of the larynx. Favorable epithelialization on the scaffold with proper reconstruction of original contours was observed in one of three operated dogs. Useful outcomes and other advantageous reconstructive factors may favor the present novel tissue engineering approach over conventional surgical approaches, although further investigations are warranted to further improve the benefits and useful outcomes established so far.

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The Laryngoscope
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Tracheal Regeneration After Partial Resection: A Tissue Engineering Approach

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Objectives: **The aims of this study are to investigate the efficiency of a tissue engineering approach to partial tracheal reconstruction and to improve epithelialization of the reconstructed trachea. The trachea must be resected in some cases of cancer or trauma. Various restructuring techniques are used, with no consensus on the best approach. Two problems that arise when treating tracheal defects by conventional techniques are an inability to regenerate ciliated epithelium at the reconstructed site and having to perform multiple procedures to achieve the desired repair. This study is designed to address these problems.** ***Study Design:*** **Preliminary, an animal experiment.** ***Methods:*** **Surgery was performed on five adult beagles under anesthesia. After the making of a longitudinal cervical skin incision, the trachea was exposed and a circular defect created. A polypropylene and collagen scaffold preclotted with peripheral blood was inserted to the defect site. Postoperatively, the site was evaluated fiberscopically, histologically, and radiographically.** ***Results:*** **All dogs did well postoperatively. Fiberscopic examination showed that the implanted scaffolds were completely covered with regenerated mucosa with capillaries in all cases. Histologic data showed ciliated epithelium regenerated at the operated site from 1 month postoperatively. Newly formed cartilage was detected in the specimens from 8 to 12 postoperative months. Computed tomography images revealed the fine luminal contour of the regenerated site.** ***Conclusions:*** **Good epithelial regeneration was observed after repair of a round tracheal resection using a simple tissue engineering technique, making the technique a good substitute for conventional approaches to tracheal reconstruction in patients with cancer or trauma.** ***Key Words:*** **Tracheal regeneration, tissue engineering, polypropylene, tracheal epithelialization.**

***Laryngoscope*, 117:497–502, 2007**

INTRODUCTION

There are many therapeutic options for reconstructing the trachea after surgical resection following diseases such as cancer, stenosis or trauma, but no perfect way has yet been established. Primary end-to-end anastomosis is generally used for reconstruction after circumferential resection followed by stenotic lesions. It requires anastomotic tension control both intra- and postoperatively.[1,2] The "trough" method is one efficient procedure that enables the partially affected laryngotracheal region to keep an air space inside, but this requires staged and skilled operations.[3,4] Both of these conventional techniques still require improvement. Damage to the donor site or the risk of transmitted diseases from the donor should also be considered when using autologous tissue or allografts. In taking these facts into consideration, prosthetic reconstructions are preferable, although immunoreaction and tissue compatibility have to be overcome.

Remarkable progress has recently been achieved in regenerative medicine. Differentiated tissues and certain organs can now be regenerated, under appropriate conditions, using a tissue engineering technique. This technique was proposed by Langer and Vacanti[5] in 1993 and routinely requires three fundamental components: cells, scaffold, and growth-regulation factors. Of these, the scaffold on which cells are proliferated and differentiated is particularly important for regenerating structured tissues and organs. If an appropriate scaffold and conditions are prepared at the site, regeneration of the tissue would be accelerated even without implantation of cells or regulation factors.[6] Using this concept, we designed an artificial scaffold and applied it to a human case.[7] In the early postoperative period of this case, however, the luminal surface was deformed and nonepithelialized. It is very important to reduce the difference in level of the reconstructed area and to epithelialize its luminal surface at an

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Editor's Note: This Manuscript was accepted for publication November 6, 2006.

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DOI: 10.1097/MLG.0b013e31802e223d

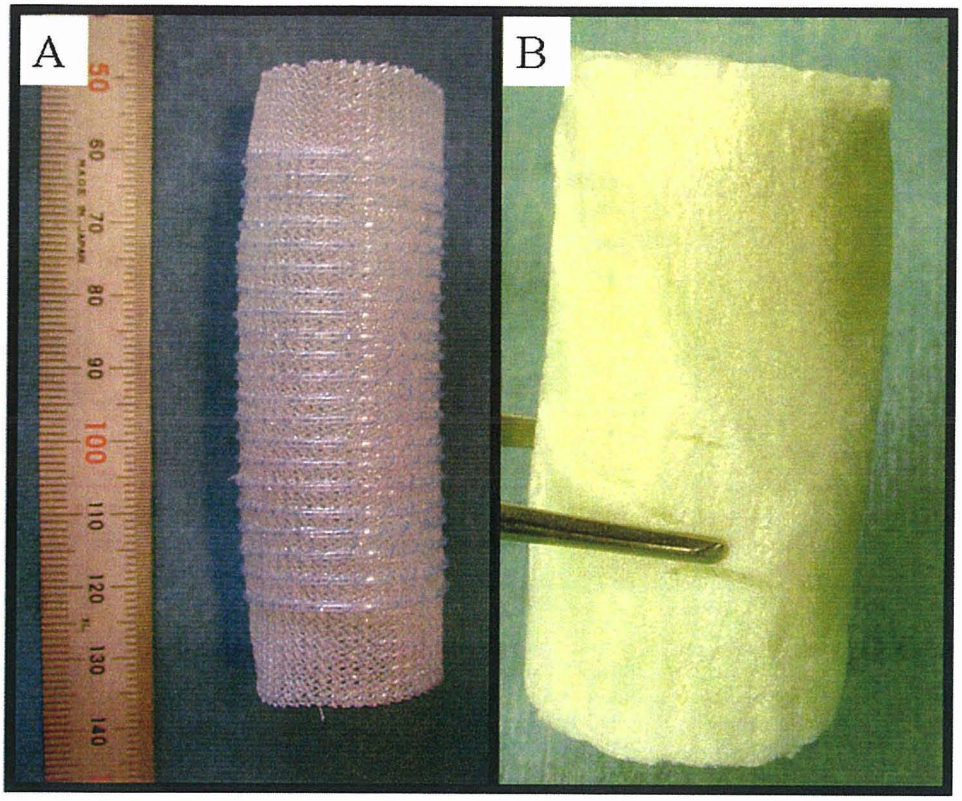

Fig. 1. (A) Polypropylene framework of artificial trachea. (B) Appearance of scaffold after spongy collagen treatment.

early postoperative stage. This study attempts to solve the problems encountered in this clinical application.

MATERIALS AND METHODS

Preparation of Scaffold and Media for Cell Growth

A single sheet of polypropylene mesh with a pore size of 260 μm (Marlex Mesh; CR Bard, Inc., Billerica, MA) was rolled and reinforced by spiral polypropylene strings (Fig. 1A). A 1% porcine dermal atelocollagen (supplied by Nippon Meatpackers, Inc., Ibaraki, Japan) preparation composed of type I (70%) and type III (30%) collagens dissolved in aqueous hydrochloric acid (pH 3.0) was used to coat both sides of this scaffold framework. This spongy collagen (Fig. 1B) would accelerate the cellular attachment and growth into the scaffold. After collagen coating, freeze drying with a freeze dryer (FDU-810, Tokyo Rikakikai Co. Ltd., Tokyo, Japan), and cross-linkage with a vacuum dry oven (VOS-300SD, Tokyo Rikakikai Co., Tokyo, Japan) were applied to the scaffold.

Animals and Surgical Procedures

Tracheal regeneration after partial resection was attempted in dogs with a revised approach, which preserved the mucosa marginally and sutured the scaffold described above to the resected site edge to edge instead of with overlaying (Fig. 2). Animal care, housing, and experimental procedures were conducted according to the Guidelines for Animal Experiments of Kyoto University. Five adult beagle dogs weighing 10 to 12 kg were anesthetized with subcutaneous (SC) injections of ketamine hydrochloride (5.0 mg/kg; Sankyo Co., Ltd, Tokyo, Japan) and xylazine hydrochloride (2.0 mg/kg; Bayer, Ltd., Tokyo, Japan) followed by tracheal intubation. The animals were then anesthetized generally with a mixture of oxygen, nitric oxide, and halothane before creation of a partial tracheal resection of 1.5 cm to 2 cm in diameter with use of a scalpel (Fig. 3A). The tracheal mucosa was preserved marginally along the cartilage defect for the acceleration of epithelialization and the prevention of scaffold dislocation.

A scaffold implant was preclotted with 5 mL of peripheral arterial blood. This resulted in the implant being completely infiltrated with blood and prevented air spaces from causing leakage. The preclotted scaffold was then trimmed to the size of the cartilage defect created during surgery and then was anastomosed to the resected boundaries of the tracheal cartilage with 3-0 absorbable sutures (Vicryl; Ethicon, Inc., Somerville, NJ) (Fig. 3B). The distance between each suture was 4 mm. Ampicillin sodium (Meiji Seika Kaisha Ltd., Tokyo, Japan) was administered (250 mg/animal; SC) for 3 days, followed by daily oral 500 mg doses for 1 week to prevent postoperative infections.

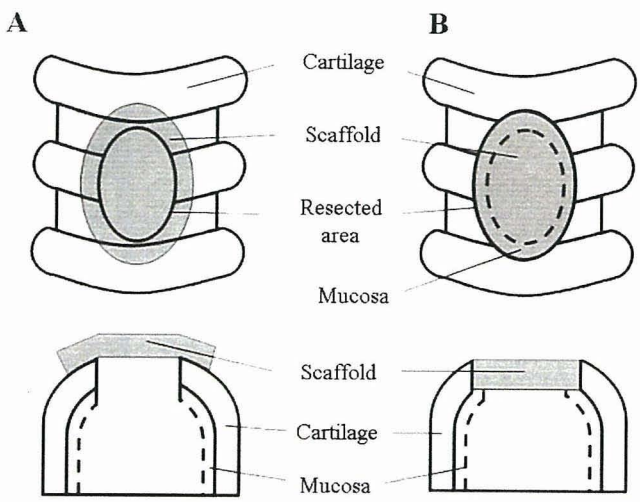

Fig. 2. Schematic of (A) our previous technique and (B) the technique described in this study.

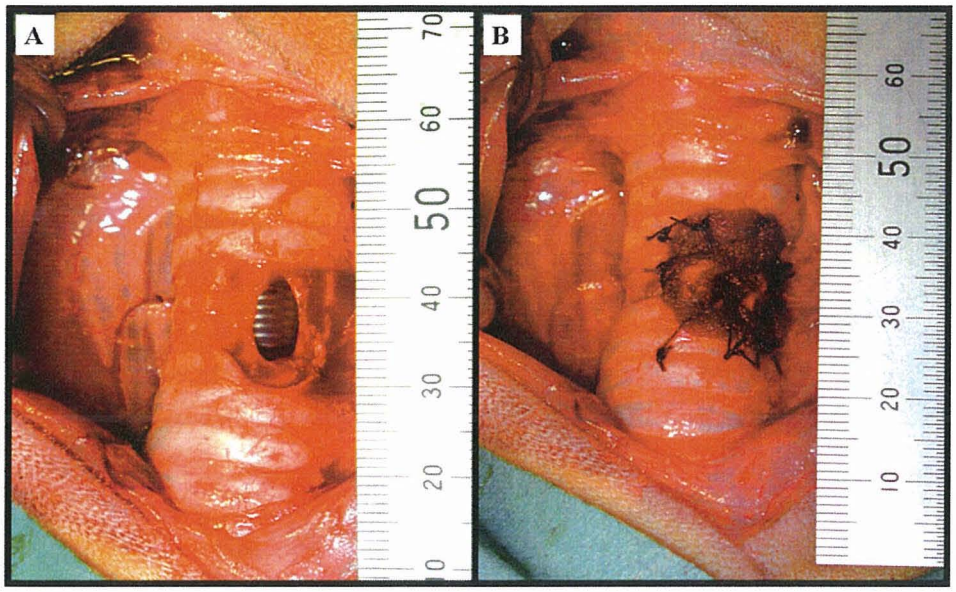

Fig. 3. (A) Patch defect approximately 1.5 cm in diameter was created on cervical trachea by scalpel. (B) Scaffold preclotted with peripheral arterial blood was trimmed and fixed to defect boundaries with bioabsorbable sutures.

Outcome Assessment

Endoscopic examinations to monitor progress of regenerative status were undertaken periodically with a video-endoscopy system consisting of a video bronchoscope (BF type 1T240, Olympus Co., Tokyo, Japan) and a video processor (CV-240, Olympus Co., Tokyo, Japan) with a light source (CLV-U40D, Olympus Co., Tokyo, Japan). Anesthesia was induced with ketamine hydrochloride and xylazine hydrochloride (see above) to facilitate these examinations.

Histologic assessments including light microscopy and scanning electron microscopy were also performed to evaluate the regenerative status of the operated site at various time points up until 1 year postoperatively. One year postoperatively, a three-dimensional image of a dog's trachea was obtained using computed tomography (CT) performed on the operated site using a helical CT scanner system (Legato Duo, GE Yokogawa Medical Systems, Tokyo, Japan).

RESULTS

The postoperative conditions of all dogs were good. None of them showed any signs of infection. Local fiberscopic findings from the five dogs showed no stenosis or

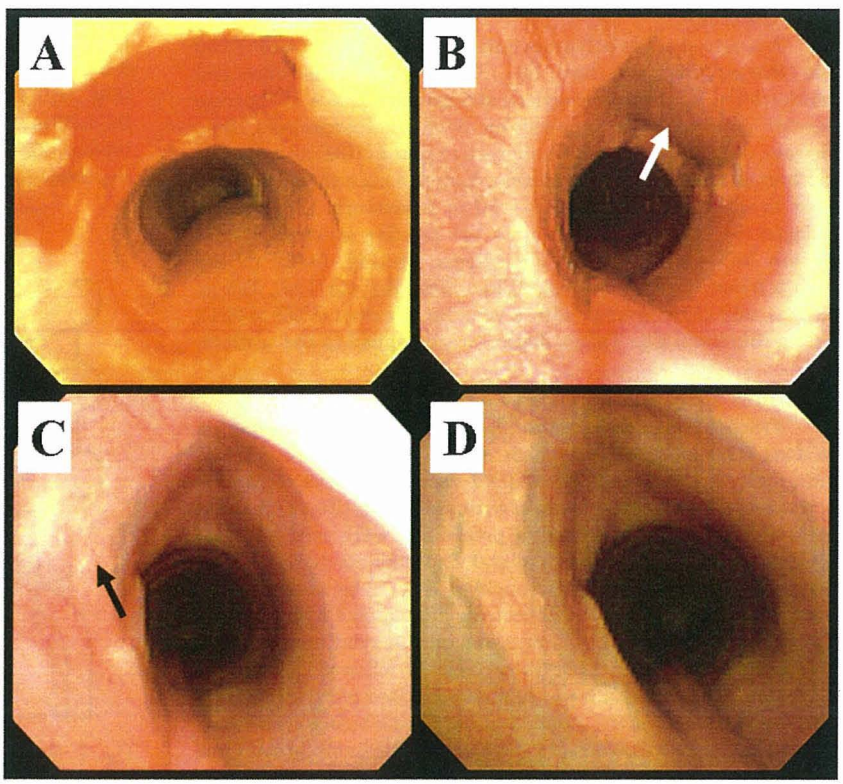

Fig. 4. (A) Preclotted and fixed scaffold implant was a good-fit on tracheal defect in a dog. (B) Implant surface was covered with soft tissues on day 7 after operation. Images 6 months (C) and 1 year (D) after operation in another dog. Implant was completely covered with regenerated mucosa without any granulation or displacement. Good vascularization was also observed in both images.

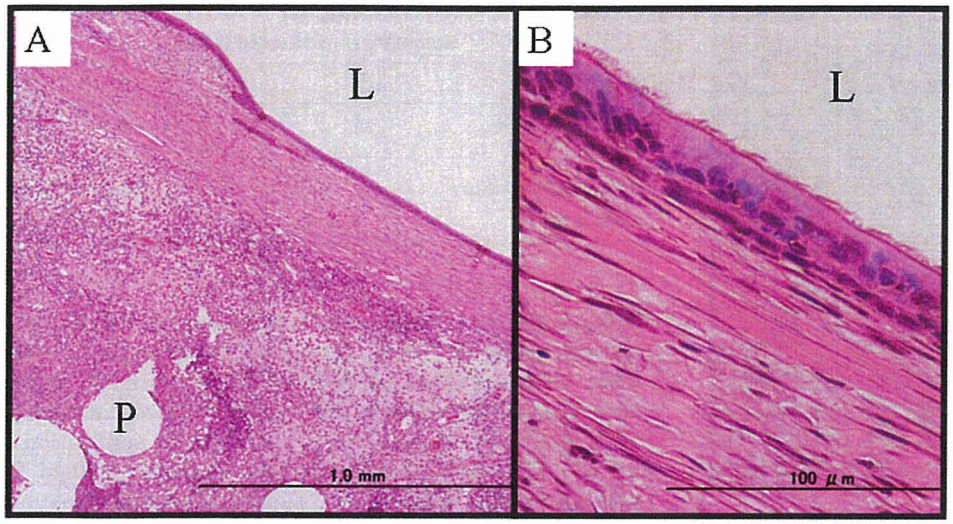

Fig. 5. Hematoxylin-eosin stained images of tissue from central portion of regenerated site 1 month after operation. (A) Many inflammatory cells are seen in submucosa. Luminal surface is completely covered with epithelial cells on layered connective tissue. (B) Ciliated epithelium is clearly observed, although concentration of cilia is low (magnification ×200). L = tracheal lumen; P = polypropylene.

granulation in the regenerated trachea. The implanted scaffolds were completely covered with newly regenerated mucosa with capillaries in all cases.

Figure 4 shows the fiberscopic images from operated dogs with typical luminal portrayal of a successfully reconstructed trachea 1 year postoperatively (Fig. 4D) compared with the tracheal image obtained just after surgery (Fig. 4A). The preclotted and fixed scaffold implant was a good fit on the tracheal defect, and the implant surface was covered with soft tissues on day 7 after surgery (Fig. 4B). Figure 4C and 4D, taken at 6 months and 1 year after surgery, respectively, show that the implant was completely covered with regenerated mucosa without any granulation or dislocation of the scaffold.

Histologic assessments reveal infiltration by many inflammatory cells around the polypropylene framework 1 month after surgery (Fig 5A). The epithelial lining was already complete at the operated site, although the concentration of cilia was low (Fig 5B).

Eight months after surgery, a fine, complete epithelial lining with newly formed immature cartilage and pseudo-ossification was detected (Fig. 6), and proliferation of ciliated epithelial cells with subepithelial glands was observed in light microscopic images (Fig. 7). A scanning electron microscopic image of regenerated cilia is shown in Figure 8. No inflammatory reaction was found in any of the specimens 8 months after surgery. A summary of histologic data is shown in Table I. One year after surgery, axial CT images at the operated sites revealed a fine luminal portrayal of the reconstructed trachea (Fig. 9A), but no obvious cartilage signal was detected in the three-dimensional images reconstructed from fine axial images (Fig. 9B).

DISCUSSION

Various treatment options to reconstruct the trachea after partial surgical resections or structural damage have been pursued for more than 50 years.[8] However, established treatments have remained controversial as surgeons continue to encounter inconsistent outcomes in tracheal reconstructions. Factors contributing to unsatisfactory outcomes include 1) lack of ciliated epithelium, which contributes to autopurification; 2) infection or foreign body reaction;

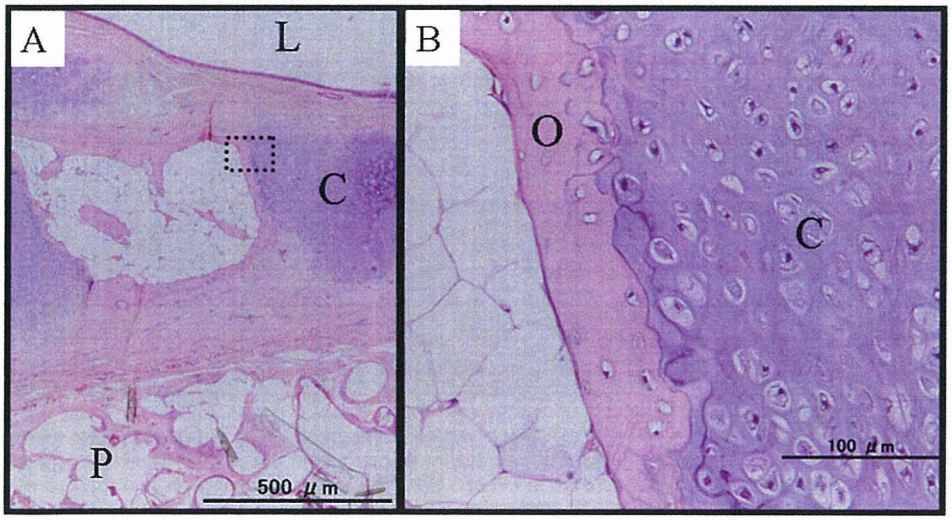

Fig. 6. Histologic images of regenerated tissue 8 months after surgery (hematoxylin-eosin). (A) Luminal surface is covered with epithelium, and an area with newly formed immature cartilage and pseudo-ossification is indicated by dotted area. Polypropylene framework of scaffold is on outer side. (B) Magnification (×100) of dotted area in A; regenerated cartilage with chondrocytes and pseudo-ossification are seen. L = tracheal lumen; C = cartilage; P = polypropylene; O = pseudo-ossification.

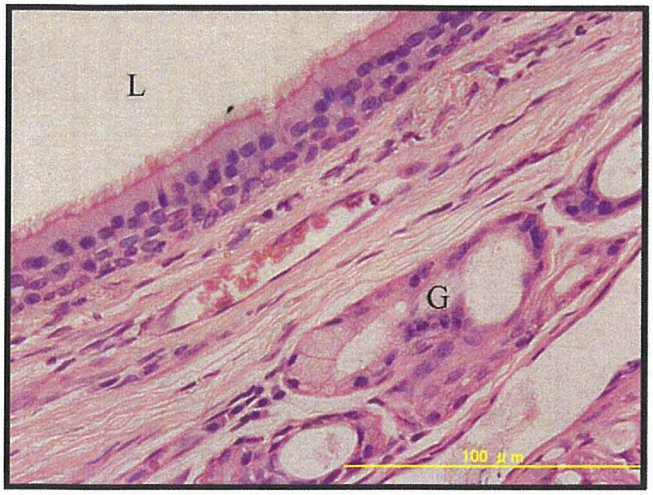

Fig. 7. Microscopic image of regenerated epithelium 8 months after surgery (hematoxylin-eosin). Proliferation of stratified ciliated epithelial cells with subepithelial glands observed. L = tracheal lumen; G = gland.

and 3) repeated surgeries including tracheostomy. Although autologous tissues[9] and homografts[10] have hitherto been used as implant materials for tracheal reconstruction, damage inflicted on the donor site or the risk of donor-recipient disease transmissions warrant a more clinically efficient approach. Moreover, in cases with tumors invading into the trachea, deformities of the reconstructed site render it difficult to monitor the tumor recurrence. Furthermore, unintentionally inflicted cosmetic handicaps in postoperated patients sometimes result in unfavorable psychologic outcomes that eventually defeat the reconstruction effort. A regenerative approach for this area is suitable to overcome these problems.

Regenerative medicine has recently been accepted as a useful clinical discipline that ensures and enhances the quality of life in patients undergoing organ reconstructions. Improved tissue engineering techniques have facilitated successful regenerations of various organs/tissues.

Fig. 8. Scanning electron microscopic image on luminal surface of scaffold. Well-regenerated cilia covers luminal surface of scaffold.

TABLE I.
Summary of Histologic Data.

Months	1	8	10	12
Epithelialization	+	+	+	+
Gland	—	+	+	+
Inflammatory cells	+	—	—	—
Cartilage	—	+	+	+

These techniques rely on three fundamental components: 1) cells acting as "seeds" for tissue regeneration; 2) scaffolds where cells can proliferate and grow; and 3) regulatory factors that mediate cell behaviors. A recent concept in in situ tissue engineering, involving the application of an in vitro innovated porous microcellular scaffold for mediating tissue repair and regeneration processes, has been proposed. Under suitable conditions, this concept would allow for the omission of component 1 or 3 or both, which would be available from tissues surrounding the operated site. Our group has been successful in regeneration of mastoid air cells,[11] cricoid area,[12] and nerves[13,14] solely by using scaffolds.

In the present study, we prepared a novel scaffold made of polypropylene and dermal collagen from porcine skin to regenerate the functional trachea after creating a partial tracheal defect. Because polypropylene is widely used as a polymer with high biocompatibility and feasible morphologic adjustability, it was appropriate for use as the framework material for this novel scaffold.

Using this tracheal prosthesis with porous-type collagen and polypropylene as a scaffold, Okumura et al.[15] have previously achieved favorable outcomes in canine circumferential tracheal regeneration, namely, favorable cellular invasion to intact collagen, proper epithelialization on the luminal side of implants, and complete integration of the scaffold with the recipient's tissue. In the clinic, not many patients require this circumferential resection. Especially in cases of head and neck malignancies, injury to the trachea is usually limited to the wall; therefore, partial tracheal resections are the procedures most frequently performed. Thus, the experimental setup of this study is more appropriate.

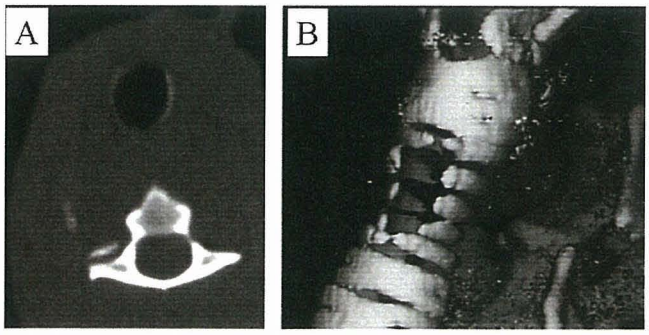

Fig. 9. (A) Axial computed tomography image at operated site reveals fine luminal portrayal of reconstructed trachea. (B) Three-dimensional reconstructed image from fine axial sections shows no marked new cartilage formation.

Omori et al.[7] have demonstrated that this polypropylene scaffold with spongy collagen has sufficient mechanical strength for regeneration in the human trachea. However, better epithelialization and reduction of deformity in early postoperative stages still needs to be achieved. We modified the previous overlaying technique into the current one, which preserved mucosa marginally and sutured the scaffold to the resected site edge to edge.

The results of fiberscopic and histologic assessments in this study revealed the efficiency of this new regenerative approach, especially in the regeneration of ciliated stratified epithelium, which contributes to autopurification of the trachea. Moreover, newly formed cartilage or pseudo-ossification was detected under microscopic examination. However, to achieve structured and layered tracheal regeneration with mature cartilage and muscle tissue, the addition of mesenchymal stem cells or growth-regulation factors may be warranted.[16–19] Once that goal is achieved, then the scaffold framework could be modified such that it is bioabsorbable or biodegradable, thus enabling us to use this scaffold in children with intractable stenotic lesions. Because the outcomes were excellent with this cost-effective tissue engineering technique and less invasive surgical approach described here, our novel scaffold and regenerative technique might be an alternative to conventional surgical approaches in tracheal reconstructions.

CONCLUSION

This preliminary study shows that using a scaffold infiltrated with peripheral arterial blood for tracheal regeneration after partial resection resulted in favorable epithelialization on the scaffold, with proper reconstruction of original contours.

Acknowledgments

The authors thank radiologic technologists Mr. Hirokazu Morimatsu and Mr. Shinya Kitano for their technical help with computed tomography examinations, and Mr. Yoshinobu Toda, a histopathologic technologist, for his advice on histologic assessments.

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TISSUE ENGINEERING
Volume 12, Number 9, 2006

Effect of Fibroblasts on Tracheal Epithelial Regeneration *in Vitro*

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ABSTRACT

Several artificial grafts for covering deficient trachea have been produced through tissue engineering. Recently, our group clinically used an artificial trachea made from collagen sponge for patients with noncircumferential tracheal resection. However, the slowness of epithelial regeneration on the surface of the artificial trachea was confirmed as one particular problem. In this study, we co-cultured tracheal epithelial cells with fibroblasts and examined effects of fibroblasts on epithelial regeneration *in vitro*. Fibroblasts activated epithelial cell proliferation and migration. In co-culture with fibroblasts, epithelial cells reconstructed pseudostratified epithelium, which was composed of ciliated, goblet, and basal cells. Furthermore, a basement membrane was reconstructed between epithelial cells and fibroblasts, and integrin β4 was also observed there. Fibroblasts rapidly increased mucin secretion by epithelial cells. These results indicate that stimulatory effects of fibroblasts on epithelial cell migration, proliferation, and differentiation would reduce the time required for covering of epithelial cells on the defect of luminal surface and hasten regeneration of morphologically and functionally normalized epithelium involving the reconstruction of basement membrane.

INTRODUCTION

IN SOME TYPES OF TRACHEAL DISEASE that cause stenosis, or in the case of malignant tumor of the thyroid gland, larynx, esophagus, or trachea, tracheal resection is required. Recently, several artificial grafts for covering deficient trachea were produced through tissue engineering.[1–4] Nakamura *et al.*[5,6] developed an artificial trachea made from collagen sponge as a scaffold with a spiral polypropylene stent and Marlex polypropylene mesh as a frame. Our group has used it clinically as a patch graft for patients with noncircumferential tracheal resection.[7] However, the slowness of epithelial regeneration on the surface of this artificial trachea is a problem. Tracheal epithelium is indispensable for providing a physical barrier to the external environment and regulates several metabolic functions of airways, including fluid and ion transport to the airway lumen, mucociliary clearance, and airway diameter.[8–10] A deficient epithelium is associated with worsening of clinical symptoms.[11,12] Thus, epithelial regeneration on the collagen sponge of the artificial trachea surface must be completed as quickly as possible.

Epithelial regeneration is achieved through epithelial cell growth and differentiation. Cell growth takes place primarily to cover defective regions and for cell differentiation for the reconstruction of a functional epithelium.[13,14] The tracheal epithelium is mainly composed of ciliated, goblet, and basal cells.[15] These cells play physiological roles to maintain airway homeostasis; the ciliated cells regulate fluid and ion content and remove extraneous particles,[16] the goblet cells produce secretions, such as mucin, which protect the airways against microbial, particulate,

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and chemical toxins that contaminate inhaled air;[17] and the basal cells serve as a pool of progenitor cells that can repopulate a damaged epithelial cell layer.[18] Thus, quick growth and appropriate differentiation of epithelial cells are important to hasten functional epithelial regeneration.

Many researchers have suggested that epithelial-mesenchymal interactions are indispensable for epithelial morphogenesis, homeostasis, and regeneration.[19–22] Epithelial regeneration primarily involves the migration of epithelial cells from the edge of the defects and their proliferation and differentiation to reconstitute normal epithelium with proper functions.[13,14] During this process, the underlying mesenchyme stimulates epithelial growth and differentiation by provision of a suitable biomatrix environment or by synthesis of diffusible factors for epithelial regeneration.[23–26] If these interactions take place, ordinary epithelial regeneration will be completed faster and more properly in the artificial grafts for covering the deficient trachea. Fibroblasts play a key role in epithelial-mesenchymal interactions and exist in the submucosal layer of tracheal epithelium and other mesenchymal tissues. Stimulatory effects of fibroblasts on morphologically normalized epithelial regeneration are observed during wound repair in the skin, oral cavity, and cornea.[20,27,28] It has been predicted that artificial grafts containing fibroblasts would cause epithelial cells to regenerate normalized epithelium. However, in the trachea, the effects of fibroblasts on epithelial regeneration are not known in detail. In the studies reported here, we show effective roles of fibroblasts in tracheal epithelial regeneration *in vitro*.

MATERIALS AND METHODS

Animals

Sprague-Dawley normal or green fluorescent protein (GFP)-transformed rats (8–10 weeks; Tg-act (enhanced GFP) Osb-CZ004, provided by Okabe Masao, Genome Information Research Center, Osaka University, Osaka, Japan) were used for this study. All animals were euthanized using pentobarbital sodium. Studies were carried out in accordance with the guidelines for animal experiments of Fukushima Medical School.

Isolation of tracheal epithelial cells and fibroblasts

The tracheas were removed from normal or GFP-transformed rats, and their lumen was filled with phosphate buffered saline (PBS) containing 0.4% pronase (Sigma Chemical, St. Louis, MO) through cannula and then incubated for 20 h at 4°C. Epithelial cells were collected by flushing the inside of the tracheal lumen with Dulbecco's modified Eagle's medium (DMEM)/F-12 (GIBCO-BRL, Grand Island, NY) supplemented with 10% fetal bovine serum (FBS; JRH Biosciences, Lenexa, KS), 10 μg/mL insulin (Sigma), 5 μg/mL transferrin (Sigma), 25 ng/mL epidermal growth factor (EGF; BD Biosciences, Bedford, MA), 50 nM retinoic acid (Sigma), 100 U/mL penicillin (GIBCO-BRL), 100 μg/mL streptomycin (GIBCO-BRL), and 2.8 g/L bicarbonate of soda ($NaHCO_3$). The epithelial cells collected using centrifugation were resuspended in the growth medium and used for each experiment with or without fibroblasts.

Tracheal fibroblasts were isolated from the tracheas using the method described by Goto *et al.*[29] Briefly, the tracheas, with epithelium denuded using pronase treatment, were minced. The minced pieces were rinsed thoroughly with PBS to remove remaining epithelial cells. Those pieces were then cultured in DMEM containing 10% FBS, and the medium was replaced every 2 days. Fibroblasts migrating from the pieces were harvested using trypsin-ethylenediaminetetraacetic acid (EDTA) solution (GIBCO-BRL). The fibroblasts collected using centrifugation were resuspended and cultured in DMEM containing 10% FBS. Non-spindle-shaped cells were mechanically removed using a Pasteur pipette under a microscope. The confluent fibroblasts were harvested using trypsin-EDTA solution and used for each experiment with epithelial cells.

Three-dimensional co-culturing of tracheal epithelial cells and fibroblasts

Collagen gels containing fibroblasts were prepared using the method described by Yang *et al.*[30] Briefly, type I collagen solution (Nitta gelatin, Osaka, Japan), 5-fold concentrated DMEM/F-12, and reconstituent buffer (25 mM N-2-hydroxyethylpiperazine-n′-2-ethanesulfonic acid, 0.15 M sodium hydroxide) were mixed at the ratio of 7:2:1, and fibroblasts were suspended in reconstituted collagen solution at a density of 10^5 or 5.0×10^5 cells/mL. A suspension of 1.0 mL was poured on a cell culture insert (BD Biosciences) and then incubated at 37°C for 1 h to allow the collagen to gel. The collagen gel was washed with growth medium, and then epithelial cells were seeded on top of the collagen gel at a density of 10^6 per insert and cultured in growth medium for 4 days. On day 4, Matsui's differentiation medium,[31] which consisted of DMEM/F-12 supplemented with 0.5 ng/mL EGF, 10 μg/mL insulin, 5 μg/mL transferrin, 0.2 μM hydrocortisone (Sigma), 10 nM triiodothyronine (Sigma), 50 nM retinoic acid, 30 μg/mL bovine pituitary extract (Sigma), 0.5 μM ethanolamine (Sigma), 0.5 μM phosphoethanolamine (Sigma), 0.5 mg/mL bovine serum albumin (Sigma), 100 U/mL penicillin, 100 μg/mL streptomycin, and 2.8 g/L $NaHCO_3$ was substituted for the growth medium. On day 5, the growth medium on the upper side was removed to establish the air–liquid interface and then cultured for 6 days. All cultures were incubated in a humid chamber at 3% carbon dioxide and 37°C, and the medium was changed every 2 days.

Scanning electron microscopy

The samples, in which tracheal epithelial cells were cultured with or without fibroblasts for 10 days, were fixed in a solution of 2% glutaraldehyde in 0.1 M phosphate

buffer, pH 7.2, washed in several changes of 5% sucrose 0.1 M phosphate buffer pH 7.2, dehydrated, critical point-dried, and coated with gold. Observations of the samples using scanning electron microscopy (JSM-5800; JOEL, Tokyo, Japan) were performed at an accelerating voltage of 10 kV.

Antibodies

The following antibodies were used as primary antibodies for immunological studies: mouse monoclonal antibodies against β-tubulin IV (1:400 dilution, Sigma), keratin 14 (1:400 dilution, Sigma), and MUC5AC (1:500 dilution, Lab Vision Corporation, Fremont, CA) and rabbit polyclonal antibodies against laminin (1:2000 dilution, LSL Corporation, Tokyo, Japan), type IV collagen (1:1000 dilution, LSL), and integrin β4 (1:200 dilution, LSL). Secondary Alexa Fluor 633-conjugated goat anti-mouse (1:400 dilution) and Alexa Fluor 543-conjugated goat anti- rabbit (1:400 dilution) were purchased from Molecular Probes (Eugene, OR).

Immunohistochemistry and analysis

The samples were fixed with 4% paraformaldehyde in PBS, pH 7.4, for 12 h and embedded in optimal cutting temperature compound (Tissue Tek; Sakura, Tokyo, Japan). The frozen sections (5 μm thick) were cut in a cryostat (CM3050; Leica, Solms, Germany). The sections were incubated with blocking solution (Dainipponn Pharmaceutical Corporation, Osaka, Japan) to saturate nonspecific sites. They were then exposed to the primary antibodies for 60 min at room temperature in blocking solution, washed, and exposed to secondary antibodies for 60 min at room temperature in blocking solution. Controls were run by omission of the primary antibody. The images of stained sections were acquired using a confocal laser scan microscope (Eclipse 80i; Nikon, Tokyo, Japan), and the positive cells were counted directly in at least 6 sections that originated from different samples. Anti-β-tubulin IV,[32] keratin14,[18] and MU5AC[33] antibodies were used for markers of cell differentiation as ciliated cells, basal cells, and goblet cells, respectively. Sections stained with hematoxylin-eosin and Alcian Blue (AB)-periodic acid shift (PAS) were observed under a light microscope (BX-51; Olympus, Tokyo, Japan).

Cell migration assay

To measure activity of epithelial cell migration, epithelial cells were cultured on the above-described collagen gels, on which a cover glass 7 mm in diameter was placed, in the growth medium. When the cover glass was removed from the gel, on which epithelial cells had reached a confluence except in the area of the cover glass, epithelial cells then begun to migrate toward the blank area. The images of the migrating areas of epithelial cells were acquired using AxioCam MRc5 (Carl Zeiss, Berlin, Germany), and the ratio of cell migrating surface against the blank area was analyzed using Scion Image β2 (Scion Corporation, Frederick, MD; http://www.scioncorp.com).

Cell proliferation assay

Collagen gels containing fibroblasts that originated from GFP-transformed rats were prepared using the above-described methods. Epithelial cells originating from normal rats were seeded on the gels at a density of 10^4 cells/well and cultured in the growth medium. After 1, 3, and 5 days of culture, the gels were rinsed gently with PBS and treated with collagenase type II (Worthington Biochemical Corporation, Lakewood, NJ) at 37°C for 10 minutes. The cells collected using centrifugation were treated with trypsin-EDTA solution at 37°C for 20 minutes to obtain dissociated cells, and the GFP-negative cells were counted as epithelial cells using a hemocytometer with fluorescent microscope (Axiovert 200; Carl Zeiss).

Mucin analysis

The amount of mucin deposition was measured using enzyme-linked lectin assay (ELLA).[34] The apical side of the epithelial cell layer was washed with PBS. The washing solutions were diluted serially with PBS, and 50 μl of each sample was incubated with bicarbonate-carbonate buffer (50 μl) at 4°C in a 96-well plate (BD Biosciences). After washing with PBS, plates were blocked with 1% bovine serum albumin and 0.1% gelatin in PBS for 1 h at room temperature and incubated for 1 h at 37°C with horseradish peroxidase-conjugated lectin (1 μg/mL, Sigma). Plates were washed 3 times with PBS. Color reaction was developed with 3,3′,5,5′-tetramethylbenzidine peroxidase solution (DAKO Corporation, Carpinteria, CA) and stopped with 1 M sulfuric acid. Absorbance was read at 450 nm. Mucin concentrations of samples were determined with a standard solution of mucin type III (Sigma). Controls were run by omission of the primary antibody.

Statistical analysis

Data are expressed as means ± standard deviation. All statistical significance was verified using analysis with Student *t* test (minimum, n = 4).

RESULTS

Influence of fibroblasts on epithelial cell growth

To study the effects of fibroblasts on cell growth, primary tracheal epithelial cells were seeded on collagen gels that contained a low or high density of fibroblasts, and the outgrowth area of epithelial cells on a blank area of epithelium was measured (Fig. 1A). In the outgrowth areas

of epithelial cells for the first 24 h, there were obvious differences between monoculture and co-culture with fibroblasts. Moreover, the difference between a high density of fibroblasts (5×10^6/well) and a low density of fibroblasts (10^6/well) steadily increased over 5 days.

The epithelial cells in co-culture with a low or high density of fibroblasts increased to 20% and 60%, respectively, above that of the monoculture after 5 days of culture (Fig. 1B). However, although 10^4 cells/well were seeded on the collagen gel, total cell number was approximately one-half after 1 day, indicating that a fraction of seeded cells adhered to the gel, as described by Goto *et al.*[29] Fibroblasts did not influence epithelial cell adhesion to collagen gels because the adherent epithelial cells were almost the same number in the monoculture as in the co-culture with fibroblasts.

Influence of fibroblasts on epithelial morphology

Epithelial cells were cultured at the air–liquid interface with or without fibroblasts in the differentiation medium. After 6 days of air–liquid interface culture, the apical surface of epithelial cells was observed using scanning electron microscopy. In the monoculture of epithelial cells, flat cells with microvilli surrounded a few ciliated cells (Fig. 2A). The ciliated epithelial surface expanded in the presence of a low density of fibroblasts (Fig. 2B). In co-culturing with a high density of fibroblasts, epithelial cells became smaller and showed well-developed cilia (Fig. 2C).

Histological examination of epithelium showed different morphogenesis with and without fibroblasts. In the monoculture of epithelial cells, the thin epithelium was composed of squamous and cuboidal cells, but mucin-secreting cells could not be identified using AB-PAS staining (Fig.3A, D). Epithelial cells in co-culture with a low density of fibroblasts formed a pseudostratified epithelium in some parts of the collagen gel with ciliated cells and mucin-secreting cells (Fig. 3B, E). Although the epithelium in the low-density co-culture was somewhat thicker than that of the monoculture, the shape of the epithelial cells was still cuboidal rather than columnar. A high density of fibroblasts increased the thickness of epithelium 2 to 3 times that in the monoculture of epithelial cells. Epithelial cells formed representative pseudostratified columnar epithelium and AB-PAS positive cells with typical goblet shape lined with ciliated cells in an appropriate manner (Fig. 3C, F).

Influence of fibroblasts on epithelial cell differentiation

To classify differentiated epithelial cells exactly, immunohistochemical study was performed. Beta-tubulin IV, keratin 14, and MUC5AC are specific markers of ciliated, basal, and goblet cells, respectively.[32,33,35] Therefore, we used their antibodies to identify differentiated epithelial cells. Anti-β-tubulin IV antibodies identified immature ciliated and well-developed ciliated cells (Fig. 4A–C). The basal cells, which were stained with anti-keratin 14 antibodies, were on the lower side of epithelium (Fig. 4D–F). In particular, the basal cells were lined up on the basement membrane in the co-culture with a high density of fibroblasts (Fig. 4F, 5C, F). In the co-culture with a low and high

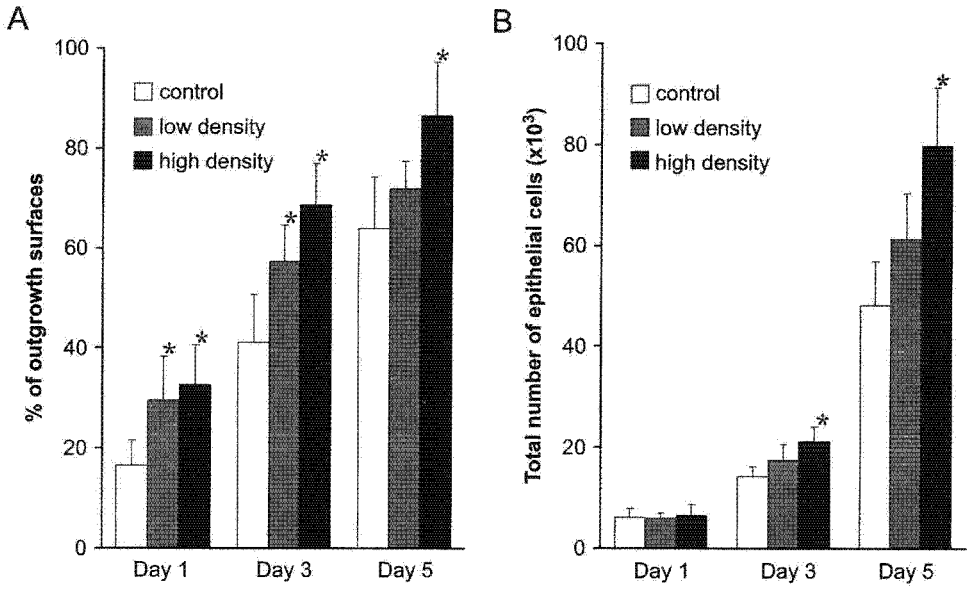

FIG. 1. Influence of fibroblasts on epithelial cell growth. (**A**) Green fluorescent protein (GFP)-negative epithelial cells were cultured with a low (106/well) or high density (5×106/well) of GFP-positive fibroblasts, and the number of GFP-negative cells was counted as epithelial cells. (**B**) Epithelial cells migrated on collagen gels that included a low or high density of fibroblasts; the covering area of epithelial cells was measured. Control shows monoculture of epithelial cells. $*p < 0.05$ compared with control.

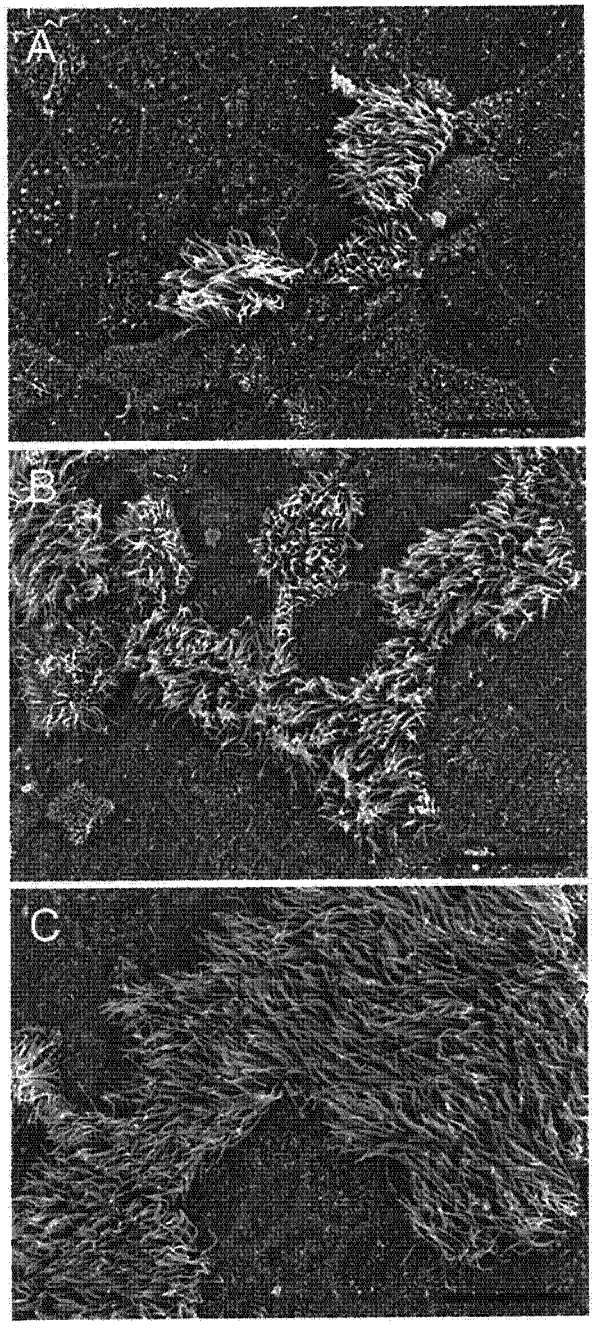

FIG. 2. Scanning electron microscopy of apical epithelial cell surfaces. Epithelial cells cultured on the collagen gel in the absence (**A**) or presence of a low (**B**) or high (**C**) density of fibroblasts for 6 days at the air–liquid interface. Well-developed cilia and expansion of ciliated epithelial surface were observed in co-culture with fibroblasts. Bar = 10 μm.

density of fibroblasts, the goblet cells were on the upper side of epithelium, and the immunoreaction against anti-MUC5AC antibodies was more intense near the apical surface of epithelium (Fig. 4G–I). However, the goblet cells in the monoculture of epithelial cells were scattered throughout the epithelium without the obvious cell polarity observed in the co-culture with fibroblasts.

To elucidate effects of fibroblast on epithelial cell differentiation, the ratios of β-tubulin IV–, keratin 14–, and MUC5AC-positive cells to total cells were examined (Fig. 6). Epithelial cells in the co-culture with a high density of fibroblasts were composed of 39.0% ciliated, 32.0% basal, and 19.4% goblet cells, versus 16.6%, 12.9%, and 3.6% in the monoculture. Fibroblasts facilitated the differentiation of epithelial cells into ciliated, basal, and goblet cells. In particular, a high density of fibroblasts increased the ratio of goblet cells more than 5 times.

Influence of fibroblasts on the reconstitution of basement membrane

Laminin and type IV collagen are major components of the basement membrane. In the monoculture of epithelial cells, both components were scattered throughout the epithelium (Fig. 5A, D). However, in the co-culture with a low and high density of fibroblasts, laminin and type IV collagen were localized at the bottom of the epithelium (Fig. 5B, C, E, F). The positive regions against both antibodies showed a complete boundary between GFP-negative epithelial cells and GFP-positive fibroblasts. Integrin β4, which is well known as an epithelial cell surface receptor of laminin,[36] existed in the surface of epithelial cells without obvious cell polarity in the monoculture (Fig. 5G). However, in co-culture with fibroblasts, integrin β4-positive regions were observed at the bottom of epithelial cell layer (Fig. 5H, I)

Influence of fibroblasts on the production of mucin

To determine whether fibroblasts affect the amount of mucin secretion in epithelial cells, secreted material was collected from the apical surface of epithelium and analyzed using ELLA. In the monoculture and co-culture with a low density of fibroblasts, the amount of mucin secretion increased slowly over time, although a high density of fibroblasts increased secretion rapidly at 7 to 9 days of culture (Fig. 7). The amount of mucin secreted in the co-culture with a high density of fibroblasts was 5 times greater than that secreted in the monoculture and 2 times greater them in the co-culture with a low density of fibroblasts at 9 days of culture. Fibroblasts stimulated mucin secretion from epithelial cells. However, at 9 to 11 days of culture, the amount of mucin secreted did not increase in co-culture with a high density of fibroblasts.

DISCUSSION

Many researchers have reported reconstitution systems for tracheal epithelium. Some of them use other kinds of cells for the reconstitution of the tracheal epithelium. Goto *et al.* seeded tracheal epithelial cells on the upper side of a human amnion membrane and fibroblasts on the lower side

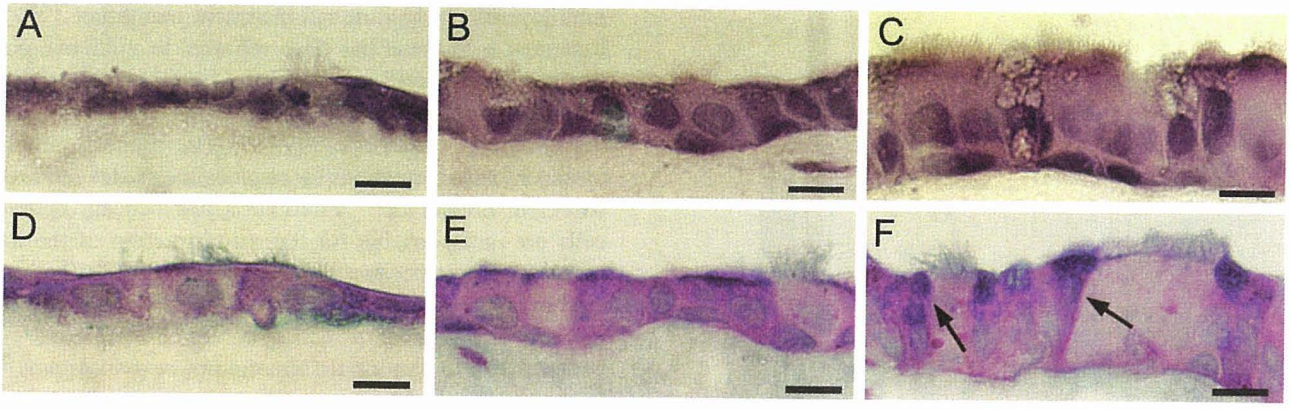

FIG. 3. Hematoxylin-eosin (**A–C**) and Alcian Blue-periodic acid shift (**D–F**) staining of epithelial cells cultured on the collagen gel in the absence (**A, D**) or presence of a low (**B, E**) or high (**C, F**) density of fibroblasts for 6 days at the air–liquid interface. Arrows show well-developed goblet cells. Bar = 10 μm.

of the membrane.[29] Le Visage *et al.* co-cultured epithelial cells with mesenchymal stem cells.[36] However, their culture system used amnion membrane or the transwell insert as a basement membrane, respectively, and epithelial cells did not contact underlying cells directly. In this study, we seeded epithelial cells directly on collagen gels containing fibroblasts to create proper epithelial–mesenchymal interactions *in vitro*. This culturing showed several stimulatory effects of fibroblasts on epithelial cells in the regeneration of normalized tracheal epithelium.

In the presence of fibroblasts, epithelial cell migration and proliferation were activated, and the time required for the covering of epithelial cells was faster than in the absence of fibroblasts. Stimulatory effects of fibroblasts were also confirmed in epithelial cell differentiation to ciliated, basal, and goblet cells. Fibroblasts secrete various growth factors, such as keratinocyte growth factor, EGF, and hepatocyte growth factor during wound repair.[37,38] Stimulatory effects of these growth factors on epithelial cell migration, proliferation, and differentiation have been reported.[39,40] In the trachea, keratinocyte growth factor has been identified that stimulates the proliferation and differentiation of epithelial cells and modulates basal expression of mucin genes, interacting with retinoic acid in a concentration-dependent manner.[41,42] EGF activates epithelial cell migration.[43] Hepatocyte growth factor influences the differentiation of epithelial cells through the activation of c-met at the basolateral surface of these cells.[44] Furthermore, Goto *et al.* showed that the condition medium of fibroblasts could stimulate epithelial cell differentiation, in particular that of goblet cells.[29] The same results were obtained in this study. Fibroblasts dramatically increased the ratio of goblet cells to total cells. These observations indicate that growth factors secreted from

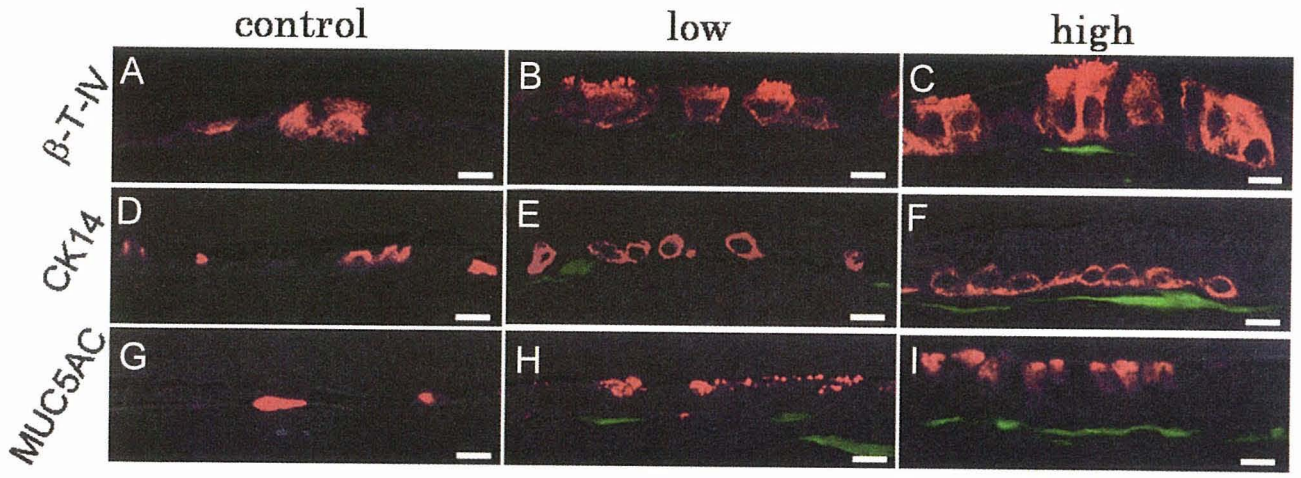

FIG. 4. Immunofluorescent staining of epithelial cells cultured on collagen gel in the absence (**A, D, G**) or presence of a low (**B, E, H**) or high (**C, F, I**) density of fibroblasts for 6 days at air–liquid interface. Identification of ciliated, basal, and goblet cells was confirmed using anti-β-tubulin IV (β-T-IV, **A–C**), keratin 14 (CK14, **D–F**), and MUC5AC (**G–I**) antibodies, respectively. Red represents positive reaction against the above-mentioned antibodies. Green represents green fluorescent protein–transforming fibroblasts. Bar = 10 μm.

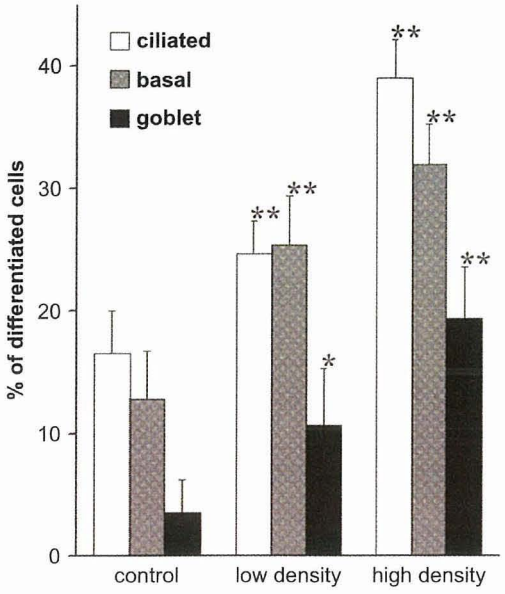

FIG. 5. Immunofluorescent staining with anti-laminin (LN, **A–C**), type IV collagen (IV col, **D–F**), and integrin β4 (β4, **G–I**) antibodies showed the reconstruction of basement membrane in monoculture (**A, D, G**) and co-culture with a low (**B, E, H**) and high (**C, F, I**) density of fibroblasts. In the 3-color images, red represents the positive reaction against the above-mentioned antibodies, green represents green fluorescent protein–transforming fibroblasts, and blue represents nuclei stained with 4′,6-diamidino-2-phenylindole. Bar = 10 μm.

fibroblasts stimulate epithelial cell behaviors, which are closely related to the time required for covering of epithelial cells on defects on the luminal surface on the artificial trachea.

Morphologically normalized epithelial regeneration requires underlying mesenchyme.[19–22] In the presence of fibroblasts, keratinocytes or oral epithelial cells reconstruct a multilayered epithelium and basement membrane.[27,45] The basement membrane has been reported to influence polarization, differentiation, migration, and proliferation of epithelial cells and plays an important role in maintaining structural tissue integrity.[46,47] The proper epithelial–mesenchymal interactions for organogenesis also require the basement membrane.[48] Fibroblasts just beneath epithelial cells are indispensable for the reconstruction of the basement membrane because the reconstruction of the basement membrane requires deposition of products of fibroblasts.[49,50] Mutations affecting basement-membrane components lead to abnormal formation of several organs.[51,52] In this study, fibroblasts existed just beneath epithelial cells (direct contact between epithelial cells and fibroblasts was not confirmed). A pseudostratified epithelium and basement membrane were observed in the presence of fibroblasts. Major components of the basement membrane, laminin and type IV collagen, were co-localized at the bottom of the pseudostratified epithelium, and the reconstituted basement membrane separated fibroblasts from the epithelial layer. In the absence of fibroblasts, the pseudostratified epithelium and apparent basement membrane were not observed. These results suggested that fibroblasts just beneath epithelial cells could play an important role in the reconstruction of pseudostratified epithelium and basement membrane.

In the presence of a high density of fibroblasts, differentiated cells showed apparent cell polarity and proper arrangement in a pseudostratified epithelium. The ciliated cells grew well-developed cilia over the apical surface. The goblet cells showed intense positive reaction against MUC5AC near the apical surface. ELLA also confirmed the increase of mucin secretion by fibroblasts. Cilia regulate fluid, and removal of extraneous particles and mucin protects the trachea surface from microbial, particulate, and chemical toxins *in vivo*.[16,17] These observations indicate that ciliated and goblet cells in co-culture with fibroblasts would have the ability to maintain the trachea surface.

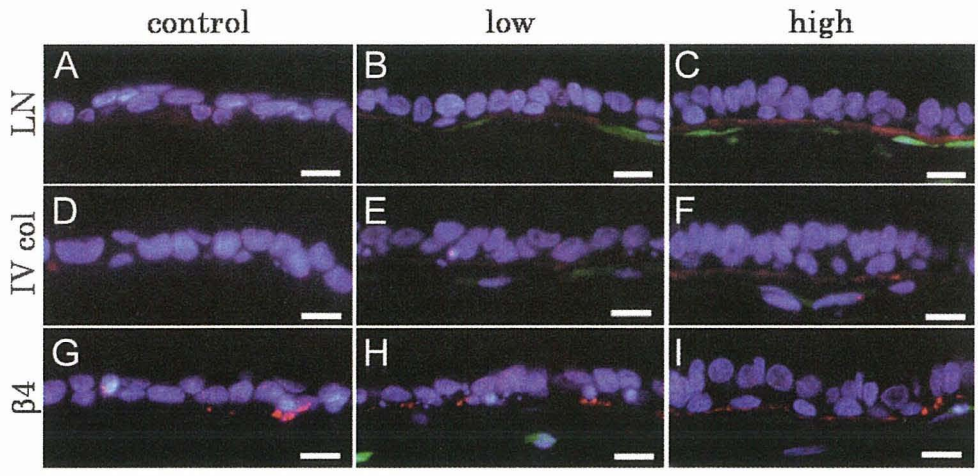

FIG. 6. The ratio of differentiated epithelial cells to total cells in monoculture (control) and co-culture with a low or high density of fibroblasts was measured. $*p < 0.05$ and $**p < 0.05$ compared with the control.

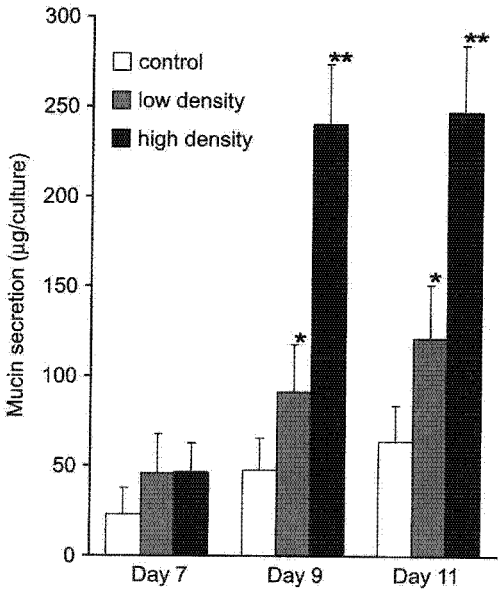

FIG. 7. Influence of fibroblasts on mucin secretion. Apical secretion was collected and quantified using enzyme-linked lectin assay. $*p < 0.05$ and $**p < 0.05$ compared with the control.

Furthermore, the basal cells were lined on the basement membrane and expressed integrin β4 at the bottom surface of the cell. The integrin β4 has been known to be located in hemidesmosomal structures that mediate stable adhesion of epidermal cells to the underlying basement membrane component laminin and regulate signaling cascades that control proliferation of basal cells through a mechanism independent of its adhesive function.[53] The basal cells in the co-culture with fibroblasts might stabilize the epithelium and function as progenitor cells, but in the absence of fibroblasts, goblet and basal cells did not show obvious cell polarity, and ciliated cells did not have well-developed cilia. These results suggested that fibroblasts stimulate functionally normalized epithelial regeneration.

In summary, our studies showed the stimulatory effects of fibroblasts on epithelial cell migration, proliferation, and differentiation, indicating that fibroblasts would reduce the time required for covering of epithelial cells on the defect of the luminal surface on the artificial trachea. Effects of fibroblasts on epithelial cell migration and differentiation seemed to be greater and sooner than effect on cell proliferation. Moreover, fibroblasts played a key role in the regeneration of morphologically and functionally normalized epithelium involving the reconstruction of the basement membrane. Fibroblasts can be seeded into our artificial trachea made from collagen sponge with or without collagen gel (data not shown). These findings suggest that fibroblasts are useful in hastening normalized epithelial regeneration on the collagen sponge of an artificial tracheal surface.

ACKNOWLEDGMENTS

This study was financially supported in part by a Health and Labor Science Research Grant for Research on Human Genome, Tissue Engineering, from the Ministry of Health, Labor and Welfare of Japan (200400069A).

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Tissue Engineering for Regeneration of the Tracheal Epithelium

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Objectives: The slowness of epithelialization on the artificial trachea that has been successfully used in humans is a problem. The purpose of this study was to develop a way to regenerate the epithelium on the surface of this artificial trachea.

Methods: In an in vitro study, isolated rat tracheal epithelial cells were seeded on a collagenous gel that was stratified on a collagenous sponge. Histologic and immunohistochemical examinations were made. In an in vivo study, we transplanted grafts with green fluorescent protein–positive tracheal epithelial cells onto the tracheal defects of normal rats. At 3, 7, 14, and 30 days after the operation, histologic and immunohistochemical examinations were made.

Results: In the in vitro study, the 3 layers — the epithelium, gel, and sponge — could be observed. The epithelium expressed cytokeratin 14, cytokeratin 18, and occludin. In the in vivo study, the artificial trachea was covered with epithelium at 3 days after operation, and then the epithelium differentiated from single- or double-stratified squamous epithelium into columnar ciliated epithelium. Green fluorescent protein–positive cells were found 3 days after operation.

Conclusions: We believe that the method used in our experiment is an effective way to regenerate the epithelium on the surface of an artificial trachea. With further experimentation, this method should be suitable for clinical application.

Key Words: regeneration, tissue engineering, trachea, tracheal epithelium.

INTRODUCTION

It is often necessary to resect tracheal lesions and to reconstruct the resulting defects for patients who have various types of tracheal disease that cause stenosis or who have a malignant tumor of the thyroid glands, larynx, esophagus, or trachea. Conventionally, autogenous tissues such as skin, nasal septal cartilage, or auricular cartilage have been used to patch noncircumferential defects. Circumferential defects have usually been reconstructed by end-to-end anastomosis, but there are no proven methods of reconstruction of tracheal defects when end-to-end anastomosis is considered impossible because of a lack of effective long-term grafts or material to replace the defects. Production of grafts for tracheal reconstruction is very important. Several articles have reported the regeneration of the trachea through tissue engineering. Teramachi et al[1] and Nakamura et al[2] developed an artificial trachea made from collagenous sponge as a scaffold and a spiral polypropylene stent and Marlex polypropylene mesh as the frame. It has been clinically used by our group[3] as a patch graft for patients with noncircumferential tracheal resection. However, the slowness of epithelialization on the surface of the artificial trachea is a particular problem. The purpose of this study was to develop a way to regenerate the epithelium on the surface of an artificial trachea to be used in tracheal reconstruction. In an in vitro study, we made a graft by covering the same collagenous sponge used in the artificial trachea with a collagenous gel layer that was then covered with a regenerated tracheal epithelium. In an in vivo study, the grafts were transplanted into the tracheal defects of rats.

MATERIALS AND METHODS

In Vitro Study. Our animal care, housing, and surgical procedures followed the Guidelines of the Animal Experiment Committee, Fukushima Medical University. For the study, all rats were painlessly sacrificed by inhalation of diethyl ether and intravenous in-

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Presented at the meeting of the American Broncho-Esophagological Association, Boca Raton, Florida, May 13-14, 2005. Dr Nomoto received Second Place in the Steven Gray Resident Research Award.

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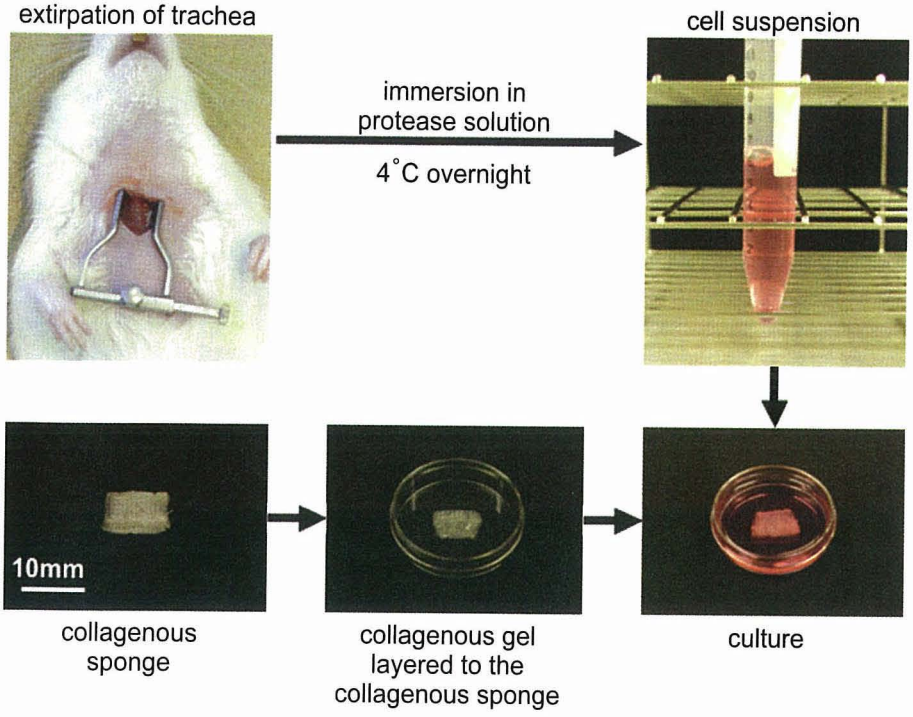

Fig 1. Procedure for making graft with collagenous sponge, collagenous gel, and tracheal epithelial cells.

fusion of a lethal dose of pentobarbital sodium.

The tracheas were harvested from wild-type 9-week-old male Sprague-Dawley rats (Fig 1), and the tracheal epithelial cells were isolated through overnight immersion in a protease solution at 4°C. After flushing, we suspended the epithelial cells in Dulbecco's modified Eagle's medium (Gibco, Invitrogen, Carlsbad, California) with 10% fetal bovine serum, penicillin G, streptomycin, and amphotericin B (antibiotic-antimycotic, Gibco). In order to make a flat surface, we layered collagenous gel on the collagenous sponge used in the artificial trachea. Collagen gel was made from type I-A collagen (Cellmatrix Nitta Gelatin Inc) and sterile reconstitution buffer (2.2 g sodium hydrogen carbonate in 100 mL of 0.05N sodium hydroxide and 200 mmol/L Hepes). The tracheal epithelial cells in the suspension were placed on the composite of collagenous sponge and gel for seeding, and cultured in a carbon dioxide incubator at 37°C for about 3 days. Sections of normal tracheas of rats were made as controls. The samples were fixed by 10% formalin, embedded in paraffin, and sliced for hematoxylin-eosin staining, or made into frozen sections for fluorescent immunostaining with monoclonal antibodies of cytokeratin 14 and cytokeratin 18, which are characteristic phenotypes of epithelium, with polyclonal antibody of occludin, which is a characteristic phenotype of tight junctions, and with 4',6-diamidine-2'-phenylindole dihydrochloride (DAPI) for nuclei.

In Vivo Study. The tracheas were harvested from 9-week-old male transgenic Sprague-Dawley rats (Tg-act[EGFP]Osb-CZ004, provided by Okabe Masao, Genome Information Research Center, Osaka University) and were transfected with genes of green fluorescent protein (GFP). In this study, we used them to distinguish donor cells from cells of recipients. The process of making the grafts was the same as in the in vitro study with the exception of using the GFP-positive epithelial cells.

Wild-type, 9-week-old, male Sprague-Dawley rats were used as recipients. They were placed under general anesthesia by intravenous infusion of pentobarbital sodium (30 mg/kg). Through a vertical skin incision and midline splitting of the sternohyoid and sternothyroid muscles, the tracheas were exposed. Defects of the cervical trachea, about 1.5 mm wide and 3 mm long, were made by electrocautery in the monopolar mode (Fig 2). The grafts were laid onto the tracheal defects with the epithelial cell layer facing into the lumen. The sternohyoid and sternothyroid muscles were then replaced over the graft. At 3, 7, 14, and 30 days after operation, rats were painlessly sacrificed, and the tracheas and muscles were extirpated en bloc. Samples were fixed by 10% formalin, embedded in paraffin, and sliced for hematoxylin-eosin staining, or made into frozen sections for fluorescent immunostaining with DAPI for nuclei.

RESULTS

In Vitro Study. On hematoxylin-eosin staining the 3 layers of the artificial grafts (the tracheal epithelial cells, collagenous gel, and collagenous sponge) were observed by microscopy (Fig 3). The epithelial cells

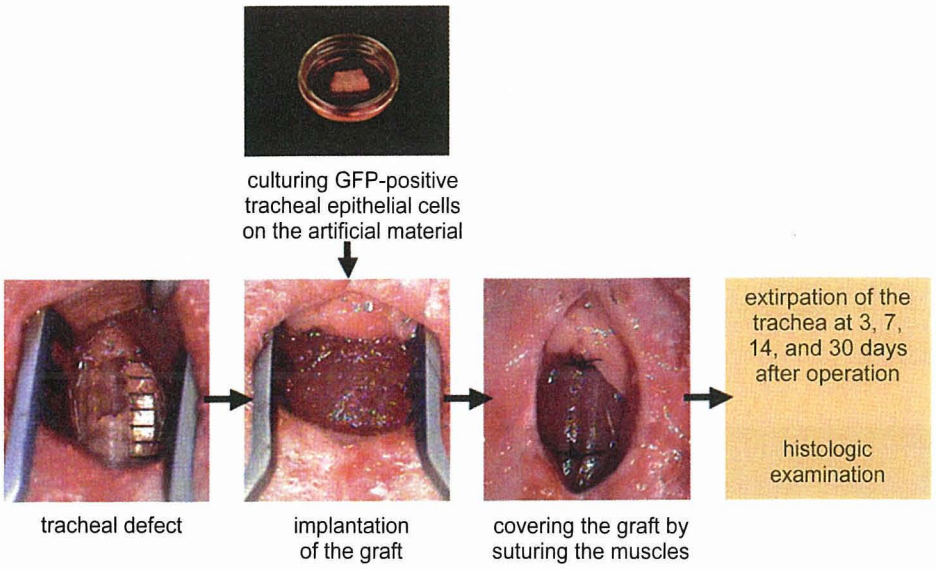

Fig 2. Procedure for transplanting grafts with collagenous sponge, collagenous gel, and green fluorescent protein (GFP)–positive tracheal epithelial cells to tracheal defects of rats.

formed a single squamous epithelium that was from 5.0 to 6.5 μm in thickness. The collagenous gel layer was from 100 to 400 μm in thickness. Cilia were not observed in the sections.

On immunohistochemical examination, cytokeratin 14 stained the cytoplasm of regenerated epithelium diffusely. It was expressed specifically in the basal layer of the normal tracheal epithelium. Cytokeratin 18 also stained the cytoplasm of regenerated epithelium diffusely. It was expressed specifically in the upper layer of the normal tracheal epithelium. Occludin sparsely stained the intercellular regions of the regenerated epithelium, in contrast to the normal tracheal epithelium, which was stained densely (Fig 4).

In Vivo Study. On hematoxylin-eosin staining, single or double-stratified squamous epithelium covering the whole surface of the gel layer of the artificial

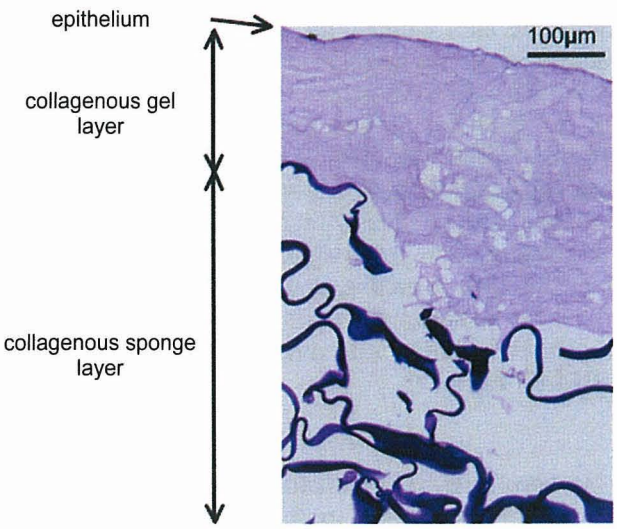

Fig 3. Microscopic image of hematoxylin-eosin staining of artificial trachea.

grafts were observed at 3 days after operation; stratified squamous epithelium was observed at 7 days, and columnar ciliated epithelium was observed at 14 and 30 days. The collagenous gel layer was well preserved at 3 days after operation; infiltration of fibroblasts, lymphocytes, and granulocytes into the gel layer were observed at 7 days. Most of the gel layer had disappeared and the subepithelial layer was regenerated at 14 and 30 days. The collagenous sponge layer had no remarkable change at 3 days after operation. Fibroblasts, lymphocytes, granulocytes, and macrophages were observed in and around the sponge at 7 days. Infiltration of a large number of those cells into the sponge and fibrosis around the sponge were observed at 14 days. A remarkable decrease in the volume of the sponge was observed at 30 days (Fig 5).

Fluorescent microscopic observation revealed GFP-positive cells located at the central area of the regenerated epithelium and a lump of collapsing gel in some sections at 3 days after operation, but none at 7, 14, or 30 days (Fig 6).

DISCUSSION

Circumferentially resected tracheas are conventionally reconstructed by end-to-end anastomosis, but this reconstruction is limited by the length of the tracheal defect. The general limits of safe resection are about half of the tracheal length in adults and probably one third in small children. Tracheal resection longer than these limits requires replacement. Many experimental and clinical studies about tracheal replacement have been done, and various artificial materials, allografts, and autogenous tissue have been reported for use in tracheal replacement.[4] Beall et al[5] reported an animal experiment using heavy Marlex mesh woven of polyethylene monofilaments as grafts for circumferential