Biomaterials xxx (2013) 1–10

Contents lists available at ScienceDirect

Biomaterials

journal homepage: www.elsevier.com/locate/biomaterials

The use of laryngeal mucosa mesenchymal stem cells for the repair the vocal fold injury

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ARTICLE INFO

Article history:
Received 10 July 2013
Accepted 1 August 2013
Available online xxx

Keywords:
Mesenchymal stem cells
Laryngeal mucosa
Vocal fold
Scar
Epiglottis

ABSTRACT

Stem cell transplantation is a kind of attractive and new approach that complements traditional restorative or surgical techniques for the regeneration of injured or pathologically damaged laryngeal tissues. However, the best cell delivery strategy remains to be identified. The objective of this study was to establish a new strategy to the healing of injured vocal fold, using laryngeal mucosa mesenchymal stem cells differentiating into myofibroblasts or fibroblasts and improving the reconstruction microenvironment in the vocal fold injury as a new alternative as seed cells for laryngeal tissue engineering. After isolation and expansion, cells were identified as adherent mesenchymal cells with substantial proliferation potential in vitro, and were also characterized by flow cytometry. The differentiation potential of mesenchymal cells was maintained during proliferation as confirmed by culturing for adipogenesis, osteogenesis and chondrocyte. When LM-MSC was transplanted into the injured vocal fold, it has the potent differentiated into myofibroblasts and fibroblasts, which could regulate extracellular matrix, block collagen and the fibronectin rapid increased, inhibit the rapidly decrease of elastic fiber and HA, decrease the microenvironment inflammatory reaction, and prevent the formation of vocal fold scar.

1. Introduction

Lesions of vocal fold can usually treated by laser under the micro-scope in current clinical treatment, however, which also makes the laminapropria of vocal fold miss and forms scars [1]. Vocal fold scarring is a major etiology of voice disorders including hoarseness, the loss of vocal control, and fatigue, which alters the normal biological and biomechanical properties of vocal fold laminapropria [2,3]. In this process, the extracellular matrix (ECM) which is of critical importance in maintaining the biological and biomechanical properties of the vocal folds, is remodeled [4,5]. Therefore, it is easy to find that the key to improve the qualities of patients' voicing is to effectively prevent scar formation. As a result, a relatively ideal tissue engineering method which could fast realize the regeneration of ECM thus recovering the ECM microenvironment for the tissue recovery of vocal fold is urgently needed.

Seed cell is an important part of tissue engineering. With the development of stem cells, there are more choices which were available in tissue engineering. However it is still difficult to find the most suitable seed cell in a specific research field, especially for larynx with limited space. The considerable therapeutic potential of mesenchymal stem cells (MSC) has been used in the larynx. At the moment, the mostly used MSC for preventative treatment of vocal scar include bone marrow mesenchymal stem cells (BMSCs) [6,7], muscle-derived stem cells (MDSCs) [8,9], adipose-derived stem cells (ADSCs) [10–13]. But, so far, all the MSCs mentioned above had their own disadvantages so as to be considered as the most suitable seed cell. The main existing difficulty lied in that all those cells were not able to form the original structure in vivo after planting [14]. The exploration for laryngeal stem cells has never been stopped: Masaru et al. revealed the existence of side population cells in the vocal fold [15,16]. Kiminori et al. demonstrated the presence of stellate cells in the adult human vocal fold for the first time [17], which located at maculae flavae of the mucosa of human vocal fold, thought to be involved in the metabolism of

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http://dx.doi.org/10.1016/j.biomaterials.2013.08.004

extra cellular matrix in the vocal fold mucosa and to form the characteristic layered structure of the human vocal fold mucosa. The macula flavae was also considered to be great important in the growth, development and ageing of the vocal fold mucosa [18–23]. Human Laryngeal mesenchymal tissues mainly include the vocal fold and surrounding mucosa tissues. So human vocal fold mesenchymal stem cells (hVF-MSC) were found, which were identified according to the basic regulation of the International Society for Cellular Therapy (ISCT), and they were initially proved that MSC from human vocal fold fibroblasts [24]. Chen et al. also found vocal fold laminapropria ECM will be up-regulated by the use of suitable vocal fold fibroblast (VFF) cell lines in appropriate model systems [25]. Taking the effect of promoting healing of injured vocal fold into consideration, compared with BMSCs, MDSCs and ADSCs, the satellite cells, VF-MSC and VFF had the advantage of reconstructing the most similar structures to the laryngeal tissues as their derivation. It is well known that epiglottis has the same structure with vocal fold, laryngeal mucosa (from epiglottis) instead of vocal fold was that the size of vocal fold was limited and vocal operation would create new scar, all of which were against to apply for laryngeal tissue. At present, no literature has reported that laryngeal mucosa (epiglottis) exist MSC the same as vocal fold [26].

In this study, laryngeal mucosa from epiglottis was got and MSC or MSC like cells were isolated from the “donation area”. And we established a more efficient and reliable method during the procedure of isolating and cultivating LM-MSC to overcome the drawbacks of vocal fold mesenchymal stem cells for laryngeal tissue engineering. The biocharacteristics of LM-MSC were detected including markers for stem cells, colony forming ability, cell proliferation and multi-directional differentiation. The ability of LM-MSC enhancing vocal fold regeneration after trauma and inhabiting the vocal fold scar formation in canine vocal fold trauma model were also studied.

2. Materials and methods

2.1. Surgery

14 canine (2 years old, female, about 10–11 kg) were used from laboratory of animal research center, Fourth Military Medical University. All experimental animals were fed under standard conditions and the experimental protocols were approved by the Animal Ethical Committee of the Fourth Military Medical University. All surgical procedures were performed under general anesthesia with intraperitoneal injection of pentobarbital. One canine was sacrificed and the epiglottis mucosa was obtained from the normal laryngeal mucosa, from which LM-MSC was isolated in vitro. The model of vocal cord wound was established by semiconductor laser resection with the help of laryngoscope, topical spray a small amount of lidocaine, the wound near sit of 2/3 vocal cord membrane, depth of the thyroarytenoid muscle, bilateral symmetry. After the surgery, four canines were used to observe the healing conditions for the bilateral vocal cord such as congestion, edema, surface line irregularity, atrophy and scar formation. The LM-MSC was used to treat the injured vocal fold. Nine canines vocal fold wound were treated by LM-MSCs which were isolated from their “donated area” epiglottis mucosa respectively. when bilateral vocal fold injury models were established successfully, left vocal cord was injected of 0.2 ml LM-MSCs and collagen mixtures (containing about 2×10^6 cells) as stem cell treatment group, while the right vocal cord was injected only 0.2 ml collagen as control group within the same animal. After surgery, all animals were placed under warm light, allowing to recover from the anesthesia, and then housed separately with access to food and water in a colony room maintained at constant temperature (19–22 °C) and humidity (40–50%) on a 12 to 12 h light/dark cycle.

2.2. Cells isolation and culture

The harvested normal laryngeal mucosa were excised and rinsed with PBS containing 1% penicillin, streptomycin and 1% amphotericin B. Muscles and other organizations were separated, then mucosa was cut into 1 cm wide stripe and put into centrifuge tubes. Dispase (Gibco BRL) was placed into the tube for 30 min at 37 °C, after that stripes were tore to remove epidermis cells. The rest of lamina propia were then minced in the Petri dish and placed in collagenase type I (3 times, Gibco BRL) for 1 h at 37 °C. After digestion, a cell suspension was obtained by removing undigested tissue pieces through a 100 holes cell strainer. The filtrate was collected and was put into the culture flask containing Dulbecco’s modified Eagle’s medium supplemented with F12 (DMEM/F12; Gibco, UK) and 10% fetal bovine serum. After 3 days, cells were adherent and passage once 70–80% confluency. Cells at passage 3 were used for all experiments.

2.3. Cell biological behavior detection

2.3.1. Flow cytometry analysis

The cells were identified by flow cytometry after detachment from the culture flasks and detached mesenchymal stem cells were washed twice with flow cytometry buffer consisting of phosphate-buffered saline (PBS), 2% bovine serum albumin (BSA), and 0.01% NaN_3 and diluted at 1×10^6 cells/ml. A total of 100 μl of each cell suspension were incubated with 10 μl of fluorescein-conjugated antibodies, or alternatively 10 μl of non-conjugated monoclonal antibody and secondary goat-anti-mouse immunoglobulin G1 (IgG1)-FITC at 4 °C for 30 min. To discriminate MSC from hematopoietic stem cells (HSC), cells were stained for CD34 (FITC) and CD45 (FITC). All other mAbs against the human antigens CD29 (FITC), CD44 (FITC), CD90 (FITC), CD105 (FITC) were described previously to be expressed on LM-MSC [27–29]. Nonspecific isotype-matched controls were used to determine background fluorescence. Cells were analyzed on Elite ESP flow cytometry (Beckman Coulter, Fullerton, CA, USA). All antibodies were purchased from eBioscience.

2.3.2. Colony forming and cell proliferation assay

The LM-MSC detached from the culture flasks were plated into 9 cm culture dishes at 2500 cells per well and cultured in DMEM/F12 medium with 10% fetal bovine serum, 1% antibiotic, antimycotic for 2 weeks in incubator at 37 °C. After cell culturing, colonies were fixed with 10% neutral-buffered formalin for an hour, washed with 1× phosphate-buffered saline and then stained by toluidine blue for 5 min. Colonies were observed and counted on phase-contrast inverted micro-scope using a 4× objective. More than 50 cells were counted as a colony. The experiment was repeated at least three times. Cell proliferation was assessed by the MTT assay. LM-MSCs were plated into 96-well tissue culture plates at 3×10^3 cells/well. The cells were allowed to grow for 8 days. Every 24 h, the medium was changed to serum-free medium, 10 μl of 0.5 mg/mL MTT was added to each well and incubated for 4 h. The medium was then discarded, formazan salts were dissolved in 100 μl of DMSO, and the plate was read at 570 nm by a microplate reader (Bio-Rad, Model 550) [30,31].

2.3.3. Differentiation in adipogenesis, osteogenesis and chondrocyte lineage

Adipogenesis, osteogenesis and chondrocyte differentiation was performed according to modified protocols described by Jones et al. [32]. Briefly, cells were seeded into 6-well plates at a density of 1×10^5 cells/cm^2 upon confluency, the culture medium was changed to adipogenic medium supplemented with 10% fetal bovine serum, 2 μM insulin (Sigma), 0.5 mM isobutylmethylxanthine (IBMX; Sigma) and 10 nM dexamethasone (Sigma); osteogenic medium containing 10% fetal bovine serum, 5 mM β-glycerophosphate (Sigma), 100 nM dexamethasone (Sigma) and 50 μg/ml ascorbic acid; chondrocyte medium supplemented with 10% fetal bovine serum, ITS, sodium pyruvate, dexamethasone, DMEM high glucose medium, TGF- β1. After 14 days and 21 days of culture, the cells were washed three times in PBS after being fixed in 4% paraformaldehyde for 10min and then incubated in 0.3% Oil Red O (Sigma) solution for 15min or in 0.1% alizarin red solution (Sigma) in Tris–HCl (pH-8.3) at 37 °C for 30min. After being washed twice in PBS, cells were routinely observed and photographed under an inverted micro-scope (BX-51, Olympus, Japan). 21 days later the cartilage cell clumps embedded and sectioned collagen type II antibody immunohistochemical staining.

2.4. Establish the vocal fold laser injured model

5 canine were fasting for 12 h, then the hindlimb muscle were injected of Sumianxin (Changchun, China) 1–1.5 mg (0.1 mg/kg), sodium pentobarbital (0.2 ml/kg) 2–3 ml. After anesthesia, the animals were fixed in the animal platform, a canine as a control group not injured, 4 canine under support laryngoscope in provoking epiglottis revealed bilateral vocal cord, topical spray a small amount of lidocaine (Jiangsu, China), a semiconductor laser (10W) was employed to resect of vocal cord membrane, bilateral symmetry, damage area of about 8 mm^2, depth of the thyroarytenoid muscle. After injury model was established, to last 3 days intramuscular gentamicin 2 ml/day. At 4 days, 2 weeks, 4 weeks and 8 weeks post-surgery, a canine was euthanatized and observed the bilateral vocal cord in healing, such as congestion, edema, surface line irregularity, atrophy and scar formation or not, respectively. Bilateral vocal cord was obtained of which the left vocal cord was paraffin scented and the vocal cord injury inflammatory response was observed by HE staining. Masson trichromatic staining, Elastin Van Giessen staining, Alcona staining and Elastin staining were used to observe collagen fibers, elastic fibers, hyaluronic acid distribution and content change in blue, respectively. The right vocal cord was frozen scented parallel immunohistochemical staining of fibronectin to observe the changes of fibronectin levels.

Please cite this article in press as: Peng H, et al., The use of laryngeal mucosa mesenchymal stem cells for the repair the vocal fold injury, Biomaterials (2013), http://dx.doi.org/10.1016/j.biomaterials.2013.08.004