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Preparation and Characterization of a Biologic Scaffold from Esophageal Mucosa

Timothy J. Keane^{1,2}, Ricardo Londono^{1,4}, Ryan M. Carey¹, Christopher A. Carruthers^{1,2}, Janet E. Reing¹, Christopher L. Dearth^{1,3}, Antonio D'Amore¹, Christopher J. Medberry^{1,2}, and Stephen F. Badylak^{1,2,3}

¹McGowan Institute for Regenerative Medicine, University of Pittsburgh, Pittsburgh, Pennsylvania, 15219, USA

²Department of Bioengineering, University of Pittsburgh, Pittsburgh, Pennsylvania, 15219, USA

³Department of Surgery, University of Pittsburgh, Pittsburgh, Pennsylvania, 15219, USA

⁴School of Medicine, University of Pittsburgh, Pittsburgh, Pennsylvania, 15219, USA

Abstract

Biologic scaffolds composed of extracellular matrix (ECM) are commonly used to facilitate a constructive remodeling response in several types of tissue, including the esophagus. Surgical manipulation of the esophagus is often complicated by stricture, but preclinical and clinical studies have shown that the use of an ECM scaffold can mitigate stricture and promote a constructive outcome after resection of full circumference esophageal mucosa. Recognizing the potential benefits of ECM derived from homologous tissue (i.e., site-specific ECM), the objective of the present study was to prepare, characterize, and assess the in-vivo remodeling properties of ECM from porcine esophageal mucosa. The developed protocol for esophageal ECM preparation is compliant with previously established criteria of decellularization and results in a scaffold that maintains important biologic components and an ultrastructure consistent with a basement membrane complex. Perivascular stem cells remained viable when seeded upon the esophageal ECM scaffold in vitro, and the in-vivo host response showed a pattern of constructive remodeling when implanted in soft tissue.

Keywords

ECM (extracellular matrix); Decellularization; Scaffold; Immune response

1. Introduction

The default mechanism of mammalian tissue repair typically results in scar tissue deposition, a protective and favorable response in most tissues. However, this scar tissue formation is associated with adverse clinical consequences including stricture in select anatomic locations such as the esophagus. Preclinical studies have shown that placement of

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Corresponding Author: Dr. Stephen Badylak, McGowan Institute for Regenerative Medicine, University of Pittsburgh, Bridgeside Point 2, 450 Technology Drive, Pittsburgh, Pennsylvania, 15219. Tel: 412-624-5253 Fax: 412-624-5253, badylaksf@upmc.edu.

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an extracellular matrix (ECM) scaffold derived from heterologous tissue is capable of restoring a functional esophagus with minimal stricture and normal esophageal motility following circumferential mucosal resection [1]. A clinical report involving patients with stage 1 esophageal adenocarcinoma corroborated this finding and provided proof-of-concept in the clinical setting [2, 3]. While heterologous ECM was successful in reducing stricture formation, the remodeled tissue did not fully reconstitute all components of normal esophageal tissue; for example, glandular tissue was absent. Delivery of the scaffold also required temporary placement of an intraluminal stent to allow integration of the scaffold with the subjacent tissue. A possible advantage of a site-specific, homologous ECM could be more rapid integration and faithful remodeling of the esophageal mucosa.

Recent work has described potential benefits of ECM scaffold materials derived from homologous tissue versus heterologous tissue when used in selected anatomic locations [4–13]. While tissue specificity is not necessary for all therapeutic applications [2, 14, 15], some studies have shown that site-specific ECM can preferentially maintain tissue-specific cell phenotypes [4–7], promote cell proliferation [6, 8], induce tissue-specific differentiation [9], and enhance the chemotaxis of lineage-directed progenitor cells [10–12]. It is plausible therefore that a site-specific esophageal mucosal ECM may promote similar effects and further improve clinical outcomes in esophageal mucosa repair. The harvesting and preparation of an ECM scaffold requires tissue specific methodologies for optimal outcomes [16–20].

Biologic scaffolds composed of ECM, when prepared by methods designed to preserve structure and composition of the native source tissue, contain bioactive molecules including growth factors (e.g., vascular endothelial growth factor (VEGF) [21], basic fibroblast growth factor (bFGF) [22]) and glycosaminoglycans (GAGs) [23]. The composition, ultrastructure, and mechanical properties of an ECM construct are affected by the methods used to decellularize the source tissue as well as the methods of sterilization and storage of such bioscaffolds [20, 24, 25]. Therefore, the methods of preparing ECM scaffolds intended for use in the repair and reconstruction of the esophageal mucosa must be carefully considered as regenerative medicine strategies are developed for this intended therapeutic application.

The objective of the present study was to prepare, characterize, and determine the in-vitro cytocompatibility and in-vivo host response of ECM derived from porcine esophageal mucosa (emECM). Esophagi were collected and decellularized by a method sufficient to meet stringent decellularization criteria: specifically no visible intact nuclei by hematoxylin and eosin staining, remnant DNA concentration less than 50 ng/mg dry weight, and DNA fragment length less than 200 basepairs [24]. Biochemical and mechanical properties of the ECM were then characterized by quantitative and qualitative measures.

2. Materials and methods

2.1. Harvest and preparation of ECM from porcine esophagus

Esophagi were harvested from market weight (240–260 lbs) pigs and split longitudinally. The mucosa and submucosa were isolated by mechanical separation from the muscularis propria. The luminal surface was gently abraded to remove squamous epithelium. The tissue that remained was composed primarily of the basement membrane, lamina propria, muscularis mucosa, and submucosa. This tissue was then subjected to a series of immersion treatments as follows: 1% trypsin/0.05% EDTA (Invitrogen, Carlsbad, CA) for 1 h at 37°C on a rocker plate, deionized water for 15 min, 1.0 M sucrose (Fisher Scientific, Pittsburgh, PA) for 30 min, deionized water for 30 min, 3.0% Triton X-100 (Sigma Aldrich, St. Louis, MO) for 48 h, deionized water for 15 min, PBS (Fisher Scientific) for 15 min, 10% deoxycholate (Sigma Aldrich) for 4 h, deionized water for 30 min, 0.1% peracetic acid

(Rochester Midland Corp., Rochester, NY) in 4.0% ethanol for 4 h, 100 U/ml DNase (Invitrogen) for 2 h on a rocker plate, followed by 15 min washes with PBS, deionized water, PBS, and deionized water. All treatments were performed at room temperature with agitation on a shaker plate at 300 RPM unless otherwise stated. For cytocompatibility evaluation and in-vivo remodeling evaluation, chemically cross-linked emECM (XL-emECM) scaffolds were used as negative controls. Chemically cross-linked bioscaffolds have been shown to consistently inhibit a constructive remodeling response [26, 27]. Cross-linking was achieved by immersion in 0.01 M carbodiimide for 24 hours with multiple subsequent washes in PBS over 48 hours. All devices were lyophilized and sterilized using ethylene oxide.

2.2. Assessment of DNA content

DNA was extracted from representative samples (n=6) of emECM. For DNA extraction, lyophilized ECM scaffolds were powdered using a Wiley Mill and filtered through a 60-mesh screen. One hundred milligrams of lyophilized, powdered emECM was digested with proteinase K digestion buffer (100 mM NaCl, 10 mM Tris-HCl (pH = 8), 25 mM EDTA (pH = 8), 0.5% SDS, 0.1 mg/mL proteinase K) at 50 °C for 24 hr. The digest was extracted twice using 25:24:1 (v/v/v) phenol/chloroform/isoamyl alcohol. DNA was precipitated from the aqueous phase at -20 °C with the addition of 2 volumes of ethanol and 0.1 volume of 3 M sodium acetate (pH = 5.2). The DNA was then centrifuged at 10,000 g for 10 min and resuspended in 1 mL of TE buffer (10 mM Tris (pH = 8), 1 mM EDTA).

The concentration of each extracted DNA sample was determined using Quant-iT PicoGreen dsDNA Assay Kit (Invitrogen) following the manufacturer's recommended protocol. A standard curve was constructed by preparing samples of known DNA concentrations from 0 to 1000 ng/mL and concentration of DNA was found by linear interpolation of the standard curve. Samples were read using SpectraMax M2 Plate Reader (Molecular Devices, Sunnyvale, CA). DNA samples were diluted to ensure their absorbance properties fell within the linear region of the standard curve.

To determine the fragment size of remnant DNA, equal concentrations of extracted DNA from each sample were separated on a 2% agarose gel containing 0.5% ethidium bromide and visualized with ultraviolet transillumination using a reference 100 bp ladder (New England BioLabs, Ipswich, MA). All assays were performed in quadruplicate.

2.3. Immunolabeling and histochemistry

A set of slides (n=6) was stained to visualize the extent of cell removal with a standard hematoxylin and eosin (H&E) protocol.

Antigen retrieval was performed for immunolabeling studies using a 0.01 M citrate buffer (pH=6) heated to 95–100°C. Slides were placed in the hot buffer for 20 min and subsequently rinsed in PBS (3 × 5 min). Sections were placed in pepsin solution (0.05% pepsin/0.01 M HCl) at 37°C for 15 minutes. After rinsing in PBS (3 × 5 min), the samples were blocked in blocking buffer (2% goat serum/1% bovine serum albumin/0.1% Triton X-100/0.1% Tween) for 1 hr at room temperature. The sections were then incubated in the blocking buffer with rabbit polyclonal collagen IV antibody (1:500 dilution, Abcam, Cambridge, UK), rabbit polyclonal laminin antibody (1:200 dilution, Abcam), or mouse monoclonal fibronectin (1:200 dilution, Abcam) overnight at 4°C in a humidified chamber. Sections were subsequently rinsed in PBS (3 × 5 min). Endogenous peroxidase activity was quenched by rinsing sections in a 3% hydrogen peroxide in methanol solution for 30 min followed by rinsing in PBS (3 × 5 min). Biotinylated goat anti-rabbit or goat anti-mouse secondary antibodies (Vector Laboratories, Burlingame, CA) were diluted 1:200 in blocking

buffer and added to the sections for 30 min at 25 °C and sections were subsequently rinsed in PBS (3 × 5 min). The slides were then incubated in detection solution (VectaStain® Elite ABC Reagent, Vector Laboratories) for 30 minutes at 37°C. After rinsing the slides, peroxidase substrate, 3,3'-diaminobenzadine (ImmPACT™ DAB, Vector Laboratories) was prepared as per manufacturer instructions and sections were incubated while being visualized under a microscope to time the color change for subsequent section staining intensities. Tissues were rinsed in water (3 × 5 min). Sections were dipped in hematoxylin (Thermo Shandon, Pittsburgh, PA) for 1 min for a nuclear counterstain and subsequently rinsed in PBS (3 × 5 min).

2.4. Sulfated glycosaminoglycan assay

Sulfated glycosaminoglycan (sGAGs) concentration in esophageal ECM samples was determined using the Blyscan Sulfated Glycosaminoglycan Assay Kit (Biocolor Ltd, Belfast, Northern Ireland). For extraction of sGAGs, lyophilized ECM scaffolds were powdered using a Wiley Mill and filtered through a 60-mesh screen. Samples were prepared by digestion of 50 mg/ml dry weight of each sample with 0.1 mg/ml proteinase K in buffer (10 mM Tris-HCl, pH 8, 100 mM NaCl, 25 mM EDTA) for 48 hr at 50°C. Digested samples were assayed following the manufacturer's protocol, and the assay was performed in duplicate on three different emECM sample.

2.5. Growth factor assay

The concentration of basic fibroblast growth factor (bFGF) and vascular endothelial growth factor (VEGF) in urea-heparin extracts of emECM samples was determined with the Quantikine Human FGF basic Immunoassay, Human VEGF Immunoassay (R&D Systems, Minneapolis, MN). Each assay for bFGF and VEGF was performed in quadruplicate. The ELISA assays are cross-reactive with porcine growth factors and do not measure activity.

2.6. Scanning electron microscopy

Scanning electron micrographs were taken to examine the surface topology of emECM. Prior to final lyophilization, samples were fixed in cold 2.5% (v/v) glutaraldehyde (Electron Microscopy Sciences, Hatfield, PA) in PBS for at least 24 hr, followed by three washes in PBS. Fixed samples were then dehydrated using a graded series of alcohol (30, 50, 70, 90, 100%) for 15 min each, followed by 15 min in hexamethylenediamine (Fisher) and subsequent air-drying. The dried samples were sputter coated with a 3.5 nm layer of gold/palladium alloy using a Sputter Coater 108 Auto (Cressington Scientific Instruments, Watford, UK) and imaged with a JEOL JSM6330f scanning electron microscope (JEOL, Peabody, MA) at 100× and 500× magnifications.

2.7. Perivascular stem cell (PVSC) culture

Perivascular stem cells isolated by flow cytometry from fetal muscle [28, 29] were used in all experiments. These cells (CD146⁺/NG2⁺/CD34⁻/CD144⁻/CD56⁻) have been previously shown to represent a distinct population of perivascular cells obtained after positive selection and stringent exclusion of hematopoietic, endothelial, and myogenic cells, and which are able to differentiate into mesodermal lineages [29, 30]. Isolated cells were cultured in high-glucose Dulbecco's modified Eagle's medium (Invitrogen) containing 20% fetal bovine serum (Thermo), 100 U/mL penicillin, and 100 µg/mL streptomycin (Sigma Aldrich) at 37°C in 5% CO₂.

In-vitro cell viability assays were performed using single layer sheets of ECM. PVSCs (0.5×10⁶) were cultured for 48 hr on 2cm diameter circular sheets of emECM or XL-emECM. Cell viability was compared to growth on tissue culture plastic (TCP) using LIVE/

DEAD® Viability/Cytotoxicity Kit (Invitrogen) following manufacturer's guidelines. Capturing 4 random fields across the emECM scaffold, the live and dead cells were imaged with green fluorescent calcein-AM (cAM) and red fluorescent ethidium homodimer-1 (EtH1), respectively. Quantification of live and dead cells was achieved using a custom image analysis algorithm developed using the cell profiler image analysis package [31, 32]. This custom algorithm identified and quantified the number of cAM⁺ (live) and EtH1⁺ (dead) cells present on the emECM scaffolds. These results were then expressed as a percentage of total cells.

2.8. In-vivo cytocompatibility

All procedures were performed in accordance with the National Institute of Health (NIH) guidelines for care and use of laboratory animals and with approval of the Institutional Animal Care and Use Committee (IACUC) at the University of Pittsburgh. Sprague Dawley rats (female; 250–350g) were anesthetized with 1.5–3% isoflurane and maintained at a surgical plane of anesthesia. The surgical site was shaved, disinfected with a betadine solution, and an incision was made into the ventrolateral abdominal wall. Bilateral partial thickness abdominal body wall defects [33] were created by excision of a 1cm² piece of tissue comprising the internal and external oblique muscles but leaving the transversalis muscle intact [26]. Size matched emECM or XL-emECM scaffolds were then sutured into the defect site using nonresorbable 4-0 prolene sutures at each of the 4 corners of the device. The skin was closed using 3-0 resorbable vicryl sutures. Rats were euthanized at 14 or 35 days post-implantation and implant sites were identified by nonresorbable sutures. The implant site containing emECM devices and adjacent tissue site were isolated and placed in 10% neutral buffered formalin. Fixed samples were paraffin embedded and cut into 6 µm sections.

The sections from 14 and 35 days post-op were stained with H&E for a qualitative and semiquantitative histomorphologic analysis that evaluated cell infiltration, multinucleated giant cells, vascularity, connective tissue, encapsulation, and scaffold degradation. Two blinded investigators scored the sections according to a previously established and validated semi-quantitative scoring method [26, 34]. Using quantitative scoring criteria (Table 1), biologic scaffolds can be grouped according to chronic inflammation and foreign body response (quantitative score < 5), early inflammation with decreased cellularity and little evidence of constructive remodeling at later time points (5 < quantitative score < 10), and early inflammation by inflammatory cells and signs of constructive remodeling at a later time point (quantitative score > 10).

Immunolabeling of macrophages was performed on tissue sections from day 14 explants. Paraffin embedded sections were deparaffinized with xylene and rehydrated through a graded ethanol series. Heat-mediated antigen retrieval was performed with 0.01 M citrate buffer (pH=6) at 95–100 °C for 25 min. The tissue sections were subjected to Tris-Buffered Saline Tween-20 (TBST) for 15 min, followed by incubation in blocking buffer (2% horse serum albumin/1% bovine serum albumin/0.05% Tween-20/0.05% Triton X-100) for 1 h. The primary antibodies, diluted in blocking buffer, were added to the slides for 16 hr at 4 °C in a humidified chamber. The slides were then washed three times in PBS prior to the addition of the secondary antibody for 1 hr in a humidified chamber at room temperature. DAPI was used as a nuclear counterstain. The primary antibodies used in this study were mouse anti-rat CD68 (1:150, AbD Serotec, Raleigh, NC), goat polyclonal CD206 (Santa Cruz Biotech, Santa Cruz, CA), rabbit anti-rat CD86 (1:150, Abcam) and mouse anti-rat CD68 (1:50, Serotec, Raleigh NC). The secondary antibodies used were Alexa Fluor® donkey anti-mouse 594 (1:200, Invitrogen), Alexa Fluor® donkey anti-goat 488 (1:200, Invitrogen) and donkey anti-rabbit IgG-PerCP-Cy5.5 (1:300, Santa Cruz). CD68 is a pan-macrophage marker. CD86 is an M1 marker. CD206 is an M2 marker. All primary

antibodies were confirmed to cross-react with rat epitopes. The sections were imaged at random fields along the interface of the native tissue and ECM scaffold. Quantification of M1/M2 polarization was achieved using a custom image analysis algorithm developed using the cell profiler image analysis package [31, 32]. This algorithm identified and quantified the number of CD68+ CD86+ (M1 phenotype) and CD68+ CD206+ (M2 phenotype) cells present within the tissue sections. Any cells that co-expressed these markers were not counted. These numbers were then expressed as a ratio of M2/M1.

2.9. Biomechanical testing

The passive biaxial mechanical properties were characterized for the native esophageal mucosa and emECM (n=8). A detailed description of the testing device and methods used for planar biaxial testing has been reported previously [35]. Briefly, samples were affixed to 250 g load cells (Model 31, Honeywell, Columbus, OH) with two loops of suture attached to each side with four hooks, and deformation was measured from a four marker array. Samples were tested in PBS at room temperature under an equibiaxial stress protocol from a 0.5 g tare load to 250 kPa after 10 cycles of preconditioning with a cycle time of 30 s. All data was referenced to the post-preconditioned free-float state. The maximum strain for each sample was then defined as the strain at the maximum tested stress of 250 kPa.

The suture retention analysis we performed according to a previously described protocol [36]. Briefly, a 2-0 prolene suture with a taper needle was passed through the specimen with a 2 mm bite depth, and tied with a square knot and the loop attached to an Instron machine, and pulled at a constant rate of 10 cm/min [36]. Two locations were tested per sample and eight samples were tested per group. Samples were thoroughly rehydrated prior to testing.

2.10. Statistical analysis

An independent samples t-test was used to determine whether the DNA, growth factor, and GAG content, and mechanics of the emECM were different than that of native esophagus ($p < 0.05$). A one-way analysis of variance (ANOVA) was used to determine differences in the percentage of viable cells in culture. Macrophage phenotype ratio between XL-emECM and emECM was compared using an independent samples t-test. A two-way ANOVA with post-hoc Tukey test was performed to determine differences in biomechanical properties with the two independent variables being axes and material. All data are reported as mean $\pm$ standard error.

3. Results

3.1. Decellularization efficacy

The degree of decellularization following the described method was assessed using previously established guidelines for decellularization [24]. No intact nuclei were visible by H&E or DAPI staining following decellularization (Fig 1C). The concentration of remnant DNA in emECM (48 ± 6.4 ng/mg) was markedly less ($p < 0.001$) than that in native esophageal tissue (855 ± 24 ng/mg) (Fig 1A). Residual DNA was present only in fragments less than 200 bp in length (Fig 1B).

3.2. Biochemical and ultrastructural characteristics of esophageal ECM

The concentration of sGAGs in emECM (226 ± 19 mg/g) was not different ($p = 0.37$) compared to the concentration of sGAGs in native esophagus (188 ± 28 mg/g) (Fig 2A). Quantification of growth factors showed no detectable levels of VEGF were present following decellularization. However, bFGF was retained after decellularization (63 ± 16 ng/g) (Fig 2B) in a lesser amount than that in native tissue (3585 ± 100 ng/g) ($p < 0.001$).

In addition to measuring the concentration of remaining sGAGs and growth factors, the preservation and spatial distribution of basement membrane proteins, collagen IV and laminin, and a non-basement membrane protein, fibronectin, was examined. Immunolabeling showed the presence of collagen IV (Fig 3B) and laminin (Fig 3D) that was predominant along the luminal surface of the emECM (marked by “L”). Positive staining for fibronectin was present and distributed throughout the emECM scaffold (Fig 3F).

Lastly, SEM images of the luminal and abluminal surface of emECM showed a smooth surface on the luminal surface of the emECM (Fig 4A,C). The abluminal surface, however, had a more textured and fibrous structure (Fig 4B,D).

3.3. Biomechanical properties

The equibiaxial stress response of the native esophagus showed anisotropic behavior with a maximum strain of 83% and 18% in the circumferential and longitudinal direction, respectively (Fig 5A,B). The emECM showed similar anisotropy, but had a lower compliance along both axes, with the circumferential strain reaching only 10.5% (Fig 5A,B). The decellularized tissue had 30% lower suture retention strength than the native esophagus (Table 2).

3.4. Cytocompatibility

When cultured on emECM and XL-emECM, quantification of PVSC cell viability in-vitro showed no difference when compared to tissue culture plastic ($p=0.67$). Both conditions resulted in over 98% viability following 48 hours in culture (Fig 6).

In-vivo host response to emECM was examined at both 14 and 35 days post-implantation in a rat abdominal body wall model. The host response to emECM scaffolds showed a robust mononuclear cell response throughout the partially degraded scaffold at 14 days (Fig 7A) and yielded a histologic score of 11.4. Along the interface between the emECM scaffold and native tissue, the macrophage response was predominantly of the M2 phenotype (Fig 8A) with a ratio of M2/M1 macrophages of 1.29 ± 0.21 (Fig 8C). By 35 days post-implantation, the original material was not identifiable by histologic evaluation and the remodeling site was composed of organized host connective tissue and islands of skeletal muscle at the periphery that extended into the center of the remodeling site (Fig 7B). Semiquantitative histomorphologic analysis of emECM at day 35 resulted in a total score of 12. In contrast, the host response to XL-emECM was characterized by little to no cellular infiltration or vasculature within the chemically cross-linked bioscaffold, a dense population of mononuclear macrophages at the host-scaffold interface, the deposition of disorganized connective tissues surrounding the implanted test article, and little to no degradation of the material at 14 days (Fig 7C). The cellular response along the scaffold and native tissue interface was shown to be predominantly macrophages of the M1 phenotype (Fig 8B) with an M2/M1 ratio of 0.19 ± 0.03 , which was less than ($p<0.001$) the M2/M1 ratio in emECM. By 35 days, the XL-emECM was still largely intact and showed no infiltration of skeletal muscle (Fig 7D).

4. Discussion

Although current clinical applications of ECM-based biologic scaffolds have included the use of devices originating from heterologous tissue sources, recent studies have suggested there may be an advantage to using ECM derived from homologous tissue (i.e., site-specific) [4–6, 10]. This concept is based upon the fact that ECM from different tissue sources have distinct and specific properties, including the ultrastructure and composition; i.e., a tissue specific microenvironmental niche.

The necessity or preference for site-specific ECM remains unknown for many therapeutic applications. Zhang et al have shown that ECM derived from liver, skin, and skeletal muscle increases the proliferation and differentiation potential for site-matched cell types [8]. Sellaro and colleagues have shown that ECM derived from liver improves the maintenance of sinusoidal endothelial cell phenotype [4] and the function of hepatocytes in-vitro [5]. More recently, porcine myocardial ECM has been shown to improve cardiac progenitor cell function in-vitro [7]. Seif-Naraghi et al have shown that injection of a hydrogel form of cardiac ECM after myocardial infarct improves cardiac function and results in increased cardiac muscle mass [37]. Although the present study showed that the emECM facilitates a constructive remodeling response in a heterologous location and excellent in-vitro cytocompatibility, any site specific benefit in the esophageal mucosa (homologous) location has not yet been tested.

The importance of effective decellularization is well recognized [24, 38]. While protocols for decellularizing the esophagus have been reported, little has been described with focus on the esophageal mucosa. Bhrany et al developed a rat full thickness esophageal scaffold that was able to support epithelial cell growth [39]. Marzaro et al decellularized intact porcine esophagus and seeded with autologous smooth muscle cells for repair of an esophageal muscularis defect [40]. Using a similar protocol, Totonelli et al decellularized intact esophagus using luminal perfusion [41]. These groups reported decellularization of the entire esophagus, including both muscularis externa and mucosa. However, the efficacy of these decellularization protocols, characterization, and cytocompatibility of the scaffold were not investigated in a comprehensive manner.

Protocols for esophageal decellularization have been reported but have been conducted using non-porcine species and/or have used harsh detergents such as sodium dodecyl sulfate (SDS) [39, 42]. SDS, as an ionic detergent, destroys the cell membrane and denatures protein—altering the collagen structure in ECM [43]. Thus, SDS has the associated drawback of ultrastructure disruption [44–46] and growth factor elimination [47]. A loss of ECM structure is also associated with variability in biomechanical properties [48]. Therefore, the use of SDS was avoided in the present study.

Studies have shown the requirement for retention of at least a portion of the submucosal tissue to promote constructive remodeling of the esophagus over stricture and scarring [1]. The use of emECM would therefore appear a more logical strategy for clinical translation. The methods of the current study thoroughly decellularized esophageal mucosa with the use of mild detergents while preserving the anisotropic mechanical properties and bioactive molecules. The described method effectively removed cellular components while maintaining ECM constituents and basement membrane proteins, collagen IV and laminin, in a contiguous pattern at the surface of the emECM material. Scanning electron micrographs of the luminal surface of emECM showed a smooth contour that was also consistent with an intact basement membrane surface. The basement membrane complex may be of importance to esophageal mucosal remodeling because of its natural function of supporting the growth of epithelial cell populations [49–51]. The emECM scaffold was cytocompatible with perivascular stem cells, which were shown to survive and proliferate when cultured on the scaffold.

The role of the host response to biologic ECM scaffolds is a topic of interest and has been reviewed in detail elsewhere [52]. Briefly, the successful therapeutic efficacy of biologic scaffolds is attributed largely to the ability of these ECM-derived materials to modulate the innate immune response in favor of a constructive remodeling outcome over scarring/encapsulation. Key mediators of the innate immune response are macrophages—a highly plastic and heterogeneous cell population [53, 54]. Appropriately prepared biologic

scaffolds have been shown to elicit a macrophage response that is predominantly of an anti-inflammatory (M2) phenotype which has been associated with a downstream constructive remodeling response (i.e., formation of functional, site-appropriate tissue) [27, 34, 55]. However, when biologic scaffolds are prepared using harsh decellularization methods, are chemically cross-linked, or are inadequately decellularized, a robust proinflammatory (M1) macrophage phenotype is observed at the in-situ interface of host tissue and ECM scaffold and ultimately results in chronic inflammation, encapsulation, and fibrosis [34, 42]. In the present study, implantation of emECM scaffolds in an established rodent model was associated with a predominant M2 macrophage response after 14 days and was shown to remodel in a constructive fashion with a histomorphologic score comparable to urinary bladder matrix (UBM-ECM) and small intestinal submucosa ECM (SIS-ECM) [26, 34, 56]. These findings are consistent with the predictive association of the M2 phenotype with constructive remodeling outcomes [34].

The objective of the present study was to develop and characterize an emECM scaffold but was limited by a number of factors. The effects of emECM on esophageal cells were not studied. Instead, perivascular stem cells were used because they are well-characterized [29] and have been used in a number of studies to evaluate the cytocompatibility of a variety of ECM scaffolds [30]. In addition, while retention of growth factor proteins was used as an indicator of the relative mildness of the decellularization protocol, the activity of the growth factors was not determined and the effect of the presence of these growth factors in the overall remodeling process is unknown. While the M2/M1 macrophage phenotype ratio has been shown to be strongly associated with a constructive remodeling response in several anatomic locations, a direct cause-effect relationship has yet to be established. Finally, the present study observed the in-vivo compatibility and constructive remodeling response of the emECM scaffold in a well-characterized abdominal wall defect model, a heterologous anatomic site. Thus, the potential benefits of ECM derived from homologous tissue (i.e., the use of emECM in an esophageal mucosal resection model) remain unknown.

5. Conclusions

Porcine esophageal mucosa was effectively decellularized with the use of a relatively mild detergent-based protocol. The emECM scaffold maintained structural proteins and an ultrastructure consistent with a basement membrane complex. Likewise, retention of sGAGs and bFGF was shown. Compared to native esophageal mucosal tissue biomechanics, the emECM scaffold was expectedly less compliant but retained similar anisotropy. The emECM biologic scaffold was conducive to stem cell viability in-vitro and was associated with a host innate immune response consisting predominantly of M2 macrophages and a more robust constructive remodeling response when compared to XL-emECM biologic scaffolds in-vivo. Future studies aimed at investigating the specific physical and/or biochemical factors responsible for the constructive remodeling outcome and the utility of an emECM biologic scaffold in an esophageal location are warranted.

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Fig 1. Decellularization efficacy

Decellularization of emECM was assessed by the amount and size of remaining DNA and histologically by hematoxylin and eosin (H&E). The amount dsDNA in emECM was less than 50ng/mg, which was significantly less (asterisk; $p < 0.001$) than native tissue (A). DNA fragment length was assessed by gel electrophoresis using a reference 100 bp ladder (B). No intact nuclei were visible after decellularization by H&E staining (C). Data represented as mean $\pm$ standard error. Scale bar = 100 μ m.

Fig 2. Retention of biologic components

Growth factor protein and glycosaminoglycans (GAGs) remain after decellularization. The amount of (A) GAGs and (B) basic fibroblast growth factor (bFGF) remaining after decellularization was measured. Data represented as mean $\pm$ standard error. Asterisk signifies $p < 0.05$.

Fig 3. Immunolabeling of collagen IV, laminin, and fibronectin in emECM samples
 Dark brown indicates positive stain. Immunolabeling of native tissue (A,C,E) is shown as a comparison to emECM (B,D,F). L indicates luminal surface. Scale bar = 200 μ m.

Fig 4. Scanning electron micrographs (SEM) of emECM surface

The luminal surface of the emECM scaffold was characterized by a smoother surface (A,C) compared to the abluminal surface which was more textured and fibrous (B,D)

Fig 5. Native and decellularized esophagus mechanical characterization

The equibiaxial stress response was characterized along the circumferential and longitudinal axes (A). The maximum strain defined at a stress of 250 kPa for both circumferential (C) and longitudinal (L) axes (B). Significant differences ($p < 0.05$) between the circumferential and longitudinal axes of the same sample are denoted as the following: (*) as different from circumferential. Significant differences between samples in each axis are denoted as the following: (^) as different from native. Data represented as mean $\pm$ standard error.

Fig 6. Cytocompatibility of emECM and XL-emECM

The viability of perivascular stem cells (PVSCs) after 48 h culture on emECM (A), XL-emECM (B), and tissue culture plastic (TCP) was assessed. Percentage of live cells was quantified and compared across groups (C). Data represented as mean $\pm$ standard error. Scale bar = 50 μ m.

Fig 7. In-vivo cytocompatibility

Tissue sections were stained with H&E at 14 and 35 days after implantation of emECM (A,B) and XL-emECM (C,D). Histomorphologic sections were evaluated and scored according to previously established criteria (E). Scale bar= 100 μ m

Fig 8. In-vivo macrophage response

Macrophage immunolabeling in emECM (A) and XL-emECM (B) in explants 14 d after implantation was quantified and represented as a ratio of M2/M1 phenotype (C). Dashed line indicates the interface of native tissue (marked by a triangle) and the surgical site. Data represented as mean $\pm$ standard error. Scale bar = 50 μ m.

Table 1

Semiquantitative scoring criteria for day 14 and day 35 explants

Day 14 Scoring Criteria	3	2	1	0
<i>Cellular Infiltration (per 40x field)</i>	>150 cells	75–150 cells	1–75 cells	0 cells
<i>Connective Tissue Organization</i>	Highly organized connective tissue present	Moderately organized connective tissue present	Unorganized connective tissue throughout disrupted original scaffold	Original scaffold intact
<i>Degradation</i>	No scaffold present	Some scaffold present	Mostly present	No degradation
<i>Encapsulation</i>	No encapsulation	Minimal encapsulation	Moderate encapsulation	Dense encapsulation
<i>Multinucleated Giant Cells (per 40x field)</i>	0 cells	1 cell	2–5 cells	>5 cells
<i>Vascularity (per 40x field)</i>	>10 vessels	6–10 vessels	2–5 vessels	0–1 vessel
Day 35 Scoring Criteria	3	2	1	0
<i>Connective Tissue Organization</i>	Highly organized connective tissue present	Moderately organized connective tissue present	Unorganized connective tissue throughout disrupted original scaffold	Original scaffold intact
<i>Degradation</i>	No scaffold present	Some scaffold present	Mostly present	No degradation
<i>Encapsulation</i>	No encapsulation	Minimal encapsulation	Moderate encapsulation	Dense encapsulation
<i>Multinucleated Giant Cells (per 40x field)</i>	0 cells	1 cell	2–5 cells	>5 cells
<i>Muscle Ingrowth</i>	Organized muscle throughout scaffold	Muscle cells present in scaffold center	Muscle cells present at scaffold periphery	No muscle ingrowth

Table 2

Native esophagus and emECM suture retention test.

	Treatment			
	Native		mECM	
	Mean	Standard Error	Mean	Standard Error
Suture Retention Strength (N)*	2.42	0.24	1.73	0.17