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.

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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

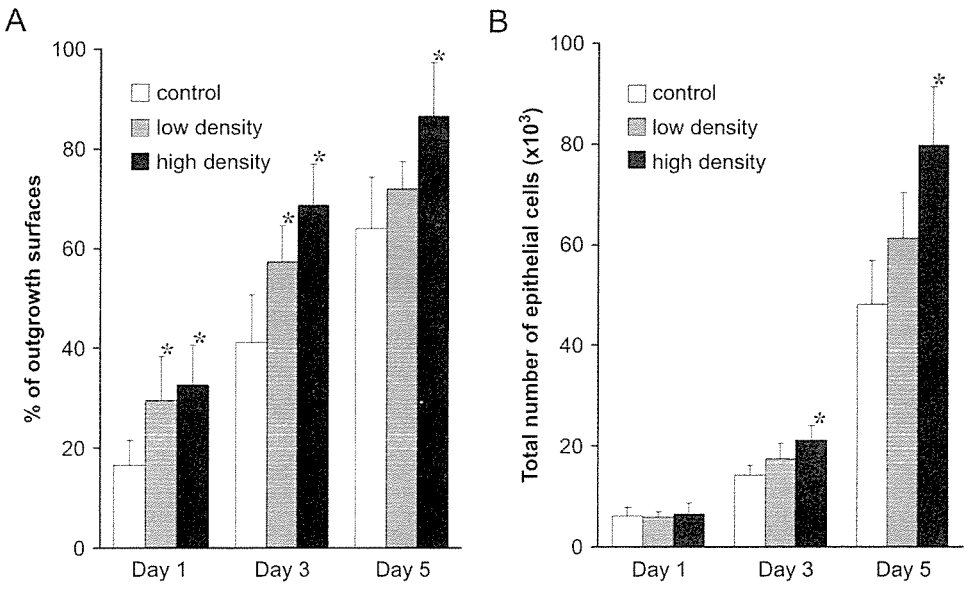

FIG. 1. Influence of fibroblasts on epithelial cell growth. (**A**) Green fluorescent protein (GFP)-negative epithelial cells were cultured with a low (106/well) or high density (5×106/well) of GFP-positive fibroblasts, and the number of GFP-negative cells was counted as epithelial cells. (**B**) Epithelial 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.

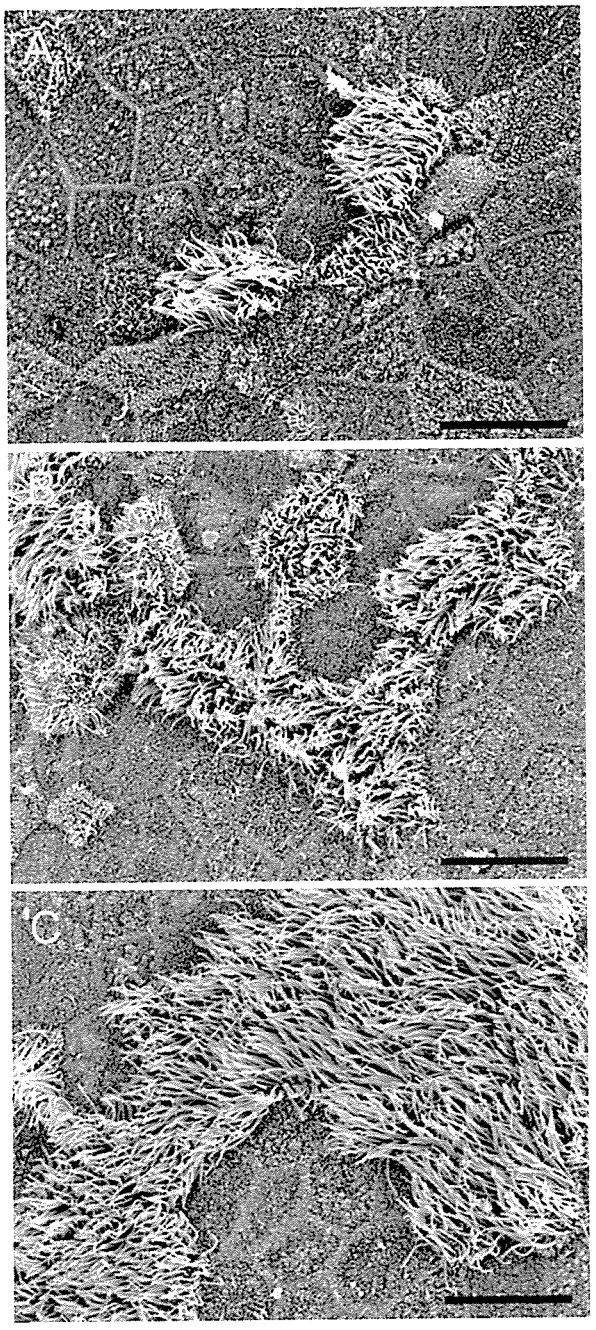

FIG. 2. Scanning electron microscopy of apical epithelial cell surfaces. Epithelial cells cultured on the collagen gel in the absence (**A**) or presence of a low (**B**) or high (**C**) density of fibroblasts for 6 days at the air–liquid interface. Well-developed cilia and expansion of ciliated epithelial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

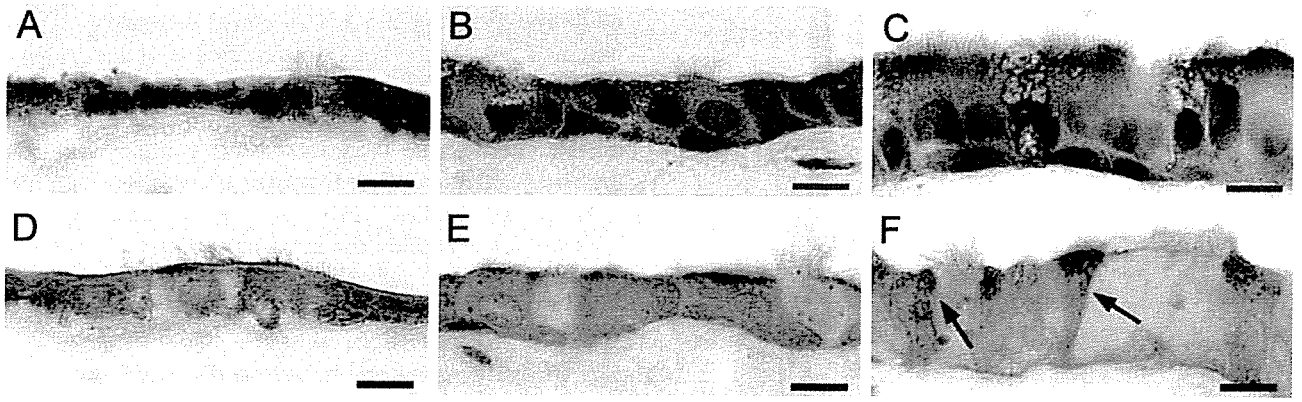

FIG. 3. Hematoxylin-eosin (**A–C**) and Alcian Blue-periodic acid shift (**D–F**) staining of epithelial cells cultured on the collagen gel in the absence (**A, D**) or presence of a low (**B, E**) or high (**C, F**) density of fibroblasts for 6 days at the air–liquid interface. Arrows show well-developed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

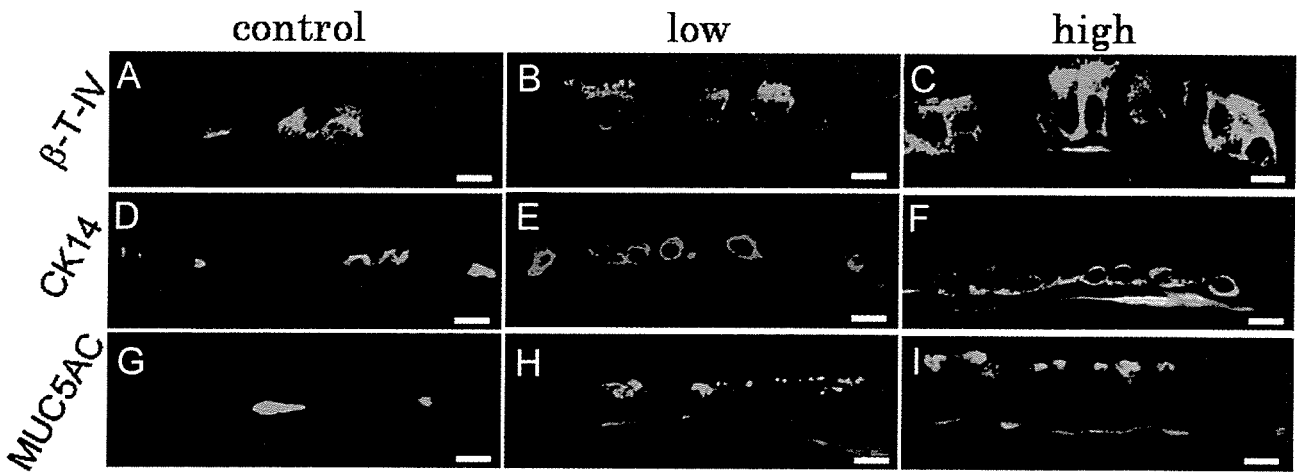

FIG. 4. Immunofluorescent staining of epithelial cells cultured on collagen gel in the absence (**A, D, G**) or presence of a low (**B, E, H**) or high (**C, F, I**) density of fibroblasts for 6 days at air–liquid interface. Identification of ciliated, basal, and goblet cells was confirmed using anti-β-tubulin 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.

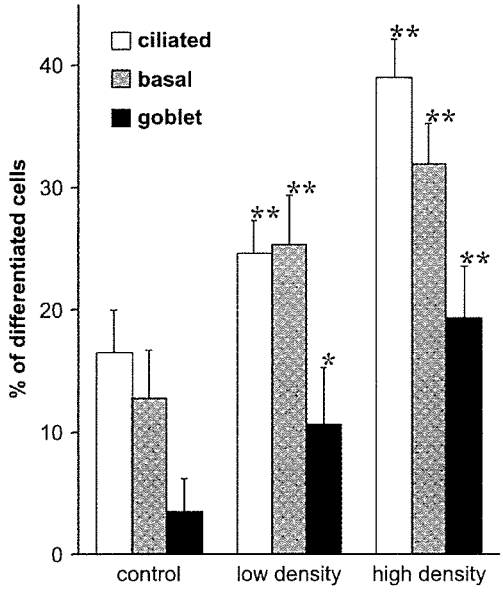

FIG. 5. Immunofluorescent staining with anti-laminin (LN, **A–C**), type IV collagen (IV col, **D–F**), and integrin β4 (β4, **G–I**) antibodies showed the reconstruction of basement membrane in monoculture (**A, D, G**) and co-culture with a low (**B, E, H**) and high (**C, F, I**) density of fibroblasts. 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.

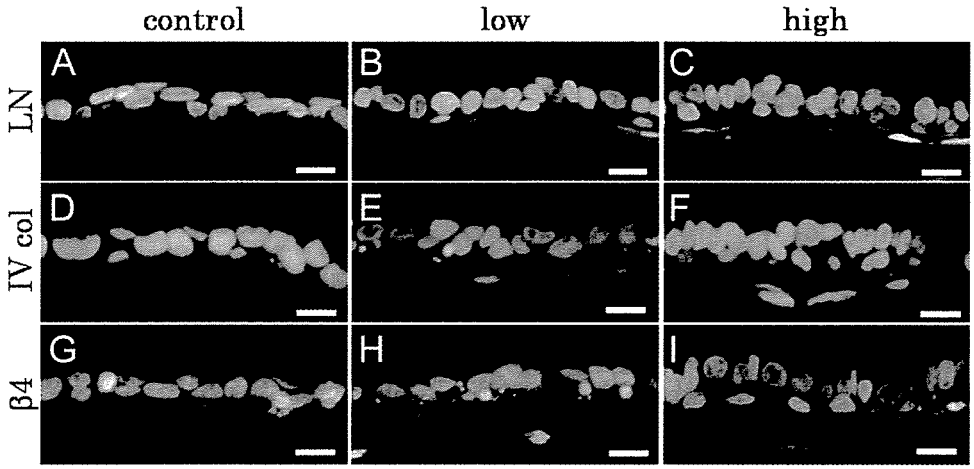

FIG. 6. The ratio of differentiated epithelial cells to total cells in monoculture (control) and co-culture with a low or high density of fibroblasts was measured. $*p < 0.05$ and $**p < 0.05$ compared with the control.

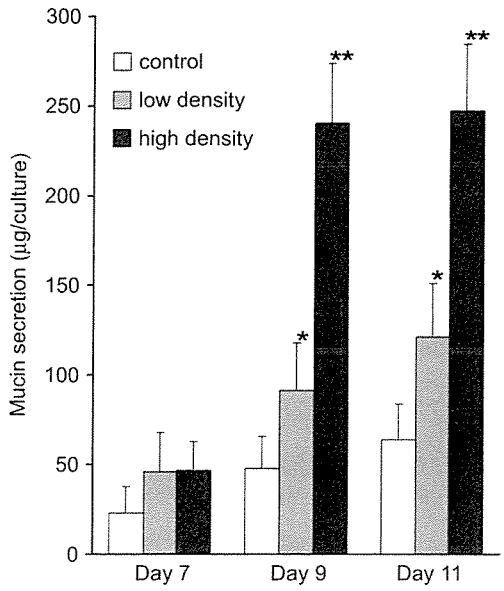

FIG. 7. Influence of fibroblasts on mucin secretion. Apical secretion was collected and quantified using enzyme-linked lectin assay. $*p < 0.05$ and $**p < 0.05$ compared with the control.

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.

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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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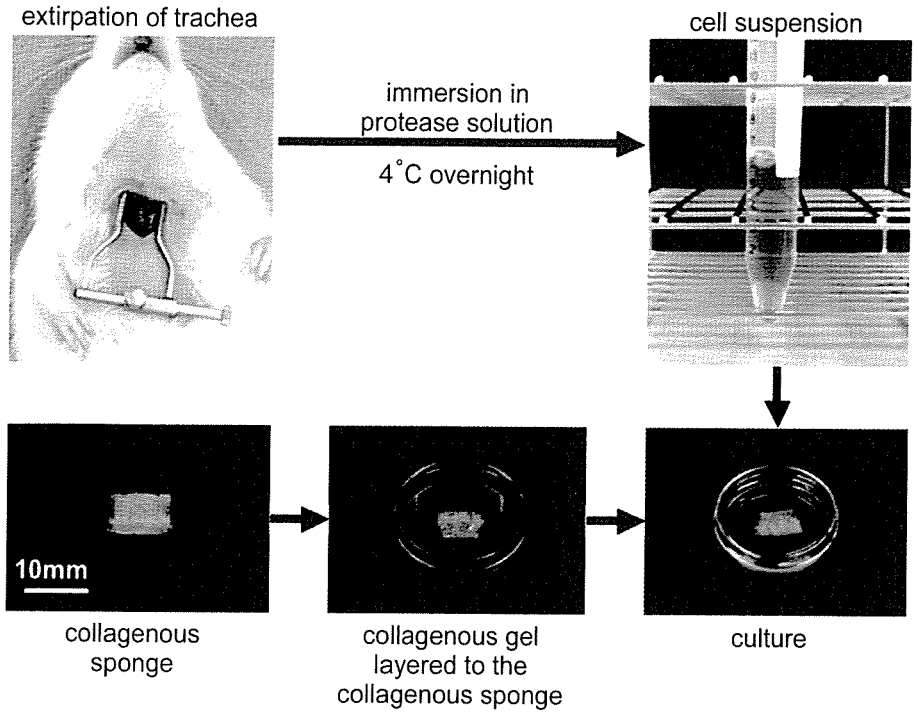

Fig 1. Procedure for making graft with collagenous sponge, collagenous gel, and tracheal epithelial cells.

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

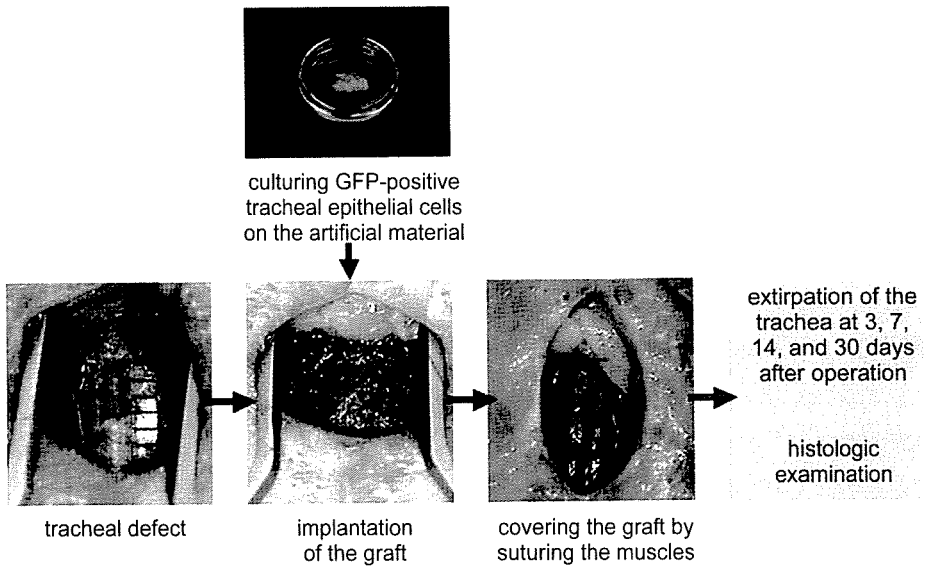

Fig 2. Procedure for transplanting grafts with collagenous sponge, collagenous gel, and green fluorescent protein (GFP)–positive tracheal epithelial cells to tracheal defects of rats.

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

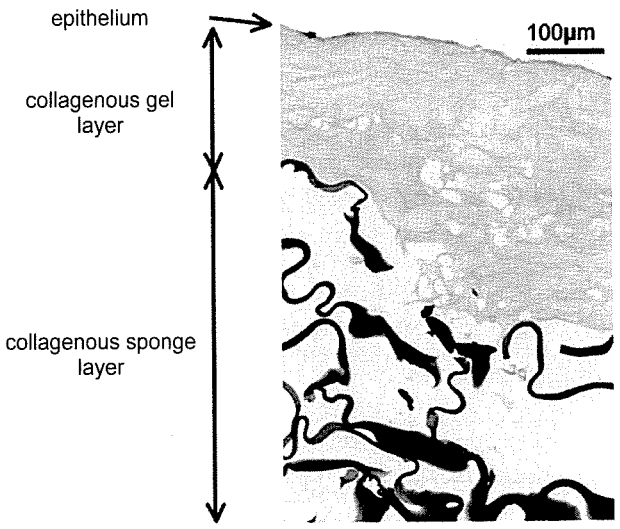

Fig 3. Microscopic image of hematoxylin-eosin staining of artificial trachea.

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

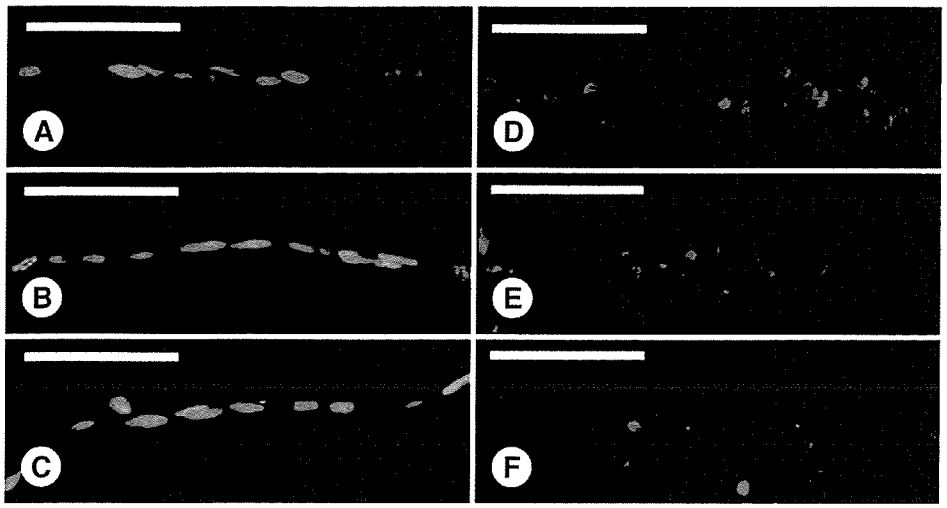

Fig 4. Microscopic images of fluorescent immunostaining of regenerated epithelium and normal tracheal epithelium. Nuclei are blue. Bars — 50.0 μm. **A-C)** Regenerated epithelial cell layers expressing **A)** cytokeratin 14 (red), **B)** cytokeratin 18 (red), and **C)** occludin (red). **D-F)** Normal tracheal epithelia expressing **D)** cytokeratin 14 (red), **E)** cytokeratin 18 (red), and **F)** occludin (red).

tracheal reconstruction in mongrel dogs. In the report, epithelialization began from either end of the grafts and slowly proceeded toward the center, which was the last area to be covered. Because of this pattern, fibrosis occurred in the center of the grafts in association with an acute inflammatory reaction that led to stenosis, resulting in the death of 4 of the 21 dogs. Okumura et al[6] reported a successful animal experiment using an artificial trachea made from spiral polypropylene stent and Marlex mesh woven of polypropylene monofilament as the frame and using collagen extracted from porcine skin for coating the frame as the scaffold for a 2-cm-long circumferential tracheal reconstruction in mongrel dogs. Confluent epithelial coverage in the dogs was confirmed histologically after the dogs were sacrificed 6 months or more after operation. Teramachi et al[1] and Nakamura et al[2] used an artificial trachea that was made from polypropylene stent and Marlex mesh as the frame and used collagenous sponge as the scaffold for carinal replacement in mongrel dogs, and reported successful results at the 5-year observation. Omori et al[3] reported the first human case of using an artificial trachea with a construction the same as used in the previous studies.[1,2] The patient had papillary carcinoma of the thyroid gland with tracheal invasion and underwent hemithyroidectomy, noncircumferential tracheal resection, and reconstruction with a patch of the artificial trachea. Under endoscopic observation, the artificial trachea was seen to be covered with epithelium after 2 months. These results indicate that epithelialization on the lumen of the artificial trachea takes a considerable length of time after reconstruction, and that the larger the replacement with artificial material, the more frequently problems originate from inflammation or excessive fibrosis. It is expected that epithelialization at an early post-reconstruction period helps to prevent problems with grafts. In this study we tried to cover the artificial prosthesis with epithelium by means of tissue engineering and examined its effectiveness for acceleration of epithelialization.

Collagen accounts for about one third of the total protein of an animal and is important not only for supporting tissue construction, but also for differentiation and formation of cells. Collagenous matrix has enabled

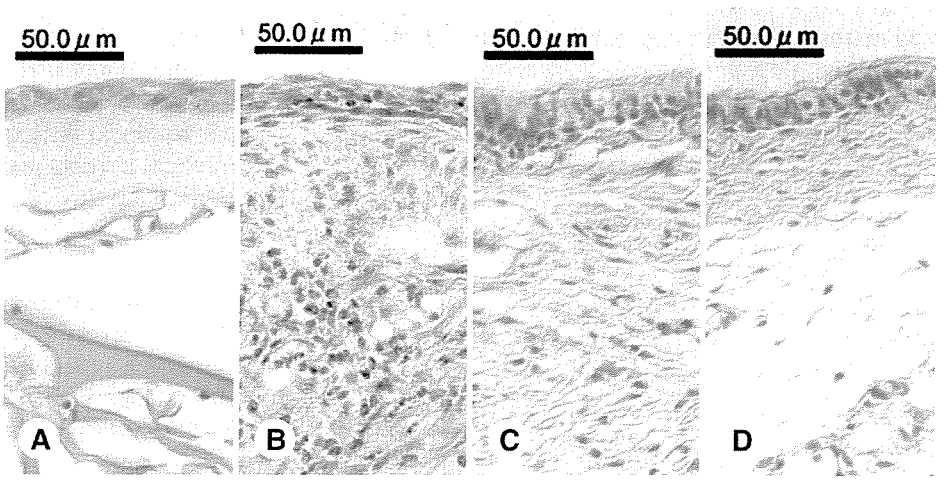

Fig 5. Microscopic images of hematoxylin-eosin staining of defects of tracheas after operation. **A)** Three days after operation. Double-stratified squamous epithelium is observed on gel. Collagenous gel layer is well preserved. **B)** Seven days after operation. Stratified squamous epithelium and infiltration of cells such as fibroblasts, granulocytes, and lymphocytes into gel are observed. **C)** Fourteen days after operation. Columnar ciliated epithelium is observed. Most of gel has disappeared, and subepithelial layer has regenerated. **D)** Thirty days after operation. Same as 14 days after operation.

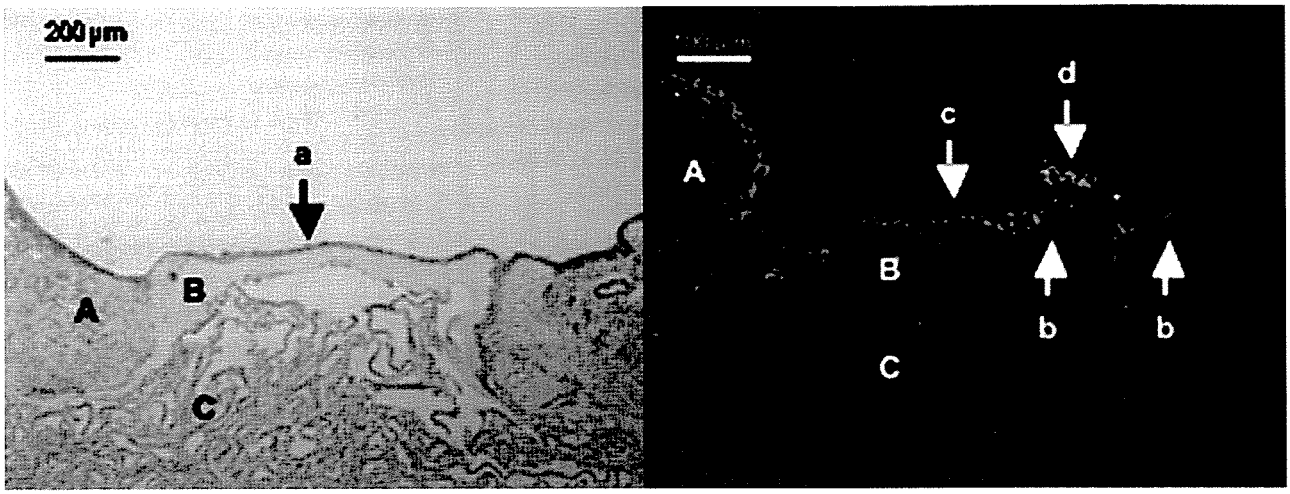

Fig 6. Microscopic images of hematoxylin-eosin staining (left) and fluorescent immunostaining (right) at 3 days after operation. GFP-positive cells (green) are located at central area of regenerated epithelium. Nucleic acid is stained by 4',6-diamidine-2'-phenylindole dihydrochloride and colored blue. A — edge of tracheal defect; B — collagenous gel layer; C — collagenous sponge layer; a — regenerated epithelium; b — epithelium with GFP-positive cells; c — epithelium with GFP-negative cells; d — lump of collapsing gel and GFP-positive epithelium.

many kinds of cells to be cultured in vitro. Cell culture on a collagenous gel — an anchorage-dependent culture called the attached collagen gel culture method — is suitable for epithelial cells. With this method, culture of tracheal epithelial cells of several species, such as rats, guinea pigs, rabbits, dogs, and humans, has been reported.[7-9] Collagen gel can be also stratified on other matrices such as collagen sponge. These two characteristics made collagen gel suitable for this study. In this study, it was confirmed that covering the surface of the prosthesis with epithelium was possible with a collagenous gel layer.

Cytokeratin is found in the cytoskeleton of epithelial cells. Cytokeratin 14 and cytokeratin 18 are markers of epithelial cells. Occludin is a marker of tight junctions that is located on cell-to-cell adhesion in epithelial and endothelial cellular sheets and plays the important role of acting as a barrier to the diffusion of solutes through the intercellular space. In our in vitro study, it was demonstrated that the regenerated epithelium on the collagen gel displayed characteristics that are found in the tracheal epithelium immunohistochemically.

In our in vivo study, the artificial trachea was covered with epithelium through the observation period, and the epithelium differentiated from single- or double-stratified nonciliated squamous epithelium to columnar ciliated epithelium. Green fluorescent protein–positive cells were partially seen at 3 days after operation, but they were not seen from 7 to 30 days. It seems that the disappearance of GFP-positive donor cells and epithelialization with the recipient's epithelial cells occurred simultaneously. One of the reasons for this disappearance may be a kind of allograft rejection. In the in vivo study, infiltration of lymphocytes and granulocytes into the gel was observed at 7 days after operation. Those inflammatory cells probably play a role in immunoreactions against the transplanted graft containing the donor's cells. The allograft rejection can be overcome by using autogenous cells such as the epithelial cells of oral or nasal mucosa, and this is an important subject from the point of view of clinical use. Another reason for the disappearance of GFP-positive donor cells is the weakness of the fixation between the epithelial cells and the collagenous gel. Air currents or the alternately changing pressure with respiration may exfoliate the epithelial layer. It is necessary to find a way to fix the epithelial layer on the collagenous gel more firmly.

In this study, it was confirmed that the donor's epithelial cells with artificial trachea survived until the recipient's epithelial cells formed the epithelium over the defect, and that collagenous gel did not disturb the regeneration of epithelium or subepithelial tissue. The size of the tracheal defects of rats was too small to evaluate the acceleration of epithelialization. The next step is to repeat the experiment using a larger model of tracheal defects and measure the acceleration of epithelialization.

CONCLUSIONS

In the in vitro study, the surface of the artificial tracheas was covered with a tracheal epithelium by a tissue engineering technique. The epithelium on the artificial trachea kept the same immunohistochemical characteristics as normal tracheal epithelium.

After transplantation of the artificial trachea with

the epithelium to the tracheal defect of the rats, the artificial trachea was covered with regenerated epithelium through the observation period. The donor's epithelial cells survived until the recipient's epithelial cells formed an epithelium over the defect.

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.

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Tissue Engineering of Small Intestinal Tissue Using Collagen Sponge Scaffolds Seeded with Smooth Muscle Cells

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ABSTRACT

In a previously reported attempt to regenerate small intestine with autologous tissues, collagen scaffolds were used without cell seeding or with autologous mesenchymal stem cell seeding. However the regenerated intestine lacked a smooth muscle layer. To accomplish regeneration of a smooth muscle layer, this present study used collagen scaffolds seeded with the smooth muscle cells (SMC) in a canine model. Autologous SMC were isolated from stomach wall and cultured. Two types of scaffolds were fabricated: in SMC (+), cultured SMCs were mixed with collagen solution and poured into a collagen sponge; and in SMC (−), SMCs were omitted. Both scaffolds were implanted into defects of isolated ileum as a patch graft. Animals were euthanized at 4, 8, and 12 weeks; for the last time point, the ileal loop had been reanastomosed at 8 weeks. At 12 weeks, the SMC (−) group showed a luminal surface covered by a regenerated epithelial cell layer with very short villi; however only a thin smooth muscle layer was observed, representing the muscularis mucosae. In the SMC (+) group, the luminal surface was covered completely by a relatively well-developed epithelial layer with numerous villi. Implanted SMCs were seen in the lamina propria and formed a smooth muscle layer. Thus, we concluded that collagen sponge scaffolds seeded with autologous SMCs have a potential for small intestine regeneration.

INTRODUCTION

SHORT-BOWEL SYNDROME (SBS) is a clinical complication of massive small bowel resection characterized by malabsorption and malnutrition. To varying degrees, patients require total parental nutrition; weaning from such alimentation is slow and depends on the area of bowel surface available.[1] The promising treatment for insufficient absorptive surface would be intestinal transplantation; however this therapy requires a donor of appropriate tissue type as well as long-term immunosuppression.[2]

Recent technological progress in the field of tissue en-

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gineering allows many tissues and organs to be regenerated,[3] and some such advances have been reported for the small intestine. Vacanti's group, using polyglycolic acid (PGA) scaffolds, successfully obtained formation of cystic structures seeded with intestinal organoids harvested from neonatal rats.[4–8] They reported that tissue-engineered small intestine improved recovery after massive small bowel resection in a rat model.[9] Other research groups have used small intestinal submucosa (SIS) as a scaffold for small intestine regeneration in rat,[10,11] rabbit,[12] and canine models.[13] Chen and Badylak found that use of SIS patches produced three regenerated layers–mucosa, smooth muscle, and a serosal covering–in a canine model.[13] Hori *et al.* aimed to regenerate small intestine with material of autologous origin and using collagen sponge as a scaffold,[14] but the regenerated intestine lacked a smooth muscle layer, which is essential for functional peristalsis and maintenance caliber of lumen.[15] In an attempt to regenerate a smooth muscle layer of the intestine, collagen sponge seeded with autologous mesenchymal stem cells was used, but any smooth muscle layer still did not regenerate.[16]

Unlike mature skeletal and cardiac myocytes, mature smooth muscle cells (SMC) retain the developmental potential to dedifferentiate, both *in vivo* and *in vitro*.[17] Tissue engineering of urinary bladder or blood vessels using scaffolds seeded with SMCs from bladder or artery were reported to develop an orderly smooth muscle layer.[18–22] SMCs from a given organ show behavior distinct from that of most other organs, particularly in terms of functional responses to various stimuli.[23] It is likely that SMCs from the gastrointestinal tract would perform better for regeneration of intestine. In the present study, we isolated SMCs from the stomach wall, which contains abundant SMCs, to investigate the effect of autologous SMC seeded onto collagen scaffolds for regeneration of small intestine compared to collagen scaffolds without cell seeding in a canine model.

MATERIALS AND METHODS

Animals and anesthesia

Female beagle dogs (under 2 years and weighing 9 ~ 11 kg) were premedicated by intramuscular administration of atropine sulfate at 0.05 mg/kg. Dogs were then anesthetized with 15 mg/kg ketamine hydrochloride and 3 mg/kg xylazine hydrochloride and incubated endotracheally. Halothane and nitrous oxide gas were used for the maintenance of anesthesia during the procedure, under mechanical ventilation. Animal care, housing, and surgery followed the Rules and Regulations of the Committee for Animal Research of the Kyoto Prefectural University of Medicine in Japan.

Isolation and culture of SMCs

Gastric SMCs were harvested from anesthetized dogs by laparotomy. A portion of smooth muscle layer was resected from the anterior wall of the stomach (1 × 1 cm), using 4-0 silk sutures to close the defect of this smooth muscle layer. Postoperatively, animals were maintained on a liquid diet and water for 24 h, followed by resumption of a full diet of dog chow.

Two techniques have already been reported for SMC isolation–explant techniques and enzymatic dispersion. In this study, SMCs were obtained by enzymatic dispersion, because the explant techniques yield a mixture of SMC and myofibroblasts.[20] Enzymatically dissociated stomach wall and SMCs were cultured according to the modified method of Kiwamoto *et al.*[24] In brief, the resected smooth muscle layer was incubated with 0.25% trypsin for 30 min at 37°C and then minced with scissors, followed by incubation with 0.1% collagenase I for 2 h at 37°C and passed through a nylon cell strainer (200 μm). Then, the cell suspension was centrifuged at 1000 rpm for 10 min. The pellets were resuspended in Dulbecco's modified Eagle's medium (DMEM), containing 10% fetal bovine serum (FBS), 100 U/mL penicillin, and 0.1 mg/mL streptomycin (all from Invitrogen, Carlsbad, CA). The suspension was placed in 100 mm dishes coated with collagen type I (Asahi Techno Glass, Chiba, Japan). Cells were grown at 37°C in an incubator with a humidified atmosphere of 5% CO_2. Culture media were changed every 2–3 days. When cultures reached about 80% confluence, cells were resuspended by dissociating them with 0.25% trypsin and 0.2% EDTA (Invitrogen). They were maintained and grown in the same culture medium.

Collagen sponge scaffolds

In this study, we used the collagen sponge Pelnac (Gunze, Kyoto, Japan), which has been used clinically as artificial skin with excellent results in regeneration of the dermis.[25,26] We therefore considered this collagen sponge well suited for promoting regeneration of the smooth muscle layer. Pelnac is composed of a silicone sheet and a sheet of collagen sponge. We used only the collagen sponge sheet, which is 3 mm thick, with a pore size of 70–110 μm and pore volume fraction of 80–95%. Tensile strength of this collagen sponge is 1.4 ± 0.23 (mean $\pm$ SD $\times 10^4$ N/m^2).

Labeling of cells and seeding on scaffolds

SMCs were labeled with chloromethy ibenzamido (Cell Tracker CM-DiI, Invitrogen) at a concentration of 1 μg CM-DiI per 1 mL of Hank's balanced salt solution (Invitrogen). DiI-labeled SMCs were detected using a fluorescence microscope (IX70, Olympus, Tokyo, Japan). SMCs were cultured in the collagen sponge as follows.

Seven volumes of acid-soluble type I collagen solution mixed with 2 volumes of a 5-fold concentration of DMEM and 1 volume of reconstruction buffer (all from Nitta Gelatin, Osaka, Japan). This mixture was kept on ice, mixed with cultured cells at a density of 5×10^6 cell/cm^3, and poured into the collagen sponge. The mixture was incubated at 37°C for 1 h to allow trapping of cells in the collagen sponge. There were collagen sponges with collagen solution containing SMCs (+) or not containing SMCs (−). The collagen scaffolds were cultured for 12 h using DMEM containing 10% FBS before implantation, as described below.

Implantation procedure

After laparotomy, two ileal loops were isolated, together with several feeding and draining vessels, while the interrupted bowel was reanastomosed using a two-layer technique with 4-0 Vicryl (Ethicon, Mountain View, CA) and 4-0 silk to maintain a normally functioning alimentary canal. We created defects (1 × 1 cm) in the small intestine at the middle of the isolated ileal loops and patched these with silicone sheets using 6-0 Vicryl sutures. The silicone sheets were necessary to protect the scaffolds from infection and digestion. SMC (+) and (−) scaffolds were placed below the silicone sheet. They were fixed in place with 6-0 Vicryl sutures (Fig. 1A) and covered on the serosal aspect with omentum. The ileal loops were used to construct a double ileostomy on both sides of the abdominal incision (Fig. 1B). The abdominal incision was closed in two layers with 1-0 silk.

Two animals were euthanized at 4 weeks after implantation, and another two at 8 weeks. The last four animals were divided into two subgroups of two dogs each. At 8 weeks, one subgroup underwent reanastomosis of the SMC (+) ileal loop, and the other subgroup underwent reanastomosis of the SMC (−) ileal loop, with the aim of preventing disuse atrophy (Fig. 1C). Dogs in both subgroups were killed at 12 weeks after implantation. Specimens were examined histologically. Postoperatively, animals were maintained on a liquid diet and water for 48 h and then returned to a full diet of dog chow. An antibiotic (cefazolin sodium, 50 mg/kg/day, i.m.) was given for 5 days.

Histologic, immunohistochemical, and immunofluorescence analyses

Samples of the isolated cells were fixed for 10 min in acetone. Collagen sponge scaffolds before implantation, and also scaffolds harvested later with tissues, were fixed with 10% buffered formalin, processed for embedding in paraffin, and sectioned at a thickness of 4 μm. These scaffolds and tissues were stained with hematoxylin and eosin. Tissues and cells were stained immunohistochemically with an Envision+/HRP detection system (Dako, Kyoto, Japan). In brief, endogenous peroxidase activity was blocked with peroxidase blocking reagent (Dako) for 5 min. Then sections or fixed cells were incubated with mouse monoclonal antibodies against α-smooth muscle actin (α-SMA), basic calponin, vimentin, desmin, von Willebrand factor (vWF), or cytokeratin MNF116 (all from Dako) for 1 h at room temperature. Then the sections or cells were incubated for 30 min at room temperature with peroxidase-labeled polymer conjugated to goat anti-rabbit or goat anti-mouse immunoglobulin. They were incubated with diaminobenzidine (DAB) chromogen for 5 min, counterstained with H&E, and mounted with a cover slip. Between the steps, sections were rinsed gently with Tris-buffered saline. Control sections were incubated in the absence of primary antibody.

In the immunofluorescence study, in order to examine the location of DiI-labeled SMCs, paraffin-embedded tis-

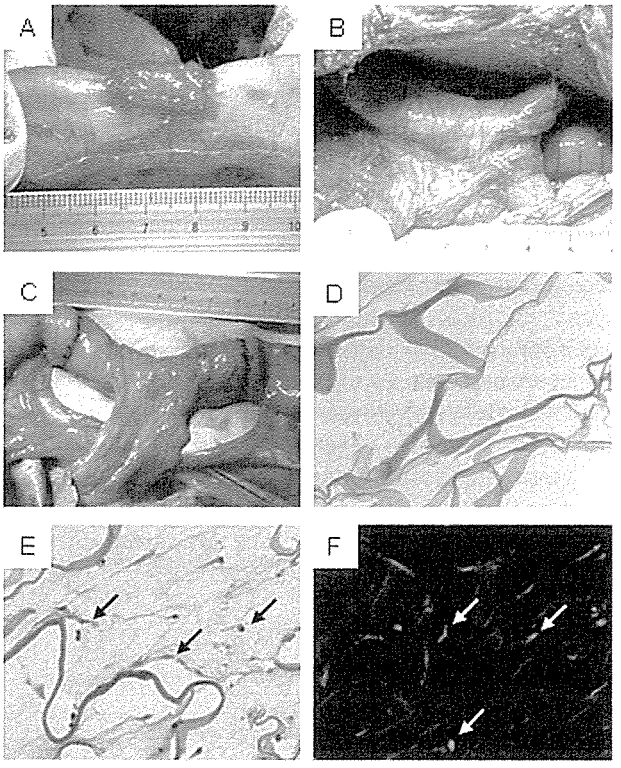

FIG. 1. Surgical technique and collagen scaffolds for SMC (+) and SMC (−) groups. **(A)** SMC (+) or SMC (−) scaffolds were implanted as patch grafts into defects created in two isolated ileal loops. **(B)** The patch graft was covered with omentum, and the two ileal loops were used to construct a double ileostomy on the anterior abdominal wall bilaterally. **(C)** At 8 weeks after implantation, the ileal loops were reanastomosed to avoid disuse atrophy. **(D and E)** Hematoxylin and eosin staining of SMC (−) **(D)** and SMC (+) scaffolds **(E)**. SMC were seeded in the lattice spaces of the collagen sponge in the SMC (+) scaffolds (arrows). **(F)** Most cells of the SMC (+) scaffolds are labeled with DiI (arrows). **(D–F)** Original magnification × 100.