Autologous tissue-engineered trachea with sheep nasal chondrocytes

Koji Kojima, MD, PhD
Lawrence J. Bonassar, PhD
Amit K. Roy, PhD
Charles A. Vacanti, MD
Joaquin Cortiella, MD

Objective: This study was designed to evaluate the ability of autologous tissue-engineered trachea shaped in a helix to form the structural component of a functional tracheal replacement.

Methods: Nasal septum were harvested from six 2-month-old sheep. Chondrocytes and fibroblasts were isolated from tissue and cultured in media for 2 weeks. Both types of cells were seeded onto separate nonwoven meshes of polyglycolic acid. The chondrocyte-seeded mesh was wound around a 20-mm-diameter × 50-mm-long helical template and then covered with the fibroblast-seeded mesh. In 2 separate studies the implants were placed either in a subcutaneous pocket in the nude rat (rat tissue-engineered trachea) or in the neck of a sheep (sheep tissue-engineered trachea). Rat tissue-engineered tracheas were harvested after 8 weeks and analyzed by means of histology and biochemistry. Sheep tissue-engineered tracheas were harvested from the neck at 8 weeks and anastomosed into a 5-cm defect in the sheep trachea.

Results: Sheep receiving tissue-engineered trachea grafts survived for 2 to 7 days after implantation. Gross morphology and tissue morphology were similar to that of native tracheas. Hematoxylin-and-eosin staining of rat tissue-engineered tracheas and sheep tissue-engineered tracheas revealed the presence of mature cartilage surrounded by connective tissue. Safranin-O staining showed that rat tissue-engineered tracheas and sheep tissue-engineered tracheas had similar morphologies to native tracheal cartilage. Collagen, proteoglycan, and cell contents were similar to those seen in native tracheal tissue in rat tissue-engineered tracheas. Collagen and cell contents of sheep tissue-engineered tracheas were elevated compared with that of normal tracheas, whereas proteoglycan content was less than that found in normal tracheas.

Conclusions: This study demonstrated the feasibility of recreating the cartilage and fibrous portion of the trachea with autologous tissue harvested from single procedure. This approach might provide a benefit to individuals needing tracheal resection.

From the Center for Tissue Engineering, University of Massachusetts Medical School, Worcester, Mass.

Read at the Eighty-first Annual Meeting of The American Association for Thoracic Surgery, San Diego, Calif, May 6-9, 2001.

This study was funded by the University of Massachusetts Medical School and Worcester Foundation for Biomedical Research.

Received for publication June 26, 2001; revisions requested Aug 13, 2001; revisions received Oct 1, 2001; accepted for publication Oct 8, 2001.

Address for reprints: Joaquin Cortiella, MD, c/o Lawrence Bonassar, PhD, Center for Tissue Engineering, Department of Anesthesiology, University of Massachusetts Medical School, 55 Lake Ave North, Worcester, MA 01603-3122 (E-mail: Lawrence.Bonassar@umassmed.edu).

J Thorac Cardiovasc Surg 2002;123: 1177-84

0022-5223/2002 $35.00+0 **12/6/121161**

doi:10.1067/mtc.2002.121161

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Extensive tracheal reconstruction is often required in patients with benign and malignant diseases. Several approaches for tracheal replacement have been described, including the use of autologous tissue,[1] autografts,[2,3] allografts,[4] prosthetic materials,[5,6] or a combination of these approaches.[7] These efforts have met with limited success because of stenosis, immunologic complications, bacterial infections, and material failure. The limitations of synthetic tracheal replacement have led to an interest in regeneration of tracheal tissue. Reconstruction of other cartilaginous structures, such as the ear and nose, has been accomplished with tissue-engineering techniques,[8] but few studies have focused on reconstruction of cartilage in the trachea. Tissue engineering endeavors to combine concepts from biology, fundamental engineering, and polymer chemistry to produce new tissue replacement materi-

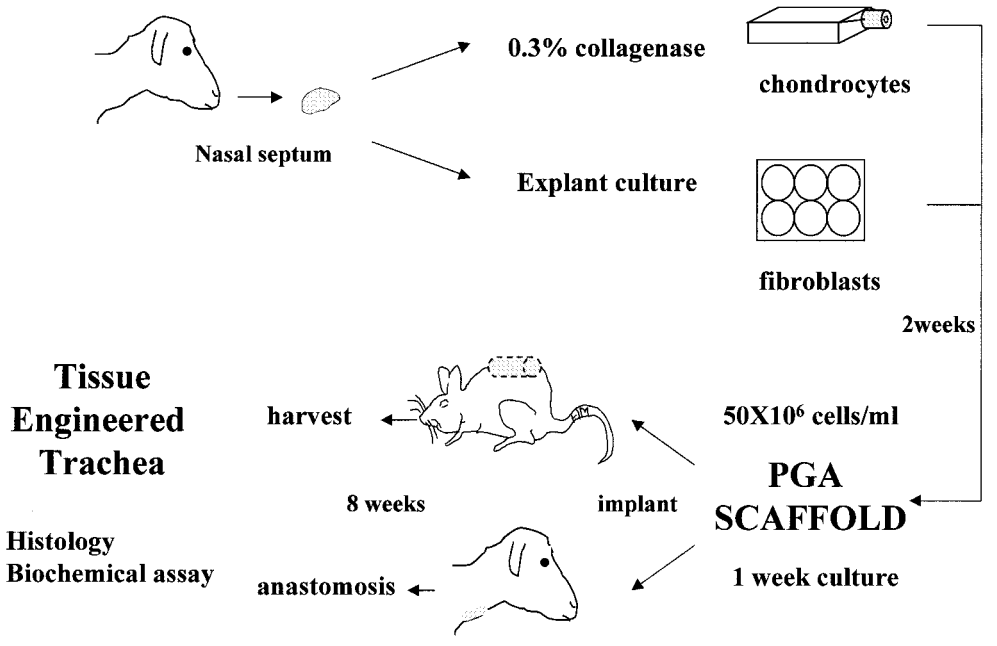

Figure 1. Schematic diagram of methods for isolation, culture, and implantation.

als. The reconstruction of the trachea according to biologic principles makes it desirable to use autologous tissue meeting the requirements of reliability, stability, and biocompatibility. Previously, we demonstrated the replacement of resected rat tracheas with a tube of tissue-engineered cartilage.[9] One of the main limitations in this approach is the specific mechanical requirement placed on the trachea. The trachea must maintain flexibility in the longitudinal direction to allow for free movement of the head, while maintaining the rigidity necessary to prevent collapse of the trachea during breathing. This is accomplished in native tissue by using the cartilaginous rings, which are not adequately modeled by a cartilaginous tube. This study was designed to evaluate the ability of autologous tissue-engineered cartilage shaped in a helix to form the structural component of a functional tracheal replacement.

Materials and Methods

Cell Isolation and Culture

Samples of sheep nasal septal cartilage (5 × 5 mm) were obtained from six 2-month-old sheep (Figure 1). Connective tissue was separated from the underlying cartilage. Chondrocytes were isolated from cartilage by means of digestion in 0.3% collagenase type II (Worthington Biochemical Corp) on a 37°C shaker for 5 to 8 hours. The resulting cell suspension was passed through a 100-μm cell strainer (Becton Dickinson and Co). Fibroblasts were obtained from connective tissue by means of culture of 2 × 2–mm explants.

Chondrocytes were cultured in Ham's F-12 media (Gibco), including 10% fetal calf serum (Gibco) with 292 μg/mL L-glutamine, 10,000 U/mL penicillin G, 10,000 U/mL streptomycin sulfate, 25 μg/mL amphotericin B, and 50 μg/mL ascorbic acid for 2 weeks. Fibroblasts were cultured in Dulbecco's modified Eagle's medium (Gibco), including 10% fetal bovine serum with L-glutamine and antibiotic-antimycotic solution. Culture media were changed every 3 days (Figure 2, *A*). After 2 weeks, once a confluent monolayer was detected, chondrocytes and fibroblasts were harvested by means of digestion with 0.05% trypsin–ethylenediamine tetraacetic acid (EDTA; Gibco). The isolated cells were counted with a hemocytometer, and their viability was determined with use of the trypan blue (Sigma-Aldrich) exclusion method.

Cell Seeding and Implantation

Chondrocyte suspensions were concentrated at 50 × 10^6 cells/mL and placed on a 100 × 10 × 2–mm nonwoven mesh of polyglycolic acid (PGA) fibers (Davis & Geck; Figure 2, *B*). Similarly, fibroblasts were concentrated at 25 × 10^6 cells/mL (Figure 2, *C* and *D*) and seeded onto a 50 × 10–mm PGA mesh. The chondrocyte-seeded mesh was placed in the grooves of a 20-mm-diameter × 50-mm-long helical template made from a Silastic ERTV mold-making kit (Dow Corning; Figure 3, *A*), and the entire template was covered with the fibroblast-seeded mesh (Figure 3, *B*). Two experimental models of tissue-engineered trachea (TET) were designed.

For rat TETs (6 implants), the implants were placed in a subcutaneous pocket in the dorsum of athymic rats, harvested, and analyzed by means of histology and biochemistry. Tissue was assayed for cartilage-specific extracellular matrix components, including proteoglycans and collagen, as well as tissue cell density. For sheep TETs (6 implants), each sheep was anesthetized with ketamine (5 mg/kg) and xylazine (0.05-1 mg/kg), intubated endotracheally, and administered isoflurane by means of a ventilator. A longitudinal incision was made in the neck of the sheep just above the sternocleidomastoid muscle. A pocket was created under this muscle, and implants were derived from cells from each sheep in

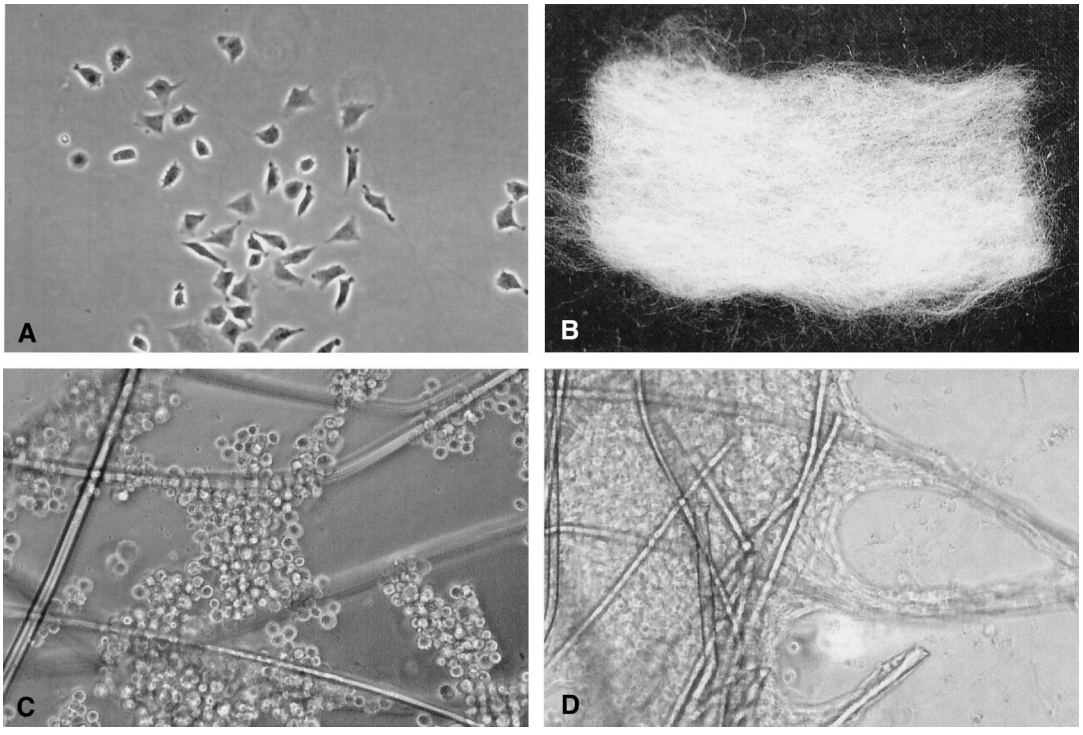

Figure 2. Phase-contrast photomicrograph of sheep nasal chondrocytes in monolayer culture (A). (Original magnification 200×.) Nonwoven PGA mesh used for chondrocyte and fibroblast growth (B). Chondrocytes attaching to PGA on day 0 (C) and growth of cells and matrix on day 7 (D).

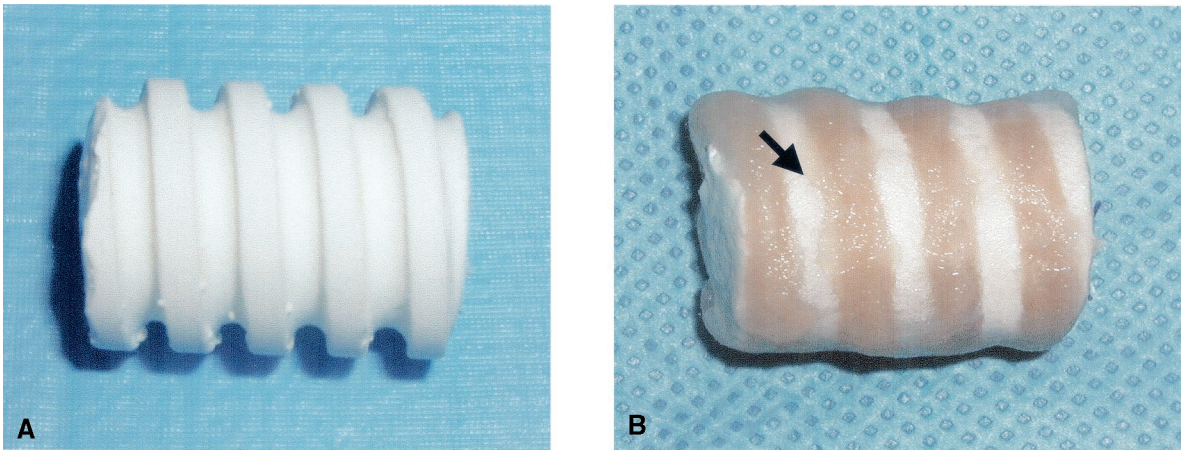

Figure 3. A, Helical template fabricated with a silicone mold-making kit. B, The chondrocyte-seeded matrix was placed in the grooves of the template *(arrow)*, and the entire template was wrapped with the fibroblast-seeded mesh.

the pocket in corresponding sheep. After 8 weeks, each sheep was anesthetized as described above. The autologous TET was harvested from the neck, and the cervical trachea was resected at 5 cm in length. The lungs were ventilated by means of a second endotracheal tube intubated through the operative field. The autologous TET was implanted with end-to-end anastomoses by using 3-0 absorbable running sutures (Figure 4). Postoperatively, each sheep was allowed to recover spontaneously. All animals received humane care in compliance with the "Principles of Laboratory Animals Care" formulated by the National Society for Medical Research and the "Guide for the Care and Use of Laboratory Animals" prepared by the National Academy of Science and published by the National Institutes of Health (publication No. 85-23, revised 1985). All animal procedures

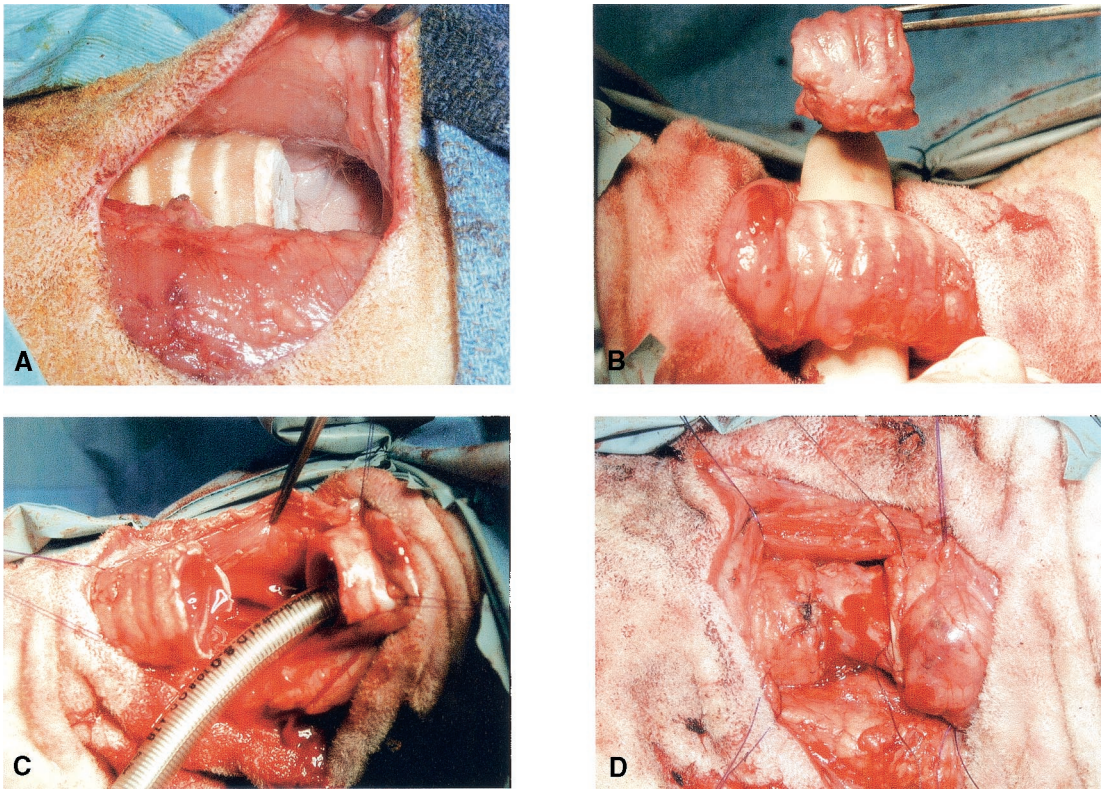

Figure 4. Implantation of TETs in sheep. A, A pocket was created under the sternocleidomastoid muscle, and the implant was placed in this pocket. B, The autologous TET was harvested from the sheep neck at 8 weeks. C, A 5-cm defect in the cervical trachea was created, and the lungs were ventilated with a second endotracheal tube inserted through the operative field. D, The sheep TET was implanted by means of an end-to-end anastomosis.

complied with the guidelines provided by the Institutional Animal Care and Use Committee of the University of Massachusetts Medical School.

Histologic Examinations

The specimens for histology were fixed in 10% phosphate-buffered formation, embedded in paraffin, and sectioned. Sections were stained with hematoxylin-and-eosin (H&E) and Safranin-O.

Biochemical Analysis

Biochemical analysis was performed on harvested tissue to quantify the level of cartilage-specific extracellular matrix components. Samples were digested by means of the addition of 1.0 mL of 100 mmol/L sodium phosphate, 10 mmol/L sodium EDTA, 10 mmol/L cysteine hydrochloride (Sigma), 5 mmol/L EDTA (BDH), and 125 μg/mL papain (Sigma). The samples were incubated at 60°C for 24 hours and stored at −20°C. The sulfated glycosaminoglycan (GAG) content of digests was quantified by means of previously described methods.[10] In brief, 10 μL of papain digest was added to 200 μL of 1,9-dimethylmethylene blue dye at pH 3.0, with absorbencies detected at 525 nm with a spectrophotometer immediately after the addition of the dye. The GAG content of the samples was determined by using a C-6-S from shark cartilage (Sigma) as a standard. The hydroxyproline contents of digests were determined by using the procedure of Stegemann and Stalder.[11] In brief, the papain digests were hydrolyzed with equal volumes of 6N HCL in 115°C for 16 to 24 hours. Chloramine T and *p*-dimethylamino-benzaldehyde were added to hydrolyzed samples, and absorbances were detected at 560 nm with a spectrophotometer immediately after the addition of the dye. The DNA content of samples was determined by quantitating the fluorescence (358/458 nm) of aliquots immediately after mixing with bisbenzimidazole dye (Hoechst 33258) with a fluorimeter.[12]

TABLE 1. Postoperative course of the sheep undergoing tracheal reconstruction with TETs

No.	Postoperative survival period	Trachea status at death	Sheep TET condition
1	7 d	Malacia	Soft
2	5 d	Malacia	Soft
3	2 d	Stenosis	Small
4	2 d	Stenosis	Small
5	2 d	Stenosis	Small
6	2 d	Stenosis	Small

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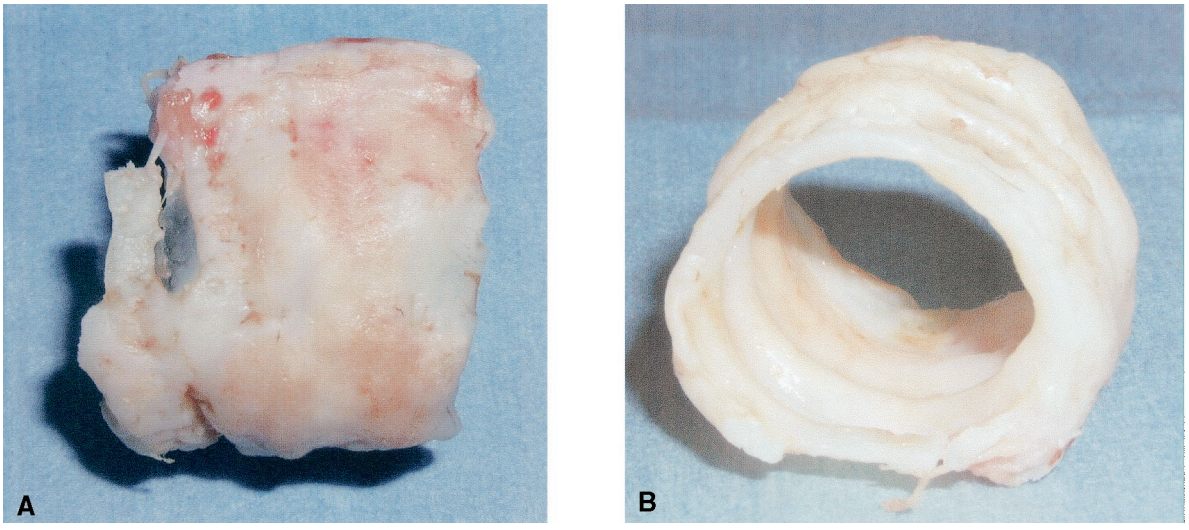

Figure 5. Lateral view (A) and frontal view (B) of a TET harvested from a nude rat at 8 weeks. In both views similarity to native tracheal tissue is evident. There is a distinct structure containing both cartilage and connective tissue, just as in the normal trachea.

Results

Sheep receiving TETs breathed spontaneously with no subcutaneous emphysema and were voluntarily ambulatory. Animal survival time ranged from 2 to 7 days (Table 1). Sheep with the longest survivals were killed as a result of extensive tracheomalacia at day 7. Animals with the shortest survivals were killed as a result of stenosis at day 2.

Gross Morphology

The gross appearance of the TET in both groups 1 and 2 was very similar to that of native trachea. There is a distinct structure containing both cartilage and connective tissue that is similar to that seen in native trachea (Figures 4, *B*, and 5).

Histology

On histologic examination, the engineered tracheal surfaces of both groups were smooth, and formation of cartilage was evident. H&E and Safranin-O stains demonstrated hyaline cartilage closely resembling that of native tracheal cartilage, with similar overall morphology. H&E staining revealed the presence of mature cartilage surrounded by connective tissue, as expected in native trachea. Safranin-O staining showed that tissue-engineered cartilage was organized into lobules with round, angular lacunae, each containing single chondrocytes (Figure 6).

Biochemical Assay

Proteoglycan content of the rat TET was similar to that found in native trachea. In the sheep TETs proteoglycan contents were lower than that found in the native trachea and in rat TETs (Figure 7).

The collagen content of both TETs was similar to that found in the native trachea (Figure 8). Total tissue DNA content was measured to quantify cell density. The cell density in sheep TETs was higher than that in the native trachea and rat TETs (Figure 9).

Discussion

A variety of materials have been used in tracheal prostheses, such as various inert materials alone[5,6] or in combination with autologous tissue,[7,13] but none of them has been effective. There is, however, a general agreement that autologous tissues are the first choice in reconstructive surgery, particularly in tracheal surgery. Several reports have described experimental tracheal reconstruction with autotransplants, but none of them has proven to be satisfactory for human use. However, this procedure is complicated by the need for long-term immunosuppressant therapy and a lack of sufficient donor tissue. Akl and colleagues[14] and Osada and Kojima[15] reported experimental reconstruction of a tracheal defect longer than 10 cm using a rotated main bronchus. The major drawback of this method is loss of part of the lung, and the method is also unable to be used for high mediastinal tracheal defects. Cavadas[1] created a suitable native trachea, which is composed of an autologous microvascular jejunal transfer with a cartilage skeleton for major tracheal reconstruction. This method is ideal but is complicated by the need for a tracheal stent and the need for a 10- to 15-cm jejunum with its vessels.

The goal of our study was to create a completely autologous TET structure composed of a cartilaginous framework with connective tissue by using tissue harvested from a single procedure. This has the potential benefit of facilitating an autologous approach for repair of segmental de-

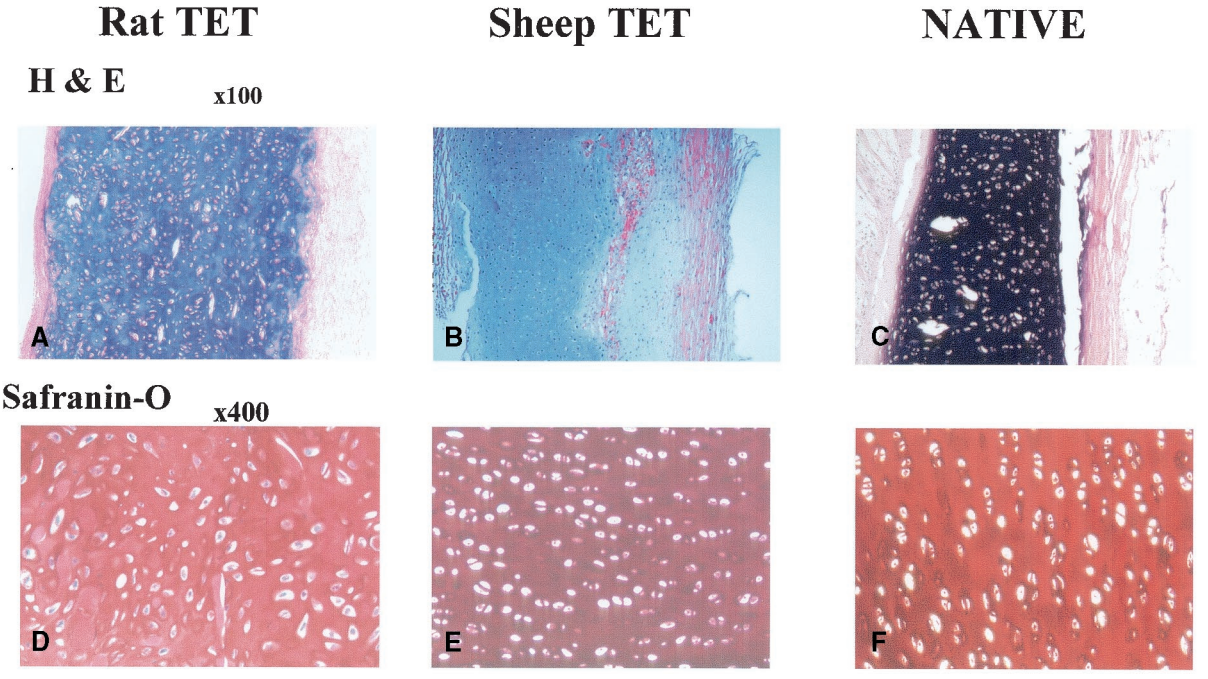

Figure 6. H&E staining: A, rat TET; B, sheep TET; C, native trachea. (Original magnification 100×). Safranin-O stains were deeply positive and indicative of abundant proteoglycan production in each group: D, rat TET; E, sheep TET; F, native trachea.

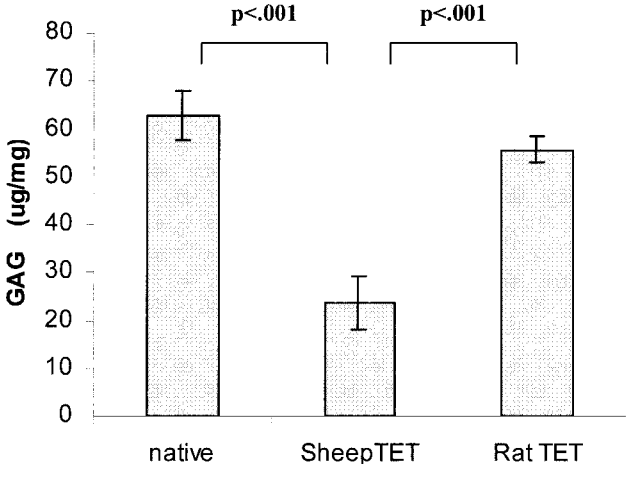

Figure 7. GAG content of native and engineered tissue (data represent n = 6 ± SD). Statistical difference was determined by means of 1-way analysis of variance.

fects. Gross morphology showed that the rat TET was similar to the native trachea and had excellent flexibility.

In contrast, sheep TETs were much less stiff and collapsed quite easily. This is consistent with the tracheomalacia observed in animals after anastomosis. The difference in stiffness between the rat TET and the sheep TET was reflected in the biochemical analysis of these tissues. Sheep TETs had a much lower GAG content than rat TETs. GAG and hydroxyproline are reflective of proteoglycan and collagen levels in these tissues. The presence of GAG in the neomatrix provides quantitative evidence of extracellular matrix production by chondrocytes seeded onto PGA. Because proteoglycans are the major structural component of the cartilage extracellular matrix responsible for tissue stiffness,[16] it is logical that the sheep TET should be less stiff as well. The mechanism behind the decreased proteoglycan content in sheep TETs is not clear, but it likely involves the inflammatory response to the implant. PGA is known to elicit a strong inflammatory response that can inhibit the formation of tissue-engineered cartilage.[17] This response is likely to involve inflammatory mediators, such as interleukin 1 (IL-1). IL-1 specifically is known to induce a degradation of cartilage that results in significant loss of tissue mechanical function.[18] This process is thought to be mediated by the IL-1–induced production of degradative enzyme that is known to alter the mechanical properties of cartilage.[19] Greater understanding of this process would aid in the analysis and design of polymer-based tissue-engineered implants, as well as in the understanding of inflammatory-based diseases of the trachea.

In future studies we intend to construct and use a larger diameter template for the helical portion of the implant. This will compensate for the animal growth that

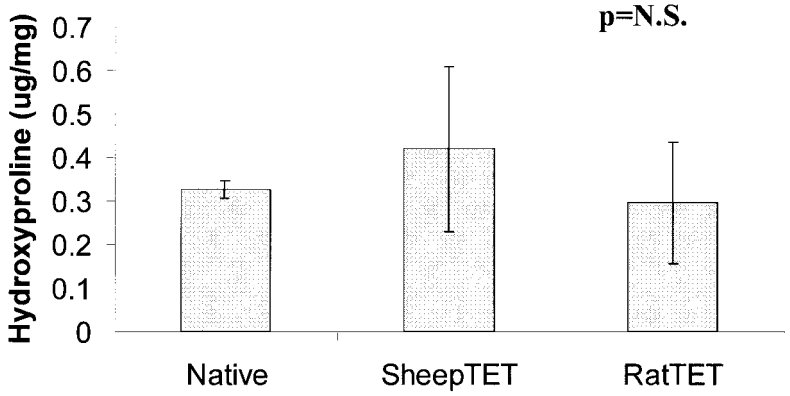

Figure 8. Hydroxyproline content of native and engineered tissue (data represent n = 6 ± SD). Data were not statistically different by means of 1-way analysis of variance. *NS*, Not significant.

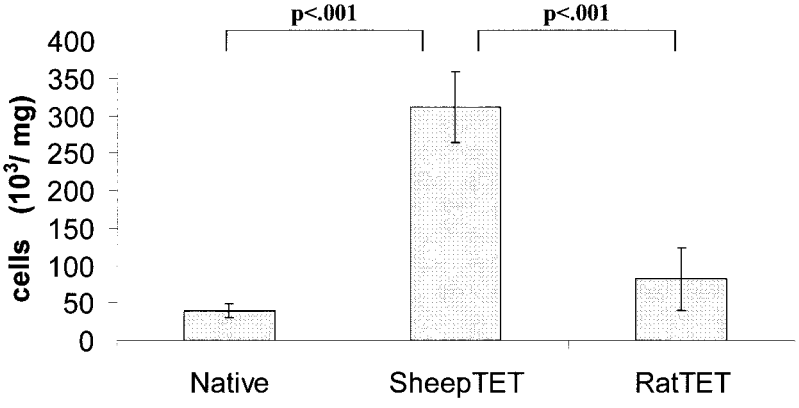

Figure 9. Cell density of native and engineered tissue (data represent n = 6 ± SD). Statistical difference was determined by means of 1-way analysis of variance.

occurs between the initial implantation and anastomosis and should prevent short-term stenosis after surgical intervention. We will also investigate the use of alternative materials as scaffolds for cartilage growth, such as alginate and pluronic. These materials have been shown to be favorable for cartilage growth in autologous models. Furthermore, we plan to maintain the vascular supply to the implant after anastomosis by preserving the surrounding muscle pedicle.

We thank Steve Carr, Carol Lovewell, and Gwenn Gaumon for their expert technical assistance and for preparation of the experiment.

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