Original Article

Fabrication of an anatomy-mimicking BIO-AIR-TUBE with engineered cartilage

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ABSTRACT

Introduction: We devised a strategy for the fabrication of an 'anatomy-mimicking' cylinder-type engineered trachea combined with cartilage engineering. The engineered BIOTUBEs are used to support the architecture of the body tissue, for long-segment trachea (>5 cm) with carinal reconstruction. The aim of the present study was to fabricate an anatomy-mimicking cylinder-type regenerative airway, and investigate its applicability in a rabbit model.

Methods: Collagen sponge rings (diameter: 6 mm) were arranged on a silicon tube (diameter: 6 mm) at 2-mm intervals. Chondrocytes from the auricular cartilage were seeded onto collagen sponges immediately prior to implantation in an autologous manner. These constructs were embedded in dorsal subcutaneous pouches of rabbits. One month after implantation, the constructs were retrieved for histological examination. In addition, cervical tracheal sleeve resection was performed, and these engineered constructs were implanted into defective airways through end-to-end anastomosis.

Results: One month after implantation, the engineered constructs exhibited similar rigidity and flexibility to those observed with the native trachea. Through histological examination, the constructs showed an anatomy-mimicking tracheal architecture. In addition, the engineered constructs could be anastomosed to the native trachea without air leakage.

Conclusion: The present study provides the possibility of generating anatomy-mimicking cylinder-type airways, termed BIO-AIR-TUBEs, that engineer cartilage in an in-vivo culture system. This approach involves the use of BIOTUBEs formed via in-body tissue architecture technology. Therefore, the BIO-AIR-TUBE may be useful as the basic architecture of artificial airways.

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1. Introduction

Currently, it is possible to fabricate engineered cartilage plates with mechanical properties mimicking those of native tracheal cartilage in an *in-vivo* culture system within 6 weeks after implantation [1]. Accordingly, we have manufactured temporary (i.e., 6 weeks after implantation) rigid scaffolds for engineered airways, until the engineered cartilage is generated to maintain airway patency [2]. Engineered cartilage for graft patch reconstruction can be fabricated through *in-situ* implantation of slow-release basic fibroblast growth factor [2]. These engineered airway internal

lumens can be autonomously epithelialized within 4 weeks after implantation. However, cartilage grafts comprising >30% of the circumference of the airway can be absorbed and replaced by fibrous tissue [3]. It is important to limit exposure to diseases caused by graft patch tracheoplasty. Therefore, the use of a cylinder-type regenerative airway in tracheal surgery is necessary.

Thus far, 14 patients have received cylinder-type bioengineered tracheas [4,5]. An enzymatically decellularized tracheal scaffold and a synthetic nanocomposite scaffold have been used with bone marrow cells in *ex-vivo* or *in-vivo* culture systems in humans. However, the fabrication and use of anatomy-mimicking cylinder-type regenerated tracheas have not yet been reported. Cartilage rings support airway patency, while the tracheal annular ligament and membranous parts of the trachea buffer the expansion and contraction, flexure, and internal pressure. Therefore, the development of an anatomy-mimicking engineered trachea is necessary for the reconstruction of a long-segment trachea (>5 cm).

Biodegradable polymers [6] or decellularized biomaterials [7] are commonly used as cylinder-type scaffolds to generate the tubular architecture. In an alternative approach, cylinder-type tissues termed BIOTUBES — consisting of autologous tissues — have been prepared using in-body tissue architecture technology (iBTA) [8]. A BIOTUBE is prepared by embedding polymer rods as a mold in subcutaneous pouches of dorsal skin, and constituting the fibrous connective tissue cover of the mold. The BIOTUBE consists of a collagen-rich extracellular matrix (maximal wall thickness: 200 μm). The inner diameter and shape of the BIOTUBE can be changed by altering the size of the polymer rod. The burst strength is >200 mmHg [8]. The BIOTUBE can be used as a scaffold for engineering small-diameter vessels [8,9]. Recently, construction of the BIOSHEET offered a sheet-shaped collagenous connective tissue. This type of tissue was applied to the development of heart valve tissue, corneal equivalent, tracheal patch, and diaphragm patch [10–12]. Thus far, the use of a BIOTUBE or BIOSHEET alone as a patch graft has demonstrated autonomous regeneration similar to that observed in the surrounding tissue. We have been developing an anatomy-mimicking cylinder-type engineered trachea, combined with cartilage engineering and the BIOTUBE produced using iBTA. The engineered cartilage serves as an equivalent to the tracheal cartilage ring, while the BIOTUBE is the equivalent of the tracheal annular ligament and membranous part. These interactive techniques allow the fabrication of a strong and supple artificial trachea. The aim of the present study was to fabricate an anatomy-mimicking cylinder-type regenerative airway using engineered cartilage and BIOTUBE formed through tissue engineering and iBTA, respectively. In addition, this study investigated the applicability of this cylinder-type regenerative airway in a rabbit model.

2. Material and methods

2.1. Cell harvesting

All procedures performed in the present experiments were approved by the Animal Care Committee of Tokyo University, Tokyo, Japan (protocol no. P-13-84). We performed the experiments in accordance with the guidelines established by the National Institutes of Health for the care and use of Laboratory animals (NIH Publications No. 8023, revised 1978). Auricular cartilage was aseptically harvested from New Zealand white rabbits weighing 0.8–1.0 kg. Briefly, female New Zealand white rabbits were anesthetized with propofol (Maruisihi Pharmaceutical Co. Ltd., Osaka, Japan) and halothane (Takeda Pharmaceutical Co. Ltd., Osaka, Japan). In the prone position, two pieces of auricular cartilage (size: 5 × 10 mm) were harvested under sterile conditions. Chondrocytes

were isolated as previously reported [1,2]. Briefly, the dissected cartilage fragments were digested in 0.3% collagenase type 2 solution (Worthington, Freehold, NJ, USA) for 90 min. The recovered cells were washed with phosphate-buffered saline (Wako Co. Ltd., Osaka, Japan). The collected cells were counted using a hemocytometer and stocked in Hamm's F12 media (Dulbecco's Modified Eagle Medium/F12; Sigma–Aldrich Corp., St Louis, MO, USA) in an incubator (37 °C; 5% carbon dioxide).

2.2. Construct assembly

Collagen sponges for 35-mm culture dishes (KOKEN Co. Ltd. Tokyo, Japan) were shaped into rings (diameter: 6 mm; width: 2 mm; and thickness: 2 mm) using a punch cutter. These collagen sponge rings were arranged onto a 6-mm diameter silicon tube at 2-mm intervals (Fig. 1a).

2.3. Fabrication of the engineered airway

Female New Zealand white rabbits were anesthetized as described earlier in this article. In the prone position, these constructs were embedded in dorsal subcutaneous pouches of the rabbits in an autologous manner (Fig. 1b). In addition, the collected chondrocytes were seeded onto collagen sponges at a density of 50×10^6 cells per cm^3, immediately prior to implantation. A non-seeded area on the collagen sponges was preserved to form the membranous part of the trachea. One month after implantation, the engineered constructs were retrieved from the dorsal subcutaneous pouches.

### 2.4. Morphological and histological examinations

The engineered specimens ($n = 3$) were harvested 1 month post implantation for morphological and histological analyses. The specimens were embedded in Tissue-Tek® OCT compound 4583 (Sakura Finetechnical Co. Ltd., Tokyo, Japan) and frozen in liquid nitrogen. Subsequently, they were cut into 7.5-μm sections and stained with hematoxylin–eosin, toluidine blue, and safranin O to confirm the presence of metachromasia.

2.5. Mechanical properties

Scanning haptic microscopy was employed to assess the mechanical properties of the engineered cartilage implants, as previously described [13,14]. Briefly, the samples ($n = 3$ per segment) were fixed using a sample holder at room temperature. A video camera was used to monitor the surface of the sample and the tip of the sensor probe. The latter was calibrated using the force-deformation method with a 1-mm diameter metal rod indenter and a silicone rubber (elastic modulus: 6–1324 kPa). Scanning haptic microscopy was performed without any preconditioning, as previously reported [13,14]. The distance between two adjacent scanned points was 5 μm. The tip radius and indentation depth of the sensor probe was 5 and 10 μm, respectively. The overall scanning speed was approximately 3 points/s. The scanning area of each image was 200×200 μm (number of pixels: 25 points). Scanning measurements were repeated for the entire wall of the engineered cartilage.

2.6. Implantation

In the supine position, the rabbit trachea ($n = 3$) was exposed through anterior longitudinal cervicotomy. In addition, cervical tracheal sleeve resection of three cartilaginous rings was
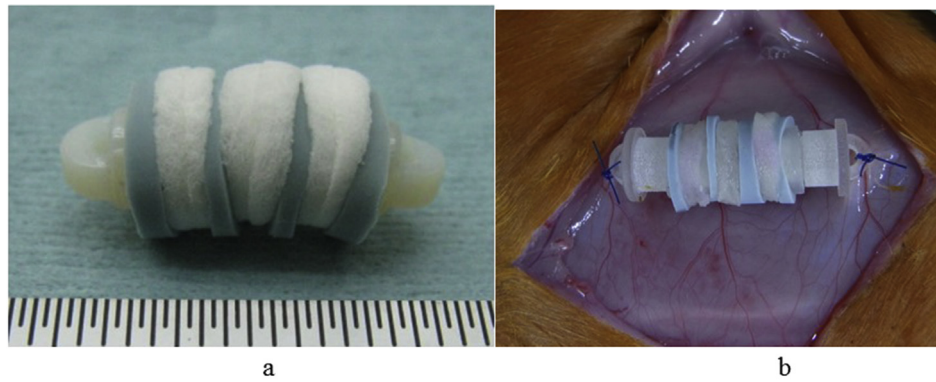
Fig. 1. (a) Collagen sponge rings were arranged on a silicon tube (diameter: 6 mm) at 2-mm intervals. These collagen sponge rings constitute the scaffold for the engineered cartilage. The BIOTUBE can be generated in the longitudinal space of the collagen sponge rings as a substitute for tracheal annular ligaments, and the non-seeded area of the collagen sponges can form the membranous part. (b) Prior to implantation, the collected chondrocytes were seeded onto collagen sponges at a density of 50×10^6 cells per cm^3. In addition, the membranous part of each sponge is the area not seeded with chondrocytes. The constructs were fixed to the superficial fascia using a 4-0 nylon suture material, to generate the engineered airway.
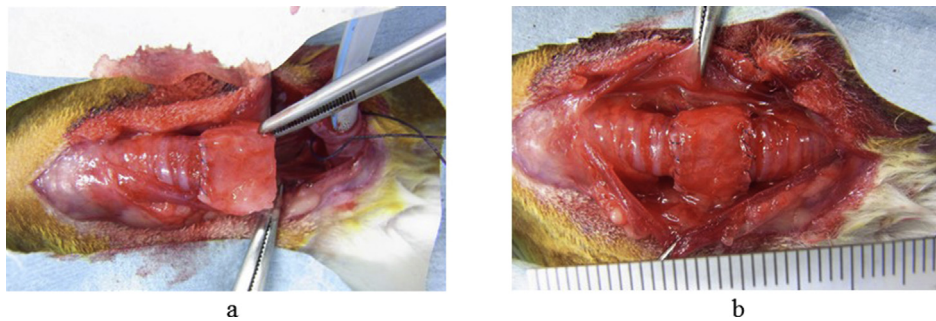
Fig. 2. (a) Cervical tracheal sleeve resection of three cartilaginous rings was performed in the cervical trachea. Endotracheal intubation was performed in the operative field for ventilation. (b) BIO-AIR-TUBEs were also implanted into defective airways through end-to-end anastomosis. The constructs were fixed in place using 6-0 monofilament running sutures. Air leakage did not occur from the cylinder-type airway or site of anastomosis. Moreover, there was no deformation of the outward appearance post implantation.
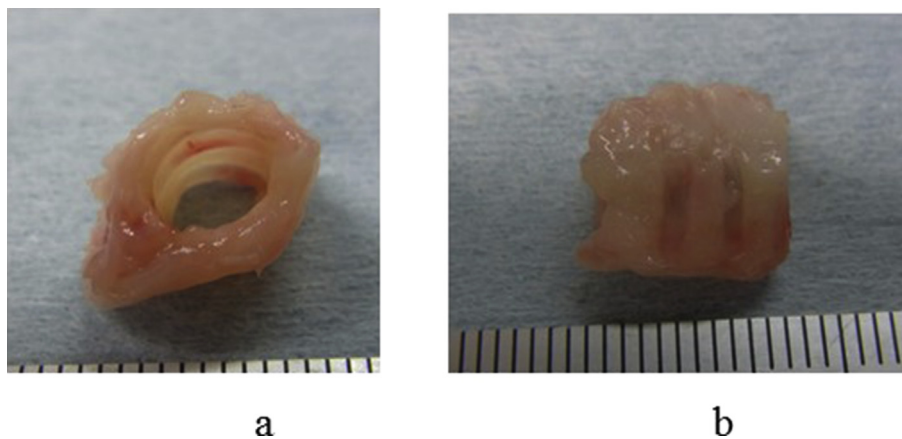

Fig. 3. (a) In the cross-sectional view, the cylinder-type engineered airway maintained its shape. (b) In the longitudinal view, the cartilage rings were transparent fibrous membranes, and the BIOTUBE was observed between the cartilage rings.

performed. Endotracheal intubation was performed in the operative field for ventilation (Fig. 2a). These engineered constructs were also implanted into defective airways through end-to-end anastomosis (Fig. 2b). The constructs were fixed in place using 6-0 monofilament running sutures (Prolene; Ethicon, Somerville, NJ, USA). Air leakage was determined *via* flooding above the construct level. In addition, the engineered airway was monitored

for deformation of its outward appearance during spontaneous breathing.

2.7. Statistical analysis

Statistical analysis was performed with the Student's t-test, using commercially available software (Microsoft Excel 2010,
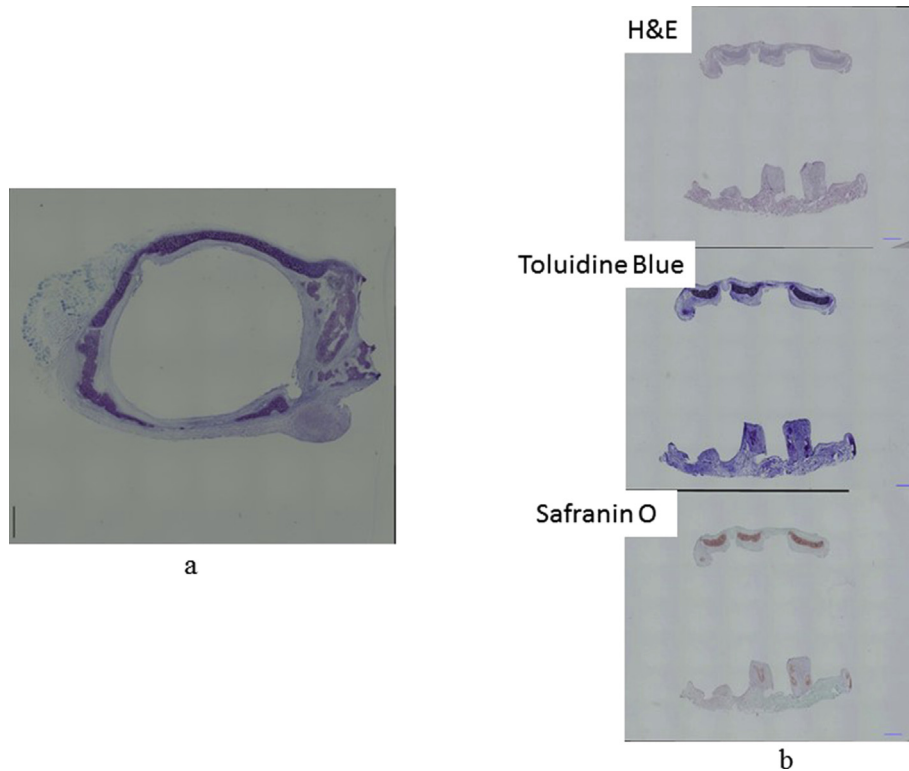
Fig. 4. (a) The engineered cartilage formed a ring-like structure similar to the tracheal cartilage rings. (b) The engineered cartilage rings were connected with fibrous connective tissue, and the equivalent of the membranous part was formed by fibrous connective tissue at the longitudinal midsection. These engineered constructs were mimicking the anatomy of the trachea.

Microsoft, Tokyo, Japan). P-values <0.05 denoted statistical significance.

3. Results

3.1. Gross findings

Four weeks after implantation, the engineered constructs were retrieved. The constructs exhibited similar rigidity and flexibility to those observed with the native trachea (Fig. 3a, b). In the cross-

Fig. 5. The means of Young's modulus for the engineered cartilage BIO-AIR-TUBE and native tracheal cartilage were 733 ± 223 kPa and 581 ± 124 kPa, respectively. Of note, the observed difference between the two types of cartilage was not statistically significant.

sectional view, the cylinder-type engineered airway maintained its shape. In the longitudinal view, the cartilage rings were transparent fibrous membranes, and the BIOTUBE was observed between the cartilage rings.
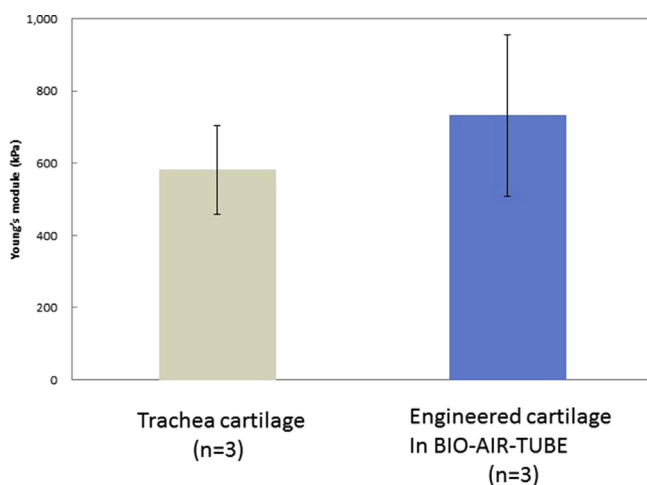
3.2. Histological findings

Histological examination of a cross section through hematoxylin—eosin, toluidine blue, and safranin O staining revealed that the engineered cartilage formed a ring-like structure, similar to the tracheal cartilage rings. The engineered cartilage rings were connected with fibrous connective tissue, and the equivalent of the membranous part was formed by fibrous connective tissue at the longitudinal midsection. These engineered constructs were anatomy-mimicking tracheas (Fig. 4a, b).

3.3. Mechanical parameters

The mechanical parameters of three engineered cartilage BIO-AIR-TUBEs (retrieved 6 weeks after implantation), and three normal tracheal cartilages from each of the three rabbits were measured. One month after implantation, the means of Young's modulus in the engineered cartilage of the BIO-AIR-TUBES and native tracheal cartilage were 733 ± 223 kPa versus 581 ± 124 kPa, respectively. However, the observed difference between the two types of cartilage was not statistically significant (Fig. 5).

3.4. Capability of the constructed airway as an artificial trachea

These engineered constructs were anastomosed to the native trachea using a relatively simple procedure (Fig. 2b). Post implantation, air leakage did not occur from the cylinder-type airway or

the site of anastomosis during spontaneous breathing. In addition, the cylinder-type engineered constructs did not exhibit deformation during spontaneous breathing and maintained airway patency.

4. Discussion

The present study documents that the construction of a cylinder-type regenerative airway, by arranging engineered cartilage inherent in the anatomical location on the BIOTUBE, shows satisfactory analogy with the tracheal anatomy. This cylinder-type engineered trachea can be anastomosed to the native trachea in an end-to-end fashion without air leakage. Moreover, airway patency can be maintained without deformation of the outward appearance. The combination of tissue engineering techniques can lead to the fabrication of complex organs. The anatomy-mimicking artificial trachea, consisting of composite engineered tissue, exhibits airway functions such as patency, flexibility, and absorption of pressure.

In 1994, Vacanti et al. [15] reported for the first time the construction of a cylinder-type tissue-engineered trachea, using a tissue-engineered cartilage cylinder without ligament in animal models. Moreover, in 2002, Kojima et al. [16] produced a tissue-engineered trachea with helical engineered cartilage. Furthermore, Macchiarini group [4,5] conducted a clinical study of a cylinder-type engineered airway using a decellularized trachea or synthetic nanocomposite scaffolds seeded with autologous epithelial cells and mesenchymal stem cells. However, the architecture of the engineered construct was unknown. In 2009, Hsu and Li et al. [17] fabricated a tissue-engineered trachea with ring-engineered cartilage in a bioreactor. Another strategy involved the use of a Marlex® (Chevron Phillips Chemical Company LP, The Woodlands, TX, USA) mesh scaffold with a collagen sponge implanted into a tracheal defect to induce re-epithelialization of the lumen and successful anastomosis through *in-situ* tissue engineering [18]. The present study was the first to investigate the fabrication of an anatomy-mimicking artificial trachea. We successfully fabricated an anatomy-mimicking engineered trachea, designated "BIO-AIR-TUBE", consisting of engineered cartilage rings and autologous fibrous connective tissue.

Cartilage engineering through the present "in-vivo" culture fabrication system yielded similar mechanical parameters to those previously reported [1]. Additionally, cartilage engineering using BIOTUBEs maintained airway patency, especially inspiratory negative pressure. The use of BIOTUBEs in BIO-AIR-TUBE showed adequate mechanical strength, high burst strength, and stretching capacity at the site of implantation. Moreover, we demonstrated that the BIO-AIR-TUBE can reconstruct the airway by means of a running suture to the native trachea without air leakage, and withstand pressure in the airway. A substitute for the ligament of the BIO-AIR-TUBE is the autologous fibrous connective tissue termed BIOTUBE. The BIOTUBE combined with engineered cartilage play a crucial role in the mechanical properties of the tracheal wall.

In our previous study, the BIOSHEET (as part of the tracheal wall substitute) could automatically generate an epithelial layer and cartilage [12]. Therefore, it may generate an epithelium lining on the luminal surface of the BIO-AIR-TUBE in mid- to long-term experiments. It is necessary to perform mid- to long-term research studies investigating the generation of an epithelial lining on the luminal surfaces of BIO-AIR-TUBEs. In addition, BIOTUBEs can be fabricated in a wide range of shapes and sizes to fit each target cylinder-type tissue. Therefore, these combined tissue engineering techniques for each tissue may be used for the fabrication of the carina. This was the first study showing that BIOTUBE compounds with engineered tissue form new composite architecture. These

combined autologously-engineered tissues may be beneficial to the function of cylinder-type airways.

The present study provides the possibility of generating cylinder-type engineered airways that engineer cartilage in an *in-vivo* culture system. This approach utilizes BIOTUBEs formed using iBTA. The BIO-AIR-TUBE maintains patency and airtightness, which is the most important function of the airway. Additionally, the BIOTUBE is equivalent to the annular ligament and membranous part of the trachea, resulting in flexibility and absorption of pressure in the BIO-AIR-TUBE. Therefore, the BIO-AIR-TUBE may be useful as the basic architecture of artificial airways. The potential clinical applications of the BIO-AIR-TUBE should be further investigated in mid- to long-term animal implantation studies.

Declarations of interest

None.

Acknowledgments

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.reth.2019.07.004.

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