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Synergistic effect of laminin and mesenchymal stem cells on tracheal mucosal regeneration

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ABSTRACT

Although several studies have been successfully undertaken of tracheal reconstruction in terms of the maintaining the framework of the graft, most cases of reconstruction failure have resulted from delayed mucosal regeneration. The purposes of this study were to evaluate whether laminin-coated asymmetrically porous membrane (APM) scaffold enhances mucosal regeneration, to compare the mucosalization capability with mesenchymal stem cell (MSC) seeded APM, and to determine whether laminin coating and MSC seeding has a synergistic effect on mucosal regeneration. We reconstructed the full-thickness anterior tracheal defect of 36 New Zealand White rabbits with the APM scaffold. MSCs were isolated from the rabbit's inguinal fat. The animals were divided into 4 groups by the presence of laminin coating on APM and application of MSC [Group I, −/− (laminin/MSC); Group II, −/+; Group III, +/−; Group IV, +/+]. Endoscopy and histologic evaluation were performed and the results were compared among the groups. The results showed that ciliated columnar epithelium was regenerated earlier in groups II and III than in group I. Furthermore, the application of laminin and MSC had synergistic effects on tracheal epithelial regeneration. These results demonstrate that tracheal reconstruction by laminin-coated APM seeded with MSCs is most effective in enhancing tracheal mucosalization, and appears to be promising strategy in the regenerative treatment of tracheal defects.

1. Introduction

The tracheal substitute as a biomedical application poses challenges due to the interface between the individual and the bacterial contaminated environment [1]. The main reasons for the failure of segmental tracheal defect reconstruction are strongly related to airway stenosis (caused by the overgrowth of granulation tissue), airway collapse (caused by framework softening), and mucus impaction (mainly caused by lack of epithelium) [2,3]. A variety of artificial tracheas have been designed for use in such cases and undergone assessment, but so far none has proved satisfactory for clinical use. In our previous study, we have reported on the feasibility of asymmetrically porous membrane (APM) as a tracheal scaffold with the specific design in which one side of the APM has nanosize pores to decrease fibroblast ingrowth into the tracheal lumen, while the other side has microsize pores to enhance integration with the surrounding tissue [4]. APM scaffold was shown to be effective in preventing fibroblastic invasion into the lumen, with adequate strength to preclude airway collapse and excellent integration with the surrounding tissue and with minimal inflammatory reaction.

However, despite the advantages as a scaffold, mucosal regeneration on the internal surface of the APM was delayed as late as 12 weeks (until sacrifice). Regarding functional restoration of trachea, regeneration of mucosa is critical because re-epithelialization is important not only for foreign body removal and humidification of inhaled air but also for preventing granulation tissue formation,

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fibrosis, and eventual stenosis [5,6]. Mucosal regeneration in the scaffold occurs in 2 steps. First, stem cell migration takes place from the neighboring normal mucosa which then proliferates to cover the defect. Second, migrated cells differentiate into functional epithelium [7–9]. Basal cells are the stem cells of the tracheal mucosa, attached to the basal lamina. Basal lamina, which is a highly organized sheet of extracellular matrix (ECM) has several functions, including directing cell migration and inducing cell differentiation [10]. Laminin is the major protein of the basal lamina, and is known to be the key substance responsible for cell migration and differentiation [11,12]. In vitro studies have indicated that laminin-rich matrices support cell migration, and normal epithelial cells migrate significantly faster on a laminin-rich matrix than on fibronectin [13].

Thus, we designed the animal experiment to take advantage of the characteristics of laminin, by modifying the internal surface of the APM by laminin coating.

Mesenchymal stem cell (MSC), which because of its immunomodulatory and trophic effect is widely used for tissue regeneration [14–16], was also used for our study by seeding it in the APM. The purposes of this study were: to evaluate whether laminin-coated APM enhanced mucosalization, to compare the mucosalization capability with MSC-seeded APM, and to demonstrate the synergistic effect of the combination of laminin and MSC in the rabbit animal model.

2. Materials and methods

2.1. Materials

Polycaprolactone (PCL; Mw 80,000 Da; Aldrich, Milwaukee, WI), tetraglycol (glycofurol; Sigma, St. Louis, MO) and Pluronic F127 (Mw 12,500 Da; BASF, Parsippany, NJ) were used to prepare asymmetrically porous membranes (APMs) with hydrophilicity. Laminin derived from human fibroblast was purchased from Sigma. All other chemicals were analytical grade and were used as received. Ultrapure grade water (>18 mΩ) was purified using a Milli-Q purification system (Millipore Co., Billerica, MA).

2.2. Fabrication and characterizations of asymmetrically porous membranes

2.2.1. APM (asymmetrically porous membrane)

Asymmetrically porous PCL/F127 membranes were prepared by an immersion precipitation method described elsewhere [17]. In brief, PCL granules were dissolved in tetraglycol at 90 °C (12 wt%) and Pluronic F127 flakes (5 wt%; PCL base) were added to the PCL solution. The hot PCL/F127 mixture solution was poured into a mold (50 mm × 50 mm × 0.4 mm) and then immersed into excess water for 1 hour at room temperature. The surface of the PCL/F127 mixture solution was immediately precipitated by contact with water and the sublayer was gradually solidified by the diffusion of water into the mixture solution (solvent–nonsolvent exchange). The precipitated APM was washed in excess water and dried in a vacuum oven at room temperature. The APMs were sterilized by ethylene oxide for the animal study. The biocompatibility of APM was proved in our previous in vivo studies for regeneration of trachea, bone and nerve [4,18,19].

2.2.2. APM coating with laminin

In order to incorporate laminin, the APMs were soaked in a laminin solution [100 μg/mL in Dulbecco's phosphate buffered saline (DPBS, pH 7.4)] at 37 °C for 1 hour. The laminin-coated APMs were washed with DPBS 3 times and freeze-dried.

2.2.3. MSCs culture and label with PKH26

All protocols and experimental design parameters were reviewed and approved by the Institutional Animal Care and Use Committee of the Seoul National University Hospital. Animal experiment protocols have been approved by the Animal Care and Use Committee of the Seoul National University Hospital and all the procedures were carried out in accordance with institutional guidelines. The adipose tissue was obtained from the subcutaneous tissue of New Zealand white rabbit and cut into small pieces. These were rinsed thoroughly with phosphate buffered solution to remove red blood cell. It was digested with 0.075% Type 1 collagenase (Worthington, Lakewood, NJ) at 37 °C for 30 minutes and neutralized by the addition of a Dulbecco's modified Eagle's medium (DMEM)/F12 containing 10% fetal bovine serum (FBS) and 1% penicillin streptomycin (Gibco-BRL, Grand Island, NY). Then, the mixture was filtered (100 um pore size, #21008-950, Falcon® Cell Strainers, Sterile, Corning®) and the filtrate was centrifuged. After 5 days, when the cells reached their optimal confluency, they were passaged. Characteristics of MSCs were confirmed by FACS analysis and chondrogenic, osteogenic, and adipogenic differentiation which were reported previously [20]. Passage 2 MSCs were labeled with the red fluorescent dye PKH26 (PKH26GL, Sigma Aldrich, St. Louis, MO) according to the manufacturer's protocol. Briefly, the detached MSCs were washed by a serum-free medium and resuspended in 1 mL of dilution buffer from the manufacturer's labeling kit. The suspension of cells was mixed with an equal volume of the labeling solution containing 2 μM PKH26 in the dilution buffer and incubated for 5 minutes at room temperature. After the reaction was stopped by adding 2 mL FBS, cells were washed 3 times with Dulbecco's modified Eagle's medium (DMEM)/F12 and observed by fluorescent microscopy.

2.2.4. APM seeding with MSC

MSCs were seeded on the external surface of APM scaffold immediately after PKH labeling. The reason why we did not seed MSCs on the luminal surface was that the internal surface of APM has nanosize pore where the cells are easily detached by deep breath or coughing after implantation. MSCs labeled with PKH26 were suspended in a complete cell culture medium at a density 3×10^5 cells/mL. A suspension of 1.0 mL was seeded on the external surface with larger pores of APM in the 24-well plate and then incubated in Dulbecco's modified Eagle's medium (DMEM)/F12 containing 10% fetal bovine serum (FBS) and 1% penicillin streptomycin (Gibco-BRL, Grand Island, NY) at 37 °C for 2 days.

2.3. Surgery

2.3.1. Animals

Thirty-six mature New Zealand white rabbits (weight range, 3.15–3.44 kg) were assigned to one of 4 groups as follows according to the graft used: 1) APM (Group I), 2) APM coated with laminin (Group II), 3) APM seeded with MSC (Group III), 4) APM coated with laminin and seeded with MSC (Group IV).

2.3.2. Procedures and reconstruction

The animals were placed in a supine position and intramuscular zoletile and xylazine (50 mg/kg and 4.5 mg/kg, respectively) used for anesthetic induction. The relevant neck areas of the rabbits were shaved and the tracheal contour palpated. After sterile preparation and draping, a midline vertical incision was made, with dissection of the fat and muscular planes performed down to the trachea. After exposing the trachea, a consecutive 3-ring, 120-degree anterior tracheal wall segment was excised with the blade (20×10 mm^2) and APM overlapping the edges of the defect by approximately 2 mm on all sides (~0.4 mm in thickness; 24×14 mm^2 in size) was sutured to the trachea using 4–0 Vicryl. In our previous study, this size tracheal defect was shown to cause severe tracheal stenosis and subsequent death in the rabbits [19], and mucosal regeneration was delayed until 12 weeks [4]. The membrane surface with micro-size pores (strap muscle contact side in application) was stained with rabbit blood before overlapping the edges of the defect and was sutured to the trachea using 4–0 Vicryl (Johnson & Johnson, New Brunswick, NJ). Thus, the membrane surface with nano-size pores was placed to face the inhaled air. The strap muscles were sutured with 4–0 Vicryl and the skin was closed using 4–0 nylon (Johnson & Johnson). Animals were observed for 2 hours postoperatively before being returned to their cages, where water and standard feed were available. Over the following 4 days, the rabbits were given 20 mg/kg kanamycin as prophylaxis. Clinical signs were monitored daily, with special attention given to weight and respiratory symptoms/signs such as cough, sputum production, wheezing, and dyspnea.

2.4. Endoscopic airway examination

Animals in each group were euthanized at 1, 2, and 4 weeks after operation for the purposes of endoscopic and histologic evaluation of the reconstructed tracheas. Immediately after euthanization, a rigid endoscope (Richards, Knittlingen, Germany) was inserted orally and fixed in position to provide the best view of the trachea. Images were taken of each animal's airway with a digital camera (E4500, Nikon, Japan) which was connected to the rigid endoscope. Luminal patency and mucosal regeneration were serially compared among the 4 groups and evaluated semi-quantitatively. Airways were graded for the maximal degree of stenosis: grade I, 0%–33% stenosis; grade II, 33%–66% stenosis; and grade III, 66%–100% stenosis (Supplemental Fig. 1) [21].

2.5. Histologic and scanning electron microscopic analyses

Biopsy samples were collected from each implant at 1, 2, and 4 weeks after operation as mentioned above. After endoscopic evaluation, these samples were fixed in a 10% neutral formalin solution, embedded in paraffin, cut at a thickness of 4 μm, and stained with hematoxylin-eosin for light microscopy. For SEM analysis, samples were prefixed by immersion in 2% glutaraldehyde in 0.1 M phosphate buffer solution, and post-fixed for 2 hours in 1% osmic acid dissolved in phosphate buffer solution. Samples were treated in a graded series of ethanol and t-butyl alcohol, dried in a freeze dryer (ES-2030, Hitachi, Tokyo, Japan), platinum coated using an ion coater (IB-5, Eiko, Japan), and observed under an FESEM (Hitachi).

2.6. Immunofluorescence

Trachea specimens were fixed in 4% paraformaldehyde and then were dehydrated and embedded in paraffin. Finally, 4 um thickness sections were prepared. After deparaffinization, the sections were pretreated with a citric acid solution (100 mM citrate, pH 6.0) and 0.5% Triton X-100. After blocking with 3% BSA, the