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Changes of chondrocyte expression profiles in human MSC aggregates in the presence of PEG microspheres and TGF-β3

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ABSTRACT

Biomaterial microparticles are commonly utilized as growth factor delivery vehicles to induce chondrogenic differentiation of mesenchymal stem/stromal cells (MSCs). To address whether the presence of microparticles could themselves affect differentiation of MSCs, a 3D co-aggregate system was developed containing an equal volume of human primary bone marrow-derived MSCs and non-degradable RGD-conjugated poly(ethylene glycol) microspheres (PEG-μs). Following TGF-β3 induction, differences in cell phenotype, gene expression and protein localization patterns were found when compared to MSC aggregate cultures devoid of PEG-μs. An outer fibrous layer always found in differentiated MSC aggregate cultures was not formed in the presence of PEG-μs. Type II collagen protein was synthesized by cells in both culture systems, although increased levels of the long (embryonic) procollagen isoforms were found in MSC/PEG-μs aggregates. Ubiquitous deposition of type I and type X collagen proteins was found in MSC/PEG-μs cultures while the expression patterns of these collagens was restricted to specific areas in MSC aggregates. These findings show that MSCs respond differently to TGF-β3 when in a PEG-μs environment due to effects of cell dilution, altered growth factor diffusion and/or cellular interactions with the microspheres. Although not all of the expression patterns pointed toward improved chondrogenic differentiation in the MSC/PEG-μs cultures, the surprisingly large impact of the microparticles themselves should be considered when designing drug delivery/scaffold strategies.

1. Introduction

Articular cartilage is a highly specialized tissue with important load-bearing functions. Mature tissue consists of a relatively sparse population of chondrocytes embedded within an abundant, well-organized extracellular matrix (ECM) [1]. Type II collagen fibers provide the tissue with tensile strength while proteoglycans (mainly aggrecan) contribute to its compressive load-bearing function. The avascular nature of articular cartilage renders it incapable of self-repair following traumatic, degenerative or inflammatory damage. Subsequently, many tissue engineering strategies are being investigated as a means to generate articular cartilage tissue *in vitro* or *in vivo* [2–5]. Common approaches involve the use of multipotent mesenchymal stem/stromal cells (MSCs) in combination with TGF-β superfamily growth factors and three-dimensional (3D) biomaterial scaffold systems [6–9]. Recent advances in the field are now focusing on ways to spatially and temporally control the availability of bioactive growth factor to enhance MSC differentiation. Specifically, the application of biomaterial microspheres as growth factor delivery vehicles for cartilage engineering is currently being explored. Microspheres generated from either poly(lactic-*co*-glycolic acid) (PLGA) or gelatin have been engineered to release TGF-β1with somewhat promising effects on improving chondrocyte differentiation in MSC aggregate cultures [10,11]. Other strategies to attempt to enhance cartilage matrix production have been reported by the application of biodegradable insulin-loaded PLGA microspheres or hyaluronic acid-based hydrogel microparticles releasing BMP-2 [12,13]. In addition, PLGA microspheres as injectable scaffolds for cartilage tissue engineering are being investigated [14] while other studies are exploring the use of 3D scaffolds generated from protein-loaded

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microspheres or the combination of bulk hydrogel scaffolds with alginate microspheres to permit slow release of biological factors [15–17]. Based on these current microsphere-based technologies to improve cartilage tissue engineering, questions arise as to the effect of microspheres themselves on the differentiation of MSCs. The goal of this study was to investigate the effect of non-degradable poly(ethylene glycol) microspheres (PEG-μs) on TGF-β3-induced differentiation of human bone marrow-derived MSCs by generating co-aggregate 3D cultures of MSCs and PEG-μs. Data obtained was directly compared to MSC aggregate cultures grown in the absence of microspheres; this MSC aggregate system has been well reported in the literature as a common *in vitro* chondrogenesis assay [18]. The rationale for using PEG-based microspheres is that PEG surfaces are noteworthy for their low degree of protein adsorption and cell adhesion [19] thus providing a relatively non-adhesive background to directly address the architectural effects of microspheres on MSC differentiation. In our system, RGD (arginine-glycine-aspartic acid) peptide was incorporated within the PEG-μs to promote cell adhesion. The reason for doing this stemmed from previous studies showing that cells within PEG scaffolds tended to undergo apoptosis due to lack of adhesion sites [20,21] and that MSC viability was improved by incorporation of RGD peptide within such scaffolds [20,22]. As others have shown that microspheres may affect ES cell differentiation [23,24], we hypothesized that the presence of microspheres would alter the MSC response to TGF-β3 due to effects of cell dilution, altered growth factor diffusion and/or cellular interactions with the microspheres themselves. These studies have important implications in the cartilage tissue engineering field by directly addressing the impact of biomaterials on MSC differentiation and set the foundations for future investigations aimed toward improving cartilage matrix production *in vitro* and *in vivo*.

2. Materials and methods

2.1. Human mesenchymal stem cells

Human bone marrow-derived mesenchymal stem cells (MSCs) from two independent donors were used in this study. One MSC primary cell line was purchased commercially (Lonza, Walkersville Inc.) and the second primary MSC cell line was isolated in-house from a bone marrow aspirate specimen obtained via an IRB-approved core facility at Washington University. Using standardized differentiation assay kits (Lonza), the multipotential capacity of both MSC cell lines (i.e. the ability to differentiate to adipocytes, osteoblasts and chondrocytes) was first confirmed before using the cells for further analyses.

2.2. Synthesis of PEG microspheres

Unless otherwise noted, all reagents were purchased from Sigma-Aldrich. *PEG Synthesis:* Eight-arm PEG-OH (PEG_8-OH; MW = 10,000; Shearwater Polymers, Huntsville, AL) was used to synthesize PEG_8-vinylsulfone (PEG_8-VS) and PEG_8-amine as previously described [25]. PEG macromonomers were dissolved separately in 200 mg/mL in Dulbecco's phosphate buffered saline (1×PBS; 8 mM sodium phosphate, 2 mM potassium phosphate, 140 mM sodium chloride, 10 mM potassium chloride, pH 7.4) and sterile filtered with 0.22 μm syringe filters (Millipore). To generate the microspheres, PEG_8-amine solutions were combined with PEG_8-VS solutions at a 1:1 ratio [26]. An equal volume of RGD peptide (Ac-GCGYGRGDSPG-NH2; GenScript, Piscataway, NJ; 20 mg/mL; 1.88 mol RGD/mol PEG) in 1×PBS was added to the PEG solutions and incubated at 37 °C for 20 min. The PEG solutions were diluted to 20 mg/mL with 1×PBS and 1.5 M sodium sulfate (in 1×PBS) to a final sodium sulfate concentration of 0.6 M at room temperature. The PEG_8-VS/PEG_8-amine solutions were then incubated above the cloud point at 70 °C for 20 min. Suspensions of microspheres (approximately 6.01 ± 1.98 μm diameter) were subsequently buffer exchanged twice in 1×PBS to remove the sodium sulfate, each time diluting the microsphere solution 3:1 with 1×PBS and titurating, centrifuging at 14,100×*g* for 2 min and removing the supernatant.

2.3. Induction of MSCs by TGF-β3 in two different in vitro culture systems

2.3.1. Stem cell aggregate cultures

MSC aggregate cultures each containing 2.5×10^5 cells were generated as described previously [18]. Aggregates were formed in 15 mL polypropylene tubes following centrifugation at 350×*g* for 10 min. Serum-free differentiation medium (500 μL) was added to each cell aggregate. This medium consisted of DMEM, 1% ITS+, 1% sodium pyruvate, 0.1 μM dexamethasone, 40 μg/mL ascorbate and TGF-β3 (10 ng/mL; Lonza, Walkersville, Inc). The lids of the tubes were loosened slightly to allow gas exchange and then incubated at 37 °C. Medium was changed every 2 days.

2.3.2. MSC/PEG microsphere co-aggregate cultures

MSCs (2.5×10^5) were combined with PEG microspheres (1:1 volume ratio of cell : microsphere suspension) in sterile 1.5 mL eppendorf tubes and gently rotated for 3 h at 37 °C to ensure uniform mixing of cells with microspheres. MSC/PEG-μs aggregates were formed by centrifugation at 350×*g* for 10 min. Pin-holes were punched in the lids of each sample tube to permit gas exchange. Prior to differentiation, MSC/PEG-μs aggregates were cultured for a short time (3 d) at 37 °C to maximize cell attachment to the microspheres and permit the cells to acclimate to the microsphere environment. During this time, a medium containing FGF-2 (known to maintain MSCs in a de-differentiated state) [27,28] was added to the aggregates [DMEM-LG containing 10% FBS (Atlas Biologicals, Fort Collins, CO) 10 ng/mL FGF-2 (Peprotech, Rocky Hill, NJ)]. Medium was then replaced with the same serum-free TGFβ3-containing differentiation medium (500 μL) as described above and replenished every 2 d until time of harvest at 12 d or 28 d. Of note, our experience with MSC culture has shown that the presence of FGF-2 in medium prior to MSC aggregate formation had no effect on how the cells responded to TGF-β3-induced differentiation (unpublished observations). All downstream analyses were carried out in triplicate for each MSC or MSC/PEG-μs aggregate differentiation experiment.

2.4. Analysis of cell viability and phenotype

2.4.1. Viability assay

Following 12 days and 28 days of TGF-β3 induction, MSC and MSC/PEG-μs aggregate cultures were stained with a combination of calcein AM (2 μM) and ethidium homodimer (4 μM) (Live/Dead cytotoxicity kit; Invitrogen) for 30 min at room temperature. Cultures were rinsed twice in 1×PBS, fixed in 4% paraformaldehyde for 2 h and processed through a gradient of 30%, 50% and 70% ethanol for paraffin embedding. Paraffin sections (10 μm) were obtained for fluorescence analysis. Calcein uptake by live cells was visualized using the fluorescein (FITC) filter set and uptake of ethidium homodimer by dead cells was analyzed using the rhodamine (TRITC) filter set. As a control, MSC/PEG-μs aggregates cultured in the absence of TGF-β3 were analyzed in order to show that ethidium homodimer staining was functional and to prove that calcein or ethidium homodimer did non-specifically stain the microspheres.

2.4.2. Safranin-O staining

Paraffin sections of aggregate cultures from day 12 or day 28 of TGF-β3 induction were treated with xylene, rehydrated through decreasing concentrations of ethanol, stained in Weigert's Hematoxylin for 5 min, washed in running water for 5 min and stained with 0.001% Fast Green for 3 min. Samples were then rinsed in 1% glacial acetic acid, stained in 0.1% Safranin O for 5 min, then dehydrated and cleared by incubation in 95% alcohol, 100% alcohol and xylene. Mounting medium was applied and stained sections were cover-slipped.

2.5. Antibodies

The following antibodies were used for detection of extracellular matrix collagens and α-smooth muscle actin (α-SMA) by immunofluorescence : type I collagen mouse monoclonal antibody (Abcam; ab6308) used at 1/1000 dilution; type II collagen rat polyclonal antisera [29] used at 1/400 dilution; type IIA/IID collagen mouse monoclonal antisera that recognizes epitopes within the exon 2-encoded domain of the IIA and IID procollagen isoforms and used at 1/500 dilution (gift from Dr Linda Sandell); type X collagen mouse polyclonal antibodies used at 1/500 dilution [30]; and α-SMA mouse monoclonal antibodies (Sigma A5228) used at 1/1000 dilution.

2.6. Immunohistochemical analysis of protein localization

MSC or MSC/PEG-μs aggregate cultures were harvested following 12 days or 28 days of TGF-β3 induction, fixed in 4% paraformaldehyde for 2 h, rinsed thoroughly in 1×PBS followed by dehydration in increasing concentrations of ethanol (30%, 50% and 70% ethanol) and then embedded in paraffin. Micro-thin (10 μm) sections were de-paraffinized, rehydrated and blocked with 1% hyaluronidase (Sigma) for 30 min at 37 °C. Sections were rinsed with 1×PBS and blocked further with 10% goat serum and then incubated overnight at 4 °C with the primary antibody diluted in 2% goat serum. Following 1×PBS washes, sections were incubated with species-specific secondary antibodies conjugated to Alexa-488 dye at a dilution of 1/200 (Invitrogen; A11001, A11006) for 45 min at room temperature. DAPI mounting medium was applied following three rinses in 1×PBS. Human embryonic limb sections (day 54 or day 67 of development) were obtained from the Laboratory of Developmental Biology at University of Washington in Seattle, under an IRB-approved protocol. Sections of these tissues were used as positive controls for all collagen antibodies to