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Solubilized extracellular matrix from brain and urinary bladder elicits distinct functional and phenotypic responses in macrophages

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ABSTRACT

Extracellular matrix (ECM) derived from a variety of source tissues has been successfully used to facilitate tissue reconstruction. The recent development of solubilized forms of ECM advances the therapeutic potential of these biomaterials. Isolated, soluble components of ECM and matricryptic peptides have been shown to bias macrophages toward a regulatory and constructive (M2-like) phenotype. However, the majority of studies described thus far have utilized anatomically and morphologically similar gastrointestinal derived ECMs (small intestine, esophagus, urinary bladder, etc.) and a small subset of macrophage markers (CD206, CD86, CCR7) to describe them. The present study evaluated the effect of solubilized ECM derived from molecularly diverse source tissues (brain and urinary bladder) upon primary macrophage phenotype and function. Results showed that solubilized urinary bladder ECM (U-ECM) up-regulated macrophage PGE2 secretion and suppressed traditional pro-inflammatory factor secretion, consistent with an M2-like phenotype. The hyaluronic acid (HA) component in solubilized U-ECM played an important role in mediating this response. Brain ECM (B-ECM) elicited a pro-inflammatory (M1-like) macrophage response and contained almost no HA. These findings suggest that the molecular composition of the source tissue ECM plays an important role in influencing macrophage function and phenotype.

1. Introduction

The use of intact extracellular matrix (ECM) scaffolds or individual components of ECM to repair, reinforce, or reconstruct injured or missing tissues has been shown to improve tissue remodeling outcomes in a wide variety of preclinical and clinical studies [1,2]. ECM scaffolds orchestrate a complex, temporally controlled series of events at the site of implantation, beginning with the recruitment of endogenous stem and progenitor cells, and transitioning to ECM scaffold degradation, deposition of new ECM, and culminating with the formation of site appropriate tissue [3–5]. Macrophages are a critical part of both the initiation and propagation of this regenerative response [6,7]. Modulation of macrophage phenotype by the bioactive molecules in ECM has been hypothesized to be one of the critical, if not the most critical, function of ECM scaffolds [8–10]. Recently, intact ECM scaffolds have been processed into soluble forms by enzymatic digestion [11,12]. These soluble forms of ECM afford many advantages including minimally invasive delivery to the site of injury and the ability to fill an irregularly shaped cavity [13–15]. Most importantly, solubilized ECM retains many of the bioactive properties of intact ECM including the ability to modulate macrophage phenotype [16,17].

Macrophages have remarkable plasticity and respond to environmental cues by altering their functional phenotype along a spectrum between the extremes of M1 and M2 [18,19]. M1 macrophages express and/or secrete a variety of pro-inflammatory markers including TNFα, IL-1β, iNOS, and TLRs; while M2 macrophages on the other hand express and/or secrete anti-inflammatory markers including IL-10, IL-1RA, CD206, and TGFβ [20,21]. M2 macrophages also appear to be more functionally diverse with several M2 subsets (e.g., M2A, M2B, M2C, etc.) that all play unique

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roles in tissue remodeling and homeostasis [22]. Phenotypically distinct macrophages participate in a temporally orchestrated fashion during the default wound healing process [23], and their absence during wound healing, or inability to shift from an M1 to an M2 phenotype, results in adverse outcomes [24–26]. *In vitro* studies have suggested that ECM degradation fragments, including cryptic peptide motifs and matricryptic sites, may contribute to constructive tissue remodeling by influencing a number of cellular functions [17,27,28] and neomatrix assembly [29,30]. Recent studies have shown that enzymatically solubilized ECM can promote an M2-like phenotype in primary bone marrow derived murine macrophages and in a human monocyte derived cell line (THP-1 cells) [31,32]. However, the ECMs utilized in these studies (small intestine ECM [SIS-ECM] and urinary bladder ECM [U-ECM]) were similar in their embryologic source (endoderm) with similar molecular compositions. No study has attempted to comprehensively compare the macrophage phenotypic profile of ECM derived from different germ layers and distinct molecular composition.

While SIS-ECM and U-ECM are rich in various types of collagens (e.g., elastin, collagen I, collagen IV, etc.); brain ECM (B-ECM) is deficient in these components but enriched with proteoglycans such as myelins, leticans, and chondroitin sulfate [12,33–35]. Both B-ECM and U-ECM contain glycosaminoglycans (GAGs) such as hyaluronic acid (HA) and sulfated GAGs [12]; however, the types of GAGs, the matricellular proteins they interact with, the molecular weight of the GAGs, and molecular interactions GAGs utilize to help orchestrate ECM assembly and cell behavior are unique in both B-ECM and U-ECM [34,35]. Given that proteoglycans, GAGs, and their degradation products are all capable of influencing macrophage phenotype and function [36–38], it is reasonable to speculate that the macrophage phenotype initiated by B-ECM could be distinct from the phenotype initiated by U-ECM.

The objective of the present study was to compare the effects of solubilized ECM from distinct tissue sources, B-ECM and U-ECM, upon macrophage phenotype and function. The role of high molecular weight HA in U-ECM in modulating macrophage function was also examined.

2. Materials and methods

2.1. Experimental overview

The purpose of this study was to compare macrophage phenotype and function in response to treatment with solubilized B-ECM and U-ECM. B-ECM and U-ECM were prepared, solubilized and exposed to primary rat bone marrow derived macrophages. A comprehensive panel of macrophage phenotypic markers was examined, including both secreted factors (TNFα, IL-1β, PGE2, nitric oxide [NO]) and functional outputs (phagocytosis, arginase activity). The ECM mediated macrophage response was compared with traditional M1 (LPS/IFNγ) and M2 (IL-4) stimuli to help classify the ECM mediated macrophage response along the M1-M2 continuum. Finally, differences in the HA content between B-ECM and U-ECM were quantified, and the role of high molecular weight HA in the phenotypic profile of U-ECM was investigated by using hyaluronidase.

2.2. Chemicals, reagents, and tissues

All chemicals used in the study were supplied by Sigma–Aldrich (St. Louis, MO) unless otherwise specified. All cell culture reagents were supplied by Life Technologies (San Diego, CA) unless otherwise specified. All animals used for the production of macrophages were supplied by Charles River (San Diego, CA) and humanely sacrificed according to the procedure outlined by the University of Pittsburgh's Institutional Animal Care and Use Committee. Both brain tissue and urinary bladder tissue were harvested from market weight pigs supplied by Tissue Source, LLC. (Lafayette, Indiana).

2.3. Preparation of solubilized U-ECM and B-ECM

U-ECM and B-ECM were prepared as previously described [12,39,40]. When prepared according to the aforementioned procedures, U-ECM is prepared as a solid, (~10 × 10 cm) hydrated sheet, while B-ECM is prepared in small pieces (~0.25–0.75 cm^3). The U-ECM sheet and B-ECM pieces were lyophilized to dryness using a LabConco Freezone 2.5 Plus lyophilizer. The dried U-ECM and B-ECM were manually cut into small pieces and ground into a powder using a Wiley Mill with a #60 mesh screen [41]. The U-ECM and B-ECM powders were solubilized by enzymatic digestion in pepsin (10 mg/mL ECM in 1 mg/mL pepsin, pepsin had ≥ 500 IU/mg) at room temperature in 0.01 M HCl for 48 h under constant stirring. To control for the effect of pepsin on macrophages (hereafter referred to as pepsin control), a solution of 1 mg/mL pepsin in 0.1 M HCl was prepared following the same procedure (constant stirring, 48 h, RT). After solubilization, the U-ECM, B-ECM, and pepsin control were neutralized to a pH of 7.4 in 1× PBS (137 mM NaCl, 2.7 mM KCl, 12 mM Phosphate) to inactivate the pepsin and prepare the solubilized material for cell culture assays. All materials were stored at −80 °C until use.

2.4. Primary macrophage isolation and culture

Primary rat bone marrow mononuclear cells were matured to macrophages as previously described [42]. Briefly, bone marrow cells were flushed from the femurs of female Lewis rats, repeatedly triturated in isolation medium (DMEM high glucose medium with 2% Penicillin–Streptomycin) and subsequently centrifuged at 300× g from 10 min. The cell pellet was then re-suspended in red blood cell (RBC) lysis buffer (155 mM NH_4Cl, 10 mM $KHCO_3$, 0.9%EDTA in distilled water) for 15 min on ice. Lysed RBCs were removed by centrifugation (300× g for 10 min) and the cell pellet was then re-suspended in macrophage maturation medium (DMEM high glucose, 10% heat inactivated FBS, 20% L929 fibroblast conditioned medium, 2% 10 mM MEM non-essential amino acids, 1% 10 mM L-glutamine, 1% 1 M Hepes Buffer, 1% Penn/Strep, 0.12% 50 mM 2-mercaptoethanol). Cells were seeded at a density of one million cells/mL in six well culture plates. Medium was changed every 2–3 days for 7 days.

The matured bone marrow derived macrophages were harvested and treated with 1 mL of StemPro® Accutase®, incubated at 37 °C for 10 min and then detached by gently pipetting. The cell suspension was collected and centrifuged for 5 min at 300× g. The cell pellet was re-suspended in macrophage culture medium (DMEM high glucose, 10% heat inactivated FBS, 2% 10 mM MEM non-essential amino acids, 1% 10 mM L-glutamine, 1% 1 M Hepes Buffer, 1% Penn/Strep, 0.12% 50 mM 2-mercaptoethanol) and plated at a density of 500,000 cells in 1 mL of medium in 12 well cell culture plates. After one hour, cytokines or solubilized ECM materials were added to the culture medium. To promote a pro-inflammatory (M1) macrophage phenotype, a final concentration of 100 ng/mL LPS and 20 ng/mL IFNγ was applied, whereas 20 ng/mL IL4 was used to drive cells toward an (M2) anti-inflammatory phenotype [43]. Solubilized U-ECM or B-ECM was applied to macrophages at two final concentrations of 1 mg/mL and 0.5 mg/mL. Pepsin control, diluted to match the final pepsin concentration in the U-ECM and B-ECM, was also applied to macrophages to determine if the pepsin enzyme was contributing to the phenotypic profile. Macrophages were cultured with the different stimuli for 48 h. Macrophages cultured in medium without any stimulus are referred to as naïve macrophages.

2.5. Arginase activity

Arginase activity was measured using macrophage lysate as previously described [44,45] with slight modification. Briefly, macrophages were lysed with 100 μL of 0.1% triton x-100 on a rocker at 150 rpm for 30 min at room temperature. Then 100 μl of 25 mM $MnCl_2$ (pH 7.2) was added to 100 μl of the cell lysate and mixed well. One hundred μl of the cell lysate/$MnCl_2$ mixture was activated at 56 °C for 10 min. Arginine hydrolysis was conducted by incubating the 100 μl cell lysate/$MnCl_2$ mixture with 100 μl of 0.5 M L-arginine (pH 9.7) at 37 °C for 1 h. The reaction was stopped with 800 μl of 18 M H_2SO_4/14.8 M H_2PO_4/H_2O mixture (1/3/7, v/v/v). The urea concentration was measured at 540 nm with a microplate reader after addition of 40 μl 0.9% α-isonitrosopropiophenone (dissolved in ethanol) followed by heating at 95 °C for 45 min. One unit of arginase activity is defined as the amount of enzyme that catalyzes the formation of 1μmole of urea/min. Known concentrations of urea solutions were added with same amount of acid mixture and α-isonitrosopropiophenone and heated at 95 °C for 45 min to generate a standard curve.

2.6. Nitric oxide quantification

Nitrite concentration was measured using Griess reagent. Fifty μL of the cell culture supernatants were mixed with 50 μl of 1% sulfanilamide and incubated for 10 min at room temperature. Fifty μl of 0.1% N-(1-naphthyl)ethylenediamine dihydrochloride (NED) solution was then added to the mixture and incubated for another 10 min at room temperature. The absorbance was measured at 540 nm with a microplate reader. Known concentrations of nitrite solutions were used to generate a standard curve.

2.7. Phagocytic ability

Macrophage phagocytic ability was measured as previously described [46] with slight modification. Culture media containing each stimulus were first removed after 48 h and cells were rinsed with PBS twice prior the addition of fluorescent macroparticles (1 μm diameter, Polysciences). Macroparticles in fresh medium were added to macrophages from all treatment groups at 1:1000 dilution for 15 min. After 15 min, media with macroparticles were removed and macrophages were rinsed with PBS twice. Macrophages were then harvested by 10 min of Accutase® treatment at 37 °C followed by gentle pipetting. Cells were then centrifuged at 300× g for 5 min and fixed in 200 μl of 4% paraformaldehyde. Flow cytometry was used to count