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Vocal Fold Fibroblast Response to Growth Factor Treatment is Age Dependent: Results From an *In Vitro* Study

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Summary: Introduction. Vocal fold (VF) fibroblasts (VFFs) are the central target in developing new strategies for treatment of VF injury and scarring. Nevertheless, only little is known about the basic biological characteristics of these cells. The aim of this study was to explore the impact of age of VFFs on the response to external growth factor stimulation.

Study Design. *In vitro* cell study using a rat model.

Methods. VFFs were extracted from young and aged rat VF 3 months after establishing unilateral injury. Resulting scar fibroblasts (SFs) and normal fibroblasts (NFs) were subsequently cultured separately with or without the addition of hepatocyte growth factor (HGF). After 24 and 72 hours, the production of hyaluronic acid (HA) was examined in the supernatant culture media using enzyme-linked immunosorbent assay.

Results. Only cultured SF and NF from young animals could be stimulated significantly in the production of HA by HGF. Within these, average percentage increase was higher in NF compared with SF.

Conclusion. The response of VFFs in cell culture to growth factors stimulation is highly depending on the age of the animals. This is another step in a nearer characterization of scar VFF and could furthermore be an important point when estimating the success of an intervention. Age-depending effects must be considered as an important factor in developing possible therapeutic agents for VF scarring.

Key Words: Vocal fold scarring–Vocal fold fibroblasts–Age depending effects–Hyaluronic acid–Hepatocyte growth factor.

INTRODUCTION

Vocal fold (VF) scarring remains an unresolved problem and a major challenge in modern laryngology. Reasons for VF scarring are numerous and comprise chronic inflammation caused by smoking or radiation therapy, external or internal trauma, chronic voice abuse, and so forth.[1] Therapeutic options are unsatisfying and limited, with conservative speech therapy and various surgical procedures being the most commonly used treatment strategies.[2] Underlying pathology is a deterioration of the microarchitecture of the highly complex trilayered VF lamina propria with a subsequent alternation of the vibration characteristics leading to dysphonia.

Vocal fold fibroblasts (VFFs) play a pivotal role in the aforementioned structural and functional changes. They represent the main cell type of the VF and have unique characteristics not shared by other types of fibroblasts. Under normal circumstances, relatively few VFF can be found in the vibrational mid-part of the VF, where they maintain structural and vibrational properties of the VF by producing extracellular matrix (ECM) components such as hyaluronic acid (HA).

After injury some VFF transform into myofibroblasts (aka. scar fibroblasts [SFs]) with altered protein expression patterns. This leads to significant changes in the production of important proteins of the ECM, such as the aforementioned HA, but also of different types of collagen and fibronectin.[3] This results in increased stiffness and viscosity of the VF, with glottic incompetence and dysphonia.[4]

VFF not only produce structural proteins and glycos-aminoglycans but also various kinds of growth factors such as hepatocyte growth factor (HGF). This is triggered by several mechanisms that are still under investigation. Local stem cells are assumed to play a central role in mediating the inflammatory responses after VF injury.[5]

A complete regeneration of the changes of the lamina propria is the subject of numerous studies[6] but could not be achieved so far. Laryngeal tissue engineering aims to change pathologic tissue characteristics also at the cellular level by various attempts including injection of somatic[7] and stem cells[8] or by injection of various cytokines and growth factors.[6] Most experiments so far showed beneficial results in terms of improved tissue viscosity and collagen reduction.

Despite many trials and experiments in this field, only little is known about the basic characteristics of the cell type that most of these studies target, the VFF. A better characterization and a better understanding of the behavior of VFF should give new insights in cellular mechanisms and inflammatory pathways.

The aim of this study was to investigate the impact of age on the cellular responses of VFF after external growth factor stimulation with HGF. This not only has effects on planning and

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pursuing further cellular experiments but should also elucidate physiology and pathophysiology of VFF. Fibrogenesis and inflammation processes presumably underlie physiological aging influences, but this has never been proved before for VFF.[9]

MATERIALS AND METHODS

Study animals consisted of eight male Sprague-Dawley rats (four—aged 3 months at the time of injury ["young group"], four—aged 11 months ["old group"]). In general, life expectancy of Sprague-Dawley rats is up to 24 months, so animals from the older group were about mid-age.[10] Initially, the right VF of all animals were injured by a syringe, the left side was left intact and served as a control. After 3 months, animals were euthanized and their larynges were excised. Methods of harvesting were performed exactly as described before.[11] Fibroblasts from uninjured (left) VF were named normal fibroblasts (NFs); fibroblasts from injured (right) VF were named scar fibroblasts (SFs). Therefore, in total, there were four different groups: NF young, SF young, NF old, and SF old. All study procedures were conducted according to the Austrian guidelines for animal experiments and were approved by the Austrian Ministry of Science.

Growth medium consisted of Dulbecco's modified Eagle's medium (DMEM; Life Technologies, Gaithersburg, MD) enriched with 10% fetal calf serum (FCS), 1% penicillin/streptomycin and 1000 mM vitamin C per well (L-Ascorbic acid 2-phosphate sesquimagnesium salt hydrate; Wako Pure Chemical Industries, Osaka, Japan).

After confluence of 90%, NF and SF of both groups were passaged using 0.5% trypsin and seeded at concentrations of 2×10^5 cells into 24 well plates (Nalge Nunc International, Rochester, NY) with 1.4 mL medium. Each sample of the primary culture (n = 8) was split in three at this time, giving 12 samples for each side (NF/SF) and 12 samples for each group (young/old). At this time, FCS concentrations in growth medium were reduced to 1%. After a starvation period of 24 hours, each group was again split into an HGF and a sham group by replacing the medium, consisting of DMEM, including 1% FCS and 1% penicillin/streptomycin with or without HGF at a concentration of 200 ng/mL. This concentration of HGF has been shown to be effective in influencing the HA production of canine VFF *in vitro*.[12]

After 24 and 72 hours, 0.5 mL of supernatant medium was sampled and immediately stored at −80. After collecting the supernatants, the cells were detached from the bottom of the wells using trypsin–ethylenediaminetetraacetic acid. The density of cells in each well was counted using the CASY Cell Counter by Roche Innovatis (Basel, Switzerland). Assessment of HA production from supernatants was carried out using enzyme-linked immunosorbent assays (TECO-Hyaluronic acid; TECOmedical group, Sissach, Switzerland for HA, ELISA Kit; USCN Life Science, Inc., Wuhan, Hubei, PR China for collagen-I-α). All assays were performed in duplicates (technical duplicates). Values of HA per well were divided by the cell count results to calculate the HA production per cell.

Statistical analysis

Differences of the means were analyzed by paired and unpaired *t* tests after proof of normal distribution by using PASW statistics 18.0 (SPSS, Inc., Sunnyvale, CA). *P* value of 0.05 was chosen as a level for statistical significance. Normal distribution was given in all parameters as confirmed by the Kolmogorov-Smirnov test. The paired *t* test allowed us to compare biological behavior of VFF of one and the same animal (right-side injured − left-side uninjured control). Biological replicates were treated as independent variables, whereas the technical duplicates were averaged.

RESULTS

All results are displayed in Table 1.

HA production without stimulation of HGF

Levels of HA increased in all settings ($NF_{y,o}$ and $SF_{y,o}$) from 24 to 72 hours as a consequence of time indicating intact culture conditions. Pair wise comparison of NF and SF (noteworthy of the same animal) showed only significant differences in cell cultures of younger animals after 24 and 72 hours (*P* always < 0.05). This is in accordance with results from our previous study.

HA production with stimulation of HGF

In the same cell settings, absolute amounts of measured HA were always higher under the administration of HGF. Again levels of HA increased in all wells as a consequence of time.

TABLE 1.
HA Per Cell [pg/mL]

	Young Group		Aged Group	
Cell Setting	24 h	72 h	24 h	72 h
NF	148 ± 63	283 ± 106*	122 ± 51	268 ± 90*
SF	252 ± 85†	416 ± 126*,†	156 ± 87	359 ± 194*
NF + HGF	205 ± 40	501 ± 134*	141 ± 42	280 ± 73*
SF + HGF	262 ± 85	660 ± 236*	197 ± 170	502 ± 211*

Abbreviations: HA, hyaluronic acid; HGF, hepatocyte growth factor; NF, normal fibroblasts; SF, scar fibroblasts.
* $P \leq 0.05$ versus 24 h in the same group.
† $P \leq 0.05$ versus NF of the same group.

Differences reached statistical significance when comparing NF_y and NF_y + HGF resp. SF_y and SF_y + HGF after 72 hours (NF: 501 vs 283 pg/mL, $P \leq 0.05$; SF: 660 vs 416 pg/mL, $P \leq 0.05$; Figure 1). Average percentage increase was noteworthy higher in NF_y (+77%) compared with SF_y (+59%).

Increases of HA production in cell cultures from the older animals did never reach statistical significance.

Effects of stimulation on cell density

There was no significant difference between cell density in stimulated and unstimulated fibroblasts in neither group (data not shown).

DISCUSSION

Laryngeal tissue engineering opened a completely new therapeutic and diagnostic field in modern laryngology. Kanemaru et al[12,13] were, in 2003 and 2005, among the first who injected mesenchymal stem cells (MSC) into canine VF right before injuring them. They observed macroscopically a better healing tendency of the VF treated with MSC compared with the sham group. Although many of these earlier trials explored changes at a macroscopic level by observing wound healing or testing viscoelastic properties, newer studies focused more on the cellular and molecular level of VF injury and scarring. Many trials performed excellent work using mesenchymal or somatic stem cells in the treatment of acute and chronic VF injury.[14,15] Tateya et al[16] studied histologic parameters in chronic VF scar and expression patterns of ECM proteins directly after VF injury in rat models.

Nevertheless, an adequate characterization of the target cells of virtually all treatment options, that is, VFF has not been performed so far but is highly desirable. Only recently studies focused on microscopic and cellular aspects of VF injury and healing: Jette et al[17] explored cell characteristics of scarred and normal human VFF from two individuals. We recently described for the first time an age effect on HA production capacities of scarred and normal VFF. We could demonstrate that age is a significant factor for VF regeneration after chronic

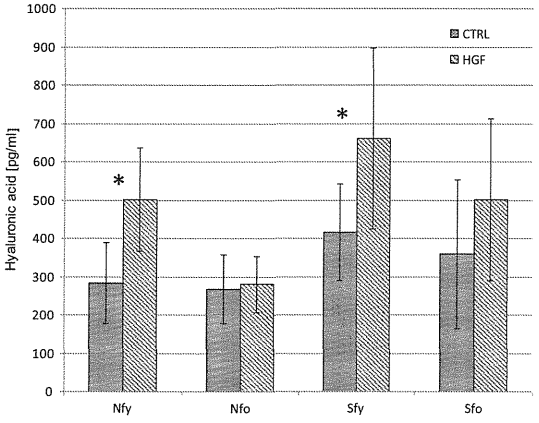

FIGURE 1. Production of HA per cell after 72 hours in different subgroups. Nfy, normal fibroblasts young group; Nfo, normal fibroblasts aged group; Sfy, scar fibroblasts young group; Sfo, scar fibroblasts aged group; HGF, hepatocyte growth factor. *$P \leq 0.05$.

VF injury: Scarred VFF of younger rats produced significantly higher amounts of HA compared with older animals.[11] This observation was already known from other fields, mainly the skin but is completely new to the larynx.[18] Hirano et al[19] were among the few who explored age effects on HA production and several other parameters. In contrast to us, they did not use mature SF, but VFF from uninjured VF.

Exploring basic cellular characteristics of VFF is an absolute precondition for a variety of applications: We and other groups aim to establish standardized cell culture settings to create a reliable *in vitro* fibrogenesis system. There is a strong need for such models as these could be a basis for exploring basic pathways of VF scarring, as well as for testing diverse antifibrotic compounds. Before going into time and money intensive larger clinical trials, an *in vitro* model based on (human) VFF can lead to faster decision about promising compounds. We, furthermore, need to learn about the physiological behavior of these cells in live organisms to understand interactions and effects when performing clinical interventions.

Growth factor therapy is one of the most promising options for the treatment of VF scarring. Pioneer work in this respect was done by Hirano et al[20]: he was the first who applied basic fibroblast growth factor (bFGF) in humans for treatment of atrophied VF. A further study by the same author reported recently beneficial effects of bFGF in a combined phonosurgical approach in the treatment of VF sulcus and scar.[21] Another very promising molecule for laryngeal application is HGF. It was studied extensively in VF cell culture and animal trials in various dosages. HGF was originally discovered as growth factor involved in the regeneration of hepatocytes. In further studies, its angiogenic, angioprotective, and antifibrotic activity in various organs like liver and kidney was shown.[22] HGF has been used in different *in vitro* and *in vivo* animal studies as a promising agent in the prevention as well as in therapy of VF scarring. In a canine model, HGF was administered immediately after VF injury[23] and in a second study, 1 month after scarring.[24] Both studies showed better vibratory properties for the treatment group than for the sham group. *In vitro* use of HGF has been shown to reduce collagen I and enhance HA production[25] and upregulate messenger RNA (mRNA) expression of endogenous HGF and HA synthases in VFF.[26] Even if all these experiments were carefully performed, age of animals was considered as an independent variable in neither trial.

Our study revealed that HA production per cell of SF in both the young and the aged groups were higher than in NF at both time points (after 24 and 72 hours). However, this was only significant in cell cultures from young fibroblasts, which is in absolute accordance with our recent article.[11] Furthermore, only NF_y and SF_y produced significantly higher amounts of HA after stimulation with HGF and this only after 72 hours. This is in contrast to the aforementioned study by Hirano et al who described stimulating properties of VFF from older rats. This might be related to a different strain, as they used Fisher 344/Brown Norway rats in their experiment. Better response of VFF from younger animals might be related to the higher number of surface receptors for HA as was shown

in fetal rat fibroblasts.[27] However, this assumption must be confirmed immunohistochemically.

An *in vitro* model of VF scarring as ours can never fully reflect *in vivo* situations for several reasons and we are aware of these limitations. Protein production patterns of VFF are further known to be vibration driven,[28] so a static model presumably yields slightly different expression patterns. We, furthermore, need to elucidate if and how higher levels of HA produced by SF of younger animals can be translated into histology, as the latter depends on the complex balance between ECM, growth factors, hyaluronidases, collagen, matrix metalloproteinases, HGF, transforming growth factor-beta, and many other factors. HA expression needs furthermore to be confirmed on the protein production level by mRNA analysis. In contrast to comparable studies, we did not evaluate the levels of collagen production, as we observed in our previous work that levels of collagen type I decrease as soon as 8 weeks after injury in a rat VF scarring model.[10,11]

CONCLUSION

Only cultured NF and SF of young animals could be stimulated by HGF to increase the production of HA, whereas this was not the case in older animals. This finding underlines the importance of age as another independent variable in the complex mechanisms of VF inflammation and fibrogenesis and lets us assume that wound healing and fibrogenesis change during the lifespan. Possible therapeutic agents must consider this finding, as well as forthcoming cellular trials dealing with VFF.

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Growth medium consisted of Dulbecco's modified Eagle's medium (DMEM; Life Technologies, Gaithersburg, MD) enriched with 10% fetal calf serum (FCS), 1% penicillin/streptomycin and 1000 mM vitamin C per well (L-Ascorbic acid 2-phosphate sesquimagnesium salt hydrate; Wako Pure Chemical Industries, Osaka, Japan).

After confluence of 90%, NF and SF of both groups were passaged using 0.5% trypsin and seeded at concentrations of 2×10^5 cells into 24 well plates (Nalge Nunc International, Rochester, NY) with 1.4 mL medium. Each sample of the primary culture (n = 8) was split in three at this time, giving 12 samples for each side (NF/SF) and 12 samples for each group (young/old). At this time, FCS concentrations in growth medium were reduced to 1%. After a starvation period of 24 hours, each group was again split into an HGF and a sham group by replacing the medium, consisting of DMEM, including 1% FCS and 1% penicillin/streptomycin with or without HGF at a concentration of 200 ng/mL. This concentration of HGF has been shown to be effective in influencing the HA production of canine VFF *in vitro*.[12]

After 24 and 72 hours, 0.5 mL of supernatant medium was sampled and immediately stored at −80. After collecting the supernatants, the cells were detached from the bottom of the wells using trypsin–ethylenediaminetetraacetic acid. The density of cells in each well was counted using the CASY Cell Counter by Roche Innovatis (Basel, Switzerland). Assessment of HA production from supernatants was carried out using enzyme-linked immunosorbent assays (TECO-Hyaluronic acid; TECOmedical group, Sissach, Switzerland for HA, ELISA Kit; USCN Life Science, Inc., Wuhan, Hubei, PR China for collagen-I-α). All assays were performed in duplicates (technical duplicates). Values of HA per well were divided by the cell count results to calculate the HA production per cell.

Statistical analysis

Differences of the means were analyzed by paired and unpaired *t* tests after proof of normal distribution by using PASW statistics 18.0 (SPSS, Inc., Sunnyvale, CA). *P* value of 0.05 was chosen as a level for statistical significance. Normal distribution was given in all parameters as confirmed by the Kolmogorov-Smirnov test. The paired *t* test allowed us to compare biological behavior of VFF of one and the same animal (right-side injured − left-side uninjured control). Biological replicates were treated as independent variables, whereas the technical duplicates were averaged.

RESULTS

All results are displayed in Table 1.

HA production without stimulation of HGF

Levels of HA increased in all settings ($NF_{y,o}$ and $SF_{y,o}$) from 24 to 72 hours as a consequence of time indicating intact culture conditions. Pair wise comparison of NF and SF (noteworthy of the same animal) showed only significant differences in cell cultures of younger animals after 24 and 72 hours (*P* always < 0.05). This is in accordance with results from our previous study.

HA production with stimulation of HGF

In the same cell settings, absolute amounts of measured HA were always higher under the administration of HGF. Again levels of HA increased in all wells as a consequence of time.

TABLE 1.
HA Per Cell [pg/mL]

	Young Group		Aged Group	
Cell Setting	24 h	72 h	24 h	72 h
NF	148 ± 63	283 ± 106*	122 ± 51	268 ± 90*
SF	252 ± 85†	416 ± 126*,†	156 ± 87	359 ± 194*
NF + HGF	205 ± 40	501 ± 134*	141 ± 42	280 ± 73*
SF + HGF	262 ± 85	660 ± 236*	197 ± 170	502 ± 211*

Abbreviations: HA, hyaluronic acid; HGF, hepatocyte growth factor; NF, normal fibroblasts; SF, scar fibroblasts.
* $P \leq 0.05$ versus 24 h in the same group.
† $P \leq 0.05$ versus NF of the same group.

Differences reached statistical significance when comparing NF_y and NF_y + HGF resp. SF_y and SF_y + HGF after 72 hours (NF: 501 vs 283 pg/mL, $P \leq 0.05$; SF: 660 vs 416 pg/mL, $P \leq 0.05$; Figure 1). Average percentage increase was noteworthy higher in NF_y (+77%) compared with SF_y (+59%).

Increases of HA production in cell cultures from the older animals did never reach statistical significance.

Effects of stimulation on cell density

There was no significant difference between cell density in stimulated and unstimulated fibroblasts in neither group (data not shown).

DISCUSSION

Laryngeal tissue engineering opened a completely new therapeutic and diagnostic field in modern laryngology. Kanemaru et al[12,13] were, in 2003 and 2005, among the first who injected mesenchymal stem cells (MSC) into canine VF right before injuring them. They observed macroscopically a better healing tendency of the VF treated with MSC compared with the sham group. Although many of these earlier trials explored changes at a macroscopic level by observing wound healing or testing viscoelastic properties, newer studies focused more on the cellular and molecular level of VF injury and scarring. Many trials performed excellent work using mesenchymal or somatic stem cells in the treatment of acute and chronic VF injury.[14,15] Tateya et al[16] studied histologic parameters in chronic VF scar and expression patterns of ECM proteins directly after VF injury in rat models.

Nevertheless, an adequate characterization of the target cells of virtually all treatment options, that is, VFF has not been performed so far but is highly desirable. Only recently studies focused on microscopic and cellular aspects of VF injury and healing: Jette et al[17] explored cell characteristics of scarred and normal human VFF from two individuals. We recently described for the first time an age effect on HA production capacities of scarred and normal VFF. We could demonstrate that age is a significant factor for VF regeneration after chronic

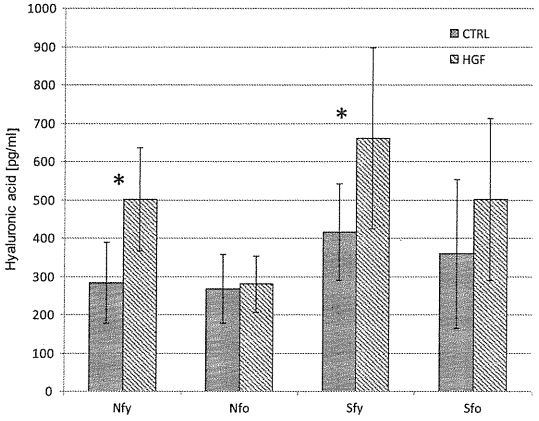

FIGURE 1. Production of HA per cell after 72 hours in different subgroups. Nfy, normal fibroblasts young group; Nfo, normal fibroblasts aged group; Sfy, scar fibroblasts young group; Sfo, scar fibroblasts aged group; HGF, hepatocyte growth factor. *$P \leq 0.05$.

VF injury: Scarred VFF of younger rats produced significantly higher amounts of HA compared with older animals.[11] This observation was already known from other fields, mainly the skin but is completely new to the larynx.[18] Hirano et al[19] were among the few who explored age effects on HA production and several other parameters. In contrast to us, they did not use mature SF, but VFF from uninjured VF.

Exploring basic cellular characteristics of VFF is an absolute precondition for a variety of applications: We and other groups aim to establish standardized cell culture settings to create a reliable *in vitro* fibrogenesis system. There is a strong need for such models as these could be a basis for exploring basic pathways of VF scarring, as well as for testing diverse antifibrotic compounds. Before going into time and money intensive larger clinical trials, an *in vitro* model based on (human) VFF can lead to faster decision about promising compounds. We, furthermore, need to learn about the physiological behavior of these cells in live organisms to understand interactions and effects when performing clinical interventions.

Growth factor therapy is one of the most promising options for the treatment of VF scarring. Pioneer work in this respect was done by Hirano et al[20]: he was the first who applied basic fibroblast growth factor (bFGF) in humans for treatment of atrophied VF. A further study by the same author reported recently beneficial effects of bFGF in a combined phonosurgical approach in the treatment of VF sulcus and scar.[21] Another very promising molecule for laryngeal application is HGF. It was studied extensively in VF cell culture and animal trials in various dosages. HGF was originally discovered as growth factor involved in the regeneration of hepatocytes. In further studies, its angiogenic, angioprotective, and antifibrotic activity in various organs like liver and kidney was shown.[22] HGF has been used in different *in vitro* and *in vivo* animal studies as a promising agent in the prevention as well as in therapy of VF scarring. In a canine model, HGF was administered immediately after VF injury[23] and in a second study, 1 month after scarring.[24] Both studies showed better vibratory properties for the treatment group than for the sham group. *In vitro* use of HGF has been shown to reduce collagen I and enhance HA production[25] and upregulate messenger RNA (mRNA) expression of endogenous HGF and HA synthases in VFF.[26] Even if all these experiments were carefully performed, age of animals was considered as an independent variable in neither trial.

Our study revealed that HA production per cell of SF in both the young and the aged groups were higher than in NF at both time points (after 24 and 72 hours). However, this was only significant in cell cultures from young fibroblasts, which is in absolute accordance with our recent article.[11] Furthermore, only NF_y and SF_y produced significantly higher amounts of HA after stimulation with HGF and this only after 72 hours. This is in contrast to the aforementioned study by Hirano et al who described stimulating properties of VFF from older rats. This might be related to a different strain, as they used Fisher 344/Brown Norway rats in their experiment. Better response of VFF from younger animals might be related to the higher number of surface receptors for HA as was shown

in fetal rat fibroblasts.[27] However, this assumption must be confirmed immunohistochemically.

An *in vitro* model of VF scarring as ours can never fully reflect *in vivo* situations for several reasons and we are aware of these limitations. Protein production patterns of VFF are further known to be vibration driven,[28] so a static model presumably yields slightly different expression patterns. We, furthermore, need to elucidate if and how higher levels of HA produced by SF of younger animals can be translated into histology, as the latter depends on the complex balance between ECM, growth factors, hyaluronidases, collagen, matrix metalloproteinases, HGF, transforming growth factor-beta, and many other factors. HA expression needs furthermore to be confirmed on the protein production level by mRNA analysis. In contrast to comparable studies, we did not evaluate the levels of collagen production, as we observed in our previous work that levels of collagen type I decrease as soon as 8 weeks after injury in a rat VF scarring model.[10,11]

CONCLUSION

Only cultured NF and SF of young animals could be stimulated by HGF to increase the production of HA, whereas this was not the case in older animals. This finding underlines the importance of age as another independent variable in the complex mechanisms of VF inflammation and fibrogenesis and lets us assume that wound healing and fibrogenesis change during the lifespan. Possible therapeutic agents must consider this finding, as well as forthcoming cellular trials dealing with VFF.

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The Laryngoscope

Regenerative Phonosurgical Treatments for Vocal Fold Scar and Sulcus With Basic Fibroblast Growth Factor

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Objectives/Hypothesis: Vocal fold scar and sulcus are still challenges. Basic fibroblast growth factor (bFGF) has proven to be effective to resolve scar tissue in animal models. This study reports the efficacy of regenerative treatments using bFGF on vocal fold scar and sulcus in human cases.

Study Design: Retrospective chart review.

Methods: Fifteen cases (7 scar; 8 sulcus) were treated by either local injection of bFGF (n = 6) or regenerative surgery using bFGF (n = 9). Injection regimen was to locally apply 10 micrograms of bFGF in 0.5 mL saline into each vocal fold under topical anesthesia repeatedly (4 times with intervals of 1 week between each injection). The regenerative surgical procedure consisted of the dissection of scar tissue and the implant of gelatin sponge with bFGF. Follow-up periods ranged from 6 months to 24 months.

Results: Maximum Phonation Time (MPT); Voice Handicap Index (VHI)−10; and Grade, Roughness, Breathiness, Asthenia, Strain (GRBAS) scale were assessed in both groups. The injection group showed significant improvement on VHI-10 and GRBAS. The regenerative surgery group showed significant improvement in all parameters. Jitter and shimmer were evaluated in the surgery group, and the results indicated improvement in six and five cases of nine cases, respectively. No major adverse effects were observed in both treatment groups.

Conclusions: Regenerative treatments using bFGF has shown to be effective for improvement of vocal function in scar and sulcus.

Key Words: Vocal fold scar, sulcus, basic fibroblast growth factor, regenerative medicine.

Level of Evidence: 4.

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INTRODUCTION

Vocal fold scar and sulcus alter the histological structure of the vocal fold mucosa and stiffen the mucosa, leading to severe intractable disturbance of the vibratory property of the vocal fold.[1] The normal vocal fold mucosa consists of the epithelium, the superficial lamina propria (SLP), and the vocal ligament. The SLP is the central portion to vibrate.[2] The normal SLP has abundant hyaluronic acid (HA) and glycosaminoglycans while fibrous proteins are few,[3] but the SLP of scarred vocal folds shows the deposition of disorganized thick collagen bundles with few HA.[4] The histological study of sulcus vocalis has indicated that the bottom of the sulcus is attached to the vocal ligament with firm collagen tissue, and the SLP is almost lost.[5] It is essential to address these histological deteriorations in order to treat these pathologies.

Several surgical approaches have been attempted to improve vocal function in cases with vocal fold scar and sulcus, including the dissection or excision of the scar/sulcus,[6] slicing technique,[7] CO_2 laser ablation with collagen injection,[8] fat implant,[9] and fascia implant.[10] Resection, excision, or laser ablation technique of the scarred tissue have been attempted, anticipating better wound healing after the procedure that may lead to the recovery of vibratory tissue property. However, the wound healing process is unpredictable. Therefore, the surgical outcome varies due to the wound healing mechanism of each case. Fat implant aims at softening the scarred SLP through the soft property of fat. Fascia implant may have more biological effects on regenerative process after the procedure, and it is performed to recreate the SLP. However, these implants did not induce better wound healing and regeneration of the tissue.

Tissue engineering aims at (or stimulates) the regenerative process of tissue using cells, scaffolds, and/or growth factors.[11] Growth factors are stimulant of growth, proliferation, migration of cells, and also affect the function of cells. Exogenous application of growth factors is believed to work as a "trigger" to jump start

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the regenerative process.[12] Basic fibroblast growth factor (bFGF) is a stimulant of the growth of fibroblasts, and induces the regeneration of mesenchymal tissues and the skin. It has been reported that bFGF reduces collagen expression in gingival fibroblasts[13]; meanwhile, it stimulates the production of HA from skin fibroblasts.[14] We have also confirmed that bFGF stimulates HA production and suppresses the production of collagen from the vocal fold fibroblasts.[15] Subsequent in vivo study using canines showed the recovery of vibratory properties of scarred vocal folds by the local injection of bFGF.[16] Histological examination indicated the recovery of HA with a reduction of disorganized collagen bundles.

Based on these laboratory experiments, we have set up the clinical application of bFGF to human cases with vocal fold scar and sulcus, using a commercial product of bFGF that was already approved by the Ministry of Health and Welfare of Japan. The current study examines the efficacy of a local injection of bFGF and regenerative surgery using bFGF on vocal outcomes of patients with vocal fold scar and sulcus.

MATERIALS AND METHODS

Patients

Fifteen cases (7 scar and 8 sulcus) were treated by either local injection of bFGF (n = 6: injection group) or regenerative surgery using bFGF (n = 9: surgery group). The injection group consisted of four males and two females, with age ranging from 43 years to 65 years old (mean 54 years old). Five cases had bilateral lesions and one case had unilateral lesion. The surgery group consisted of five males and four females, with age ranging from 22 years to 75 years old (mean 50 years old). Eight cases had bilateral lesions and one case had unilateral lesion (Table I). No smoking history was confirmed in all cases.

TABLE I.
Demographic Data of Patients.

Case	Gender	Age	Disease	Side	Follow-up (months)
Injection group					
1	M	43	sulcus	bilateral	24
2	M	48	sulcus	bilateral	12
3	F	56	scar	bilateral	6
4	M	56	scar	unilateral	8
5	F	59	scar	bilateral	6
6	M	65	sulcus	bilateral	12
Surgery group					
7	F	22	sulcus	bilateral	18
8	M	34	sulcus	bilateral	9
9	M	37	sulcus	bilateral	8
10	M	42	sulcus	bilateral	12
11	F	52	scar	bilateral	6
12	M	60	Sulcus	bilateral	9
13	M	62	scar	bilateral	10
14	F	69	scar	unilateral	15
15	F	75	scar	bilateral	6

Drug Information

A commercial form of human recombinant bFGF (Fiblast, Kaken Co., Tokyo) was prepared. The active ingredient of this drug is Trafermin (a recombinant genetically engineered form of human bFGF). The supplied drug information shows that this drug stimulates growth and the proliferation of endothelial cells and fibroblasts, and contributes to the improvement of wound healing by stimulating angiogenesis and the formation of proper granulation tissue. Adverse effects were reported in 1.5% of cases, including pain, rash, and itching at the application site. It is not advisable to apply the drug to the site of malignant tumors because of concerns that the drug might stimulate growth of the tumor. Information on the half-life of this drug is not provided.

Fiblast was approved for the treatment of skin ulcers and bed sores by the Japanese Ministry of Health in 1991 and has been widely used on human patients in spray form. No serious adverse effects have been reported.

Injection Protocol

Ten micrograms of bFGF dissolved in 0.5 mL saline were injected transorally into one side of the vocal fold. Injection was performed unilaterally or bilaterally depending on the site of lesion. The pharynx and larynx were completely anesthetized with 4% lidocaine administered with an atomizer. The injection was performed using a curved injection needle under transnasal fiberscopic monitoring of the larynx. Possible allergic response, including edema of the vocal fold, was examined 1 hour after the injection. The injection was repeated 4 times with an interval of 1 week between each injection. The first checkup of the effects was performed 3 months after the initial injection by means of a perceptive voice quality assessment and stroboscopic examination; if there were few effects, another cycle of injection was indicated. In the end, the mean number of injection was 6 times in the current case series.

Patients were instructed to rest their voices on the day of the injection and allowed to phonate after the next day.

Regenerative Surgical Procedure

The regenerative surgical procedure consisted of dissection of the scar and implant of the gelatin sponge with bFGF (Fig 1). Under direct laryngoscopy, superficial cordotomy was performed at lateral portion of scar or sulcus. The microflap was elevated, and then scar tissue in the SLP was dissected until the epithelium was totally detached from the vocal ligament. An appropriately sized gelatin sponge soaked in bFGF solution (100 microg/mL) was implanted into the pocket between the epithelium and the ligament. The incision was fixed with fibrin glue.

Patients were instructed to rest their voices for 1 week after the surgery, following the instruction for routine phonomicrosurgery.

Indication of Injection and Surgery

Regenerative surgery was basically recommended for patients with scar or sulcus because dissection of scar tissue is regarded as primarily essential. However, since surgery requires a longer period of voice rest, and it may also take a longer period until the completion of postoperative wound healing, injection was indicated for cases with mild scar or for those who did not accept surgery.

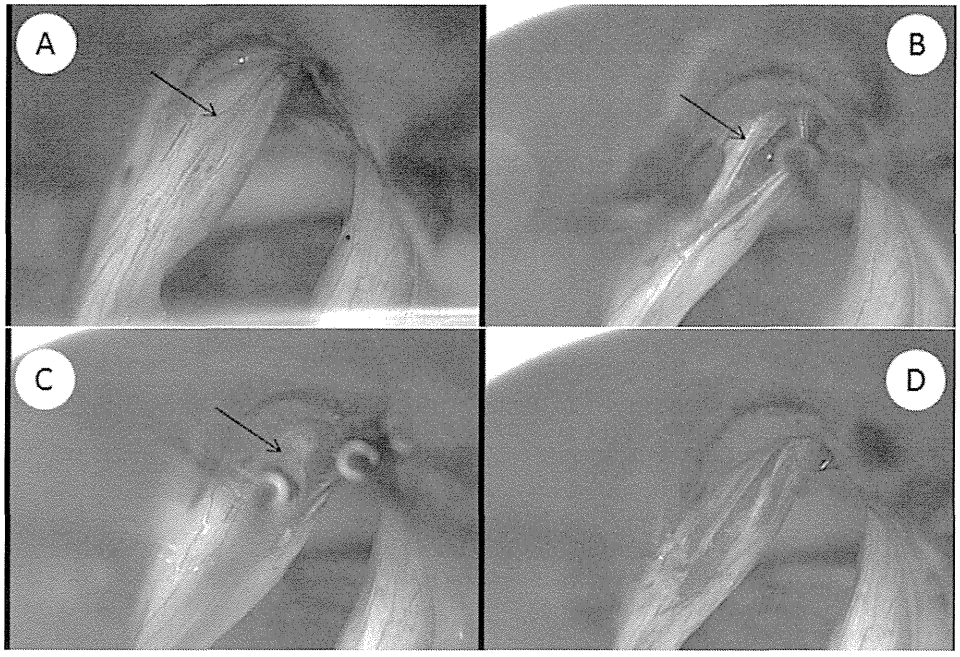

Fig. 1. Regenerative surgical procedure. (A) Direct endoscopic view of vocal fold sulcus. Arrow = sulcus. (B) Elevation of microflap and dissection of scar tissue in the superficial lamina propria. Arrow = vocal ligament. (C) Implant of gelatin sponge soaked in basic fibroblast growth factor solution. Arrow = gelatin sponge. (D) Fixation of incision with fibrin glue.

Assessment of Vocal Outcomes

Vocal outcomes were evaluated at least 6 months after the final procedures. Follow-up periods varied from 6 months to 24 months (mean period of 11 months).

Maximum phonation time; voice handicap index (VHI)−10; and grade, roughness, breathiness, asthenia, strain (GRBAS) scale were assessed in both groups. GRBAS scale was evaluated by two listeners, one laryngologist (SH), and one speech pathologist (MK) with thorough experience in clinical practice, independently. This scale was first developed by the Japanese Society of Logopedics and Phoniatrics, and became popular worldwide.[17] The GRBAS scale is scored from 0 to 3, in which 0 = within normal limits, 1 = slight, 2 = moderate, and 3 = severe. The ratings of the five subscales (G, R, B, A, S) were summed and the mean rating-score between two listeners was calculated. The inter-rater reliability between the listeners was made using Cronbach's alpha, and the result showed significant correlation with a correlation coefficient of $r = 0.8$ ($P < 0.001$).

Acoustic analysis was performed for surgery group. Acoustic analyses evaluated amplitude perturbation quotient (APQ; shimmer) and pitch perturbation quotient (PPQ; jitter) during vowel phonation using a Multi-Dimensional Voice Program (MDVP), Model 5105 (KayPentax, Lincoln Park, NJ).

Statistical Test

Statistical tests for pre- and posttreatment data were completed for each parameter using paired t test. A P value of less than 0.05 was considered significant.

Degree of improvement in maximum phonation time (MPT), VHI-10, and GRBAS was compared between both groups. The degree of improvement was calculated by the formula: Degree of improvement = |postoperative value − preoperative value|/preoperative value. Statistical analysis was performed using unpaired t test.

RESULTS

All cases showed no allergic response or any severe adverse effects. Minor adverse effects were hyperemia of the vocal fold and temporary rough voice. Mild hyperemia was found in 12 cases of 15 cases, including one case with severe hyperemia. This reaction disappeared within a few months. Temporary rough voice was observed in two cases, which retained for a few months and disappeared with time.

Injection Group

Injection group showed significant improvement on VHI-10 and GRBAS ($P = 0.008$, 0.01, respectively) (Fig 2). MPT was improved postoperatively; however, it did not reach statistical significance.

Figure 3 demonstrates stroboscopic findings in a representative case (case 4) of a 56-year-old male teacher at a high school. He underwent biopsy for leukoplakia on the left vocal fold at the prior hospital, and severe dysphonia occurred after the procedure. He was referred to us 2 years after the initial biopsy because his voice continued to be severely disturbed. Figure 3A indicates the stroboscopic findings at the first visit, showing a focal scar on the left side that hardly vibrated. After unilateral injection of bFGF was completed on the left vocal fold, his voice did not change for 6 months. However, his voice became much better at 8 months after the injection. The stroboscopic findings showed disappearance of the focal scar, with improved vibration of the vocal folds. MPT was improved from 10 to 38 seconds, and VHI-10 was reduced from 32 to 5.

Surgery Group

Regenerative surgery group showed significant improvement in MPT, VHI-10, and GRBAS ($P = 0.014$, 0.035, and 0.002, respectively) (Fig 4). Jitter and shimmer was also improved in six and five cases of nine cases respectively, although the improvement did not reach statistical significance.

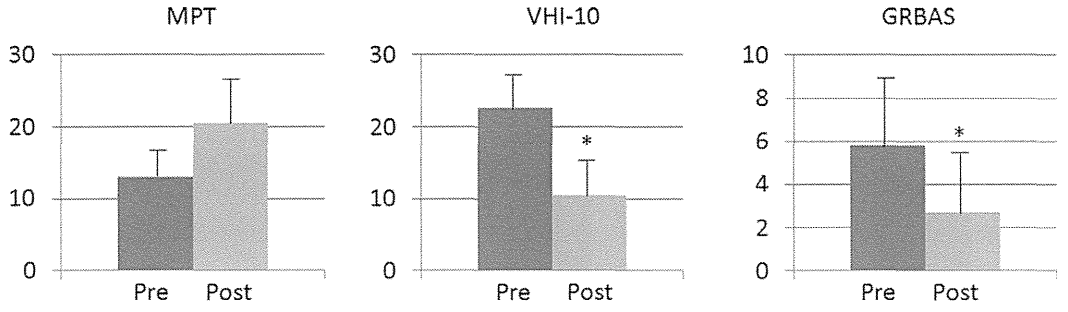

Fig. 2. Pre- and postoperative value of vocal parameters in injection group. Significant improvement was noted in Voice Handicap Index-10 and Grade, Roughness, Breathiness, Asthenia, Strain Scale ($P = 0.008$, 0.01, respectively).

Figure 5 indicates pre- and postoperative stroboscopic findings on a representative case (case 13). He was a 62-year-old male patient. He underwent bilateral cordectomy for dysplastic lesions on the vocal folds at the prior hospital, and his voice became worse postoperatively. He was referred to us 1 year after the surgery. The stroboscopic findings showed severely disturbed vibration on both sides of the vocal folds (Fig [5]A). Bilateral procedure of regenerative surgery was performed. The stroboscopic findings indicated much better vibratory properties of both vocal folds, with complete glottic closure 10 months after surgery (Fig [5]B). MPT was improved from 15 to 35 seconds, and VHI 10 was improved from 14 down to 6.

Degree of Improvement

Comparison of the degree of improvement between the injection group and the surgery group showed no statistical difference in MPT, VHI-10, and GRBAS ($P = 0.874$, 0.073, 0.274, respectively).

DISCUSSION

Tissue engineering and regenerative medicine, innovations developed at the end of the 20th century, aim to regenerate lost organs and recover their function. The principle concept is to regenerate tissues using cells, growth factors, and scaffolds. The cell is the most critical part that directly regenerate tissues. Although the development of embryonic stem cell (ES cell) and induced pluripotent stem cell (iPS cell) have been exciting and promising materials, there have been several concerns or problems to be solved, including ethical issues and tumorigenesis. Autologous mesenchymal stem cells derived from bone marrow or adipose also have received widespread attention because they have multipotency to differentiate into several cells, regardless of the type of

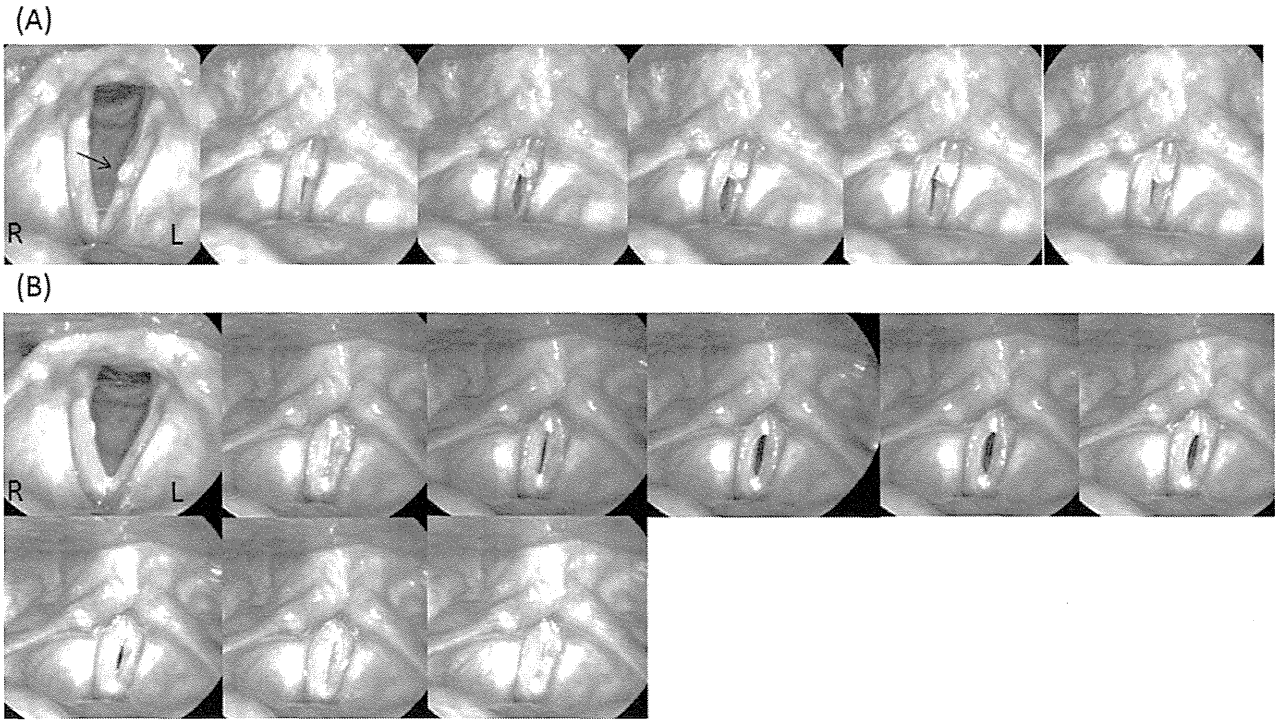

Fig. 3. Stroboscopic findings of representative case (4) in injection group. (A) Preoperative findings, indicating focal scar tissue on the left vocal fold that severed mucosal vibration. Arrow = scar. (B) 8 months after injection. Focal scar disappeared and vibratory function was much improved.

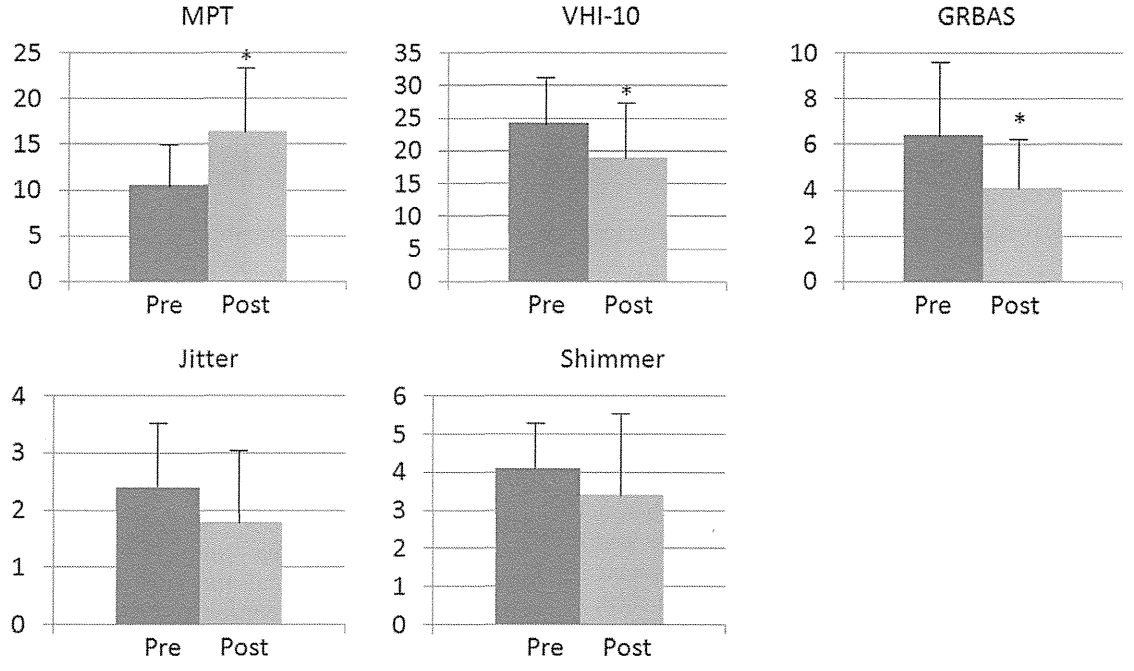

Fig. 4. Pre- and postoperative value of vocal parameters in surgery group. Significant improvement was noted in Maximum Phonation Time, Voice Handicap Index-10, and Grade, Roughness, Breathiness, Asthenia, Strain scale ($P = 0.014$, 0.035, and 0.002, respectively).

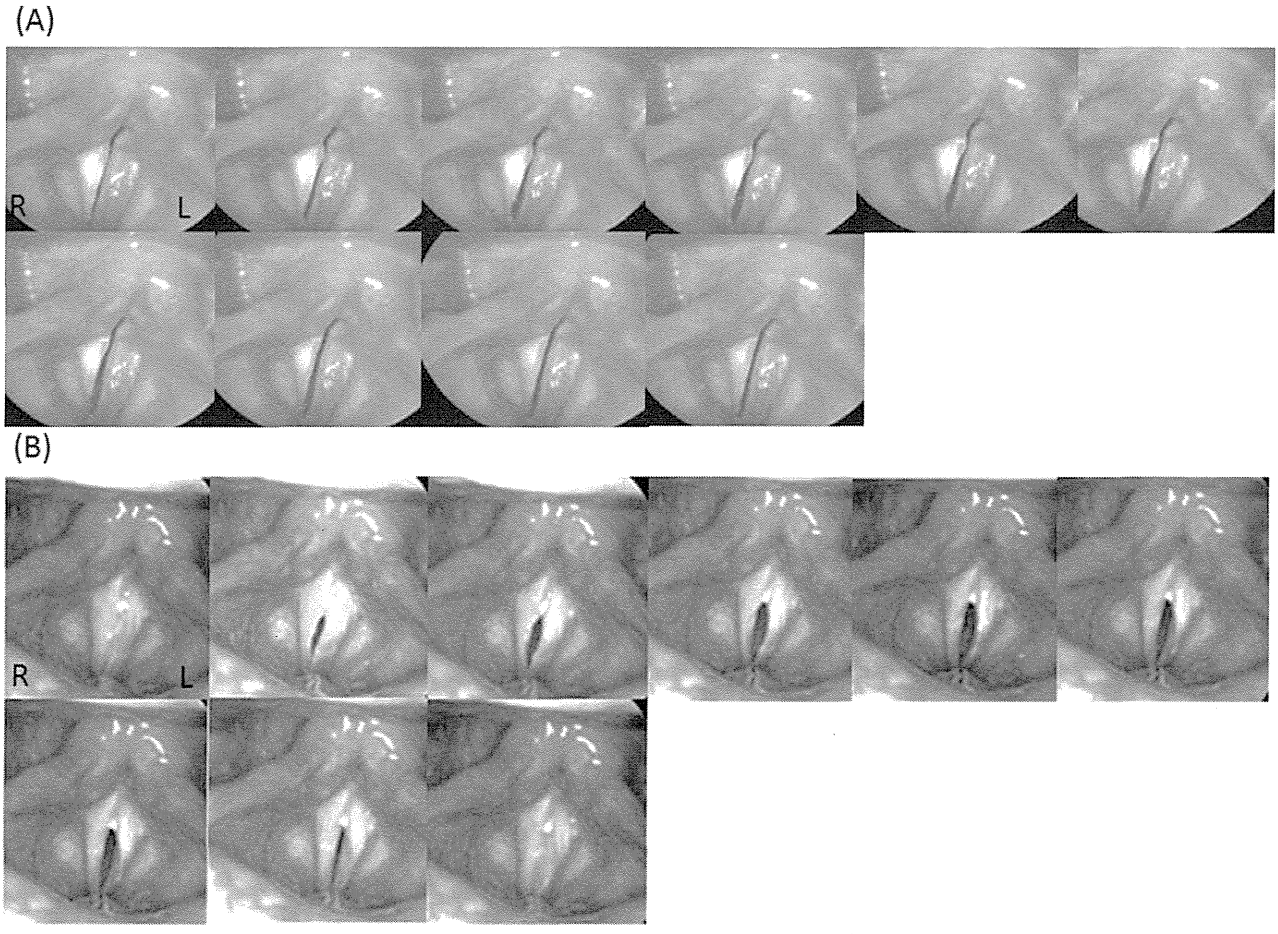

Fig. 5. Stroboscopic findings of representative case (13) in surgery group. (A) Preoperative findings, indicating bilateral scar that severed mucosal vibration. (B) 10 months after surgery. Vibratory function was much improved.

original germ layer. Kanemaru et al. first reported the use of bone marrow-derived mesenchymal stem cell (MSC) for the regeneration of injured vocal folds in a canine model.[18] They reported better wound healing of injured vocal folds after injection of MSC in histological and morphological examination. They also found that injected MSC differentiated into several types of cells, including the epithelial, mesenchymal, and muscle cells.[19] The similar reports have been published by other investigators using rabbit vocal fold scar models.[20,21] Adipose-derived stem cell (ASC) has also been proven to have a good potential to regenerate injured vocal folds in canine and rabbit models.[22,23] These cells are promising for regeneration of the vocal fold; however, the clinical application is still restricted.

Growth factors are another strong tool to induce tissue regeneration by stimulating and controlling cells. We have reported therapeutic potentials of hepatocyte growth factor (HGF) and basic fibroblast growth factor (bFGF) for vocal fold scar. Our previous in vitro studies have demonstrated that both HGF and bFGF stimulated the production of hyaluronic acid (HA) from vocal fold fibroblasts, while also reducing collagen production.[15,24] Subsequent in vivo studies using canine and rabbit have revealed that both growth factors restored injured and scarred vocal folds.[16,25] HGF was further revealed to be effective for the treatment of chronic vocal fold scar.[26] HGF has been found to be a strong regenerative factor that contributes to embryogenesis, angiogenesis, and organogenesis.[27] It has been proven to regenerate several organs such as liver, kidney, lung, etc. HGF likely may have more potential to regenerate the vocal fold than bFGF, but the clinical application of HGF is still difficult because of the lack of a good manufacturing practice (GMP)-compatible product. Because bFGF is a stimulant of growth and proliferation of fibroblasts that stimulates rapid wound healing, possibly with less scarring, its clinical application is more feasible because of the existence of a GMP-compatible product that was approved by Japanese ministry. The company is also making efforts to distribute the product worldwide.

Injection of bFGF

We primarily recommend regenerative surgery for cases with vocal fold scar or sulcus, but repeated injection of bFGF is still indicated for selected cases. There are several ways to apply growth factors, including injection in the form of solution, drug delivery system, and gene therapy. We have reported that drug delivery system of HGF, coupled with gelatin hydrogel, showed considerable effects for the treatment of scar of canine vocal folds.[28] It has been speculated that exogenous growth factors can work more effectively when they retain in the tissue for longer period. In this sense, the drug delivery system or gene therapy is regarded as superior to injection of the solution because the solution is absorbed quickly. However, the clinical application of a drug delivery system or gene therapy is still under restriction and injection of the solution is more clinically feasible. In the current study, injection was indicated chiefly for mild cases of scarring, but we did not expect that the injection worked well for such a severe case, as shown in a representative case (case 4). Exogenous growth factors are regarded to work as a trigger to change the phenotype of the target cells; once the cells change their function, regenerative effects may occur gradually with time. Indeed, in case 4 few effects were observed on the scar of the vocal fold at 6 months after injection; resolution of scar tissue appeared at 8 months.

We have reported that a local injection of bFGF showed regenerative effects on aged atrophy of the vocal fold in human cases.[29] The present study has confirmed that bFGF injection is also useful for scarred vocal folds. Histologically, aged atrophy and scar have similar characteristics, such as reduction of HA and over-deposition of collagen, which should be one of the reasons why bFGF works for scar as well as atrophy of the vocal fold.

Regenerative Surgery With bFGF

The current surgery includes two aims: one is to remove scar tissues in the SLP, and the other is to induce better wound healing postoperatively. The second aim should be the key for successful results, and the combined use of gelatin sponge and bFGF was adopted to achieve this aim. Several materials have been used as implantable materials, such as fascia, acellular dermis,[30] and atelocollagen sponge.[31] Gelatin sponge is another absorbable material; thus, it was used in order to temporarily keep the space for newly generated SLP in this study. Moreover since gelatin can keep bFGF for a certain period by attaching firmly to each other, gelatin may work as a drug delivery system of bFGF.[28] It was expected that gelatin sponge with bFGF could contribute to induction of better wound healing, which would lead to regeneration of the vocal fold. The current results were encouraging, with significant improvement of phonatory functions in terms of MPT, VHI-10, and GRBAS. Acoustic parameters (jitter, shimmer) also demonstrated improvement in more than half of cases, although there was no statistical significance in these parameters. It is necessary to clarify how consistently this strategy works in a larger series of patients in the future.

Injection or Regenerative Surgery

The present study showed no significant difference in the degree of improvement of MPT, VHI-10, or GRBAS between the injection group and the surgery group. It is difficult to conclude which strategy is superior, or whether or not both treatments have similar effects, because the selection of cases was biased. Randomized control study should be needed to clarify this aspect. It is at least suggested that injection of bFGF solution still has some regenerative effects on the scarred vocal folds, as well as regenerative surgery with implant of gelatin sponge and bFGF.

Adverse Effects

No major adverse effects were observed in either injection or regenerative surgery. Hyperemia of the vocal

fold and temporal rough voice were observed in both treatment groups. Hyperemia was thought to occur because of the strong effect of angiogenesis of bFGF. Blood supply is inevitable to induce regeneration, therefore, hyperemia is regarded as a sign of regenerative effects of bFGF. Rough voice might occur because of hyperemia or temporal imbalance of tissue property between both vocal folds during regenerative process.

CONCLUSION

Injection of bFGF or regenerative surgery with implant of gelatin sponge and bFGF was performed for human patients with vocal fold scar or sulcus. Both treatments resulted in the significant improvement of vocal parameters, including MPT, VHI-10, and GRBAS. It is suggested that bFGF has regenerative effects for vocal fold scar and sulcus. Further study is needed to explore the most consistent strategy with bFGF in large series of cases.

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