International Journal of Pediatric Otorhinolaryngology 76 (2012) 345–351

Contents lists available at SciVerse ScienceDirect

International Journal of Pediatric Otorhinolaryngology

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

Tracheal reconstruction by mesenchymal stem cells with small intestine submucosa in rabbits

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ARTICLE INFO

Article history:
Received 8 August 2011
Received in revised form 6 December 2011
Accepted 7 December 2011
Available online 30 December 2011

Keywords:
Tracheal reconstruction
Mesenchymal stem cells
Small intestine submucosa
Tracheal stenosis

ABSTRACT

Aim: The increasing number of newborns requiring intubation and artificial ventilation in the sophisticated premature and intensive care units of recent years has been followed by a concomitant increase in the number of children who develop tracheal stenosis as a sequela of prolonged intubation, with a consequent increasing need for tracheal surgical repair. The aim of this study was to evaluate tracheal reconstruction by monolayered autologous mesenchymal stem cells (MSCs) with small intestine submucosa (SIS) in a rabbit model.

Methods: Twelve male rabbits were randomly divided into three groups: rabbits with tracheal defects without reconstruction (untreated group, $n = 4$), rabbits with tracheal defects given porcine small intestine submucosa graft (SIS group, $n = 4$), and rabbits with tracheal defects that underwent transplantation of monolayered mesenchymal stem cells on SIS (SIS+MSC group, $n = 4$). Histological and endoscopic analyses were performed by hematoxylin–eosin staining (H&E), Prussian blue staining and endoscopy.

Results: Tracheal stenosis in the SIS+MSC group was minimal, compared to the untreated group and SIS group. Specimens obtained from the untreated and SIS groups showed severe infiltration of inflammatory cells and granulation tissue formation into the trachea. In the SIS+MSC group, however, minimal infiltration of the inflammatory cells and granulation tissue formation were observed. Twelve weeks following the operation, regeneration of pseudostratified columnar epithelium was confirmed by H&E staining with minimal inflammatory cell infiltration in the SIS+MSC group. Moreover, Prussian blue staining clearly demonstrated the presence of labeled MSCs in the regenerated tissue of SIS+MSC group.

Conclusions: These results demonstrate that tracheal reconstruction by MSCs with SIS is effective in rabbits with tracheal defects with minimal mortality and morbidity, which appears to be a promising strategy in the treatment of tracheal defects.

1. Introduction

Congenital and acquired tracheal stenosis continues to be challenging problems. The number of premature and intensive care infants and children who require intubation and artificial ventilation has increased dramatically in recent years, with a concomitant increase in the number of children who develop tracheal stenosis as a sequel of prolonged intubation [1]. Tracheal replacement using prosthetic or biological substitutes has yielded unsatisfactory results thus preventing their clinical application [2]. Failure of these methods is mainly due to restriction to the donor sites, immunologic complications, bacterial infections, material failure, and tracheal lumen obstruction [3,4].

Recently, cell sheet engineering has been extensively developed using temperature-responsive culture surfaces [5–8]. These cell sheets enable cell-to-cell connections and maintain the presence of adhesion proteins because enzymatic digestion is not needed [9–11]. So the use of cell sheets has the advantage that an entirely natural neotissue assembled by the cells, with mature extracellular matrix (ECM), can be engineered. However, it is not easy to make the cell sheets from the commercialized temperature-responsive culture surface, and it is needed to individualize the culture condition and lifting condition according to the kinds of cells. So, other groups advocate making the cell sheet with cell scrappers [12]. Previous studies showed that esophageal wound healing after mucosal resection and submucosal dissection improved with significantly fewer inflammatory cells within the operated areas following transplantation of oral mucosal epithelial cell sheets, which significantly prevented esophageal stenosis [7]. Therefore, cell sheet

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doi:10.1016/j.ijporl.2011.12.005

transplantation may be a promising strategy for the prevention of tracheal obstruction following tracheal reconstruction.

Mesenchymal stem cells (MSCs) are desirable cell source for tracheal reconstruction. They are multi-potent adult stem cells that reside within the bone marrow microenvironment [13]. MSCs can differentiate not only into osteoblasts, chondrocytes, neurons, cardiomyocytes, skeletal muscle cells, and vascular endothelial cells, but also into ciliated epithelium cells [14,15]. MSCs can be isolated also from adipose tissue that is typically abundant and can be harvested easily [16,17]. More excitingly, MSCs suppress immune response in a variety of *in vitro* and disease models, and may thus be of benefit to patients with autoimmune disorders or transplant rejection [18]. During the past several years, MSCs derived from adult tissue have rapidly moved from *in vitro* and animal studies into clinical trials as a therapeutic modality potentially applicable to a wide range of disorders, including head and neck reconstruction [19]. The clinical role of tissue-derived MSCs, though not well understood, holds promise for many therapeutic applications in regenerative medicine and reconstruction.

Cell sheets offer an alternative approach to tissue engineering, with a number of advantages. However, the greatest drawback in using cell sheets is their inferior mechanical properties, resulting in extensive contraction and difficulty in handling. To eliminate these shortcomings, Zhou et al. [20] and Chen et al. [21] utilized cell sheet techniques in conjunction with composite scaffolds in bone engineering and Ng and Hutmacher [12] produced dermal constructs by culturing fibroblast sheets in combination with 3D matrices. In this study, we chose porcine small intestine submucosa (SIS) as a bioabsorbable scaffold for MSCs. SIS is a rigid, cell-free collagen matrix consists of 90% protein and 10% lipid, and has been processed to remove immunogenic factors. SIS has been used in sheet form in multiple *in vivo* settings as a graft material, including bladder [22] and urethra reconstruction [23], heart valve replacement [24], diaphragmatic defect repair [25], abdominal wall defect repair [26], and regeneration of meniscal tissue [27]. Histologic evaluation has demonstrated the material's ability to allow for complete epithelialization and mesenchymal cell infiltration with ingrowth of a robust microvascular network [25]. Gubbels et al. demonstrated the reconstruction of a sublethal rabbit tracheal defect using SIS with no mortality and minimal morbidity [28].

The objectives of this study were to evaluate the ability of monolayered MSCs with SIS using cell sheet technology to (1) maintain airway patency, (2) integrate into the tracheal wall, (3) avoid granulation tissue formation, and (4) allow for mucosalization with respiratory epithelium when used to reconstruct a tracheal defect.

2. Materials and methods

2.1. Study protocol

Twelve ($n = 12$) male New Zealand white rabbits (Koatech Laboratory Animal Company, Korea) weighing 2.6–3.0 kg were randomly divided into three groups: rabbits with tracheal defect without reconstruction (untreated group, $n = 4$), rabbits with tracheal defect given SIS graft (SIS group, $n = 4$) and rabbits with tracheal defect that underwent transplantation of monolayered MSCs on SIS (SIS+MSC group, $n = 4$). All protocols were performed in accordance with the guidelines of the Animal Care Ethics Committee of Dongguk University Ilsan Hospital and NIH guidelines.

2.2. Isolation and cultivation of MSCs from adipose tissue

Rabbits were anesthetized with a combination of zoletile 50 (50 mg/kg) and xylazine (4.5 mg/kg) administrated intramuscularly. Subcutaneous adipose tissues were taken from the inguinal area and minced into small pieces. The pieces were digested with 0.075% type 1 collagenase (Worthington, Lakewood, NJ, USA) for 30 min at 37 °C. The solution was then neutralized with Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% FBS and 1% antibiotics (Gibco, Grand Island, NY, USA). The suspension was filtered through 100 μm nylon mesh, and the high-density fraction was sedimented after centrifugation at 1200 rpm for 5 min. The pellet was re-suspended and cultured in a growth medium at 37 °C with 5% CO_2. Non-adherent cells were removed by changing growth media after 24 h. The obtained cells were cultured for 3 passages in growth medium at 37 °C. Confluent MSCs were harvested with trypsin-EDTA solution (Gibco, Grand Island, NY, USA) and were used for each experiment.

2.3. Ferumoxide labeling of MSCs and Prussian blue staining

"Ferumoxides, a superparamagnetic iron oxide contrast agent (Feridex®, Advanced Magnetics Inc., Rochester, IN, USA), was used for magnetic labeling of MSCs [29]. Ferumoxides (25 μg/mL) and poly-L-lysine (PLL, 0.75 μg/mL, Sigma–Aldrich, St. Louis, MO, USA) were mixed together with MSC medium and incubated at room temperature for 60 min [30–32]. The MSCs were then incubated for 24 h to allow uptake of ferumoxides by the cells. Prussian blue staining was performed additionally on the MSC group to identify the presence of MSCs tagged with Feridex after the regeneration process, as described previously [33]. Briefly, the embedded trachea was cut longitudinally into 3 μm thick sections and incubated for 30 min with 1% potassium ferrocyanide in 3% hydrochloric acid, washed, and counterstained with nuclear fast red. Labeled MSCs are identified as blue area on Prussian blue staining.

2.4. MSCs preparation and transplantation

MSCs at the third or fourth passage from adipose tissue were used to form a monolayered autologous cell sheet. Cells (2×10^4 per well) were loaded into temperature-responsive culture multidishes (Nunc Surface, Thermo Fisher Scientific Inc., Rochester, NY, USA) and incubated at 37 °C. The medium was replaced three times per week. After confluently cultured cells were labeled with ferumoxides, MSCs on the temperature-responsive dishes were incubated at 20 °C. By 50 min, MSCs detached spontaneously as monolayered cell grafts. Immediately after detachment, the monolayered cell grafts were gently transferred on to an SIS sheet (Surgisis®, Cook Biotech Inc., West Lafayette, IN, USA).

For transplantation of the monolayered autologous MSC sheet, each rabbit underwent anesthesia with an intramuscular injection of zoletile (50 mg/kg) and xylazine (4.5 mg/kg). Surgery was performed as follows (Fig. 1). The animal was placed in the supine position. The relevant area was shaved and disinfected. A vertical incision was made at the midline of the neck. The strap muscles were separated in the midline until the tracheal cartilages were encountered. A 3-ring, 120-degree anterior tracheal wall defect was then made in the tracheal rings and a graft was placed in the trachea. SIS grafts with MSCs facing inwards (SIS+MSC group) or without MSCs (SIS group) were sutured to the trachea using 5–0 vicryl (Johnson & Johnson, New Brunswick, NJ, USA). The graft overlapped the edges of the defect by 2–3 mm on all sides. The skin incision was closed using 4–0 nylon (Johnson & Johnson, New Brunswick, NJ, USA). The untreated group underwent no tracheal reconstruction after defect creation.

Postoperatively, the animals were observed for 2 h before being returned to their cages, where water and standard feed were available. For the following 3 days, the rabbits were given 20 mg/kg kanamycin as prophylaxis. Clinical signs were monitored daily, with special attention to weight, cough, sputum production, wheezing, and dyspnea.