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Original Research Article

Proteomic composition and immunomodulatory properties of urinary bladder matrix scaffolds in homeostasis and injury

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ABSTRACT

Urinary bladder matrix (UBM) is used clinically for management of wounds and reinforcement of surgical soft tissue repair, among other applications. UBM consists of the lamina propria and basal lamina of the porcine urinary bladder, and is decellularized as part of the process to manufacture the medical device. UBM is composed mainly of Collagen I, but also contains a wide variety of fibrillar and basement membrane collagens, glycoproteins, proteoglycans and ECM-associated factors. Upon application of the biomaterial in a traumatic or non-traumatic setting in a mouse model, there is a cascade of immune cells that respond to the damaged tissue and biomaterial. Here, through the use of multicolor flow cytometry, we describe the various cells that infiltrate the UBM scaffold in a subcutaneous and volumetric muscle injury model. A wide variety of immune cells are found in the UBM scaffold immune microenvironment (SIM) including F4/80$^+$ macrophages, CD11c$^+$ dendritic cells, CD3$^+$ T cells and CD19$^+$ B cells. A systemic IL-4 upregulation and a local M2-macrophage response were observed in the proximity of the implanted UBM. The recruitment and activation of these cells is dependent upon signals from the scaffold and communication between the different cell types present.

1. Introduction

Urinary bladder matrix (UBM), a decellularized extracellular matrix (ECM), is used clinically in a variety of applications [1–5]. Clinical indications of the commercialized UBM include reinforcement of abdominal wall repair [6,7], management of diabetic ulcers [8], gastrointestinal tissue reinforcement [9], urologic and gynecologic surgical reinforcement, skin wounding [10], and management of deep, partial thickness burns [11]. Off-label, the material, and other ECM-derived scaffolds, have been used in treatment of larger soft tissue defects such as skeletal muscle repair [5,12,13], breast reconstruction [14], dural repair, subcutaneous injection with or without PRP (platelet-rich plasma) to induce hair growth [15], and tendon repair. These varied applications capitalize on a pro-regenerative host remodeling response that is induced when ECM scaffolds such as UBM are implanted within the body, although the determinants of this response are still being investigated.

ECM scaffolds are created by treating native tissue, often porcine or human, with a variety of acids and detergents to remove the majority of cellular components and leave behind a complex structural and signaling scaffold [4,16]. A large array of tissues have been decellularized for tissue engineering applications including but not limited to, urinary bladder, small intestinal submucosa, cardiac muscle, demineralized bone, and amnion. Urinary bladder matrix in particular can be synthesized either as a sheet (used for large surfaces) or particulate (used in skin wound management) and are currently being investigated in other forms such as hydrogels [3,17–19]. As they are derived from native tissue, ECM scaffolds such as UBM carry a structural and biochemical

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complexity that cannot be mimicked synthetically, and are likely an important factor in successful scaffold remodeling.

Current evaluations of UBM composition have shown that is composed primarily of collagens, interspersed with proteoglycans ECM sequestered growth factors, and cytokines [18,20,21]. While the constituents of these scaffolds can greatly affect their biologic activity and performance in the clinical setting, comprehensive characterization of their composition is incomplete. Currently, the primary methods used for determining components of ECM scaffolds are through histological staining, chemical assays, and enzyme-linked immunosorption assays (ELISA) of target proteins such as growth factors. Further understanding of the components of these scaffolds can be achieved through proteomic analysis [22], but the chemical nature of ECM proteins have made such characterizations challenging and incomplete. Contrary to typical substrates for proteomic analysis, ECM scaffolds are highly hydrophobic, difficult to solubilize, and are protease resistant, requiring more stringent protocols to prepare fragments that can be detected by mass-spectrometry. However, with a distinct profile of the proteins found in ECM scaffolds, we can better characterize the results seen in the clinic with the scaffold composition.

The proteomic composition of a material can be correlated with alterations in cell function, including immune responses [23–25]. Immune cells are the first responders to injury and biomaterial implantation. The immune microenvironment created by a scaffold will alter the presence of various cytokines and growth factors that can contribute to stem cell differentiation and tissue regeneration [26]. UBM materials are clinically implanted in areas of acute injury (*i.e.* an inflammatory environment) and in areas of chronic injury or to reinforce tissue (*i.e.* a more homeostatic environment). Each of these represent very different immune milieus that may influence the host response to the implanted UBM.

A wide variety of immune cells have been implicated in regeneration of murine muscle, liver, and salamander limbs [27–29]. In muscle tissue, we have shown the importance of Th2 polarized T cells [30], and Heredia et al. described the importance of eosinophils in muscle recovery after cardiotoxin injury [27]. In both cases, if elements of the type-2 immune response were depleted, there was a reduction in functional muscle fiber formation, replaced with fibrosis, ectopic adipogenesis and small irregularly shaped muscle fibers. Eosinophils have also been implicated as a major mediator of liver regeneration [28]. In the salamander, depletion of macrophages results in full inhibition of limb regeneration and deposition of a thick collagenous extracellular matrix at the limb bud [29]. Characterizing the scaffold immune microenvironment (SIM) of clinical grade materials would allow insight into the cell dynamics that could be expected in a clinical setting. In the context of extracellular matrix scaffolds, studies have demonstrated that these materials induce a pro-regenerative M2-macrophage phenotype that is dependent upon signaling from Th2 T cells [17,30,31]. The present study provides a detailed characterization of the proteomic composition of clinically utilized UBM-ECM scaffolds and of immune cell recruitment/polarization in sites of injury vs homeostasis.

2. Materials and methods

2.1. Mechanical testing and scanning electron microscopy

Sterile UBM particulate (MicroMatrix®) was obtained from ACell, Inc. (Columbia, MD). Rheology was performed with an Ares G2 rheometer (TA Instruments New Castle, DE). Experiments were conducted at physiological temperatures (37 °C) and performed in sequence. Freshly made samples of different UBM concentrations were loaded in 400 mg quantities on the rheometer stage. Experiments were conducted at a rheometer gap of 1.5 mm, using a 25 mm parallel plate geometry. Determination of linear viscoelastic region by a frequency sweep conducted from 0.1 rad/s to 15 rad/s at a constant oscillation strain of 0.5%, followed by a strain sweep at constant oscillation frequency 15 rad/s was performed. Finally, storage and lost moduli were observed over time using constant oscillation frequency 15 rad/s and oscillation strain 0.5%. Three samples per group were tested under the same rheological protocol. Samples were kept hydrated during these rheology experiments using a solvent trap.

Collagen from bovine tendon (Sigma) was obtained from a commercial supplier to use as a material control. Collagen and UBM samples were scanned using a double furnace, power compensation differential scanning calorimeter (DSC 8500, Perkin Elmer) fitted with an intracooler. Samples were individually weighed, placed in, and crimp-sealed in manufacturer supplied aluminum pans and lids. Sample pans were placed in the DSC and heated at 5 °C per minute from 20 °C to 90 °C. An empty pan was used as reference.

The structure and surface topography of UBM particles was evaluated using scanning electron microscopy (SEM). Particles were adhered to aluminum stubs with copper tape and sputter coated with 20 nm Au/Pd. SEM was performed using LEO 1530 Field Emission Scanning Electron Microscope (Carl Zeiss, Jena, Germany) operating at 1–20 kV.

2.2. Proteomics

UBM proteomic composition was determined by mass spectometry of tryptic peptides derived from the matrix components of three separate manufacturing lots. UBM particles were suspended in 9 M urea (pH 8, 30 mM HEPES buffer) at a concentration of 2.5 mg ECM/ml, vortexed for 60 s, reduced with dithiothreitol (DTT, 5 mM final concentration), and sonicated (20 W output, 5 s on 20 s off for 12 cycles). Further reduction was performed by heating samples to 60 °C for 20 min followed by alkylation with iodoacetic acid (10 mM final concentration). Sample urea concentration was adjusted to 8 M with HEPES buffer for digestion with Lys-c (Wako Chemicals) for 4 h at 37 °C with agitation (1:110 ECM:Lys-c dry wt. ratio). Sample urea concentration was then further reduced to 2.5 M with HEPES buffer for digestion with trypsin (Promega Gold) overnight at 37 °C with agitation (1:50 trypsin:ECM dry wt. ratio). Small aliquots of samples were collected before and after enzymatic digestion for size evaluation by SDS-gel electrophoresis. Following digestion, samples were centrifuged for 10 min at 16,000 × *g* to confirm the absence of remaining insoluble material, and the supernatant collected.

Tryptic peptides for each lot of material were analyzed in triplicate runs using an Orbitrap Fusion Lumos mass spectrometer (Thermo Scientific). Peptides were separated by on-line reverse phase chromatography consisting of EASY-Spray analytical column (2 µm, 50 cm, Thermo Scientific) over a 150 min gradient. The data was acquired in data dependent manner in 'top speed' mode over 3 s. MS1 scans were acquired in Orbitrap analyzer at 120,000 resolution followed by MS2 scans also acquired in the Orbitrap analyzer at 60,000 resolution. Peptides were fragmented in HCD mode at 35% collision energy. Dynamic exclusion of 30 s was included in the method.

Peptide search, protein identification and label free quantification was carried out using MaxQuant software (Supplementary Table S1). Peptide search parameters were as follows: precursor mass error of 5 PPM and fragment mass error of 0.05 Da was allowed. Cysteine carbamidomethylation was used as static modification while methionine oxidation and acetylation of protein N terminal were used as dynamic modifications. Refseq73 protein database for the species *Sus scrofa* with common contaminant proteins was used for peptide search. 1% FDR rate at PSM level and protein level was permitted. Replicate runs for each lot were combined for label free quantification using the intensity-based absolute quantification (iBAQ) method. The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium *via* the PRIDE partner repository with the dataset identifier PXD005400 [32]. *via* . Gene level annotation was applied for each protein match and gene symbols beginning with LOC were manually matched to current gene annotations if available. Protein gene products