Journal of Pediatric Surgery (2009) **44**, 896–901

Journal of Pediatric Surgery

www.elsevier.com/locate/jpedsurg

Esophagus tissue engineering: in vitro generation of esophageal epithelial cell sheets and viability on scaffold

Amulya K. Saxena*, **Herwig Ainoedhofer**, **Michael E. Höllwarth**

Department of Pediatric and Adolescent Surgery, Medical University of Graz, A-8036 Graz, Austria

Received 4 January 2009; accepted 15 January 2009

Key words:
Esophagus;
Tissue engineering;
Squamous epithelium;
Collagen;
Culture

Abstract

Purpose: Management of long gap esophageal atresia poses challenges. The surgical techniques for esophageal replacement are associated with complications and high morbidity. The aim of this study was to develop protocols to obtain single layer sheets of esophageal epithelial cells (EECs) and to investigate their survival on collagen scaffolds.

Methods: Esophageal epithelial cells were sourced from adult Sprague-Dawley rats. Briefly, the esophagus was treated with dispase to separate the epithelial layer and further trypsined to obtained EEC. The esophageal epithelial cells were cultured in vitro and seeded on to new generation of 3-dimensional collagen scaffolds.

Results: Esophageal epithelial cells organized after 48 hours in culture and formed clusters after 72 to 96 hours. Organization of the EEC was completed after 7 days in culture and characteristic sheets of EEC with the histologic morphology of mature esophageal epithelium were obtained after 14 days of culture. Immunohistochemistry demonstrated pure EEC culture using cytokeratin (CK-14) markers. The esophageal epithelial cells transferred on to collagen polymers demonstrated excellent viability after 8 weeks of in vitro culture.

Conclusion: Successful protocols for EEC isolation and proliferation have been established. The engineering of sheets of EEC and the viability of EEC on collagen scaffolds for 8 weeks in vitro, which are prerequisites for esophagus tissue engineering, was demonstrated.

Esophageal atresia is relatively a common congenital malformation occurring in 1:3000 to 5000 births [1]. Although the surgical repair to bridge the gap by primary anastomosis is successful in most cases, reconstruction of long gap esophageal atresia still poses challenges in surgical management. In patients with long gap atresia, delayed repair (after tacking the end of the esophagus to the prevertebral fascia or the Foker technique), myotomy, and esophageal replacement with strategies that include gastric, jejunum, or colon transposition are the possible options [2-4]. Unfortunately, these strategies are associated with a high rate of short and long-term complications that include leakage, stricture, elongation, and malnutrition because of shortening of the gastrointestinal tract [5-7].

Presented at the 40th Annual CAPS Meeting, August 21-24, 2008, Toronto, Ontario, Canada.

* Corresponding author. Tel.: +43 316 3853762; fax: +43 316 3852775.
E-mail address: amulya.saxena@meduni-graz.at (A.K. Saxena).

0022-3468/$ – see front matter © 2009 Elsevier Inc. All rights reserved.
doi:10.1016/j.jpedsurg.2009.01.019

The need for viable alternatives exists, and a tissue-engineered esophagus could overcome these limitations.

The esophagus is composed of the following 3 layers: the mucous layer, submucosa, and muscularis externa. One component of the mucous layer is the epithelial lining of stratified squamous cells. Within the stratified squamous epithelial lining, the state of differentiation varies spatially, with the basal layer containing epithelial progenitor cells and the outer layer consisting of epithelial cells that are terminally differentiated. As cells move from the inner to the outer layers, cytokeratin (CK) expression changes and proliferative ability decreases [8]. The submucosa of the esophagus consists of dense irregular connective tissue. Esophageal glands are concentrated in the cranial half of the esophagus in this layer and consist of compound tubulo-alveolar glands that secretes a slightly acid mucus to lubricate the lumen. The muscularis externa is bilaminar with an inner concentric and outer longitudinal arrangement. Remarkably and atypically, the type of muscle forming the muscularis externa changes from skeletal muscle proximally to smooth muscle distally. Hence, although the esophagus is a simple tubular organ, it presents tremendous variations in histologic morphology along its entire length (from the pharynx to the gastroesophageal junction) that offer challenges in tissue engineering.

Attempts at tissue engineering of the esophagus have demonstrated the feasibility of esophageal regeneration. In vivo esophageal repair has been performed with patch or circumferential implantation of synthetic as well as natural scaffolds such as: polyglycolic acid, acellular porcine aorta, small intestine submucosa, and a silicone/collagen hybrid [9-13]. Studies using patch grafts have shown that coverage of grafts by adjacent epithelium is a slow process that requires 3 to 4 weeks. On the other hand, circumferential grafts have been associated with complications ranging from stricture to dilation, including little or no muscle regeneration in these implants. Furthermore, when autologous epithelial cells were seeded into the silicone/collagen hybrid scaffold, epithelialization occurred within a period of 2 weeks after implantation and was associated with increased regeneration of the mesenchymal tissue [14]. Studies have also investigated the in vitro interaction of esophageal epithelial cells with scaffolds such as collagen and/or polyglycolic acid scaffolds [15,16].

Because treatment of pathologic conditions such as long gap esophageal atresia requires a larger patch of tissue-engineered esophagus, the purpose of this study was to isolate esophageal epithelial cells and to produce sheets of esophageal epithelium in vitro. Furthermore, this study aimed to evaluate the viability of isolated esophageal cells seeded under low density on the new generation 3-dimensional collagen polymers and to determine the period these cells could be maintained viable on collagen polymers in vitro, which is a prerequisite for future composite scaffold-hetrocellular esophagus tissue engineering.

1. Materials and methods

1.1. Epithelial cell isolation and characterization

Rat esophageal epithelial cells (REECs) were isolated by modifying a previously published protocol [17]. Briefly, a rat esophagus was harvested from a Sprague-Dawley rat as approved by the Animal Care and Use Committee at Ministry of Science and Research, Vienna, Austria. The esophagus was rinsed well in 4°C PBS (Phosphate buffered saline) (Sigma-Aldrich, St Louis, Mo) containing antibiotics (100 U/mL of penicillin G sodium, 100 mg/mL of streptomycin sulfate, and 0.25 mg/mL of amphotericin B; Sigma-Aldrich, St Louis, Mo) and incubated overnight at 4°C in dispase (50 caseinolytic U/mL; BD Biosciences, Bedford, Mass) containing antibiotics. The next day, the epithelium was mechanically separated from the connective tissue and cut longitudinally using an operating microscope or loops to magnify the structures. The epithelium was then treated for 10 minutes with 0.05% trypsin in 0.53 mmol/L of EDTA (Sigma-Aldrich, St Louis, Mo) at 37°C, followed by continual pipetting for 10 minutes. Culture media plus 10% fetal bovine serum (Sigma-Aldrich, St Louis, Mo) was added to neutralize the trypsin, and the cells were pelleted by centrifugation (1000 revolutions per minute for 5 minutes). Finally, the cells were resuspended in culture media and plated on collagen-precoated 24-well plates. The culture media consisted of EpiLife basal media (Cascade Biologics, Portland, Ore) to which calcium was added to reach a concentration of 0.06 mmol/L calcium, supplemented with Human Keratinocyte Growth Supplement (Cascade Biologics, Portland, Ore), bovine pituitary extract (0.2%), insulin (5 μg/mL), hydrocortisone (0.18 μg/mL), human epidermal growth factor (0.2 ng/mL), transferrin (5 μg/mL), triiodothyronine (6.51 ng/mL) (Sigma-Aldrich, St Louis, Mo), and glutamine-penicillin-streptomycin/amphotericin-B (Sigma-Aldrich, St Louis, Mo). Cells were grown at 37°C in 5% carbon dioxide. The media was changed every alternative day.

To confirm REEC identity, cultured cells were fixed in acetone (Merck, Darmstadt, Germany) and stained using an anti–CK-14 antibody (sc-23878, Santa Cruz Biotechnology, Santa Cruz, Calif). The control consisted of a sample incubated with concentration-matched mouse IgG (sc-2025, Santa Cruz Biotechnology, Santa Cruz, Calif) instead of the primary antibody. Cells were then incubated with a (Fluorescein isothiocyanate)-conjugated rabbit antimouse (F0232, Dako, Glostrup, Denmark) and To-Pro-3-iodide (Invitrogen, Carlsbad, Calif) was used as a nuclear counterstain. Cells were imaged using a fluorescent microscope.

1.2. Collagen scaffold

OptiMaix-3D collagen scaffolds (OptiMaix 3D001315, Matricel GmbH, Herzogenrath, Germany) 13 mm in diameter and 1.5-mm thick were used as scaffolds for

REEC seeding. OptiMaix-3D scaffolds provide a unique 3-dimensional matrix for in vitro investigation because of a patented manufacturing process that involves unidirectional solidification and freeze-drying of an aqueous dispersion containing 1.5 weight percentage of porcine collagen (mainly type I) and low amounts of elastin [18]. Briefly, the collagen dispersion is cooled and frozen using a defined temperature gradient that is maintained during cooling. This temperature gradient creates a 1-dimensional heat flow in the direction of the temperature gradient. By adding an organic acid, ice crystals are induced to grow in a dendritic crystal morphology without side branches. These finger-shaped ice crystals sublime during freeze-drying that result in oriented pores. Most collagen fibers in the dispersion are concentrated between the ice fingers thereby forming the final structure of the scaffold. Through this process, a controlled, homogeneous, and well-organized pore structure with pore sizes between 25 and 100 μm can be produced (Fig. 1).

1.3. Cell seeding protocol

OptiMaix is sterilized using ethylene oxide because it does not distort the porous structure and is packaged for direct use. Scaffolds were removed from sterile packaging and sewn into tubes to mimic the esophagus structure (Fig. 2). Care was taken during bending of the scaffold to prevent tears because of excessive and forceful bending. Also, care was taken while suturing under operating microscope using microsurgical instruments to avoid tears in the scaffold at the entry and exit point of the sutures. All tubes were precoated with 25 mg/mL collagen (11179179001, Roche Diagnostics, Mannheim, Germany) for 1 hour at 37°C and 7-day-old REECs were seeded on the inner surface of the sewn tubes at a low density of 60,000 cells/cm^2. The tubes were attached to the tissue culture dishes using collagen. The cells were allowed to adhere for 30 minutes before introducing media into the wells. Precaution was taken to provide media sufficient enough to cover the scaffold, to prevent its dislodgement from the culture plates initially. Media was changed every alternative day, and disks were removed in regular weekly intervals (1, 2, 3, 4, 5, 6, 7, and 8 weeks) for histochemical analysis.

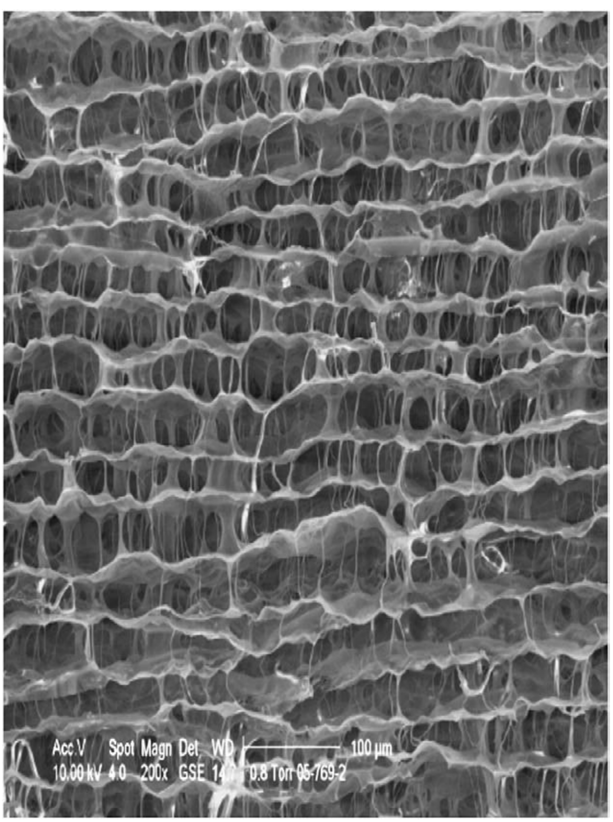

Fig. 1 SEM image of OptiMaix 3D collagen scaffolds with pore diameters of 50 μm (original magnification, ×200; bar, 100 μm) showing geometric orientation of the scaffold produces using patented unidirectional solidification and freeze-drying technique.

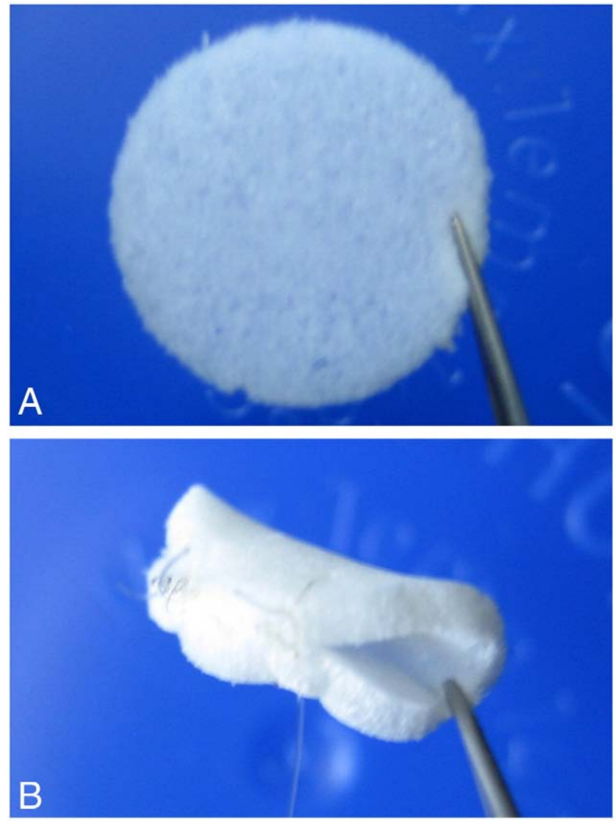

Fig. 2 A, OptiMaix 3D collagen scaffold disks 13-mm diameter and 1.5-mm thickness. B, Disks are folded and sutured using monofilament sutures as tubes for seeding of REEC on the inner surface.

2. Results

Rat esophageal epithelial cells were successfully isolated and cultured. The cells displayed a spherical morphology

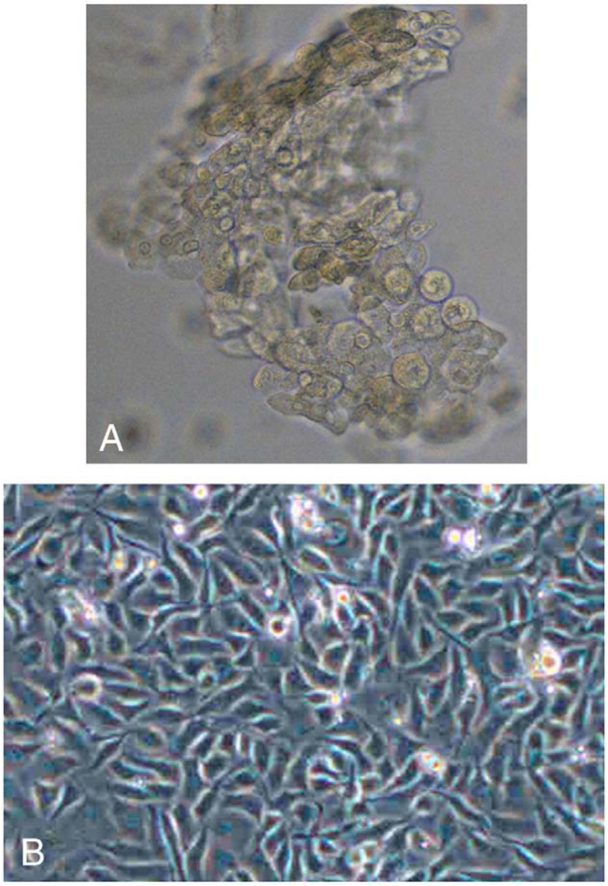

Fig. 3 A, Epithelial cell formed cluster on plates that were not coated and (B) spread out as cobblestone-shaped cells on plates that were precoated with collagen (phase contrast, original magnification ×60).

after isolation when placed in culture. After 1 week, cells in non-precoated culture dishes congregated to form clusters (Fig. 3A), whereas in precoated dishes, REEC exhibited a cobblestone morphological characteristic of epithelial cells

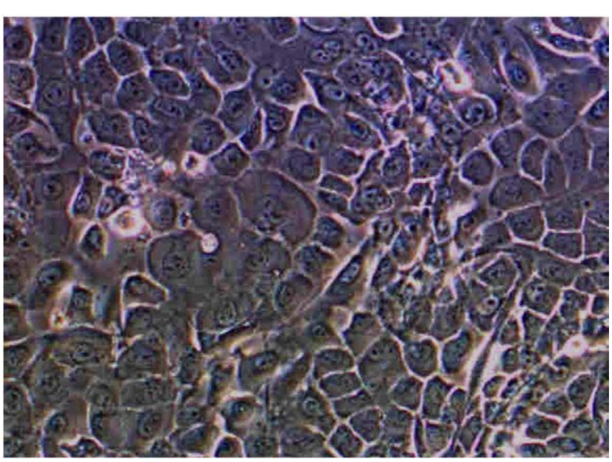

Fig. 4 Single sheet of esophageal epithelial layer spread across the tissue culture plates after 14 days of in vitro culture (phase contrast, original magnification ×60).

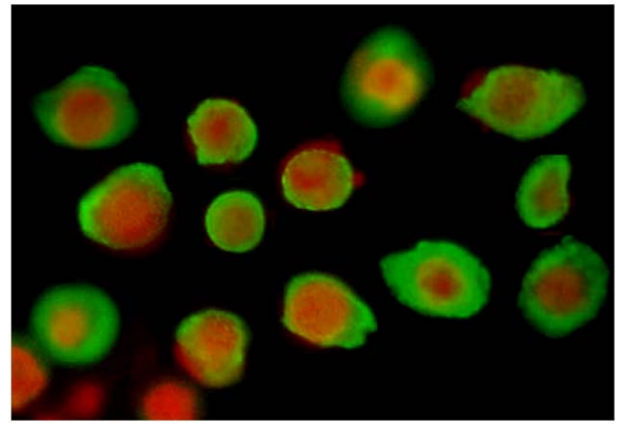

Fig. 5 Positive expression of REECs using CK-14 markers (immunofluorescence) after 7 days of in vitro culture (oil, original magnification ×100).

(Fig. 3B). Cells maintained further in culture differentiated to mature epithelium after 14 days and filled the entire wells with a single squamous epithelial layer (Fig. 4). Squamous epithelial cells that were stained using CK marker CK-14 after 7 days in culture demonstrated pure cultures and positive staining of cells (Fig. 5).

Rat esophageal epithelial cells were seeded successfully on the inner surface of OptiMaix 3D scaffolds that were

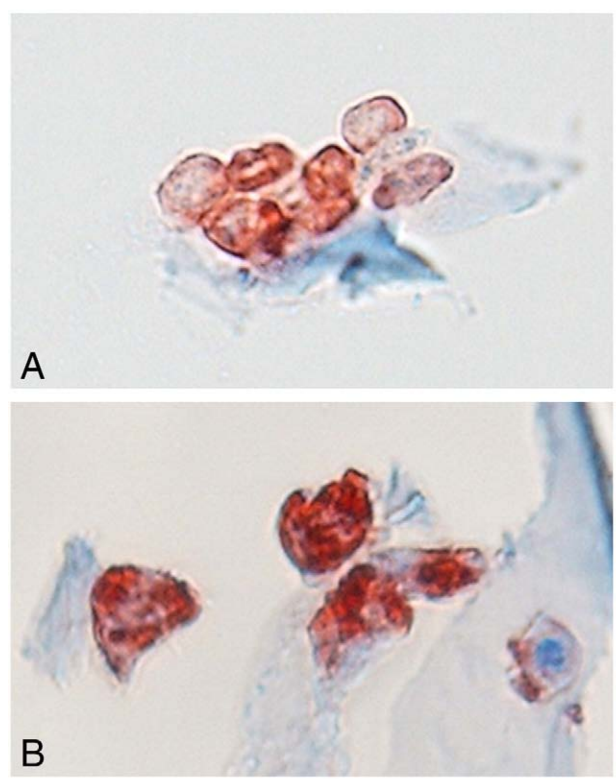

Fig. 6 A, Cluster of REECs on collagen scaffolds after 8 weeks of in vitro culture, demonstrating positive expression for CK-14 (immunohistochemistry) and (B) isolated cells (oil, original magnification ×100).

folded into tubes mimicking the esophagus. Scaffold suturing was successful using 5-0 monofilament sutures with care taken to apply controlled tension during knot tying while approximating the edges of the disk. Caprosyn (Syneture, Norwalk, Conn) 5-0 monofilament absorbable sutures were found to be the ideal to produce tubes from OptiMaix 3D (13-mm diameter and 1.5-mm thickness) disks.

Cell-polymer constructs removed at 1, 2, 3, 4, 5, 6, 7, and 8 weeks of in vitro culture were examined histologically for viable cells and investigated for expression of CK-14. Rat esophageal epithelial cells seeded on scaffolds expressed CK-14 on all cell-polymer constructs investigated up to the eighth week of in vitro culture, with most of the cells adherent to the inner surface of the scaffold tube where they were initially seeded. No cell migration through the thickness of the scaffold was observed. Rat esophageal epithelial cells were found to survive in groups as well as in isolation on the polymer scaffold even after 8 weeks (Fig. 6). Furthermore, immunohistochemical evaluation confirmed the presence of only one cell type (REEC) on the scaffold, confirming the purity of the isolated cells.

3. Discussion

There is a need for tissue engineering of esophageal tissue as it has widespread application for the pediatric and adult patients. Long gap atresia, cancer, Barrett's esophagus, and esophagus strictures and stenosis (corrosive esophagitis after alkaline ingestions) are some pathologic states that may necessitate esophagus replacement [19,20]. However, attempts to replace the esophagus with natural, synthetic, and experimental substitutes have been futile because of problems such as leakage, infections, or stenosis being associated with them [21-23].

Esophagus is a muscular tube that transverses 3 anatomical planes (neck, thorax, and abdomen) and serves as a conduit to transport food and fluids from the mouth to the stomach. It comprises of 3 main kinds of cells, that is, squamous epithelial cells, fibroblasts, and smooth muscle cells. Attempts to tissue engineer the esophagus requires composite scaffolds that will ensure the proper orientation of these cells with the distribution of the epithelial cells toward the lumen, fibroblasts within the submucosal, and circular and longitudinal smooth muscles toward the periphery. Before composite scaffolds are reconstituted, viability of each of the 3 cell types must be evaluated on scaffolds in in vitro conditions. Furthermore, for the engineering of long-term implants, which neither leak nor had stenosis, epithelialization over the entire lumen of the implanted scaffold will be of paramount importance [24]. Because epithelial cells are considered to be the most important of the class of esophageal cells in providing natural esophageal functions in tissue-engineered substitutes, it is necessary to evaluate the formation of epithelial layers under in vitro conditions and to investigate the sustained viability of these cells on scaffolds.

The in vitro production of single sheet of esophageal epithelial layer on tissue culture plates shows promising results in the attempts to tissue engineer esophagus epithelium. Confluence of the individual cells to form epithelial sheets in this study was evident from the seventh day in culture with mature morphology of squamous epithelial cells evident from the 14th day. Collagen precoating of tissue culture dishes has a positive influence on the formation of sheets and the even distribution of cells [25]. However, tissue culture plates that were not precoated showed weak attachment of cells. Groups of cells in closer proximity to each other demonstrated cluster formation and growth of cells in layers above the cells that were attached. These 2 observations are important because epithelial sheets may be transferred to scaffolds to (*a*) cover an entire surface thus preventing leaks in tissue-engineered constructs and (*b*) be layered on top of one another to engineer multilayered esophageal epithelial sheets.

Because complete epithelialization of the scaffold is necessary to prevent leaks, it is necessary to investigate the survivability of epithelial cell on scaffolds before in vivo implantation. Furthermore, complete epithelialized scaffolds of larger sizes will require the viable epithelial cells toward the center of the scaffolds. The in vitro survivability of epithelial cells on porous scaffolds (used for this purpose), up to 8 weeks after seeding, demonstrated the ability of these cells to exist isolated or in groups throughout the scaffold. Also, the expression of CK-14 in the epithelial cells was confirmed, which demonstrated phenotypic preservation 8 weeks after seeding on scaffolds maintained in vitro. Cytokeratin-14 of the seeded epithelial cells demonstrated positive expression in the entire cell population and verified the purity of the cell population seeded.

Scaffold designing and production using synthetic materials (to mimic natural materials that demonstrate better organization of esophageal epithelial cells) will play a major role in the progress of esophagus tissue engineering [26]. Although attention is being focused on the esophageal epithelial layer, better understanding of the complex mechanical properties of the nonlinear and anisotropic esophageal tissue with regard to axial extension and its correlation to pressure-radial-axis force will be necessary [27] to produce scaffolds that meet the required mechanical demands. At the same time, the viscoelasticity of the esophageal tissue will have to be estimated during ramp and relaxation phases [28]. Besides mechanical properties, orientated scaffold architecture will have to be developed to support the myoarchitecture of the esophageal wall. Recent studies to investigate the myoarchitecture of bovine esophageal wall with diffusion spectrum imaging and tractography have shown that esophageal myoanatomy consists of crossing myofibers exhibiting a decreasing degree of helicity as a function of axial position and that this unique geometric construct provides a mechanism to resist distension [29].

Using a biopsy to obtain cells, expanding them in culture, seeding them onto a biopolymer, and using it for tissue repair in host are the goals of tissue engineering [30]. Esophageal epithelial sheet formations and the survival of esophageal epithelial cells for a period of 8 weeks on scaffolds in vitro, using this principle, demonstrate promising results as part of the efforts in producing a tissue-engineered esophagus.

Acknowledgment

This research is funded by the European Union within the sixth Framework Program (EuroSTEC; LSHC-CT-2006-037409). We thank Prof Wout Feitz (Radboud University Medical Center, Nijmegen, the Netherlands), Dr Ingo Heschel (Matricel GmbH, Herzoganrath, Germany) along with Mrs Anna Kuess and Ms Kristina Kofler (Medical University of Graz, Austria) for the valuable contributions toward this study.

References

[1] Clark DC. Esophageal atresia and tracheoesophageal fistula. Am Fam Physician 1999;59:910-6.

[2] Spitz L, Ruangtrakool R. Esophageal substitution. Semin Pediatr Surg 1998;7:130-3.

[3] Cusick EL, Batchelor AA, Spicer RD. Development of a technique for jejunal interposition in long-gap esophageal atresia. J Pediatr Surg 1993;28:990-4.

[4] Raffensperger JG, Kuck SR, Reynolds M, et al. Intestinal bypass of the esophagus. J Pediatr Surg 1996;31:38-46.

[5] Spitz L. Esophageal atresia- lessons I have learned in a 40-year experience. J Pediatr Surg 2006;41:1635-40.

[6] Cauchi JA, Buick RG, Gornall P, et al. Oesophageal substitution with free and pedicled jejunum: short- and long-term outcomes. Pediatr Surg Int 2007;23:11-9.

[7] Arul GS, Parikh D. Oesophageal replacement in children. Ann R Coll Surg Engl 2008;90:7-12.

[8] Squier CA, Kremer MJ. Biology of oral mucosa and esophagus. J Natl Cancer Inst Monogr 2001;29:7-15.

[9] Kajitani M, Wadia Y, Hinds MT, et al. Successful repair of esophageal injury using an elastin based biomaterial patch. ASAIO J 2001;47: 342-5.

[10] Isch JA, Engum SA, Ruble CA, et al. Patch esophagoplasty using AlloDerm as a tissue scaffold. J Pediatr Surg 2001;36:266-8.

[11] Takimoto Y, Nakamura T, Yamamoto Y, et al. The experimental replacement of a cervical esophageal segment with an artificial prosthesis with the use of collagen matrix and a silicone stent. J Thorac Cardiovasc Surg 1998;116:98-106.

[12] Badylak S, Meurling S, Chen M, et al. Resorbable bioscaffold for esophageal repair in a dog model. J Pediatr Surg 2000;35:1097-103.

[13] Grikscheit T, Ochoa ER, Srinivasan A, et al. Tissue-engineered esophagus: experimental substitution by onlay patch or interposition. J Thorac Cardiovasc Surg 2003;126:537-44.

[14] Natsume T, Ike O, Okada T, et al. Experimental studies of a hybrid artificial esophagus combined with autologous mucosal cells. ASAIO Trans 1990;36:M435-7.

[15] Sato M, Ando N, Ozawa S, et al. An artificial esophagus consisting of cultured human esophageal epithelial cells, polyglycolic acid mesh, and collagen. ASAIO J 1994;40:M389-92.

[16] Miki H, Ando N, Ozawa S, et al. An artificial esophagus constructed of cultured human esophageal epithelial cells, fibroblasts, polyglycolic acid mesh, and collagen. ASAIO J 1999;45:502-8.

[17] Oda D, Savard CE, Eng L, et al. Reconstituted human oral and esophageal mucosa in culture. In-Vitro Cell Dev Biol Anim 1998;34: 46-52.

[18] Kuberka M, von Heimburg D, Schoof H, et al. Magnification of the pore size in biodegradable collagen sponges. Int J Artif Organs 2002; 25:67-73.

[19] Patrick Jr CW, Mikos AG, McIntire LV. Frontiers in tissue engineering. New York: Elsevier Science; 1998.

[20] American Cancer Society. Esophagus Cancer Detailed Guide. www.cancer.org; 2007.

[21] Takimoto Y, Nakamura T, Teramachi M, et al. Replacement of long segments of the esophagus with a collagen silicone composite tube. ASAIO J 1995;41:M605-8.

[22] Chen MK, Badylak SF. Small bowel tissue engineering using small intestinal submucosa as a scaffold. J Surg Res 2001;99:352-8.

[23] Lindberg K, Badylak SF. Porcine small intestinal submucosa (SIS): a bioscaffold supporting in vitro primary human epidermal cell differentiation and synthesis of basement membrane proteins. Burns 2001;27:254-66.

[24] Zhu Y, Leong MF, Ong WF, et al. Esophageal epithelium regeneration on fibronectin grafted poly(L-lactide-co-caprolactone) (PLLC) nanofiber scaffold. Biomaterials 2007;28:861-8.

[25] Herzog C, Zhuang L, Gorgan L, et al. Tissue- and developmental stage-specific activation of alpha 5 and alpha 6 (IV) collagen expression in the upper gastrointestinal tract of transgenic mice. Biochem Biophys Res Commun 2003;311:553-60.

[26] Beckstead BL, Pan S, Bhrany AD, et al. Esophageal epithelial cell interaction with synthetic and natural scaffolds for tissue engineering. Biomaterials 2005;26:6217-28.

[27] Yang W, Fung TC, Chian KS, et al. 3D Mechanical properties of the layered esophagus: experiment and constitutive model. J Biomech Eng 2006;128:899-908.

[28] Yang W, Fung TC, Chian KS, et al. Viscoelasticity of esophageal tissue and application of a QLV model. J Biomech Eng 2006;128:909-16.

[29] Gilbert RJ, Gaige TA, Wang R, et al. Resolving the three-dimensional myoarchitecture of bovine esophageal wall with diffusion spectrum imaging and tractography. Cell Tissue Res 2008;332:461-8.

[30] Vacanti JP, Morse MA, Saltzmann WM, et al. Selective cell transplantation using bioabsorbable artificial polymers as matrices. J Pediatr Surg 1988;23:3-9.