Acta Biomaterialia 49 (2017) 140–151

Contents lists available at ScienceDirect

Acta Biomaterialia

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

Full length article

Anatomical region-dependent enhancement of 3-dimensional chondrogenic differentiation of human mesenchymal stem cells by soluble meniscus extracellular matrix

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ARTICLE INFO

Article history:
Received 4 August 2016
Received in revised form 12 November 2016
Accepted 18 November 2016
Available online 19 November 2016

Keywords:
Meniscus
Extracellular matrix
Fibrochondrogenesis

ABSTRACT

Extracellular matrix (ECM) derived from decellularized tissues has been found to promote tissue neogenesis, most likely mediated by specific biochemical and physical signaling motifs that promote tissue-specific differentiation of progenitor cells. Decellularized ECM has been suggested to be efficacious for the repair of tissue injuries. However, decellularized meniscus contains a dense collagenous structure, which impedes cell seeding and infiltration and is not readily applicable for meniscus repair. In addition, the meniscus consists of two distinct anatomical regions that differ in vascularity and cellular phenotype. The purpose of this study was to explore the region-specific bioactivity of solubilized ECM derived from the inner and outer meniscal regions as determined in 2D and 3D cultures of adult mesenchymal stem cells (MSCs). When added as a medium supplement to 2D cultures of MSCs, urea-extracted fractions of the inner (imECM) and outer meniscal ECM (omECM) enhanced cell proliferation while imECM most strongly upregulated fibrochondrogenic differentiation on the basis of gene expression profiles. When added to 3D cultures of MSCs seeded in photocrosslinked methacrylated gelatin (GelMA) hydrogels, both ECM fractions upregulated chondrogenic differentiation as determined by gene expression and protein analyses, as well as elevated sulfated glycosaminoglycan sGAG content, compared to ECM-free controls. The chondrogenic effect at day 21 was most pronounced with imECM supplementation, but equivalent between ECM groups by day 42. Despite increased cartilage matrix, imECM and omECM constructs possessed compressive moduli similar to controls. In conclusion, soluble meniscal ECM may be considered for use as a tissue-specific reagent to enhance chondrogenesis for MSC-based 3D cartilage tissue engineering.

Statement of Significance

The inner region of the knee meniscus is frequently injured and possesses a poor intrinsic healing capacity. Solubilized extracellular matrix (ECM) derived from decellularized meniscus tissue may promote homologous differentiation of progenitor cells, thereby enhancing fibrocartilage formation within a meniscal lesion. However, the meniscus possesses regional variation in ultrastructure, biochemical composition, and cell phenotype, which may affect the bioactivity of soluble ECM derived from different regions of decellularized menisci. In this study, we demonstrate that urea-extracted fractions of ECM derived from the inner and outer regions of menisci enhance chondrogenesis in mesenchymal stem cells seeded in 3-dimensional photocrosslinkable hydrogels and that this effect is more strongly mediated by inner meniscal ECM. These findings suggest region-specific bioactivity of decellularized meniscal ECM.

1. Introduction

The menisci of the knee, crescent-shaped fibrocartilaginous tissues interposed between the articular surfaces of the femur and tibia, must resist compressive, tensile, and shear forces in

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http://dx.doi.org/10.1016/j.actbio.2016.11.046
1742-7061/

order to efficiently distribute tibiofemoral contact stresses and maintain joint health [1,2]. As with other musculoskeletal tissues, the complex structure of the meniscus imparts a unique function, allowing the meniscus to facilitate efficient articulation of the tibiofemoral joint. A gradient of decreasing collagen type II and proteoglycan content exists when moving from inner meniscal regions towards the periphery, while the outer region is principally composed of aligned collagen type I fibers capable of resisting hoop stresses arising from joint loading [3–7]. Much like hyaline cartilage, the inner region of the meniscus is avascular, while blood vessels infiltrate the outer 10–30% of the meniscus width [8]. The region-specific differences in ultrastructure and biochemical composition correspond to differences in cell phenotype; inner meniscal cells possess a round morphology reminiscent of articular chondrocytes while cells of the outer meniscus are found between aligned collagen fibers, similar to fibroblasts of tendon or ligament [1,2]. Similarly, cells of the inner region express higher levels of collagen type II and aggrecan while cells of the outer region express greater collagen type I [9].

Given the rapid onset of joint degeneration experienced with the loss of meniscal function [10,11], coupled with the limited availability and narrow inclusion criteria associated with meniscal allograft transplantation, tissue engineers have sought to develop novel biomaterials to serve as a meniscus substitute [12,13]. Decellularized menisci derived from animal sources have been explored, as the removal of cellular material could mitigate an adverse immune response while preservation of tissue ultrastructure and biochemical composition could maintain meniscal function and promote region-specific differentiation of infiltrating host cells. However, the dense collagenous extracellular matrix (ECM) of native menisci necessitates relatively harsh decellularization protocols with resulting losses in proteoglycan content and the associated compressive moduli of inner meniscal regions [14,15]. Despite these alterations in ultrastructural and biochemical properties, infiltration of seeded cells is still limited [16,17].

Enzymatic digestion (e.g., pepsin) of decellularized meniscus ECM can produce a thermoresponsive hydrogel capable of delivering exogenous cells to a meniscal lesion, while theoretically retaining meniscus-specific bioactive motifs capable of directing fibrocartilaginous neotissue formation [18]. However, Lin et al. [19] reported that pepsin digestion of ECMs offered negligible advantage over collagen type I in terms of promoting proliferation, migration, and multilineage differentiation of mesenchymal stem cells (MSCs), likely due to the proteolytic removal of functional components. On the other hand, a urea-soluble fraction of ECM has been demonstrated in several studies to promote differentiation of MSCs towards tissue-specific (i.e., homologous) phenotypes [19–21]. Most recently, we demonstrated that urea-soluble extracts of the inner and outer meniscus ECM could promote region-specific gene expression of MSCs seeded in a photocrosslinked polyethylene glycol diacrylate (PEGDA) hydrogel [22]. Expanding on these findings, this study explores the effect of urea-soluble extracts of the inner and outer meniscus ECM in promoting chondrogenic/fibrochondrogenic differentiation of MSCs seeded in a visible light (VL) photocrosslinked methacrylated gelatin (GelMA) hydrogel. GelMA hydrogels have been shown to support robust chondrogenesis of encapsulated MSCs [23] while the use of a VL-activated photoinitiator obviates concerns of UV light-induced mutagenesis or cytotoxicity [24]. We hypothesized that the ECM extracts would promote region-specific cell phenotypes, with the inner and outer ECM extracts respectively enhancing chondrogenic and fibrochondrogenic differentiation of MSCs seeded in GelMA hydrogels.

2. Methods

2.1. Overview of experimental design

Urea-soluble extracts from the decellularized ECM of inner and outer regions of juvenile bovine menisci were isolated and characterized. The biological effects of ECM extracts on human bone marrow MSCs were evaluated in both 2D and 3D cultures in vitro. MSCs were cultured on 2D plastic in the presence of ECM-supplemented media; assays for cell morphology, metabolism, and gene expression were performed up to 7 days of culture. For 3D constructs, MSCs were encapsulated in ECM-enhanced GelMA hydrogels and cultured for up to 42 days to assess the region-specific bioactivity of the ECM extracts, as evaluated on the basis of gene expression, histology, immunohistochemistry, biochemical composition, and mechanical properties.

2.2. Meniscus ECM preparation

2.2.1. ECM decellularization

Menisci were procured from hindlimbs of 6–8 week old cows (Research 87, Boylston, MA) and stored in a protease inhibitor solution (phosphate-buffered saline, PBS; 5 mM ethylenediaminetetraacetic acid, EDTA; 0.5 mM phenylmethylsulfonyl fluoride, PMSF) at −20 °C until use. Once thawed, menisci were halved, coarsely minced (Fig. 1A-C), and decellularized by adapting a previously established method [21]. Briefly, 4 g of minced tissue was agitated for 24 h at 4 °C in 40 ml of protease inhibitor solution containing 1% Triton X-100 (Sigma-Aldrich, St. Louis, MO, USA), followed by 3 washes (30 min each at 4 °C) in PBS. Subsequently, 40 ml of Hanks Buffered Salt Solution (HBSS, Thermo Fisher Scientific, Pittsburgh, PA, USA) supplemented with 200 U/ml DNase and 50 U/ml RNase (Worthington, Lakewood, NJ, USA) was added to the tissue, with continuous agitation for 12 h at room temperature. The tissue was washed six times in PBS, as above, before freezing and subsequent lyophilization. Native and decellularized tissues were evaluated for histological appearance, cellular content, and biochemical composition, including total collagen and sulfated glycosaminoglycan (sGAG) contents, as described below.

2.2.2. Histology of native and decellularized ECM

Native and decellularized tissues were fixed in 10% phosphate-buffered formalin, serially dehydrated, embedded in paraffin, and then sectioned (6 μm thickness) with a microtome (Leica RM2255, Leica Biosystems, Buffalo Grove, IL, USA). Samples were rehydrated and stained with haematoxylin & eosin (H&E, Sigma-Aldrich) or 4′,6-diamidino-2-phenylindole, dilactate (DAPI, Life Technologies, Carlsbad, CA, USA). H&E-stained samples were imaged using an Olympus SZX16 stereo microscope while DAPI-stained sections were examined with an Olympus CKX41 inverted microscope using fluorescent excitation at 405 nm.

2.2.3. Biochemical composition of native and decellularized ECM

To determine the biochemical composition of native and decellularized tissues, dried samples were digested overnight at 65 °C at a concentration of 10 mg/mL in a digestion buffer (pH 6.0) containing 2% papain (v/v, from Papaya latex, Sigma-Aldrich), 0.1 M sodium acetate, 0.01 M cysteine HCl, and 0.05 M EDTA. The pH was then adjusted to 7.0 through addition of concentrated NaOH. sGAG content was quantified with a Blyscan Assay according to the manufacturer's instructions (Biocolor, Carrickfergus, United Kingdom). dsDNA content was determined using the Quant-iT Picogreen dsDNA assay (Life Technologies). Total collagen content was determined using a modified hydroxyproline assay. Briefly, 200 μL of each sample was hydrolyzed with an equal volume of