Journal of Controlled Release 179 (2014) 42–51

Contents lists available at ScienceDirect

Journal of Controlled Release

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

A chondromimetic microsphere for *in situ* spatially controlled chondrogenic differentiation of human mesenchymal stem cells

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ARTICLE INFO

Article history:
Received 12 September 2013
Accepted 22 January 2014
Available online 31 January 2014

Keywords:
Microspheres
Delivery vehicle
Mesenchymal stem cell
Chondrogenesis
Osteoarthritis
Tissue engineering

ABSTRACT

Human mesenchymal stem cells (hMSCs) have been identified as a viable cell source for cartilage tissue engineering. However, to undergo chondrogenic differentiation hMSCs require growth factors, in particular members of the transforming growth factor beta (TGF-β) family. While *in vitro* differentiation is feasible through continuous supplementation of TGF-β3, mechanisms to control and drive hMSCs down the chondrogenic lineage in their native microenvironment remain a significant challenge. The release of TGF-β3 from an injectable microsphere composed of the cartilage-associated extracellular matrix molecule hyaluronan represents a readily translatable approach for *in situ* differentiation of hMSCs for cartilage repair. In this study, chondromimetic hyaluronan microspheres were used as a growth factor delivery source for hMSC chondrogenesis. Cellular compatibility of the microspheres (1.2 and 14.1 μm) with hMSCs was shown and release of TGF-β3 from the most promising 14.1 μm microspheres to control differentiation of hMSCs was evaluated. Enhanced accumulation of cartilage-associated glycosaminoglycans by hMSCs incubated with TGF-β3-loaded microspheres was seen and positive staining for collagen type II and proteoglycan confirmed successful *in vitro* chondrogenesis. Gene expression analysis showed significantly increased expression of the chondrocyte-associated genes, collagen type II and aggrecan. This delivery platform resulted in significantly less collagen type X expression, suggesting the generation of a more stable cartilage phenotype. When evaluated in an *ex vivo* osteoarthritic cartilage model, implanted hMSCs with TGF-β3-loaded HA microspheres were detected within cartilage fibrillations and increased proteoglycan staining was seen in the tissue. In summary, data presented here demonstrate that TGF-β3-bound hyaluronan microspheres provide a suitable delivery system for induction of hMSC chondrogenesis and their use may represent a clinically feasible tissue engineering approach for the treatment of articular cartilage defects.

1. Introduction

Controlling mesenchymal stem cell (MSC) differentiation within a complex *in vivo* milieu represents a challenging task requiring finely tuned growth factor delivery to initiate cellular signalling networks [1,2]. Biomaterials have been used as model delivery systems to enhance MSC differentiation. In addition to releasing signalling molecules, biomaterials can provide a substrate for cell attachment. Encapsulation of bioactive molecules can be achieved through chemical crosslinking or simple protein adsorption. This typically exploits direct charge–charge interactions between growth factors and matrices or *via* intermediate proteins such as heparin [3]. Such material approaches include microspheres, hydrogels, scaffolds or combination of scaffolds/hydrogels and microspheres [4,7]. Microspheres derived from materials such as alginate [8] chitosan [9,10] and chondroitin sulphate [11] have been described as appropriate carriers for coordinated release of growth and differentiation factors. By combining TGF-loaded alginate microspheres within a hyaluronic acid hydrogel, Bian et al. demonstrated chondrogenic differentiation of human MSCs (hMSCs) [8]. Similarly, porcine chondrocytes embedded in a chitosan scaffold containing TGF-β1-loaded chitosan microspheres showed increased proliferation and differentiation with increased production of extracellular matrix (ECM) *in vitro* [10]. Chitosan microspheres have also been used to deliver peptides with potential to direct rabbit MSC osteogenic differentiation [9]. Moreover, synthetic microspheres composed of poly(lactic-co-glycolic acid) (PLGA) have shown promise for controlled chondrogenic induction of hMSCs [12,13].

Encapsulation of both cells and growth factors is another method of investigation [14]. This study used hMSCs and an antibody specific for

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http://dx.doi.org/10.1016/j.jconrel.2014.01.023

bone morphogenetic protein encapsulated in alginate microspheres for bone regeneration. Recently, chemical modification of the surface of PLGA microspheres was shown to affect hMSC differentiation. The addition of amine groups induced hMSC osteogenesis while hydroxyl modification promoted chondrogenic differentiation [15]. The use of microspheres to direct differentiation is not limited to hMSCs [16,17]. For example, gelatin microspheres incorporating different growth factors have been shown to influence lineage commitment of embryonic stem cells [18].

A key advantage of microspheres is the potential for minimally invasive local delivery allowing for optimal construct-tissue integration [19]. Bioactive microspheres, loaded with therapeutic drugs or signalling agents, offer the capacity for controlled and sustained delivery to stimulate tissue regeneration/differentiation [20,21]. As discussed above, one application where the use of microspheres for controlled growth factor delivery may be beneficial is the treatment of articular cartilage defects. hMSCs have been described as an attractive cell source for cartilage regeneration due to their chondrogenic potential and the accessibility of source tissues in comparison to chondrocytes [22]. However, hMSC chondrogenesis *in vitro* requires the repeated addition of TGF-β, usually the 1 or 3 isoform [23]. Whether MSCs are implanted immediately after incorporation into scaffold/hydrogels or pre-differentiated as a bioengineered cartilage construct prior to *in vivo* implantation [24], the availability and/or cost of TGF-β limits the *in vivo* potential of this approach. This limitation is of particular relevance in the context of osteoarthritis where current cartilage tissue engineering applications cannot meet the requirement for repair of large defects or resurfacing of the whole joint [24]. Previous studies using microspheres to promote chondrogenic differentiation of hMSCs have generally focused on materials that are not considered native to articular cartilage [8,9,12]. Herein, we describe the development and characterisation of a chondromimetic hyaluronan (HA) microsphere, a system which we believe is more compatible with cartilage ECM. HA is a critical component of the cartilage ECM and plays a role in lubricating the joint and mechanical support. It also acts to modulate the function of various cells including MSCs and chondrocytes [25,27]. Given that HA is an essential component of matrix, we believe that these microspheres will mimic native cartilage tissue more closely than previously utilised materials. Furthermore, the approach we have taken supports the control of microsphere size [28] compared with previously reported studies [12,29], an important consideration for a clinically desirable intra-articular injectable delivery system.

The release of TGF-β3 from microspheres signifies a translatable approach for *in situ* differentiation of hMSCs. Moreover, the use of microspheres would allow for potential functionalization of their surface with antibodies/peptides for site-specific targeting to enable a more localized concentration of growth factor with reduction in potential off-target effects [30,31]. For the purpose of this study we examined the application of HA microspheres loaded with TGF-β3 to induce chondrogenic differentiation of hMSCs in 3D pellet culture [23] *in vitro* and in an *ex vivo* osteoarthritic cartilage explant system.

2. Materials and methods

2.1. Materials

All reagents were purchased from Sigma–Aldrich unless otherwise stated.

2.2. Preparation and characterisation of hollow HA microspheres

HA microspheres were prepared using a previously reported methodology with modifications [28,32] (Fig. 1A). Briefly, polystyrene beads (PS; 1 ± 0.4 μm and 10.5 ± 2.5 μm GENTAUR, Brussels) were incubated in 2 mg/ml poly(allylamine) in 0.9% sodium chloride (NaCl) for 1 h at room temperature (RT) with agitation followed by incubation with 2 mg/ml HA solution (851 kDa-1190 kDa, LifeCore, USA). Residual nonadsorbed polyelectrolyte was removed by repeated washing with 0.9% NaCl. Following layer deposition, microspheres were stabilised by chemical cross-linking. HA carboxylic groups were activated using water-soluble carbodiimide, 1-Ethyl-3-[3-dimethylaminopropyl] carbodiimide hydrochloride and *N*-hydroxysulfosuccinimide, both at concentrations of 0.2 M in 0.1 M 2-(N-morpholino)ethanesulfonic acid, 0.9% NaCl buffer (pH 4.7). Polystyrene cores were removed by treating with tetrahydrofuran. For *in vitro* tracking, microspheres were labelled with fluorescein isothiocyanate–dextran (FITC) (1.0 mg/mL). Collagen (COLL) microspheres, prepared as previously reported were included as a microsphere control in initial experiments [28].

The size and surface uniformity of HA, control PS and COLL microspheres were evaluated by scanning electron microscopy (SEM) using the Hitachi S2600N Variable Pressure SEM with an electron acceleration voltage of 10 kV. 10 μl sample was placed on aluminium stubs, allowed to air dry and gold-coated prior to analysis using a sputter coater. The size distribution of microspheres and surface charge (zeta potential) were measured using the Zetasizer Nano ZS (Malvern instruments, UK).

2.3. Isolation and characterisation of hMSCs

hMSCs were isolated from bone marrow harvested from the iliac crest of healthy donors (18–30 years) with approval from the National University of Ireland Galway and University College Hospital ethics committees and after informed consent. Characterisation of surface receptors was performed using CD105, CD73, CD90 (positive) and CD34, CD45 (negative). Tri-lineage differentiation capacity was determined using standard chondrogenic, adipogenic and osteogenic differentiation assays [33]. MSCs derived from three separate donors, were utilised for experiments and maintained in Minimum Essential Medium Alpha Medium supplemented with 10% foetal bovine serum (FBS) and 1% penicillin/streptomycin (P/S). All cells were cultured at 37 °C and 5% CO_2, unless stated otherwise.

2.4. Interaction of hollow HA microspheres with hMSCs

hMSCs were seeded at 2.5×10^4 cells/cm^2 and incubated with varying concentrations (20, 50, 100, 200 μg/ml) of HA microspheres for 48 h. Cell proliferation and metabolic activity were determined using the Quant-iT™ PicoGreen® dsDNA and alamarBlue® assays (Invitrogen) following manufacturer's instructions. As a control for donor variability, hMSCs grown on tissue culture plastic were included as were PS as an additional control. Metabolic activity was measured by absorbance (550/595 nm) while DNA, isolated by digestion of cells for four hours at 60 °C using papain (1 mg/ml in 50 mM sodium phosphate, pH 6.5, containing 2 mM *N*-acetyl cysteine and 2 mM EDTA) was detected by measuring fluorescence (485/535 nm) on a microplate plate reader (Wallac 1420 Victor 3, Perkin-Elmer Inc.).

2.5. Monocyte activation in response to HA microspheres

Human monocytic leukaemia THP-1 cells were plated at a density of 1×10^5 cells/cm^2 and cultured in RPMI 1640 supplemented with 10% FBS, 5 mM L-glutamine and 1% P/S. Cells were incubated with 200 μg/ml HA, COLL or PS microspheres for 12 h at 37 °C. Stimulation of THP-1 cells with 0.1 μg/ml lipopolysaccharide (LPS) was used as a positive control for monocyte responses. THP-1 cells stimulated with LPS were additionally incubated with HA microspheres to evaluate potential anti-inflammatory properties. Levels of tumour necrosis factor alpha (TNF-α), secreted into the medium, was measured by an enzyme-linked immunosorbent assay (ELISA) (Human TNF-alpha R&D Systems) as per the manufacturer's instructions and absorbance read at 450 nm.