Effect of Dermal Fibroblasts on Long-Term Maintenance of Regenerating Xenotransplanted Human Esophageal Epithelial Cells in Immunodeficient Mice

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Objective: Artificial esophagus of various materials has been tried without satisfactory results. Effects of fibroblasts derived from human dermis on cultured human esophageal epithelial cells were investigated regarding their preservation over time.

Methods: Human esophageal epithelial cells (EE) were subcutaneously injected either alone or with human esophageal fibroblasts (EF) or human dermal fibroblasts (DF) into the flank of the BALB/cA-*nu*, *scid* mice of 8 to 12 weeks of age. At 10 days, 3, 6 and 48 weeks after transplantation, randomly chosen mice were sacrificed and the rates of nodule formation at the injected sites were compared. Nodules were examined histologically by hematoxylin and eosin staining and immunohistochemically with anti- Ki-67 and anti-p63 antibodies.

Results: The rate of subcutaneous epidermoid cyst formation at 10 days was 33% for EE, and 67% for EE with EF, and 100% for EE with DF transplantation. At 6 weeks after transplantation, subcutaneous cysts were not found for EE alone or EE with EF, but were detected in 100% of the mice transplanted EE with DF and still preserved at 12 months.

Conclusions: Long-term preservation of regenerated esophageal epithelium *in vivo* after transplantation of cultured esophageal epithelial cells is possible by co-transplantation of human dermal fibroblasts.

Key words: esophagus, cultured epithelial cells, fibroblasts, artificial, immunohistochemistry

INTRODUCTION

For the reconstruction of the esophagus after esophagectomy, the stomach, jejunum and colon have been used as a substitute. However, these reconstructive surgeries show significant morbidity and are often complicated with postoperative malnutrition [1, 2]. Although many efforts have been made to replace the esophagus by substitutes made of artificial materials, no satisfying result has been reported [3, 4]. Meanwhile, a hybrid type bio-engineered artificial esophagus has been on trial [5-8]. The bio-engineered esophagus consists of cultured human esophageal epithelial cells, polyglycolic acid mesh and collagen. Recently, it was reported that fibroblasts enhanced proliferation of esophageal epithelial cells of the bio-engineered esophagus both *in vitro* and *in vivo* [7, 9, 10].

Although the bio-engineered esophagus consisting of the patient's own cells is considered as a promising esophageal substitute, several obstacles should be overcome [11]. One of these major problems is whether regenerating esophageal tissues can be maintained for a long term *in vivo*. Because the esophageal epithelium is a self-renewing tissue, both preservation of esophageal stem cells and proper controls of proliferation and differentiation of epithelial cells are essential for long-term maintenance of bio-engineered esophagus.

Previously, we reported the necessity of human dermal fibroblasts for long-term maintenance of regenerating human skin after transplantation of cultured human epidermal keratinocytes to immunodeficient mice [12, 13]. This supporting function of dermal fibroblasts could not be found in fibroblasts originating from other organs or species [12, 13].

Therefore, we investigated the effects of fibroblasts derived from human dermis or esophageal submucosal tissues on esophageal epithelium regenerating after xenotransplantation of cultured human esophageal epithelial cells in immunodeficient mice.

MATERIALS AND METHODS

Cell Culture

Human esophageal mucosal tissues and skin biopsies were obtained from surgical specimens of 4 different individuals with informed consent [14]. Esophageal epithelial cells were cultured as follows: mucosal tissues were incubated with 2,000 U/ml neutral protease (Dispase II; Godo Shusei Co., Tokyo, Japan) at 37℃ for 40 min. Then the esophageal epithelium was separated from the esophageal specimen. The separated epithelial sheets were washed with Ca^{++} and Mg^{++}-free phosphate buffered saline, PBS(-), and treated with 0.25% trypsin solution at 37℃ for 5 min. Epithelial cells were dissociated by vigorous pipetting and washed

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with PBS containing 10% fetal calf serum (FCS; JRH Bioscience, Lenexa, CS). The cells were centrifuged and washed twice with PBS (-).

Approximately 1×10^6 epithelial cells were resuspended in modified MCDB 153 medium (KGM; Clonetics Corp., Mountain View, CA) containing 0.15 mM $CaCl_2$, 140μg/ml bovine pituitary extract, 0.5μg/ml hydrocortisone, 0.1 ng/ml human recombinant epidermal growth factor, 5μg/ml insulin, 0.1 mM phosphoethanolamine, 0.1 mM ethanolamine and 100 ng/ml cholera toxin (List Biological Lab. Inc., Campbell, CA). The cells were placed in a 25-cm^2 tissue culture flask. After 5 to 7 days of incubation in a 5% CO_2 incubator, the cells were harvested, subcultured 3 times and used for experiments (esophageal epithelial cells, EE).

After separation of the esophagus, the remaining esophageal tissues were washed twice with PBS (-). The tissues were implanted on tissue culture dishes in minimal essential medium alpha (MEM-alpha; Gibco Oriental, Tokyo, Japan) containing 20% FCS and incubated for 7 to 10 days at 37℃ in a 5% CO_2 incubator. Separated esophageal submucosal tissues were minced and cultured in MEM-alpha containing 20% FCS. Fibroblasts derived from esophageal submucosal tissues were subcultured 4 times and used for experiments (esophageal fibroblasts, EF).

Explant culture of dermal tissues was done the same as that of esophageal mucosal tissues. Proliferating dermal fibroblasts were serially subcultured in MEM-alpha containing 15% FCS. Dermal fibroblasts subcultured 3 or 4 times were used for transplantation (dermal fibroblasts, DF).

The protocols of this investigation conformed to *The Guide for the Care* and *Use of Laboratory Animals* published by the U.S. National Institutes of Health for studies involving experimental animals and to the principles outlined in the Declaration of Helsinki. The protocols were approved by the Institutional Review Board of Tokai University School of Medicine.

Transplantation of the cells into immunodeficient mice

To minimize immune response to grafts, double mutant immunodeficient mice (BALB/cA-*nu*, *scid*), produced as follows, were used. CB-17-*scid* mice (female) were mated with BALB/cA-*nu* mice (male). Then their progeny were mated with each other to produce BALB/cA-*nu/nu* and BALB/cA-*scid/scid* double homozygotes. Thereafter, they were crossed successively by the cross-intercross method for up to 6 generations. They were maintained under a specific pathogen free environment. Mice of 8 to 12 weeks of age were used in this study.

The cultured epithelial cells and fibroblasts were harvested by trypsinization. The cells were washed with PBS (-) containing 10% FCS and then washed twice with PBS (-). Approximately 2×10^6 cells were resuspended in 200 μl of PBS (-). Mice of 8 to 10 weeks of age were used in the experiments.

EE alone, EE + EF, or EE + DF were injected subcutaneously into the flank area of the BALB/cA-*nu*, *scid* mice. EE and DF of each injection were originated from different individuals.

At 10 days, 3, 6 and 48 weeks after transplantation, randomly chosen mice were sacrificed under ether anesthesia, and formation of nodules at the injected sites was observed. Subcutaneous nodules were removed for histological analyses. Nodules were fixed with 10% formaldehyde solution and embedded in paraffin wax. Thin sections of the specimens were stained with hematoxylin and eosin, or studied by immunohistochemical staining.

Immunohistochemical staining

Immunohistochemical staining was performed on 5-μm-thick sections. As primary antibodies, the following mouse monoclonal antibodies were used: anti- Ki-67 (clone MIB-1, 1:100 dilution; Dako A/S, Copenhagen, Denmark), and anti-p63 (clone 4A4, 1:3200 dilution; PharMingen International, San Diego, CA).

Deparaffinized and dehydrated sections were immersed in 0.3% hydrogen peroxide (H_2O_2) in methanol for 30 min to abolish endogenous peroxidase activities. For detecting Ki-67 antigen and p63, the sections were pretreated with autoclave heating (ES-215, High-pressure steam sterilizer, TOMY, Japan) at 121℃ for 4 min for antigen retrieval. Nonspecific binding was abolished with diluted normal sheep serum (Cosmo Bio Co. Ltd., Tokyo, Japan). Next, the sections were overlaid with primary monoclonal antibodies optimally diluted with 1% bovine serum albumin containing phosphate-buffered saline (PBS) and left overnight at 4℃ in a moist chamber. Immunoreactivities were detected by the peroxidase-labeled streptavidine biotin method (Dako A/S) with modifications. After washing with PBS, biotinylated anti-mouse Ig $(Fab)_2$ antibody at 1:100 (Amersham International plc., Buckinghamshire, UK) was applied for 60 min at room temperature. The sections were then treated with streptavidine-conjugated horseradish peroxidase for 30 min at room temperature. Reaction products were visualized using diaminobenzidine tetra hydrochloride reaction for 4 min in Tris buffer. Counter staining was performed using hematoxylin. The number of Ki-67 positive cells in a 100×100 μm area was counted in 10 randomly selected epithelia of different transplanted mice and compared.

RESULTS

Rate of cyst formation after transplantation into immunodeficient mice

Table 1 indicates the rate of subcutaneous epidermoid cyst formation after transplantation of the cells. At 10 days after transplantation of EE without fibroblasts, subcutaneous cysts were formed in 1/3 (33%) of the mice at the injected sites, while cysts were detected in 2/3 (67%) of the mice transplanted EE with EF and 3/3 (100%) of the mice when EE were transplanted with DF.

The rate of cyst formation in the mice transplanted EE alone or EE with EF significantly decreased with time in comparison with the one transplanted EE with DF. At 6 weeks after transplantation of EE alone or EE with EF, no subcutaneous cyst was found. In contrast, cyst formation was detected in 4/4 (100%) of the mice transplanted EE with DF. A cyst was still observed in one of the two mice transplanted EE with EF at 12

Table 1 Rate of subcutaneous epidermoid cyst formation after infection of esophageal epithelial cells with or without fibroblasts into BALB/cA-*nu*, *scid*

		After transplantation			
Transplanted cells*	N	10 days	3 weeks	6 weeks	48 weeks
EE alone	23	1/3 (33%)	1/7 (14%)	0/3 (0%)	n.d.**
EE+EF	25	2/3 (67%)	2/7 (29%)	0/3 (0%)	0/2 (0%)
EE+DF	25	3/3 (100%)	5/6 (83%)	4/4 (100%)	1/2 (50%)

* EE, human esophageal epithelial cells; EF, human esophageal fibroblasts; DF, human dermal fibroblasts.
** Not determined.

Table 2 Ki-67 positive cell populations in regenerated esophageal epithelia at 10 days after xenotransplantation into BALB/cA-*nu, scid*

	Transplanted cell*		
	EE alone	EE+EF	EE+DF
Ki-67 antigen positive cells**(mean ± SD)	0.4±0.7	1.6±1.8	5.4±2.5***

* EE, human esophageal epithelial cells; EF, human esophageal fibroblasts; DF, human dermal fibroblasts.
** Mean number of Ki-67 positive cells counted in a 100 × 100 μm area in 10 randomly selected epithelia.
*** Difference between EE+DF and other two transplantation schemes are statistically significant ($p<0.01$).

months after transplantation. These results clearly indicate that DF facilitated long-term maintenance of the xenotransplanted EE *in vivo*.

Histology of the nodules in early post- transplantation periods

EE were injected subcutaneously into BALB/cA-*nu*, *scid* mice with or without fibroblasts. At 10 days after transplantation, each nodule was examined by hematoxylin and eosin and Ki-67 staining (Fig.1). The inner surface of the cysts was lined with stratified squamous epithelium resembling that of human esophagus in EE+DF mice (Fig.1A). The basal portion of the esophagus consisted of cuboidal cells. Deposition of homogenous basophilic substances, resembling that of esophageal collagen tissues, was seen in the surrounding connective tissues. The nodules formed after injection of EE alone or mixed EE+DF cells showed epidermoid cysts. The stratification was uneven although each layer could be identified (Fig.1E), but the cells of the outer layer of the cysts not always showed the typical cuboidal shape of basal cells. Comparison of Ki-67 staining among these three groups revealed that Ki-67 positive cells were frequent in the basal layer of the esophageal epithelium in EE+DF transplanted mice. Moreover in these mice, the positive cells were found even in the supra-basal layer. Population of Ki-67 positive cells was compared among three groups at 10 days after transplantation (Table 2). EE+DF cells transplanted mice were proven to have positive cells statistically more frequently.

Long-term maintenance of the cysts

Histology of the cysts was examined. The cysts remaining in the mice at 3 or 6 weeks after injection of EE with EF contained atrophic esophageal epithelia containing few Ki-67 positive cells. In contrast, the cysts in the mice transplanted EE with DF maintained for 12 months their mature epithelium-like structures that appeared nearly identical to the epithelial structure of normal human esophagus (Fig. 2A). Each layer of the epithelium, including the cuboidal basal layer, was clearly identified (Fig. 2B), although papilla formation of the esophageal epithelium was not observed. Immunohistochemical staining studies showed both Ki-67 antigen and p63 positive cells in the basal and supra-basal layers of the epithelium (Fig. 2D, 2F), consistent with the immunolocalization in the normal esophageal epithelium (Fig. 2C, 2E).

DISCUSSION

One of the reasons for the poor nutritional status of postoperative patients after esophagectomy for esophageal cancer or neonatal esophageal atresia is attributable to the utilization of the stomach as a substitute.

The stomach is the organ most frequently applied as a substitute after esophagectomy because it has rich blood supply, enabling to avoid anastomotic failure, and it is close enough to the esophagus to be extended to the neck.

When the stomach is used for esophageal reconstruction, the function of the stomach is affected, as the stomach is, in most of the cases, changed its form to a roll, the ability as an alimentary reservoir is damaged, and the postoperative patient cannot take much amount of food anymore. In addition, the denervated gastric tube has decreased secretive ability, and the pulled up stomach can readily cause reflux esophagitis of the remnant esophagus. Thus the substitute other than the stomach is desirable, leaving the stomach in its place and preserving the alimentary reservoir and gastric digestive ability. The colon has been used occasionally, but is not a standard due to technical difficulties. Thus the most desirable way not to damage the upper alimentary function is to utilize an artificial esophagus. Artificial esophagi made of artificial materials were invented and tried in the early years [15], followed by a bio-engineered esophagus using the esophageal components. Persistence of an artificial organ depends on its proliferating ability.

This study showed that the esophageal epithelium regenerated *in vivo* after transplantation of cultured esophageal epithelial cells can be preserved for a long term when co-transplanted with human dermal fibro-

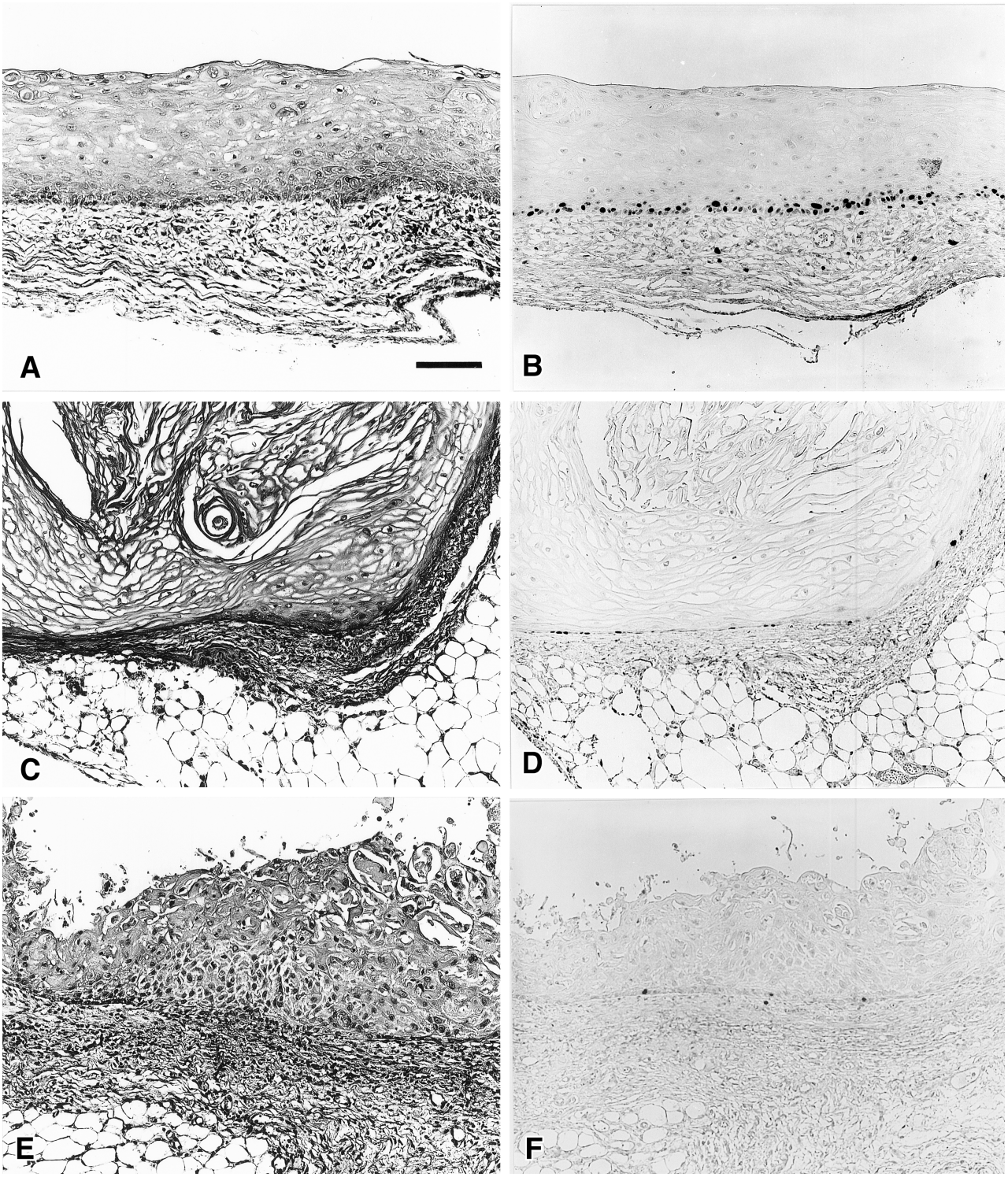

Fig. 1. Histological appearance of the regenerated esophageal epithelia at 10 days after xenotransplantation of EE+DF (**A**, **B**), EE+EF (**C**, **D**) or EE alone (**E**, **F**). Hematoxylin and eosin staining (**A**, **C**, **E**) and immunostaining for Ki-67 antigen (**B**, **D**, F). Bar indicates 100μm.

blasts. Transplantation of cultured esophageal epithelial cells alone or even co-transplantation of fibroblasts originating from esophageal submucosal tissues only transiently induced esophageal epithelium-like tissues and, as indicated by the low rates of Ki-67 positive cells, the regenerated epithelium regressed with time, resulting in short-term preservation.

Squamous epithelium is maintained by perpetual self-renewal, where epithelial stem cells play a major role [16, 17]. Esophageal epithelial stem cells are thought to reside in the inter-papillary basal layer, and transient amplifying cells generated from those stem cells seem to migrate off the tips of the papillae and supra-basal layers after asymmetrical proliferation, differentiate and become stratified. When human esophageal epithelial cells are cultured *in vitro* on acellular esophageal submucosa with intact basement membranes, this asymmetrical proliferation important

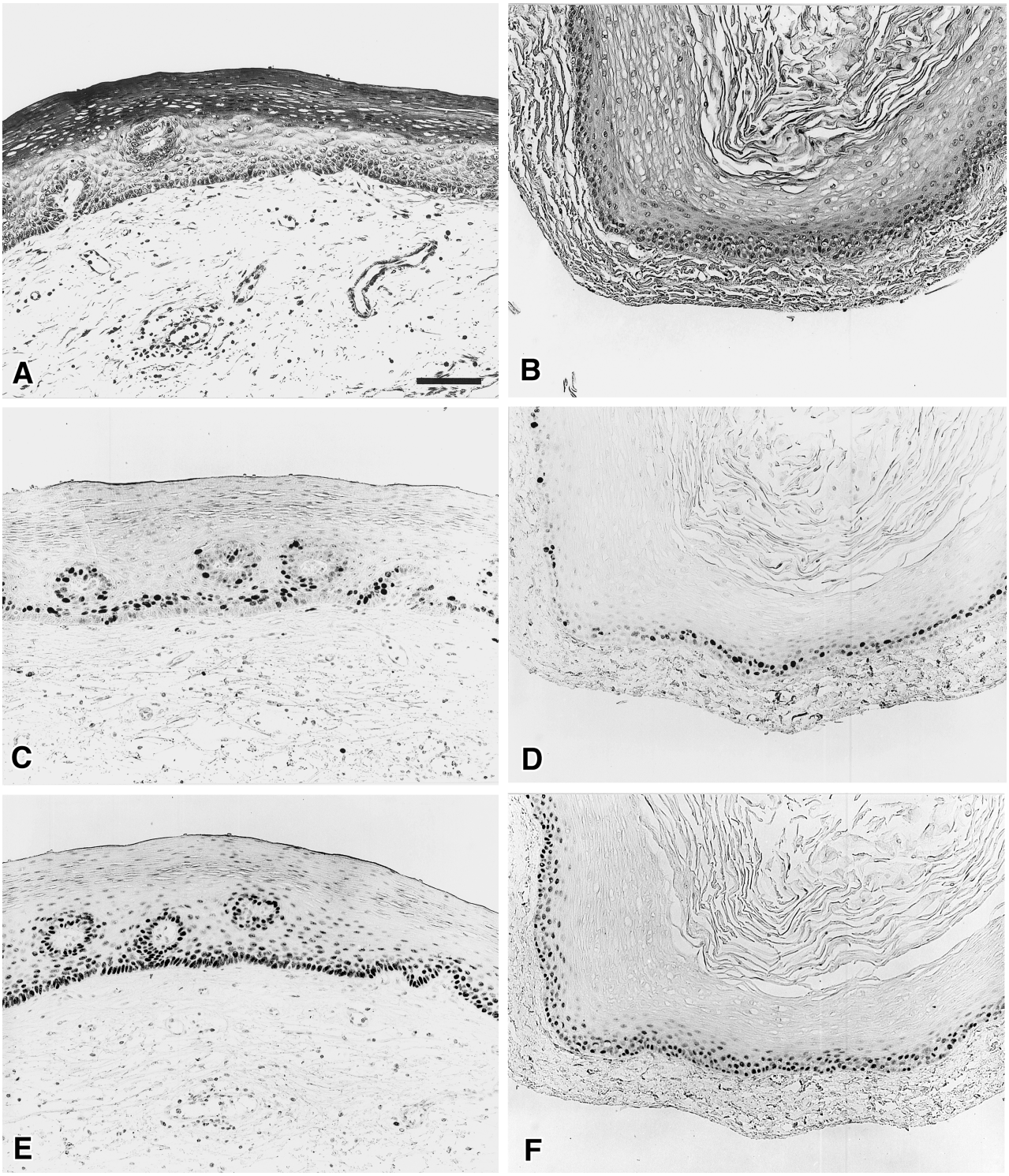

Fig. 2. Histology of normal human esophageal epithelium (**A**, **C**, **E**) and the regenerated esophageal epithelium at 48 weeks after transplantation of EE+DF (**B**, **D**, **F**) in BALB/cA-*nu*, *scid*. Hematoxylin and eosin staining (**A**, **B**) and immunostaining for Ki-67 antigen (**C**, **D**) and p63 (**E**, **F**).

to maintain the function of the stem cells is predominant in the basal layer [17, 18]. Also, the papillary architecture persists in the reconstituted esophagus. In contrast, when human esophageal epithelial cells are grown on acellular dermis, no papilla is formed and basal cell division is predominantly symmetrical. Seery and Watt hypothesize that the difference is attributable to the composition of the basement membrane [17, 19].

One of the causes of esophageal submucosal cells failing to sustain the esophageal epithelium in the present study is attributed to a lack or paucity of the indispensable categories of cell derived for the present research from the culture of esophageal submucosal tissues. Nevertheless, we could show the superiority of dermis-derived fibroblasts to esophageal submucosal fibroblasts for post-transplantational self-renewal.

In our experiments, the regenerated esophageal epithelium was maintained on dermal fibroblast-derived

tissues for 12 months, when many Ki-67 positive cells were detected in the basal and supra-basal layers. Furthermore, p63, a putative marker of epithelial stem cells, was detected on the basal layer cells. These results indicate the possibility of permanent self-renewal of esophageal epithelium on the regenerated dermal fibroblast-derived tissues.

The role of epithelial-mesenchymal interactions is reported to be important in the differentiation and maintenance of epithelial tissues. In our study, the cultured esophageal epithelial cells were transplanted with fibroblasts *in vivo*, which resulted in regeneration of epithelial cells, indicating that the interactions of these cells were maintained. Migration of esophageal epithelial stem cells on the dermal tissues *in vivo* should be further studied regarding the basement membrane proteins in the regenerated esophageal epithelium, especially the localization of laminin isoform and the asymmetric division of basal layer cells.

Long-term maintenance of regenerated esophageal tissues is indispensable for the development of hybrid artificial esophagus, but is rarely investigated. The present study shows that the possibility of long-term maintenance of artificial esophagus made of cultured human esophageal epithelial cells combined with human dermis-derived fibroblasts, and that maintenance is not possible with epithelial cells alone.

The present study concerns the regeneration and maintenance of esophageal mucosal structure, and this esophageal mucosa can be applied as a graft to the resected area after endoscopic resection, which would prevent stricture with scar formation [20].

At the same time, the role of esophagus, which is viewed only as an alimentary passage from mouth to stomach, must incorporate another function, namely peristalsis, in the next stage of the investigation. This movement is autonomically regulated by smooth muscles and nerves, both of which should be integrated in an artificial esophagus. This means not only mucous membranes, muscles, and nerves combined in place, but also the functioning as an organ via cooperation by each other. After development of anatomical esophagus, the artificial esophagus should be studied further from the physiological viewpoint before its clinical application

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