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Fabrication of a Neotrachea Using Engineered Cartilage

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Abstract

Objectives—Surgical management of long-segment tracheal stenosis is an ongoing problem. Many types of tracheal prostheses have been tried but with limited success because of immune rejection, graft ischemia, or restenosis. Tissue engineered cartilage may offer a solution to this problem, although scaffolds, which are currently often used for support, can lead to biocompatibility problems. This study investigated the feasibility of scaffold-free cartilage to tissue engineer a vascularized neotrachea in rabbits.

Study Design—Animal study.

Methods—Autologous neotracheal constructs were implanted in the abdomen of six New Zealand white rabbits. Auricular chondrocytes were used to engineer scaffold-free cartilage sheets. A muscle flap raised from the external abdominal oblique muscle and the engineered cartilage were wrapped around a silicone stent to fabricate a vascularized neotrachea in vivo. In two of the six rabbits, a full thickness skin graft was used to create an epithelial lining. The constructs were harvested after either 6 or 10 weeks.

Results—All neotracheal constructs were healthy with well-vascularized and integrated layers. The implanted engineered cartilage underwent a remodeling process, forming a solid tracheal framework. Constructs harvested after 10 weeks proved to have significantly better mechanical properties than after 6 weeks and were comparable with the rabbit's native trachea.

Conclusion—Scaffold-free engineered cartilage can successfully fabricate a well-vascularized, autologous neotrachea with excellent mechanical properties. The results suggest that this approach can be used to reconstruct tracheal defects in rabbits.

Keywords

Scaffold-free cartilage; cartilage tissue engineering; tracheal reconstruction; tracheal substitute; engineered trachea

INTRODUCTION

Long-term intubation and tracheostomy are by far the most common causes of tracheal stenosis despite continued improvements in the care of ventilated patients. The treatment of choice for symptomatic benign stenoses is segmental tracheal resection and primary end-to-end anastomosis.¹ Although the success rate of this procedure is approximately 90%, it is not

generally recommended for long-segment stenoses (>50% of total tracheal length in adults or one third in children).²⁻⁴ Long-term tracheotomy is often the only management available to these patients, who would, therefore, benefit greatly from a reliable tracheal substitute.

Tissue engineering presents a promising technique to create a functional tracheal substitute that may overcome many difficulties other tracheal substitutes could not. To achieve a sound tracheal framework, fabrication of large constructs must meet certain basic biomechanical requirements. Most cartilage tissue engineering approaches use biodegradable three-dimensional scaffolds, which are important for neocartilage formation by promoting chondrocyte attachment, cell redifferentiation, and production of extracellular cartilage matrix.⁵ Clinically, engineered cartilage has been used for repair of articular cartilage defects, but until now, no studies have reported successful implantation of engineered cartilage in the head and neck area.⁶ When implanted in an extra-articular environment, scaffolds frequently suffer from poor biocompatibility, which may initiate an immune response and lead to degradation of the engineered cartilage, thereby changing its original three-dimensional shape.⁷

A new method to engineer large cartilage sheets without the use of scaffold material has been developed in this laboratory. The purpose of this study was to investigate the feasibility of using these scaffold-free cartilage sheets for engineering a vascularized neotrachea in a rabbit model and to evaluate its suitability for future tracheal reconstruction.

MATERIALS AND METHODS

Cell Culture

Six New Zealand white adult male rabbits, weighing 3.0 to 3.5 kg and 12 to 14 months of age, were used for harvest of 5×5 mm pieces of auricular cartilage under sterile conditions. The perichondrium was carefully removed to minimize potential contamination with fibroblastic cells. The cartilage was cut into approximately 1 mm^3 pieces, sequentially digested, and expanded in culture, as previously described.⁸ Medium was replaced every 3 to 4 days, confluent plates were trypsinized, and the cells frozen in expansion medium containing 10% dimethyl sulfoxide (Sigma-Aldrich, St. Louis, MO) and stored in liquid nitrogen for the following experiments.

Fabrication of Scaffold-Free Cartilage Sheets

Prior to the bioreactor culture, the chondrocytes were thawed, seeded at $5,000 \text{ cells/cm}^2$, and expanded in 175 cm^2 culture flasks. Nonadherent cells were removed by changing growth media after 4 days. Cells were passaged by standard methods using trypsin after reaching confluence and subcultured. Chondrocytes from the second passage were used to form scaffold-free engineered cartilage. Expanded cells were counted and 5.0×10^7 cells resuspended in 15 mL of chondrogenic defined medium loaded into an OptiCell culture chamber (Biocrystal, Westerville, OH). The medium consisted of Dul-becco's Minimal Essential Medium with 4.0 g/L glucose supplemented with 1% ITS + Premix (BD Biosciences, San Jose, CA), $37.5 \text{ } \mu\text{Mg/mL}$ ascorbate-2-phosphate (Wako Chemicals, Richmond, VA), and 10^{-7} mmol/L dexamethasone (Sigma-Aldrich, St. Louis, MO). In addition, the media contained 2 mmol/L L-glutamine, antimycotic-antibacterial supplements (10,000 U/mL penicillin G sodium, 10,000 $\mu\text{Mg/mL}$ streptomycin sulfate, and 25 $\mu\text{Mg/mL}$ amphotericin B in 0.85% saline), nonessential amino acids, and sodium pyruvate (Invitrogen, Carlsbad, CA), each at 1%. Medium was replaced three times per week. After 3 weeks, the cartilage sheet was transferred into a 150 mm large culture dish where it was allowed to float freely. After 3 weeks in the dish, the sheets had reached adequate strength to be used for neotracheal fabrication (Fig. 1).

Surgical Procedure

Neotracheal constructs were implanted into the abdomen of six New Zealand white rabbits. The animal care committee of Case Western Reserve University of Cleveland, Ohio, approved the protocol. The rabbits were anesthetized by intramuscular injection of ketamine hydrochloride (70 mg/kg) and xylazine (7 mg/kg). The abdomens were shaved and disinfected with 10% povidone-iodine and 70% ethanol and positioned supine. In two of the six animals, a composite neotrachea was produced by using a full thickness skin graft to create an epithelial tracheal lining. After the ear was disinfected, a 3 × 2 cm skin graft was harvested from the base of the cavum conchae, defatted, and wrapped around a silicone tube, epithelial side in, and secured with 5-0 Vicryl. A silicone tube, measuring 30 mm in length by 7 mm in diameter, was used as a template to maintain the C-shape of the engineered tracheal cartilage while it matured in vivo. For implantation of the neotrachea, the abdominal skin was incised midline and the overlying tissue separated from the external oblique muscle. Two parallel incisions, each 3 cm long, were made in the external abdominal oblique muscle, and a bipedicaled flap was raised. The muscle belly of the flap, which acted as a vessel carrier for the tracheal construct, was rolled around the implant and closed with absorbable sutures (Fig. 2). The scaffold-free cartilage sheet was then removed from the culture dish, folded four times (creating 9 layers) to increase total tracheal cartilage thickness and thus provide sufficient structural support, wrapped around the construct once, and secured with 4-0 Vicryl sutures. The neotrachea was then buried under the abdominal skin, serving as coverage and providing the engineered cartilage with a rich blood supply.

Postoperatively, the animals were observed for approximately 2 hours before being returned to their cages, where water and standard feed were available. For the following 3 days, the rabbits were given 10 mg/kg enrofloxacin as a prophylaxis. The animals were monitored for signs of infection and were weighed weekly. After 6 weeks, three of the six implants, including the constructs with the skin graft, were harvested. The remaining three neotracheal constructs were harvested after 10 weeks.

Macroscopic Assessment and Histology

After harvesting the constructs, the silicone tube was removed, and the neotrachea was evaluated macroscopically. Although no formal biomechanical measurements were made, the neotrachea's rigidity and flexibility were evaluated manually by first bending it 45 degrees and then compressing the trachea with forceps until the lumen was completely closed. The trachea was then released and recompressed (10 times) while inspecting for deformation between each cycle. For comparison, native tracheas from rabbits, which were available from a separate study, underwent the same testing. The implants were then fixed in formalin, embedded in paraffin, cut into 5 µm thick sections, and representative slides stained with Mallory's Haidenhein, hematoxylineosin, and safranin-O. The specimens were examined on a bright field microscope (Leika DM6000B, Wetzlar, Germany), and images were recorded.

RESULTS

Clinical Findings

In total, six rabbits underwent abdominal implantation of an engineered neotrachea. All animals survived the experiments, and none showed any wound infection or signs of inflammation at the implantation site.

Macroscopic Assessment and Evaluation of Mechanical Properties

All six neotracheal constructs were viable, and there were no overt signs of necrosis or shrinkage. All layers of the composite neotrachea, including cartilage, muscle flap, and skin

graft, appeared to be healthy and integrated, suggesting that adequate blood supply had developed. All neotracheas retained the luminal shape provided by the silicon tube and were comparable in size with that of a native rabbit trachea (Fig. 3). The two neotracheas in which a full thickness skin graft was used showed distinct hair growth and production of sebum. Because multiple layers were used for fabrication of the neotrachea, one distinct difference was that the total wall thickness was greater than that of the native trachea.

Neotracheas were assessed for their mechanical properties by bending and compression (Fig. 4). Implants that had been in vivo for 6 weeks appeared to be significantly softer than the 10 week samples. After 10 weeks, neotracheal constructs proved to be stiff, yet flexible, for all tested directions and appeared comparable with the rabbit's native trachea. After each compression and bending cycle, the neotracheas immediately regained their original shape, and the cartilaginous framework retained its configuration.

Histology

No signs of acute or chronic inflammation were detected in any of the six specimens. A fibrous capsule containing multiple capillaries and larger blood vessels invading the skin graft had formed in the muscle flap. All layers used for fabrication of the neotracheal construct showed integration. The skin grafts used in two implants contained hair follicles and sebaceous glands, resulting in some sebum production. After 6 weeks in vivo, all cartilage layers appeared to have annealed with each other. The engineered cartilage had undergone a considerable remodeling process because most layers had produced large amounts of matrix and become significantly thicker or had integrated with the adjacent layers with no visible remnants of the original layers (Fig. 5A). However, a few sheet layers remained rather thin and had not produced as much matrix. The cartilage architecture looked very similar to native auricular cartilage, with a typical organization of chondrocytes. After 10 weeks, all cartilage sheet layers had integrated into each other, and a solid tracheal framework up to 2 mm in thickness with a native tissue-like C-shape had developed (Fig. 5B). Moreover, areas where the chondrocytes had become hypertrophic or where small amounts of bone had formed could be seen. In some cases, healthy looking, nonhypertrophic cartilage was located adjacent to mature bone.

DISCUSSION

This rabbit model was designed to determine the suitability of using autologous scaffold-free engineered cartilage sheets for the fabrication of a neotrachea. Six rabbits underwent subcutaneous implantation of a neotracheal construct in the abdomen using a muscle flap for vascularization and internal lining. Viable, integrated, and biocompatible neotrachea was formed, which proved to have a solid, stable, trachea-like framework with excellent mechanical properties, thus suggesting that it could likely be used for segmental tracheal reconstruction.

The problem of tracheal reconstruction in cases where primary end-to-end anastomosis cannot be performed remains unsolved. Many attempts to find a suitable substitute, including allografts and prosthetic materials, have been unsuccessful. The failure is primarily related to graft ischemia, immune rejection, poor epithelization, or chronic infection.² In this light, the ideal tracheal graft material would be a well-vascularized composite graft consisting of autologous tissue, which could provide adequate support and is not subject to rejection. Tissue engineering offers the potential to meet all of these criteria by using the patient's own cells, thus avoiding many adverse affects described for other tracheal substitutes. However, the trachea is a complex organ, and, thus, engineering a functional substitute is a significant challenge.

Cartilage tissue engineering techniques usually rely on scaffolds onto which the cells are applied and then begin the process of redifferentiation. Scaffolds have been researched

extensively in an effort to optimize cartilage formation, biocompatibility, and degradation properties, but none of the investigated materials has proven to be clinically acceptable when implanted extra-articularly. Many biodegradable scaffolds, if used in vivo, cause inflammatory cell invasion, leading to damage and failure of the engineered cartilage implants. Kojima et al.⁹ performed a tracheal reconstruction with an autologous tissue engineered trachea in a sheep model using polyglycolic acid scaffolds, and all animals had to be euthanized within 7 days because of tracheomalacia or stenosis. In our laboratory, we have used a hyaluronan-based scaffold in the past to engineer cartilage implants for laryngotracheal reconstruction in a rabbit model and found that the scaffold had initiated a foreign body immune response, resulting in a degradation of the cartilage implant.¹⁰ A big hurdle for scaffold-free cartilage engineering is that it relies on the cells' intrinsic capability to form a three-dimensional environment, which allows them to redifferentiate and produce extracellular matrix without the assistance of a scaffold. In addition, there is the issue of fabricating the cartilage into different sizes and shapes without using a scaffold to function as a backbone. Therefore, it is clear that an engineered trachea composed of scaffold-free cartilage will face many challenges and as yet unsolved problems.

We recently developed a method to engineer large scaffold-free cartilage sheets measuring 65 × 75 mm with a maximum thickness of only 0.25 mm. To obtain a stable tracheal framework, the cartilage needed to be thicker. To overcome this problem, the cartilage sheets were folded several times prior to being used for fabrication of a neotrachea. In preliminary in vivo experiments (data not shown), we found that to form a mechanically stable structure, it was necessary to increase the cartilage thickness by folding the cartilage sheet four times to create a total of nine layers. Based on preliminary results from 3 and 5 week subcutaneously implanted samples, it was determined that at least 6 weeks would be needed to form mechanically sound cartilage tissue, so neotracheal implants were harvested 6 and 10 weeks postimplantation. As the results show, with increasing time in vivo, the cartilage layers remodeled, integrated, and produced large amounts of extracellular cartilage matrix. After 6 weeks in vivo, the appearance of the engineered cartilage was comparable with that of native auricular cartilage, but, on compression, the tracheal framework was rather soft and most likely not suitable to be used as a tracheal substitute. However, after 10 weeks, a solid tracheal framework with excellent mechanical properties had developed, which was comparable with the rabbit's native trachea. Despite multiple compression and flexion maneuvers during evaluation of the mechanical properties, the neotrachea retained its shape and lumen size, suggesting that it would be feasible for circumferential tracheal reconstruction without instability problems.

Surprisingly, the 10 week neotracheal constructs showed areas where the engineered cartilage had stimulated bone formation. In contrast to mesenchymal stem cells, which have the potential to differentiate into mesenchymal tissue, auricular chondrocytes of rabbits were not expected to produce bony tissue. Auricular cartilage does produce type X collagen, a component of the extracellular cartilage matrix that is associated with endochondral bone formation, yet native auricular cartilage never ossifies, so this result was unexpected. These results suggest that engineered cartilage made from auricular chondrocytes may turn into a hybrid of bone and cartilage when implanted in vivo. However, from a clinical point of view, the bone formation might be advantageous if the goal is to produce a stable substitute for segmental tracheal reconstruction.

To be successful and to obtain an adequate engineered tracheal construct that maintains its shape and size, it is essential to establish a rich blood supply. Many different prefabrication techniques have been described in the past for tracheal reconstruction to provide the tracheal substitute with optimal circulation and to prepare it for second stage tracheal surgery.¹¹⁻¹² In this study, we used a bipedicle flap of the external abdominal oblique muscle as a vessel carrier, which was positioned on the luminal side of the neotrachea. This promoted the

formation of an intrinsic blood supply and helped to avoid central necrosis. If used for reconstruction, a well-vascularized muscle flap would not only help to protect the underlying cartilage from infection but may also provide an adequate substrate for respiratory mucosa to rapidly migrate from the native trachea. Engineering a functional respiratory epithelium remains an unsolved problem; therefore, current methods rely on the migration of ciliated mucosa from either end of the native trachea.¹³ Some authors believe that a surface of respiratory mucosa is desirable for a tracheal substitute, but this may not be essential.² Clinically, muscle and myocutaneous flaps have been used with success in the past for tracheal and laryngotracheal reconstruction.^{14,15} Also, the use of squamous epithelium grafts for tracheal substitutes has been a common approach, and in a recent animal study, transformation of buccal mucosa into ciliated columnar epithelium was observed after 6 months.¹² In our study, two neotracheal constructs were engineered using a full thickness skin graft. After 6 weeks, the skin graft appeared to be integrated and healthy; however, because the engineered cartilage required at least 10 weeks in vivo to become adequately stiff, it is questionable whether the use of a skin graft is a feasible approach for creating a functional composite neotrachea. Any skin graft that is buried subcutaneously for 10 weeks or more will most likely not be able to survive and deteriorate. Also, intratracheal hair growth and sebum production may pose the risk of a tracheal infection and of mucous stasis. Instead, a tracheal substitute consisting of autologous cartilage and vascularized muscle tissue internally, as presented here, may be adequate for reconstruction.

CONCLUSION

This study demonstrates the feasibility of scaffold-free engineering methods to produce cartilage tissue, which can be used to fabricate a vascularized neotrachea. After 10 weeks in vivo, the neotracheal constructs showed excellent rigidity and flexibility very similar to the mechanical properties of a rabbit's native trachea. Further research is necessary to evaluate 1) the long-term development of the neotrachea in vivo and 2) its performance after tracheal reconstruction.

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BIBLIOGRAPHY

1. Grille HC, Mathisen DJ. Surgical management of tracheal strictures. *Surg Clin North Am* 1988;68:511–524. [PubMed: 3375956]
2. Grille HC. Tracheal replacement: a critical review. *Ann Thorac Surg* 2002;73:1995–2004. [PubMed: 12078821]
3. Mitchell JD, Mathisen DJ, Wright CD, et al. Clinical experience with carinal resection. *J Thorac Cardiovasc Surg* 1999;117:39–53. [PubMed: 9869757]
4. Weidenbecher M Jr, Weidenbecher M, Iro H. Segmental tracheal resection for the treatment of tracheal stenose. *HNO* 2007;55:21–28. [PubMed: 16601994]
5. Raghunath J, Rollo J, Sales KM, Butler PE, Seifalian AM. Biomaterials and scaffold design: key to tissue-engineering cartilage. *Biotechnol Appl Biochem* 2007;46:73–84. [PubMed: 17227284]
6. Marcacci M, Berruto M, Brocchetta D, et al. Articular cartilage engineering with Hyalograft C: 3-year clinical results. *Clin Orthop Relat Res* 2005;435:96–105. [PubMed: 15930926]
7. Christophel JJ, Chang JS, Park SS. Transplanted tissue-engineered cartilage. *Arch Facial Plast Surg* 2006;8:117–122. [PubMed: 16549738]

8. Henderson JH, Welter JF, Mansour JM, Niyibizi C, Caplan AI, Dennis JE. Cartilage tissue engineering for laryngotracheal reconstruction: comparison of chondrocytes from three anatomic locations in the rabbit. *Tissue Eng* 2007;13:843–853. [PubMed: 17394383]
9. Kojima K, Bonassar LJ, Roy AK, Vacanti CA, Cortiella J. Autologous tissue-engineered trachea with sheep nasal chondrocytes. *J Thorac Cardiovasc Surg* 2002;123:1177–1184. [PubMed: 12063466]
10. Weidenbecher M, Henderson JH, Tucker HM, Baskin JZ, Awadallah A, Dennis JE. Hyaluronan-based scaffolds to tissue-engineer cartilage implants for laryngotracheal reconstruction. *Laryngoscope* 2007;117:1745–1749. [PubMed: 17690606]
11. Okumus A, Cizmeci O, Kabakas F, Kuvat SV, Bilir A, Aydin A. Circumferential trachea reconstruction with a prefabricated axial bio-synthetic flap: experimental study. *Int J Pediatr Otorhinolaryngol* 2005;69:335–344. [PubMed: 15733592]
12. Suh SW, Kim J, Baek CH, Han J, Kim H. Replacement of a tracheal defect with autogenous mucosa lined tracheal prosthesis made from polypropylene mesh. *ASAIO J* 2001;47:496–500. [PubMed: 11575825]
13. Shi H, Xu Z, Qin X, Zhao X, Lu D. Experimental study of replacing circumferential tracheal defects with new prosthesis. *Ann Thorac Surg* 2005;79:672–677. [PubMed: 15680856]
14. Chen WX, Ruan YY, Cui PC, Sun YZ. Long-term results of the sternohyoid myocutaneous rotary door flap for laryngotracheal reconstruction. *Ann Otol Rhinol Laryngol* 2002;111:93–95. [PubMed: 11800377]
15. Fleischer A, Khafif R. Reconstruction of the mediastinal trachea with a tubed pectoralis major myocutaneous flap. *Plast Reconstr Surg* 1989;84:342–346. [PubMed: 2748748]

Fig. 1. Scaffold-free cartilage sheet after 6 weeks in culture. Prior to implantation, the sheets were folded to create a tracheal framework of adequate thickness.

Fig. 2.

Fabrication of neotrachea. A flap of the external abdominal oblique muscle, serving as the vessel carrier, was raised and rolled around a silicon tube. The engineered cartilage sheet was folded, wrapped around the muscle-silicone construct, and secured with absorbable sutures. To obtain C-shaped tracheal framework, a small gap was left between the cartilage ends. The rigid silicone tube functioned as a template for the lumen as cartilage matured in vivo.

Fig. 3. Cross section of a rabbit's native trachea (left) and engineered neotrachea (right), 10 weeks after implantation. In some areas, the engineered tracheal cartilage was thicker than the native cartilage. M = muscle flap; C = engineered tracheal framework.

Fig. 4. Assessment of the mechanical properties of an engineered neotrachea. After 10 weeks in vivo, flexibility and rigidity, as tested by bending and compression, were excellent and comparable with the rabbit's native trachea.

Fig. 5. Histologic cross sections of the neotracheal construct, 6 weeks (A) and 10 weeks (B) after implantation. With increasing time in vivo, the cartilage sheet layers remodeled and formed a stiff cartilage framework. After 6 weeks in vivo, the engineered cartilage had produced large amounts of extracellular matrix. In most areas, the architecture of engineered cartilage was similar to native auricular cartilage. After 10 weeks, solid, C-shaped tracheal framework had developed; however, in some areas, bony tissue was found adjacent to nonhypertrophic cartilage. M = muscle flap; CS = cartilage sheet; * = remodeled cartilage sheets; B = bone; C = engineered tracheal cartilage.