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Influence of select extracellular matrix proteins on mesenchymal stem cell osteogenic commitment in three-dimensional contexts

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ABSTRACT

Growth factors have been shown to be powerful mediators of mesenchymal stem cell (MSC) osteogenic differentiation. However, their use in tissue engineered scaffolds not only can be costly but also can induce undesired responses in surrounding tissues. Thus, the ability to specifically promote MSC osteogenic differentiation in the absence of exogenous growth factors via the manipulation of scaffold material properties would be beneficial. The current work examines the influence of select extracellular matrix (ECM) proteins on MSC osteogenesis toward the goal of developing scaffolds with intrinsically osteoinductive properties. Fibrinogen (FG), fibronectin (FN) and laminin-1 (LN) were chosen for evaluation due to their known roles in bone morphogenesis or bone fracture healing. These proteins were conjugated into poly(ethylene glycol) diacrylate (PEGDA) hydrogels and their effects on encapsulated 10T½ MSCs were evaluated. Specifically, following 1 week of culture, mid-term markers of various MSC lineages were examined in order to assess the strength and specificity of the observed osteogenic responses. PEG–LN gels demonstrated increased levels of the osteogenic transcription factor osterix relative to day 0 levels. In addition, PEG–FG and PEG–LN gels were associated with increased deposition of bone ECM protein osteocalcin relative to PEG–FN gels and day 0. Importantly, the osteogenic response associated with FG and LN appeared to be specific in that markers for chondrocytic, smooth muscle cell and adipocytic lineages were not similarly elevated relative to day 0 in these gels. To gain insight into the integrin dynamics underlying the observed differentiation results, initial integrin adhesion and temporal alterations in cell integrin profiles were evaluated. The associated results suggest that α_2, α_v and α_6 integrin subunits may play key roles in integrin-mediated osteogenesis.

1. Introduction

Mesenchymal stem cells (MSCs) are being increasingly recognized as a viable cell source for bone regeneration applications due to their ability to be expanded in vitro and to differentiate into a number of cell lineages. MSC differentiation is known to be influenced by a range of environmental stimuli, among the most potent of which are growth factors. However, the use of exogenous growth factors in tissue engineering scaffolds not only can be costly but also can induce undesired responses in surrounding tissues. Thus, MSC-based bone regeneration strategies would benefit from the identification of scaffold material properties which intrinsically promote osteoblast lineage progression in the absence of exogenous growth factors.

A number of two-dimensional (2-D) studies have demonstrated MSC osteogenic differentiation to be tightly regulated by cellular interactions with the surrounding extracellular matrix (ECM) [1–13]. However, comparatively little is known regarding the effects of various ECM components in regulating MSC osteogenesis in three-dimensional (3-D) scaffold environments [14–16]. This is significant since recent studies suggest that effects observed in two dimensions may not be indicative of the effects of the same scaffold variables in more biomimetic 3-D culture systems [17–19]. Therefore, the current work focuses on elucidating the influence of select ECM constituents on MSC osteogenic differentiation in 3-D contexts.

Towards this goal, we incorporated specific ECM molecules into hydrogel scaffolds designed to have moduli within the "osteogenic" range identified in the 3-D human and mouse MSC studies of Huebsch et al. [20]. In selecting molecules for examination, we chose to focus on several ECM proteins associated with bone morphogenesis (fibronectin [21] and laminin-1 [22,23]) and/or bone fracture

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healing (fibrinogen [24]). These proteins were then conjugated into poly(ethylene glycol) diacrylate (PEGDA) hydrogel networks. PEGDA hydrogels were selected as the base scaffold due to the broad tunability of their mechanical properties and their previous use in bone regeneration applications [25–28]. In addition, pure PEGDA hydrogels function as biological "blank slates" in that they do not significantly adsorb cell adhesive serum proteins and therefore do not intrinsically promote cell adhesion [29]. Thus, cell interactions with PEGDA gels are initially isolated to the proteins specifically tethered to the scaffold as well as the interactions supported by these proteins (e.g. growth factor binding).

In the present study, 10T½ MSCs were encapsulated within PEGDA hydrogels containing defined amounts of fibronectin (FN), fibrinogen (FG) or laminin-1 (LN). The levels of various markers of osteoblast, chondrocytic, smooth muscle cell and adipocytic fates were then monitored with time in culture toward assessing the strength and specificity of observed osteogenic responses. Due to the critical role of integrins in transducing the signals provided by glycoproteins such as FN, FG and LN [30], initial integrin adhesion profiles as well as temporal alterations in cell integrin profiles were also characterized.

2. Material and methods

2.1. Polymer synthesis and characterization

2.1.1. PEG diacrylate synthesis

PEGDA was prepared as previously described [31] by combining 0.1 mmol ml^{-1} dry PEG (10 kDa, Fluka), 0.4 mmol ml^{-1} acryloyl chloride and 0.2 mmol ml^{-1} triethylamine in anhydrous dichloromethane and stirring under argon overnight. The resulting solution was washed with 2 M K_2CO_3 and separated into aqueous and dichloromethane phases to remove HCl. The organic phase was subsequently dried with anhydrous $MgSO_4$, and PEGDA was precipitated in diethyl ether, filtered and dried under vacuum. Acrylation of the PEG end hydroxyl groups was characterized by proton nuclear magnetic resonance (^{1}H-NMR) to be ~95%.

2.1.2. Synthesis of acrylate-derivatized proteins

Proteins FN (human plasma, BD Biosciences), FG (human plasma, Sigma Aldrich) and LN (mouse, BD Biosciences) were lightly functionalized in their folded state by reaction with acryloyl-PEG-N-hydroxysuccinimide (ACRL-PEG-NHS, 3.4 kDa, Nektar) at a 1:2 M ratio at pH 8.5 [31]. The resulting acrylate-derivatized products were purified by dialysis against a 100 kDa membrane, lyophilized and stored at −20 °C until use. ACRL–PEG conjugation to the target proteins was confirmed using ^{1}H-NMR. A representative ^{1}H-NMR spectrum for acrylate-derivatized FG is shown in Supplementary Fig. 1.

To confirm the ability of the modified proteins to be incorporated within PEGDA hydrogel networks, hydrogel precursor solutions were prepared with 0.5 mg ml^{-1} protein and 100 mg ml^{-1} PEGDA. Following the addition of 10 μl ml^{-1} of a 300 mg ml^{-1} solution of UV photoinitiator 2,2-dimethoxy-2-phenyl-acetophenone (DMAP) in N-vinylpyrrolidone (NVP), gels were polymerized by 4 min exposure to long-wave UV light (Spectroline, ~6 mW cm^{-2}, 365 nm). The gels were then immersed in PBS overnight, after which they were transferred to a 0.12 M NaOH solution to hydrolyze the PEGDA crosslinks and release encapsulated protein. The levels of protein released were compared to the levels in the precursor solution using the CBQCA assay (Invitrogen), and the average level of protein incorporation was found to be consistent across protein types at 86.7 ± 7.2%. In addition, to assess the ability of cells to interact with proteins incorporated into the hydrogel network, 10T½ cells were seeded onto the surface of each gel formulation. Cell adhesion and spreading were confirmed for each PEG–ECM gel type (Supplementary Fig. 2).

2.2. Cell culture, initial characterization and encapsulation

Cryopreserved 10T½ mouse MSCs (American Type Culture Collection; ATCC) at passage 2 were thawed and expanded in monolayer culture per ATCC protocols. Prior to encapsulation, cells were maintained at 37 °C and 5% CO_2 in Dulbecco's modified Eagle's medium (DMEM, Hyclone) supplemented with 10% heat-inactivated fetal bovine serum (FBS, Hyclone). Cells at passage 4–6 were termed "day 0" and were harvested and allocated for either protein extraction, integrin blocking studies or hydrogel encapsulation.

2.2.1. Protein extraction

Proteins were extracted from day 0 10T½ cells by the addition of Trizol (Invitrogen) per manufacture's protocols. The resulting solutions were centrifuged, and each supernatant was mixed with chloroform (Sigma), vigorously shaken for 15 s and centrifuged. The lower protein-rich phenol–chloroform phase of each sample ($n = 4$) was mixed with ethanol to precipitate residual DNA. The resulting phenol–ethanol phase was transferred to a 3.4 kDa SnakeSkin dialysis membrane (Pierce). The solution was dialyzed for ~60 h at 4 °C against an aqueous solution of 0.1% sodium dodecyl sulfate (SDS), with buffer exchange every 18 h. By the end of the third 18 h dialysis period, the samples had partitioned into three phases: (1) a supernatant, (2) a globular mass and (3) a colorless, viscous liquid. The globular mass, containing the bulk of sample proteins [32], was resuspended in PBS containing 0.5% SDS and 1% Triton X-100. The isolated sample proteins were subsequently used in quantitative ELISA assays.

2.2.2. Integrin blocking studies

Standard adhesion blocking studies were performed to determine the integrin alpha subunits through which the 10T½ cells initially interacted with the PEG–ECM gels. In brief, functionalized FG, FN and LN were resuspended in PBS at 100 μg ml^{-1}, after which they were applied to a 96-well, high protein binding plate at 100 μl per well for 12 h at 4 °C. The wells were then blocked with 3% bovine serum albumin (BSA). Harvested 10T½ cells were washed with PBS and resuspended in serum-free DMEM supplemented with 1 mM Ca^{2+} and 1 mM Mg^{2+}. Cells were then exposed to 50 μg ml^{-1} of α_1, α_2, α_5, α_v or α_6 integrin antibodies or to 50 μg ml^{-1} of appropriate negative control antibodies for 30 min at room temperature. Further details regarding antibodies are given in Supplementary Table 1. The cell suspensions were subsequently applied to the coated wells at 10,000 cells cm^{-2}. Following 30 min incubation at 37 °C and 5% CO_2, wells were rinsed three times with PBS. Adherent cells were then lysed, and the number of adherent cells in each well was measured using a lactate dehydrogenase assay kit (Roche). Percentage inhibition was evaluated relative to the corresponding negative control. At least five sample wells per antibody were analyzed for each protein type.

2.2.3. Cell encapsulation and culture

Hydrogels were fabricated by preparing: (1) a 20 wt.% PEGDA solution in HEPES-buffered saline (HBS) and (2) separate solutions of 1 mg ml^{-1} acrylate-derivatized FN, FG or LN in HBS. A 300 mg ml^{-1} solution of DMAP in NVP was added at 2% (v/v) to the PEGDA mixture. The PEGDA and protein solutions were then separately sterilized by filtration, after which each protein solution was mixed with an equal volume of the 20 wt.% PEGDA solution. Harvested 10T½ cells were resuspended in the resulting precursor solutions at 1×10^6 cells ml^{-1}. The cell suspensions were then

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