Biomaterials 33 (2012) 2916–2925

Contents lists available at SciVerse ScienceDirect

Biomaterials

journal homepage: www.elsevier.com/locate/biomaterials

Biologic scaffold composed of skeletal muscle extracellular matrix

Matthew T. Wolf [a,b], Kerry A. Daly [b,c], Janet E. Reing [b], Stephen F. Badylak [a,b,c,*]

[a] Department of Bioengineering, University of Pittsburgh, Pittsburgh, PA 15213, USA
[b] McGowan Institute for Regenerative Medicine, University of Pittsburgh, Pittsburgh, PA 15219, USA
[c] Department of Surgery, University of Pittsburgh, Pittsburgh, PA 15213, USA

ARTICLE INFO

Article history:
Received 31 October 2011
Accepted 31 December 2011
Available online 20 January 2012

Keywords:
ECM (extracellular matrix)
Muscle
Scaffold
Regenerative medicine
Animal model

ABSTRACT

Biologic scaffolds prepared from the extracellular matrix (ECM) of decellularized mammalian tissues have been shown to facilitate constructive remodeling in injured tissues such as skeletal muscle, the esophagus, and lower urinary tract, among others. The ECM of every tissue has a unique composition and structure that likely has direct effects on the host response and it is plausible that ECM harvested from a given tissue would provide distinct advantages over ECM harvested from nonhomologous tissues. For example, a tissue specific muscle ECM scaffold may be more suitable for constructive remodeling of skeletal muscle than non-homologous ECM tissue sources. The present study describes an enzymatic and chemical decellularization process for isolating skeletal muscle ECM scaffolds using established decellularization criteria and characterized the structure and chemical composition of the resulting ECM. The results were compared to those from a non-muscle ECM derived from small intestine (SIS). Muscle ECM was shown to contain growth factors, glycosaminoglycans, and basement membrane structural proteins which differed from those present in SIS. Myogenic cells survived and proliferated on muscle ECM scaffolds *in vitro*, and when implanted in a rat abdominal wall injury model *in vivo* was shown to induce a constructive remodeling response associated with scaffold degradation and myogenesis in the implant area; however, the remodeling outcome did not differ from that induced by SIS by 35 days post surgery. These results suggest that superior tissue remodeling outcomes are not universally dependent upon homologous tissue derived ECM scaffold materials.

1. Introduction

Biologic scaffold materials composed of extracellular matrix (ECM) are typically produced by decellularization of mammalian tissues such as urinary bladder, dermis, or small intestine [1] and have been shown to facilitate the functional reconstruction of several tissue types [2,3] including the lower urinary tract [4,5], heart and vascular structures [6,7], esophagus [8,9], and musculoskeletal tissues [10–13], among others. The mechanisms by which constructive remodeling occurs include the recruitment of multipotential stem and progenitor cells to the site of scaffold placement [14,15], promotion of a favorable M2 macrophage phenotype at the host tissue/bioscaffold interface [16], regional angiogenesis [17], and mitogenesis [15,18]. These tissue derived biologic scaffolds are frequently used in non-homologous anatomic sites, but recent studies have suggested that biologic scaffolds derived from site specific homologous tissues such as liver and lung may be better suited for constructive tissue remodeling than non-site specific tissue sources [19–25].

Muscle tissues, including cardiac, skeletal, and smooth muscle, respond favorably when biologic scaffolds are used for their reconstruction following injury [11,12]. To date, there have been several attempts to isolate and process skeletal muscle ECM (M-ECM) [26–33]. Most of these attempts have involved the decellularization of intact rodent muscles or the extraction of rodent muscle ECM proteins, with varying degrees of success. DeQuach et al. [33] did show that proteins extracted from a decellularized porcine muscle matrix retain bioactivity. None of these studies have provided a detailed characterization of the intact M-ECM scaffold derived from a large animal tissue source, nor have any of these studies applied stringent decellularization criteria in the development of the decellularization process. The objectives of the present study were to (1) determine a method for decellularization of skeletal muscle and characterize the structure and composition of the resulting ECM, and (2) to compare the in-vitro bioactivity and in-vivo remodeling properties of skeletal muscle

* Corresponding author. McGowan Institute for Regenerative Medicine, 450 Technology Drive Suite 300, University of Pittsburgh, Pittsburgh, PA 15219, USA. Tel.: +1 412 624 5253; fax: +1 412 624 5256.
E-mail address: badylaks@upmc.edu (S.F. Badylak).

0142-9612/$ – see front matter © 2012 Elsevier Ltd. All rights reserved.
doi:10.1016/j.biomaterials.2011.12.055

ECM vs. non-muscle ECM, specifically SIS, in a rodent model of abdominal wall muscle repair.

2. Materials and methods

2.1. Overview of study design

Canine skeletal muscle was harvested and decellularized by enzymatic and chemical methods. The resulting M-ECM was then assessed for biochemical and structural composition, the cell response *in vitro,* and the *in vivo* remodeling characteristics in a rat abdominal wall defect model. ECM composed of porcine small intestinal submucosa (SIS) was used for comparison purposes. All animal experiments were conducted in accordance to University of Pittsburgh Institutional Animal Care and Use Committee (IACUC) regulations and guidelines.

2.2. Preparation of M-ECM and SIS

Whole quadriceps and hamstring muscle groups were isolated from mongrel dogs and frozen at −80 °C immediately following sacrifice. While frozen, entire muscle groups were then sliced into 2.25 mm thick sheets transverse to the alignment of muscle fibers. The muscle slices were then thawed and trimmed of all macroscopic vasculature, fat, and connective tissues, rinsed in deionized water for 1 h and lyophilized.

The lyophilized muscle was decellularized following the protocol summarized in Table 1. Briefly, the muscle was subjected to lipid extraction by placement in a 2:1 (v/v) solution of chloroform/methanol (Fisher, Waltham, MA) for 2 h under a constant stir rate [34]. The muscle was rehydrated using a graded series of ethanol and then exposed to a series of enzymatic and chemical treatments to remove cellular materials in a spinner flask at a stir rate of 70RPM. These treatments consisted of: 0.2% Trypsin/0.2% EDTA for 2 h (at 37 °C and pH = 7.6), 2% sodium deoxycholate for 5 h, fresh 2% sodium deoxycholate for 14–16 h, 1% Triton-X 100 for 1 h, and finally 0.1% (w/v) peracetic acid/4% (v/v) ethanol for 2 h followed by extensive rinsing. The muscle tissue was washed with deionized water and 2× phosphate buffered saline (PBS) between each step. The M-ECM scaffolds were lyophilized for storage. Scaffolds used for tissue culture or *in vivo* implantation were terminally sterilized with ethylene oxide (16 h cycle at 50 °C in a Series 3plus EOGas Sterilizer, Anderson Sterilizers, Inc. Haw River, NC).

Porcine small intestine (jejunum) was obtained from 6 month old pigs from the local abbatoir and prepared as previously described [35]. In brief, the majority of the mucosa and the entire serosa, and muscularis externa layers of the intestine were mechanically delaminated from the intestine. The remaining submucosa, muscularis mucosa and stratum compactum layers were then washed with water and treated with 0.1% (w/v) peracetic acid/4% (v/v) ethanol for 1 h. SIS was then rinsed extensively, lyophilized, and sterilized with ethylene oxide.

2.3. Determination of decellularization

Decellularization was defined as fulfilling the following criteria for DNA content: having less than 50 ng dsDNA/mg ECM dry weight, having all residual DNA fragments be less than 200 base pair in size, and lacking visible nuclei after histologic staining with 4′,6-diamidino-2-phenylindole (DAPI) [1,36,37]. Immediately after processing, M-ECM samples ($n = 7$) were fixed in 10% neutral buffered formalin. Samples were then embedded in paraffin, surface sectioned, and then stained with H&E and DAPI for detection of nuclei multispectrally at 200× magnification. (Nuance multispectral imaging, CRi, Cambridge, MA) Additional non-fixed samples were used to quantify the amount of double stranded DNA using the PicoGreen assay (Invitrogen, Carlsbad, CA). DNA was extracted from powdered M-ECM by digesting in 0.1 mg/ml proteinase K (Sigma, St. Louis, MO) at 50 °C for 24 h. Samples were then purified with two phenol/chloroform/isoamyl alcohol (25:24:1 v/v) extractions. After ethanol precipitation and drying, the DNA was resuspended in 1 ml TE buffer (pH = 8.0) then quantified using the PicoGreen assay according to the manufacturer's instructions. The size of the extracted DNA fragments was determined after separation by 2% agarose gel electrophoresis.

Table 1
Summary of the steps in the decellularization of skeletal muscle tissue including the chemical treatment and time of exposure.

Chemical	Length of treatment
Chloroform/methanol (2:1 v/v)	2 h
Graded series of alcohol (100,90,70,50,0)	30 min ea.
0.2% Trypsin/0.2% EDTA at 37 C	2 h
Deionized water, 2× PBS	30 min ea.
2% sodium deoxycholate	5 h
Deionized water, 2× PBS	30 min ea.
2% sodium deoxycholate	14–16 h
1% Triton X-100	1 h
Deionized water	30 min
0.1% (w/v) peracetic acid/4% ethanol (v/v)	2 h
1× PBS	30 min ea. (twice)
Deionized water	30 min ea. (twice)

2.4. Scanning electron microscopy

Scanning electron micrographs were taken to examine the surface topology of M-ECM and native muscle tissue. 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. Lipid fixation was performed in 1% (w/v) osmium tetroxide (Electron Microscopy Sciences) for 1 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 25×, 500×, 1000×, and 10,000× magnifications.

2.5. Sulfated glycosaminoglycan quantification

Sulfated glycosaminoglycan (GAG) content in different preparations of M-ECM ($n = 7$), SIS ($n = 4$), and native muscle tissue ($n = 3$) was determined using the Blyscan Sulfated Glycosaminoglycan Assay Kit (Biocolor Life Sciences, Carrickfergus, UK). For each sample, 25 mg/ml of powdered ECM in 100 mM Tris (pH = 7.5) was digested with 0.1 mg/ml proteinase K (Sigma) at 50 °C for 24 h with gentle agitation. The digested scaffold was then assayed following the manufacturer's instructions.

2.6. Protein extraction & growth factor quantification

Soluble proteins were extracted from different preparations of M-ECM ($n = 5$), SIS ($n = 3$), and native muscle tissue ($n = 2$) and analyzed for growth factor content. Soluble proteins were extracted from 300 mg of powdered ECM or tissue in 7 ml of a urea-heparin buffer (2M urea, 50 mM Tris, 5 mg/ml heparin, 10 mM N-ethylmaleimide, 5 mM benzamidine, and 1 mM phenylmethylsulfonyl fluoride at pH = 7.4). Samples in urea-heparin buffer were gently agitated for 20–24 h at 4 °C, after which they were centrifuged for 30 min at 3000*g* and the supernatant collected. The remaining pellet was resuspended in freshly prepared urea-heparin buffer and the extraction process repeated. Each extract was then dialyzed against 80–100× volume of deionized water using 3500MWCO Slide-A-Lyzer dialysis cassettes (Pierce, Rockford, IL) for 24 h, with water changes after 4 and 8 h. The recovered extracts were analyzed for total protein recovered using the BCA protein assay (Pierce) and frozen at −80 °C until further use.

The isolated protein extracts were quantified for vascular endothelial growth factor (VEGF) and basic fibroblast growth factor (bFGF) with human ELISAs (R&D systems, Minneapolis, MN) following the manufacturer's instructions. Canine, porcine, and human bFGF and VEGF show a high level of sequence homology and extractions from native canine tissue were found to be reactive with the human ELISA kits used.

2.7. ECM staining and immunolabeling

M-ECM, SIS, and native muscle tissue were fixed in formalin, embedded in paraffin, and then cut into 5 μm sections. Standard histologic stains were performed using Herovici's Polychrome (staining for collagen Types I & III) and Movat's Pentachrome (staining for elastin, collagen, and GAGs). Immunolabeling studies were also conducted for the presence of basement membrane proteins laminin, type IV collagen, and fibronectin.

For immunolabeling, slides were deparaffinized followed by epitope retrieval in 10 mM citrate buffer (pH = 6.0) at 95 °C for 15 min. Endogenous peroxidase activity was quenched by incubation in a 3% (v/v) hydrogen peroxide/methanol solution for 30 min at room temperature. Non-specific antibody binding was blocked with 2% normal goat serum in PBS (Vector, Burlingame, CA) for 1 h at room temperature. Tissues were then labeled with primary antibodies overnight at 4 °C. Antibodies were raised in rabbit against human laminin (1:50, L9393, Sigma), type IV collagen (1:100, T59106R, Meridian Life Science Inc., Saco, ME), and fibronectin (1:300, F3648, Sigma) and were diluted in the blocking solution. Sections were then rinsed in PBS and incubated in a biotinylated goat anti-rabbit IgG secondary antibody (1:100, Vector) diluted in blocking solution for 2 h at room temperature. Sections were rinsed as before and incubated in the Vectastain ABC reagent (Vector) for 30 min at room temperature and then exposed to a diaminobenzadine substrate (ImmPact DAB, Vector) until appropriate staining developed. Staining was stopped by rinsing sections in deionized water followed by counterstaining with hematoxylin. Antibody isotype controls were used in the place of the primary antibody to determine the presence of non-specific staining.