Acta Biomaterialia 9 (2013) 6041–6051

Contents lists available at SciVerse ScienceDirect

Acta Biomaterialia

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

Induction of mesenchymal stem cell chondrogenesis by polyacrylate substrates

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ARTICLE INFO

Article history:
Received 12 September 2012
Received in revised form 4 December 2012
Accepted 5 December 2012
Available online 11 December 2012

Keywords:
Mesenchymal stem cell
Cell culture
Chondrocyte
Polyacrylate
Surface chemistry

ABSTRACT

Mesenchymal stem cells (MSCs) can generate chondrocytes in vitro, but typically need to be cultured as aggregates in the presence of transforming growth factor beta (TGF-β), which makes scale-up difficult. Here we investigated if polyacrylate substrates modelled on the functional group composition and distribution of the Arg-Gly-Asp (RGD) integrin-binding site could induce MSCs to undergo chondrogenesis in the absence of exogenous TGF-β. Within a few days of culture on the biomimetic polyacrylates, both mouse and human MSCs, and a mesenchymal-like mouse-kidney-derived stem cell line, began to form multi-layered aggregates and started to express the chondrocyte-specific markers, Sox9, collagen II and aggrecan. Moreover, collagen II tended to be expressed in the centre of the aggregates, similarly to developing limb buds in vivo. Surface analysis of the substrates indicated that those with the highest surface amine content were most effective at promoting MSC chondrogenesis. These results highlight the importance of surface group functionality and the distribution of those groups in the design of substrates to induce MSC chondrogenesis.

1. Introduction

Over the years, many studies have explored the potential of different stem cell types, such as MSCs [1], to generate chondrocytes in vitro, the long-term aim being to determine if stem-cell-derived chondrocytes have the potential to repair osteoarthritic lesions [2,3]. In order to develop culture conditions to direct stem cells to differentiate to chondrocytes in vitro, it is important to understand the mechanisms that regulate the differentiation of these cells in vivo. Using this understanding will lead to the possibility of designing substrates that promote differentiation down this route.

One of the best studied models of chondrogenesis is the developing limb bud [4,5], where chondrocyte differentiation is initiated by the migration and subsequent condensation of mesenchymal cells to form tightly packed aggregates [4]. TGF-β signalling is important in these initial stages, its key role being to up-regulate the expression of the extracellular matrix (ECM) molecule, fibronectin, the cell adhesion molecule, N-cadherin, and the transcription factor, Sox9 [6–8]. Fibronectin is critical for the migration of the limb bud mesenchymal cells, and together with N-cadherin and Sox9, plays an important role in the aggregation process [9–11]. Functional analysis of the role of fibronectin in chondrogenesis has shown that the integrin-binding Arg-Gly-Asp (RGD) motif of fibronectin is critical for mesenchymal cell aggregation [11,12]. Mesenchymal cells within the aggregates then start to differentiate to become proliferating chondrocytes that express the chondrocyte-specific ECM proteins, collagen II and aggrecan [5]. Sox9 is crucial at this stage, being required to induce the genes that encode these ECM proteins [6,9,13]. As the limb bud matures, most of the chondrocytes in the developing long bones become hypertrophic, finally being replaced by bone tissue, and only chondrocytes at the ends of the long bones differentiate to hyaline cartilage. The mechanisms that control the differentiation of early proliferating chondrocytes to either hypertrophic or hyaline chondrocytes are not fully understood, but hypertrophic chondrocytes stop expressing Sox9 and begin to express mineralization markers [9], whereas articular hyaline chondrocytes continue to express Sox9 and collagen II [14].

Various types of stem cells, including MSCs [1], embryonic stem cells [15], amniotic-fluid-derived stem cells [16] and dermis isolated adult stem cells (DIAS cells) [17], have chondrogenic potential in vitro. However, most in vitro studies have focused on the regulation of MSC chondrogenesis. The standard method for inducing MSCs to undergo chondrogenesis in vitro involves growing the cells in micromass or pellet culture for several weeks in the presence of TGF-βs [1,18]; typically, TGF-β1, 2 or 3 are used to induce chondrogenesis, and BMP2, 4 or 6 are sometimes added as chondrogenic enhancers [19]. Under these conditions, MSC chondrogenesis resembles that which occurs in the developing

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http://dx.doi.org/10.1016/j.actbio.2012.12.007

limb bud; for instance, proliferating chondrocytes expressing Sox9 and collagen II start to differentiate centrally, and with time, some of these undergo further differentiation to become hypertrophic chondrocytes [20]. Peripheral cells within the MSC pellets express mineralization markers, such as alkaline phosphatase and osteocalcin, resembling the periosteal cells of the limb bud [20]. Although the standard method for MSC chondrogenesis is effective, the requirement for pellet culture and expensive growth factors makes scale-up problematic. Previous studies have shown that various types of biomaterial substrates can promote chondrogenesis without the need for pellet culture [21–28]. Nevertheless, in the aforementioned studies, there was still a requirement for TGF-β supplementation. More recently, matrix stiffness has been shown to have an impact on MSC differentiation; for instance, Park et al. have shown that in contrast to stiff substrates, soft substrates can induce MSC chondrogenesis to some extent, even in the absence of TGF-β, though the authors noted that adipocytes also differentiated under these conditions [29].

The aim of this study was to investigate if novel biomimetic polyacrylate substrates, designed to mimic the functional composition and distribution of the RGD integrin-binding site, were able to induce chondrogenesis of three stem cell types, namely mouse MSCs (mMSCs), mouse-kidney-derived stem cells (KSCs) and human MSCs (hMSCs) in the absence of exogenous growth factors. These substrates are complex multi-monomeric, acrylic based polymers, each monomeric unit containing specific functional groups, the composition and distribution of which can be discretely modified in terms of the starting monomer ratio in order to tailor the surface properties. The polymers are synthesized by free radical polymerization using proprietary controlled process techniques to ensure that the functional group chemistries of the constituent monomers are evenly distributed throughout the polymeric backbone. The composition and distribution of the functional group chemistries along the polymeric backbone influence the charge, charge density, hydrophobic/hydrophilic balance and surface stereochemistry of the resultant coating [30]. These substrates attempt to model the guanidinium and carboxyl groups, and their spatial distribution, using pendant amine and carboxyl or hydroxyl side groups distributed along a polyacrylate backbone.

2. Materials and methods

2.1. Substrate preparation

The following polyacrylates were fabricated using the Biomer Technology Ltd (BTL) proprietary polymerization technique: BTL15, ESP03, ESP04, ESP07. Each batch was tested for consistency of chain length by gel permeation chromatography. Polyacrylates differed in the proportion and distribution of amine, carboxyl and hydroxyl functional groups and the degree of steric hindrance present in the polymer chain, modulated by a ratio of ethyl to butyl side groups. All polyacrylate materials were supplied by BTL. Each of the polyacrylate-coated discs (Borosilicate Glass Co. UK) were prepared from the same w/w concentration of polymer in solvent using the same dip coating programme, at a thickness of ~2 μm. The mechanical properties were not measured, but as the polyacrylates are all rigid glassy materials, their mechanical properties are expected to be similar. Substrates were sterilized prior to cell culture with ultraviolet light (265 nm).

2.2. Dynamic contact angle (DCA)

DCA was recorded using the Willhelmy method with a Cahn DCA322 microbalance and analysis software WinDCA32 (Thermo Cahn, USA). Advancing and receding contact angle was calculated for each sample in distilled water (72.6 dyne cm^{-1}).

2.3. X-ray photoelectron spectroscopy (XPS)

XPS was conducted using an NCESS ESCA300 XPS spectrometer (VG Scienta). Survey, valence, C1s, O1s and N1s spectra were analysed at take-off angles of 45° and 15° at 150 eV, 0.8 mm slit, 1.8 kW. To prevent the samples from charging due to emission of photoelectrons, the sample surface was bombarded with a low-energy electron flood gun (Scienta FG300) within the analysis chamber, with the gun settings adjusted for optimum spectral resolution. Spectra curves were analysed by curve fitting, conducted using CasaXPS (Casa software Ltd) and OriginPro 7.5 SR6 (OriginLab Corporation, MA, USA).

2.4. Surface amine assay

The relative amine content of substrates was measured using sulfo-NHS-Biotin (Thermo) to bind to surface amines, followed by streptavidin-horse radish peroxidase (Bioscience) to generate a colorimetric enzyme assay with 3,3′,5,5′-tetramethylbenzidine (TMB) (Sigma). Sulfo-NHS-Biotin is typically used to biotinylate proteins and antibodies for detection, immobilization or purification [31,32]. Substrates were incubated with a 1 mg ml^{-1} solution of sulfo-NHS-biotin in PBS for 1 h, washed once in 50 mM glycine in PBS, and after washing with PBS, were incubated in 10 mM Streptavidin-HRP (Sigma) in PBS for 30 min, followed by three washes in PBS. Substrates were then incubated with TMB solution (Sigma) for 15 min. The reaction was halted by adding 1 N HCl (Sigma) and the optical density read at 450 nm. Specificity was confirmed by blocking with NHS-acetate (Thermo), under the same conditions.

2.5. Cell culture

For routine culture, D1 mMSCs (ATCC®) and H6 mKSCs [33] were maintained in MSC medium (Dulbecco's modified Eagle medium (DMEM, Invitrogen), 10% FCS (PAA), 2 mM L-glutamine (Invitrogen)). Primary hMSCs (Lonza Walkersville Inc.) were cultured using MSCGM™ BulletKit® (Lonza), according to the manufacturer's guidelines. For culture on polyacrylate substrates, mMSCs, mKSCs and hMSCs were seeded onto 15 mm diameter glass discs (polyacrylate-coated or uncoated controls) in 24-well plates (Nunc) in 250 μl droplets containing 1×10^4 cells (mMSC and mKSC) or 5×10^3 cells (hMSC). Medium was topped up to 0.5 ml after 1 day, then replaced every 3 days. For MSC chondrogenesis in standard pellet culture, a protocol adapted from Peister et al. was used [34]. In brief, 2×10^5 mMSCs in 0.5 ml 10% (v/v) FCS were transferred to a 15 ml falcon tube and pelleted by centrifugation. After 1 day, medium was replaced with chondrogenic medium (high-glucose DMEM supplemented with 10 ng ml^{-1} TGFβ3, 500 ng ml^{-1} BMP-6 (Sigma), 0.1 μM dexamethasone (Sigma), 50 μg ml^{-1} ascorbate-2-phosphate (Sigma), 40 μg ml^{-1} proline (Invitrogen), 100 μg ml^{-1} pyruvate (Invitrogen), 50 mg ml^{-1} ITS + 3 liquid supplement (Invitrogen)). hMSCs were cultured as above, except that they were grown in hMSC chondro bulletkit (Lonza) supplemented with 10 ng ml^{-1} TGFβ3, according to the manufacturer's guidelines.

2.6. Immunofluorescence

Cells were fixed using 4% (w/v) paraformaldehyde, and immunofluorescence and microscopy were performed as previously described [35]. The primary antibodies used were as follows: anti-mouse/human type II collagen antibody (CIICI; Hybridoma bank, NIH), anti-mouse osteocalcin (OG1; Santa Cruz) and anti-human osteocalcin (Osteocalcin; Santa Cruz).