Injection of Human Mesenchymal Stem Cells Improves Healing of Vocal Folds after Scar Excision—A Xenograft Analysis

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Objectives: Using a xenograft model the aim was to analyze if injection of human mesenchymal stem cells (hMSC) into the rabbit vocal fold (VF), after excision of an established scar, can improve the functional healing of the VF.

Study Design: Prospective design with an experimental xenograft model.

Methods: The VFs of 12 New Zealand rabbits were injured by a bilateral localized resection. After 9 weeks the scar after the resection was excised and hMSC were injected into the VFs. After another 10 weeks 10 VFs were dissected and stained for histology. Lamina propria thickness and relative content of collagen type I were measured. Viscoelasticity of 14 VFs at phonatory frequencies was quantified by a simple-shear rheometer. The hMSC survival was determined using a human DNA specific reference probe, that is, FISH analysis.

Results: The viscoelastic measurements, that is, dynamic viscosity and elastic shear modulus for the hMSC-treated VFs, were found to be similar to those of normal controls and were significantly lower than those of untreated controls (P < .05). A significant reduction in lamina propria thickness was also shown for the hMSC treated VFs compared with the untreated VFs (P < .05). This histologic finding corresponded with the viscoelastic results. No hMSC survived 10 weeks after the injection.

Conclusions: Human mesenchymal stem cells injected into the rabbit VF following the excision of a chronic scar, were found to enhance the functional healing of the VF with reduced lamina propria thickness and restored viscoelastic shear properties.

Key Words: Vocal fold, scar, viscoelasticity, mesenchymal, stem cells.

Level of Evidence: 5

INTRODUCTION

Tissue defects in the vocal fold (VF) caused by trauma, surgical procedures, or postradiotherapy, often heal with scar formation. The scar tissue causes stiffness of the lamina propria rendering disturbed viscoelastic properties in the VF. A scarred VF causes severe voice problems. Treatment is difficult, and besides voice therapy, injections are used for augmentation of the VF scar defects. Different injectable substances have been used, for example, human/bovine collagen, autologous fat, hyaluronic acid and autologous implantation of fascia. Tissue engineering approaches, such as injection of hepatocyte growth factor and autologous fibroblasts, have shown improved viscoelastic properties/vibratory characteristics in scarred VFs, but presently there are no effective methods to prevent scarring of VF defects or to heal VF scars.

Human mesenchymal stem cells (hMSCs) have a multilineage potential to develop into myelosupportive tissue, muscle, cartilage, and bone. Autologous MSCs injected in scarred canine and rat VFs have been shown to differentiate into epithelial and muscle cells and macroscopically heal the VFs well. hMSCs have also been shown to have immunomodulative properties reducing the inflammatory response of lymphocytes and T cells. In two previous xenograft studies using hMSCs injected in injured VFs of rabbits, we found a survival rate of the hMSCs, engraftment, of 0.18% after 1 month and no survival of hMSCs after 3 months. Both studies also showed that the VFs treated with hMSCs gained significantly improved viscoelastic function, measured as dynamic viscosity and elastic modulus compared with...
untreated VFs. The relative amount of collagen type I and lamina propria (Lp) thickness as a measurement of Lp scarring were significantly reduced for the hMSC-treated VFs compared to the untreated VFs and the improvements were sustainable over 3 months. Using a xenograft rabbit model, the aim of this study was to analyze if the results in our previous studies showing improved healing of injured VFs after an injection of hMSCs into the injured VFs are transferable to a clinical-like situation of excising an established scar followed by an immediate injection of hMSCs into the scar-excised VF.19

MATERIALS AND METHODS

The principal study design has been used by several investigators.2,7,5,12,17

United States and Swedish principles and protocols of laboratory animal care were followed. The experiment was approved by the local ethics committee of the Karolinska Institute, Sweden. Twelve female New Zealand white rabbits (body weight [bw] 3.0–4.0 kg) were used in the experiment. Additional data for five normal VFs were collected from a data bank from earlier experiments and were used in the histologic analyses.

VF Scarring and Scar Excision

Ten animals were anesthetized for each procedure. Two animals remained untreated and were used as normal controls in the rheology measurements.

After premedication with glycopyrolate (0.1 mg/kg s.c.) and Hypnorm® (fentanyl citrate 0.3 mg/mL mixed with fluanisonum 10 mg/mL, 0.3 mL/kg i.m., Janssen Pharmaceutica, Beerse, Belgium) the animals were anesthetized with diazepam (2 mg/kg i.v.). The laryngeal structures and the mobility of the cricoarytenoid joints of the animals were anesthetized with diazepam (2 mg/kg i.v.). The laryngeal structures and the mobility of the cricoarytenoid joints were found normal at examination by means of a modified 4.0-mm pediatric laryngoscope (model 8576E, Karl Storz Endoscope, Tutlingen, Germany) and a Storz-Hopkins 0° 2.7-mm rigid endoscope (model 7218A). A digital video recorded on a computer was made of the VFs before and after the scarification and the scar excision procedure (Richard Wolf video camera No 5512 and a Canopus ADVC100 digital video converter, Reading, UK). The scarring procedure was performed with a 1.5-mm microcup forceps (MicroFrance Medtronic, Düsseldorf Germany) excising the mucosa (Lp) and the superficial layer of the thyroarytenoid muscle. After 9 weeks a scar excision procedure was performed with a 2-mm microcup forceps (MicroFrance Medtronic, Düsseldorf, Germany) excising all visible scarred tissue. All 12 animals were sacrificed after another 10 weeks. Twenty VFs were operated upon and the remaining four VFs were left as normal controls together with the five VFs from the data bank, that is, total n = 24 + 5.

hMSC Preparation and Characterization

hMSCs were isolated and expanded from bone marrow (BM) taken from the iliac crest of healthy volunteers as previously described.16,17 Heparinized BM was mixed with a double volume of phosphate-buffered saline (PBS), centrifuged at 900 x g, resuspended, and layered over a Percoll gradient (1.073 g/mL; Sigma-Aldrich, St. Louis, MO). The mononuclear cells were collected from the interface, washed, and resuspended in HMSC medium consisting of Dulbecco’s modified Eagle’s medium-low glucose (DMEM-LG) (Life Technologies, Gaithersburg, MD), supplemented with 10% fetal bovine serum (FBS) (Sigma-Aldrich) and 1% antibiotic–antimycotic solution (Life Technologies). The serum lot was selected on the basis of optimal cell growth and differentiation. The cells were plated in culture flasks (Becton Dickinson Biosciences, Bedford, MA) at a density of 160,000 cells/cm². Nonadherent cells were removed after 48 to 72 hours and the adherent cells were cultured. When >70% confluence was reached, the cells were detached by trypsin and ethylenediamine-tetraacetic (EDTA) (GibcoBRL, Grand Island, NY) and replated at a density of 4,000 cells/cm². The cells were harvested in passage five and classified as MSCs based on their ability to differentiate into fat, bone, and cartilage and by flow cytometric analyses (positive to CD73, CD90, CD105, and CD80).
Immunohistochemistry for Collagen Type I Staining

Staining was performed as previously described. Briefly, slides were deparaffinized in xylene, rehydrated in alcohol, and blocked in PBS containing 3% bovine serum albumin (BSA). Slides were incubated with a primary antibody (antibody 6308, Abcam, Cambridge, UK), followed by incubation with a secondary antibody (no. A21127 Jackson ImmunoResearch labs Inc., West Grove, PA). Sections were rehydrated in ethanol and xylene and mounted with Vectashield containing DAPI (Vector Labs Inc., Burlingame, CA). The relative contents of collagen type I in the VFs were measured from the digitized stains after a color filtering and normalization process with Photoshop (version 8.0) and a custom-made software (Hans Larsson, Karolinska Institute, Department of Logopedics and Phoniatrics).

Lp Thickness

Measurements of the Lp thickness were made on the digitized hematoxylin-eosin image of each sample (custom-made software Hans Larsson, Karolinska Institute, software Image Pro Plus® version 3.0 Media Cybernetics). The thickest parts of the Lp were measured at three spots for each VF. If a tendency of polyp formation was seen, six measure points including the polyp were used. All measurement values were then used in the statistical evaluation.

The Lp of two of the hMSC-treated VF were damaged in the preparation processes and were left out in the Lp thickness measurements, resulting in n = 5 for hMSC-treated, n = 3 for untreated (scar + NaCl), and n = 5 for normal VFs.

Verhoeff Staining

Verhoeff staining was performed to detect elastin. The slides were deparaffinized and rehydrated as above and incubated in Verhoeff solution, containing haematoxylin, ferric chloride, and potassium iodine, for 1 hour at room temperature. Then differentiated in 2% aqueous ferric chloride for 2 minutes and subsequently treated with 5% sodium thiosulphate for 1 minute and counterstained with Van Gieson solution (HT254, Sigma-Aldrich, Inc.). Finally, the slides were dehydrated, mounted, and digitized as described for the collagen staining.

Alcian Blue Staining

Alcian blue staining was performed to detect mucopolysaccharides and glycosaminoglycans, for example, hyaluronic acid. The slides were deparaffinized and rehydrated in series of alcohol and incubated in alcin blue stain (Alcian blue kit, #SS012, BioGenex, CA), for 1 hour at room temperature and counterstained with nuclear fast red solution for 5 minutes. Finally, the slides were dehydrated, mounted, and digitized as described for the collagen staining.

Fluorescence in situ hybridization—FISH analysis—for persistence of transplanted hMSCs.

Detection of human cells in the VFs was done with a human DNA-specific reference probe, similar to an antibody, linked to a fluorescent molecule, that is, FISH analysis.

The FISH analysis was performed as previously described. Briefly, slides were deparaffinized in xylene and rehydrated in alcohol, followed by pretreatment with pepsin and hybridization overnight at 38°C with the human specific fluorescent probe (Spectrum Red human genomic DNA, Vysis Inc., Burlingame, CA). The detected human cells were counted.

Viscoelastic Measurements

The linear viscoelastic shear properties of VF tissues have been quantified in previous studies. In this study, a controlled-strain, linear, simple-shear rheometer based on the EnduraTEC ElectroForce 3200 mechanical testing system (Bose Corporation, ElectroForce Systems Group, Eden Prairie, MN) was used. The rheometer was capable of empirical measurements of viscoelastic shear properties at phonatory frequencies, following validation previously. In the rheometer, a specimen was sandwiched between an upper plate and a lower plate separated by 0.5 to 1.0 mm. A translational displacement of a prescribed amplitude and frequency was applied to the specimen through the upper plate. The shear force resulting from the viscoelastic response of the specimen upon oscillatory shear deformation was detected by a piezoelectric force transducer attached to the lower plate. All rheometric measurements were performed in the linear viscoelastic region (at 1%–2% strain) over the frequency range of 1 to 250 Hz. The elastic shear modulus ($G'$, in Pa) and the dynamic viscosity ($\eta'$, in Pa · s) were derived as functions of frequency according to the theory of linear viscoelasticity. The elastic (storage) modulus ($G'$) represents a measure of the specimen's stiffness under shear deformation, whereas the dynamic viscosity is a measure of the specimen's resistance to shear flow, or energy dissipation.

Measurements of $G'$ (Pa) and $\eta'$ (Pa · s) as functions of frequency, $f$ (in Hz) were plotted in log-log scale as shown in Figure 1a and b. Curve-fitting regression was then performed.
for each curve to examine the relationships between $G'$ and $f$ and between $\eta'$ and $f$. The linear model was used for both $G'$ and $\eta'$, that is, $log(G')$ or $log(\eta') = B_0 + B_1 \cdot log(f)$, where $B_0$ and $B_1$ are coefficients of parameterization. The curve-fitting estimations, based on least-squares regression analysis, resulted in highly significant findings using the ANOVA $F$ test in all cases ($P < .001$). The significant values of the $F$ test suggested that the variation explained by the model was not due to chance. Goodness of fit was also estimated by the coefficient of determination, $R^2$. The $R^2$ statistic is a measure of the strength of association between the observed and model-predicted values for both $log(G')$ and $log(\eta')$. The values of $R^2$ were high for each regression model indicating goodness of fit ($R^2 > .86$ for $log(G')$ and $R^2 > .96$ for $log(\eta')$).

**Statistical Analyses**

For the histologic measurements each single value was included when differences between the various groups were estimated. Differences between two groups were assessed using Mann–Whitney $U$ test. Calculations whether or not, the dynamic viscosity and the elastic modulus, respectively, differed between normal VFs, hMSCs treated VFs, and untreated scarred controls, were performed with the binomial test. In regression analysis the $F$ test was used. When a direction of the difference between two samples could be predicted as to their means, that is, the mean of a sample 1 was “greater than” or “less than” that of sample 2, the test was one tailed, otherwise it was two tailed. Statistical significance was considered when $P < .05$.

**RESULTS**

**Viscoelastic Analyses**

**Dynamic viscosity, $\eta'$ (Pa $\cdot$ s).** Scarring significantly increased the tissue dynamic viscosity compared to normal VFs ($P = .01$). Treatment with hMSC significantly decreased the dynamic viscosity compared with the untreated scarred controls (scar + NaCl) ($P = .03$), and hMSC-treated VFs were not significantly different from the unscared controls, that is, normal VFs ($P = .2$) (Fig. 1a).

**Elastic shear modulus, $G'$ (Pa).** Scarring also significantly increased the tissue elastic modulus indicating stiffer VFs, compared to normal VFs ($P = .003$). Treatment with hMSCs significantly decreased the elastic modulus in comparison with the untreated scarred controls (scar + NaCl) ($P < .001$). No significant difference was found between the hMSC-treated VFs and the unscared controls, that is, normal VFs ($P = .7$) (Fig. 2).

**FISH Analysis for Persistence of Transplanted hMSCs**

No hMSCs were detected in any of the treated VFs according to FISH analysis.

**Histologic Analyses**

**Lp thickness.** A significant reduction in Lp thickness was shown between hMSC-treated VFs and untreated VFs (scar + NaCl) ($P = .01$). There was no significant difference between hMSC-treated VFs and normal VFs. A significant difference was shown between normal VFs and untreated VFs (scar + NaCl) ($P * < .001$) (Fig. 2).

**Collagen type I staining.** No significant reduction in the relative content of collagen type I was found between hMSC-treated VFs and untreated VFs. But neither were there any significant differences between hMSC-treated VFs and normal VFs. Normal VFs showed significantly lower relative collagen type I compared with untreated VFs ($P < .05$).

**Hematoxylin-eosin staining.** The VFs were characterized into four categories depending on the grade of scarring, that is, fibrosis. Grade A showed no or minimal signs of fibrosis. Grade B showed a focal or noncompact fibrosis in the Lp or superficial vocal muscle. Grade C showed a more compact fibrosis in the Lp and superficial muscle, and Grade D a compact fibrosis in Lp and superficial muscle as well as fibrosis in the deeper part of the vocal muscle (Fig. 3).

The untreated VFs were placed in group C and D, one in group B ($n = 3$). One of the hMSC ($n = 7$)-treated VF was placed in group C, none in group D. When the A and B groups were compared with the C and D groups, there was a clear tendency, although not significant, that the hMSC-treated VFs were placed in the A and B groups and the untreated VFs in the C and D groups.

**Verhoeff Staining**

The stainings were judged visually and blindly for each sample. The differences in elastin content between the groups were minute and without significance. However, the VFs treated with hMSC showed a normal distribution or a tendency to slightly increased elastin content in both the Lp and the superficial muscle. In the untreated VFs, samples of both increased and reduced elastin content were seen.

**Alcian Blue Staining**

The VFs were analyzed by Alcian blue similar to the Verhoeff stainings. Alcian blue stained hyaluronic acid was distributed in a very scarce patchy manner in the Lp and the superficial muscle of the normal VF.
VFs treated with hMSC showed a tendency of increased amount and distributed mainly anteriorly and near the cartilage. The untreated VFs showed almost no hyaluronic acid, and if present, it was found at the anterior or posterior end of the VF.

DISCUSSION

The results were consistent with those of our earlier 1-month and 3-month studies, and show that hMSC injected in an injured rabbit VF, improve the functional healing process with significantly improved histologic and viscoelastic properties of the VF. The present study shows that the results are also applicable after a scar excision has been made. The results indicate that the method of excising a scar and thereafter injecting hMSC in the VF in order to restore its viscoelastic function has the potential to work in a clinical setting. Moreover, with the results from the earlier 3-month study, the improved healing can be expected to be sustainable over time.

The hMSC did not survive 10 weeks in the VFs of rabbits. This is interpreted as a positive finding as it means that no hMSC lingered in the VF after the healing process was completed. If transferable to a clinical setting this is advantageous as it is preferable that no potent cells remain in the tissue after healing. This, however, needs to be shown also in an autologous environment.

hMSCs have been shown to be immunosuppressive, reducing lymphocyte proliferation and formation of cytotoxic T cells, as well as enhancing the secretion of

![Fig. 3. Representative hematoxylin-eosin stainings of (1) normal vocal fold (VF), showing none, or to the left minimal loose connective tissue superficially under the lamina propria (Lp). Some inflammatory cells in the space of Lp, seen as black dots (group a). (2) Human mesenchymal stem cells-treated VF, showing loose connective tissue that tends to split up some superficial muscle (Sm) bundles but deep muscle (Dm) is unaffected. Very few inflammatory cells (group b). (3) Untreated VF (scar + NaCl) showing compact connective tissue/fibrosis/ in deep Lp expanding into Sm and down into the Dm. Extensive amount of inflammatory cells deep into Sm and Dm. The Group, a, b, and c, refer to grade of fibrosis classification described in the text. Arrows mark the border between Lp and Sm (10× magnifications, scale bar 1 mm).](image)

![Fig. 4. Human mesenchymal stem cell-treated vocal fold with a damaged part of the epithelium and lamina propria (damaged in the preparation). (a) Verhoeff staining for elastin shows an extremely thick Lp rich in grayish elastin fibers extending down into the superficial muscle (Sm), but no fibrosis is seen. Deep muscle (Dm) is unaffected. (b) The same VF stained with Alcian blue for hyaluronic acid. The bluish hyaluronic acid is richly distributed in the Lp and the superficial part of the Sm. Arrows mark the border between Lp and Sm. Arrowhead marks lamina flava posterior. Dark blue is reference colored cartilage.](image)
CONCLUSIONS

A reduced inflammatory response induced by the hMSC seems to have contributed to the improved healing as measured by a significant reduction of Lp thickness indicating reduced fibrosis. A lower grade of fibrosis was also seen in the histologic pictures of the hMSC-treated VFs compared to the untreated controls. The hMSC-treated VFs when histologically classified tended to be placed in the groups with the least fibrosis when a four-grade scale of classification was used. The scarred nontreated controls tended to be placed in the groups with most fibrosis.

One of the two hMSC-treated VFs that was excluded in the Lp thickness measurements due to the Lp being partially split up in the preparation process, showed by Verhoeff staining, extensive elastin production in the Lp and also some in the superficial vocal muscle. In the Alcian blue staining this VF was also found to be rich in hyaluronic acid both in the Lp and the superficial part of the vocal muscle. The VF, however, showed promising viscoelastic shear properties. This might be due to the hMSCs having stimulated the creation of a structured scaffold, in which elastin is a part, in the injured Lp. The hMSC could also enhance the functional healing of the VF with reduced Lp thickness indicating reduced fibrosis.

In this study immunosuppression was used to reduce the host-versus-graft reaction. This may have influenced the inflammatory reaction and also affected the hMSCs. In a study comparing the healing process with and without the calcineurin inhibitor tacrolimus, we did not find any improved healing with immunosuppressant alone (unpublished data). If the immunosuppressant has had any effect on the hMSC, it is reasonable to believe it has been negative. If so, the hMSCs could have the potential to show even further improved results in an autologous environment.

CONCLUSIONS

The hMSCs, when injected into the scarred rabbit VF after a scar excision has been made, were shown to enhance the functional healing of the VF with reduced Lp thickness and restored viscoelastic properties of the VF. The injected hMSCs did not seem to survive for 10 weeks in the rabbit VF.

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BIBLIOGRAPHY