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CONCISE REVIEW

Esophageal tissue engineering: from bench to bedside

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For various esophageal diseases, the search for alternative techniques for tissue repair has led to significant developments in basic and translational research in the field of tissue engineering. Applied to the esophagus, this concept is based on the *in vitro* combination of elements judged necessary for *in vivo* implantation to promote esophageal tissue remodeling. Different methods are currently being explored to develop substitutes using cells, scaffolds, or a combination of both, according to the severity of lesions to be treated. In this review, we discuss recent advances in (1) cell sheet technology for preventing stricture after extended esophageal mucosectomy and (2) full-thickness circumferential esophageal replacement using tissue-engineered substitutes.

Keywords: regenerative medicine; esophagus; tissue engineering; decellularized scaffold; cell sheet technology; clinical trials

Introduction

Tissue engineering (TE) is a field of regenerative medicine originally introduced in the 1990s by Langer and Vacanti.[1] It is a multidisciplinary field that requires close collaborations between different fields, such as medicine, cell and molecular biology, biochemistry, and physics. TE consists of constructing an *ex vivo* substitute for normal tissues using cells and scaffolds for subsequent *in vivo* implantation. Different approaches using stem or differentiated cells and natural or synthetic scaffolds have been used separately or in combination.

The esophagus is a complex organ composed of four layers (innermost mucosa, submucosa, muscularis propria, and adventitia) and different cell types, including epithelial, glandular, and muscle cells.[2] Therefore, based on the nature and depth of damage caused to the esophagus, different TE techniques have been evaluated for repair. To prevent stricture formation after extensive mucosectomy performed for superficial carcinoma or Barrett's esophagus, cell sheet technology appears to be the most appropriate TE technique and does not involve the use of scaffolds. However, when full-thickness circumferential esophageal replacement is necessary, such as to treat esophageal atresia, a combination of a scaffold and cells is the most effective method for inducing regeneration of all layers of the esophagus and recovering its functionality.[3]

Our review focuses on the two main applications used in TE for esophageal repair: (1) cell sheet technology for preventing stricture after extended esophageal mucosectomy and (2) the production of biomaterials for full-thickness circumferential esophageal replacement. Regulatory issues related to clinical applications are also discussed.

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Tissue engineering for superficial esophageal lesions

Stricture formation is the main complication occurring after extensive endoscopic submucosal dissection (ESD) of superficial esophageal neoplasms.[4] These strictures require multiple interventions that significantly alter patients' quality of life.

Steroids, either injected locally[5] or administered orally,[6] have shown efficacy for stricture prevention, but their use raises safety concerns because they may cause esophageal perforation and infectious morbidity. Other stricture-preventing drugs include Tranilast, an antiallergy drug that also inhibits collagen synthesis and interleukin-6 production *in vitro*. This drug was shown to reduce the rate of stricture more effectively compared with preventive endoscopic dilation.[7]

Currently, two different TE approaches are available for preventing ESD-induced stricture. The first, developed by Badylak *et al.*,[8] involves application of an ECM with a stent calibration. In five patients after submucosal resection for a superficial carcinoma, wounds were covered with an extracellular matrix composed of xenogeneic ECM derived from porcine small intestine (Surgisis®; Cook Biotech Inc.) under the cover of a temporary stent to examine stricture prevention. Patient outcomes were jeopardized by a high rate of stent migration and subsequent requirement for dilatation. However, the normal mature squamous epithelium was restored in all patients, and patients were returned to a normal diet without significant dysphagia.

The second approach is cell sheet technology, which involves culturing cells on temperature-responsive polymers that change their physical properties based on temperature.[9] As an example, poly(*N*-isopropylacrylamide), which is widely used in TE, is hydrophobic at temperatures greater than 37 °C, allowing cells to attach and proliferate (Fig. 1A). Once the temperature is reduced to below 32 °C, the polymer changes its physical state and becomes hydrophilic.[10] These physical changes allow for the spontaneous detachment of cells as cell sheets. Unlike enzymatic detachment, thermoresponsive polymers preserve the components of the cell ECM, as well as cell morphology and functionality[11] (Fig. 1B).

As a proof of concept, it was demonstrated in a canine model that after hemi-circumferential endoscopic mucosal resection, cell sheets composed of autologous oral mucosal epithelial cells prevented host inflammation and enhanced the healing process.[12] More recently, a porcine model of ESD was used to show that using allogenic adipose tissue–derived stromal cells as the cell sheet prevented the formation of esophageal strictures after hemi-circumferential mucosal resection.[13]

In a previous safety and feasibility study in humans, tissue-engineered epithelial cell sheets were produced by culturing oral mucosal epithelial cells from 10 patients on temperature-responsive culture dishes followed by transplantation of the sheets onto ulcer surfaces in each patient following ESD.[14] Transplantation of the cell sheet into the lumen was carried out using an endoscopic mucosal resection tube. The autologous oral mucosal epithelial cell sheet was attached to polyvinylidene difluoride support membranes, which in turn were grasped by endoscopic forceps and carefully maneuvered onto the ulcer site through the endoscopic mucosal resection tube. Gentle pressure was applied on the cell sheet using endoscopic forceps to stably adhere it to the wound bed after 10 minutes. This procedure was repeated several times to fully cover the

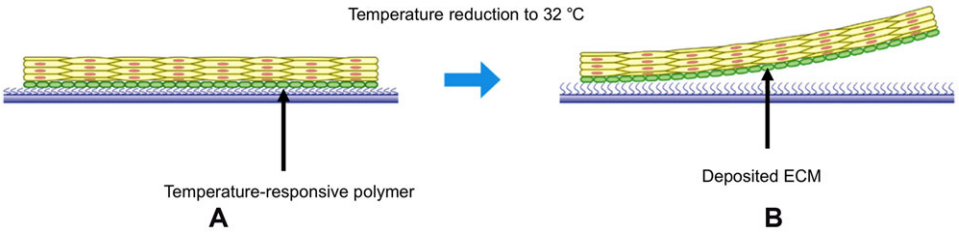

Figure 1. Cell sheet technology using temperature-responsive cell culture dishes. (A) Confluent cultured cells on temperature-responsive cell culture surface at 36 °C. (B) The hydrophobic surface of a temperature-responsive culture dish can be converted to a hydrophilic surface. Cell sheets are harvested by reducing the temperature to 20 °C. The extracellular matrix is maintained under the cell sheet.

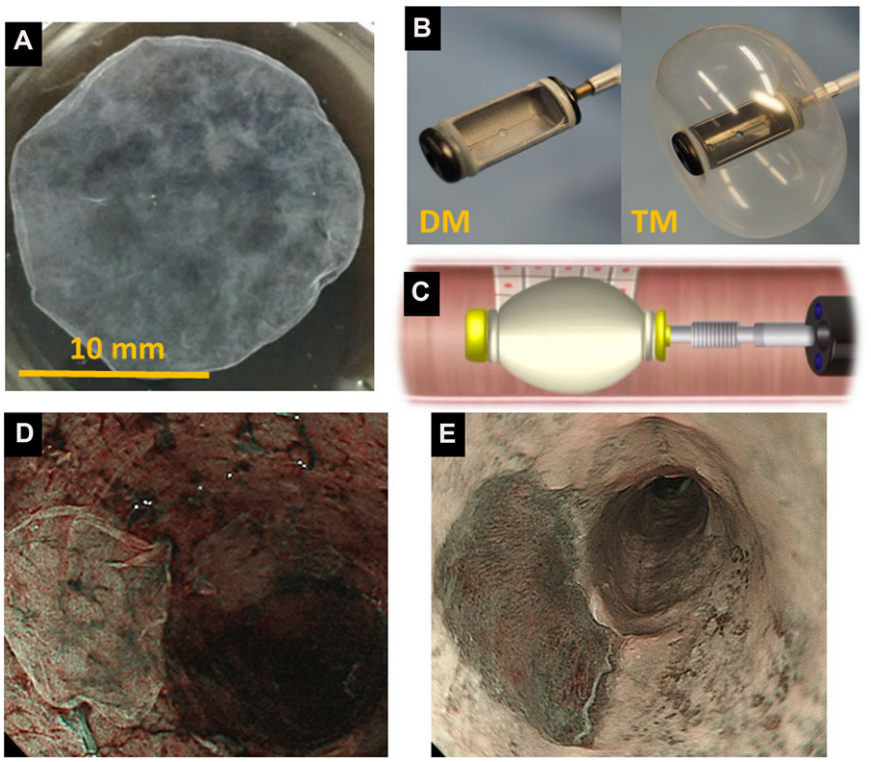

Figure 2. **Transplantation of epithelial cell sheets. (A) Epithelial cell sheet, (B) delivery device, (C) schematic of transplantation, (D) endoscopic view immediately after transplantation (endoscopic narrow-band imaging), and (E) endoscopic view at 2 weeks after transplantation (endoscopic narrow-band imaging). DM denotes delivery mode and TM denotes transplantation mode.**

ulcer with cell sheets.[15] This study showed that human autologous epithelial cell sheets (Fig. 2A) can be produced reproducibly, transplanted safely (Fig. 2D), and used to promote early re-epithelialization (Fig. 2E). The median re-epithelialization time for this method was 3.5 weeks. Only one patient, who had a full circumferential ulceration expanding into the gastroesophageal junction, experienced stricture formation.

The main limitation of this method is the difficulty in properly transporting and transplanting the cell sheets into the esophageal lumen. In response, a new endoscopic device (Fig. 2B and C) was developed and produced using a 3D printer.[16] To prevent losing or damaging the cell sheet during delivery through the oral cavity and pharynx, a vacuum system is used to hold the sheet within the device's protective walls. Once positioned on the ulcer surface, pressure is applied to expand the membrane attached to the cell sheet, thereby providing a rapid, simple, and accurate means of transplantation. Based on this prototype, a medical device was approved by the Japanese Pharmaceuticals and Medical Devices Agency. Additionally, this delivery device has acquired CE marking.

Although the clinical results were highly encouraging, the molecular mechanisms underlying the healing of wound ulcer sites after transplantation of cell sheets are poorly understood. To determine the mechanism of action, comprehensive gene expression profiles of epithelial cell sheets produced using Good Manufacturing Practice were analyzed by next-generation sequencing. This work revealed that the therapeutic effects of epithelial cell sheets mainly resulted from three biological activities: (1) enhancement of epithelial keratinocyte migration, (2) prevention of inflammatory immune cell infiltration into wound ulcer sites, and (3) inhibition of fibrosis.

Together, these findings indicate that cell sheet therapy is a viable treatment option. Accordingly, clinical trials have been planned to investigate

the effectiveness of autologous, fabricated epithelial cell sheets after large-sized ESD for superficial esophageal neoplasms in Japan and Europe. In Japan, a multicenter phase III clinical trial for treating superficial esophageal squamous cell neoplasms with autologous epithelial cell sheets is currently underway. This multicenter, single-arm clinical trial is registered under the number NCT02866019 and has recruited nine patients.

Full-thickness esophageal TE using scaffolds and stem cells

After esophagectomy performed during cancer treatment, esophageal replacement is typically performed using gastric or colonic transplants. The treatment of benign diseases, such as long-gap esophageal atresia or caustic strictures refractory to endoscopic dilation, often involves whole esophageal replacement. However, these treatments promote early mortality and morbidity, as well as cause disabling symptoms related to late complications, including anastomotic strictures, reflux, and delayed conduit emptying, which impair patients' development and quality of life.[17,18] Additionally, the failure of these techniques can result in critical situations that preclude further reconstructive attempts because of the lack of appropriate esophageal substitutes. Therefore, developing alternative procedures for esophageal reconstruction is very important.

Attempts to perform esophageal replacement using artificial nonabsorbable materials, such as Teflon,[19] polyethylene terephthalate (Dacron),[20] expanded polytetrafluoroethylene,[21] or silicone,[22] have failed because these materials are not biocompatible, resulting in chronic infection, anastomotic leakage, material extrusion, and strictures. Although it has been performed in the past, esophageal allotransplantation is not a realistic option because of the complexity of the esophageal vascular anatomy and the necessity for chronic immunosuppression. In contrast, tissues such as the pleura, aorta, and pericardium have been used as auto- or allografts with relative success.[23]

Advances in TE in animal models and, more recently, in humans have made this concept a viable option for full-thickness esophageal wall replacement in the near future.[24–26] Several experimental models have been used to develop ideal treatments, with studies initially investigating the use of acellular matrices.

The matrix plays a major role in inducing cell integrity, proliferation, and differentiation through its tissue-specific biochemical and biomechanical properties. It provides a scaffold and delivers the signals necessary for the function and repair of a tissue.[27] Therefore, for TE purposes, an ideal matrix should be biocompatible and biodegradable as well as replaced by a matrix produced by host cells.

Currently, a wide range of biodegradable synthetic polymers, including polyester-based aliphatic polymers (polylactic acids, poly-L-lactic acid, polycaprolactone, polyglycolic acid, poly-D,L-lactic acid, and poly-L-lactide-co-caprolactone), can be tested for esophageal TE.[28] However, these synthetic polymers are weakly biocompatible and frequently induce foreign body reactions, leading to fistula or stricture.

The most encouraging results for esophageal TE were obtained using natural matrices and components of the ECM, such as collagen scaffolds. These matrices induce fewer proinflammatory reactions and less fibrosis, which are limiting factors in tissue remodeling.

Collagen matrices have been widely used for full-thickness circumferential esophageal replacement in several animal models. Takimoto *et al.*[29] and Yamamoto *et al.*[22] carried out esophageal replacement in dogs using collagen matrices supported by a silicon endostent for 4–8 weeks. Early mucosal regeneration and the appearance of submucosal glands, as well as a few islands of smooth muscle cells, were reported at 1 year. However, soon after the stent was removed, the animals developed strictures and a new stent was placed after stricture dilation.

For circumferential replacement of the cervical esophagus in a rabbit model, Saito *et al.*[30] implanted a collagen substitute in the latissimus dorsalis muscle for 3 weeks before using the substitute to bridge a full-thickness circumferential defect. In this experiment, all animals died within 3 weeks from aspiration caused by stricture in the graft area. When porcine small intestinal submucosa (SIS) was used for esophageal replacement in piglets by Doede *et al.*[31] and rats by Lopes *et al.*,[32] both studies reported stricture development in the SIS area and no muscle cell colonization was observed.

Using esophageal decellularized scaffolds, several groups developed techniques for fully removing

cells while preserving the ECM. For example, Ozeki *et al.* developed a protocol for decellularization of rat esophagi involving treatment with deoxycholic acid and DNase under constant agitation and reported that when the decellularized esophagi were reseeded with esophageal epithelial cells, the morphology and protein expression of the introduced cells were identical to those of native esophageal cells.[33] However, this study was limited to a small animal model, and the protocol requires modifications to decellularize an esophagus large enough to be used in humans. In contrast, Totonelli *et al.* decellularized porcine esophagi by conducting cycles of incubation in deionized water at 4 °C, followed by detergent and DNase treatment at room temperature with perfusion through the lumen. They characterized their decellularized scaffold by conducting DNA and collagen quantification, immunohistochemistry, and electron microscopy and reported that full decellularization was obtained only after three cycles.[34] However, this scaffold was not seeded with cells and its cytotoxic and proinflammatory properties were not evaluated. No decellularized esophageal scaffold has been fully characterized from a clinical perspective. After development, full *in vitro* validation and evaluation by a preclinical trial, such a scaffold in a large animal model, may have the potential for TE in human patients. Currently, no acellular matrix alone allows for full-thickness esophageal regeneration. This has directed research toward the use of cellular matrices obtained by seeding cells of interest, such as epithelial cells, muscle cells, and mesenchymal stem cells (MSCs).

Initially, a hybrid approach based on an *in vitro* combination of different cell types and/or matrices appeared to show great promise.[35,36] The murine esophagus was fully regenerated using a tubular polyglycolic acid scaffold seeded with esophageal organoid units. To prepare the esophageal organoid units, samples of human and mice esophagi were harvested and enzymatically digested to obtain heterogeneous multicellular clusters. As the esophagus is an organ with multiple cellular origins and no clear stem cell niches have been defined, these organoids provide a complex cell source allowing for regeneration of all layers. The organoids proliferated in the scaffold *in vitro*; when implanted *in vivo*, these substitutes regenerated all layers of the esophagus.[37] In a recent study using a porcine model, La Francesca *et al.* showed that full-thickness circumferential esophageal regeneration was achieved with synthetic polyurethane electrospun grafts seeded with autologous adipose-derived MSCs. The polyurethane graft was not integrated into the newly synthetized tissue and was removed after 3 weeks, followed by luminal administration of autologous platelet-rich plasma and MSCs.[38]

Nakase *et al.* showed that a substitute composed of oral keratinocytes and fibroblasts cultured on a human amniotic membrane and sheeted on polyglycolic acid filled with smooth muscle tissue successfully replaced the thoracic esophagus without stent calibration.[35] In another study, using a porcine model, an acellular SIS scaffold was seeded with skeletal myoblasts, covered with a human amniotic membrane, and seeded with oral epithelial cells.[39] After a 2-week maturation period in the greater omentum (Fig. 3) to promote early vascularization,[24,35] this substitute was used to bridge 5-cm circumferential esophageal defects in minipigs. Under the temporary cover of an esophageal endoprosthesis, both the recovery of nutritional autonomy and tissue remodeling toward an esophageal phenotype were observed.[40]

Current interest in using MSCs for esophageal regeneration is based on the multipotency of such cells, that is, their ability to develop into several cell types and differentiate into the osteocyte, chondrocyte, and adipocyte lineages,[41] which is of particular interest for esophageal TE. Moreover, MSCs secrete a variety of growth factors, cytokines, and exosomes[42] that contribute to the induction of neovascularization and regulation of immune cell activities, as well as exhibit chemoattractive and anti-inflammatory properties.[43] In a patch esophagoplasty model in dogs, Tan *et al.* demonstrated that bone marrow MSCs on an SIS scaffold (Surgisis®; Cook Biotech Inc.) can promote re-epithelialization, revascularization, and muscular regeneration.[44] In minipigs, it has also been demonstrated that after circumferential replacement of the esophagus, the presence of autologous MSCs accelerated mature re-epithelialization and the initiation of muscle cell colonization.[45] Future studies characterizing the secretome of these MSCs may reveal the mechanism underlying tissue repair by MSC-seeded matrices.

The first case of successful full-thickness circumferential replacement of the esophagus by TE in humans was reported in 2016. In this study, the esophagus of a 24-year-old man perforated by a

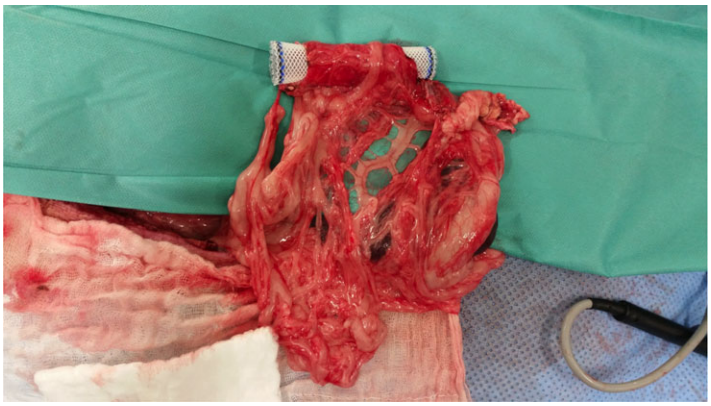

Figure 3. **Vascularized small intestine submucosa (SIS) scaffold seeded with mesenchymal stem cells after a 2-week maturation period in the greater omentum.**

metal plate placed in his cervical spine after a severe accident was repaired using AlloDerm®, a commercially available dermal ECM, sprayed with autologous platelet-rich plasma to promote stem cell recruitment. The matrix was rolled around a nonbiological stent, which was then introduced into the defect area to prevent strictures. Three years after surgery, the stent was removed; the following year, the patient showed full nutritional autonomy.[46]

Regulatory aspects

An important aspect that must be considered while developing a substitute for esophageal TE in human patients is the regulatory restrictions on these methods. In the European Union, different categories of laws are applied based on the final product. For instance, decellularized scaffolds or any other biomaterials alone would fall into the category of medical devices,[47,48] and thus validation of the product would require analyses to determine product sterility, biomechanical properties, and scaffold stability in the body.[49] Additionally, cell sheets or a combination of cells and a scaffold can be categorized as Advanced Therapy Medicinal Products, meaning that they are "medicines based on genes, tissues, or cells, offering groundbreaking new opportunities for the treatment of disease and injury."[50] These types of products are highly regulated[51] and require further thorough analysis to determine the reproducibility of the procedure, state of the cells alone or on a scaffold, their genomic stability, their distribution in the body once transplanted, and their outcome in a complex biological environment.

Thus, unlike *in vitro* and preclinical experimental trials, using a combination of cell types and scaffolds may make it more difficult to conduct clinical trials compared with a single cell type and scaffold. It is therefore important to thoroughly examine from the beginning of substitute development the cell types and matrices for inducing esophageal regeneration.

Conclusion

TE has become a promising alternative to traditional methods of esophageal repair, either for superficial defects following ESD or after esophagectomy. Cell sheet technology is a feasible and efficient method for preventing esophageal stricture following hemicircumferential ESD in animal models and humans. For full-thickness esophageal replacement, the use of cellularized biocompatible matrices has shown the best outcome in translational models. Current clinical techniques for organ replacement by TE as well as additional translational research protocols for esophageal replacement in large animals will result in authorization from health agencies for future clinical trials.

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Competing interests

The authors declare no competing interests.

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