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The Ability of Conditioned Media From Stem Cells to Repair Vocal Fold Injuries

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INTRODUCTION
Vocal fold scarring after injury can cause voice problems. No definite treatment for vocal fold scarring has been established, although a number of treatments have been attempted, including voice therapy, replacement of the extracellular matrix (ECM) of the vocal fold, a tissue-engineering approach using several types of cells and growth factors, and new surgical techniques.1–5 However, the ECM does not return to normal after replacement. Therefore, the prevention of scar formation is important in vocal fold wound healing.6

Mesenchymal stem cells (MSC) are widely used in regeneration medicine. MSC have several advantages: for example, they can be harvested from various sites and are hypoimmunogenic. Many studies have reported that MSC improves the healing of injured vocal folds.7–12 Previous studies have revealed that human nasal inferior turbinate-derived mesenchymal stem cells (hTMSC) exhibit MSC-like features.13,14 However, stem cell therapy has potential limitations, such as an immunological response, high cost, persistence, cell-handling issues, and malignant transformation.15,16

Recently, conditioned media (CM) has emerged as a treatment candidate for overcoming these limitations and a replacement for stem cell therapy. This concept is based on increasing evidence that the therapeutic ability of stem cells is mainly mediated by CM.17 This study investigated whether the injection of hypoxia-induced 25-fold concentrated conditioned media (hCM) from hTMSC leads to regeneration in injured vocal folds and compared the effects of hCM to those of hTMSC. Quantitative real-time polymerase chain reaction (PCR) and histologic examination were used to analyze the early phase of wound healing in animal xenograft models.
**MATERIALS AND METHODS**

**Isolation and Culture of hTMSC**

The isolation and culture of hTMSC were performed as described previously. The procedure was approved by the institutional review board of Daejeon St. Mary's Hospital, Catholic University of Korea (DC15TISI0022). With the informed consent of each patient, inferior turbinate tissues discarded after septoplasty and partial turbinectomy were obtained. Tissue was obtained from a patient with a history of nasal polyp surgery. Tissue fragments were minced to 1 cm and then grown under hypoxic conditions (5% CO2) for 24 hours. The medium was then concentrated 25-fold according to a previously introduced method using centrifugation and ultrafiltration units (Millipore, Bedford, MA) with a 3-kDa cutoff. Concentrated CM, obtained under either normoxic or hypoxic culturing conditions, are herein termed normoxia-conditioned media and hypoxia-conditioned media (hCM), respectively. Each medium was stored at −80°C until use.

**Hypoxic Conditioned Media From hTMSC**

hTMSC at passage 3 were seeded at 1 × 10^6 cells in 100-mm dishes and then grown at 37°C to confluency. After serum deprivation, hTMSC was cultured under either normoxic or hypoxic conditions. Then, the medium was replaced by growth medium for proliferation experiments. Following the designated incubation time interval, 10 μL of MTT reagent was added to each well and incubated for 4 hours. Finally, optical density measures were evaluated at 450 nm.

**Cell Proliferation Assay**

The proliferation of hTMSC was measured by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay using an Ez-Cytox Cell Viability Assay Kit (DoGen, Seoul, Republic of Korea) according to the manufacturer’s instructions. Cells were seeded in 96-well flat-bottomed plates at a concentration of 1 × 10^4 cells per well in serum-free medium and kept for 24 hours under normoxic or hypoxic conditions. Then, the medium was replaced by growth medium for proliferation experiments. Following the designated incubation time interval, 10 μL of MTT reagent was added to each well and incubated for 4 hours. Finally, optical density measures were evaluated at 450 nm.

**Western Blot Analysis**

hTMSC (passages 3–5) was washed three times with ice-cold PBS and then treated with radiolabeled proteins. The proliferation of hTMSC was measured by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay using an Ez-Cytox Cell Viability Assay Kit (DoGen, Seoul, Republic of Korea) according to the manufacturer’s instructions. Cells were seeded in 96-well flat-bottomed plates at a concentration of 1 × 10^4 cells per well in serum-free medium and kept for 24 hours under normoxic or hypoxic conditions. Then, the medium was replaced by growth medium for proliferation experiments. Following the designated incubation time interval, 10 μL of MTT reagent was added to each well and incubated for 4 hours. Finally, optical density measures were evaluated at 450 nm.
buffer (Elpis Biotech, Daejeon, Korea) containing protease inhibitor cocktail tablets (Roche Diagnostics GmbH, Mannheim, Germany). The samples were centrifuged at 13 thousand revolutions per minute for 15 minutes at 4°C, and then the supernatants were collected as whole-cell lysates. Protein concentrations were estimated using the bicinchoninic acid assay (Pierce, Rockford, IL) with a bovine serum albumin standard. Equal amounts of protein (20 μg) were separated by electrophoresis on a 10% sodium dodecyl sulfate-polyacrylamide gel and transferred electrophoretically onto a nitrocellulose membrane (Pall Corp., Ann Arbor, MI). After blocking with 5% nonfat powdered milk, the membrane was incubated overnight at 4°C with primary antibodies directed against hypoxia-inducible factor (HIF)-1α (Abcam, Cambridge, MA) and vascular endothelial growth factor (VEGF) (Abcam) and then incubated with horseradish peroxidase-conjugated anti-rabbit IgG (Santa Cruz Biotechnology, Santa Cruz, CA). Positive bands were detected and analyzed by chemiluminescence technology using ChemiDoc XRS + (Bio-Rad Laboratories, Hercules, CA). As loading controls, each membrane was probed with glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (Cell Signaling Technology, Beverly, MA).

Animal Experiments

This study was approved by the Animal Ethics Committee of Catholic University of Korea (permit no. CMCDJ-AP-2016-005). The animals were cared for in accordance with our established institutional guidelines.

In total, 60 Sprague-Dawley rats were used in this study, with 12 rats in each of the two experimental groups (the hCM group and the hTMSC group) and 12 rats in each of the two control groups (the media group and the PBS group). Three rats in

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[Figures and tables described in the text are not included, as the natural text representation is focused on the content and context rather than the visual elements.]
each group were used for histologic examination at 3 weeks after injury. All animals were anesthetized via an intraperitoneal injection of ketamine hydrochloride (100 mg/kg) and xylazine hydrochloride (10 mg/kg) and then placed in a semi-vertical position on a custom-made platform. The larynx was visualized with a pediatric endoscope. The right-side vocal folds were injured with microscissors until the thyroarytenoid muscle was exposed. This surgical procedure was described previously.18 The left vocal fold remained untreated (the normal group).

All injections were performed using a syringe equipped with a 30-gauge long needle under direct vision employing a pediatric endoscope. The right-side vocal folds were injured with microscissors until the thyroarytenoid muscle was exposed. This surgical procedure was described previously.18 The left vocal fold remained untreated (the normal group).

RNA Isolation and Real-Time PCR

Vocal fold specimens were dissected away from the larynx and homogenized with TissueLyser II (Qiagen, Valencia, CA). Total RNA was isolated using TRizol reagent (Invitrogen, Carlsbad, CA) and treated with ribonuclease-free deoxyribonuclease I (Qiagen) to minimize contamination from genomic DNA. The quantity of total RNA was determined using a NanoDrop ND-1000 spectrophotometer (NanoDrop, Wilmington, DE). Reverse transcription was performed using Reverse Transcriptase Premix (Elpis Biotech) according to the manufacturer’s recommended reaction protocol. Reactions were performed with a Veriti 96-well Thermal Cycler (Applied Biosystems, Forster City, CA) using the following parameters: 25°C for 10 minutes, 37°C for 60 minutes, 94°C for 5 seconds, and 4°C for 5 minutes. Specific primer sequences are displayed in Table I. Real-time PCR was performed using ABI 7500 FAST (Applied Biosystems) in a 20-μL reaction mixture containing Power SYBR Green PCR Master Mix (Applied Biosystems), 500 nM forward and reverse primers, and 1 μL of cDNA. The real-time PCR protocol was executed for

Fig. 3. The mRNA expression ratios of several genes involved in the organization of vocal folds in the normal, PBS, and hTMSC groups on postinjury day 7. hTMSC = human nasal inferior turbinate-derived mesenchymal stem cells; PBS = phosphate-buffered saline.
40 cycles, each of which consisted of denaturation at 95°C for 15 seconds and annealing at 60°C for 60 seconds. In the initial cycle, samples were incubated at 95°C for 10 minutes. Triplicate PCRs were run, and GAPDH mRNA expression was analyzed for each sample for normalization.

Histopathological and Immunohistochemical Examination

Vocal fold specimens from each group were harvested for histological analysis at 3 weeks after injury. Specimens were embedded in paraffin blocks and then sectioned at 4 μm using a microtome. Next, slides in each group were stained with hematoxylin and eosin, Masson’s trichrome (for collagen), or alcian blue (for hyaluronic acid (HA)) using standard pathology department protocols. The slides were analyzed by light microscopy, and a blinded examiner measured collagen and HA densities using the ImageJ software (National Institutes of Health, Bethesda, MD) for the quantification of histological images.

Statistics

All statistical analyses were performed with the aid of the SPSS 18.0 for Windows (SPSS Inc., IBM Corp., Armonk, NY). The Mann-Whitney test was used to explore the significance of differences between two groups, and the Kruskal-Wallis test was employed to compare more than three groups. A P value less than 0.05 was considered statistically significant. All results are expressed as means ± standard deviations.

RESULTS

Hypoxia-Induced Cell Proliferation

hTMSC were cultured under both normoxic and hypoxic conditions, and the proliferation rates and HIF-1α and VEGF protein levels were compared. The hTMSC proliferated about 1.23 times more under hypoxic (1% O2) than normoxic conditions, and the difference was significant.

Fig. 4. Comparison of mRNA expression ratios between the hCM and hTMSC groups on postinjury day 7. hCM = hypoxia-induced conditioned media (25-fold) group; hTMSC = human nasal inferior turbinate-derived mesenchymal stem cell group; M = media (25-fold) group; PBS = phosphate-buffered saline group.
Western blotting demonstrated that the hypoxic condition upregulated the HIF-1α and VEGF protein levels in hTMSC (Fig. 1).

**Gene Expression in the hCM Group at 1 Week After Injury**

The PCR results showed that the expression levels of mRNAs encoding hyaluronan synthase (HAS) 1, HAS 2, hepatocyte growth factor (HGF), and fibroblast growth factor (FGF) 2 were significantly upregulated in the hCM group compared with the normal and media groups. The gene encoding HAS 3 was upregulated compared with the media group but not the normal group. The gene encoding transforming growth factor (TGF)-β1 was significantly upregulated in the hCM group compared with the normal group but not the media group. The gene encoding procollagen type III (COL III) was significantly downregulated in the hCM group compared with the media group, whereas the gene encoding fibronectin (FN) was upregulated in the media group compared with the normal group. However, there was no difference between the hCM group and the normal group (Fig. 2).

**Gene Expression Analysis: Comparison of the hCM and hTMSC Groups at 1 Week After Injury**

In the hTMSC group, the mRNA expression levels of HAS 1, HAS 2, HAS 3, HGF, and FGF 2 were significantly upregulated compared with the normal and PBS groups.
**TGF-β1** was significantly upregulated in the hTMSC group compared with the normal group but not the PBS group. **COL III** mRNA expression was significantly upregulated in the PBS group compared with the normal and hTMSC groups. **FN** mRNA expression was upregulated in the PBS group compared with the normal group but not the hTMSC group (Fig. 3). There was no difference between the hCM and hTMSC groups in the mRNA expression of **HAS 1, HAS 2, HAS 3, COL III, FN, TGF-β1, FGF 2,** and **HGF** (Fig. 4).

**Gene Expression Analysis of the hCM and hTMSC Groups at 2 Weeks After Injury**

There were no significant differences between the hCM, media, and PBS groups in the mRNA expression levels of **HAS 1, HAS 2, HAS 3, COL III, FN, TGF-β1, FGF 2,** and **HGF**. However, a trend toward increasing mRNA expression of **HAS 1, HAS 2,** and **HAS 3** was observed in the hCM group (Fig. 5).

PCR at 2 weeks after injury showed similar results in the hTMSC and PBS groups compared with the hCM and media groups. Also, there were no differences between the hCM and hTMSC groups in the mRNA expression of **HAS 1, HAS 2, HAS 3, COL III, FN, TGF-β1, FGF 2,** and **HGF** (Fig. 6).

**Histological Examination**

Histological analysis was performed at 3 weeks after injury. Densitometry results from histology showed that the hCM and hTMSC groups exhibited better antifibrotic effects than the media group. Mason’s trichrome staining showed that collagen deposition was significantly reduced in the hCM and hTMSC groups compared to the media group. The hCM group
also had less collagen deposition compared to the hTMSC group. HA was detected by alcian blue staining. The hCM and hTMSC groups had significantly increased HA deposition compared to the media group. There was no significant difference between the hCM and hTMSC groups (Fig. 7).

**DISCUSSION**

Preventing vocal fold scarring remains an unsolved problem. Many attempts have been made to reduce vocal fold fibrosis after injury. Recently, stem cell therapy has been widely used in regenerative medicine. Since autologous bone marrow-derived stem cells were first implanted into the injured vocal folds of dogs by Kanemaru et al., various kinds of stem cells such as adipose tissue-derived stem cells and embryonic stem cells have been reported to be effective in vocal fold regeneration after injury. Other studies have reported short survival times of implanted stem cells without any differentiation and engraftment. The implanted stem cells seemed to exert their regenerative effects through a paracrine mechanism. Our previous study on vocal regeneration with hTMSC also supports a paracrine mechanism.

The regeneration mechanism for implanted stem cells in injured vocal folds has not been fully explained. Some studies have reported that implanted stem cells survived for more than 1 month and suggested that improved vocal fold healing was due to stem cell differentiation. Other studies have reported short survival times of implanted stem cells without any differentiation and engraftment. The implanted stem cells seemed to exert their regenerative effects through a paracrine mechanism. Our previous study on vocal regeneration with hTMSC also supports a paracrine mechanism.

The injection of CM without stem cells has been studied in various contexts but not in terms of vocal fold regeneration. As far as we know, this study is the first report on the injection of CM alone in the treatment of injured vocal folds in an animal model.

In this study, the secretion of paracrine factors by hTMSC was enhanced by hypoxic culture conditions and concentration. Hypoxic culture conditions have been reported to upregulate HIF-1α and cell proliferation, and increased HIF-1α protein expression has been associated with an increased expression of paracrine factors in stem cells. In this study, hypoxia-induced CM containing paracrine factors were concentrated about 25-fold (hCM). A previous study revealed that the survival period of hTMSC transplanted to injured vocal folds was more than 2 weeks but not more than 1 month. Therefore, transplanted stem cells are believed to exhibit a paracrine effect continuously for at least 2 weeks. However, the duration of the effect of hCM injected into the vocal folds is limited due
to diffusion and decreasing concentrations. To solve this problem, in this study the hCM were injected twice a week.

In this study, PCR showed that the results of hCM injection twice in the first week after vocal fold injury were equivalent to those of the transplantation of hTMSC at 1 week after injury. At 2 weeks after injury, PCR showed no significant difference between the experimental groups (hCM and hTMSC groups) and the control groups (media and PBS groups). Therefore, for at least 2 weeks after injury, the hCM group was equivalent to the hTMSC group in mRNA expression.

The fact that host immunity was not controlled could be a limitation of this study. Some previous studies controlled host immunity to transplanted xenograft stem cells and reported that the implanted stem cells survived for more than 4 weeks.9,19,30 Host immunity might affect the survival of implanted xenograft stem cells.24 The survival period of implanted stem cells also could affect the regeneration of injured vocal folds.31 Therefore, additional studies are needed to investigate how host immunity influences the survival of implanted xenograft stem cells and to compare the results of stem cell implantation and hCM in an immune-controlled host. The concentration technique using an ultrafiltration unit with a 3-kDa cutoff could be another limitation. Several studies have applied a technique for concentrating CM using ultrafiltration units.16,32,33 Most materials in CM, such as growth factors, cytokines, hormones, and genetic materials, could be concentrated. However, materials under the 3-kDa cutoff, such as amino acids and nucleotides, were removed and not concentrated. Further studies are needed to clarify the function of materials under 3-kDa.

CONCLUSION

The injection of hCM into injured vocal folds twice per week produced an antibioretic effect in the early phase of wound healing. The results were equivalent to those with hTMSC for up to 2 weeks. These results provide a foundation for future clinical use of hCM for vocal fold regeneration.

BIBLIOGRAPHY