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Preparation and characterization of a biologic scaffold from esophageal mucosa

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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.

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 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

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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 h with multiple subsequent washes in PBS over 48 h. 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 h. 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 min. 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 h 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 min 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 h 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 h, 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% CO_2.

In-vitro cell viability assays were performed using single layer sheets of ECM. PVSCs (0.5×10^6) were cultured for 48 h on 2 cm 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