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Tissue-engineered trachea for airway reconstruction

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Abstract

Introduction—Scaffold-free cartilage has been used to engineer a biocompatible and stable neotrachea that, when implanted into the abdomen, produces a vascularized neotrachea with excellent mechanical stability. The purpose of this animal study was to determine if neotracheal constructs implanted in the neck, could successfully be used for segmental tracheal reconstruction.

Design—Animal study

Material and methods—Auricular chondrocytes from New Zealand White rabbits were used to engineer scaffold-free cartilage. Engineered cartilage and a strap muscle flap were wrapped around a silicone tube and implanted paratracheally. Twelve and 14 weeks post-implantation neotracheas were used to reconstruct a 20mm tracheal defect. In 2 of the 6 rabbits, the neotrachea with its intraluminal situated strap muscle flap was dropped into the defect followed by an end-to-end anastomosis; in 2 animals the muscle flap was partially followed immediately by tracheal reconstruction; and in the remaining 2 animals the muscle flap was partially removed, the silicone re-inserted and the construct temporarily re-implanted to allow formation of a fibrous lining over the exposed cartilage followed by tracheal reconstruction 6 weeks later.

Results—All implanted neotracheal constructs were well vascularized and mechanically sound. Following tracheal reconstruction, none of the animals showed immediate signs of respiratory distress, however, 2 rabbits died after 24 hours due to extensive endotracheal muscle flap edema. The remaining 4 rabbits developed fibrous stenosis of the neotrachea and died after 12 to 39 days.

Conclusion—Tissue engineered neotracheas proved to have adequate stability, but lacked adequate endotracheal lining which lead to neotracheal stenosis.

Introduction

The gold standard for treatment of tracheal stenosis is a simple segmental resection followed by an end-to-end anastomosis [1]. In contrast, long-segmented tracheal stenosis exceeding more than 50% in adults or 30% in infants are usually considered unresectable very often resulting in a tracheotomy as the only alternative long-term treatment option.

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In this light it is evident that the need for tracheal replacement is great. The spectrum ranges from autologous tissue, autografts, allografts, to prosthetic material, all of which carry the risk of immune rejection, poor graft integration, graft ischemia or rejection [2]. Clinically, the use of these prostheses has therefore been limited. Tissue engineering has the potential to overcome many of these problems, however, thus far several hurdles need to be taken to make tracheal tissue engineering successful. In order to fabricate a sound tracheal framework, most cartilage engineering approaches entail the use of absorbable scaffolds, which encourage cell attachment, redifferentiation and cartilage production, but have shown to elicit a foreign body reaction with tissue loss once implanted in an immune competent animal [3]. We recently developed a technique to engineer scaffold free cartilage, which was successfully used to fabricate a biocompatible, autologous neotracheal constructs in rabbits [4]. The constructs, which were implanted up to 12 months, formed a vascularized tracheal substitute with excellent rigidity and flexibility very similar to the mechanical properties of a rabbit's native trachea. Using a similar tracheal engineering approach, the purpose of this study was to determine neotracheal suitability and functionality for segmental tracheal reconstruction in rabbits.

Material and Methods

Cell culture

Six New Zealand White adult male rabbits, weighing 3.0–3.5 kg and 12 to 14 months of age, were used to harvest a 5×5 mm piece 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 mm³ pieces, sequentially digested and expanded in culture as previously described [3]. Medium was replaced every 3 to 4 days, confluent plates were trypsinized, the cells frozen in expansion medium containing 10% dimethyl sulfoxide (Sigma-Aldrich, St. Louis, MO) and stored in liquid nitrogen for following experiments.

Fabrication of scaffold-free cartilage sheets

Prior to the bioreactor culture, the chondrocytes were thawed, seeded at 5,000 cells/cm² and expanded in 175 cm² culture flasks. Non-adherent 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 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 a custom designed bioreactor, which allowed fabrication of 1mm thick scaffold-free cartilage sheets. Briefly, the bioreactor is a 4×4 cm semi-open chamber holding a gas and water permeable membrane. Cells were applied onto the membrane, where they started to form a coherent cell layer, which over the course of 4 weeks, developed a cartilage sheet. Culture medium was changed every other day. The medium consisted of Dulbecco's Minimal Essential Medium with 4.0 g/L glucose supplemented with 1% ITS+Premix™ ([BD Biosciences, San Jose, CA], 37.5 µg/mL ascorbate-2-phosphate [Wako Chemicals, Richmond, VA], and 10⁻⁷ M dexamethasone [Sigma-Aldrich, St. Louis, MO]). In addition, the media contained 2 mM L-glutamine, antimycotic-antibacterial supplements (10,000 U/ml penicillin G sodium, 10,000 µg/ml

streptomycin sulfate, and 25 µg/ml amphotericin B in 0.85% saline), nonessential amino acids and sodium pyruvate (Invitrogen, Carlsbad, CA), each at 1%. After 4 weeks, the cartilage sheet was peeled off the bioreactor membrane and transferred into a 150 mm large culture dish, where they were allowed to float freely. After an additional 4 weeks in the dish, the sheets had reached a thickness of approximately 1mm with adequate strength for neotracheal fabrication.

Surgical procedure

Neotracheal fabrication—A total of six New Zealand White rabbits received a neotracheal construct each, which was implanted paratracheally into the neck. The protocol was approved by the animal care committee of Case Western Reserve University of Cleveland. The rabbits were anesthetized by an intramuscular injection of ketamine hydrochloride (70 mg/kg) and xylazine (7 mg/kg). Their necks were shaved and disinfected with 10% povidone-iodine and 70% ethanol and positioned supine. A midline neck incision was made, strap muscles were identified, a bipediced muscle flap consisting of both sternohyoid muscles raised, wrapped around a 7.5mm wide and 30 mm long sterile silicone tube and secured with 5-0 Vicryl sutures (Fig. 1). Three layers of autologous scaffold free cartilage, each 1mm thick, were wrapped around the silicone-muscle construct leaving a gap at the posterior aspect and secured with 4-0 Vicryl creating a 3 layered construct consisting of a silicone tube, muscle flap and engineered cartilage. The bipediced, sternohyoid muscle flap with its blood supply from superior and inferior served as a vessel carrier for the neotrachea's intrinsic vascularisation. The construct was placed paratracheally and the neck was closed using absorbable sutures. 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.

Tracheal reconstruction—Segmental tracheal reconstruction was performed 12–14 weeks following neotracheal implantation. A group of 2 rabbits underwent tracheal reconstruction at a time to allow modifying the surgical technique if needed. For segmental tracheal reconstruction, the rabbits were anesthetized and prepped as previously described. A midline incision was made, and trachea and neotrachea identified and exposed. The silicone tube was removed and neotracheas manually assessed for adequate mechanical stability prior reconstruction. A 2cm long segment of the cervical, native trachea was resected after identifying the laryngeal recurrent nerve. Care was taken not to penetrate the esophagus. In order to prevent muscle flap compression at the tracheal end-to-end anastomosis, the diameter of the neotracheal framework was designed 1mm larger than the diameter of the native trachea. Upper and lower end-to-end anastomosis was done by using 4-0 Vicryl (Fig. 2). All knots were placed extraluminally to minimize granulation tissue. An airtight anastomosis was ensured and the wound closed with 4-0 vicryl sutures in layers. Rabbits were recovered as described above and antibiotics given as described above. In addition, the animals received a daily dose of 0.3 mg/kg dexamethasone for 5 days. Rabbits were monitored for signs of respiratory distress respiratory. If stridor and nasal flaring were noted, rabbits airway was evaluated under sedation using a 0° rigid endoscope (Karl-Storz, Germany). If necessary, tracheal secretions were suctioned and fibrotic-stenotic segments

dilated using rubber dilators. Based on the postoperative results of group 1, which included flap edema with tracheal obstruction, the surgical technique was slightly modified for the following 2 rabbits (group 2). One side of the sternohyoid muscle flap was resected prior performing the end-to-end anastomosis in order to improve its venous drainage, which resulted in a partially denuded neotracheal cartilage. Both rabbits experienced endotracheal scarring with stenosis. In order to allow a smooth, vascularised, fibrous capsule to form over the denuded cartilage, in the remaining 2 rabbits (group 3) the silicone tube was re-inserted after the partial resection of the muscle flap. Tracheal reconstruction was performed 6 weeks later.

Macroscopic assessment and histology—Tracheas were harvested, fixed in formalin, embedded in paraffin, cut into 5 micron thick sections, and representative slides stained with Hematoxylin-Eosin and Safranin-O. The specimens were examined on a bright field microscope (Leika DM6000B, Germany) and images were recorded.

Results

Clinical Findings

A total of 6 rabbits underwent multi-step tracheal reconstruction, which included fabrication and implantation of engineered neotracheas for 12 to 14 weeks in vivo, followed by segmental tracheal reconstruction. None of the rabbits' neotracheas showed signs of a wound infection and there were no signs of rejection. All neotracheal frameworks maintained their stability and mechanical integrity throughout with no change in shape or size. All 6 animals expired between 1 and 39 days due to tracheal obstruction for the following reasons: rabbits of group I (n=2), experienced venous obstruction of the muscle flap with edema and tracheal obstruction, and died after 24 hours. Rabbits of group II survived 12 and 39 days, and rabbits of group III 14 and 29 days. All 4 rabbits of groups II and III revealed intraluminal fibrosis with a cicatricial stenosis as determined endoscopically (Fig. 3).

Histology

Externally, no signs of acute or chronic inflammation were detected in any of the 6 specimens. A fibrous capsule containing multiple capillaries and larger blood vessels surrounding the neotracheas externally had formed. The engineered cartilage had undergone a considerable remodeling process as the cartilage layers had integrated into each other with a C-shaped tracheal framework up to 2.5 mm thick. In some areas of the engineered neotracheas cartilage looked much like native auricular cartilage with a typical organization of chondrocytes and healthy cartilage. Safranin O staining indicated that the engineered neotracheal cartilage contained less extracellular glycosaminoglycan than native tracheal cartilage. Some smaller areas of the neotracheal framework revealed hypertrophic chondrocytes. There were also areas, where cartilage had turned into bone. In group I, the muscle flap appeared to be edematous resulting in a complete lumen obstruction. In groups 2 and 3, the muscle flap was viable and intact. Fibrous tissue had developed circumferentially within the lumen resulting in a cicatricial stenosis with its point of maximum in the center of

the neotrachea. Respiratory mucosa had migrated into the neotracheal lumen transitioning into a thin layer of non-keratinizing epithelium towards the center of the neotracheal lumen.

Discussion

In a rabbit model, a neotrachea was fabricated by using engineered cartilage derived from autologous auricular chondrocytes combined with a prelaryngeal muscle flap. After 12–16 weeks in vivo a well-vascularised neotrachea with a stable neotracheal framework had developed, which was used to perform segmental tracheal reconstruction. In all cases neotracheas proofed to have adequate mechanical stability to function under physiological conditions, however, all 6 rabbits expired due to tracheal obstruction.

Tracheal defects of more than 1/3 of the total tracheal length in children or 50% in adults present a major problem for clinicians, because effective treatment options are missing. Simple resection is usually not an option since a tension free anastomosis is essential to prevent anastomosis failure or re-stenosis. A large spectrum of tracheal substitutes has been explored over last few decades, all of them with limited success due to complications, such as lack of integration and epithelialisation or immune rejection. Langer and Vacanti's review in 1993 seemed to boost the trend of tissue-engineering approaches in this field [5]. The use of biodegradable matrices has been a common approach for cartilage tissue engineering, however, it has been reported that they can initiate an immune response, if used in vivo, with possible cartilage degradation [3]. As a result tracheal tissue engineering was unable to show long-term survival of the implants [6]. Recently, Macchiarini and colleagues performed a tracheal transplantation in 30-year-old women with left bronchial stenosis. They used a human cadaveric trachea as matrix loaded with autologous chondrogenic mesenchymal stem cells and performed a 6 cm long tracheal reconstruction. Their tracheal implant remained patent for 4 months. The main drawback of their proposed reconstruction is the lack for an attempt to vascularise the construct prior reconstruction since avascular tissue is known for its unpredictable healing and tracheal stenosis may occur.

According to Belsey, the tracheal replacement should have lateral rigidity and longitudinal flexibility, biocompatibility, good integration, and an internal lining consisting of ciliated respiratory epithelium [7]. The proposed factor of tracheal flexibility is of rather relative importance. The main challenge in regards to mechanical requirement of tracheal conduits is to maintain a patent airway and to prevent tracheal collapse. Several studies have studied the mechanics of tracheas or tracheal rings, however, none of them were able to quantify their biomechanical properties relevant to in vivo situations or to predict functionality under physiologic conditions [8]. In the authors' opinion, tracheal substitutes should have sufficient stability to keep the airway patent, but they do not have to meet the biomechanical properties of a native trachea. In this study, neotracheas did not undergo mechanical testing prior reconstruction, however, their in vivo application for tracheal reconstruction revealed that all 6 tracheas had excellent stability. None of the neotracheas collapsed, shrank or showed significant change in their configuration up to 6 weeks following tracheal reconstruction. Tracheal stenosis following tracheal reconstruction is a major concern in particular in a growing child with a small tracheal airway.

As many previous reports on tracheal replacement have pointed out, complete reepithelialisation is a key factor for the longterm success of a functional tracheal substitute. It will allow mucocilliary clearance, protect the underlying cartilage and prevent fibrosis and tracheal stenosis. Since the amount of autologous respiratory epithelium available for transplantation is limited, this neotracheal model would ideally have consisted of tissue engineered tracheal cartilage lined with engineered respiratory mucosa prior to reconstruction. Several experimental studies have shown that it is possible to culture and transplant respiratory epithelium, and maintaining its tissue specific characteristics. However, to date, engineered respiratory epithelium is lacking mechanical stability and until this problem has been solved, tissue engineering of a fully functional tracheal segment remains unsolved [13, 14].

Myocutaneous flaps, such as the rotatory door flap, have successful been used to treat non-circumferential tracheal stenosis. The flap creates an immediate, highly vascularized lining for the airway supporting epitheliasation [9]. Similarly, in this study, a bi-pediced, prelaryngeal muscle flap with a rich superior and an inferior blood supply was used, which was supposed to serve 2 purposes: To encourage intrinsic vascularisation of the neotrachea after implantation, and secondly, to protect the underlying cartilage from infection and to promote rapid epithelial ingrowth from the upper and lower native tracheal ends once used for reconstruction. The strap muscle flap was positioned extraluminally in relation to the native trachea, but intraluminally in regards to the neotrachea. In order to prevent muscle flap compression at the anastomosis during tracheal reconstruction, the diameter of the neotracheal framework was designed 1mm larger than the native trachea. All 6 implanted constructs had developed into viable, vascularized neotracheas with adequate stiffness for tracheal reconstruction. There were no signs of resorption or inflammation. Difficulties were encountered during reconstruction due to the bulk of the flap pedicles, which resulted in torque of the flap vessels while rotating neotracheas of group I into the tracheal defect. Both rabbits died within 24 hours due to muscle flap edema with endotracheal obstruction. This problem was successfully addressed by removing one side of the prelaryngeal muscle flap prior reconstruction, however, a rough endotracheal surface was created resulting in scar formation (group II). In an attempt to minimize fibrosis, a vascularised smooth fibrous capsule was allowed to form over the previously denuded cartilage area prior to tracheal reconstruction (group III), which proofed not to prevent tracheal stenosis as fibrosis developed circumferentially. Due to the rabbit's small tracheal airway tracheal stenosis increased their airway resistance leading to respiratory distress rather early on following reconstruction. All 4 rabbits underwent multiple tracheal dilations without any prolonged success.

Topical application of mitomycin has been reported to reduce cicatricial scarring and has commonly been used as part of the management in patients with tracheal and laryngotracheal stenosis [10]. In experimental studies mitomycin C has been shown to inhibit collagen production and thus fibrosis, however, more recent reports also indicate that it may postpone, but not prevent tracheal stenosis [11]. However, in particular the pediatric patient population may benefit from its application. As the tracheal airflow increase is proportional to the fourth power of the tracheal radius increase, the children's symptoms may improve precipitously with the tracheal growth. Montgomery T-tubes are commonly

used in the setting of tracheal reconstruction to support a reconstructed area or to prevent tracheal stenosis [12]. These tubes, which usually cause little tissue reaction, are often left in place for several weeks and are removed after the initial postoperative healing phase hoping for the fibrosis not to proceed and to mature. Despite their successful application in many cases, it has to be kept in mind that fatal complications including displacement with asphyxia have been reported.

Conclusion

A vascularised and biocompatible neotrachea has been fabricated using engineered, scaffold-free cartilage. Neotracheal constructs proofed to have an engineered, cartilaginous framework with excellent mechanical stability to function under physiologic conditions, however, all 6 rabbits expired due to tracheal obstruction. Tracheal scarring with stenosis remains a major problem for tracheal tissue engineering. Current research is underway to investigate alternatives to provide neotracheal constructs with an epithelial lining.

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Fig. 1.

Paratracheal implantation of a neotrachea. A strap muscle flap, consisting of both sternohyoid muscles, is raised and wrapped around a silicone tube (A). Three layers of scaffold-free, engineered cartilage sheets are folded around the tube-muscle construct (B). Following paratracheal implantation, the intraluminal muscle flap will provide the cartilage with instant blood supply. Once used for reconstruction it will function as a layer to protect the cartilage and promote epitheliasation. T= native trachea, M= strap muscle flap, CS= engineered cartilage sheets, arrow= internal jugular vein and common carotid artery

Fig. 2.

Tissue engineered trachea used for segmental tracheal reconstruction. After 12–14 weeks in vivo, a vascularised neotrachea had formed consisting of a prelaryngeal muscle flap, which lined the stable framework intraluminally (A). The neotracheal framework was designed 1mm larger than the native tracheal lumen, in order to prevent compression of the muscle flap at the anastomosis (B). The neotracheal construct was rotated along the axis of the bipedicle flap into the defect followed by an airtight end-to-end anastomosis. M=pedicle of strap muscle flap, C=engineered neotracheal cartilage, NT=neotrachea, arrow=upper end-to-end anastomosis.

Fig. 3. Endoscopic (A) and macroscopic (B) view of a cicatricial stenosis within the neotracheal lumen 39 days following reconstruction.

Fig. 4.

Histologic sections of the neotracheal construct 29 days following segmental tracheal reconstruction. A significant amount of scar tissue has formed within the tracheal lumen resulting in a pinhole stenosis. There were no signs of an acute or chronic rejection. (A) The longitudinal section reveals purposely chosen difference in diameter between the larger neotrachea and the smaller native trachea. The pedicle of strap muscle flap can be identified as it travels through the anastomosis. Respiratory epithelium has migrated into the neotracheal lumen transitioning into non-keratinizing epithelium towards the stenosis. (B) The cross section at the level of the tracheal stenosis reveals a C-shaped, cartilaginous framework. The engineered cartilage layers have mostly remodeled and formed a stiff, C-shaped tracheal framework. Some areas revealed bony metaplasia. Non-keratinizing epithelium lines the stenosis.

M=muscle flap, arrow=transition point from respiratory to non-keratinizing epithelium, T=native tracheal rings, C=engineered neotracheal cartilage, B=bone.