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

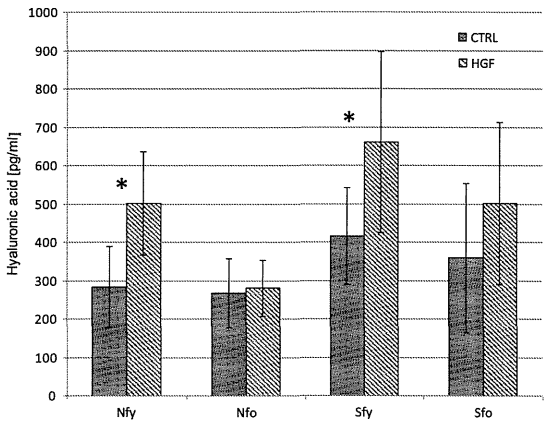

FIGURE 1. Production of HA per cell after 72 hours in different subgroups. Nfy, normal fibroblasts young group; Nfo, normal fibroblasts aged group; Sfy, scar fibroblasts young group; Sfo, scar fibroblasts aged group; HGF, hepatocyte growth factor. *$P \leq 0.05$.

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.

Acknowledgments

The authors thank Richard Ackbar PhD for discussing and proofreading.

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Eur Arch Otorhinolaryngol (2014) 271:1107–1112
DOI 10.1007/s00405-013-2722-7

LARYNGOLOGY

Age effects on extracellular matrix production of vocal fold scar fibroblasts in rats

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Received: 22 July 2013 / Accepted: 18 September 2013 / Published online: 28 September 2013

Abstract Vocal fold (VF) fibroblasts are the central subject of interest in fibrogenesis and wound healing after VF injury. Scar fibroblasts (SF) exhibit an aberrant production of several extracellular matrix (ECM) components which lead either to VF fibrosis or scarless wound healing. This study aimed to investigate the role of age at the time of injury on ECM production of SF. This is designed as an animal study. VF injury was established unilaterally in eight male Sprague-Dawley rats [3 months of age ($n = 4$), 11 months of age ($n = 4$)], while the other side was left intact. Three months after injury the larynges were excised and fibroblasts were extracted from VF [normal fibroblasts (NF)–scar fibroblasts (SF)] and cultured in vitro. After first passage, VF fibroblasts were plated in 24-well plates and levels of hyaluronic acid (HA) and collagen type I were determined enzymatically from supernatant after 24 and 72 h. Cultured SF from younger animals produced significantly higher levels of HA compared to NF fibroblasts from the same animals. HA concentrations of the older animals did not differ significantly between the NF and SF cultures, but the range in SF cultures was large. In contrast to previous studies, we found that even 3 months after VF injury cultured SF from young animals expressed higher levels of HA in comparison to SF from older animals. No difference in collagen levels were observed between the younger and older animals. Age of animals is an essential factor during VF healing and has to be considered for study design.

Keywords Vocal fold scar · Scar fibroblasts · Rat vocal fold scar model

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Introduction

Following VF injury, the damaged epithelium and lamina propria undergo a dramatic change in size, cell density, composition and morphology [1]. Main histological features of scarred VF are disorganized thick collagen and elastin bundles, loss of important extracellular matrix (ECM) constituents and subsequent volume deficiency, reduced VF pliability and glottal insufficiency [2]. VF fibroblasts—as the main cell type—undergo a morphological transformation into scar fibroblasts (SF) and a change in production of ECM proteins which has a detrimental effect on the micro-architecture of the lamina propria and thus on vibrational behavior [3]. ECM components of importance are hyaluronic acid (HA), different types of collagen (mainly types I and III), elastin and fibronectin. HA as a main ECM component acts as shock absorber during phonation and plays an important role in maintenance of tissue viscosity [4]. Furthermore, HA has a significant impact on wound healing and scar formation. We know from scarless fetal wound healing from organs other

than the VF, that elevated levels of HA play a central role [2]. HA is found physiologically at higher concentrations in the superficial layer of the lamina propria and is already decreased at early stages of wound healing [5]. Welham et al. showed that in chronically scarred rat VF, the concentrations of HA are reduced as a result of the aforementioned fibroblast transformation. On the other hand, they described elevated levels of collagen types I and III in chronically scarred VF [6].

For practical reasons, animal models are very common in studying molecular mechanisms of VF scarring. However, we have learned from these studies that many species show different responses to injury and differ significantly during the healing process. The rat model has been well studied and characterized by Tateya et al. [7] and it has been proven to be of special value as the rats' lamina propria is tri-layered and thus can be compared well to the human VF.

So far the age of animals at the time of VF injury was not a special subject of interest. We hypothesize that age at the time of injury should be regarded as another independent variable, as wound healing in general is supposed to be strongly age-dependent [8]. Such a finding can have both theoretical and clinical implications. Treatment of chronic VF scars remains still a challenge, as we know from former studies that tissue remodeling slows significantly down after 2 months [9]. Exploring the biological behavior of mature VF scar fibroblasts can therefor lead to a better understanding and new therapeutic facilities.

There is a strong need for a robust in vitro model of VF scarring that would allow screening of antifibrotic compounds before going into larger clinical trials. A careful characterization of the employed cells is an absolute prerequisite for such a model. Among many other factors, the age of animals at time of injury is of considerable importance, but has not been explored so far.

We furthermore wanted to investigate current cell-culture conditions and methods. We know from reports in scar research other than the VF, that collagen matrix formation is very slow under in vitro conditions and can be enhanced and stabilized by adding ascorbic acid to provide a better conversion of de-novo synthesized procollagen to collagen [10]. Even if this seems to be very obvious, it has never been reviewed in trials dealing with scar VF.

Materials and methods

Study animals consisted of eight male Sprague-Dawley rats [4: 3 months old at time of injury (young group), 4: 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 [11]. Under general anesthesia [Ketamine (25 mg/kg) and Xylazine hydrochloride (4.5 mg/kg)] the right VF of all rats were injured endoscopically using a 23G syringe. The injury was confirmed by exposure of the thyroarytenoid muscle as described previously [12]. The left VF was left intact and served as a control. This specific study design allowed us to control for variations in cell behavior between the animals. Procedures were well tolerated by the rats, all showed normal feeding and drinking behavior the next day.

Three months later, rats were humanely euthanized by intracardiac injection of Ketamine 100 mg/ml after sedation with isoflurane (5 %) in an induction chamber with an oxygen flow of 1.5 l/min. This time point was chosen as it is generally accepted that it takes 28–42 days to form a mature scar in rats [9]. All study procedures were conducted according to the Austrian guidelines for animal experiments and were approved by the Austrian Ministry of Science.

Under stereo-magnification, epithelium and underlying lamina propria of both VF were carefully excised: specimens were minced, and fibroblasts were extracted and cultured following established tissue-explant protocols. Fibroblasts from uninjured (left) VF were named normal fibroblasts (NF); fibroblasts from injured (right) VF were named scar fibroblasts (SF). Therefore, in total there were four different groups: NF young, SF young, NF old, SF old.

Growth medium consisted of DMEM (Dulbecco's modified Eagle's medium (Life Technologies, Gaithersburg, MD, USA) enriched with 10 % fetal calf serum (FCS), 1 % penicillin/streptomycin and 1,000 mM vitamin C per well (L-ascorbic acid 2-phosphate sesquimagnesium salt hydrate, Wako Pure Chemical Industries, Osaka, Japan). Procedures were repeated in NF and SF of young animals without addition of ascorbic acid to reveal given differences in collagen matrix deposition.

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 (Nalgen Nunc International, NY, USA) 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 24 and 72 h 0.5 ml of supernatant medium was sampled and immediately stored at −80°. Medium was replaced after 24 h.

Assessment of HA and collagen-I production from supernatants was carried out using enzyme-linked immunosorbent assays (TECO-Hyaluronic acid; TECOmedical group, Sissach, Switzerland for HA, resp. ELISA Kit USCN Life Science Inc. for collagen-I-α). All assays were performed in duplicates (technical duplicates).

Statistical analysis

Differences of the mean were analyzed by paired and unpaired t tests after proof of normal distribution using PASW statistics 18.0 (SPSS Inc.). 0.05 was chosen as level for statistical significance. Normal distribution was given in all parameters as confirmed by the Kolmogorov–Smirnov test. Biological replicates were treated as independent variables, whereas the technical duplicates were averaged.

Results

Hyaluronic acid

Cultured SF of the young group produced significantly higher levels of HA compared to the uninjured contralateral side [170 vs. 105 ng/ml after 24 h ($p = 0.003$), respectively. 305 vs. 195 ng/ml ($p = 0.001$) after 72 h]. HA production of SF cultures from old animals did not differ to production levels from the contralateral, uninjured side at either measuring point. HA levels were higher in absolute dimensions in SF cultures from young animals at both measuring points compared to older animals, but this was not statistically significant (170 vs. 117 ng/ml after 24 h, $p = 0.3$) (see Table 1; Figs. 1, 2). Importantly, testing of media-only (cell-free) controls demonstrated that neither DMEM nor fetal calf serum interfered with the assays.

Collagen-I

For collagen-I there was no difference between the younger and the older animals at both 24 and 72 h. Also inter group deviations did not differ significantly in either group (see Table 2; Figs. 3, 4).

Ascorbic acid

In a subgroup (NF and SF of young rats), we added no ascorbic acid to the growth medium and compared it with exactly the same setting containing ascorbic acid. In neither setting were detected differences in collagen-I (p always >0.05) (see Table 3).

Table 1 Levels of HA in ng/ml

Group	24 h (mean ± SD)		72 h (mean ± SD)	
NFy	105 ± 36	$p = 0.003$	195 ± 48	$p = 0.001$
SFy	170 ± 37		305 ± 45	
NFo	90 ± 19	$p = 0.4$	197 ± 31	$p = 0.7$
SFo	117 ± 90		177 ± 145	

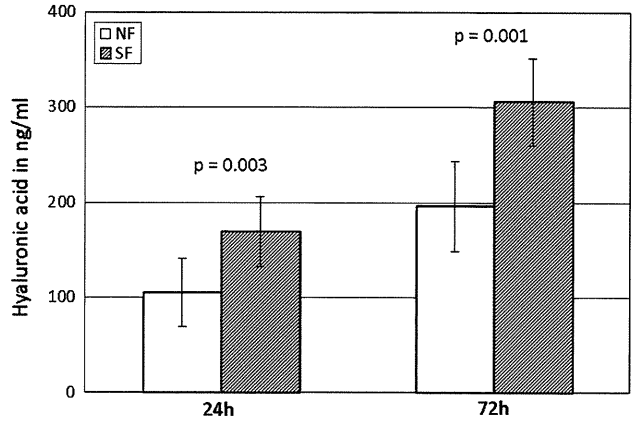

Fig. 1 Levels of hyaluronic acid at 24 and 72 h between NF and SF of young animals

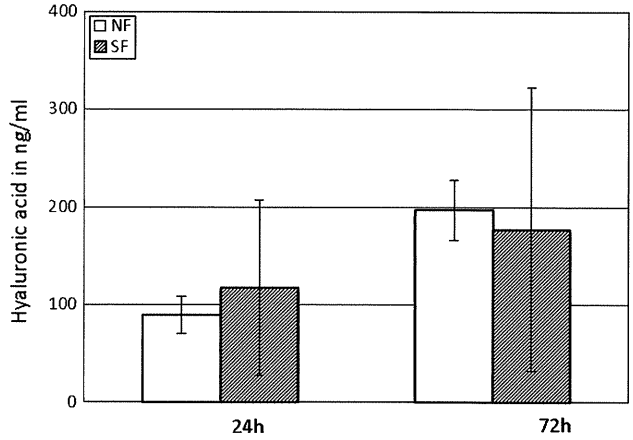

Fig. 2 Levels of hyaluronic acid at 24 and 72 h between NF and SF of old animals

Table 2 Levels of collagen-I in ng/ml

Group	24 h (mean ± SD)		72 h (mean ± SD)	
NFy	283 ± 59	$p = 0.1$	443 ± 63	$p = 0.2$
SFy	413 ± 181		361 ± 62	
NFo	253 ± 118	$p = 0.5$	377 ± 83	$p = 0.4$
SFo	275 ± 65		544 ± 396	

Discussion

Following VF injury, three distinct phases of wound healing can be distinguished: inflammatory phase, proliferative phase and remodeling phase [13]. Despite recent increase in knowledge it is not completely understood, which are the cellular mechanisms that either lead to scarred VF or to scarless wound healing. As a consequence it was still not possible to date to resolve mature VF scars or blockade fibrogenesis after VF injury.

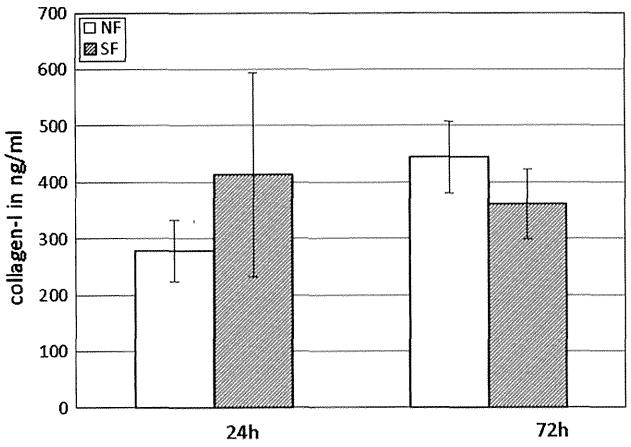

Fig. 3 Levels of collagen-I at 24 and 72 h between NF and SF of young animals

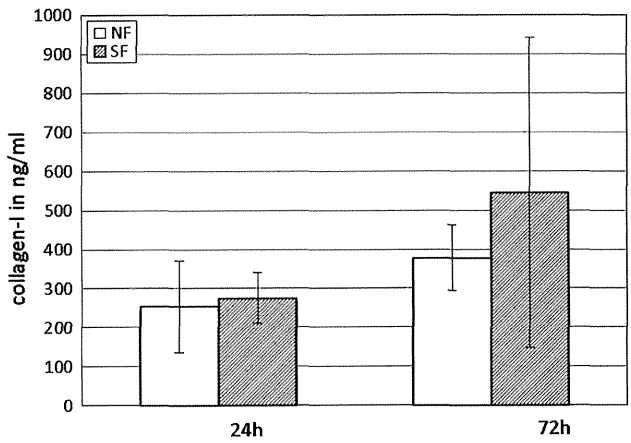

Fig. 4 Levels of collagen-I at 24 and 72 h between NF and SF of old animals

Table 3 Levels of collagen-I in ng/ml in growth medium without ascorbic acid

Group	24 h (mean ± SD)	72 h (mean ± SD)
NFy	244 ± 59	443 ± 63
SFy	310 ± 84	555 ± 127

Cell culture developed as a useful tool in laryngeal scar research. VF fibroblasts are at the center of research as they mediate the inflammatory and fibrotic cascade after VF injury by aberrant fibroblast activity and specific gene expression [14]. VF fibroblasts have unique characteristics not shared by other types of fibroblasts. Scar fibroblasts (aka myofibroblasts) are characterized by a different cell phenotype, they occur at lower cell densities and have a different expression profile of several ECM components, such as HA, fibronectin, collagen and decorin [15]. The use of human VF fibroblasts in cell-culture models would be optimal, but the availability of these cells is very restricted and virtually impossible to obtain from live donors.

As a consequence studies focused so far on animal studies employing rats, mice, dogs and rabbits [7, 16]. We learned from these that there are significant inter-species variations that make it difficult to compare results. Rat models are ubiquitous in VF scar research as they provide several advantages: The rat VF lamina propria contains a tri-layered structure which is similar in morphology and fibrous protein composition to humans. Furthermore, the formation of chronic VF scar has been reported to take 2 months following injury in the rat, whereas it takes up to 6 months in rabbits [6].

HA plays a major role during wound healing and is secreted by VF fibroblasts. Wound repair in HA rich environment is thought to represent a regenerative-like repair [5]. The presence of higher levels of HA during early stages of wound healing might minimize scar formation. HA levels undergo significant time dependent changes after VF injury. During early phases of wound healing HA levels were found to be decreased compared to the uninjured side [5]. In rabbit and canine models HA levels were found to be similar in scarred and unscarred VF 6 months after injury [17]. Our results confirm this for the rats that were 8 months of age at time of the injury. In contrast to this we found significantly increased HA levels in SF cultures of younger rats. VF of younger animals preserved elevated levels of HA even 3 months after injury which is supposed to create an optimal environment for wound healing. Having this in mind, the aforementioned in vivo study by Welham et al. [6] described HA depletion in Sprague-Dawley rats VF 2 months after injury (4 months old at time of injury). In contrast to our study, they determined HA content only histologically and only in one age group. HA levels were higher in absolute dimensions in SF cultures from young animals at both measuring points compared to older animals, but this was not statistically significant, thus we cannot make a proper statement about this. We can only assume that the lack of significance was due to the fact that we compared SF of different animals in this setting, whereas we used one and the same animal for the first analysis. HA concentrations of the older animals did not differ significantly between the NF and SF cultures, but the range in SF cultures was large. Consequently, the fibroblasts in older animals might have a different response profile to the injury and animal to animal variation is great.

Alternation in VF fibroblast collagen production and collagen deposition is another key parameter in several models. Rat studies showed that levels of collagen type I and III increased starting as soon as 3 days after injury [7, 16]. Whereas it generally assumed that VF scarring is characterized by increased collagen deposition in the

lamina propria, some authors described decreased collagen with increased stiffness [18]. We know from fibrogenesis studies other than the VF that collagen deposition in cell culture is very limited due to several reasons: first, unprocessed procollagen accumulates in the cell culture medium due to a tardy procollagen C-proteinase/BMP1 activity in aqueous culture conditions. Secondly, ascorbate is a crucial co-substrate of the enzymes responsible for the post-translational hydroxylation of prolyl and lysyl residues for rendering the collagen triple helix thermostable and for regular extracellular cross-linking [19]. Addition of ascorbic acid, in form of a magnesium salt resulted in enhanced production and deposition of collagen on the cell layer [20]. This aspect was not reviewed in VF scar models so far. Even under administration of higher amounts of ascorbate as found in literature we did not detect different levels of collagen in either group, which is in accordance with literature for the tested time span of 3 months. Tateya and co-workers described decreasing levels of collagen type I as soon as 8 weeks after injury in a rat VF scarring model. Only levels of collagen type III remained elevated after 12 weeks [9].

We are fully aware that the results from our in vitro trial offer preliminary data that concentrate on cell culture and cannot be directly transferred into in vivo conditions. Several points remain to be elucidated. Among those we need to clarify 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 (MMP), hepatocyte growth factor (HGF), transforming growth factor beta (TGF-β) and many other factors. HA expression needs furthermore to be explored on the protein production level by mRNA analysis.

Further work should be undertaken to create standardized cell culture settings and to create a valuable in vitro fibrogenesis system. Production of stable collagen molecules is an important point in this respect which could be achieved by addition of charged and neutral macromolecules into culture medium. This approach (macro-molecular crowding) has led to promising in vitro fibrogenesis models [21, 22].

Conclusion

SF of younger animals preserved elevated levels of HA in vitro even 3 months after VF injury under cell culture conditions. This may contribute to a better understanding of VF fibroblast behavior during the different phases of wound healing. Age of animals is an important factor, when planning in vitro studies VF wound healing and fibrogenesis. Addition of ascorbic acid to the growth medium showed no effect on collagen matrix deposition by SF or NF.

Acknowledgments The authors would like to thank Richard Ackbar, PhD for discussing and proofreading. This project was supported by the Styrian government.

Conflict of interest None.

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The Laryngoscope

Restoration of Scarred Vocal Folds Using 5 Amino Acid-Deleted Type Hepatocyte Growth Factor

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Objectives/Hypothesis: Our previous studies demonstrated a regenerative effect of recombinant human hepatocyte growth factor (HGF) on vocal fold scarring using full-length HGF. However, clinical application has not yet been achieved because of the lack of a good manufacturing practice (GMP) for full-length HGF. Another natural form of human HGF, 5 amino acid-deleted type HGF (dHGF), has been newly produced under a GMP procedure. In the current study, we investigated the effect of dHGF in comparison with full-length HGF for the treatment of vocal fold scars using a canine model.

Study Design: Prospective animal experiment.

Methods: The vocal folds of nine beagles were unilaterally injured. Four weeks after injury, the vocal folds were treated with an intracordal injection of full-length HGF (full HGF group), dHGF (dHGF group), or phosphate-buffered saline (sham group). Vibratory and histological examinations were performed for each group 6 months after injury.

Results: Vibratory examinations demonstrated significantly lower phonation threshold pressure and a higher ratio of normalized mucosal wave amplitude in both the full HGF and dHGF groups as compared to the sham group. Histological examination showed restoration of hyaluronic acid in both the full HGF and dHGF groups as compared to the sham group. No significant differences were observed for each parameter between the full HGF group and the dHGF group.

Conclusions: dHGF showed the same potential for regenerative effects on vocal fold scars as full-length HGF. dHGF should be applicable for human clinical trials in patients with vocal fold scars.

Key Words: Vocal fold scarring, 5 amino acid-deleted type hepatocyte growth factor.

Level of Evidence: NA

***Laryngoscope*, 124:E81–E86, 2014**

INTRODUCTION

The vocal fold has an ideal viscoelasticity that enables rapid mucosal vibration to produce sounds. Vocal fold scars develop as a consequence of injury and inflammation of the mucosa, which causes the folds to stiffen and difficulties in vibration.[1] Once the vocal fold is scarred, severe dysphonia or aphonia occurs. Previous histological studies using animals and humans revealed increased deposition of disorganized collagen, a reduction of hyaluronic acid, elastin, decorin, and fibromodulin, and an increase of fibronectin.[2–6] Thick collagen deposition and loss of hyaluronic acid (HA) are regarded as particularly detrimental and need to be addressed in the treatment of vocal fold scars.

Hepatocyte growth factor (HGF) was originally identified and cloned as a potent mitogen for mature hepatocytes.[7,8] It is multipotent and is able to regenerate several organs and tissues due to its strong angiogenic, antifibrotic, and antiapoptosis activities.[7,8] Human HGF has two natural forms: one is full-length HGF composed of 697 amino acid residues and the other is a 5 amino acid-deleted type HGF (dHGF), which lacks the F-L-P-S-S amino acid residues in the first kringle domain.[7,9,10] We have demonstrated that full-length HGF stimulates protein production and gene expression of extracellular matrix molecules in the vocal fold mucosa.[11–16] Specifically, full-length HGF stimulates HA and matrix metalloproteinase-1 synthesis by fibroblasts in the vocal folds. This function is regarded as having a therapeutic potential for treatment of vocal fold scars. In addition, our previous animal experiments demonstrated that locally applied full-length HGF improves the vibratory properties of scarred vocal folds. There was restoration of histology including digestion of collagen and deposition of HA.[17,18] We also confirmed that delivery of full-length HGF with a gelatin hydrogel strengthened the regenerative effects, even for chronic scars of the vocal fold.[19, 20] Together, these findings demonstrate the

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Editor's Note: This Manuscript was accepted for publication August 26, 2013.

This study was supported by the Advanced Research for Medical Products Mining Program of the National Institute of Biomedical Innovation (NIBIO).

The authors have no other funding, financial relationships, or conflicts of interest to disclose.

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DOI: 10.1002/lary.24413

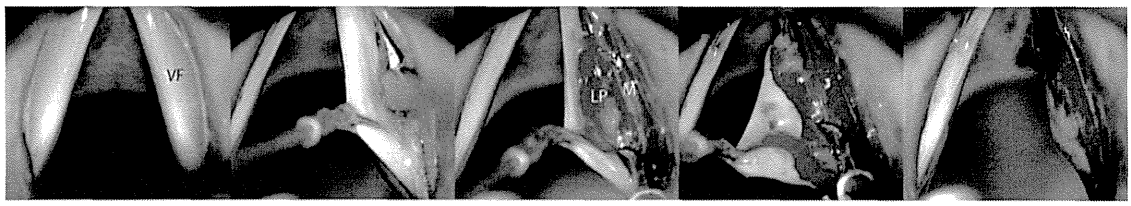

Fig. 1. Surgical procedure. The vocal folds were unilaterally scarred by stripping the entire layer of the lamina propria down to the muscle under a direct laryngoscope. LP = lamina propria; M = muscle; VF = vocal fold. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

regenerative effects of full-length HGF on vocal fold scars. However, clinical application has not yet been achieved because of a lack of a good manufacturing practice (GMP) for full-length HGF.

Recently, recombinant human dHGF has been produced by a GMP procedure. It was confirmed that dHGF has the same biological activities as full-length HGF, and it was slightly more potent under specific conditions.[10] However, it is unclear whether dHGF has the same effects on vocal fold scars as full-length HGF. In the current study, we examined the regenerative effects of dHGF on scarred vocal folds using a canine model and compared it with full-length HGF.

MATERIALS AND METHODS

Animals

Nine beagles weighing 8 to 13 kg were used in this study. All experimental protocols were approved by the Animal Committee of the Graduate School of Medicine, Kyoto University. Animal care was provided under the supervision of the Institute of Laboratory Animals of the Graduate School of Medicine, Kyoto University.

Preparation of HGF Injection

Full-length recombinant human HGF (full HGF) was purchased from Sigma-Aldrich Co. (St. Louis, MO). dHGF was supplied by Kringle Pharma, Inc. (Osaka, Japan). Both full HGF and dHGF were diluted with phosphate-buffered saline containing 0.5 % human serum albumin to avoid adsorption loss.

Surgical Procedure

The surgical procedures that were used for generating vocal fold injuries have been established in previous studies.[17,19,20] All animals were sedated under general anesthesia with an intramuscular injection of ketamine hydrochloride (15 mg/kg) and xylazine hydrochloride (6 mg/kg). The glottis was visualized using a direct laryngoscope and the vocal folds were unilaterally scarred by stripping the entire layer of the lamina propria down to the muscle with microscissors and microforceps (Fig. 1). The contralateral vocal folds were kept intact as normal controls. The sides for scarring were randomly selected.

Injection Procedure

At 4 weeks after the initial injury, the scarred vocal folds were treated with a transcordal injection of one of three different materials: 1) full HGF (full HGF group, 0.5 mL, 1 μg/mL), 2) dHGF (dHGF group, 0.5 mL, 1 μg/mL), or 3) phosphate-buffered saline (sham group, 0.5 mL). The same injection was repeated twice with an interval of 1 week between injections for each group.

Experimental animals were euthanized 6 months after the initial injury. They were sedated as described above and euthanized with an intracardiac injection of pentobarbital sodium (25 mg/kg). Larynges were harvested immediately and used for vibratory examinations and were subsequently subjected to histological examination.

Vibratory Examination of Excised Larynges

Vocal fold vibrations were examined using an excised larynx setup described in previous studies.[20] For better visualization of the vocal fold, supraglottic structures such as the epiglottis, false vocal folds, and aryepiglottic folds were removed after resection of the superior portion of the thyroid cartilage. The arytenoid cartilages were sutured together, and an arytenoid adduction procedure was performed bilaterally using 3-0 Prolene to close the glottis. Once the larynx was mounted, an endotracheal tube was inserted and clamped to prevent air leaks. Air was pumped through the tube to generate vocal fold vibrations. Larynges were irrigated with saline throughout the experiment to keep the vocal folds moist. A high-speed digital imaging system (Memrecam Ci; NAC Image Technology, Osaka, Japan) was used to record vocal fold vibrations from the superior view. The camera was mounted 50 cm above the larynx, and the image was displayed on a monitor. The images were recorded at a frame rate of 2,000 to 4,000 frames per second, and the images were then scanned into a computer (Fig. 2).

Phonation threshold pressure (PTP) was used as one of the functional parameters of the mucosal vibration. PTP, which is regulated by factors such as vocal fold thickness, property, and glottal width, was defined as the minimum amount of subglottal pressure required to initiate vocal fold oscillation. Further, the amplitude of the mucosal wave was measured to evaluate the mucosal vibration and elasticity of the vocal fold structures, namely the lamina propria, using ImageJ software (National Institutes of Health, Bethesda, MD) (Fig. 3). The distance (d1) from the midline of the glottis to the edge of the vocal fold was measured at the anteroposterior middle portion of the vocal fold during the closed phase. The closed phase was determined based on the motion of the upper and lower lips of the vocal folds. The same distance (d2) was measured at the maximum open phase. The mucosal wave amplitude was defined by subtracting d1 from d2 and was normalized by dividing the subtracted digit by the anteroposterior length of the glottis (L), which was measured from the anterior commissure to the vocal process. The following formula was used: normalized mucosal wave amplitude (NMWA) = (d2 − d1)/L.

To compare the NMWA of the treated side with the normal side, we calculated the ratio of the NMWA. The following formula was used: the ratio of NMWA = (NMWA of the treated vocal fold)/(NMWA of the control vocal fold, contralateral side).

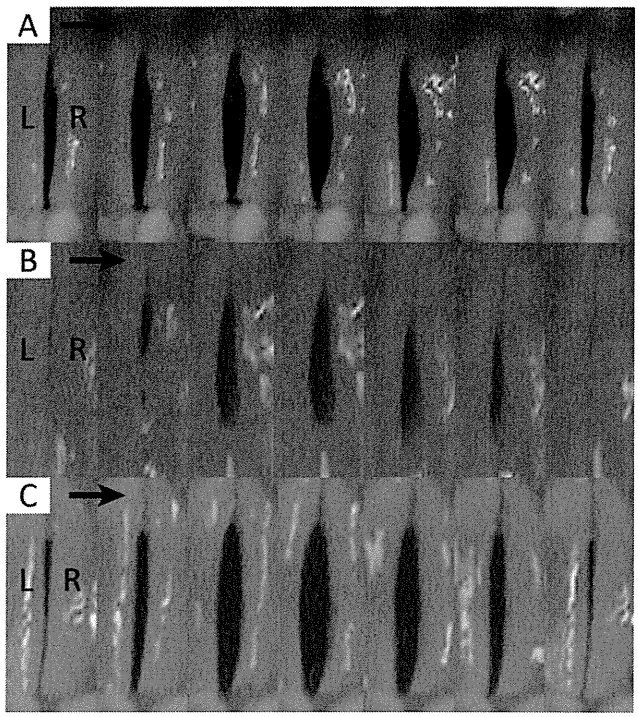

Fig. 2. Digital high-speed images of vocal fold vibratory patterns during vibratory examination of the excised larynx. (A) Sham group. (B) Full-length hepatocyte growth factor (full HGF) group. (C) 5 amino acid-deleted type HGF (dHGF) group. The full HGF-treated vocal folds and dHGF-treated vocal folds showed better mucosal vibration and a smaller glottal gap during the closed phase than the sham-treated vocal folds. L = normal side; R = treated side.

Normalized glottal gap (NGG) was calculated to evaluate glottis closure during the closed phase. The glottal gap area (a) was measured using ImageJ software and was normalized by dividing it by L^2. The following formula was used: $NGG = a/L^2 \times 100$.

Histological Examination

After vibratory examination, the larynges were fixed in 10% formaldehyde for tissue examination. Larynges were subsequently embedded in paraffin, and 5-μm-thick serial sections were prepared in the coronal plane from the anteroposterior middle portion of the vocal folds. Elastica van Gieson staining was performed to identify collagen and elastin. Alcian blue staining was performed on serial sections with or without hyaluronidase digestion to identify HA. Images were captured with a Biorevo BZ-9000 microscope (Keyence Corp., Osaka, Japan). The sections were examined at 4× to 40× magnification. On Alcian blue-stained sections, the blue-stained areas of the lamina propria were measured using software that automatically measures an area with a designated color threshold (Biorevo BZ-H1C and BZ-H1M; Keyence Corp.). The color threshold was designated by the examiner. The ratio of the stained area to the total area of the lamina propria was then calculated for each section. The amount of HA in the lamina propria was determined by subtracting the ratio of the blue-stained area of the section with hyaluronidase digestion from that of an adjacent section without digestion.[21] These assessments were performed in a blinded fashion, in which the examiners were not informed of the group to which each slide belonged.

Statistical Analysis

Statistical analyses were performed using one-way factorial analysis of variance (ANOVA) followed by a post hoc Scheffé test. Statistical significance was defined as $P < .05$.

RESULTS

Vibratory Examination of Excised Larynges

In digital high-speed images of vocal fold vibratory patterns during vibratory examination, the injured vocal folds in the full HGF group and the dHGF group showed better vibration than the sham-treated vocal folds, which had limited vibratory movement (Fig. 2). The vocal folds of the full HGF and dHGF groups resembled the normal vocal folds on the contralateral side.

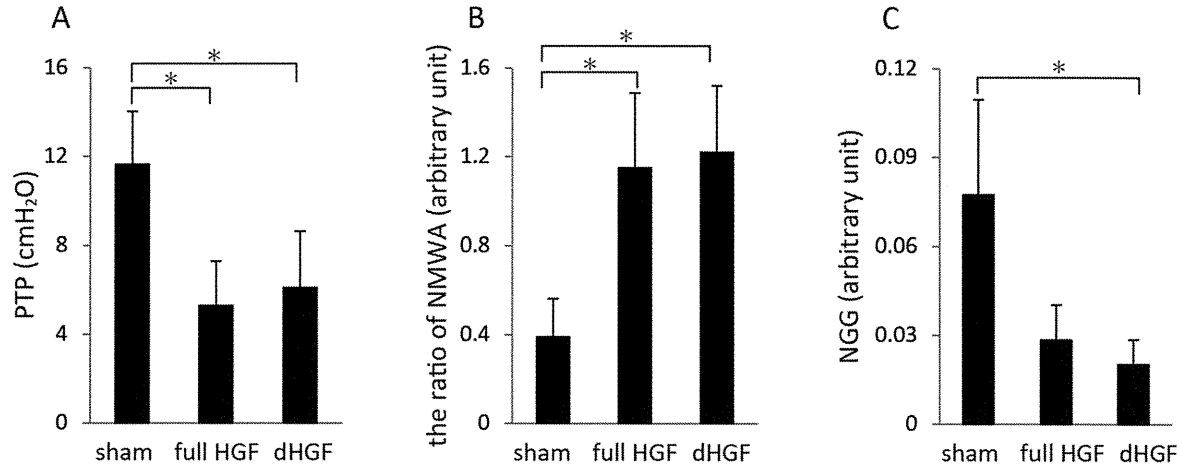

Fig. 3. Results of the vibratory examinations. The full hepatocyte growth factor (HGF) group and the 5 amino acid-deleted type HGF (dHGF) group showed significantly lower phonation threshold pressure (PTP) (A) and a higher ratio of normalized mucosal wave amplitude (NMWA) (B) than the sham group. The dHGF group showed a smaller normalized glottal gap (NGG) (C) than the sham group. No significant differences were observed for each parameter between the full HGF group and the dHGF-treated group. *Significant difference ($P < .05$) compared to the sham-treated group (one-way factorial analysis of variance, post hoc Scheffé test).

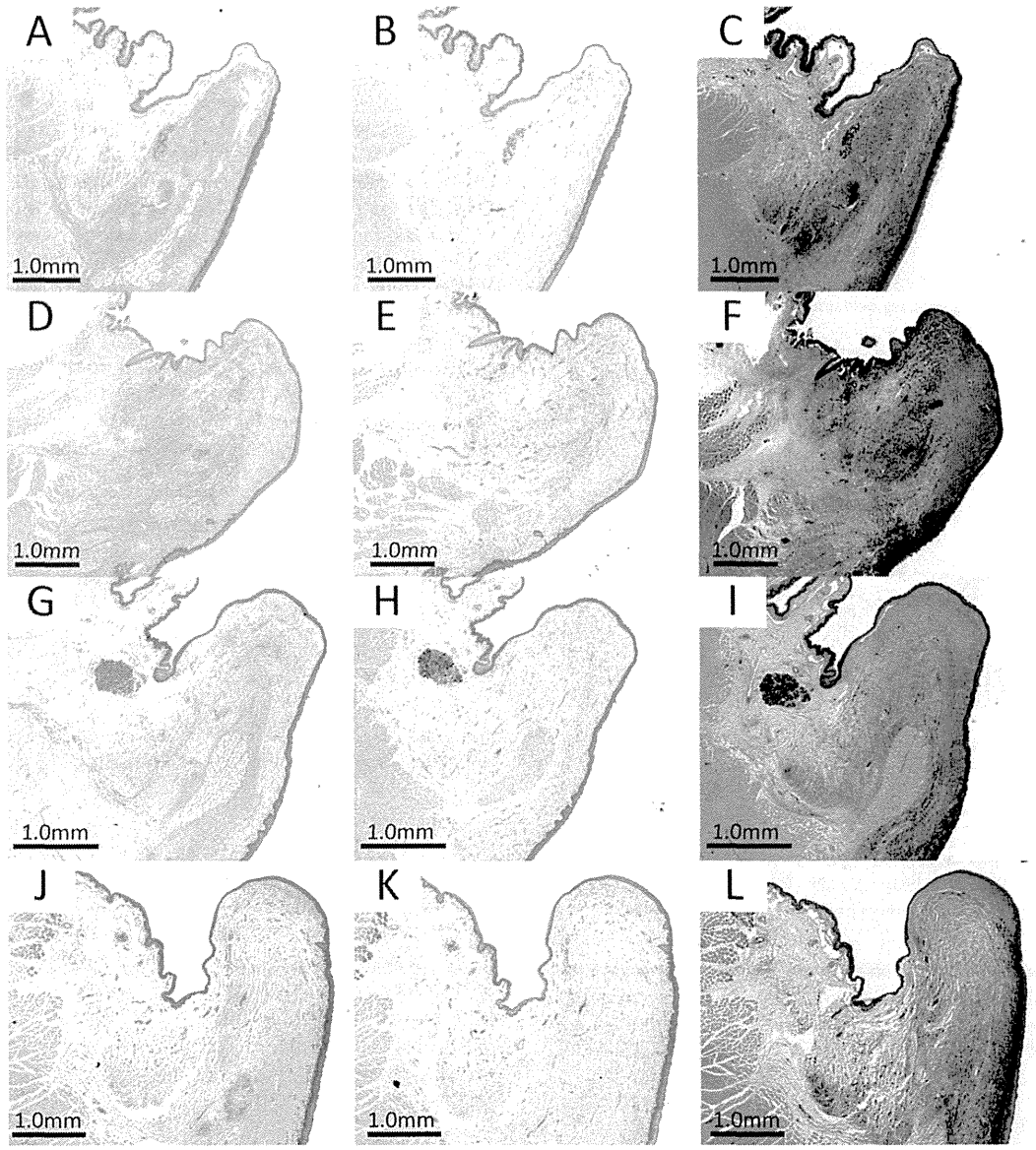

Fig. 4. Histological findings (coronal sections of vocal folds). Alcian blue staining without hyaluronidase digestion (A, D, G, J) and with hyaluronidase digestion (B, E, H, K), and Elastica van Gieson staining (C, F, I, L) of the normal group (A, B, C), the sham-treated group (D, E, F), the full hepatocyte growth factor (HGF)-treated group (G, H, I), and the 5 amino acid-deleted type HGF (dHGF)-treated group (J, K, L). The full HGF-treated group and the dHGF-treated group showed restoration of hyaluronic acid compared to the sham-treated group. Collagen deposition (purple in Elastica van Gieson staining) of the full HGF-treated group and the dHGF-treated group was minimal compared to the sham-treated group. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

Both the full HGF group and the dHGF group showed significantly lower PTP (Fig. 3A) and a higher ratio of NMWA (Fig. 3B) than the sham group. The full HGF group showed significantly smaller NGG (Fig. 3C) than the sham group. No significant differences were observed for each parameter between the full HGF group and the dHGF group.

Histological Examination

Alcian blue staining and the hyaluronidase digestion technique (Fig. 4) showed that deposition of HA in sham-treated vocal folds was reduced compared to the normal folds. Both the full HGF group and the dHGF group showed better restoration of HA, which resembled normal folds, than the sham-treated folds, although no significant difference was observed for the ratio of HA among the three groups (Fig. 5). The results also indicated better recovery of HA in the dHGF group than the full HGF group, although the difference did not reach statistical significance.

Elastica van Gieson staining showed that collagen deposition in both the full HGF group and the dHGF group was minimal compared to the sham group.

DISCUSSION

Human HGF has two natural forms: full-length HGF and dHGF. Nucleotide sequence analysis of cDNA clones for human HGF has demonstrated that HGF

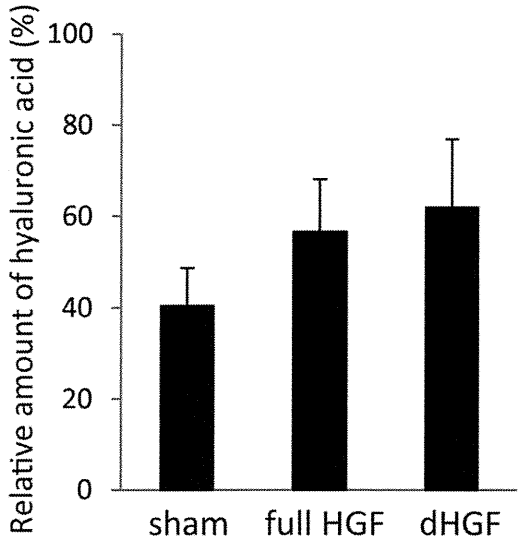

Fig. 5. The relative amount of hyaluronic acid. No significant difference was observed in the relative amount of hyaluronic acid among the three groups, although there was a tendency that the 5 amino acid-deleted type HGF (dHGF)-treated group showed a higher amount of HA compared to the sham-treated group and the full hepatocyte growth factor (HGF)-treated group.

consists of six major domains including a hairpin region and four kringle domains in the alpha chain, and a serine protease-like domain in the beta chain. dHGF lacks 5 amino acids in the first kringle domain compared to full-length HGF.[7,9,10] We have confirmed the regenerative effects of HGF on vocal fold mucosa in several animal studies including rats, rabbits, and canines using full-length HGF. These findings demonstrate the regenerative effects of full-length HGF on vocal fold scars and suggest that clinical trials are warranted, but this has been hampered by the lack of GMP-compatible full-length HGF.

Recently, recombinant human dHGF was produced by Chinese hamster ovary cells transfected with human dHGF cDNA under a GMP procedure. It has been suggested that the deletion of five amino acids in the first kringle domain might affect the biological activity of HGF.[10] However, it was reported that dHGF had higher mitogenic activity than full HGF for rat hepatocytes.[10,22] Furthermore, Shima et al.[10] investigated the differences between the biological activities of full HGF and dHGF in an in vitro study and reported that dHGF was more potent than full HGF for the stimulation of DNA synthesis in epithelial cells, whereas full HGF was more potent than dHGF in the stimulation of DNA in mesenchymal cells. However, it is widely recognized that both full HGF and dHGF have similar biological activities. Most experimental animal studies that examine the therapeutic effects of HGF in various diseases have been conducted using recombinant human dHGF. Moreover, clinical trials of recombinant HGF for the treatment of kidney disease and amyotrophic lateral sclerosis are currently in process using recombinant dHGF. Considering the advantage of the existence of a GMP-compatible product, we anticipate the clinical application of dHGF for the treatment of vocal fold scars.

The current study indicates a comparable activity of dHGF as full HGF. A dHGF injection to scarred vocal folds resulted in improvement of PTP, mucosal wave, and glottis competence. PTP is a marker that indicates the viscoelasticity of the tissues, because stiffer tissues require more pressure to be vibrated. Reduction of PTP means restoration of pliability of the mucosa. Consequently, the mucosal wave amplitude tended to become larger with complete glottic closure, which is similar to normal vocal fold vibrations. Only the dHGF group showed a statistically significant difference in glottic closure (NGG) as compared to the sham group.

The histological examinations demonstrated restoration of HA and reduction of collagen deposition in both the dHGF and full HGF groups. Because HA is regarded as a critical molecule to maintain ideal viscoelasticity of the vocal folds, restoration of HA is thought to be important to recover mucosal vibratory potency. There was a trend that dHGF induced more HA deposition as compared to full HGF, although there was no significant difference between the two HGFs, probably because of the small number of animals used in this study.

The current results are encouraging because dHGF generated at least the same power of regeneration of vocal fold scars as full HGF. dHGF shows promise for use in human clinical trials in patients with vocal fold scars using the GMP product currently used in other clinical trials.

CONCLUSION

dHGF showed a comparable potential for regenerative effects on vocal fold scarring as full HGF in terms of improvement of phonation threshold pressure, mucosal wave amplitude, glottis competency, as well as at the histological level. It is highly desirable to confirm the efficacy of dHGF as a GMP product in human clinical trials for patients with vocal fold scars.

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