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Thermally labile components of aqueous humor potently induce osteogenic potential in adipose-derived mesenchymal stem cells

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ABSTRACT

Adipose-derived mesenchymal stem cells (ASCs) hold promise for use in cell-based therapies. Their intrinsic anti-inflammatory properties are potentially useful for treatments of inflammatory conditions such as uveitis, while their ability to differentiate along multiple cell lineages suggests use in regenerating damaged or degenerated tissue. However, how ASCs will respond to the intraocular environment is poorly studied. We have recently reported that aqueous humor (AH), the fluid that nourishes the anterior segment of the eye, potently increases alkaline phosphatase (ALP) activity of ASCs, indicating osteogenic differentiation. Here, we expand on our previous findings to better define the nature of this response. To this end, we cultured ASCs in the presence of 0, 5, 10, and 20% AH and assayed them for ALP activity. We found ALP activity correlates with increasing AH concentrations from 5 to 20%, and that longer treatments result in increased ALP activity. By using serum free media and pretreating AH with dextran-coated charcoal, we found that serum and charcoal-adsorbable AH components augment but are not required for this response. Further, by heat-treating the AH, we established that thermally labile components are required for the osteogenic response. Finally, we showed myocilin, a protein present in AH, could induce ALP activity in ASCs. However, this was to a lesser extent than untreated 5% AH, and myocilin could only partially rescue the effect after heat treatment, documenting there were additional thermally labile constituents of AH involved in the osteogenic response. Our work adds to the understanding of the induction of ALP in ASCs following exposure to AH, providing important insight in how ASCs will be influenced by the ocular environment. In conclusion, increased osteogenic potential upon exposure to AH represents a potential challenge to developing ASC cell-based therapies directed at the eye.

1. Introduction

Mesenchymal Stromal/Stem Cells (MSCs) have been derived from a variety of tissue sources including adipose tissue (Bourin et al., 2013). MSCs are characterized by expression of CD73, CD90, CD105, CD146 surface markers, lack expression of CD31, CD34, or CD45 surface markers, and are capable of tri-lineage differentiation into adipocytes, osteocytes, chondrocytes (Dominici et al., 2006; Horwitz et al., 2005). Additionally, MSCs do not express MHC II, making them ideal for allogenic as well as autologous cell therapies. Low immunogenicity and their differentiation potential positions MSCs for regenerative medicine applications including bone and tendon repair (Caplan, 2013). Furthermore, MSCs have also been shown to be immunomodulatory and have been investigated in clinical trials for the treatment of immune mediated diseases (Nauta and Fibbe, 2007; Voswinkel et al., 2013). Adipose-derived MSCs (ASCs) are an especially promising source for use in regenerative medicine, as they can be acquired in high numbers from minimally invasive lipectomy procedures (Zuk et al., 2001).

The promise of cell therapies in general and ASC therapy specifically is no less apparent in the eye (Joe and Gregory-Evans, 2010;

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Rajashekhar, 2014). Degenerative ocular diseases such as diabetic retinopathy, age-related macular degeneration (AMD), and glaucoma are candidates for cell replacement therapies. Additionally, these diseases have known immune components in their progression, which could potentially be ameliorated by the immunosuppressive effects of ASCs. Several recent publications have highlighted the potential for cell therapy in the eye. MSCs have been successfully differentiated in retinal progenitor cells (Moviglia et al., 2012), keratocyte-like cells (Arnalich-Montiel et al., 2008; Liu et al., 2012; Park et al., 2012), corneal endothelial-like cells (Joyce et al., 2012), retinal pigmented epithelium cells (Vossmerbaeumer et al., 2009), and photoreceptors (Kicic et al., 2003). Further, MSCs have been used to treat models of glaucoma (Johnson et al., 2010; Manuguerra-Gagne et al., 2013), retinopathy (Chung et al., 2011; Inoue et al., 2007; Jiang et al., 2014; Machalinska et al., 2013), autoimmune uveoretinitis (Li et al., 2013; Zhang et al., 2011), and corneal wounds (Arnalich-Montiel et al., 2008; Jia et al., 2012; Oh et al., 2008; Yao et al., 2012). The bulk of these studies focus on how MSCs influence the ocular environment and limited consideration is given to how the ocular environment influences the behavior of MSCs.

We have recently reported that aqueous humor (AH), the fluid that nourishes the tissues adjoining the anterior segment of the eye, potently stimulates osteogenic potential in ASCs (Morgan et al., 2014). AH is a complex mixture of proteins, lipids, salts, and other small molecules (Cousins et al., 1991; De Berardinis et al., 1965; Duan et al., 2010; Edwards et al., 2014; Greiner et al., 1991; Iyer et al., 2012; Knisely et al., 1994; Lee et al., 1977; Rao et al., 2000; Russell et al., 2001; Tripathi et al., 1989). Several of these solutes have been previously implicated in osteogenesis, including ascorbate and glucocorticoids (Bellows et al., 1987, 1986; Herbertson and Aubin, 1995; Maniatopoulos et al., 1988; Tenenbaum and Heersche, 1982), growth-factor like lipids such as lysophosphatidic acid (LPA) (Liu et al., 2010), and the protein myocilin (Kwon et al., 2013). Understanding this effect is important to ensuring the safety and efficacy of ASC administration to the eye. Further, illuminating the osteogenic potential of AH may provide insight into the calcification of anterior chamber tissues such as the trabecular meshwork, believed to play a role in the progression of glaucoma (Borras and Comes, 2009; Gonzalez et al., 2004, 2000; Vittitow and Borras, 2004; Xue et al., 2007, 2006). In this study, we expanded on our previous results to define the effects of AH dose and treatment duration. We further identified the importance of thermally labile and charcoal adsorbable components in AH to the differentiation process. Finally, we showed myocilin by itself can increase osteogenic potential, although this induction was not as robust as that of complete AH.

2. Methods

2.1. Preparation of AH and myocilin

AH was extracted using a 25-gauge needle from enucleated bovine eyes shipped overnight on ice (Pel-freez, Rogers, AR). AH was sterile filtered, aliquoted, and stored at −20 °C until use. Some AH was heat treated (AH/HT) to denature protein components by heating it to 90 °C for 30 min. AH was also treated with dextran-coated charcoal (AH/DCC) to reduce the concentration of hormones and lipids (Herbert et al., 1965; Keane et al., 1968; Lee et al., 1998). Briefly, 20 mg DCC (Sigma–Aldrich, St. Louis, MO) was added per 1 mL AH and incubated with rocking overnight at 4 °C. The sample was then centrifuged at 5,000 *g* for 15 min and sterile filtered to remove the DCC.

Full length human MYOCILIN cDNA was cloned into the pCS2-FLAG vector as described (Kwon et al., 2009) and used for transient transfection of HEK293 cells. Transfection was performed with Lipofectamine 2000 (Invitrogen, Carlsbad, CA) according to the manufacturer's protocol. Serum-containing medium was replaced by serum-free medium 14–16 h after transfection, and cells were incubated for 48 h. Conditioned medium was collected and myocilin-FLAG protein was purified using anti-FLAG M2 agarose beads according to the manufacturer's instructions (Sigma, St. Louis, MO). Myocilin was further purified by ion-exchange chromatography using HiTrap-SP FF 1-ml columns (GE Healthcare). The purity of the isolated myocilin was analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Two closely migrated bands with mobilities corresponding to myocilin were observed after Coomassie blue staining of the gel, similar to shown in Fig. 1 of Kwon et al. (2009).

2.2. Cell culture

Primary cultures of ASCs were cultured from human donor adipose tissue as previously described (Chung et al., 2012; Morgan et al., 2014; Toupadakis et al., 2010; Wood et al., 2012). Briefly, 10–13 g of fat was minced and incubated with rocking 2 h at 37 °C in 50 mL of PBS (Invitrogen, Carlsbad, CA) with 0.1% collagenase/1% bovine serum albumin (Worthington, Lakewood, NJ). The tissue was then centrifuged to remove the lipid layer and repeatedly washed with PBS. Cell pellets were re-suspended with low glucose DMEM supplemented with 10% FBS and 1% penicillin/streptomycin (Life Technologies, Carlsbad, CA), plated, and incubated at 37 °C, 5% CO_2. Cells were passaged at 70% confluence and maintained in the supplemented DMEM, henceforth referred to as full media.

For experiments, cells were plated at 50,000 cells per well in a 24-well plate in full media and allowed to attach overnight. Cells were rinsed with PBS and placed in either full or serum free DMEM with AH or myocilin supplements. To avoid disrupting the cell monolayer, half-volume media exchanges were performed twice weekly. At either 2 or 3 weeks, the cells were briefly fixed in 4% formaldehyde and rinsed in PBS.

2.3. Staining and imaging of cells

Immediately after fixation, cells were stained for ALP activity as previously described (Morgan et al., 2014). Briefly, they were stained for 15 min with 0.1% naphthol AS-MX phosphate (Sigma) and 0.1% fast red violet LB (Sigma) dissolved in 56 mM 2-amino-2-methyl-1,3-propanediol (pH 9.9; Sigma). In the initial dose response experiments, cells were costained for the presence of sulphated acid proteoglycans using Alcian blue after ALP staining (Asahina et al., 1993). Briefly, the cells were rinsed in 0.1 N HCl (pH 1.0), stained for 15 min with 1% w/v Alcian Blue 8GX (Sigma) in 0.1 N HCl, and rinsed with 0.1 N HCl to remove non-specific staining. Following staining, the coverslips were rinsed in PBS and mounted on slides for imaging. Coverslips were imaged using a Nikon DS-Fi1 color camera attached to a Nikon Diaphot inverted microscope. Six random 0.89 mm^2 fields were taken of each coverslip. ALP activity was quantified and averaged across the six images using custom analysis programs written in the MATLAB (Mathworks, Natick, MA) software package.

2.4. Statistics

All experiments were performed on ASCs isolated from three donors. Data for each donor were normalized. For each experiment, significance was assessed on the normalized values by one way ANOVA and Fisher's post-hoc test. Levels of significance are denoted throughout the manuscript by *** = $p < 0.001$, ** = $p < 0.01$, and * = $p < 0.05$. All bar graphs are shown as mean ± SEM.