Development of a composite and vascularized tracheal scaffold in the omentum for *in situ* tissue engineering: a canine model

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Received 7 March 2014; received in revised form 24 April 2014; accepted 2 May 2014

Abstract

**OBJECTIVES:** We herein report on development of a composite (synthetic and biological) tracheal scaffold with vascularized autologous connective tissue in the omentum, followed by *in situ* tissue engineering of the composite scaffold with the pedicled omentum. In this preliminary report, we focus on development and evaluation of the vascularized autologous connective tissue in the omentum.

**METHODS:** In animal experiment 1, a polypropylene framework as a synthetic component was placed in the omental sac for 3 weeks and another was placed in the pouch of Douglas as a control in five beagle dogs. In animal experiment 2, a polypropylene framework placed in the omental sac for 3 weeks was compared with a polypropylene framework coated with porcine atelocollagen, which was also placed in the omental sac in another five dogs, to investigate whether the coating of porcine atelocollagen contributes to development of more vascularized connective tissue. Macroscopic, radiological and histological evaluations were performed for developed autologous connective tissue on the frameworks, with a focus on its thickness and capillary vessels.

**RESULTS:** In animal experiment 1, the polypropylene framework in the omentum developed a composite tracheal scaffold with homogeneous and significantly thicker (2.6 ± 0.5 vs 1.2 ± 0.4 mm, *P* < 0.0001) connective tissue in which more capillary vessels per 10-power field of view (3.5 ± 2.2 vs 0 ± 0, *P* = 0.015) were identified, compared with the control in the pouch of Douglas. In animal experiment 2, the omental developed significantly thicker connective tissue on the polypropylene framework coated with porcine atelocollagen (3.6 ± 0.7 vs 2.2 ± 0.4 mm, *P* < 0.0001) in which not significantly more capillary vessels were identified (3.5 ± 2.2 vs 5.0 ± 2.7, *P* = 0.12), compared with the framework that was not coated.

**CONCLUSIONS:** Placement of the polypropylene framework in the omental sac resulted in development of homogeneous and vascularized autologous connective tissue on the polypropylene framework for a composite tracheal scaffold. The framework coated with porcine atelocollagen did not show an additional benefit in inducing vascularization. This preliminary report will be followed by the long-term evaluations of *in situ* tissue engineering of the composite tracheal scaffold.

**Keywords:** Trachea · Omentum · Prosthesis

INTRODUCTION

A variety of artificial tracheas have been designed and assessed for inter-position, but so far none has proved satisfactory for clinical use [1]. The first human case for a circumferential airway replacement was performed by Macchiariini *et al.* in 2008 using a decellularized human tracheal allograft that was cellularized with epithelial cells and mesenchymal stem cell-derived chondrocytes by *in vitro* tissue engineering [2]. However, a non-circumferential (only a cartilaginous portion of a trachea) replacement for invading thyroid malignancy was performed by Omori *et al.* in 2002, using a polypropylene mesh framework coated with porcine atelocollagen and *in situ* tissue engineering [3], which was originally developed in our laboratory.

For a successful airway replacement, a scaffold, which can be a biological (allograft/homograft), synthetic or composite [4], requires epithelial cellularization by *in vitro* [2, 5], *in situ* [6] or heterotrophic [7] tissue engineering, which can be promoted by bioactive molecules [8].

We developed an airway scaffold that is a polypropylene framework (as a synthetic component) coated with porcine atelocollagen (as a biological component) for *in situ* tissue engineering in tracheobronchial replacements [9, 10]. Our previous attempts to replace circumferentially the airway with this type of prosthesis in
canine models revealed that its durability extends past 5 years, whereas long-term observations showed incomplete epithelialization on the prosthesis and anastomotic stenosis [9, 10]; similar findings were also noted in the first human circumferential airway replacement in the follow-up [11].

To improve incomplete epithelialization and anastomotic stenosis, we applied a pedicled omentum in tracheobronchial reconstruction procedures; however, the omental transposition procedure alone has not been sufficient to resolve incomplete epithelialization and anastomotic stenosis [9, 10, 12].

In this study, we report and evaluate the development of a composite (synthetic and biological) tracheal prosthesis with vascularized autologous connective tissue developed by the omentum. *In situ* tissue engineering of the composite scaffold with the pedicled omentum (Fig. 1) will be evaluated and described in our next report.

**MATERIALS AND METHODS**

*Polypropylene framework for a tracheal scaffold*

A polypropylene mesh framework was made using a method described in a previous report [10]. The framework is a 0.8-mm-thick polypropylene mesh (Marlex mesh; CR Bard, Inc., Billerica, MA, USA) cylinder that is 25 mm long and has an internal diameter of 20 mm, which is reinforced with four rings of polypropylene monofilament string (1 mm in diameter). This cylindrical mesh framework was exposed to a corona discharge at 9 kV for 5 min to render its surface hydrophilic (Fig. 2A); this was used in animal experiment 1. In animal experiment 2, the polypropylene framework was coated with a 5-mm-thick porcine atelocollagen layer with a pore size ranging from 100 to $500 \times 10^{-6}$ m (Fig. 2B).

![Figure 1: In situ tissue engineering of a composite tracheal scaffold, placed with a pedicled omentum through a substernal route. (A) Pedicled omental flap containing a composite and vascularized tracheal scaffold, (B) anastomosed with a native trachea (the proximal anastomosis is circled).](image1)

![Figure 2: The polypropylene frameworks and their intra-abdominal placements. (A) A polypropylene framework prior to placement; (B) a polypropylene framework coated with porcine atelocollagen prior to placement; (C) a polypropylene framework (indicated by an arrow) placed in the omentum in animal experiment 1; (D) a polypropylene framework placed in the pouch of Douglas as a control in animal experiment 1; (E) a polypropylene framework (a solid arrow) and a polypropylene framework coated with porcine atelocollagen (a dotted arrow) placed in the omentum in animal experiment 2; (F) an illustration of animal experiment 2.](image2)
The porcine atelocollagen layer was developed as follows [10]: the polypropylene framework was placed in a Teflon mould. Then, 1% collagen solution (Nippon Meat Packers, Inc., Osaka, Japan) was stirred at 8000 rpm for 15 min, and then poured into the space between the outer mould and the inner tube, and then freeze-dried. During this freeze-drying process, the cast collagen became a porous structure with a pore size range of 100 to 500 $\times 10^{-6}$ m. A 5-mm-thick collagen layer was formed on both internal and external luminal surfaces. Finally, the prosthesis was heated at 140°C under vacuum pressure for a 24-h dehydrothermal treatment session to induce moderate cross-linkage of the collagen molecules.

Animal experiments


Five adult beagle dogs, weighing 8–14 kg, were anaesthetized with an intramuscular administration of 15 mg/kg ketamine hydrochloride and 7 mg/kg xylazine and then intubated with an endotracheal tube. Mechanical ventilation was maintained using inhalational sevoflurane. A 500-mg dose of ampicillin was injected intramuscularly prior to the skin incision. In each dog, a polypropylene framework was placed in the omental sac after making a slit on the omental peritoneum via an upper mid-line laparotomy (Fig. 2C), and another polypropylene framework was placed in the pouch of Douglas as a control (Fig. 2D). Both were extracted 3 weeks afterwards via a reoperative laparotomy. After sectioning each specimen into two transversely, the thickness of the developed connective tissue was measured with a ruler at three different points (every 120°) on the circumference.

Animal experiment 2. Development of a composite scaffold in the omentum (from polypropylene frameworks versus from polypropylene frameworks coated with a porcine atelocollagen layer).

Another five adult beagle dogs, weighing 8–14 kg, were anaesthetized in the same way as above. A 500-mg dose of ampicillin was injected intramuscularly prior to the skin incision. Two types of framework, as mentioned above, were both placed in the omental sac (Fig. 2E and F). Both were extracted 3 weeks afterwards as in Animal experiment 1, and the thickness of the developed connective tissue was measured in the same way.

All surgical procedures were performed by board-certified surgeons (Masatsugu Hamaji and Fumitsugu Kojima) in accordance with the Guide for the Care and Use of Laboratory Animals published by the National Institutes of Health (NIH Public-ation No. 85-23, revised 1985). The experimental protocol was approved by the Animal Experimental Committee of Kyoto University.

Following first laparotomies (for placement of polypropylene frameworks) or second laparotomies (for extraction of the frameworks), all the dogs received the same regular care as preoperatively. Macroscopic findings at reoperative laparotomies were obtained with a focus on developed connective tissue.

Radiological analysis of developing composite scaffolds

To follow-up the process of developing composite scaffolds in the omentum, four dogs were randomly selected from animal experiment 2 for abdominal computed tomography (CT). All CT images from the diaphragm to the hip joints were obtained with a 16-row multidetector CT scanner (Alexion 16, Toshiba Medical systems, Tochigi, Japan) with an intramuscular administration of ketamine and xylazine on postoperative day (POD) 0, 7, 14 and 21. The images were obtained in the helical mode with 120 kV voltage, 50 mA per section, a 512 $\times$ 512 matrix and 7-mm slice thickness. All images were interpreted by a board-certified radiologist (Sho Koyasu).

Histological analysis of developed connective tissue on the frameworks

In animal experiment 1, two scaffolds developed from polypropylene frameworks in the pouch of Douglas and two scaffolds developed from polypropylene frameworks in the omentum (from two dogs) were sent for histological analysis by light microscopy after staining with haematoxylin and eosin (H&E), Masson trichrome (MT) and alpha-smooth muscle actin (α-SMA). The number of capillary vessels was counted in three randomly selected 10-power fields of view after staining with H&E and compared between the two kinds of scaffold. In animal experiment 2, two scaffolds developed from polypropylene frameworks in the omentum and two scaffolds developed from polypropylene frameworks coated with porcine atelocollagen in the omentum were analysed in the same way. The number of capillary vessels was counted in three randomly selected 10-power fields of view and compared between the two kinds of scaffold. All specimens were examined by a board-certified pathologist (T.T.).

Statistical analysis

Descriptive statistics for continuous variables are reported as mean ± standard deviation. For the comparison of continuous variables, the Mann–Whitney U-test was used as appropriate. All statistical tests were two-sided, and a P-value <0.05 was defined as statistically significant. JMP version 10.0.1 software (SAS Institute, Cary, NC, USA) was used for all analyses.

RESULTS

Clinical courses and macroscopic findings of developed composite scaffolds

All dogs uneventfully survived the sequential laparotomies in animal experiments 1 and 2.

In animal experiment 1, on reoperative laparotomies, there were minimal to mild adhesions between the abdominal wall and the omentum without peritoneal fluid. No migration was noted in frameworks placed in the omentum, whereas two (40%) polypropylene frameworks placed in the pouch of Douglas were noted to have cephalad migrations; the others involved a portion of the small bowels in the lumen (Fig. 3A, in animal experiment 1). The omentum was found to be focally hyperplastic around the framework. Regarding scaffolds developed from polypropylene frameworks in the pouch of Douglas, only thin layers (1.2 ± 0.4 mm) of connective tissue developed on the frameworks (Fig. 3B), with a minimal area of the mesh exposed, whereas homogeneously thick
layers (2.6 ± 0.5 mm) developed on the frameworks in the omentum without any area exposed (Fig. 3C). Serous fluid was contained in the framework in the omentum. There was a statistically significant difference in the thickness of the connective tissue between scaffolds developed in the omentum and those in the pouch of Douglas (2.6 ± 0.5 and 1.2 ± 0.4 mm, \( P < 0.0001 \)).

In animal experiment 2, the omentum was found to be focally hyperplastic around the frameworks (Fig. 3C). Regarding the polypropylene frameworks or polypropylene frameworks with conjugated porcine atelocollagen from the omentum, connective tissue developed on the frameworks without any mesh exposed (Fig. 3D). Both of the developed scaffolds appeared to be waterproof and airtight and contained serous fluid. There was a statistically significant difference in the thickness of developed connective tissue between polypropylene frameworks uncoated and those with with porcine atelocollagen (2.2 ± 0.4 and 3.6 ± 0.7 mm, respectively, \( P < 0.0001 \)).
Radiological findings of developing composite scaffolds in the omentum on abdominal computed tomography

Representative images are shown in Fig. 4. The polypropylene framework in the omentum was recognized as containing fluid that increased in volume over time (Fig. 4A–D). The polypropylene frameworks coated with porcine atelocollagen in the omentum are shown in Fig. 4E–H, and the coating porcine atelocollagen on the framework is recognized as a layer of air mixed with soft tissue density on the lumen on POD 0 (Fig. 4E, arrow). This layer was replaced gradually with soft tissue density (Fig. 4F–H).

Histological findings of the connective tissue developed on the frameworks

In animal experiment 1, the number of capillary vessels in a 10-power field of view was 0 ± 0 in connective tissue of scaffolds developed in the pouch of Douglas and 4.5 ± 3.0 in the connective tissue of ones developed in the omentum, with a statistically significant difference (P = 0.015).

In animal experiment 2, the number of capillary vessels in a 10-power field of view was 3.5 ± 2.2 in the connective tissue of scaffolds developed from polypropylene frameworks in the omentum versus 5.0 ± 2.7 in the connective tissue of scaffolds developed from polypropylene frameworks coated with porcine atelocollagen in the omentum, but the difference was not statistically significant (P = 0.15).

The connective tissue developed on the polypropylene frameworks in the omentum in animal experiment 1 showed almost the same histological findings as that on the frameworks in the omentum in animal experiment 2. Therefore, images from animal experiment 2 are shown.

Shown in Fig. 5 are microscopic (original magnification ×10) examinations. In Fig. 5D and G, fibromuscular fibres developed and surrounded the polypropylene mesh (M) with irregular capillary vessels (arrows in magnified parts). All sections with MT staining suggested that the connective tissue was rich in collagenous fibres (Fig. 5B, E and H), produced by fibroblasts, shown with α-SMA staining (Fig. 5C, F and I).

DISCUSSION

Non-circumferential (cartilaginous portion only) replacement of a human airway with a prosthesis has already been successfully performed after resection of a cartilaginous portion of cervical trachea in 2002 [3], whereas a circumferential replacement of human airway is more challenging and was first performed by Macchiarini et al. in 2008 with a decellularized human tracheal graft that was cellularized by in vitro tissue engineering [2].

An airway prosthesis comprises a scaffold that is biological, synthetic or composite (biological and synthetic) and has tissue-engineered epithelial cells on it. It is still controversial which type of scaffold is the most suitable and safe to use in clinical practice.
of scaffold and which type of engineering are most suitable for developing an airway prosthesis. Regarding the scaffold, the advantages of a biological one included its biocompatibility and its excellent environment for cellularization by tissue engineering, whereas a synthetic scaffold has the advantages of no dependency on donors and easy handling. As for tissue engineering (prior to grafting), in vitro or heterotropic, it is expensive and requires technology for cell culture and implantation, whereas in situ tissue engineering (following grafting) has no cells on the scaffold at first and, therefore, depends on the anastomosis as a sole source for epithelial cellularization.

Airway stenosis near the anastomoses is one major problem regarding an airway prosthesis in either humans [11] or a canine model [6, 9, 10]. Macchiarini et al. experienced one (11.1%) with a stenosis out of nine patients in the long-term follow-up [4], while awaiting long-term (>1 year) follow-up of heterotropic tissue engineering [7]. In a canine model, the long-term incidence of anastomotic stenosis ranged from 25 to 38% without omental transposition. To resolve anastomotic stenosis and incomplete epithelialization, the omentum was previously applied in our laboratory and by other groups to wrap the anastomosis of a prosthesis by taking advantage of its high vasculature [11–13]. The omental transposition procedure at airway grafting lowered somewhat the incidence of an anastomotic stenosis [9], but did not resolve it completely [9, 10, 12].

The omentum is known to not only vascularize other tissues, but also modulate inflammatory reactions, through which it produces connective tissue either in acute inflammatory reactions or in the subacute wound healing process [14]. Our findings in animal experiment 1 confirmed that the functions of the omentum can be applied to developing a composite scaffold for in situ tissue engineering, because vascularization in the scaffold is a key to successful epithelial cellularization on the scaffold [15]. Owing to its rich vasculature, the omentum appears to be superior to muscles, which are potential alternatives, in developing a composite and vascularized tracheal scaffold.

In this preliminary study, an attempt was made to develop a novel method for developing vascularized connective tissue on a synthetic framework, which could provide an excellent environment as a composite scaffold for in situ tissue engineering following grafting. In animal experiment 1, the control that was placed in the pouch of Douglas showed no capillary vessels in the specimen sections or an unreliable thickness (1.2 ± 0.4 mm) given that the thickness of the polypropylene mesh was 0.8 mm. In Animal experiment 2, porcine alcelocollagen coating the polypropylene framework failed to show an additional benefit in vascularizing the polypropylene framework. Although the polypropylene framework coated with porcine atelocollagen in the omentum did develop thicker autologous connective tissue (3.6 ± 0.7 mm) on the scaffold, the thickness appears more than required, given that the thickness of a normal trachea is estimated to be 1–3 mm on CT [16]. Moreover, too thick connective tissue might be a barrier to neck movements. These findings suggest that porcine atelocollagen coating the polypropylene framework does not have additional benefits in developing a composite and vascularized scaffold.

The serial CT images on the polypropylene scaffold successfully identified and followed up the process of developing autologous connective tissue, which apparently plateaued between 14 and 21 days from placement. The radiological findings suggested that the development process can be tracked to some degree but revealed limitations in estimating the thickness.

The limitations of the study included use of canine models as a substitute for human subjects. In addition, the omentum can be unreliable in cases with a history of prior laparotomy and/or an upper abdominal procedure. Prior to the second stage (tracheal grafting), 3 weeks are required for connective tissue development, which is not ideal for emergent or semiemergent treatments.

Our results suggest that development of a composite tracheal scaffold with vascularized autologous connective tissue is feasible. We need to evaluate carefully the long-term outcomes of in situ tissue engineering in further studies, but this composite tracheal scaffold could be a reasonable alternative to our previous scaffolds or to those developed by other groups. Our next report will focus on long-term outcomes of in situ tissue engineering of the composite scaffolds following grafting.

**Conflict of interest:** none declared.

**REFERENCES**


