Grafts of Porcine Small Intestinal Submucosa with Cultured Autologous Oral Mucosal Epithelial Cells for Esophageal Repair in a Canine Model

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The acellular porcine small intestinal submucosa (SIS) has been successfully used for esophagoplasty. However, it does not lead to a complete epithelialization in a canine model. A cellular component may be required for better reconstruction. The present study was undertaken to investigate the feasibility and effectiveness of the combination of SIS and autologous oral mucosal epithelial cells (OMECs) for esophageal reconstruction. The OMECs harvested from beagle dogs were cultured and propagated, and the 3rd passage cells were seeded on a single-layer SIS. Male beagle dogs were subjected to surgical resection to produce cervical esophageal defects (5 cm in length, 180° in range). SIS with or without OMECs was patched on the esophageal defects. Barium esophagram, immunohistochemistry, and histology were performed to evaluate the therapeutic effectiveness. Four weeks after surgery, the histological examination showed a complete re-epithelialization and almost no inflammation in the SIS with OMECs group. But in the SIS group, only a partial epithelialization was observed along with inflammation. Eight weeks after surgery, the squamous epithelium was found to cover the entire graft surface in both groups; however, the muscular regeneration was observed in the SIS with OMECs, but not in the SIS group. The graft of SIS combined with autologous OMECs promotes re-epithelialization and muscular regeneration. It is an effective alternative method for esophageal repair. Exp Biol Med 234:453–461, 2009

Key words: epithelial cell; esophagus; extracellular matrix; regeneration; small intestinal submucosa; tissue engineering

Introduction

The incidence of esophageal cancer has increased rapidly during the past decade or so. In 2005, about 500,000 new cases of esophagus cancer developed (1). Surgical resection is generally considered a standard treatment in the early stage of the disease. The tissues from the stomach, jejunum or colon are used as a substitute repair for the esophageal defect (2), but the incidence of complications is relatively high, and the mortality rate is up to 4% (3). To overcome those disadvantages, an alternative treatment is needed. Many attempts have been made to develop proper materials for an artificial esophageal prosthesis. Naturally derived acellular tissue scaffold of collagen-rich extracellular matrix (ECM) contains a complex mixture of structural and functional proteins (4). Recently, a diversity of ECMs, such as decellularized esophagus (5–7), decellularized urinary bladder submucosa (8), acellular dermal grafts (9), gastric acellular matrix (10), and aortal acellular matrix grafts (11), have been investigated and used for esophageal repair in animal models. The natural collagen-rich ECM has more advantages in enhancing epithelial cells attachment than a synthetic one (12). They contain vascular endothelial growth factor and basic fibroblast growth factor (13, 14), which promote neovascularization. Furthermore, the entire ECM acts as a scaffold for migration of host cells and tissue, and leads to the regeneration of esophageal tissue *in vivo*.

The porcine small intestinal submucosa (SIS) is one kind of ECM-based collagen material, which is harvested

This work was supported by National High Technology Research and Development Program of China (Research and Development of Tissue Engineered Esophagus), New Century Excellent Talents in University (NCET-07–575).

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Received January 3, 2009.
Accepted January 13, 2009.

DOI: 10.3181/0901-RM-5
1535-3702/09/2344-0453$15.00

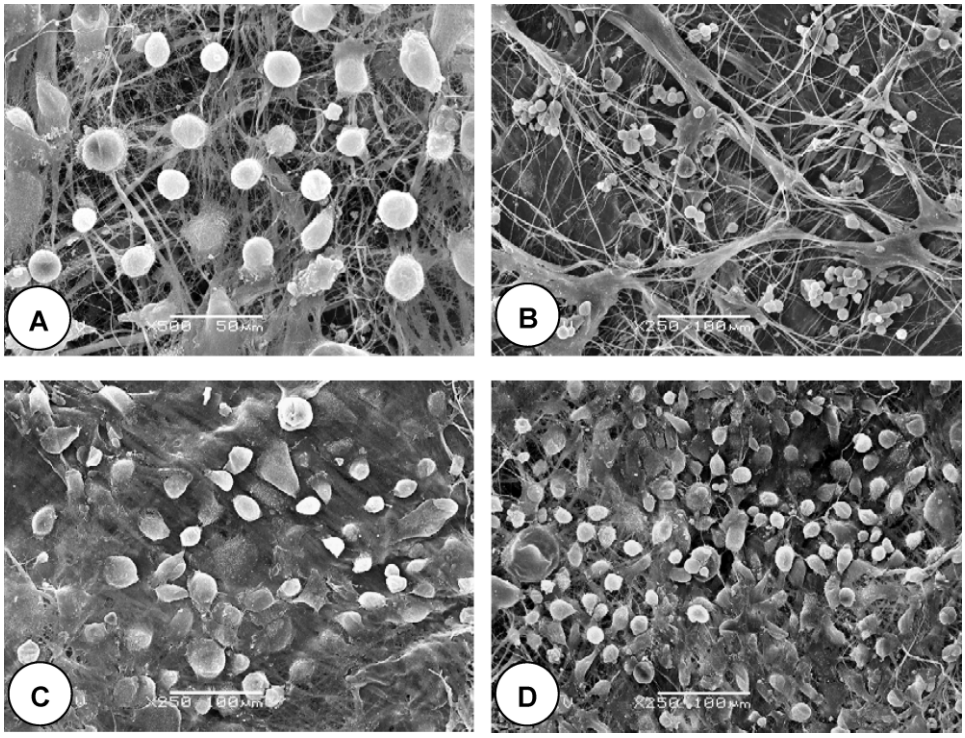


Figure 1. SEM examination of the cultured OMECs on SIS. (A) The OMECs stretched on the matrix surface on day 2 after seeding. (B) The OMECs began to form round-shaped colonies on day 3. (C) The OMECs in SIS reached confluence on day 7. (D) The OMECs displayed multilayers and arranged in a form of cobblestone on day 14 after co-cultivation.

from the submucosal layer of porcine small intestines. SIS can induce regeneration of various tissues (15). The acellular SIS was successfully used for esophagoplasty and the tissue-specific construction or remodeling resembled the original esophagus in appearance and histology (16, 17). However, SIS only caused partial epithelialization for the esophagoplasty at 35 days after surgery in a canine model (16). Timely regeneration of epithelium was important for esophageal repair because the prolonged epithelial regeneration was an important factor for the artificial esophageal stenosis (18).

Harvested the esophageal epithelial cell itself is an ideal cell source. However, the pre-existing fibrotic, inflammatory or malignant changes limit the use of the esophageal tissues. Therefore, an alternative cell source for a tissue-engineered esophagus would be more useful. Recently, cultured oral mucosal epithelial cell sheets have successfully reconstructed the ocular surfaces in animal experiments and clinical practice (19–22). The cell sheets can also promote the esophageal ulcer healing and prevent the postoperative esophageal stenosis (23). However, some disadvantages have been noticed with the cell sheets. The cell sheets cannot promote the entire or full-thickness tissue regeneration of the esophagus due to lack of a three-dimensional structure. They can only be used for the repair of esophageal mucosal or submucosal defects.

To overcome the shortcomings of SIS alone and cell sheets discussed above, this study was undertaken to evaluate the feasibility and effectiveness of the combination of autologous oral mucosal epithelial cells (OMECs) and SIS for the esophageal repair in a beagle dog model. The data obtained demonstrate that the graft of SIS combined with autologous OMECs promotes re-epithelialization and muscular regeneration, and is more effective than SIS alone for esophageal repair.

Materials and Methods

Experimental Animals. Beagle dogs were purchased from Laboratory Animal Academy of Sichuan Medical Sciences Institute (license number, SCXK2004–15). The dogs were given standard dog food and free access to water. All animal experimental procedures were approved by Sichuan University Animal Care and Use Committee, following the Principles of Laboratory Animal Care formulated by the National Society for Medical Research.

Experimental Design. Twelve male beagle dogs (one year old, 10 kg) were divided into two groups. All of the dogs were subjected to cervical esophageal patch defect (5 cm in length, 50% of the circumference). The defect was immediately patched with SIS combined with autologous OMECs (SIS with OMECs group, $n = 6$), or SIS (SIS group, $n = 6$).

SIS Preparation. The preparation of SIS was based on the instructions for the standard procedures described

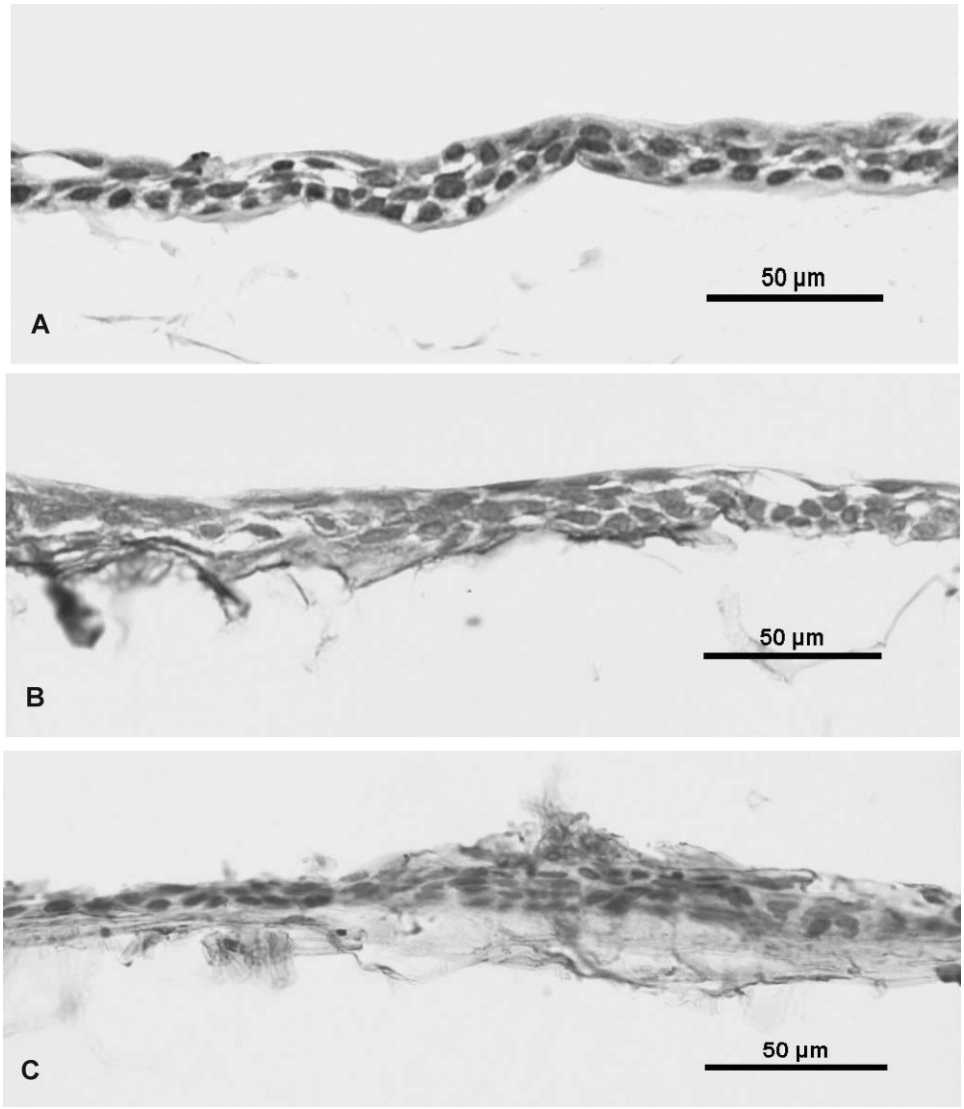


Figure 2. Formation of epithelial layers after co-culturing OMECs with SIS for 2 weeks. (A) H&E staining and (B) Masson's trichrome staining showed 3- or 4-layer stratification of the epithelial cells. The stratified cells on the SIS consisted of cuboidal basal cells, flattened middle cells and polygonal, flattened superficial cells, similar to the native esophageal mucosal epithelia. (C) Protein expression of the seeded OMECs was positive for AE1+AE3, which confirmed their epithelial origin.

previously (24). Briefly, a segment of fresh porcine jejunum was obtained from a local slaughter house. After carefully washing in water, the tunica mucosa, the serosa and tunica muscularis of the segment were mechanically removed. The remaining intestinal submucosa tube was split longitudinally and sectioned in lengths of approximately 10 cm. The SIS obtained was thoroughly rinsed in a saline solution to remove the resident cells. The SIS sheets were sterilized in 0.1% peracetic acid. Finally, the resultant SIS was vacuum-sealed into hermetic packaging, and terminally sterilized by ethylene oxide.

Harvesting and Culturing of OMECs. The oral mucosal tissues 10 mm × 10 mm in size were excised from the bilateral buccal cavity of each dog and the wounds were sutured. The OMECs were isolated according to the protocol published previously (19). Briefly, the submucosal connective tissues were removed with scissors. The remaining tissues were incubated in Defined Keratinocyte-SFM (DK-SFM, Gibco, New York, USA) containing 0.22% dispase I (Gibco) overnight at 4°C. On the following day, the epithelial layers were separated from the underlying tissues and were treated with 0.25% trypsin-0.1% EDTA (Sigma, St. Louis, Missouri, USA) for 10 min at 37°C to isolate the cells. The enzymatic digestion was stopped by washing with DK-SFM culture media containing 10% fetal bovine serum (FBS). The cell suspension was filtered through a nylon mesh (100 mm) to remove the tissue debris. The oral epithelial cells (2×10^5 cells/ml) were then seeded into a 50-ml culture flask. The culture flask was kept in a humidified atmosphere of 5% CO_2 at 37°C. The media were

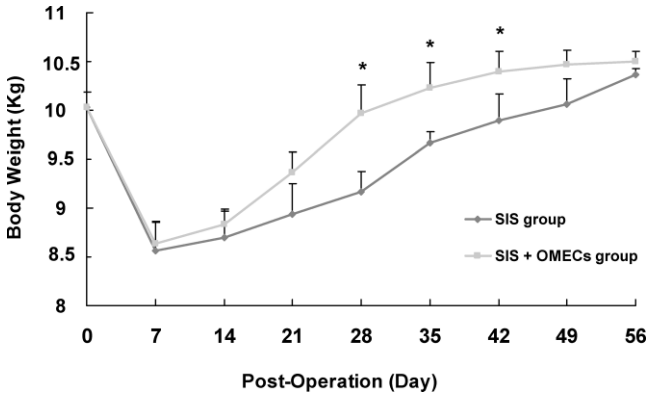


Figure 3. Changes in body weight (mean ± SD) from 0 to 56 days between the SIS and SIS with OMECs group. The weight loss occurred rapidly 7 days after operation in all the dogs. The body weight was regained 56 days after operation in the SIS group, and was regained 28 days in the SIS with OMECs group. There were significant differences in weight gain (* $P < 0.05$) at day 28, 35, and 42 time-point between the two groups.

changed every 2 days. Once the cells reached about 90% confluence, they were passaged. The 3rd passaged cells were harvested for seeding into SIS.

Seeding of OMECs into SIS. The sterilized SIS, 5 cm in length and 2.5 cm in width, was immersed into the DK-SFM culture media containing 10% FBS for 24 h. The 3rd passaged OMECs in suspension (1×10^7 cells) were seeded into SIS and incubated in the DK-SFM culture media with 10% FBS for 1 week to allow the cells to adhere and proliferate in SIS. The culture media were changed every 2 days. To evaluate the quality of the SIS combined with OMECs, the specimens of the final SIS and OMECs co-culture products were fixed in 10% neutrally buffered formalin, and processed into a 4-mm-thick paraffin-wax-embedded section for the hematoxylin and eosin (H&E) staining, Masson's trichrome staining, and the immunohistochemical analysis. H&E and Masson's trichrome staining were performed by a conventional method. For the immunohistochemical analysis, the deparaffinised sections were treated with primary antibodies for AE1+AE3 (mouse monoclonal antibody, Abcam, UK) in 1:100 dilution overnight at 4°C. All the sections were then stained with the streptavidin/HRP ABC detection system (Boster, China). Other SIS specimens were fixed in 2% glutaraldehyde and 1% osmium tetroxide for the scanning electron microscopy (SEM) as described previously (25).

Surgical Procedures. Each dog received an intramuscular injection of 0.04 mg/kg atropine and 15 mg/kg ketamine for premedication, followed by an intravenous injection of thiopental sodium (14–20 mg/kg). Anaesthesia was maintained by inhalation of isoflurane and oxygen through an endotracheal tube. A ventral cervical midline incision was made, and the esophageal exposure was accomplished by a blunt procedure. The removed section of the esophagus was around 5 cm in length and 2.5 cm in width. The defect site was patched with one layer SIS with the autologous OMECs ($n = 6$) or SIS only ($n = 6$). The grafts were sutured to the adjacent normal esophagus with a 5–0 Prolene in a continuous, simple suture pattern. The incision was closed anatomically with a 5–0 absorbable (polyglycolic acid) suture in an interrupted suture pattern. The specimens of the excised normal esophagus were placed in 10% neutral buffered formalin for later use as the controls for the immunohistochemical evaluation. Each dog was given intravenous fluids (60 ml/kg) containing antibiotics (cefazolin, 500 mg, intravenously) for 3 days after the operation. The dogs were not given any food by mouth

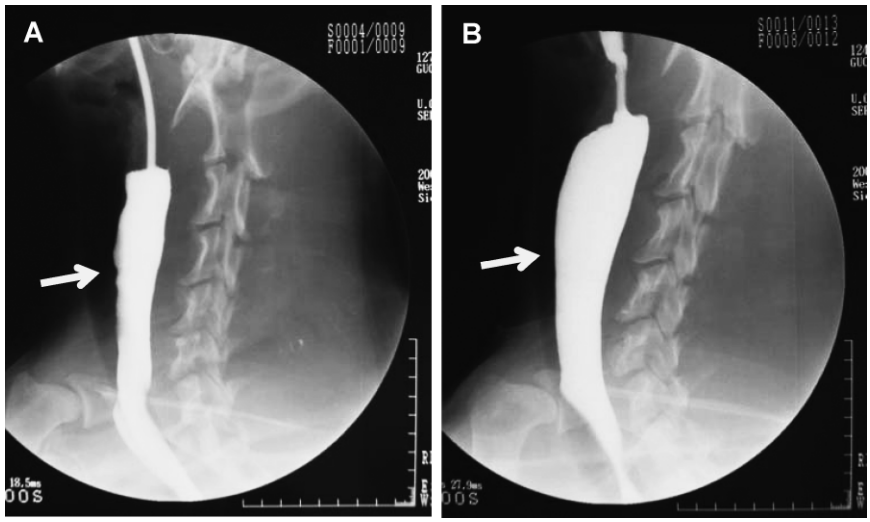


Figure 4. The contrast esophagram 2 weeks after operation. There was no evidence for leakage, stricture or diverticulum formation in either group. But the esophageal lumen surface in the SIS with OMECs group (B) was smoother than the one in the SIS group (A). The arrowhead indicates the site of the patch graft.

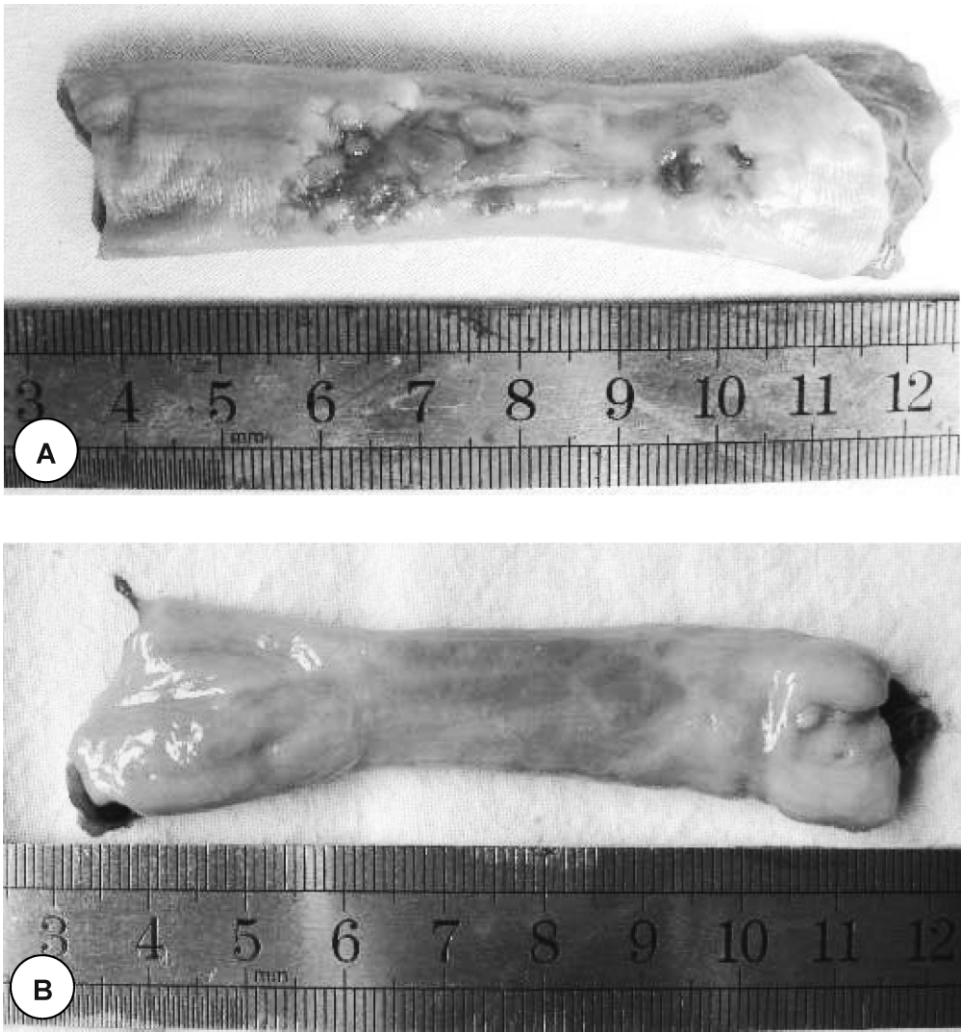


Figure 5. Gross anatomical observation of the removed esophageal segment 4 weeks after operation. (A) The partial fibrous mesh protrusion was observed in the SIS group. (B) The lumen surface of the graft was smooth in the SIS with OMECs group.

for 3 days after surgery. Then, the animals started to eat soft dog food for 7 days, followed by a return to normal diet.

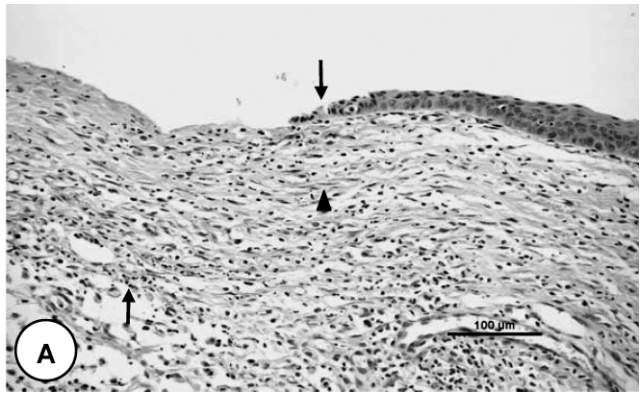
Esophageal Function Assessment, and Histological, Ultrastructural and Immunohistochemical Examinations. All the dogs were subjected to barium esophagram 2 weeks after the operation to assess esophageal function. Body weight was also closely monitored. The dogs were sacrificed at the 4- and 8-week intervals. Esophageal tissue specimens were obtained. Some of the specimens were fixed in 10% neutrally buffered formalin for H&E and Masson's trichrome staining. Other specimens were prepared for immunohistochemical analysis; the deparaffinised sections were treated with 1:100 dilution of AE1+AE3 (Abcam), and 1:1000 anti-S100 (mouse monoclonal antibody, Sigma, St. Louis, Missouri, USA) overnight at 4°C.

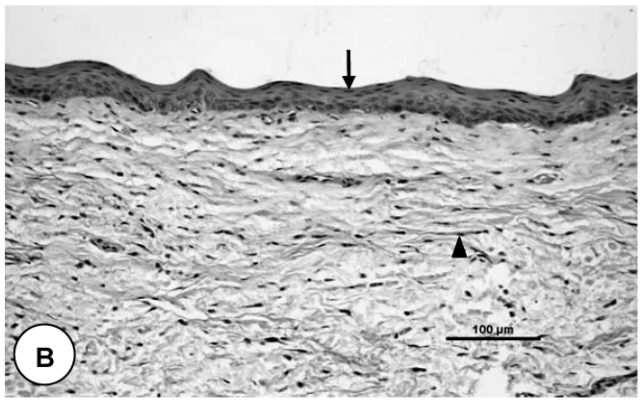
Analysis of Inflammation. The inflammatory cells from the H&E-stained postoperative tissue sections were counted under the microscope. In the 12 randomly selected areas in each of the sections, the inflammatory cells were counted in the field of view.

Statistical Analysis. Data are presented as mean ± standard deviation (SD). The statistical analysis was performed by the nonparametric test with the SPSS II software. The probability (P) value less than 0.05 was considered statistically significant.

Results

Adherence and Proliferation of OMECs on SIS. The OMECs were successfully isolated and cultivated. The cells started to adhere 24 h after being seeded in primary cultures and they grew in colonies and expanded from the second day. After 4 days in cultures, the cells showed a polygon shape, and were closely connected. The cultures reached 80–90% confluence on the fifth day and were then passaged. The immunohistochemical analysis showed that the cultured OMECs were





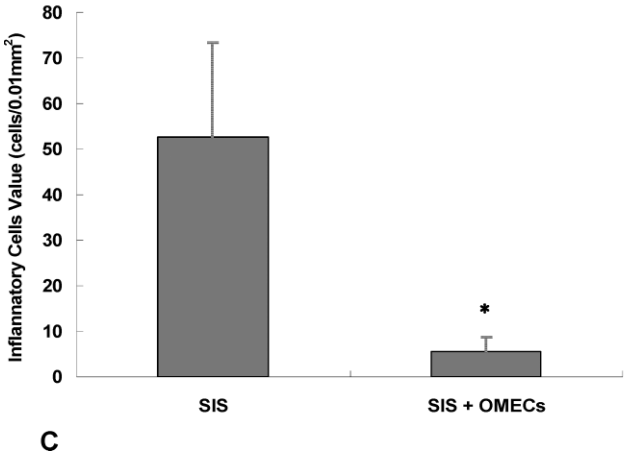


Figure 6. Epithelialization and postoperative inflammation of the two groups 4 weeks after operation. (A) The H&E staining showed only the partial immature squamous epithelium coverage (down arrowhead ↓) in the central graft in the SIS group. There was a considerably higher accumulation of the inflammatory cells (up arrowhead ↑) and disorganized fibroblastic cells (triangular arrowhead ▲). (B) The H&E staining showed the intact squamous epithelial coverage (down arrowhead ↓) with underlying well-organized fibroblastic cells (triangular arrowhead ▲) and almost no inflammation in the SIS with OMECs group. (C) Comparison of the number of inflammatory cells presented in the surgical sites between the SIS and SIS with OMECs groups. There was a significant difference between the two groups (* $P < 0.01$).

AE1+AE3 positive (data not shown), which confirmed their epithelial origin.

The 3rd passage OMECs were seeded into SIS, and 24 h after co-cultivation, the cells adhered and began to spread on SIS. SEM examination showed that the cells stretched on the matrix surface on day 2 (Fig. 1A) and began to form round-shaped colonies on day 3 after seeding on SIS (Fig. 1B). The cultures of OMECs in SIS reached confluence after 1 week (Fig. 1C), and became multi-layer 2 weeks after the co-cultivation; the cells were arranged like a cobblestone in morphology (Fig. 1D). H&E or Masson's trichrome staining of the SIS combined with OMECs showed the stratification of 3–4 layers of the epithelial cells (Fig. 2A, B). The expression of AE1+AE3 was detectable (Fig. 2C). The data obtained above indicated that OMECs on the SIS closely resembled the native esophageal epithelia.

Therapeutic Effects of SIS or SIS with OMECs. All the dogs survived and the cervical wounds remained intact with no evidence of infection or fistula formation. None of the dogs developed dysphasia, vomiting or salivation. The weight loss occurred rapidly 1 week after the operation in all the dogs. The body weight was regained 8 weeks after the operation in the SIS group. However, in the SIS with OMECs group the body weight was regained 4 weeks after the operation (Fig. 3). The barium esophagrams showed that there was no evidence of leakage, stricture or diverticulum formation 2 weeks after the operation. However, the esophageal lumen surface at the site of the patch graft in the SIS with OMECs group was smoother than that of the SIS group (Fig. 4A, B).

The patch graft sites were visible in all the dogs sacrificed 4 weeks after the operation. The lumen surface of the segmental esophageal sample was exposed. A partial fibrous mesh protrusion was observed in the SIS group (Fig. 5A), but the lumen surface of the graft was smooth in the SIS with OMECs group (Fig. 5B). Eight weeks after the operation, the patch graft fused into the esophageal tissue in both groups. The graft site could be identified only on palpation or be indicated by the marked silk sutures in the surrounding area.

Histological and Immunohistochemical Findings. Four weeks after the operation, there was only a partial epithelial coverage of the grafts but a large number of inflammatory cells in the SIS group (Fig. 6A). However, a grade 3 epithelial lining, as judged based on the criteria published previously (17), was observed in 100% (3/3) of the dogs examined in the SIS with OMECs group (Fig. 6B). Only a slight inflammation was detected in the SIS with OMECs group (Fig. 6C).

Eight weeks after the operation, it showed many new blood vessels formed, but no regeneration of muscle cells (Fig. 7A). Only a few short bundles of skeletal muscle from adjacent normal skeletal muscle extended to the graft. The organized undulate collagen fibers were observed by Masson's trichrome staining (Fig. 7B) in the SIS group. However, the submucosal tissues were well organized, and

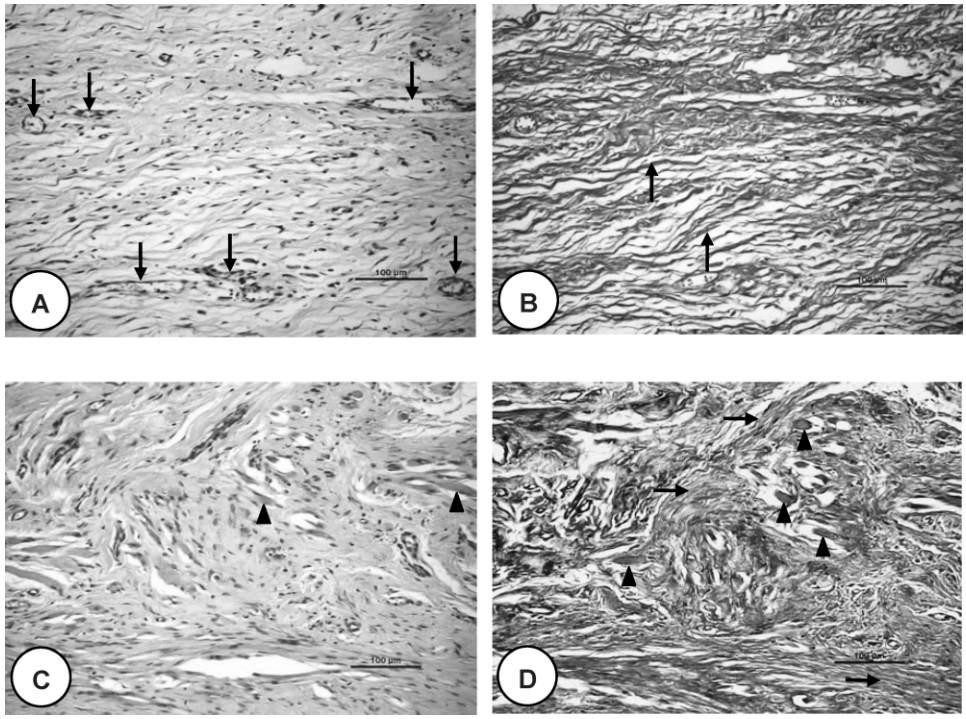


Figure 7. Histological findings 8 weeks after operation. (A) The H&E staining showed many new blood vessels formation (down arrowhead ↓) but no regeneration of muscle cells among the collagen fibers in the SIS group. (B) The organized undulated collagen fibers (up arrowhead ↑) were observed by Masson's trichrome staining in the SIS group. (C) The H&E staining showed many clusters of the muscle cells (triangular arrowhead ▲) in the SIS with OMECs group. (D) The Masson's trichrome staining showed the clusters of muscle cells (triangular arrowhead ▲) surrounded by the collagenous connective tissue matrix (horizontal arrowhead →) in the SIS with OMECs group.

many long bundles of the skeletal muscles from the adjacent skeletal muscle extended to the graft in the SIS with OMECs group. There were many clusters of the muscle cells (Fig. 7C), which were surrounded by the collagenous connective tissue matrix (Fig. 7D). The squamous epithelium covered the entire graft surface and the epithelial stratification was identical to that of the adjacent normal esophageal epithelium; however, the nerve fibers were stained negative for anti-S100 in all the specimens in both groups.

Discussion

The data obtained from the present study show that we have successfully developed a tissue-engineered esophageal patch using isolated autologous OMECs seeded on SIS. Both SIS alone or SIS loaded with autologous OMECs showed effectiveness in esophageal repair following an artificial defect simulating clinical operation of tumor removal; however, the SIS with OMECs demonstrated more advantages over the SIS only. The SIS with OMECs not only resulted in a faster recovery as demonstrated by barium esophagram and body weight gain, but also promoted re-epithelialization and skeletal muscle regeneration.

The use of the graft of SIS loaded with autologous OMECs for esophageal repair is a combination of SIS and OMECs sheet repair of esophageal defects. Both the SIS and OMECs sheet have been used separately and proven their effectiveness in animal models (16, 23). However, the use of SIS alone has shown a limitation in epithelialization in esophageal repair in a canine model (16). The OMECs sheet can only be used for the repair of the esophageal mucosal or submucosal defect. This study presented a first attempt to combine the advantages of the SIS and OMECs sheet, as well as to overcome the shortcomings of their individual application. The results demonstrate the feasibility and advantages of the combination.

The esophageal innermost covering epithelial cells can serve as a barrier or a protective layer. After the superficial layer of the epithelial cells was terminally differentiated, the basal layer of the epithelial cells can replenish them. The constituents of the naturally occurring ECM scaffolds such as SIS are almost identical to the constituents of the basement membrane (the basal layer) that supports the squamous epithelial cell growth (16). Both the esophageal epithelial cells and OMECs are squamous epithelial cells, and they are similar in morphology and function. Therefore, SIS loaded with OMECs provides both the basal layer and the protective barrier of the esophageal patch.

SIS alone or SIS loaded with OMECs adequately supported the healing process of the esophageal defect. None of the dogs developed dysphasia, vomiting or salivation. The barium esophagrams showed that there was no evidence for leakage, stricture or diverticulum in either SIS with OMECs group or SIS group. However, the

regain of body weight of the dogs after the surgery was faster in the SIS with OMECs group than that in the SIS group. Importantly, there was a complete re-epithelialization and almost no inflammation in the SIS with OMECs group 4 weeks after the operation, but there was only a partial epithelialization along with evident inflammation in the SIS group. The promotion of re-epithelialization would greatly improve the healing process. Importantly, it would prevent artificial esophageal stenosis (26). In addition, the transplantation of autologous OMECs reduced the inflammatory reaction. This result is consistent with that observed in previous studies (23). The inflammatory reaction may result in a delayed regain of body weight in the SIS group.

In addition to the promotion of faster re-epithelialization, the treatment with SIS loaded with OMECs resulted in an evident extending of the bundles of the muscle cells and an appearance of island muscle cells in the connective tissue 8 weeks after the operation. The muscle cells generation was not observed in the SIS group. Although it would be possible that a prolonged observation may reveal the appearance of isolated muscle cells in the connective tissue of the SIS group, the faster muscle regeneration had a positive impact on esophageal repair, constituting another advantage of the SIS loaded with OMECs.

Many attempts had been made to create a tissue-engineered esophagus using either synthetic materials or collagen-based ECMs as a tissue scaffold. These ECM scaffolds have been demonstrated as a promising treatment option for the esophageal repair. However, epithelialization of the graft was slower (9, 16), leading to the risk of stenosis in addition to no muscle regeneration (18). Another problem associated with the use of the ECM scaffold without any cells was the lack of effectiveness in the reconstitution of the tubular structures, especially those that would require the peristalsis for the normal function. A cellular component as a composite element was suggested to be required in the clinical practice for the tissue engineering (4). The data presented here thus demonstrate the feasibility of the ECM graft with OMECs for an effective repair of esophageal defects.

There was no regeneration of the nerve fibers found in the present study. However, we speculate that if the observation time was long enough, the regeneration of the nerve fibers might be observed. In addition, a prolonged observation would have generated more information related to whether the SIS alone group also shows muscle regeneration. We consider this is a limitation of the present study, which will be addressed in our future studies.

In summary, the present study using a canine model demonstrates the feasibility and effectiveness of SIS loaded with OMECs for esophageal repair. The data obtained suggest it would be an alternative option for clinical treatment of esophageal defects, and which has more advantages over the existing approaches. However, in future studies, a prolonged observation in the same animal model should generate further information to demonstrate its potential for clinical application.

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