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Small intestine submucosa and mesenchymal stem cells composite gel for scarless vocal fold regeneration

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ABSTRACT

The purpose of this study is to demonstrate scarless vocal fold (VF) regeneration by using a composite gel composed of small intestine submucosa (SIS) and mesenchymal stem cells (MSCs). A scar was made with an electrocoagulator on both VFs in 24 rabbits, followed by injection of either MSCs, SIS, or MSCs-SIS composite gel in the right side VF, while the left side VF was left untreated. VF scars were evaluated with *in vivo* fluorescence live imaging system (IFLIS), endoscopy, histology, and videokymography (VKG) after eight weeks. IFLIS demonstrated that SIS enabled the MSCs to survive and be engrafted in the VF. The histological analysis showed increased hyaluronic acid accumulation and controlled collagen deposition by MSCs-SIS composite gel. VKG analysis showed more favorable vibrations of MSCs-SIS injected VF, compared to other treatment group. In conclusion, the injectable SIS supplied a niche for the MSCs to stably settle down in scarred VFs and helped to regulate ECM synthesis. The ECM remodeling underwent by the surviving MSCs eventually led to the functional improvement of the VF. The results of the present investigation suggest that SIS-MSCs composite gel is a plausible biomaterial for prolonged survival of MSCs in VFs and promotes scarless VF healing.

1. Introduction

Vocal fold (VF) scarring is commonly caused by trauma, exposure to chemical components, glottic infection, or iatrogenically by surgical treatment [1]. VF scarring results in a variety of voice disorders, including hoarseness, loss of vocal control, and vocal fatigue [2]. VF scarring is attributed to a distortion of biomechanical properties of the VF lamina propria (LP) [3]. In the LP of a scarred VF, the components, density, and organization of extracellular matrix (ECM) is remodeled, which eventually leads to the deterioration of VF vibratory function [4]. Current studies have shown that abnormal ECM synthesis, such as excessive collagen accumulation and decrease of elastin as well as hyaluronic acid (HA), was frequently found in various VF scarring animal models [5,6]. Therefore, a possible key point in scarless regeneration of the VF may be control of wound healing through regulation of ECM production.

A biological approach using tissue engineering techniques has been considered as a potential treatment strategy for VF scarring [7,8]. Foremost, one of the most vital components in using tissue engineering for the treatment of VF scarring is the choice of optimal cells, which play a paramount role in proper ECM synthesis [9]. Various cells such as mesenchymal stem cells (MSCs), fibroblasts, or embryonic stem cells have been attempted in cell therapy for wound healing [10,11]. Among these cells, MSCs have the advantages of a low risk of immunologic rejection, control of mechanical properties through ECM synthesis and accumulation, and easy expansion based on the standard culture method [12,13]. For VF regeneration, various MSCs originating from bone marrow, adipose tissue or laryngeal mucosa have been utilized and showed promising results in suppressing the inflammatory response, improvement of viscoelasticity or ECM remodeling [12–14]. In addition to cells, a suitable and injectable scaffold, which functions as a structural support system and delivery vehicle to maintain cell survival and metabolism in scar tissue, is also important. Many researchers have investigated the efficacy of various materials

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including collagen, fibrin or HA in wound healing [15,16], but none have yet shown satisfying results. Small intestine submucosa (SIS), derived from the submucosal layer of porcine intestine, is an acellular, naturally occurring collagenous ECM material [17]. SIS consists of collagen, glycosaminoglycans, fibronectins, chondroitin sulfates, heparins, heparin sulfates and hyaluronic acids, as well as cytokines such as basic fibroblast growth factor (bFGF), transforming growth factor-β (TGF-β), epidermal growth factor (EGF), vascular endothelial growth factor (VEGF) and insulin-like growth factor-1 (IGF-1) [18]. It is well known that SIS plays an important role in tissue regeneration [19,20]. In this study, we intended to investigate whether the injection of MSCs combined with SIS could induce scarless regeneration of VF. We evaluated the therapeutic efficacy of MSCs and the interaction of MSCs and SIS on VF regeneration.

2. Materials and methods

2.1. Isolation and culture of bone marrow derived MSCs

The isolation and culture of bone marrow derived MSCs was performed as described previously [21]. The bone marrow from tibia and femur of New Zealand white rabbits (300 g body weight) was aspirated, washed with PBS and separated through a 100 μm cell strainer to remove tissue debris. The filtered cell suspensions were centrifuged and then the cell pellet was re-suspended in alpha-Minimal Essential Medium supplemented with 10% fetal bovine serum, 100 U/mL penicillin G, and 100 mg/mL streptomycin (all from Gibco, Grand Island, NY, USA). The cells were seeded in culture plates (1.5×10^7 cells per 150 mm dish) and cultured in a monolayer for one week at 37 °C under 5% CO_2. The attached cells were retrieved using 0.05% trypsin-ethylenediaminetetraacetic acid (EDTA) and re-plated at 1.5×10^6 cells per 150 mm dish. The third passage cells were then applied for injection.

2.2. Prepartion of SIS

SIS was prepared using a previously reported method [17,22]. Briefly, sections of porcine jejunum were harvested from market pigs (Finish pig, F1; Land race + Yorkshire, around 100 kg at 6 months) within 4 h of sacrifice. SIS was extracted from the porcine jejunum by removal of fat through mechanical removal of the tunica serosa and tunica muscularis, followed by careful washing with a saline solution. After washing with a saline solution, it was freeze-dried at −80 °C for 48 h using a freeze dryer (FD 8505, Ilshinlab, Daejeon, Korea). The dried SIS was pulverized using a freezer mill (6700, SPEX Inc., USA) at −198 °C to yield SIS powder of 10–20 μm size. The obtained SIS powder was stirred for 48 h in an aqueous solution consisting of 3% acetic acid and 0.1% pepsin, and then neutralized by using sodium bicarbonate. The neutralized solution was followed by freeze-drying to yield the final SIS powders. The obtained SIS powder was sterilized using ethylene oxide (EO) gas. The final SIS suspension was dispersed in phosphate buffered saline (PBS) to yield the concentrations (20 wt%) of SIS.

2.3. Fluorescence labeling and preparation of MSCs with SIS

The attached cells were retrieved using 0.05% trypsin-EDTA and then washed with media. The MSCs were suspended in the Vybrant® DiD cell-labeling solution (Invitrogen, Carlsbad, CA, USA) at a concentration of 1 μL/1×10^5 cells and incubated for 20 min at 37 °C under 5% CO_2. The labeled cells were washed with media and then 2×10^7 cells re-suspended with 20% SIS (W/V). The cell tracing of the labeled MSCs was analyzed using FOBI® *in vivo* fluorescence live imaging system (IFLIS; NeoScience, Seoul, South Korea).

2.4. Animal surgical procedures and injection of biomaterials

All animal care and experimental procedures were approved according to the Guidelines for Animal Experiments of Ajou University. The rabbits were divided into 3 groups of 8 animals according to the injected biomaterial; MSCs (2×10^7 cells) only, SIS only and MSCs (2×10^7 cells) mixed with SIS. The rabbits were anesthetized with tiletamine (8.0 mg/kg; Virbac Ltd., Carros, France), zolazepam (8.0 mg/kg; Virbac Ltd.) and xylazine hydrochloride (1.5 mg/kg; Bayer, Seoul, Korea), and both VFs were fully visualized with a 4 mm rigid endoscope (Karl Storz, Tuttlingen, Germany). A scar was consistently made on each VF with a Bovie electrocoagulator. Then, using a 25G long needle, 50 μL of the respective material was injected into the deep layers of LP of the right VF, while the left VF remained untreated.

2.5. Endoscopic and histologic analysis

Endoscopic examination was performed to analyze the structural change of the VFs and regenerative progress of the injected site 8 weeks postoperatively on anesthetized rabbits.

After the endoscopic examination, animals were sacrificed. The larynges were resected and fixed with 10% formaldehyde solution for 24 h. The constructs were dehydrated, and then embedded in paraffin wax. The embedded samples were sectioned serially at a thickness of 4 μm and stained with hematoxylin-eosin (H&E), alcian blue and Masson's trichrome.

2.6. Tracing of injected MSCs and quantitative analysis of fluorescence-labeled MSCs

To trace the DiD-labeled MSCs, the larynges were embedded in OCT compound for frozen section. The frozen samples were sectioned serially at a thickness of 4 μm and the nucleus were stained with Hoechst 33342 (Invitrogen) as a counterstain. The stained samples were captured using fluorescence microscope (Olympus FV1000, Japan). Three slides were made for each specimen, and DiD-labeled MSCs were counted from three randomly chosen sites from each slide with Metamorph® NX image software (Molecular Devices, Sunnyvale, CA, USA) and then averaged.

2.7. Scar index measurement

Picro-sirius red staining was performed to analyze the fibrosis in the VFs as previously described [23–25]. Birefringence patterns were evaluated under polarized light microscopy to determine collagen density, pattern, and maturation. The scar index as fibrosis index was quantified by comparing the ratio of orange-red to yellow-green staining by means of Metamorph® NX image software.

2.8. Videokymographic analysis

The larynges were acquired for *ex-vivo* analysis of the mucosal wave pattern before histologic examination. The supraglottic tissue of the larynx was removed to retain visual field, and then the arytenoid cartilages were sutured together to close the VFs. The larynx was mounted on the nozzle, through which air passed from the air flow generator to induce VF vibration. The movement of the mucosal waves was recorded through high-speed digital imaging system (NX4-S2, Integrated Design Tools, Tallahassee, FL, USA) at 5000 frames per second (fps) under moisturized conditions. Images of each sample were repeatedly recorded three times and the kymographic result was averaged. The kymograph was automatically produced by Metamorph® NX image software. The amplitude of the VF was analyzed by measuring the image pixels between the opened and closed phases on kymography. All results were normalized relative to the amplitude of the untreated left VF [14,26].

2.9. Statistical analysis

Statistical analysis between the comparative groups was performed with one-way analysis of variance (ANOVA), and specific inter-data differences between mean values were identified using the Tukey-HSD test. Each group's mean value was analyzed with both coefficient of correlation and student *T* test. $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$.

3. Results

3.1. SIS allowed MSCs to survive longer by providing a favorable environment

In this study, we first traced DiD-labeled MSCs to evaluate the precision of the injection and stability in the original injected region of the VF. The rabbits were followed by *in vivo* imaging system FOBI® for up to 8 weeks. The fluorescence- labeled MSCs were observed on the initial injected site in both MSCs with SIS group (maximum intensity: 243) and MSCs only group (maximum intensity: 252) at two days after the injection (Fig. 1A: a–c, B: a–c). Interestingly, they were detected until 3 weeks after the injection in MSCs with SIS groups (maximum intensity: 185), while the signal was not detected in MSCs only group (maximum intensity: 4) (Fig. 1A: d–f, B: d–f). After 8 weeks, *in vivo* fluorescent signal was not observed in any of the groups.

However, on histologic examination, fluorescence of the injected cells could be detected in the tissue specimen even after 8 weeks. The DiD-labeled MSCs were clearly found on the right VF in both injected groups at 8 weeks postoperatively (Figs. 2 and 3), whereas DiD-labeled MSCs was not detected on the contralateral left scarred VF. Furthermore, the injected DiD-labeled MSCs were mainly distributed in the LP of the VF (Fig. 2 b, d; white arrow). Quantification of fluorescence-labeled MSCs demonstrated that a significantly dominant signal was detected in MSCs with SIS (161.17 ± 7.93) than MSCs only group (41.26 ± 11.32) (Fig. 3, $P^{***} < 0.001$, Difference 3.91 fold).