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The combined influence of substrate elasticity and surface-grafted molecules on the *ex vivo* expansion of hematopoietic stem and progenitor cells

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ABSTRACT

Umbilical cord blood (UCB) is an attractive source of hematopoietic stem and progenitor cells (HSPCs) for transplantation. However, the low number of HSPCs from a single UCB donor limits the direct transplantation of UCB to patients. Because little is known about the effects of the physical microenvironment on HSPC expansion, we investigated the *ex vivo* expansion of HSPCs cultured on biomaterials with different elasticities and grafted with different nanosegments. Polyvinylalcohol-co-itaconic acid (PVA–IA)-coated dishes with different stiffnesses ranging from a 3.7 kPa to 30.4 kPa storage modulus were used. Fibronectin or an oligopeptide (CS1, EILDVPST) was grafted onto the PVA–IA substrates. High *ex vivo* fold expansion of HSPCs was observed in the PVA–IA dishes grafted with fibronectin or CS1, which displayed an intermediate stiffness ranging from 12.2 kPa to 30.4 kPa. The fold expansion was more than 1.4 times higher than that cultured in tissue culture polystyrene dishes (TCPS, 12 GPa). Furthermore, HSPCs cultured in fibronectin or CS1-grafted PVA–IA-coated dishes with a stiffness of 12.2–30.4 kPa generated more pluripotent colony-forming units (CFU-GM and CFU-GEMM) than those in TCPS dishes. This result indicates that both the physical and biological properties of biomaterials affect the *ex vivo* expansion of HSPCs.

1. Introduction

Increasing evidence suggests that the physical microenvironments of stem cells, in addition to soluble biological factors, help to direct stem cell fate during proliferation and differentiation [1]. Although biological cues, such as growth factors, small biologically active molecules, and the extracellular matrix (ECM), have been demonstrated to determine stem cell fates during differentiation and pluripotency [2–4], investigators have also begun to evaluate the potential importance of physical cues in this process, such as the stiffness of cell culture substrates and mechanical forces [5–9]. Human mesenchymal stem cells (hMSCs) tend to efficiently differentiate into specific tissue lineages when they are cultured on biomaterials with an elasticity similar to the tissue of interest. Engler et al. reported hMSC differentiation fates following culture on collagen-coated polyacrylamide hydrogels of different

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stiffnesses [5]. Softer materials with a stiffness similar to the brain, at approximately 0.3 kPa, tended to cause cells to express neuronal morphologies and neural markers (P-NFH, β-III tubulin), whereas stiffer materials, of approximately 10 kPa, led to similar results to those seen in muscle-guided hMSCs and tended to cause cells to express myogenic markers (MyoD) in Engler's study [5]. Furthermore, rigid materials with a stiffness of approximately 35 kPa, similar to collagenous bone, induced the expression of the osteogenic marker Runx2.

Although some researchers have reported that the stiffness of the cell culture substrate (matrix) is an important factor in the differentiation of hMSCs in 2-D culture [1,5], which demonstrates that the substrate matrix guides hMSC differentiation fates, it should be noted that several researchers have reported conflicting results and have offered different, but intriguing theories about the effect of substrate elasticity on hMSC differentiation [10,11]. It has been suggested that Engler's landmark study [5], in which it was demonstrated that the substrate matrix guides hMSC differentiation fate, can be verified under limited conditions in which stem cells are cultured on hydrogels with immobilized collagen type I in a 2-D system. In this microenvironment, the ECM (e.g., collagen type I) can penetrate the hydrogel surface to some extent, whereas on solid substrates, the ECM cannot penetrate the substrate surface. Hydrogels of varying stiffness lead to differences in ECM anchoring densities, thereby altering the mechanical feedback from the ECM to the stem cells. It appears that this mechanical feedback leads stem cells toward specific differentiation lineages or causes them to remain undifferentiated.

Embryonic stem cell (ESC) pluripotency and differentiation fates have also been reported to be dictated by the microenvironment of the ESC culture [12]. Chowdhury et al. reported that mouse ESCs (mESCs) could maintain pluripotency when cultured in the absence of exogenous leukemia inhibitory factor (LIF) on soft substrates (0.6 kPa) that matched the intrinsic stiffness of mESCs, whereas mESCs did not maintain pluripotency in conventional stiff culture polystyrene dishes (12 GPa) coated with collagen type I or on hydrogels with much stiffer moduli [13].

There are many notable investigations addressing the effects of substrate stiffness (elasticity) on the pluripotency and differentiation fates of hMSCs and ESCs [1,5]. However, little is known about the effect of substrate stiffness on the pluripotency fate and proliferation of hematopoietic stem and progenitor cells (HSPCs) [9]. This lack of evidence motivated us to investigate the effect of *ex vivo* expansion of HSPCs on the elasticity of hydrogels grafted with biologically active nanosegments. Several types of biological cues (e.g., biological nanosegments such as fibronectin (FN) and collagen) grafted on cell culture dishes have been reported to promote the *ex vivo* expansion of HSPCs [14–20]. Feng et al. investigated the *ex vivo* expansion of HSPCs on FN-immobilized and collagen-immobilized polyethylene terephthalate (PET) meshes [21]. Culture of HSPCs on FN-immobilized PET meshes yielded the greatest expansion of the HSPCs and long-term culture-initiating cells. FN appears to be preferable to other types of ECM for the *ex vivo* expansion of HSPCs [21]. One of the most important adhesion domains in FN is the connecting segment-1 (CS1, EILDVPST) motif, which is recognized by surface receptors on early hematopoietic progenitors. Jiang et al. investigated the *ex vivo* expansion of HSPCs from umbilical cord blood (UCB) on PET films with immobilized CS1 [22]. They reported that cells cultured on the CS1-immobilized film displayed positive engraftment after 10 days of *ex vivo* expansion from only 600 $CD34^+$ cells [22]. In a previous study by our group [23], the effect of nanosegment species (e.g., FN, CS1, RGDS, RGES, and nanosegments with amino groups) grafted on polystyrene culture dishes on the *ex vivo* expansion of HSPCs was systematically investigated. HSPCs cultured in dishes grafted with CS1 showed greater expansion and more pluripotent colony-forming units (CFUs) (i.e., CFU-granulocytes, erythroids, macrophages, and megakaryocytes [CFU-GEMM]) than those in FN-grafted RGDS-grafted, and polyamine-grafted dishes [23]. However, these previous studies did not investigate the effect of the elasticity of the cell culture substrates on the *ex vivo* expansion of HSPCs on FN- and CS1-grafted dishes.

Holst et al. cultured mouse and human HSPCs on tropoelastin substrates, an elastic biomaterial. They reported that culture of HSPCs on tropoelastin led to two to threefold expansion of HSPCs [9]. They suggested that substrate elasticity and tensegrity were important mechanisms influencing HSPC culture. However, the substrate these authors used for HSPC culture, tropoelastin, is a naturally derived protein that is not chemically defined. Furthermore, they did not evaluate the stiffness (Young's modulus or storage modulus) of tropoelastin [9]. Therefore, it is difficult to evaluate the pure physical effect of the tropoelastin biomaterial on the *ex vivo* expansion of HSPCs in their work.

In this study, we developed synthetic hydrogels consisting of poly(vinyl alcohol-co-itaconic acid) (PVA–IA) grafted with FN and CS1 to evaluate the physical effect of substrate stiffness on the pluripotency and proliferation fates of hematopoietic stem and progenitor cells. These hydrogels were prepared with different elasticities by controlling the crosslinking intensity (time) with glutaraldehyde. The elasticity of the PVA–IA hydrogels can be varied using the same polymeric main chain, but different crosslinking intensities. FN and CS1 can be easily grafted with the carboxylic acid group of PVA–IA via N-(3-dimethylaminopropyl)-N′-ethylcarbodiimide hydrochloride (EDC) and N-hydroxysuccinimide (NHS) chemistry in an aqueous solution. PVA–IA hydrogels grafted with or without FN and CS1 are transparent, thus allowing the morphology of HSPCs cultured on the PVA–IA hydrogels to be evaluated using microscopy techniques similar to those employed when using conventional tissue culture polystyrene (TCPS) dishes. The goal of this study was to investigate the optimal elasticity of PVA–IA hydrogels grafted with FN and CS1 for the *ex vivo* expansion of HSPCs.

2. Materials and methods

2.1. Materials

Fibronectin (FN, human, F2006) and poly(vinyl alcohol-co-vinyl acetate-co-itaconic acid) (PVA–IA, 480223) were purchased from Sigma–Aldrich (St. Louis, MO, USA). The oligopeptide CS1 (EILDVPST) was obtained from MDBio, Inc. (Piscataway, NJ, USA). TCPS dishes (diameter = 35 mm, 35-3001) were purchased from Becton Dickinson (Franklin Lakes, NJ, USA). N-(3-Dimethylaminopropyl)-N′-ethylcarbodiimide hydrochloride (EDC, 03450), N-hydroxysuccinimide (NHS, 13062), and glutaraldehyde (25% in water, G5882) were obtained from Sigma–Aldrich (St. Louis, MO, USA). Serum-free medium (StemSpan™ SFEM), the StemSpan™ CC110 cytokine cocktail, and MethoCult™ GF H4434 were purchased from STEMCELL Technologies Inc. (British Columbia, Canada). An anti-CD34 antibody conjugated to PE (A07776), an anti-CD45 antibody conjugated to FITC (A07782), and an anti-CD38 antibody conjugated to FITC (A07778) were obtained from Beckman Coulter, Inc. (Brea, CA, USA). Optilyse C (IM1401), 7-AAD (A07704), and flow-count bead solutions (7547053) were purchased from Beckman Coulter, Inc. (Miami FL, USA). MiniMACS separator (130-090-312), FcR blocking reagent (human, 130-059-901), and CD34 MicroBeads (130-046-702) were obtained from Miltenyi Biotec, Inc. (Bergisch-Gladbach, Germany). The other chemicals employed were of reagent grade and were used without further purification, which were purchased from Sigma–Aldrich (St. Louis, MO, USA). Ultrapure water produced by a Milli-Q system (Millipore Corporation, Billerica, MA, USA) was used throughout the experiments.

2.2. Preparation of crosslinked PVA–IA hydrogel dishes

PVA–IA with 1.3 mol% itaconic acid showing a degree of hydrolysis of 97.7% and a degree of polymerization of 1750 was dissolved to 0.05 wt% for cell culture experiments or 0.5 wt% for rheometer measurements in ultrapure water, then agitated for two days and subsequently kept at room temperature for one day to ensure that no air bubbles were present in the solution [24,25]. A 3 mL aliquot of the PVA–IA solution was then added a 35 mm TCPS dish and dried for a week to produce a film