Mini Review

Bio-engineered scaffold with fibroblasts for tracheal regeneration in a rabbit model

Wataru Okano, MD[1)], Yukio Nomoto, MD[1)], Ken Kobayashi, PhD[2)], Masao Miyake, PhD[3)], Teruhisa Suzuki, MD[1)], Yasuhiro Tada, MD[1)], Tatsuo Nakamura, MD[4)], Mutsumi Watanabe, MD[1)], and Koichi Omori, MD[1,*)]

[1)]Department of Otolaryngology, School of Medicine, Fukushima Medical University, Fukushima, Japan
[2)]Department of Pharmacology, Keio University School of Medicine, Tokyo, Japan
[3)]Department of Cell Science, Institute of Biomedical Sciences, School of Medicine, Fukushima Medical University, Fukushima, Japan
[4)]Department of Bioartificial Organs, Institute for Frontier Medical Sciences, Kyoto University, Kyoto, Japan

Some patients with malignant or stenotic inflammatory lesions of the trachea require tracheal resection and reconstruction. Conventionally, it is difficult to reconstruct tracheal defects by either end-to-end anastomosis or autologous tissue implantation.

Recently, a few studies on tracheal regeneration using scaffolds have been reported. Collagen-conjugated prothesis have been used for tracheal reconstruction in the clinical application. The problem of this tracheal prosthesis is delay of epithelial regeneration to avoid possible infection.

A bio-engineered scaffold consisted of collagen sponge and polypropylene mesh with fibroblasts was developed for accelerating tracheal regeneration.

The regenerated tracheas were examined by bronchoscopic findings, histological finding and measurement of average thickness of the regenerated trachea. A bio-engineered scaffold was observed to induce more rapid re-epithelization in the large tracheal defect of a rabbit model. This method appears to be feasible for clinical use after further experiments investigating its efficacy and safety.

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* Correspondence should be addressed to:
Koichi Omori, MD, Department of Otolaryngology, Fukushima Medical University School of Medicine, 1 Hikarigaoka, Fukushima 960-1925, Japan. Phone: +81-24-548-2111, Fax: +81-24-548-3011, e-mail: omori@fmu.ac.jp

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Introduction

Some patients with malignant tumors or stenotic inflammatory lesions in the trachea require surgical resection of a portion of tracheal wall and reconstruction of the defect. Conventionally, the tracheal defects were reconstructed by either end-to-end anastomosis or autologous tissue implantation using skin or cartilage from the nasal septum, auricle or costal cartilage. The problems of the clinical availability of the reconstruction of tracheal tissue are separation, stenosis, and infection of the reconstructed trachea. Artificial tracheas such as silicon, marlex mesh, and stainless steel wire mesh had been tried, but ended unsuccessful results because of separation and stenosis[1-3]. Recently, however, several studies have reported the development and usefulness of the application of tissue engineering technology for the tracheal regeneration[4-10].

Kojima et al made autologus tissue-engineered trachea with sheep nasal chondrocytes[5]. The chondrocytes and fibroblasts were seeded on their polyglycolic acid scaffolds. The postoperative sheeps were killed as results of extensive tracheomalacia or stenosis within one week. Macchiarini et al reported clinical implantation of tissue engineered trachea[6]. This trachea was retrieved from a donor who was died of cerebral haemorrhage, and they removed donor's HLA antigen. Recipient's epithelial cells and chondrocytes were combined with this donor's trachea. Recipient was implanted this tissue engineered trachea, and have been free from any troubles for 4 months.

Previously, our group had made an artificial trachea that was made from a collagen-conjugated prosthesis reinforced by polypropylene mesh frame and a polypropylene ring, which is further sandwiched with collagen sponge extracted from porcine skin[9]. The collagen sponge was absorbed on day 28 in rat models after the epithelium had formed[11]. Therefore, the degradability of collagen sponge was optimized for the tracheal regeneration. Omori et.al have successfully applied this tracheal prosthesis to patients with noncircumferential tracheal resection for up to 3 years[10,11]. However, it was noted that delay of epithelial regeneration on the luminal surface of the prosthesis remained a problem. To overcome this delay, Nomoto et al[13,14] incorporated fibroblast-containing collagen gel in this scaffold. Using rat model, it was demonstrated that this new bio-engineered scaffold is effective in accelerating the differentiation and establishment of the tracheal mucosa *in vitro* and *in vivo*[11,12].

The goal of current study is the application of the bio-engineered scaffolds to patients. To this end, next step is to evaluate whether this scaffold can also be effectively applicable to larger tracheal defects. Therefore, we used a rabbit model, in which the trachea was about 10 times larger than that of rat, and modified new artificial trachea with polypropylene mesh and ring for the rabbit model. The novelty of this result was a foundation for the next step research on the application of this bio-engineered scaffold to patients.

Fabrication of the bio-engineered scaffold

The prosthesis is of a cylindrical type and is made of polypropylene mesh reinforced with a continuous polypropylene ring, which is attached to the outside of the mesh by melting at several points and further fixed. The polypropylene rings provided the tube with reinforcement against compression. The procedure for the production of the tracheal prosthesis was described by Nakamura et al[9]. In our previous study using rat model, the artificial trachea did not involve polypropylene mesh and ring. In our current study using rabbit model, the artificial trachea involved polypropylene mesh and ring for supporting tracheal frame.

The method of collecting tracheal fibroblasts was described by Kobayashi et al[12]. For denudation of the tracheal epithelium, the extirpated tracheas were immersed in protease solution at 4°C overnight, and rinsed with phosphate-buffered saline solution. After denudation, the tracheas were minced into small pieces, placed on culture dishes, and cultured. After culturing for about 5 days, the pieces were removed and the fibroblasts were harvested.

Fibroblasts were suspended in a collagenous solution that was a mixture of type I collagen, fivefold concentrated DMEM and sterile reconstitution buffer at a ratio of 7:2:1. The density of fibroblasts in the collagenous solution was 5.0×10^5 cells per millilitre[12-14]. This density of fibroblasts showed apparent cell polarity, proper arrangement, and well-developed cilia in the previous report[9]. In fabrication of the bio-engineered scaffolds, the collagen solution containing the fibroblasts was layered on the inner surface of collagen sponge, which was then incubated at 37°C for at least 30 minutes, allowing the collagen solution to form a gel[12-14] (Fig.1B; Bio-engineered model). Current procedure of cell seeding was coating of gel with fibroblasts on the sponge instead of co-culturing epithelial cells with fibroblasts. As a control, a collagenous sponge was prepared without the addition of fibroblasts and collagenous gel (Fig.1A; Control model).

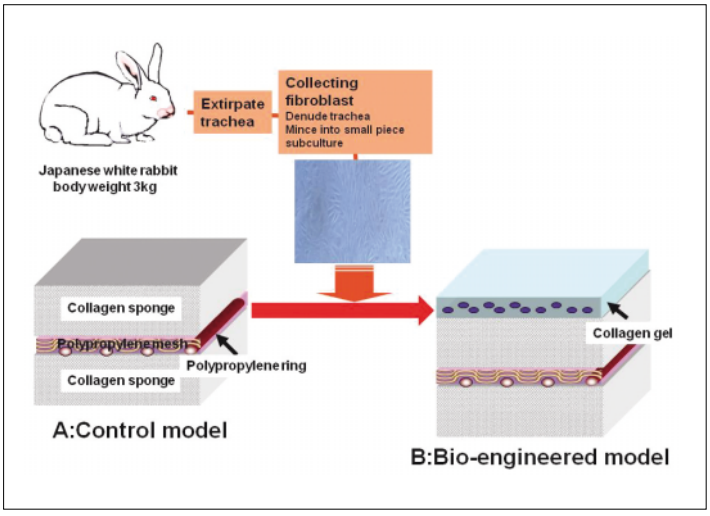

Fig.1 Fabrication of the bio-engineered scaffold

The implanted trachea was extirpated from a rabbit. We collect fibroblasts, and subculture. As a control, a collagenous sponge was prepared without the addition of fibroblasts and collagenous gel (Fig.1A; Control model). The collagenous solution containing fibroblasts was layered on the collagenous sponge (Fig.1B; Bio-engineered model).

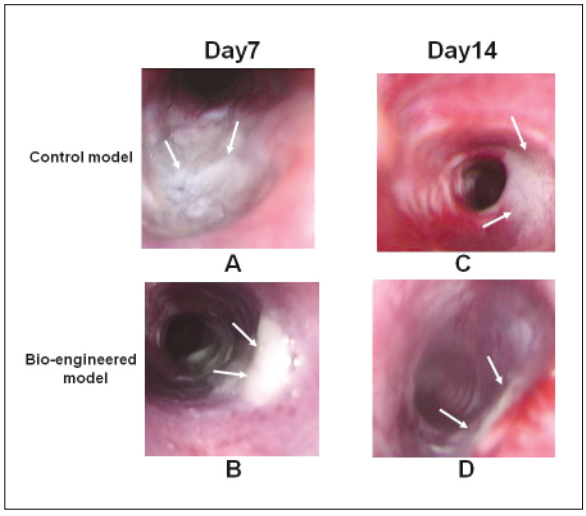

Fig.2 Bronchoscopic findings

A: Control model on day 7 post-implantation. Collagenous scaffold is exposed.

B: Bio-engineered model on day 7 post-implantation. The arrows showed that collagen gel on the collagen sponge.

C: Control model on day 14 post-implantation. The arrows showed that the surface of the implants appeared smooth.

D: Bio-engineered model on day 14 post-implantation. The arrows showed that the surface of the implants appeared smooth.

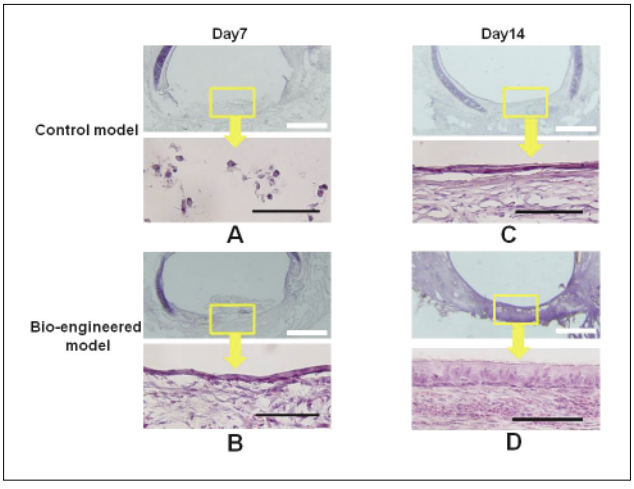

Fig.3 HE staining on day 7 and 14 post-implantation

Upper sections: original × 8, white bars = 2mm

lower sections: original × 400, black bars = 100 μm

A: Control model on day 7 post-implantation. Collagenous scaffold is exposed.

B: Bio-engineered model on day 7 post-implantation. The surface was covered by a stratified squamous epithelium. Slight granulation was observed on the surface.

C: Control model on day 14 post-implantation. The surface was covered by a stratified squamous epithelium. The surface was smooth.

D: Bio-engineered model on day 14 post-implantation. The surface was mostly covered by the columnar cuboidal ciliated epithelium.

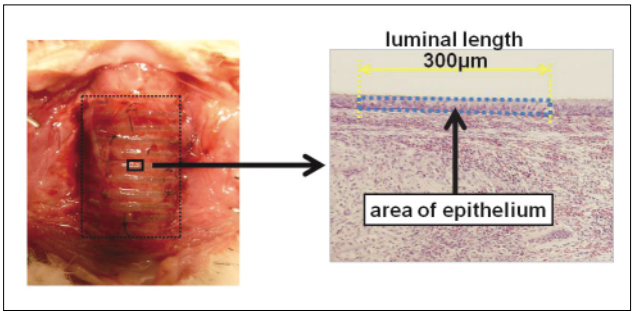

Fig.4 Measurement of average thickness of the regenerated epithelium

Tissue specimens were chosen from the center of the regenerated trachea in the HE strained samples. The areas of the regenerated epithelium were measured using Scion Image β2 software. The average thickness (μm) of the regenerated epithelium was obtained by dividing the area of the regenerated epithelium by the luminal length (300 μm in length).

Implantation of the bio-engineered scaffolds into tracheal defects

The cervical trachea was exposed through a midline incision in the neck under general anesthesia. Tracheal defects of about 5.0 mm in width and 9.0 mm in length were prepared.

The bio-engineered scaffolds group (n＝8) and control scaffolds group (n＝8) were implanted into 16 rabbits. Four rabbits from each group were examined on day 7 and 14 post-implantation. The evaluation time was selected based on the previous reports demonstrating that the period of two weeks was required in rats for recovery of the functional tracheal epithelium[12,14]. No rabbit died as a result of the operation. There were no problems such as separation, stenosis, and infection of the reconstructed trachea. The tissue integrity of epithelium regenerated by this scaffold was sufficiently obtained.

Bronchoscopic examination

On day 7 and 14 post-implantation, the luminal surface of the trachea around the implanted graft was examined using a bronchoscope. Bronchoscopic examination revealed neither stenosis nor granulation in the region of the anastomosis between implanted grafts and original trachea in all rabbits examined.

On day 7 post-implantation, the luminal surface appeared unepithelized in the control scaffold group (Fig.2A). In the bioengineered model, implanted grafts were covered by the connective tissue. A redden tissue in the edge of implanted grafts indicated induction of vascularization (Fig.2B).

On day 14 post-implantation, the implanted graft was covered by the epithelium with smooth surface in the control model (Fig.2C). Bronchoscopic examination showed a similar degree of epithelization in the bio-engineered model (Fig.2D).

Histological examination

The extirpated samples of the trachea were fixed with 4% paraformaldehyde, and embedded in paraffin. The samples were sectioned into 5 μm for hematoxylin-eosin (H-E) staining.

On day 7 post-implantation, collagen sponge was exposed on the surface of the tracheal lumen in the control scaffold group (Fig.3A). On the other hand, the surface was covered by a stratified squamous epithelium in the bio-engineered model (Fig.3B). Slight granulation was observed on the surface.

On day 14 post-implantation, the surface of the tracheal lumen appeared smooth without any columnar ciliated epithelium in the control model (Fig.3C). On the other hand, the surface was mostly covered by the cuboidal ciliated epithelium in the bio-engineered model (Fig.3D).

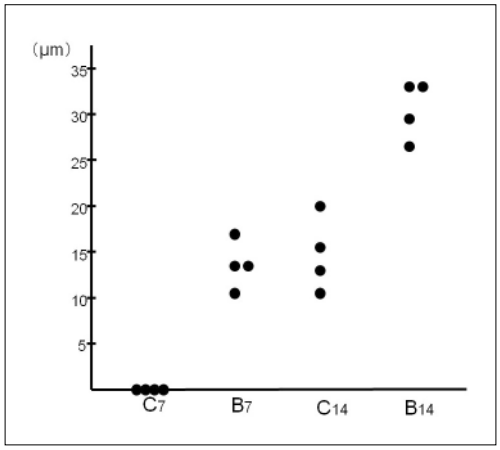

Fig.5 Average thickness of the reconstructed epithelium

C7: Average thickness of the regenerated epithelium in control group on day 7 (n＝4)

B7: Average thickness of the regenerated epithelium in bio-engineered scaffold group on day 7 (n＝4)

C14: Average thickness of the regenerated epithelium in control group on day 14 (n＝4)

B14: Average thickness of the regenerated epithelium in bio-engineered scaffold group on day 14 (n＝4)

Average thickness of regenerated epithelium

The average thickness of the regenerated epithelium was measured to estimate the cellular proliferative potential of fibroblasts in the epithelium. Tissue specimens (300 μm in length) were chosen from the center of the tracheal defect in the HE strained samples. The areas of the regenerated epithelium in the tracheal defects were measured using Scion Image β2 software (Scion Corporation, Frederick, MD; U.S.A http://www.scioncorp.com). The average thickness (μm) of the regenerated epithelium was obtained by dividing the area of the regenerated epithelium by the luminal length (300 μm) (Fig.4).

The average thickness of regenerated epithelium was expressed as mean ± SD. On day 7 post-implantation, the control model was unepithelized (0 μm) in control model (n ＝ 4). Whereas, the thickness in the bio-engineered model was 14 ± 2.0 μm (n ＝ 4). On day 14 post-implantation, the thickness in the control model and bio-engineered model was 14 ± 2.3 μm (n ＝ 4), and 30 ± 3.7 μm (n ＝ 4) (Fig.5).

In the bio-engineered model group, the average thickness of

the regenerated epithelium was greater than that in the control model group for day 7 and day 14. The average thickness of bio-engineered model on day 7 is similar to that of control model on day 14. The results indicate that the tracheal fibroblasts accelerated regeneration of the tracheal epithelium by about 1 week.

Function of fibroblast for tracheal regeneration

Fibroblasts are distributed in the connective tissue, such as the dermis and submucosa, throughout the body, including the subepithelial layer. Dermal fibroblasts are thought to release several kinds of cytokines and modulate epidermalization[15-18]. For this reason, dermal fibroblasts are clinically used with artificial dermis as a cultured dermal substitute, and several products are commercially available. Few studies have reported on interactions between tracheal fibroblasts and the tracheal epithelium from the viewpoint of healing tracheal defects. Goto et al[19] reported that reconstruction of the tracheal epithelium was achieved by co-culturing tracheal epithelial cells and fibroblasts by use of an amnion membrane without any exogenous growth or differentiation factors *in vitro*. Kobayashi et al[12] demonstrated that tracheal epithelial proliferation, morphological and immunohistochemical differentiation of the tracheal epithelial cells, formation of a basement membrane and production of mucin were accelerated by co-culturing tracheal epithelial cells with tracheal fibroblasts *in vitro*. From those reports, the fibroblasts were effective in accelerating normalized epithelial regeneration on the collagen sponge on the surface of the artificial trachea[10-13].

The examination of bio-engineered scaffolds for larger defects is necessary for assessing the potential for clinical use, because the larger tracheal defects in a rabbit model may require a longer period for epithelization than that in a rat model.

From endoscopic and histological examinations after implantation of bio-engineered scaffolds, fibroblasts were effective in accelerating proliferation and differentiation of tracheal epithelium in rabbit models.

The surface of tracheal lumen was covered by the cuboidal ciliated epithelium in the bio-engineered model. Although expectoration of sputum was not analyzed, the regenerated cilia may play an important role for expectorating sputum.

Further study

Autologous implantation is free from the problem of graft rejection. Usage of patient's fibroblasts is an essential point for clinical use. Autologous implantation will be required in further study.

Conclusion

Conventionally, it is difficult to reconstruct tracheal defects by either autologous tissue implantation or end-to-end anastomosis.

Recently, a few studies on tracheal regeneration using scaffolds have been reported. Collagen-conjugated prothesis have been used for tracheal reconstruction in the clinical application. The problem of this tracheal prosthesis is delay of epithelial regeneration.

A bio-engineered scaffold consisted of collagen sponge and polypropylene mesh with fibroblasts was developed for accelerating tracheal reconstruction. Bio-engineered scaffolds were observed to induce more rapid re-epithelization in the large tracheal defect models. This method appears to be feasible for clinical use after further experiments investigating its efficacy and safety.

Acknowledgements

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