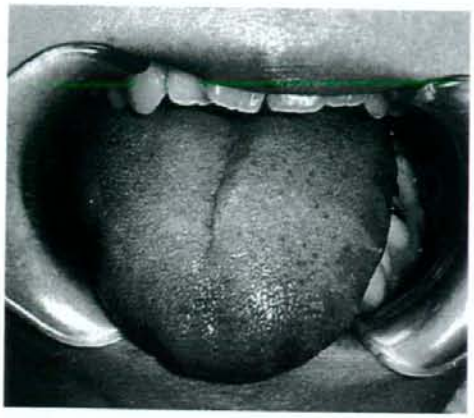

FIGURE 1. Photograph of the patient's tongue at first admission. Taste buds on the right side are completely diminished.

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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.

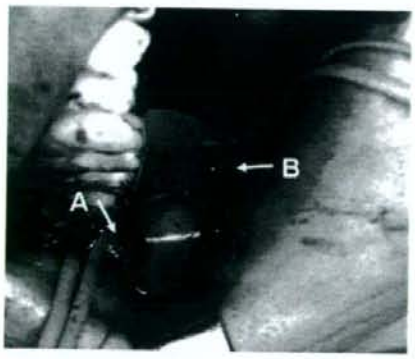

FIGURE 2. Intraoral view of the patient just before repair. The left side represents the maxilla and the right side the mandibular region. Two stump-like enlargements of the cut nerve end are observed. Arrows *A* and *B* indicate the neuroma on the proximal and distal sites, respectively, of the damaged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

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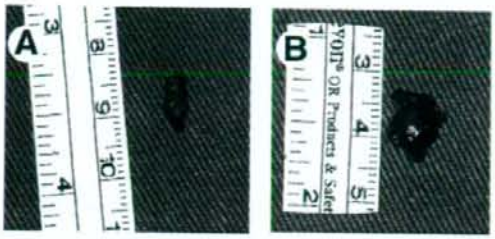

FIGURE 3. Neuromas resected from the distal (*A*) or proximal (*B*) end of the injured lingual nerve.

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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-

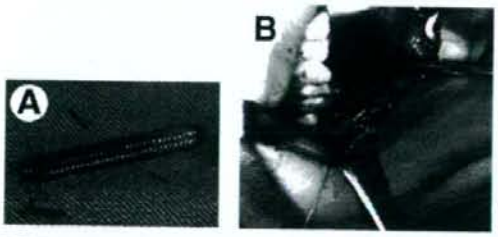

FIGURE 4. *A*, PGA collagen (polyglycolic acid-collagen) tube. Outer diameter 5 mm and length 5 cm. *B*, Intraoral view of the patient just after finishing reconstruction using the tube.

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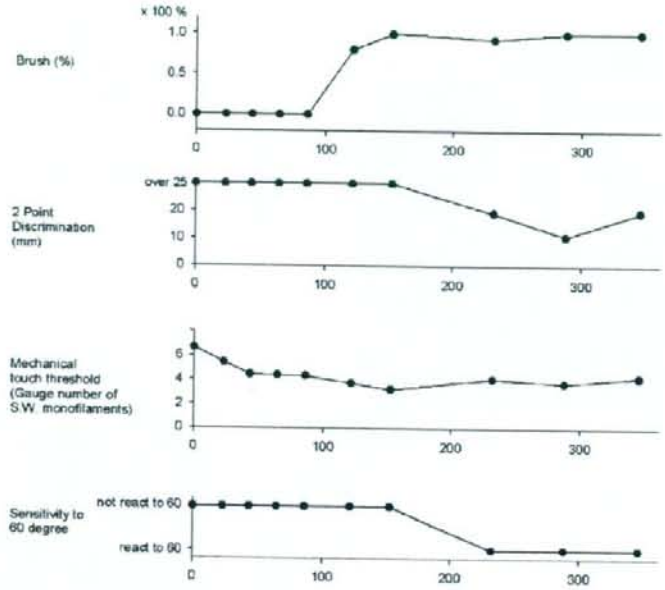

FIGURE 5. Changes in sensory parameters evaluated after operation. Brush stroke perception rate, 2-point discrimination, and mechanical touch threshold measured by Semmes-Weinstein monofilaments; and thermal perception are presented. Abscissa represents days after operation.

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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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