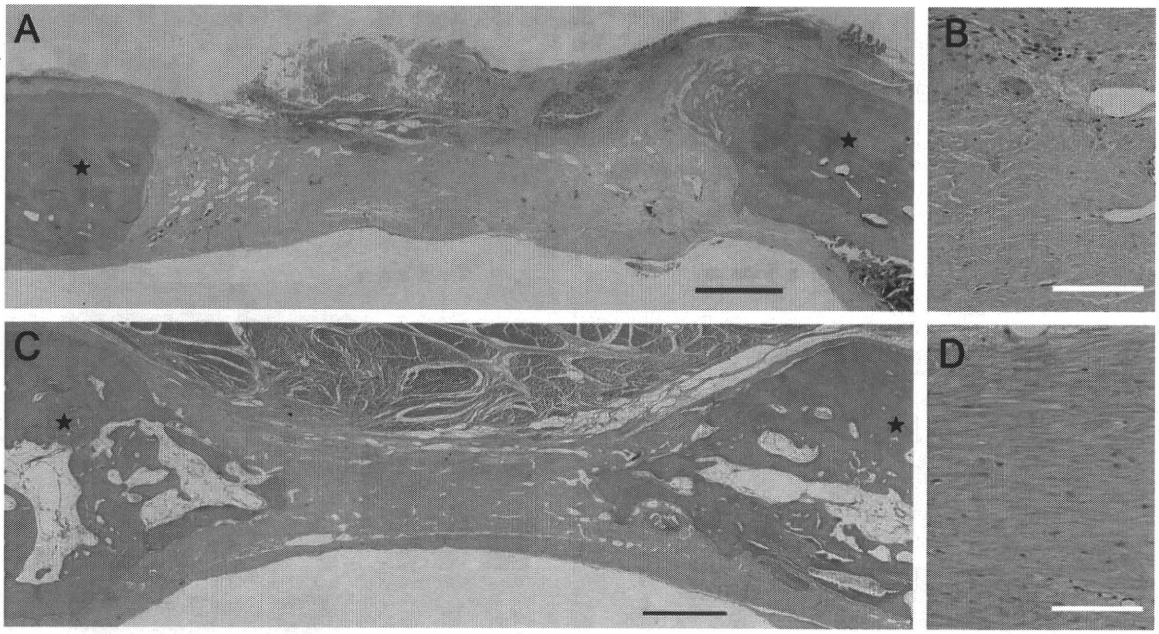

Figure 4. Histological results of group I (A and B) and II (C and D) at 3 months. Bone defects remained at the gap. In both groups, the gaps were covered with fibrous tissue (A and C). Bone neogenesis was observed around the edge of the original bone (C).Asterisk, original bone; red bars = 1 mm; white bars = 100 μm.

amount of residual β-TCP was significantly reduced in group IV compared with group III ($p < 0.01$, Figure 8). In the optimally regenerated holes of group IV at 6 months, new bone connected to existing cranial bone (Figure 6E). However, in some holes in group IV it was also observed that the superficial side of the regenerating area beneath the GBR membrane did not form bone but developed fibrous tissue instead (Figure 6C and G). Figures 7, 8 and Table II show the ratios of regenerated bone area and residual β-TCP.

Discussion

In our previous studies, we introduced the effectiveness of a composite scaffold made of β-TCP with collagen coating [1,2] and BSCs [2], but there were problems that remained to be solved before application to clinical situations. Here, experimental revisions were made, such as fixing the flap to prevent defluxion of the composite scaffold, and the effectiveness of GBR was investigated.

Table I. Findings in groups I and II at 3 months.

Parameter	Group I	Group II
Fibrous tissue intrusion ($p < 0.01$)		
None	0	5
Partial (0–50%)	0	3
Full (=50%)	8	0
Maintenance of regenerative space (NS)		
70–100%	0	0
30–70%	0	4
0–30%	8	4

Fibrous tissue intrusion significantly decreased in group II ($p < 0.01$). Regenerative site was kept <70% of the original area in group II.

GBR was first mentioned in 1959 by Hurley et al., who used the concept in a spinal fusion model [13]. Currently this concept is widely recognized and used in oral dental surgery. Expanded polytetrafluoroethylene (E-PTFE), polylactic acid (PLA), polyglycolic acid (PGA), and collagen are often used as barrier membranes for GBR [5,14]. CA, which is more cost-effective than the above materials, is also reported to be an effective material for GBR and is superior to collagen [8–10]. The aim of a GBR membrane is to prevent fibrous tissue intrusion or infection into the bone defect and to maintain the regenerative space [4,5].

Alginate shows an excellent tissue response and does not incite an inflammatory response. In addition, its thickness and tensile strength can be adjusted with various concentrations of aqueous sodium alginate and calcium chloride solutions. The degradation time of CA can also be regulated, because the thickness of the reaction product of CA changes with the concentration of the reaction liquids. Here, the reaction product of 1–3% aqueous sodium alginate solution and 3% aqueous calcium chloride solution was maintained for 2–4 weeks in

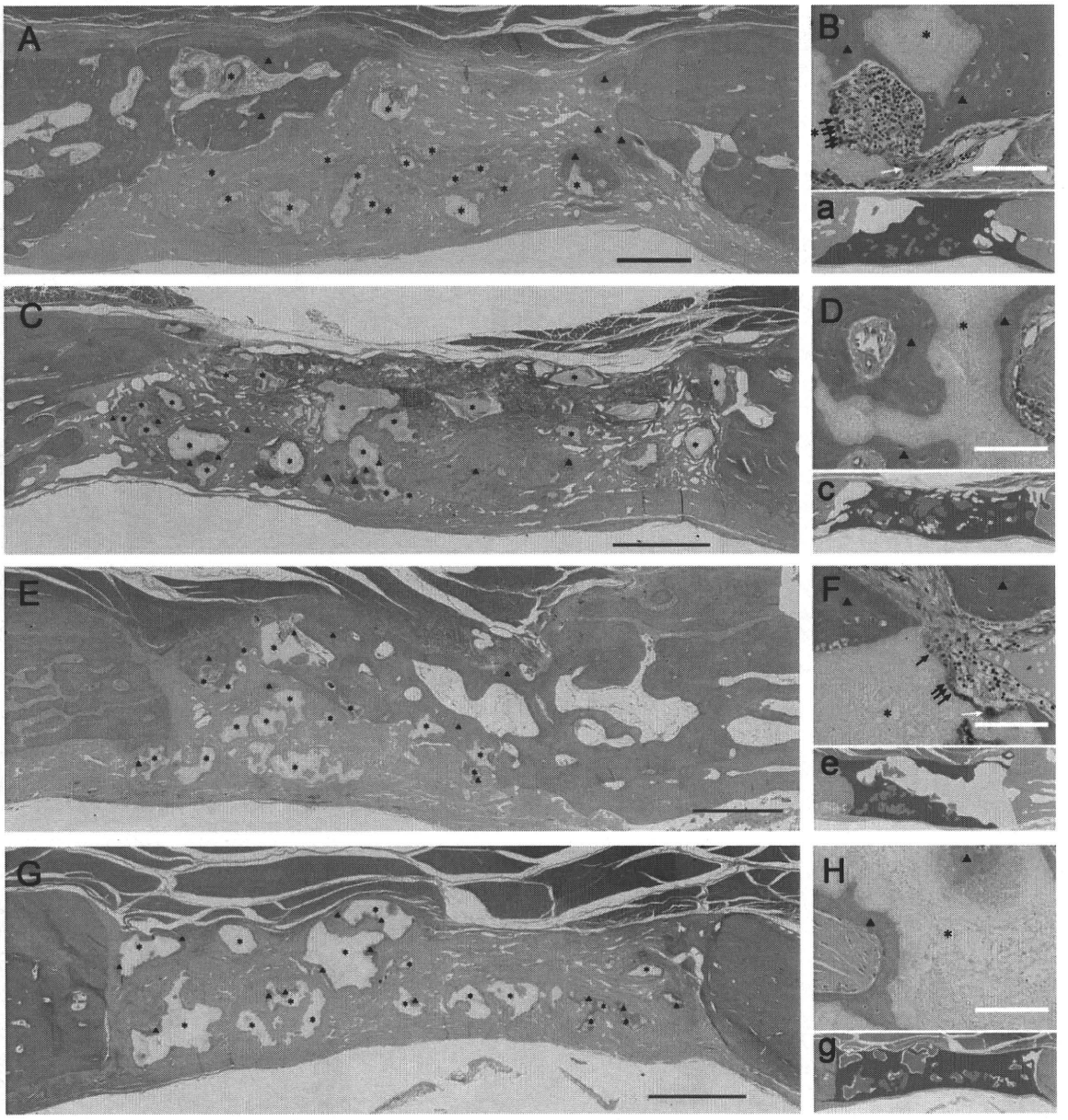

Figure 5. Histological results of group III: (A–D) 3 month model; (E–H) 6 month model. (A, B, E, F) The findings for the drilled hole with optimal bone regeneration for each model. (C, D, G, H) The findings for the hole with unfavorable regeneration. (a, c, e, g) corresponding to figures with capital letters, respectively. Yellow areas, new bone; gray areas, β-TCP; blue areas, fibrous tissue; asterisk, β-TCP; triangle, new bone; black arrow, osteoblasts; white arrow, osteoclast; red bars = 1 mm, white bars = 100 μm.

vivo [10,15]. In addition, CA was reported to become a sustained release carrier if it is used as microspheres. There are reports on the sustained release of vascular endothelial growth factor (VEGF), basic fibroblast growth factor (b-FGF), and transforming growth factor-beta (TGF-β) encapsulated in CA [16–19]. From another standpoint, these results would indicate that CA membrane may act as the barrier against regulatory factors over a period. In this study, CA membrane plays a possible role in blocking the supply of regulatory factors from the superficial tissues which are rich in blood supply. In wound healing, neovascularization is a crucial process, and VEGF is one of the most important factors for angiogenesis. In wound healing of tubular bone, VEGF has been shown to be highly expressed in angioblasts, osteoprogenitors, and osteoblasts for 11 days after injury [20]. VEGF was also reported to have synergistic interactions with both BMP4 and BMP2, improving

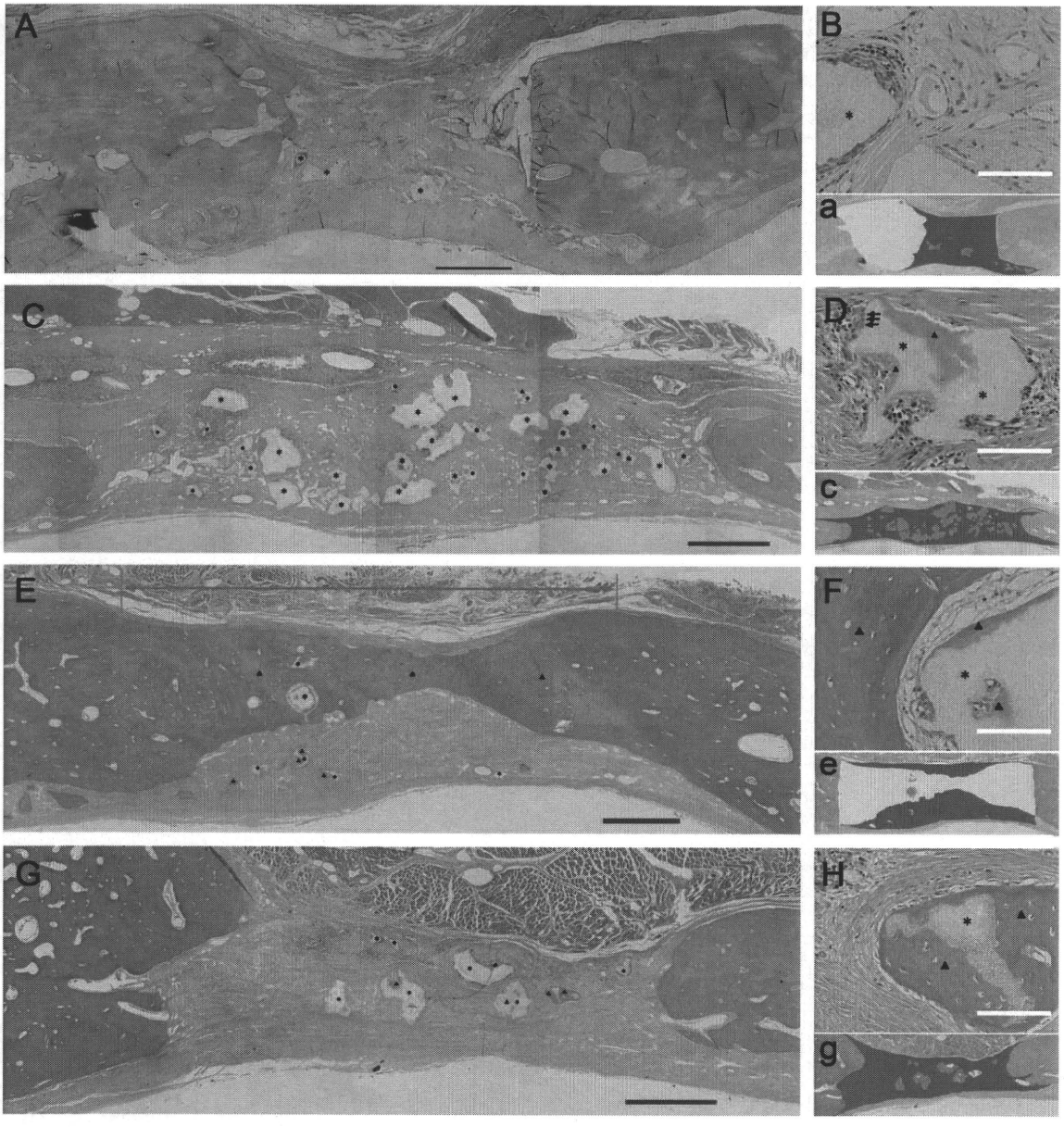

Figure 6. Histological results of group IV: (A–D) 3 months model; (E–H) 6 months model. (A, B, E, F) The findings for the drilled hole with optimal bone regeneration for each group. (C, D, G, H) The findings for the hole with unfavorable regeneration. (a, c, e, g) corresponding to figures with capital letters, respectively. Yellow areas, new bone; gray areas, β-TCP; blue areas, fibrous tissue. (E) Arrows show the site where full-thickness hole had been made. Asterisk, β-TCP; triangle, new bone; black arrow, osteoblasts; red bars = 1 mm; white bars = 100 μm.

BMP2-induced bone formation and bone healing through modulation of angiogenesis [21].

The overall results of this study suggest that a barrier membrane made of CA had both advantages and disadvantages for cranial bone regeneration. Prevention of fibrous tissue intrusion from the superficial layer and preservation of the scaffold were advantages to using CA membrane revealed by group IV. However, the CA barrier membrane did not always contribute to cranial bone regeneration. At 3 months, bone regeneration was significantly delayed at the regenerative site in group IV, while bone regeneration was seen to occur gradually at the superficial layer in group III. The reason for this may be that the different environments between cranial bone and mandible or tubular bone may cause different outcomes. In mandible or tubular bone, the GBR membrane-covered area has the rich

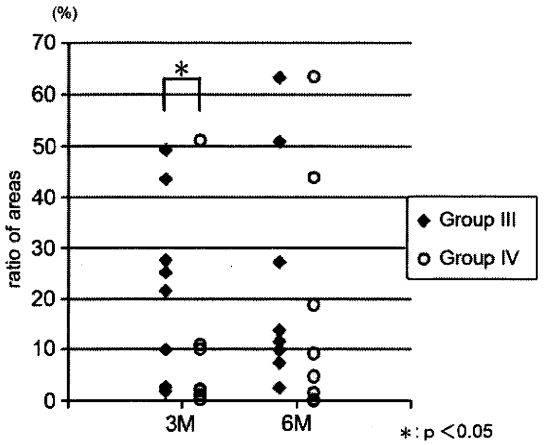

Figure 7. Image analysis results for new bone (without β-TCP). As compared to the position of the original bone, the regenerated area was defined as 100%. At 3 months, less new bone formation was observed in group IV than in group III.

bone marrow bed behind the defect. In contrast, cortical bone, containing little bone marrow, occupies almost all of the cranial bone. In this experimental model, a full-thickness defect in cranial bone was made. Accordingly, even though the barrier membrane acted properly, important bone regeneration regulatory factors might be insufficient within the barrier. In other words, the supplementary supply of vessels or regulatory factors from the superficial structures may be indispensable in cranial bone regeneration.

Superficial early bone neogenesis in the optimal regeneration cases of group III, the many residual β-TCP particles in group IV at 3 months, and the fibrosis beneath the barrier membrane of group IV at 6 months may all indicate the importance of

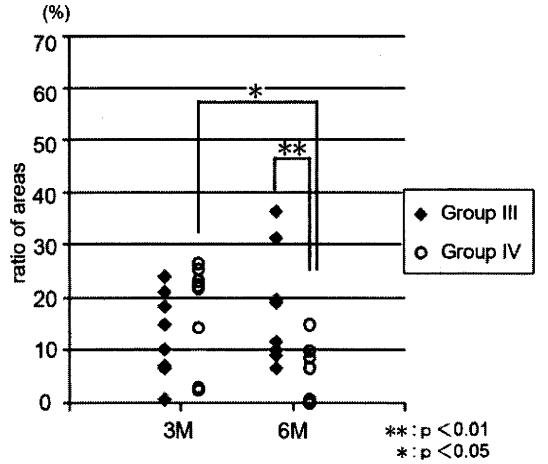

Figure 8. Image analysis results for residual β-TCP. As compared to the position of the original bone, the regenerated area was defined as 100%. The amount of residual β-TCP was significantly reduced in group IV compared with group III at 6 months.

Table II. Image analysis results.

Group	Follow-up	New bone (%): average ± SD	β-TCP (%): average ± SD
Group III	3 months	22.7 ± 17.6	12.9 ± 8.1
	6 months	23.3 ± 22.2	18.0 ± 10.9
Group IV	3 months	9.8 ± 17.3	17.3 ± 9.8
	6 months	17.7 ± 23.6	5.1 ± 5.7

As compared to the position of the original bone, the area of regeneration was defined as 100%. The ratio of the areas of new bone without β-TCP and that of β-TCP was calculated for each group.

neovascularization from the superficial structures in the early phases of regeneration. This may include regulation of factors such as VEGF for regeneration of cranial bone, as this bone type does not have rich bone marrow and vascular system.

In this experiment, we investigated assured cranial bone regeneration, applying the concept of GBR using a CA membrane, which functioned as a barrier membrane and resolved problems from prior experiments. However, other disadvantages were revealed with the application of the barrier membrane to the cranial bone regeneration. In order to regenerate cranial bone reliably, other methods or techniques for protecting defluxion of scaffolds, or techniques for inducing early angiogenesis are needed. When these issues are resolved, conclusive cranial bone regeneration will be provided.

Conclusion

Cranial bone regeneration with GBR was studied in canine models. A GBR membrane made of CA prevented fibrous tissue intrusion, but did not always accelerate cranial bone regeneration.

Acknowledgements

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References

[1] Kishimoto M, Kanemaru S, Yamashita M, Nakamura T, Tamura Y, Tamaki H, et al. Cranial bone regeneration using a composite scaffold of Beta-tricalcium phosphate, collagen, and autologous bone fragments. Laryngoscope 2006;116: 212–6.

[2] Umeda H, Kanemaru S, Yamashita M, Kishimoto M, Tamura Y, Nakamura T, et al. Bone regeneration of canine skull using bone marrow-derived stromal cells and beta-tricalcium phosphate. Laryngoscope 2007;117:997–1003.
[3] Hori Y, Nakamura T, Matsumoto K, Kurokawa Y, Satomi S, Shimizu Y. Experimental study on in situ tissue engineering of the stomach by an acellular collagen sponge scaffold graft. ASAIO J 2001;47:206–10.
[4] Kay SA, Wisner-Lynch L, Marxer M, Lynch SE. Guided bone regeneration: integration of a resorbable membrane and a bone graft material. Pract Periodontics Aesthet Dent 1997;9:185–94; quiz 196.
[5] Zellin G, Gritli-Linde A, Linde A. Healing of mandibular defects with different biodegradable and non-biodegradable membranes: an experimental study in rats. Biomaterials 1995;16:601–9.
[6] Queiroz TP, Hochuli-Vieira E, Gabrielli MA, Cancian DC. Use of bovine bone graft and bone membrane in defects surgically created in the cranial vault of rabbits. Histologic comparative analysis. Int J Oral Maxillofac Implants 2006; 21:29–35.
[7] Schantz JT, Hutmacher DW, Ng KW, Khor HL, Lim MT, Teoh SH. Evaluation of a tissue-engineered membrane-cell construct for guided bone regeneration. Int J Oral Maxillofac Implants 2002;17:161–74.
[8] He H, Huang J, Shi J, Ping F, Chen G, Dong Y. Haversian remodeling in guided bone regeneration with calcium alginate film in circular bone defect model of rabbit. Artif Cells Blood Immobil Biotechnol 2007;35:533–42.
[9] Jianqi H, Hong H, Lieping S, Genghua G. Comparison of calcium alginate film with collagen membrane for guided bone regeneration in mandibular defects in rabbits. J Oral Maxillofac Surg 2002;60:1449–54.
[10] Ueyama Y, Ishikawa K, Mano T, Koyama T, Nagatsuka H, Suzuki K, et al. Usefulness as guided bone regeneration membrane of the alginate membrane. Biomaterials 2002;23: 2027–33.
[11] Attwood AI. Calcium alginate dressing accelerates split skin graft donor site healing. Br J Plast Surg 1989;42:373–9.
[12] Nakamura T, Inada Y, Fukuda S, Yoshitani M, Nakada A, Itoi S, et al. Experimental study on the regeneration of peripheral nerve gaps through a polyglycolic acid-collagen (PGA-collagen) tube. Brain Res 2004;1027:18–29.
[13] Hurley LA, Stinchfield FE, Bassett AL, Lyon WH. The role of soft tissues in osteogenesis. An experimental study of canine spine fusions. J Bone Joint Surg Am 1959;41A:1243–54.
[14] Simion M, Fontana F, Rasperini G, Maiorana C. Vertical ridge augmentation by expanded-polytetrafluoroethylene membrane and a combination of intraoral autogenous bone graft and deproteinized anorganic bovine bone (Bio Oss). Clin Oral Implants Res 2007;18:620–9.
[15] Ueyama Y, Koyama T, Ishikawa K, Mano T, Ogawa Y, Nagatsuka H, et al. Comparison of ready-made and self-setting alginate membranes used as a barrier membrane for guided bone regeneration. J Mater Sci Mater Med 2006;17: 281–8.
[16] Gu F, Amsden B, Neufeld R. Sustained delivery of vascular endothelial growth factor with alginate beads. J Control Release 2004;96:463–72.
[17] Lee KW, Yoon JJ, Lee JH, Kim SY, Jung HJ, Kim SJ, et al. Sustained release of vascular endothelial growth factor from calcium-induced alginate hydrogels reinforced by heparin and chitosan. Transplant Proc 2004;36:2464–5.
[18] Edelman ER, Mathiowitz E, Langer R, Klagsbrun M. Controlled and modulated release of basic fibroblast growth factor. Biomaterials 1991;12:619–26.
[19] Mierisch CM, Cohen SB, Jordan LC, Robertson PG, Balian G, Diduch DR. Transforming growth factor-beta in calcium alginate beads for the treatment of articular cartilage defects in the rabbit. Arthroscopy 2002;18:892–900.
[20] Uchida S, Sakai A, Kudo H, Otomo H, Watanuki M, Tanaka M, et al. Vascular endothelial growth factor is expressed along with its receptors during the healing process of bone and bone marrow after drill-hole injury in rats. Bone 2003; 32:491–501.
[21] Peng H, Usas A, Olshanski A, Ho AM, Gearhart B, Cooper GM, et al. VEGF improves, whereas sFlt1 inhibits, BMP2-induced bone formation and bone healing through modulation of angiogenesis. J Bone Miner Res 2005;20:2017–27.

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Effect of Exogenous Hepatocyte Growth Factor on Vocal Fold Fibroblasts

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

Objectives: We have previously demonstrated the therapeutic potential of hepatocyte growth factor (HGF) in the treatment of vocal fold scarring, although how exogenous HGF affects gene expression of endogenous HGF or extracellular matrix components in the vocal fold fibroblasts remains unclear. In this in vitro study, we aimed to clarify this aspect in order to better understand the effects of HGF on the vocal folds.

Methods: Fibroblasts were obtained from the lamina propria of the vocal folds of 5 Sprague-Dawley rats and were cultured with HGF at concentrations of 100, 10, 1, and 0 ng/mL. The cells were collected on days 1, 3, and 7, and the expression of endogenous HGF, its receptor c-Met, transforming growth factor–β1 (TGF-β1), procollagen types I and III, and hyaluronic acid synthase (HAS)–1 and HAS-2 messenger RNAs (mRNAs) was examined by real-time reverse transcription–polymerase chain reaction.

Results: The expression of endogenous HGF and HAS-1 mRNAs increased significantly when exogenous HGF was administered at a concentration of 1 ng/mL. On day 1, the expression of TGF-β1 and HAS-2 mRNAs increased significantly in response to 1 ng/mL HGF.

Conclusions: Exogenous HGF triggered the up-regulation of endogenous HGF, TGF-β1, HAS-1, and HAS-2 mRNAs in vocal fold fibroblasts.

Key Words: gene expression, hepatocyte growth factor, vocal fold fibroblast.

INTRODUCTION

Vocal fold scarring occurs after injury, inflammation, or phonosurgery of the vocal fold. It disrupts the layered structure of the lamina propria, changing the biomechanical properties of the vocal fold. In other words, scarring stiffens the viscoelastic shear properties of the vocal fold mucosa that are essential for optimal vibration, and it reduces mucosal wave propagation, resulting in severe dysphonia.

For treatment of vocal fold scarring, various kinds of therapeutic strategies, such as voice therapy, injection therapy, and a medial shift of the vocal folds, have been attempted to soften the vocal fold and to restore its normal properties; however, to date, there is no optimal strategy to restore the scarred vocal fold. Therefore, development of a new regenerative pathway to restore the normal properties of the vocal fold is needed.

Previous studies on vocal fold scarring have reported the changes in the organization and distribution of extracellular matrix (ECM) components such as dense and/or disorganized type I collagen deposition, decreased elastin and decorin, increased fibronectin, and occasional decreases of hyaluronic acid (HA).[1-3] These histologic changes stiffen the vocal fold and cause hoarseness. Therefore, a correction of the distribution of ECM components is needed to restore the vocal fold after scarring. Since the ECM components are primarily synthesized by fibroblasts,[4] it is thought that controlling the fibroblasts will be an effective strategy to correct these adverse histologic changes.

Hepatocyte growth factor (HGF) is a multifunctional polypeptide that plays a significant role in embryogenesis, angiogenesis, organ regeneration, and wound healing. It has strong antifibrotic activity and has been shown to contribute to the prevention or complete resolution of fibrosis in liver, kidney, and lung in animal models.[5] Another study also reported

From the Department of Otolaryngology–Head and Neck Surgery, Graduate School of Medicine, Kyoto University (Kishimoto, Hirano, Suehiro, Tateya, Kanemaru, Ito), and the Department of Bioartificial Organs, Institute of Frontier Medical Sciences, Kyoto University (Nakamura), Kyoto, Japan.

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Correspondence: Shigeru Hirano, MD, PhD, Dept of Otolaryngology–Head and Neck Surgery, Graduate School of Medicine, Kyoto University, Sakyo-ku, Kyoto 606-8507, Japan.

that HGF has therapeutic potential in the management of vocal fold scarring because it increases HA production and decreases collagen production in vocal fold fibroblasts.[6] However, it is still unclear how the exogenous HGF affects the gene expression of endogenous HGF or ECM components in the vocal fold fibroblasts. In the present study, we aimed to clarify this aspect to better understand the effects of HGF on vocal fold fibroblasts.

MATERIALS AND METHODS

Animals. Five Sprague-Dawley rats (male; 16 to 30 weeks old; Japan SLC Inc, Hamamatsu, Japan) were used in this study. All experimental procedures were conducted in accordance with Kyoto University guidelines.

Isolation and Culture of Rat Vocal Fold Fibroblasts. The isolation and culture methods used were those that have been established from previous studies.[7,8] The animals were anesthetized by diethyl ether inhalation and then were humanely euthanized by intracardiac injection of pentobarbital sodium. The larynx was dissected out immediately, and the lamina propria and epithelium of the vocal folds, excluding the maculae flavae, were removed with the aid of a dissecting microscope. The excised tissue was minced into small pieces in a 10-cm Petri dish and then cultured with 10 mL Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum and antibiotics, at 37°C under 5% carbon dioxide. The medium was refreshed twice a week until the cells grew to confluency.

Subculture With HGF. The fibroblasts at the first passage were subcultured on 12- or 6-well microplates with the culture medium described above. After the cells grew to 80% to 90% confluency, human recombinant HGF (PeproTech Inc, Rocky Hill, New Jersey) was added into paired wells at concentrations of 100, 10, and 1 ng/mL. As a control, wells without HGF were prepared. The cells were harvested at days 1, 3, and 7. The culture media were not changed over 7 days and were allowed to diminish over time naturally.

Extraction of Total RNA and Complementary DNA Synthesis. Cells lysates were prepared by passing the collected cells through a 21-gauge needle 10 times. Total RNA was isolated from the lysates with RNeasy Mini Kit (Qiagen, Valencia, California). To eliminate genomic DNA contamination, we treated the total RNA with DNaseI (Ambion, Austin, Texas). The quantity and quality of the RNA were evaluated by measuring the A260/280 ratio of the RNA samples and visualizing 18S and 28S ribosomal RNA bands on an agarose gel.

TABLE 1. PRIMER SEQUENCES

β2-Microglobulin	
Forward	5'-TCA CAC TGA ATT CAC ACC CAC CGA-3'
Reverse	5'-TGA TTA CAT GTC TCG GTC CCA GGT-3'
HGF	
Forward	5'-ACA AGG GCT TTC CAT TCA CT-3'
Reverse	5'-CCA GTA GCA TCG TTT TCT CG-3'
c-Met	
Forward	5'-TCC TGT GGC TGA GAA AGA GA-3'
Reverse	5'-GGG CAC TTA CAA GCC TAT CC-3'
TGF-β1	
Forward	5'-GCT TCA GTG CTC ACT GCT CT-3'
Reverse	5'-GGG ACT GAT CCC ATT GAT TT-3'
Procollagen type I	
Forward	5'-AGG CAT AAA GGG TCA TCG TGG CTT-3'
Reverse	5'-AGT CCA TCT TTG CCA GGA GAA CCA-3'
Procollagen type III	
Forward	5'-ATG AGC TTT GTG CAA TGT GGG ACC-3'
Reverse	5'-ACT GAC CAA GGT AGT TGC ATC CCA-3'
HAS-1	
Forward	5'-TAG GTG CTG TTG GAG GAG ATG TGA-3'
Reverse	5'-AAG CTC GCT CCA CAT TGA AGG CTA-3'
HAS-2	
Forward	5'-ACT GGG CAG AAG CGT GGA TTA TGT-3'
Reverse	5'-AAC ACC TCC AAC CAT CGG GTC TTC TT-3'

HGF — hepatocyte growth factor; c-Met — receptor of HGF; TGF-β1 — transforming growth factor–β1; HAS — hyaluronic acid synthase.

Complementary DNAs were synthesized by reverse transcription (RT) using TacMan Reverse Transcription Reagents (Applied Biosystems Inc, Foster City, California). Reactions were performed with the GeneAmp polymerase chain reaction (PCR) system 9700 (Applied Biosystems Inc) with the following parameters: 25°C for 10 minutes, 48°C for 30 minutes, 95°C for 5 minutes, and 4°C for 5 minutes.

Real-Time RT-PCR. The primers used in this study are listed in Table 1. The expression of endogenous HGF, the HGF receptor c-Met, transforming growth factor–β1 (TGF-β1), procollagen types I and III, hyaluronic acid synthase (HAS)–1, HAS-2, and β2-microglobulin (a constitutively expressed gene included as a control) messenger RNAs (mRNAs) was examined. Primers were synthesized by Hokkaido System Science Co, Ltd, Sapporo, Japan.

Real-time RT-PCR was performed in a final volume of 20 μL according to the manufacturer's protocols; the reaction mix comprised template complementary DNA, 10 μL of Power SYBR Green Master Mix (Applied Biosystems Inc), 0.25 μmol/L final concentration of each primer, and ribonuclease-free water. The amplification profile was as follows: ura-

TABLE 2. RESULTS OF TWO-WAY FACTORIAL ANALYSIS OF VARIANCE

	Main Effect		
	Concentration	*Time Point*	*Interaction*
HGF	0.002	0.003	0.898
c-Met	0.887	<0.001	0.985
TGF-β1	0.021	<0.001	0.026
Procollagen type I	0.711	0.370	0.984
Procollagen type III	0.904	0.567	0.948
HAS-1	0.001	0.338	0.964
HAS-2	<0.001	<0.001	<0.001

Data are p values.

cil-*N*-glycosylase activation at 50°C for 2 minutes, enzyme activation at 95°C for 10 minutes, and 40 cycles of denaturing at 95°C for 15 seconds and annealing at 60°C for 1 minute. Fluorescence was detected with the ABI Prism 7000 (Applied Biosystems Inc). Each mRNA value was normalized to that of the β2-microglobulin control mRNA.

Statistical Analysis. The data of duplicate cultures were averaged before combining them across cell lines. The effects of exogenous HGF administration on gene expression of vocal fold fibroblasts were examined with 2-way factorial analysis of variance (ANOVA) and a subsequent Scheffé post hoc test. When interactions were present between the concentration and the time point, 1-factor ANOVA with Scheffé post hoc testing was used to examine the simple main effects of concentration at each time point. Values of p of less than 0.05 were considered to be statistically significant.

RESULTS

Table 2 shows the results of 2-way factorial ANOVA, which revealed an interaction effect between the concentration of HGF and the day the lysates were collected for the expression of TGF-β1 and HAS-2 mRNAs.

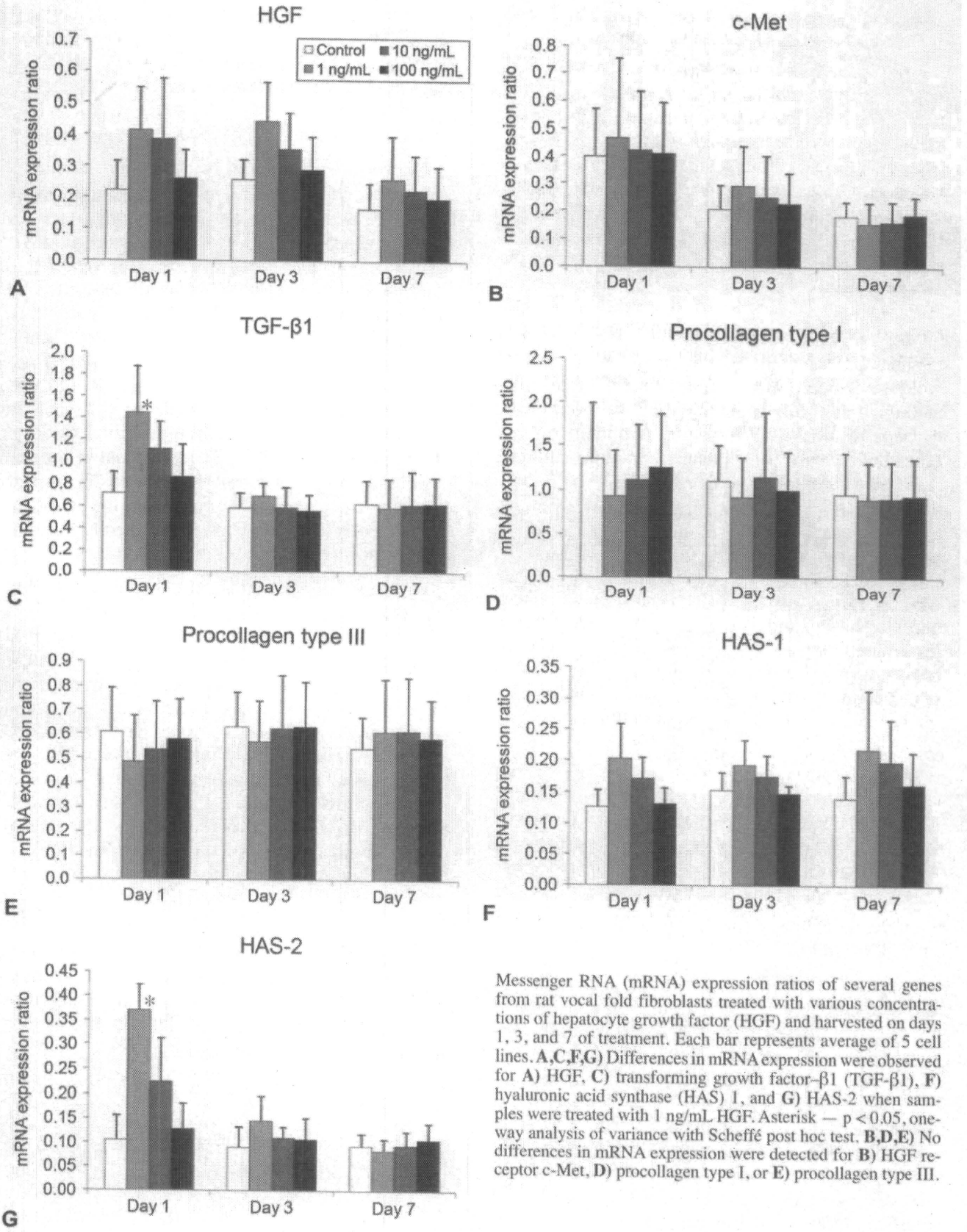
The Figure shows the temporal changes of mRNA expression ratios for several genes responding to different concentrations of exogenously applied HGF. Each bar represents the average of the 5 cell lines. As for the expressions of endogenous HGF and HAS-1 mRNAs, 2-way factorial ANOVA demonstrated a significant main effect of concentrations of exogenous HGF, and post hoc testing revealed that the expression of endogenous HGF ($p = 0.007$) and HAS-1 ($p = 0.006$) mRNAs increased significantly at an HGF concentration of 1 ng/mL. However, there were no significant mRNA expression differences for c-Met or procollagen type I or III at any concentration.

One-way ANOVA with post hoc testing was used to examine the expression of TGF-β1 and HAS-2. It showed that on day 1 the expression of TGF-β1 ($p = 0.013$) and HAS-2 ($p < 0.001$) mRNAs increased significantly at an HGF concentration of 1 ng/mL.

DISCUSSION

The treatment of vocal fold scarring remains a therapeutic challenge.[9] Many therapeutic strategies have been attempted to restore vocal fold viscoelastic shear properties; however, there is still no optimal strategy to prevent or treat vocal fold scarring. Previous studies on vocal fold scarring demonstrated histologic changes in the organization and distribution of ECM components, and disruption of the layer structure in the lamina propria.[1-3] In injured vocal folds, fibroblasts produce various kinds of ECM components to repair the wound and cytokines to control ECM production. Since the distribution of ECM is the key to determining the tissue properties of the vocal fold, a better understanding of how to control the responses of vocal fold fibroblasts at the molecular level is needed for the development of new therapeutic strategies.

We have focused on HGF as a candidate for growth factor therapy to control ECM production by vocal fold fibroblasts in the management of vocal fold scarring.[6,10-14] A multifunctional polypeptide, HGF has strong antifibrotic activity and has been used to prevent or restore fibrosis in the liver, kidney, and lung in animal models.[5] Hirano et al[6] have reported that HGF stimulates HA production and suppresses collagen type I production from normal canine vocal fold fibroblasts — a finding that suggested that HGF may have a therapeutic potential for scarred vocal folds. Subsequent in vivo studies examined the effects of HGF injection on the prevention and treatment of vocal fold scarring in rabbit[12] and canine[13] models. In those studies, HGF solution was locally injected into injured or scarred vocal folds, and the results indicated improvement in histologic and vibratory properties. It is curious why merely injecting an HGF solution works so well to restore histologic alterations of scar tissues, when such a solution is usually dissolved and metabolized quickly within a day. A possible explanation may be the existence of autocrine effects of HGF on vocal fold fibroblasts. Indeed, HGF autocrine mechanisms have been reported in some organs,[15-17] in which HGF and c-Met are detected in the same cells. Here, the growth factors are synthesized locally and activate the receptors on the cells from which they were produced. Our hypothesis was that exogenous HGF may at least trigger an up-regulation of endogenous HGF in vocal fold fibroblasts, which in turn achieves long-term biological effects inside the tissue. The present



Messenger RNA (mRNA) expression ratios of several genes from rat vocal fold fibroblasts treated with various concentrations of hepatocyte growth factor (HGF) and harvested on days 1, 3, and 7 of treatment. Each bar represents average of 5 cell lines. **A,C,F,G)** Differences in mRNA expression were observed for **A)** HGF, **C)** transforming growth factor–β1 (TGF-β1), **F)** hyaluronic acid synthase (HAS) 1, and **G)** HAS-2 when samples were treated with 1 ng/mL HGF. Asterisk — $p < 0.05$, one-way analysis of variance with Scheffé post hoc test. **B,D,E)** No differences in mRNA expression were detected for **B)** HGF receptor c-Met, **D)** procollagen type I, or **E)** procollagen type III.

study was designed to test this aspect, as well as the effects of HGF on the gene expression of various ECM components.

The HGF receptor, c-Met, is a heterodimeric tyrosine kinase receptor that consists of an extracellular subunit and a transmembrane subunit.[18,19] The

biological activities of HGF appear only when HGF couples with c-Met on the target cell membrane.[20] In this study, the administration of exogenous HGF had no significant effect on the expression of c-Met mRNA, whereas the expression of endogenous HGF mRNA increased significantly with the administration of exogenous HGF at a concentration of 1 ng/mL. This result suggests that the administration of exogenous HGF triggers the up-regulation of endogenous HGF mRNA. Although the expression of c-Met in normal fibroblasts was not regulated by the administration of exogenous HGF, it may be affected in different phenotypes of fibroblasts, such as fibroblasts from injured vocal folds. Further study is needed to test our hypothesis.

As mentioned above, a previous in vitro study suggested that HGF suppresses collagen type I production.[6] In this study, however, there were no significant differences in the expression of procollagen type I and III mRNAs at any concentration. Fibroblasts isolated from a normal vocal fold might be relatively quiescent, and different results might be achieved when we use fibroblasts isolated from injured vocal folds. Moreover, it is well known that HGF stimulates the collagenase activity of fibroblasts, which decreases the accumulation of collagen inside the tissue. Therefore, further study is needed to clarify the control mechanisms of collagen content in vocal folds by examining the effects of HGF on the expression of collagenase, as well as procollagens.

Hyaluronic acid is a kind of glycosaminoglycan distributed throughout the epithelia and connective tissues, and is concentrated in the vocal folds and other specialized tissues.[21] It plays a major role in wound healing, inhibiting collagen synthesis in human dermal fibroblasts, and is regarded as a potent antifibrotic molecule.[22] It is distributed in the superficial layer of the lamina propria of the vocal fold,[23] and is thought to be the most important ECM component for maintaining the biomechanical properties of the lamina propria. In this study, as compared to controls, HAS-1 mRNA expression was significantly higher when HGF was applied at a concentration of 1 ng/mL, and HAS-2 mRNA expression was significantly higher at a concentration of 1 ng/mL on day 1. In a previous study, Hirano et al[6] showed an increase in HA production by canine vocal fold fibroblasts using enzyme-linked immunosorbent assay at 24 and 48 hours after the administration of HGF, which is consistent with our results. Furthermore, Ohno et al[24] investigated the in vivo effects of HGF on ECM gene expression during wound healing of injured rat vocal folds, and reported significantly higher expression of HAS-2 on posttreatment day 14.

It is unclear why administration of exogenous HGF in our study increased the expression of TGF-β1 mRNA without up-regulating procollagen mRNA levels. Transforming growth factor–β1 is a cytokine that is known to induce fibrogenesis and counteract HGF in wound healing.[25] Previous in vivo studies have reported that HGF inhibits the expression of TGF-β1,[26,27] whereas a study of primary rat hepatic stellate cell cultures showed that exogenous HGF increases the expression of TGF-β1.[28] In addition, other studies have indicated no significant effects of HGF on TGF-β1 expression in human skin fibroblasts[29] or in a hepatic stellate cell line.[30] It is possible that these contrasting results on the interaction between HGF and TGF-β1 may be due to differing conditions among the experiments.

It is well known that the effects of growth factors often depend on their concentration. The data in this study indicate that the fibroblasts of the vocal fold were stimulated by the administration of HGF at lower concentrations, and suggest that the effect of exogenous HGF is to trigger the up-regulation of endogenous HGF expression in the vocal folds, possibly in an autocrine manner. Further studies are needed to prove the mechanisms of HGF's effect on vocal fold fibroblasts, including an investigation of whether HGF functions in an autocrine loop, and to determine the appropriate concentration of HGF to use for clinical applications.

CONCLUSIONS

We performed in vitro experiments to investigate the effects of exogenous HGF on the gene expression of vocal fold fibroblasts. The expression of endogenous HGF, TGF-β1, HAS-1, and HAS-2 mRNAs was significantly up-regulated with the application of exogenous HGF. These results indicate that exogenous HGF triggers the up-regulation of endogenous HGF, which can contribute to remodeling mechanisms of the vocal fold, accompanied by stimulation of HA production.

REFERENCES

1. Hirano S, Minamiguchi S, Yamashita M, Ohno T, Kanemaru SI, Kitamura M. Histologic characterization of human scarred vocal folds. J Voice (in press).

2. Rousseau B, Hirano S, Scheidt TD, et al. Characterization of vocal fold scarring in a canine model. Laryngoscope 2003;113:620-7.

3. Rousseau B, Hirano S, Chan RW, et al. Characterization of chronic vocal fold scarring in a rabbit model. J Voice 2004;

18:116-24.

4. Hirano M, Sato K, Nakashima T. Fibroblasts in human vocal fold mucosa. Acta Otolaryngol 1999;119:271-6.

5. Matsumoto K, Nakamura T. Hepatocyte growth factor (HGF) as a tissue organizer for organogenesis and regeneration. Biochem Biophys Res Commun 1997;239:639-44.

6. Hirano S, Bless D, Heisey D, Ford C. Roles of hepatocyte growth factor and transforming growth factor beta1 in production of extracellular matrix by canine vocal fold fibroblasts. Laryngoscope 2003;113:144-8.

7. Thibeault SL, Li W, Gray SD, Chen Z. Instability of extracellular matrix gene expression in primary cell culture of fibroblasts from human vocal fold lamina propria and tracheal scar. Ann Otol Rhinol Laryngol 2002;111:8-14.

8. Thibeault SL, Li W, Bartley S. A method for identification of vocal fold lamina propria fibroblasts in culture. Otolaryngol Head Neck Surg 2008;139:816-22.

9. Hirano S. Current treatment of vocal fold scarring. Curr Opin Otolaryngol Head Neck Surg 2005;13:143-7.

10. Hirano S, Bless DM, Heisey D, Ford CN. Effect of growth factors on hyaluronan production by canine vocal fold fibroblasts. Ann Otol Rhinol Laryngol 2003;112:617-24.

11. Hirano S, Bless DM, Massey RJ, Hartig GK, Ford CN. Morphological and functional changes of human vocal fold fibroblasts with hepatocyte growth factor. Ann Otol Rhinol Laryngol 2003;112:1026-33.

12. Hirano S, Bless DM, Rousseau B, et al. Prevention of vocal fold scarring by topical injection of hepatocyte growth factor in a rabbit model. Laryngoscope 2004;114:548-56.

13. Hirano S, Bless DM, Nagai H, et al. Growth factor therapy for vocal fold scarring in a canine model. Ann Otol Rhinol Laryngol 2004;113:777-85.

14. Ohno T, Hirano S, Kanemaru S, et al. Drug delivery system of hepatocyte growth factor for the treatment of vocal fold scarring in a canine model. Ann Otol Rhinol Laryngol 2007;116:762-9.

15. Wordinger RJ, Clark AF, Agarwal R, et al. Cultured human trabecular meshwork cells express functional growth factor receptors. Invest Ophthalmol Vis Sci 1998;39:1575-89.

16. Sheehan SM, Tatsumi R, Temm-Grove CJ, Allen RE. HGF is an autocrine growth factor for skeletal muscle satellite cells in vitro. Muscle Nerve 2000;23:239-45.

17. Yang XM, Toma JG, Bamji SX, et al. Autocrine hepatocyte growth factor provides a local mechanism for promoting axonal growth. J Neurosci 1998;18:8369-81.

18. Wormstone IM, Tamiya S, Marcantonio JM, Reddan JR. Hepatocyte growth factor function and c-Met expression in human lens epithelial cells. Invest Ophthalmol Vis Sci 2000;41:4216-22.

19. Jinnin M, Ihn H, Mimura Y, Asano Y, Yamane K, Tamaki K. Effects of hepatocyte growth factor on the expression of type I collagen and matrix metalloproteinase-1 in normal and scleroderma dermal fibroblasts. J Invest Dermatol 2005;124:324-30.

20. Hirano S, Thibeault S, Bless DM, Ford CN, Kanemaru S. Hepatocyte growth factor and its receptor c-Met in rat and rabbit vocal folds. Ann Otol Rhinol Laryngol 2002;111:661-6.

21. Hansen JK, Thibeault SL. Current understanding and review of the literature: vocal fold scarring. J Voice 2006;20:110-20.

22. Croce MA, Dyne K, Boraldi F, et al. Hyaluronan affects protein and collagen synthesis by in vitro human skin fibroblasts. Tissue Cell 2001;33:326-31.

23. Gray SD, Titze IR, Chan R, Hammond TH. Vocal fold proteoglycans and their influence on biomechanics. Laryngoscope 1999;109:845-54.

24. Ohno T, French LC, Hirano S, Ossoff RH, Rousseau B. Effect of hepatocyte growth factor on gene expression of extracellular matrix during wound healing of the injured rat vocal fold. Ann Otol Rhinol Laryngol 2008;117:696-702.

25. Inoue T, Okada H, Kobayashi T, et al. Hepatocyte growth factor counteracts transforming growth factor-beta1, through attenuation of connective tissue growth factor induction, and prevents renal fibrogenesis in 5/6 nephrectomized mice. FASEB J 2003;17:268-70.

26. Matsuda Y, Matsumoto K, Ichida T, Nakamura T. Hepatocyte growth factor suppresses the onset of liver cirrhosis and abrogates lethal hepatic dysfunction in rats. J Biochem 1995;118:643-9.

27. Matsuda Y, Matsumoto K, Yamada A, et al. Preventive and therapeutic effects in rats of hepatocyte growth factor infusion on liver fibrosis/cirrhosis. Hepatology 1997;26:81-9.

28. Ikeda H, Nagoshi S, Ohno A, Yanase M, Maekawa H, Fujiwara K. Activated rat stellate cells express c-Met and respond to hepatocyte growth factor to enhance transforming growth factor beta1 expression and DNA synthesis. Biochem Biophys Res Commun 1998;250:769-75.

29. Sherriff-Tadano R, Ohta A, Morito F, et al. Antifibrotic effects of hepatocyte growth factor on scleroderma fibroblasts and analysis of its mechanism. Mod Rheumatol 2006;16:364-71.

30. Ozaki I, Zhao G, Mizuta T, et al. Hepatocyte growth factor induces collagenase (matrix metalloproteinase-1) via the transcription factor Ets-1 in human hepatic stellate cell line, J Hepatol 2002;36:169-78.

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Atelocollagen Sponge as a Stem Cell Implantation Scaffold for the Treatment of Scarred Vocal Folds

Satoshi Ohno, MD; Shigeru Hirano, MD, PhD; Ichiro Tateya, MD, PhD; Shin-ichi Kanemaru, MD, PhD; Hiroo Umeda, MD, PhD; Atsushi Suehiro, MD; Yoshiharu Kitani, MD; Yo Kishimoto, MD; Tsuyoshi Kojima, MD; Tatsuo Nakamura, MD, PhD; Juichi Ito, MD, PhD

Objectives: Treatment of vocal fold scarring remains a therapeutic challenge. Our group previously reported the efficacy of treating injured vocal folds by implantation of bone marrow–derived stromal cells containing mesenchymal stem cells. Appropriate scaffolding is necessary for the stem cell implant to achieve optimal results. Terudermis is an atelocollagen sponge derived from calf dermis. It has large pores that permit cellular entry and is degraded in vivo. These characteristics suggest that this material may be a good candidate for use as scaffolding for implantation of cells. The present in vitro study investigated the feasibility of using Terudermis as such a scaffold.

Methods: Bone marrow–derived stromal cells were obtained from GFP (green fluorescent protein) mouse femurs. The cells were seeded into Terudermis and incubated for 5 days. Their survival, proliferation, and expression of extracellular matrix were examined.

Results: Bone marrow–derived stromal cells adhered to Terudermis and underwent significant proliferation. Immunohistochemical examination demonstrated that adherent cells were positive for expression of vimentin, desmin, fibronectin, and fsp1 and negative for beta III tubulin. These findings indicate that these cells were mesodermal cells and attached to the atelocollagen fibers biologically.

Conclusions: The data suggest that Terudermis may have potential as stem cell implantation scaffolding for the treatment of scarred vocal folds.

Key Words: atelocollagen, bone marrow–derived stromal cell, cell implantation, mesenchymal stem cell, scaffold.

INTRODUCTION

Vocal fold scarring can occur after inflammation, injury, phonosurgery, or radiotherapy. It causes hoarseness and decreases the quality of life. Histologically, thick, disorganized collagen bundles and disorganized, fragmented elastin fibers have been seen in the lamina propria.[1,2] Hence, the viscoelastic properties of the lamina propria are affected, and phonation disorders are observed. There is no satisfactory treatment for vocal fold scarring, and it remains a therapeutic challenge to treat scarred vocal folds.

To treat or prevent this disorder, our group has explored a novel approach to regenerate vibratory tissues by using tissue engineering methods.[3-6] Tissue engineering consists of 3 elements: cells, growth factors, and scaffold. It is believed that tissue regeneration will be achieved when appropriate cells are provided in an optimal scaffold.[7] We have previously reported the efficacy of implantation of bone marrow–derived stromal cells (BSCs) containing mesenchymal stem cells (MSCs) into injured vocal folds.[3] In that study, BSCs were injected in a 2% collagen solution into the injured vocal folds, and the morphological and histologic results showed improved wound healing, suggesting that BSCs might be useful for restoration of vocal fold mucosa. We also confirmed, in a subsequent study,[8] that injected BSCs differentiated into several lineages of mature cells, including endothelial, mesenchymal, muscular, and cartilaginous cells.

Cell therapy is an attractive strategy for tissue regeneration, but one of the important aspects of cell therapy is keeping the cells at the site and maximizing their function. In this sense, a collagen solution may not be ideal, because the solution is eas-

From the Department of Otolaryngology–Head and Neck Surgery, Graduate School of Medicine (Ohno, Hirano, Tateya, Kanemaru, Umeda, Suehiro, Kitani, Kishimoto, Kojima, Ito), and the Department of Bioartificial Organs, Institute for Frontier Medical Sciences (Nakamura), Kyoto University, Kyoto, Japan.

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Correspondence: Satoshi Ohno, MD, Dept of Otolaryngology–Head and Neck Surgery, Graduate School of Medicine, Kyoto University, Sakyo-ku, Kyoto 606-8507, Japan.

ily diluted and absorbed. Therefore, it is important to choose an adequate scaffold to enhance the efficacy of implantation of stem cells. Terudermis (Terumo Co, Tokyo, Japan) is a dehydrothermally cross-linked atelocollagen sponge derived from calf dermis. Strong fibrillar atelocollagen and heat-denatured atelocollagen, which promotes excellent cell migration, are mixed at a ratio of 9 to 1 and lyophilized to create a layer of collagen sponge about 5 mm thick.[9] The sponge has many large pores to permit cell entry and is degraded gradually in vivo by endogenous collagenase.[9,10] In the current study, we examined the feasibility of using this atelocollagen sponge as scaffolding for BSCs, with particular attention focused on the proliferative and differentiative responses of BSCs.

MATERIALS AND METHODS

Harvest and Cultivation of BSCs. GFP (green fluorescent protein) mice were anesthetized by intramuscular administration of ketamine hydrochloride (8.0×10^{-4} mg/g; Sankyo Co, Tokyo) and xylazine hydrochloride (5.0×10^{-4} mg/g; Bayer, Tokyo) and painlessly sacrificed, and both femurs were resected. Bone marrow was flushed from both femurs by means of a syringe with a 23-gauge needle. The resulting cell suspension was placed in a 10-cm-diameter tissue culture dish with 10 mL of Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum (FBS) and an antibiotic-antimycotic mixture (Gibco, Invitrogen Co, Carlsbad, California). The cells were incubated in 5% carbon dioxide at 37°C (MCO-17AIC carbon dioxide incubator, Sanyo Co, Osaka, Japan) for 72 hours to allow BSCs to adhere to the bottom of the dish. After 3 days, the culture medium containing unnecessary nonadherent cells, including hematopoietic cells and waste products, was removed, leaving behind only adherent cells on the bottom of the dish. Fresh medium was then added and exchanged twice a week until the cells reached confluence. When BSCs were confluent, the cells were lifted enzymatically from the dish with 0.25% trypsin with ethylenediaminetetraacetic acid (EDTA; Gibco, Invitrogen) and were passaged to further expand the BSC culture. Just before seeding, BSCs were again detached from the bottom of the dish with 0.25% trypsin with EDTA. After neutralization of the trypsin solution and thorough washing of the cells with DMEM containing 10% FBS and antibiotic-antimycotic mixture, the BSCs were counted with a hemocytometer (BrightLine, Becton Dickinson, Franklin Lakes, New Jersey) and collected by centrifugation at 3,500 rpm for 3 minutes (LX-120 centrifuge, Tomy Co, Tokyo).

Three-Dimensional Cultivation of BSCs. We diluted BSCs to 4.0×10^6 cells/mL with DMEM containing 10% FBS and antibiotic-antimycotic mixture. Two hundred thousand cells were seeded into 5 × 5-mm atelocollagen sponge (Terudermis; Terumo Co) by means of a syringe with a 24-gauge needle. Thereafter, the Terudermis with BSCs was set in nontreated 24-well microplates (Iwaki Glass Co, Funahashi, Japan) and incubated in 5% carbon dioxide at 37°C for 5 days. Cells were observed by fluorescence microscopy (Eclipse TE300, Nikon, Tokyo) at a magnification of ×20. The samples were divided into 3 groups: day 1, day 3, and day 5 groups (6 per group). At each time point, BSCs were detached from Terudermis with collagenase B (Roche Diagnostics GmbH, Mannheim, Germany) after rinsing with phosphate-buffered saline solution to wash out non-adhered cells and counted with a hemocytometer. Samples from the day 1 and day 5 groups were embedded in OCT compound (Sakura Finetek USA, Inc, Torrance, California) and frozen at –80°C for histologic examination.

Histologic Examination. To assess the fate of the adherent BSCs, we performed histologic and immunohistochemical analyses. Seven-micrometer-thick serial sections were prepared for hematoxylin-eosin staining or immunohistochemical staining. Immunohistochemical staining was performed with monoclonal antibodies against vimentin (a marker of mesenchymal cells; Santa Cruz Biotechnology, Inc, Santa Cruz, California), fibronectin (a marker for cell adhesion; Abcam Inc, Cambridge, Massachusetts), fsp1 (fibroblast-specific protein 1, a marker for fibroblasts; Santa Cruz Biotechnology), beta III tubulin (a marker for ectodermal cells; Covance, Berkeley, California), and desmin (a marker for muscle cells; Thermo Fisher Scientific, Waltham, Massachusetts). Each slide was treated with phosphate-buffered saline solution and then blocked with 5% normal goat serum, and then incubated with each antibody overnight at 5°C.

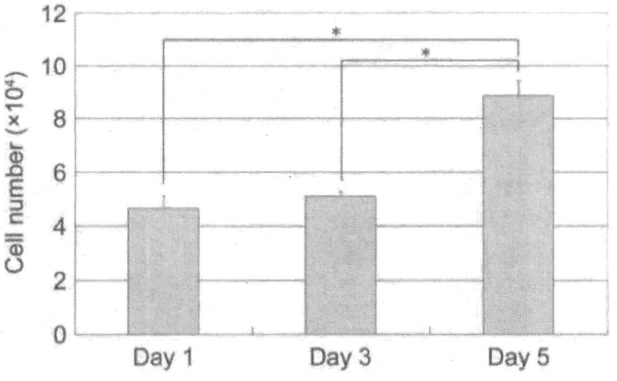

Fig 1. Adherent bone marrow–derived stromal cells (mean ± SE) within atelocollagen sponge. Number of cells significantly increased after 5 days (n = 6). Asterisk — $p < 0.001$ by Tukey's test).

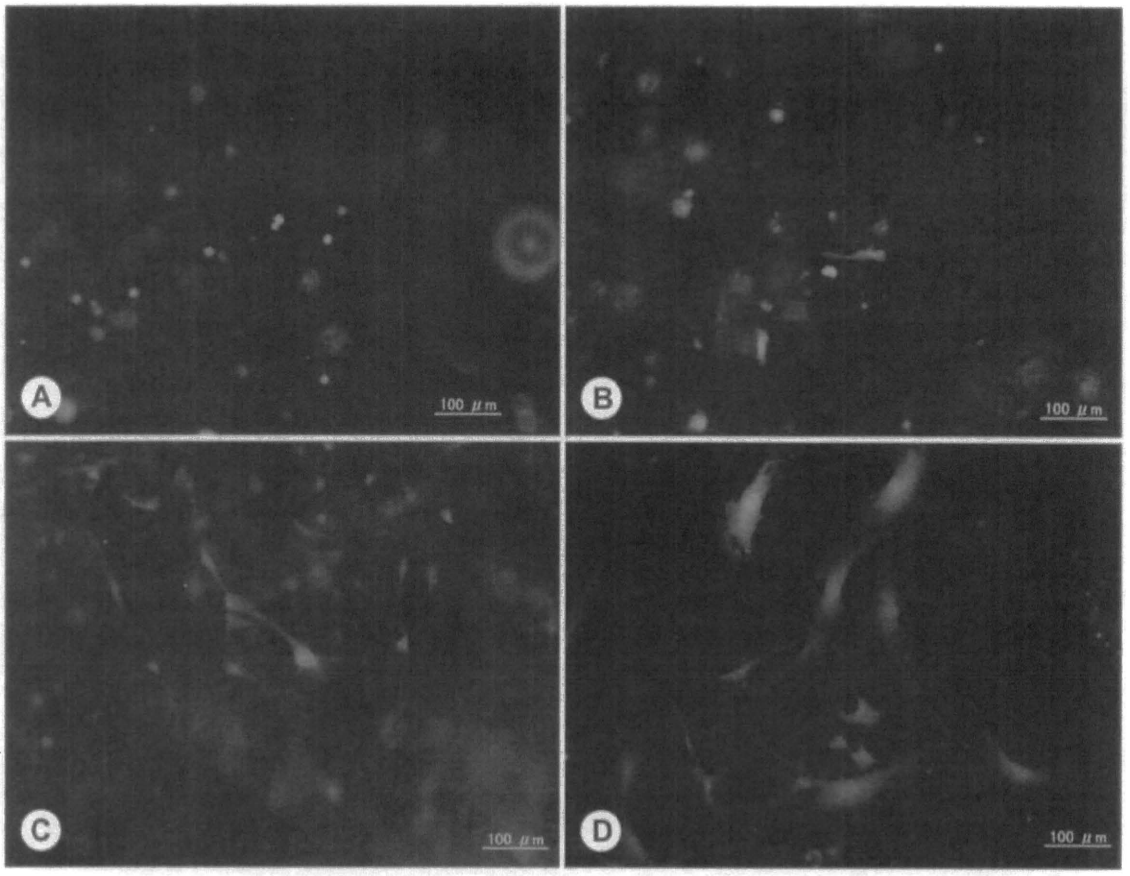

Fig 2. Morphology of bone marrow–derived stromal cells on atelocollagen sponge. Cellular extension was observed from day 1. Intercellular network developed in time-dependent manner. **A)** Day 0. **B)** Day 1. **C)** Day 3. **D)** Day 5.

Alexa Fluor 546 (Invitrogen Detection Technologies, Eugene, Oregon) was used as a secondary antibody. We used 4',6-diamidino-2-phenylindole dihydrochloride (DAPI) to stain cell nuclei in each specimen. Microscopic images of stained cells were captured by fluorescence microscopy (DM2500B, Leica Microsystems, Wetzlar, Germany). All sections were examined at 40× magnification.

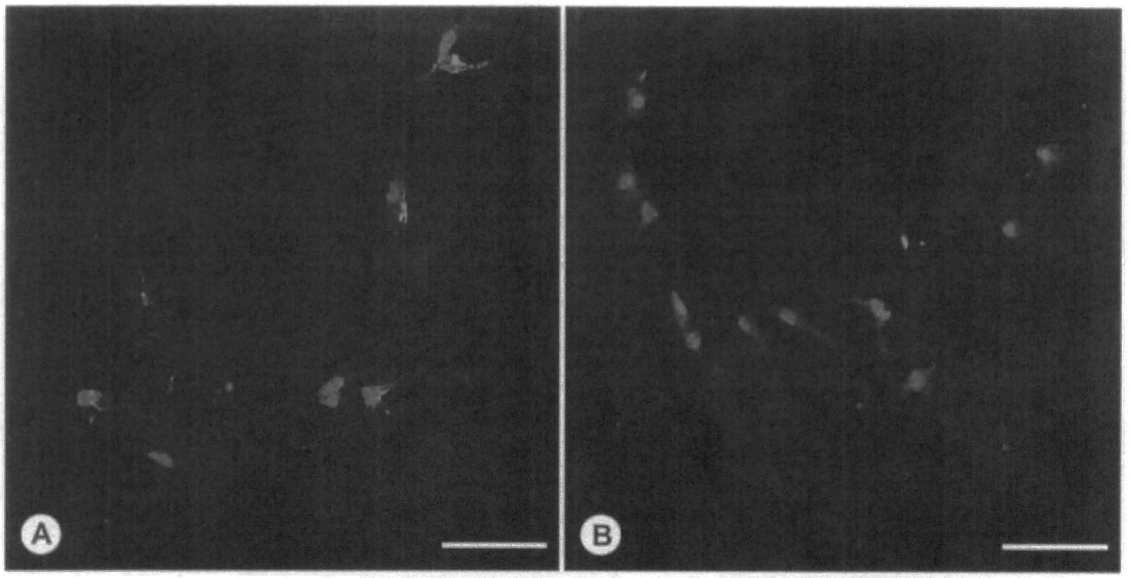

Fig 3. Immunohistochemical analyses of cultured cells. Blue — 4',6-diamidino-2-phenylindole dihydrochloride (DAPI); red — fibronectin; bars — 50 μm. Fibronectin expression was **A)** seen on day 1 and **B)** clearly observed in each cell on day 5.

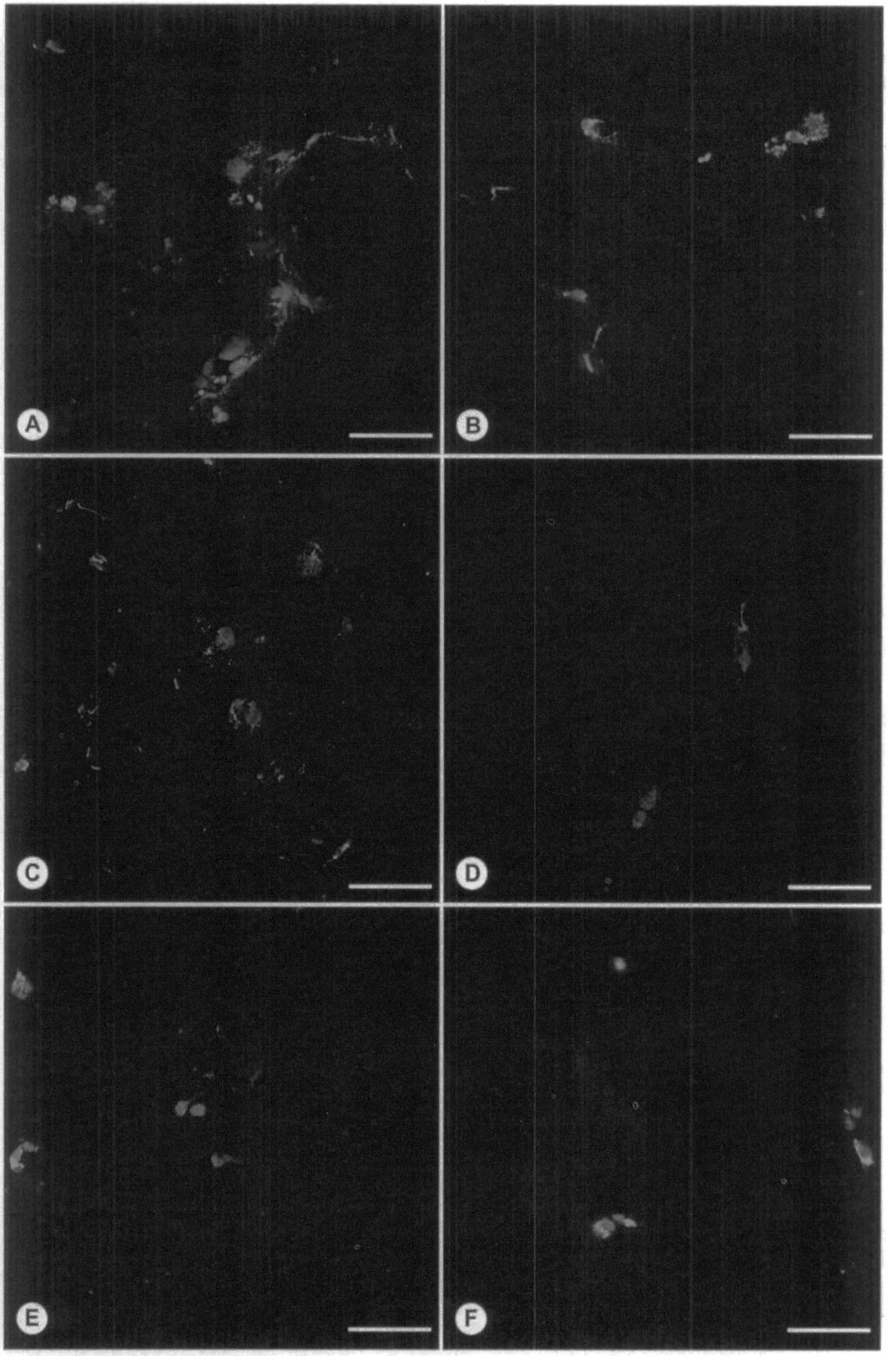

Fig 4. Immunohistochemical analyses of cultured cells. Blue — DAPI; red — fsp1, vimentin, and desmin; bars — 50 μm. Expression of **A,B)** fsp1, **C,D)** vimentin, and **E,F)** desmin was seen on day 1 and was maintained through day 5.

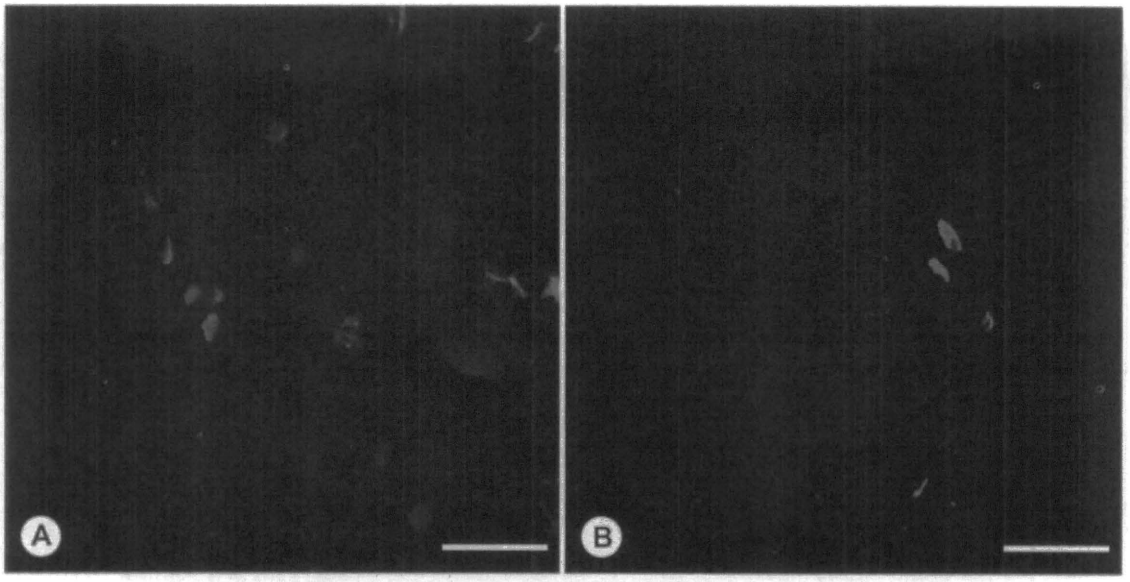

Fig 5. Immunohistochemical analyses of cultured cells. Blue — DAPI; red — beta III tubulin; bars — 50 μm. Beta III tubulin expression was not seen on **A)** day 1 through **B)** day 5.

Statistical Analysis. Statistical comparisons of the numbers of BSCs were performed between the groups with Tukey's test. Probability (p) values less than or equal to 0.05 were considered statistically significant.

RESULTS

Survival and Proliferation of BSCs. Approximately 20% of the cells seeded into the atelocollagen sponge adhered to its matrix. The number of cells on the atelocollagen sponge tended to increase by day 3 and significantly increased by day 5 (day 1 versus day 5, $p < 0.001$; day 3 versus day 5, $p < 0.0001$; Fig 1). Intercellular network formation was seen on day 1 and developed thereafter in a time-dependent manner (Fig 2).

Immunohistochemistry. Attached cells were positive for vimentin, desmin, fibronectin, and fsp1 and negative for beta III tubulin. These findings indicate that these cells were mesodermal cells that were attached to the atelocollagen fibers biologically. Marker expression was seen on day 1 and did not significantly change through day 5 (Figs 3-5).

DISCUSSION

Bone marrow–derived stromal cells include a large population of MSCs, which are multipotent and can differentiate into several types of cells such as neuronal, adipose, muscle, and endothelial cells, as well as mesenchymal cells.[11-13] Thus, BSCs are thought to be a useful candidate for regeneration of various organs. We have previously demonstrated the potential of BSCs to restore injured vocal folds.[3] A scaffold is important for successful use of cells, and an appropriate scaffold for cells implantable to the vocal fold requires 3 elements: biosafety, degradation in vivo, and biocompatibility. Terudermis was approved for clinical use in the treatment of injured dermis and epidermis by the Ministry of Health in Japan, and has been proven to stimulate tissue recovery of injured skin or mucosa without any significant adverse effects. Also, it has been confirmed that Terudermis is gradually degraded in vivo by endogenous collagenases. In this study, we examined the biocompatibility of Terudermis with BSCs.

The present results showed that approximately 20% of seeded cells attached to Terudermis on day 1. The cells were found to proliferate and produce an extracellular matrix (ECM) network. Fibronectin is produced by mesenchymal cells, and is a major component of the ECM. It is the most effective ECM molecule for promoting adhesion of MSCs to tissue culture surfaces.[14] In this study, fibronectin expression was seen on day 1, indicating that injected BSCs had adhered to the atelocollagen surface within a day. This observation indicates that Terudermis may be an appropriate material for BSC adherence.

Grayson et al[14] reported that MSCs exhibit 3 phases of growth: lag, log, and plateau. In nonwoven polyethylene terephthalate 3-dimensional (3-D) culture, the lag phase persisted for about 5 days, whereas in 2-dimensional (2-D) culture it persists for 2 days. In this study the lag phase was considered to persist for 3 days. This is a relatively short time for 3-D culture and close to the time for 2-D culture. Thus, atelocollagen sponge appears to be a good 3-D material for proliferation of BSCs.

One of the advantages of 3-D culture of MSCs is their enhanced production of ECM. It was shown that in 3-D culture, the number of cells increased less than in 2-D cultures, but the cells expressed more ECM proteins.[14] ECM production is an important aspect of vocal fold regeneration, because the vocal fold consists of various ECM components, including hyaluronic acid. Hyaluronic acid is widely distributed in the lamina propria of the vocal fold, and plays a significant role in maintaining the viscoelastic properties of the vocal fold.[15] Our previous study has indicated that BSCs expressed hyaluronic acid synthase at a significantly higher level than vocal fold fibroblasts in rats, which was considered to be beneficial for the vocal fold.[16] The current 3-D culture of the cells using Terudermis may have good potential to support hyaluronic acid production.

Earlier studies showed that MSCs in 3-D culture did not differentiate into any mature cell types when no inductive factors were applied, and that they maintained multilineage potential.[13] In this study, cell marker expression did not change through the first 5 days, suggesting that the extent of differentiation of BSCs did not change in the scaffolding.

As mentioned above, tissue engineering consists of the 3 elements of cells, scaffold, and growth factors. The present study suggested the usefulness of a combination of BSCs and atelocollagen sponge. Future experimentation would be warranted to examine the effects of implanted BSCs and the atelocollagen sponge cultured in the presence of pluripotent growth factors.

CONCLUSIONS

The present in vitro study examined the biocompatibility of atelocollagen sponge (Terudermis) with BSCs. The data showed that BSCs adhered to the atelocollagen sponge and proliferated, forming an ECM network. These results suggest that Terudermis has good biocompatibility with BSCs, and may become an appropriate scaffold in cell therapy for regeneration of the vocal fold mucosa.

REFERENCES

1. Hirano S. Current treatment of vocal fold scarring. Curr Opin Otolaryngol Head Neck Surg 2005;13:143-7.

2. Hansen JK, Thibeault SL. Current understanding and review of the literature: vocal fold scarring. J Voice 2006;20:110-20.

3. Kanemaru S, Nakamura T, Omori K, et al. Regeneration of the vocal fold using autologous mesenchymal stem cells. Ann Otol Rhinol Laryngol 2003;112:915-20.

4. Hirano S, Bless DM, Rousseau B, et al. Prevention of vocal fold scarring by topical injection of hepatocyte growth factor in a rabbit model. Laryngoscope 2004;114:548-56.

5. Hirano S, Bless DM, Nagai H, et al. Growth factor therapy for vocal fold scarring in a canine model. Ann Otol Rhinol Laryngol 2004;113:777-85.

6. Ohno T, Hirano S, Kanemaru S, et al. Drug delivery system of hepatocyte growth factor for the treatment of vocal fold scarring in a canine model. Ann Otol Rhinol Laryngol 2007;116:762-9.

7. Langer R, Vacanti JP. Tissue engineering. Science 1993;260:920-6.

8. Kanemaru S, Nakamura T, Yamashita M, et al. Destiny of autologous bone marrow–derived stromal cells implanted in the vocal fold. Ann Otol Rhinol Laryngol 2005;114:907-12.

9. Koide M, Osaki K, Konishi J, et al. A new type of biomaterial for artificial skin: dehydrothermally cross-linked composites of fibrillar and denatured collagens. J Biomed Mater Res 1993;27:79-87.

10. Bessho K, Murakami K, Iizuka T. The use of a new bilayer artificial dermis for vestibular extension. Br J Oral Maxillofac Surg 1998;36:457-9.

11. Hachisuka H, Mochizuki Y, Yasunaga Y, et al. Flow cytometric discrimination of mesenchymal progenitor cells from bone marrow–adherent cell populations using CD34/44/45(–) and Sca-1(+) markers. J Orthop Sci 2007;12:161-9.

12. Pasquinelli G, Tazzari P, Ricci F, et al. Ultrastructural characteristics of human mesenchymal stromal (stem) cells derived from bone marrow and term placenta. Ultrastruct Pathol 2007;31:23-31.

13. Jiang Y, Jahagirdar BN, Reinhardt RL, et al. Pluripotency of mesenchymal stem cells derived from adult marrow. Nature 2002;418:41-9. [Erratum in Nature 2007;447:879-80.]

14. Grayson WL, Ma T, Bunnell B. Human mesenchymal stem cells tissue development in 3D PET matrices. Biotechnol Prog 2004;20:905-12.

15. Gray SD, Titze IR, Chan R, Hammond TH. Vocal fold proteoglycans and their influence on biomechanics. Laryngoscope 1999;109:845-54.

16. Ohno T, Hirano S, Kanemaru S, et al. Expression of extracellular matrix proteins in the vocal folds and bone marrow derived stromal cells of rats. Eur Arch Otorhinolaryngol 2008;265:669-74.

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

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

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

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

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

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

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

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

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

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

MATERIALS AND METHODS

Collagen

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

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

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

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