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Vascular differentiation of bone marrow stem cells is directed by a tunable three-dimensional matrix

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ABSTRACT

Microenvironmental cues are critical in regulating cell behavior and fate. The roles that matrix mechanical signals play in regulating cell behavior have recently been elucidated. An artificial matrix that can maintain the appropriate characteristics for transplanted stem cells is therefore needed to achieve a desired cell phenotype. The objective of this study was to develop a three-dimensional (3-D) matrix with tunable physical and mechanical properties and investigate their effects on mesenchymal stem cell (MSC) differentiation towards vascular cell types. In this study we developed an extracellular microenvironment by modifying fibrinogen with various polyethylene glycol (PEG) derivatives. We hypothesized that adjusting the type of PEG derivative to modify the resultant physical and mechanical characteristics of fibrin would allow us to create a tunable system for use in culture or in vivo in conjunction with a regenerative medicine strategy. Human MSC (hMSC) were entrapped into PEGylated fibrin matrices at a density of 50,000 cells ml^{-1}. Cell phenotypes were confirmed by immunofluorescent staining as well as the use of oligonucleotide arrays. Vascular phenotypes were correlated with measured mechanical properties and fiber diameters of the PEGylated fibrin matrices. Blocking studies were performed to identify mechanistic factors controlling MSC differentiation through selected blocking of matrix degradation or cell contraction. Cell–matrix interactions were also examined in vivo. Our results demonstrate that transdifferentiation of MSC towards an endothelial cell phenotype is profoundly affected by the 3-D matrix microenvironment. Our work provides a predictive road map for the creation of fibrin-based matrices that support robust endothelial cell gene expression and tubulogenesis.

1. Introduction

Stem cells have great potential for treating vascular injury and disease through tissue engineering and regenerative medicine [1–3]. Successful application of stem cell therapy requires control over the specific kinds of cells into which stem cells differentiate. Only recently has the importance of the microenvironment on stem cell fate been demonstrated. Microenvironmental cues critical for regulating cell behavior and fate include both biochemical signals, such as soluble factors, and physical signals, such as shear stress and the elastic properties of the environment. The roles that mechanical signals play in regulating cell behavior have been reported by Engler et al. [4]. Their work suggested that an artificial matrix that can maintain the appropriate compliance for transplanted stem cells is needed to achieve a desired cell type.

Mesenchymal stem cells (MSC) have received attention because of their ability to differentiate towards connective tissue cell types [5]. There is mounting evidence that MSC have an unexplored plasticity to differentiate towards cardiovascular cell types, including endothelial cells [5–8]. MSC have been shown to form tubes in vitro on artificial extracellular matrix (ECM) (Matrigel) under growth factor stimulation or hypoxia [9–11]. MSC have also been shown to stimulate vascular in-growth into Matrigel plugs in vivo in the absence of exogeneous stimulation [9]. To date in vitro studies have not demonstrated spontaneous tube formation on fibrin-only gels by a defined population of MSC [12]. There is a body of literature, however, demonstrating that microvascular endothelial cells are able to form tubes within three-dimensional (3-D) fibrin gels and that this process is enhanced by various stimuli [13,14].

Stem cells, such as MSC and endothelial progenitor cells (EPCs) from bone marrow, have been evaluated in vivo for the treatment of ischemia following in vitro expansion [15]. Human clinical trials have evaluated autologous MSC infused following myocardial infarction and reperfusion therapy [16]. Work by Skalak's group demonstrated that while transplanted stem cells from bone marrow did participate in newly sprouting vessels, they did not demonstrate significant transdifferentiation towards endothelial or smooth muscle phenotypes [17]. It remains unclear whether

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vascular progenitor cell types provide a purely paracrine role or differentiate and are incorporated into the newly formed blood vessels [9,18,19]. The issue of matrix contribution to vascularization remains largely unexplored [20].

It has become increasingly clear that stem cell specification is regulated by local cues present in the microenvironment, however, methods developed so far can only examine cell–matrix interactions in a two-dimensional manner. Systematic evaluation of stem cell phenotype in a three-dimensional setting remains a significant challenge due to the increased complexity. Our prior work has demonstrated spontaneous tube formation by MSC within PEGylated fibrin matrices [21–24]. In the current study we further developed an ECM microenvironment by modifying fibrinogen with various PEG derivatives which resulted in differences in measured physical and mechanical properties. We hypothesized that adjusting the characteristics of the 3-D matrix would allow the system to be tuned in order to direct stem cell differentiation (Fig. 1). The current work characterizes the cell population following PEGylated fibrin culture and explores the dependency of vascular differentiation on matrix characteristics.

2. Materials and methods

2.1. PEGylation of fibrinogen and fibrin cross-linking

The following PEG derivatives were used to PEGylate fibrinogen: difunctional *N*-hydroxysuccinimide (NHS)–PEG (500 Da, Pierce Biotechnology, Rockford, IL), difunctional benzoyltriazole carbonate (BTC)–PEG (3400 Da, Nektar, San Carlos, CA), difunctional succinimidyl carbonate (SC)–PEG (3400 Da, Sunbio, Anyang City, South Korea) and difunctional succinimidyl methyl butanoate (SMB)–PEG (3400 Da, Nektar). Each of the PEG derivatives was added to an equal volume of porcine fibrinogen (No. F2629, Sigma–Aldrich, St. Louis, MO) at 40 mg ml^{-1} in Tris-buffered saline (TBS), pH 7.8 (Sigma), at a molar ratio of 10:1. The reactions were carried out at 37 °C in TBS at pH 7.6 for 12 h. Each reaction product then underwent gelation by adding an equal volume of a solution of human thrombin (25 U ml^{-1} in 40 mM $CaCl_2$) (No. T4393, Sigma). The unbound free PEG derivatives were rinsed with TBS after gelation. The final concentration of fibrinogen in all the resulting gels was 10 mg ml^{-1} and that of thrombin was 12.5 U ml^{-1}.

2.2. Rheology

Rheology was performed using a rheometer (Physica MCR300, Paar Physica Inc.) with a plate–plate geometry to study small strain oscillatory shear behavior. The cell-free gel samples were formed in a round mold (500 μl in volume and 25 mm in diameter) and transferred to the bottom plate of the rheometer. The upper plate was then lowered to a measuring gap size of 0.1 mm. An amplitude sweep was performed in order to confirm that the parameters (frequency and strain) were within the linear viscoelastic regime of the solutions. After a short pre-shear period dynamic oscillating measurement was initiated at a constant frequency of 0.5 Hz. The values of the final equilibrated storage modulus (G′) were recorded.

2.3. Swelling

Fibrin and PEGylated fibrin samples (100 μl in volume, 8 mm in diameter) were individually immersed in TBS solution, pH 7.6, and maintained at room temperature. The diameters of individual cylinders were measured using a stage micrometer under a dissecting microscope (Leica S6D) and recorded at regular intervals up to 24 h. The swelling ratio is reported as $Q = D/D_0$, where D is the final recorded diameter at equilibrium swelling and D_0 is the initial diameter immediately following cross-linking.

2.4. Scanning electron microscopy (SEM)

Fibrin and PEGylated fibrin samples were removed from wells, washed in deionized (DI) water and dehydrated in serial dilutions of ethanol. Following dehydration, gels were dried on a critical point dryer, sputtered with gold for 30 s using a sputter coater and then visualized using a scanning electron microscope (S-4700 field emission microscope, Hitachi). The fiber diameter was taken by averaging the diameters of fibers at 18 points throughout each of three images.

2.5. Human MSC culture and seeding

Human mesenchymal stem cells (hMSC) (Cambrex, East Rutherford, NJ) were cultured in serum-containing MSCBM medium (Cambrex) supplemented with MSCGM SingleQuots (Cambrex)

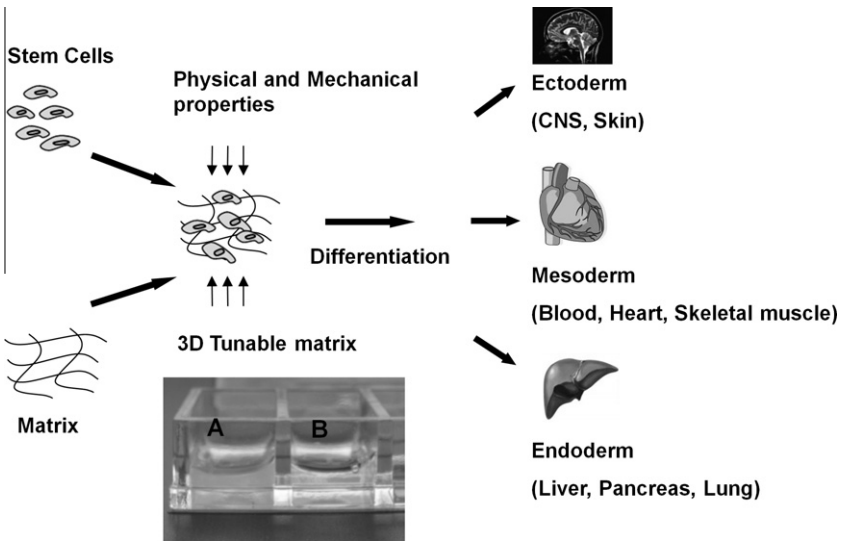

Fig. 1. Schematic of a controlled 3-D matrix culture system for directing stem cell differentiation. The 3-D cell culture matrix in a 4-well chamber slide: (A) fibrin; (B) PEGylated fibrin.

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