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Decellularized ECM effects on human mesenchymal stem cell stemness and differentiation

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ABSTRACT

Microenvironment extracellular matrices (ECMs) influence cell adhesion, proliferation and differentiation. The ECMs of different microenvironments have distinctive compositions and architectures. This investigation addresses effects ECMs deposited by a variety of cell types and decellularized with a cold-EDTA protocol have on multipotent human mesenchymal stromal/stem cell (hMSC) behavior and differentiation. The cold-EDTA protocol removes intact cells from ECM, with minimal ECM damage and contamination. The decellularized ECMs deposited by cultured hMSCs, osteogenic hMSCs, and two smooth muscle cell (SMC) lines were tested for distinctive effects on the behavior and differentiation of early passage ('naïve') hMSC plated and cultured on the decellularized ECMs. Uninduced hMSC decellularized ECM enhanced naïve hMSC proliferation and cell motility while maintaining stemness. Decellularized ECM deposited by osteogenic hMSCs early in the differentiation process stimulated naïve hMSCs osteogenesis and substrate biomineralization in the absence of added dexamethasone, but this osteogenic induction potential was lower in ECMs decellularized later in the osteogenic hMSC differentiation process. Decellularized ECMs deposited by two smooth muscle cell lines induced naïve hMSCs to become smooth muscle cell-like with distinctive phenotypic characteristics of contractile and synthetic smooth muscle cells. This investigation demonstrates a useful approach for obtaining functional cell-deposited ECM and highlights the importance of ECM specificity in influencing stem cell behavior.

1. Introduction

Adult multipotent bone marrow-derived mesenchymal stromal cells, also known as mesenchymal stem cells (MSCs), can differentiate into a variety of lineages including osteoblasts, chondrocytes, myoblasts, and adipocytes (Pittenger et al., 1999). Isolated MSCs that are expanded and banked *ex vivo* have great potential for use in human cell therapies and regenerative medicine (Johnson et al., 2012; Tang et al., 2013). Isolated MSCs currently are used to treat animal arthritis and cardiac problems, despite limited understanding of the biological mechanisms by which local administration of MSCs decreases inflammation and contributes to tissue regeneration. Challenges in stem cell banking and usage include developing protocols to overcome loss of stemness *ex vivo*, without raising the potential for the cells to become malignant, and to induce endogenous stem cell differentiation into specific cell types *in vivo* (Rosland et al., 2009). In addition to potential for clinical usage, isolated MSCs also provide a valuable model system with which to investigate how stem cells could interact with implanted biomaterials *in vivo*.

Important drivers of stem cell behavior and differentiation include the microenvironments or niches to which the cells are exposed. Cell microenvironments play vital roles in regulating cell proliferation (Williams et al., 2008), migration (Hung et al., 2012), and differentiation (Martino et al., 2009; Rowlands et al., 2008). The composition, architecture, and physical properties of microenvironment extracellular matrix (ECM) and its bound growth factors and other ligands provide specific physical and chemical cues that influence distinctive stem cell behaviors (Wipff et al., 2007). Cells respond to microenvironment physical properties such as ECM modulus and topography, for example, through mechanotransduction mechanisms that convert mechanical responses into intracellular biochemical signals (Reilly and Engler, 2010).

To improve biocompatibility and better mimic the protein composition of *in vivo* cell microenvironments, non-biological 2D and 3D *in vitro* culture substrates can be coated with single ECM proteins such as fibronectin (FN), collagen, or laminin or with more complex solubilized ECM protein mixtures such as Matrigel®. Although these coated surfaces support proliferation and differentiation of numerous cell types, they lack the specific

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compositional and architectural complexity of ECMs secreted and assembled by cells. ECMs deposited by cells in culture and then decellularized may better replicate cell-specific features of ECM architectures and presentation of associated bioactive factors and perhaps satisfy the requirement for low immunogenicity if introduced into a body (Badylak and Gilbert, 2008). Several studies have demonstrated that decellularized ECM obtained by cell-lysis protocols is better than standard cell culture substrates and substrates coated with single ECM components for increasing stem cell proliferation while maintaining stem cell multipotency for differentiation into several cell types including osteoblast and adipocytes (Lai et al., 2010, 2012; Ng et al., 2014; Sun et al., 2011).

Most approaches to decellularize cell-deposited ECM have a significant drawback. Enzymatic detachment of intact cells by treatment with proteases such as trypsin and collagenase designed to recover viable cells, for example, may damage the remaining ECM and its bound factors. Cell lysis protocols that include treatment with detergent (Decaris and Leach, 2011), alkali (Bass et al., 2007), or freeze/thaw cycles (Deutsch and Guldberg, 2010) can contaminate the remaining ECM with intracellular debris that may negatively affect subsequent cell interaction with the ECM or induce immunological reactions if implanted.

The purpose of this investigation was to investigate the effects of decellularized ECMs that were initially assembled by undifferentiated hMSCs, osteogenic hMSCs, and two smooth muscle cell lines on 'naïve' human bone marrow MSCs (hMSCs) growth and differentiation. ECMs from the osteogenic hMSCs and the two smooth muscle cell lines were chosen to determine whether they could influence the behavior of mesenchymal stem cells that might home to and interact with implantable devices such as orthopedic implants and arterial stents, respectively. Our initial attempts to investigate effects of cell-assembled ECM on stem cell proliferation, maintenance of stemness, and differentiation using ECMs decellularized by Triton-X-100 cell lysis yielded poor and highly variable results (results not shown), which spurred us to develop a protease-detergent-free method for removing intact cells from the ECM they secreted and assembled. This method involves incubating cell cultures in EDTA-PBS at 4 °C until the cells round up and detach from the underlying ECM. Removal of the detached but intact cells leaves ECM that is largely undamaged by added protease and uncontaminated with the intracellular debris that cells release when lysed with detergent or other lysis protocols. To minimize ECM damage and contamination, the cell-deposited ECMs were decellularized using a simple and effective protease- and detergent-free method involving cold EDTA removal of intact cells.

Our results demonstrate that decellularized ECMs assembled by the different cell types have distinctive effects on naïve hMSCs. ECM deposited by uninduced hMSCs enhances the proliferation and preservation of stemness of naïve hMSCs whereas ECM deposited by osteogenic hMSCs induces naïve hMSC differentiation into osteoblasts, despite the absence of added differentiation factors. Additionally, ECMs deposited by the two smooth muscle cell lines induce naïve hMSCs to exhibit distinctive phenotypic characteristics of smooth muscle cells.

2. Materials and methods

2.1. Cell culture and differentiation

The hMSCs used for this investigation were obtained from a 37 year old female donor by the Texas A&M Health Science Center College of Medicine Institute for Regenerative Medicine at Scott & White Hospital, through which hMSC supply is supported by grant P40RR017447 from the NCRR-NIH. The shipped P1 stage cells were immediately subcultured on arrival in the lab and stored frozen at the P2 stage. Before commencing experiments, the multipotentiality of the source hMSCs was confirmed with a CFU formation assay and by differentiation into osteoblasts and adipocytes. All experiments described here were done with P2-P5 stage cells. The hTERT-immortalized myometrial smooth muscle cell line was provided by Dr. James Olcese (Florida State University College of Medicine) and transfected to express mCherry. The A7R5 rat aorta smooth muscle cell line and the U2OS human osteosarcoma cell line originally were purchased from ATCC and then cultured in the lab for several years under recommended conditions. All cells were cultured in standard medium (SM) composed of alpha-MEM modified medium supplemented with 2.2 g/L $NaHCO_3$, 10% (16.5% for hMSCs) fetal bovine serum (Atlanta Biologicals), and an antibiotic-antimycotic supplement containing 100 units/ml penicillin G, 100 μg/ml streptomycin, 0.25 μg/ml amphotericin B, and 10 μg/ml gentamicin (all from Invitrogen/GIBCO, unless otherwise noted) at 37 °C in 5% CO_2.

hMSCs assayed for differentiation potential after initial growth for 3 days on decellularized ECM or directly on Tissue Culture Plastic (TCP) were recovered by trypsinization, replated on TCP, and cultured in Bone Differentiation Medium (BDM) or Fat Differentiation Medium (FDM) for an additional 21 days.

Differentiation of hMSCs into osteoblasts was induced 24 h after plating by replacing the standard culture medium with BDM, in which the standard culture medium was supplemented with 10 nM Dexamethasone, 20 mM β-glycerophosphate, and 50 μM L-ascorbic acid 2-phosphate (all from Sigma), as described in the Institute for Regenerative Medicine hMSC Manual accompanying the cells. Differentiation of hMSCs into adipocytes was induced 24 h after plating by replacing the standard culture medium with FDM, in which the standard culture medium was supplemented 0.5 μM Dexamethasone, 0.5 μM isobutylxanthine, and 50 μM indomethacin as described by Sekiya et al. (2002).

2.2. ECM decellularization

To obtain decellularized ECM, cell cultures, in which the cells were plated at a density of 1×10^4 cells per well (high density) on coverslips or directly on the TCP in a six-well plate, were cultured under specified conditions for various specified periods of time. To decellularize the deposited ECM, the cultures were washed twice with phosphate-buffered saline (PBS) that was pre-chilled to 4 °C and then incubated in 1 mM EDTA-PBS at 4 °C for 12–24 h, which caused cell rounding. The rounded cells were detached by agitating the culture dish and gently rinsing with fresh cold-EDTA. After aspirating the detached cells, the surface was washed once with cold PBS before use for plating fresh cells. For ease of reference, the decellularized ECMs are designated according to the culture condition under which the ECM was deposited. Decellularized ECMs deposited by hMSCs cultured for 3 days in standard growth medium or in BDM medium, for example, are designated 3d-hMSC-ECM and 3d-BDM-hMSC-ECM, respectively. To assay activities of the decellularized ECM, freshly thawed hMSCs ('naïve') were plated onto the decellularized ECMs at 1×10^4 cell per well.

2.3. Atomic Force Microscopy (AFM) imaging of ECM topology

Images of dry ECM surface topographies in ambient air were recorded using the AC mode of an Asylum MFP-3D AFM unit equipped with a 20 nm radius TR400PSA tip (spring constant $\sim 0.02\ N\ m^{-1}$), an ARC2 controller (Asylum Research Inc., Santa Barbara, CA), and Igor Pro software. The tip cantilever was tuned to resonate 10% below its resonance frequency.