Published in final edited form as:

Acta Biomater. 2013 December ; 9(12): 9258–9269. doi:10.1016/j.actbio.2013.08.012.

Covalent immobilization of SCF and SDF1 α for *in vitro* culture of hematopoietic progenitor cells

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Abstract

Hematopoietic stem cells (HSCs) are currently utilized in the treatment of blood diseases, but widespread application of HSC therapeutics has been hindered by the limited availability of HSCs. With a better understanding of the HSC microenvironment and the ability to precisely recapitulate its components, we may be able to gain control of HSC behavior. In this work we developed a novel, biomimetic PEG hydrogel material as a substrate for this purpose and tested its potential with an anchorage independent hematopoietic cell line, 32D clone 3 cells. We immobilized a fibronectin-derived adhesive peptide sequence, RGDS; a cytokine critical in HSC self-renewal, stem cell factor (SCF); and a chemokine important in HSC homing and lodging, stromal derived factor 1 α (SDF1 α), onto the surfaces of poly(ethylene glycol) (PEG) hydrogels. To evaluate the system's capabilities, we observed the effects of the biomolecules on 32D cell adhesion and morphology. We demonstrated that the incorporation of RGDS onto the surfaces promotes 32D cell adhesion in a dose dependent fashion. We also observed an additive response in adhesion on surfaces with RGDS in combination with either SCF or SDF1 α . In addition, the average cell area increased and circularity decreased on gel surfaces containing immobilized SCF or SDF1 α , indicating enhanced cell spreading. By recapitulating aspects of the HSC microenvironment using a PEG hydrogel scaffold, we have shown the ability to control the adhesion and spreading of the 32D cells and demonstrated the potential of the system for the culture of primary hematopoietic cell populations.

Keywords

hydrogel; poly(ethylene glycol); hematopoietic cell; stem cell factor; stromal derived factor 1 α

1. Introduction

Hematopoietic stem cells (HSCs) have been successfully transplanted into humans for fifty years to treat blood diseases such as leukemia [1–3]. The creation of a robust system to culture HSCs *ex vivo* would aid in the optimization of current treatment regimens and facilitate the development of new HSC therapeutics. *In vivo*, HSCs reside in a

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microenvironment, or niche, within the marrow of long bones. Complex interactions between HSCs, stromal cells, the extracellular matrix, and signaling molecules help to maintain an intricate balance between HSC quiescence, self-renewal, and differentiation. One of the key regulators in the niche is adhesion to ECM proteins. HSCs adhere to fibronectin (FN) and other extracellular matrix proteins through integrins such as VLA-4 (very late antigen-4, $\alpha 4\beta 1$), VLA-5 (very late antigen-5, $\alpha 5\beta 1$), $\alpha V\beta 3$, LFA1, [4–6]. Several integrins can bind to the essential amino acid sequence, RGD, of FN. Because of its specificity, this short oligopeptide can be utilized to mimic some of the adhesive properties of intact FN proteins. These interactions are not only important in cell adhesion and spreading but are also implicated in many signaling pathways within the cells, such as those that regulate hematopoiesis and proliferation [7–9].

Other molecules critical to maintaining HSCs in the niche include stem cell factor (SCF) and stromal derived factor 1 α (SDF1 α). SCF binds to and activates the c-kit receptor expressed on the surfaces of HSCs and exists in both soluble and membrane-bound forms [10–12]. Membrane- or surface-bound SCF has been shown to prolong c-kit activation [13–16], an interaction that is critical in HSC maintenance and self-renewal [17]. SDF1 α binds to C-X-C chemokine receptor type 4 (CXCR4) on HSC membranes. SDF1 α , similarly to SCF, can be found in both membrane-bound and soluble forms in the niche [18]. It plays an integral role in the homing of HSCs to the bone marrow as well as mobilization of the cells to injury sites [19–21]. Signaling between surface bound SDF1 α and CXCR4 was shown to maintain a pool of HSCs in the niche, leading to the proposal that this protein supports self-renewal of stem cell populations [22].

Incorporating niche molecules such as these into biomaterials, we can begin to culture HSCs *in vitro*. Several groups have begun to explore different bioactive scaffolds for the culture of HSCs. RGD, LDV (a peptide sequence that can bind the $\alpha 4\beta 1$ integrin), and FN covalently conjugated to PET films showed successful *ex vivo* expansion of human CD34⁺ cells [8,23]. Others have focused on the effects of the mechanical properties on hematopoietic cell behavior cultured on substrates like FN-functionalized poly(ethylene glycol) diacrylate (PEG-DA) hydrogels, collagen, or collagen-functionalized poly(acrylamide) [24,25].

Another approach is the fabrication of biomaterial wells for HSC culture. This is advantageous because it allows containment of anchorage independent HSCs and facilitates interactions between HSC surface receptors and molecules presented on the well surface. Kurth *et al.* (2009) and Kurth *et al.* (2011) have immobilized ECM molecules onto poly(dimethylsiloxane) (PDMS) microcavities to study the relationship between these molecules and HSC fate [26,27]. Kobel *et al.* and Lutolf *et al.* have demonstrated the ability to generate poly(ethylene glycol) hydrogel well surfaces to study single HSC proliferation kinetics [28,29]. Lutolf *et al.* used microcontact printing to functionalize the well surfaces with a variety of proteins to investigate the effects of specific molecules on HSC division and engraftment.

One disadvantage of the system described by Lutolf *et al.* is the manner in which the wells are functionalized. The PEG prepolymer solution is molded against PDMS pillars inked with PEG-modified Protein A to functionalize the entire well surface. A chimeric protein is then added to the wells, binding to Protein A via its Fc fragment [28]. While the need to PEGylate proteins does potentially impact bioactivity, a photopolymerization strategy would enable direct patterning of PEGylated biomolecules on the well surfaces [30–34]. Previous work has shown spatial presentation of specific adhesive ligands or niche proteins to HSCs to be critical [35]. The need to use chimeric proteins in Lutolf's strategy also limits the molecules that can be incorporated onto the well surfaces. Finally, Kobel *et al.* and Lutolf *et al.* used the wells as a tool to gain a better understanding of the kinetics of HSC proliferation

and the effects of cell division on engraftment potential as opposed to generating therapeutic populations of HSCs.

We have built on the work of Kobel *et al.* and Lutolf *et al.* by using photopolymerizable PEG-DA hydrogel wells as a substrate for the development of an *ex vivo* HSC niche. Unmodified PEG-DA hydrogels are biologically inert though the polymer matrix can easily be modified with bioactive elements such as adhesive peptide sequences, degradable elements, and whole proteins [36–46]. The ability to selectively incorporate these biomolecules in the matrix allows for significant control over the cell microenvironment in both two and three-dimensions. To recapitulate aspects of the HSC niche in the current work, RGDS, SCF, and SDF-1 α were covalently immobilized onto the surfaces of PEG-DA hydrogels that were fabricated into culture wells. To evaluate the efficacy of the newly designed materials, we observed the adhesion and morphology of 32D cells, an interleukin-3 dependent myeloid hematopoietic progenitor cell line that expresses integrins binding RGD [47,48] as well as both c-kit and CXCR4 (Figure S1). Through the incorporation of RGDS, SCF, and SDF1 α onto the substrate surface we were able to influence 32D cell adhesion and total cell area on the hydrogel surfaces and believe that further optimization of the system will result in the ability to support HSC adhesion and growth during *in vitro* culture.

2. Materials and Methods

All chemicals were obtained from Sigma-Aldrich (St. Louis, Missouri) unless noted.

2.1 Synthesis of PEG-DA

PEG-DA was synthesized as described previously [34,43,45,46,49,50]. Briefly, 6 kDa poly(ethylene glycol) (PEG) was reacted with acryloyl chloride at a molar ratio of 4:1 (PEG:acryloyl chloride) and triethylamine (TEA) at a molar ratio of 2:1 (PEG:TEA) in anhydrous dichloromethane (DCM). The resulting product was rinsed with K₂CO₃ and allowed to phase separate overnight. The organic layer containing PEG-DA was collected and dried with MgSO₄, and then rotary evaporated and precipitated in diethyl ether. The precipitate was filtered and lyophilized. A sample of the resultant polymer was dissolved in chloroform, and acrylation was confirmed with proton nuclear magnetic resonance analysis.

2.2 Synthesis of PEG-RGDS

3400 MW acrylate PEG-succinimidyl carboxymethyl (PEG-SCM, Laysan, Arab, AL) was reacted with RGDS peptide (American Peptide, Sunnyvale, CA) at a molar ratio of 1:1.1 (PEG-SCM:RGDS) in dimethylsulfoxide with *N,N*-Diisopropylethylamine overnight at 4°C as previously described [34,44]. The resulting product was purified by dialysis and lyophilized. The conjugation was verified using gel permeation chromatography.

2.3 Synthesis of PEG-SCF and PEG-SDF1 α

An alternative PEG derivative compatible with aqueous conjugation was used to PEGylate SCF and SDF1 α . 3400 MW acrylate PEG-succinimidyl valerate (PEG-SVA, Laysan) was reacted with carrier-free murine SCF (R&D, Minneapolis, MN) at a molar ratio of 42:1 (PEG-SVA:SCF) and with carrier-free murine SDF1 α (R&D) at a molar ratio of 18:1 (PEG-SVA:SDF1 α). The reactions were performed in phosphate buffered saline (PBS) overnight at 4°C, pH 8.0. To confirm conjugation, a Western blot was performed on the PEGylated and unmodified proteins using a 15% Tris-HCl precast polyacrylamide gel (BioRad, Hercules, CA) as previously described [43–46]. Primary antibodies included a rabbit polyclonal SCF and a rabbit polyclonal SDF1 α (Abcam, Cambridge, MA). The secondary antibody used was a goat polyclonal rabbit IgG conjugated to horseradish peroxidase.

(Abcam). For detection, the ECL chemiluminescent Western blotting analysis system (GE Healthcare, Chalfont St. Giles, UK) was employed.

2.4 Cell culture

Murine 32D clone 3 cells (32D cells, ATCC, Manassas, VA), an IL-3 dependent myeloid progenitor line, were cultured in RPMI-1640 media (Invitrogen, Carlsbad, CA) supplemented with 2 mM L-glutamine, 10% IL-3 culture supplement (Becton Dickinson, Franklin Lakes, NJ), 10% heat-inactivated fetal bovine serum (FBS), 100 U/L penicillin, and 100 mg/L streptomycin. Cells were maintained at 37 °C at 5% CO₂.

2.5 Bioactivity of PEGylated proteins

To evaluate PEG-SCF bioactivity, 32D cells were seeded into tissue culture polystyrene plates at a density of 5000 cells/cm² in three formulations of media: control media (RPMI-1640 with 2 mM L-glutamine, 10% IL-3 culture supplement, 10% heat-inactivated FBS, 100 U/L penicillin, and 100 mg/L streptomycin), control media with SCF (200 ng/ml) and control media with PEG-SCF (200 ng SCF/ml). After 24 h, the wells were imaged, and cell number was calculated using ImageJ software (NIH, Bethesda, MD).

To evaluate the bioactivity of PEGylated SDF1 α , a migration assay utilizing transwells with 3 μ m pores was performed. One of three media formulations was added to the well plate below the transwell: control media (RPMI 1640 containing 10% heat-inactivated FBS, 10% IL-3 culture supplement, and 1% penicillin/streptomycin), control media with SDF1 α (100 ng/ml), or control media with PEG-SDF1 α (100 ng SDF1 α /ml). 32D cells were seeded into the top of the transwell chamber in control media at 6000 cells/cm², and the number of cells that migrated to the bottom well was determined after 72 hr.

2.6 Fabrication of PEG-DA culture wells

To create PEG-DA hydrogel wells for cell culture, we first used microfabrication techniques to fabricate photoresist pillars as previously described [51]. The resulting photoresist masters had 500 μ m high pillars with pillar diameters of 5.34 mm. To form hydrogel wells, PEG-DA was dissolved in HEPES-buffered saline (HBS, 10% w/v) at pH 7.4, and 10 μ l/ml of photoinitiator (2,2-dimethoxy-2-phenyl acetophenone: 300 mg/ml in *n*-vinylpyrrolidone, NVP) were added to the polymer solution. The PEG solution was then sterilized via filtration (0.2 μ m). The hydrogel mold consisted of two SigmaCote treated glass slides, one modified with the SU-8 pillars, that were separated with a 1 mm PTFE spacer and clamped together. The polymer solution was injected into the mold and crosslinked using long wavelength UV light (365 nm, 10 mW/cm²) for 45 s/3.75 cm² creating a PEG-DA base hydrogel with wells. A schematic of the fabrication is shown in Figure 1A. The PEG-DA hydrogel wells were soaked in sterile PBS with 0.1% NaN₃ overnight to allow swelling and maintain sterility.

2.7 Surface immobilization of RGDS, SCF, and SDF1 α

A PEG-RGDS solution (or PEG-RGDS/PEG-SCF or PEG-RGDS/PEG-SDF1 α mixture) containing 10 μ l/ml of photoinitiator (described above) was added to each well of a hydrogel containing 4 wells. The hydrogel wells were then exposed to UV light for 3 min. to covalently attach the RGDS (and SCF or SDF1 α if included). The gels were then rinsed and soaked in sterile PBS containing 0.1% NaN₃. A schematic of the immobilization process is displayed in Figure 1B. To remove any NaN₃ before cell seeding, gels were rinsed twice in PBS for 1 h at 37°C and once in culture media for 1 h at 37°C. The mesh size of a 10%, 6 kDa PEG-DA hydrogel can be approximated as 3.3 nm (A description of how mesh size was determined can be found in the Supplemental Material). The minimum hydronamic radius of

PEGylated protein can be estimated using the formula $R_{\min}=0.066 \cdot MW^{1/3}$ [52]. For SCF this was 2.6 nm and for SDF1 α it was 2.4 nm. Due to the similarity in mesh size and hydrodynamic radii and the short exposure time to the solution (~ 3 minutes), we do not anticipate that a significant amount of PEGylated protein diffuses into the gel.

2.8 Quantification of SCF and SDF1 α on surfaces

To confirm surface protein conjugation on hydrogels, varying concentrations of SCF and SDF1 α (100, 200, and 400 ng/cm²) in combination with PEG-RGDS (25 μ g/cm²) were reacted with surfaces of hydrogel wells. After UV exposure, the solution was removed from each hydrogel well, and gels were soaked in PBS. A sandwich ELISA was performed on the removed reactant solution and soak solutions using Quantikine ELISA kits for SCF and SDF1 α (R&D) [45].

To quantitatively determine the amount of SCF and SDF1 α adsorbed to tissue culture polystyrene (TCPS) wells, the coating solution was collected and assessed with Quantikine ELISA kits. An ELISA was also performed on a the well plate to confirm adsorption to the plate surface.

2.9 Seeding cells in hydrogel and TCPS wells

32D cells were seeded into all wells at 5000 cells/cm². The experimental groups consisted of hydrogel wells with surface immobilized: RGDS (2.5, 25, and 250 μ g/cm²); RGDS (25 μ g/cm²) and SCF (400 ng/cm²); and RGDS (25 μ g/cm²) and SDF1 α (400 ng/cm²). PEG-DA wells (0 μ g/cm² RGDS) served as controls. Each group consisted of 8 gel wells. Cells were also seeded into tissue culture wells coated with the following solutions: FN (1 μ g/cm²); FN (1 μ g/cm²) and SCF (400 ng/cm²); FN (1 μ g/cm²) and SDF1 α (400 ng/cm²); SCF (400 ng/cm²); and SDF1 α (400 ng/cm²). Each group consisted of 4 wells. Cells were maintained in culture for 6 days at 37°C with 5% CO₂.

2.10 Evaluation of cell adhesion

To evaluate 32D cell adhesion, hydrogel and TCPS wells were rinsed by the addition of fresh media after 48 h in culture. Phase contrast images of each well were captured in randomly selected locations using a Zeiss Axiovert 135 inverted microscope (Zeiss, Oberkochen, Germany) and Jenoptik ProgRes C5 charge-coupled device (CCD) camera (Jenoptik, Jena, Germany). Cells were counted using ImageJ software.

2.11 Evaluation of cell spreading

Cells were fixed with 4% paraformaldehyde, permeabilized with 0.1% Triton X-100, and blocked in PBS containing 1% bovine serum albumin. Cells were then incubated with Alexa-Fluor 488 phalloidin (Invitrogen), which stains actin filaments, and counterstained with DAPI to visualize cell nuclei. Cells were imaged using epifluorescent microscopy on a Zeiss Axiovert 135 inverted microscope. Cell area and circularity were determined using ImageJ software.

2.12 Statistical analysis

One-way ANOVA and Tukey's post-hoc analyses were performed to evaluate statistical differences between groups using a 95% probability level ($p < 0.05$).

3. Results

3.1 PEGylation of Proteins

The conjugations of SCF and SDF1 α to PEG were confirmed with a Western blot (Figure 2). In the PEG-SCF lane, a protein smear beginning at 28 kDa demonstrated successful conjugation to the polymer by an increase over the molecular weight of the soluble cytokine (Figure 2A). A faint band at 18 kDa in the PEG-SCF lane signified a low fraction of unconjugated protein in the final product. Rather than forming a single band on the blot, these types of reactions generate a product with a range of molecular sizes due to the polydispersity of the PEG reactant and also because multiple polymer chains will attach to the proteins [44–46]. Similar results for PEG-SDF1 α are shown in Figure 2B, where the conjugated PEG-protein product showed as a smear beginning at 14 kDa, a significant shift from the 8 kDa unmodified protein.

3.2 SCF and SDF1 α bioactivity after PEGylation

The addition of large or multiple PEG chains to a protein can alter the bioactivity of a protein particularly if a high number of chains are covalently attached to the protein or the PEG chains are tethered at the protein's reactive site [53–56]. In order to ensure that SCF and SDF1 α remained bioactive after PEGylation, we exposed 32D cells to soluble forms of PEG-SCF and PEG-SDF1 α and evaluated the effects on proliferation and migration, respectively. The addition of both unmodified SCF and PEG-SCF to culture media resulted in significant increases in cell numbers compared to control wells without any form of the protein (Figure 3A). The addition of SDF1 α and PEG-SDF1 α to the media promoted significant 32D cell migration through the transwell membrane (Figure 3B), though migration in response to PEG-SDF1 α was significantly reduced compared to the native protein.

3.3 Biomolecule incorporation onto hydrogel and TCPS surfaces

To confirm that the proteins were covalently immobilized on well surfaces, ELISAs were conducted. The results indicated that approximately 80% of the PEG-SCF available for reaction was conjugated to the hydrogel with the PEG-SDF1 α immobilization slightly higher at 98%. The surface concentrations of SCF and SDF1 α used in experiments (400 ng/cm²) resulted in 73.5 ± 6.7 ng/well and 88.3 ± 0.5 ng/well, respectively.

On TCPS wells, over 99 % of the proteins were adsorbed to the plate surface. The coating solution used (400 ng/cm²) resulted in 127.997 ± 0.0093 ng/well (SCF), 127.104 ± 0.0891 ng/well (SCF + FN), 127.992 ± 0.0014 ng/well (SDF1 α), and 127.983 ± 0.0059 ng/well (FN SDF1 α). ELISAs on the coated TCPS plates confirmed the presence of SCF and SDF1 α on the TCPS surfaces.

3.4 32D cell adhesion on RGDS modified hydrogels

Figure 4 displays the average number of adherent 32D cells per square centimeter on the PEG-RGDS hydrogels after 48 h, with increases in RGDS concentration corresponding to higher levels of attachment. On hydrogels with no bioactive components, 874 ± 316 cells/cm² were adherent. Upon addition of $2.5 \mu\text{g}/\text{cm}^2$ RGDS, $1,572 \pm 1,194$ cells adhered, and with $25 \mu\text{g}/\text{cm}^2$ RGDS the adherent cell number increased to $4,102 \pm 3,196$ cells/cm² though neither of these changes was significant. At $250 \mu\text{g}/\text{cm}^2$ RGDS, there was a significant increase in 32D cell adhesion to $10,299 \pm 1,328$ cells/cm². These numbers were found to be statistically similar to 32D cell adhesion to FN coated TCPS plates (Figure S2).

3.5 32D cell adhesion on hydrogels with surface immobilized SCF and SDF1 α

Compared to the samples containing the intermediate concentration of RGDS (25 $\mu\text{g}/\text{cm}^2$) alone, gels also presenting surface immobilized SCF had significantly more adherent 32Ds after 48 h: $4,102.0 \pm 3,196.1$ cells/ cm^2 on RGDS hydrogels versus $12,236 \pm 2,389$ cells/ cm^2 with the SCF and RGDS combination. We also observed a significant increase in adherent 32D cells when SDF1 α (400 ng/ cm^2) was added to the surfaces of gels already modified with 25 $\mu\text{g}/\text{cm}^2$ RGDS (Figure 4). After 48 hours, the number of cells on gels with SDF1 α was comparable to that on SCF surfaces at $11,839 \pm 4,722$ cells/ cm^2 . At the higher concentration of RGDS (250 $\mu\text{g}/\text{cm}^2$), the effects of SCF and SDF1 α were masked by those of the adhesive peptide with no further increase in cell number with addition of the proteins. These were statistically similar to 32D cell adhesion levels on SCF- and SDF1 α -coated TCPS plates (Figure S2).

3.6 32D cell morphology on hydrogel surfaces functionalized with SCF and SDF1 α

The morphology of 32D cells on hydrogel surfaces was quantified in fluorescent images obtained following DAPI/phalloidin staining (Figure 5). On gels with RGDS alone, the cells appeared round and exhibited minimal spreading (Figure 5B–D), similar to those seen on the unmodified PEG-DA gels (Figure 5A). A definitive morphological change was observed in cells when SCF or SDF1 α was present on the surface (Figure 5E–F) as compared to gels containing RGDS alone. In these experimental groups, 32D cells were well dispersed across the entire surface of the gels and more spread with many exhibiting distinct filopodia as indicated by the white arrows in Figure 5. While there were 32D cells present on TCPS wells with adsorbed SCF or SDF1 α , the cells remained small and rounded compared to hydrogels with covalently immobilized SCF or SDF1 α (Figure 6).

A quantification of the cell images revealed that the average cell area varies widely within each group (Figure 7). This large distribution of cell size can be attributed to the varying degrees of spreading seen in the 32D population regardless of culture surface. When the proteins were covalently immobilized to hydrogel surfaces, there were significant increases in average cell size from 261 ± 175 μm^2 with 250 $\mu\text{g}/\text{cm}^2$ RGDS to 450 ± 275 μm^2 and 367 ± 247 μm^2 for SCF and SDF1 α , respectively. The data was also plotted as a histogram to obtain a better sense of the distribution of cell sizes (Figure 8A). A similar cell size distribution was seen between all three RGDS concentrations with most cell sizes falling between 0–300 μm^2 . With the addition of SCF and SDF1 α , the cell size distribution broadened and the average cell size centered on 300–400 μm^2 . There was also an increase in the percentage of cells that were larger than 500 μm^2 (30% for SCF and 20% for SDF1 α) when compared to gels containing only or no RGDS (less than 10% for these groups). In contrast, 32D cells cultured in TCPS wells with physioadsorbed proteins were significantly less spread (Figure 7). The histogram shows that the 32D cell area centered around 200 μm^2 (Figure 8B). There was also a shift of the spread to the left with more than 99% of the cells smaller than 500 μm^2 in all groups.

Measurements of circularity were also generated to determine cell morphology (Figure 9). 32D cells on surfaces with low concentrations of RGDS (0 or 2.5 $\mu\text{g}/\text{cm}^2$) retained a round shape as denoted by circularity values of 0.82 ± 0.12 or 0.82 ± 0.11 respectively. As the RGDS concentration increased from 2.5 to 25 $\mu\text{g}/\text{cm}^2$ and from 25 to 250 $\mu\text{g}/\text{cm}^2$, circularity decreased significantly (0.77 ± 0.15 and 0.72 ± 0.13 respectively) indicating a higher degree of cell spreading as more RGDS was immobilized. Upon addition of SCF or SDF1 α onto gel surfaces, circularity again dropped significantly (0.64 ± 0.13 and 0.68 ± 0.12 respectively) demonstrating the effect of these proteins on hematopoietic cell behavior. Cells cultured on TCPS wells with physioadsorbed proteins, were significantly rounder than cells cultured on hydrogel wells functionalized with RGDS and SCF or RGDS and SDF1 α .

All circularity values were greater than 0.71: 0.71 ± 0.11 ($1 \mu\text{g}/\text{cm}^2$ FN), 0.77 ± 0.10 ($1 \mu\text{g}/\text{cm}^2$ FN + SCF), 0.75 ± 0.12 ($1 \mu\text{g}/\text{cm}^2$ FN + SDF1 α), 0.74 ± 0.11 (SCF), and 0.76 ± 0.11 (SDF1 α).

4. Discussion

HSCs have proved successful in clinical applications to treat blood diseases and cancers, but they remain difficult to study for applications in other types of treatments due to their limited availability and inability to be expanded effectively *in vitro*. There is a need to engineer the HSC microenvironment to better control and understand HSC self-renewal and differentiation [57]. Several attempts have been made to expand the cells using biofunctional materials to mimic the complex HSC microenvironment [8,23–29,58–62]. However, a system that provides for both HSC expansion and long-term maintenance of their multipotent state has not been developed.

PEG-DA hydrogels are ideal materials for the culture of HSCs because we can easily control and modulate the biochemical components and mechanical properties of the scaffold [24,25,63]. In this work the niche components RGDS, SCF, and SDF1 α were PEGylated and immobilized on the surface of PEG-DA hydrogel well surfaces and assayed for their effects on cell adhesion and morphology of a model cell line, 32D cells, in an effort to demonstrate the feasibility of this approach in directing hematopoietic progenitor cell behavior. PEGylation has previously been shown to extend the half-lives of biomolecules [53,56], and our group has PEGylated several proteins critical in angiogenesis and used these molecules to promote and support blood vessel formation within PEG hydrogels for several weeks [43–46,64].

To ensure that the PEGylated versions of SCF and SDF1 α maintained their bioactivity, we observed the effects of SCF on 32D cell proliferation and SDF1 α on 32D cell migration. There was no significant difference between the PEGylated and unmodified forms of SCF suggesting that the PEG chains do not substantially affect the bioactivity of the protein. However, the presence of the PEG chains appeared to diminish SDF1 α bioactivity as demonstrated by a statistical decrease in cell migration in the PEG-SDF1 α experimental group when compared to the positive control. Nonetheless, substantial bioactivity was retained, and this was sufficient for the desired studies.

PEG-RGDS was immobilized onto the hydrogel surfaces to mimic the *in vivo* interactions between HSCs and ECM proteins in the stem cell niche, since adhesive interactions of this nature have been shown to retain HSCs in the niche and affect their fate [35,65,66]. In previous studies, RGD tethered to PEGylated glass surfaces via avidin-biotin binding successfully promoted hematopoietic cell adhesion [9], and a cyclic form of the peptide was similarly effective in inducing progenitor cell adhesion when incorporated into solid lipid monolayers [67,68]. RGD has also been shown to promote proliferation of HSCs when conjugated to poly(ethylene terephthalate) substrates [8].

32D cells cultured on PEG-DA substrates, containing no biofunctional components, demonstrated minimal adhesion. When PEG-RGDS was incorporated onto the surfaces of the hydrogel wells, we observed a dose dependent response. Previous work with a variety of cell types has shown adhesion to be sensitive to surface ligand concentration [42,69–71]. For example, Gonzalez *et al.* utilized PEG-DA hydrogels with RGDS peptide incorporated into the bulk to culture neutrophils and showed a similar increase in adhesion when increasing the peptide concentration [42]. Our work demonstrates a similar ability to affect hematopoietic cell adhesion by altering the RGDS concentration in hydrogel wells. At the low peptide concentration ($2.5 \mu\text{g}/\text{cm}^2$), minimal cell adhesion, comparable to that seen on

un-modified PEG-DA hydrogels, indicated that this amount of RGDS is insufficient to enable surface adhesion. By increasing the RGDS surface concentration from 25 to 250 $\mu\text{g}/\text{cm}^2$, we saw a correlative increase in 32D cell adhesion reaching a similar number of adherent cells as on a FN-coated TCPS plate. Further, the significant increase in cell adhesion with 250 $\mu\text{g}/\text{cm}^2$ RGDS compared to lower surface RGDS concentrations (2.5 and 25 $\mu\text{g}/\text{cm}^2$) and unmodified hydrogel controls suggested that 32D cells require binding multiple RGDS molecules to adhere to gel surfaces with enough strength to withstand rinsing. In the niche, hematopoietic cells express integrins, which bind specifically to the RGDS sequence. Integrin activation by RGDS can act as a positive feedback loop resulting in the expression of additional integrins and enhanced cell adhesion [28]. Adhesive interactions retain cells in the HSC niche and help maintain them in a multipotent state. As cells differentiate they lose the ability to adhere to FN and travel out of the niche to sites of injury [72–76]. Thus, the ability to promote cell adhesion to our synthetic niche is critical to the successful expansion of clinically relevant HSCs. To exert finer control of HSC behavior, RGDS ligands could be patterned on the hydrogel surface [30,34]. Previous work has shown that the spatial presentation of cyclic RGD ligands significantly affects HSC adhesion, integrin distribution, and signal transduction [35].

Fibronectin works with various cytokines in the niche to promote cell adhesion [7]. When an RGDS concentration of 25 $\mu\text{g}/\text{cm}^2$ was immobilized on hydrogel well surfaces with SCF and SDF1 α , 32D adhesion increased significantly. As described in Section 1, SCF is a cytokine that promotes HSC self-renewal and maintenance while SDF1 α is a chemokine that recruits circulating HSCs and is thought to maintain a pool of undifferentiated HSCs [16,22,77–80]. Several studies have shown that SCF can enhance adhesion of hematopoietic cells to FN [81,82]. Additional evidence suggests that the binding of SCF to the c-kit receptor activates integrins on the cell surface to promote adhesion to RGDS [78]. Furthermore, Lévesque *et al.* found that in several minimally adherent hematopoietic progenitor cell lines, exposure to SCF resulted in increased avidity of integrins to FN [83]. We hypothesize that SCF is activating additional integrins on the 32D cells to enable a greater number of cells to adhere. In addition, due to the covalent immobilization of the SCF in this synthetic niche, the prolonged maintenance of 32D cell adhesion may be due to sustained activation of the c-kit receptor as bound SCF cannot be internalized [13–15,84]. In previous work, SCF was non-covalently immobilized onto microparticle coated plates and successfully stimulated hematopoietic cell proliferation; however, it was gradually released from the particles over time with a half-life of five days [85]. The immobilization strategy utilized in this work can potentially increase the half-life of SCF and thus allow longer activation of the c-kit receptor and maintenance of cell adhesion.

Activation of the CXCR4 receptor on HSC surfaces by immobilized SDF1 α has been shown to activate integrins and help retain HSCs in the niche [86]. We hypothesize that immobilization of SDF1 α onto the gel surface upregulated the expression of these integrins in 32D cells. Similar to SCF, the increase in integrins allows more 32D cells to bind to RGDS molecules presented on the gel surface. The immobilization of SDF1 α on the gel surface is critical, as soluble SDF1 α has previously been shown to be less effective in promoting hematopoietic cell adhesion [87]. Again, we believe that our functionalization methods can increase the half-life of SDF1 α and in turn prolong cell adhesion.

When compared to hydrogel wells with the highest concentration of RGDS (250 $\mu\text{g}/\text{cm}^2$), the addition of SCF and SDF1 α to hydrogel well surfaces—with either 25 $\mu\text{g}/\text{cm}^2$ or 250 $\mu\text{g}/\text{cm}^2$ of RGDS—did not significantly affect 32D cell adhesion. This is likely because the final result of each of these factors is activation of a common integrin. With a finite number of integrins available for cell surface display, direct activation of integrins in the presence of high levels of adhesive peptide could be saturating the response, thus negating any potential

effect via c-kit or CXCR4. Similarly, when comparing the covalently immobilized proteins in hydrogel wells to proteins coated on TCPS plates, there was not a significant difference in 32D cell adhesion. This was not surprising as the 32D cells express integrins that bind to FN and can also adhere to serum proteins adsorbed to TCPS plates [47,48]. In addition, cell adhesion was evaluated at an early timepoint (48 h) before significant turnover of the proteins on the TCPS plate. Though these proteins did not significantly affect cell adhesion when presented in combination with high RGDS or FN concentrations, SCF and SDF1 α did influence 32D cell morphology.

32D cells on surfaces with either SCF or SDF1 α were significantly more spread than cells on surfaces with RGDS alone, indicating a greater degree of cell spreading. Cells on surfaces with no biofunctional components or RGDS alone also had a significantly rounder shape and fewer filopodia when compared to 32D cells on hydrogel substrates with SCF or SDF1 α . As mentioned above, it is known that binding to these molecules activates multiple integrins on the cell surface [78,86–88]. The integrins then cluster and can connect the RGD molecules to the cells' actin cytoskeleton allowing the cells to migrate and spread on the gel surfaces [89]. Integrin activation acts in a positive feedback loop causing a sustained upregulation of integrin expression and resulting in larger cell spread areas [90].

Many of the 32D cells on gels functionalized with SCF or SDF1 α also had a motile morphology indicated by filopodia extending outward from the cell centers. The binding of c-kit to SCF is known to promote cell motility [91–93], and previously, Fruehauf *et al.* observed podia formation and associated 32D cell motility in response to stimulation with SDF1 α on FN coated glass surfaces [18]. SDF1 α is also known to be a major modulator of HSC migration through its interaction with the CXCR4 receptor on HSC surfaces, and recent work has shown that stimulation with SDF1 α induces actin polymerization and migration in human CD34⁺ cells [94]. This migration depends on the activation of the VLA4 and VLA5 integrins which bind to FN in the HSC niche [86]. The 32D podia formation seen on our surface modified gels suggests that SCF and SDF1 α have not only upregulated cell adhesion but may have also triggered the 32D cells to migrate across the hydrogel surfaces via the interactions between integrins and the RGD peptide. This migratory phenotype could be beneficial in the culture of primary HSCs, as HSCs that are used in clinical applications must be able to home to the bone marrow environment and engraft to successfully repopulate the immune system. A migratory morphology *in vitro* can be indicative of the potential mobility of cells upon implantation. Previous work has shown that HSCs that were more migratory *in vitro* displayed higher degrees of engraftment [94,95].

Interestingly, the 32D cells did not respond similarly to physioadsorbed SCF and SDF1 α on TCPS well plates. The 32D cells were significantly less spread and more circular than 32D cells cultured on the hydrogels with covalently immobilized proteins. This could be due to the fact that covalent immobilization of SCF and SDF1 α can lead to sustained activation of c-kit and CXCR4 respectively and subsequent upregulation of integrins which allows the cells to spread and migrate [78,83,86–88]. Alternatively, the differences in the surfaces' mechanical properties may be influencing cell morphology [24,25,63]. Previous work has shown elastic modulus to influence the behavior of 32D cells, and the softer hydrogel environment could support cell spreading.

Combining these results with the cell adhesion data, we have demonstrated the ability to stimulate specific hematopoietic cell behavior via covalent immobilization of biomolecules on the hydrogel surface. We selected HSC niche components known to influence hematopoietic cell behavior to evaluate the possibility of designing PEG hydrogels to function as synthetic HSC microenvironments. We believe our findings validate the further development of bioactive PEG hydrogel wells for primary HSC expansion.

5. Conclusions

In this work, we designed biomimetic PEG-DA hydrogels for the *in vitro* culture of hematopoietic progenitor cells. By incorporating RGDS, SCF, and SDF1 α onto hydrogel surfaces, we were able to recapitulate important properties of the HSC microenvironment and alter 32D cell adhesion and spreading. This same strategy can be used to incorporate other niche molecules into the system to more finely tune the material to match properties of the *in vivo* HSC microenvironment. Currently, HSCs cannot be maintained in culture for extended periods of time without proceeding to differentiation or apoptosis. The development of synthetic scaffolds to control HSC behavior and growth will be critical in exploring the potential of HSC in novel therapeutics, which require large numbers of these multipotent cells. Biomimetic hydrogels are optimal platforms for such a culture system because we can easily render them bioactive and control cell interactions by selecting specific molecules to include in the matrix. Future work will investigate the use and optimization of this system for the expansion of primary HSCs, and the effectiveness of the scaffold will finally be determined by evaluating the differentiation potential of the cells after culture in the synthetic niche.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

This work was supported by an NSF GRF (MLC) and NIH R01 EB005173. Thank you to Dr. Melissa McHale for reviewing the manuscript.

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Figure 1. Microfabrication and Functionalization of PEG-DA hydrogel wells

A. To generate PEG-DA wells, SU-8 2100 photoresist was spin-coated onto glass slides. The photoresist slab was then exposed to UV light through a high resolution photomask. After removing the unexposed photoresist, PEG-DA wells were molded between a glass slides and the slide containing the SU-8 pillars. B. The bottom surfaces of the wells were biofunctionalized by pipetting an aqueous solution containing PEGylated biomolecules and a photoinitiator into the wells and exposing the material to UV light for 3 minutes. (Note: not to scale)

Figure 2. Western blot confirming PEGylation of SCF and SDF1α

A. In the SCF lane, a band at 18 kDa corresponds to the molecular weight (MW) of the extracellular domain of SCF. In the PEG-SCF lane, there is a smear beginning at a MW of ~28 kDa. The increase in MW confirms the addition of PEG chains to the SCF molecule, and the resulting smear indicates that some SCF molecules have multiple PEG chains. B. There is a band present in the SDF1α lane near 8 kDa. In the PEG-SDF1α lane you can see a smear beginning around 14 kDa indicating the protein has been successfully conjugated to PEG chains.

Figure 3. Bioactivity of PEGylated SCF and SDF1α

The bioactivity of the PEGylated proteins was evaluated by culturing 32D cells in media supplemented with either no protein, the natural occurring form of the protein, or the PEGylated version of the protein. For SCF, the effects on 32D cell proliferation and for SDF1α the effects on 32D cell migration were evaluated. A. In the SCF bioactivity assay, the percent change in 32D cell number after 5 days was significantly higher when SCF or PEG-SCF was added to the media. We saw no significant difference between the PEGylated and unPEGylated versions of the protein (n=8). B. In the migration assay, when SDF1α or PEG-SDF1α was added to the media, we saw an increase in 32D cell migration. However, the PEG chains appeared to interfere with the protein function as the migration of 32D cells in the presence of PEG-SDF1α was significantly reduced compared to that demonstrated with SDF1α (n=54). Data reported as average $\pm$ standard deviation, * denotes significance compared to control, and # denotes significance compared to PEG-SDF1α (p<0.05).

Figure 4. Adherent 32D cells on hydrogel well surfaces functionalized with biomolecules
 32D cells were cultured on surfaces with covalently immobilized RGDS, SCF, and SDF1α. As the concentration of RGDS was increased, there was a corresponding significant increase in cell adhesion. With the addition of SCF (400 ng/cm²) or SDF1α (400 ng/cm²) to the surface of gels with medium RGDS concentrations (25 µg/cm²), there was a significant increase in cell adhesion compared to the peptide alone. However, with 250 µg/cm² RGDS on the surface, the addition of SCF or SDF1α did not similarly increase the number of adherent cells. Data presented as average ± standard deviation, * indicates statistical significance compared to 0 µg/cm² RGDS, 2.5 µg/cm² RGDS, and 25 µg/cm² RGDS (n=4–8, p<0.05).

Figure 5. 32D cell morphology in bioactive PEG hydrogel wells

Representative images of 32D cells on hydrogel surfaces. Actin filaments are stained with phalloidin (green) and nuclei are stained with DAPI (blue). Higher concentrations of RGDS on the surface resulted in more adherent 32D cells on the gels. With the covalent incorporation of SCF and SDF1 α on the surfaces, the cells appeared more spread, and distinct filopodia can be seen extending from many of the cells (denoted by white arrows). (A. PEG-DA, B. 2.5 $\mu\text{g}/\text{cm}^2$ RGDS, C. 25 $\mu\text{g}/\text{cm}^2$ RGDS, D. 250 $\mu\text{g}/\text{cm}^2$ RGDS, E. 25 $\mu\text{g}/\text{cm}^2$ RGDS + 400 ng/cm 2 SCF, F. 25 $\mu\text{g}/\text{cm}^2$ RGDS + 400 ng/cm 2 SDF1 α ; Scale bars = 25 μm).

Figure 6. 32D cell morphology in TCPS wells with physioadsorbed proteins

Representative images of 32D cells on hydrogel surfaces. Actin filaments are stained with phalloidin (green) and nuclei are stained with DAPI (blue). Cells appeared rounded and small in all groups. (A. $1 \mu\text{g}/\text{cm}^2$ FN, B. $1 \mu\text{g}/\text{cm}^2$ FN + $400 \text{ ng}/\text{cm}^2$ SCF, C. $1 \mu\text{g}/\text{cm}^2$ FN + $400 \text{ ng}/\text{cm}^2$ SDF1 α , D. $400 \text{ ng}/\text{cm}^2$ SCF, E. $400 \text{ ng}/\text{cm}^2$ SDF1 α ; Scale bars = $25 \mu\text{m}$).

Figure 7. Quantification of 32D cell morphology in hydrogel and TCPS wells

A) 32D cells spread to a greater extent on hydrogels modified with RGDS (25 $\mu\text{g}/\text{cm}^2$) and SCF (400 ng/cm^2) or SDF1 α (400 ng/cm^2) compared to RGDS alone gels and TCPS wells with physioadsorbed proteins. The wide distribution of cell size is due to the heterogeneity of 32D cells. (Black bars are hydrogel wells while grey bars are TCPS wells. Bars represent mean $\pm$ standard deviation. Bars that do not share a common letter are significantly different, $n=105$ (0 $\mu\text{g}/\text{cm}^2$ RGDS), 193 (2.5 $\mu\text{g}/\text{cm}^2$ RGDS), 2454 (25 $\mu\text{g}/\text{cm}^2$ RGDS), 863 (250 $\mu\text{g}/\text{cm}^2$ RGDS), 617 (SCF), 631 (SDF1 α), 622 (1 $\mu\text{g}/\text{cm}^2$ FN), 1177 (1 $\mu\text{g}/\text{cm}^2$ FN + SCF), 839 (1 $\mu\text{g}/\text{cm}^2$ FN + SDF1 α), 881 (SCF), 472 (SDF1 α), $p < 0.05$).

Figure 8. 32D cell area distribution

A. Cell area distribution on hydrogel wells. On all three RGDS concentrations most cells were between 100 – 200 μm^2 . On hydrogel surfaces with SCF or SDF1 α there was a shift to the right and a broadening of the cell area distribution compared to RGDS alone. B. Cell area distribution on TCPS wells. On physioadsorbed biomolecules, cell area was centered around 100 – 200 μm^2 . The distribution of cell sizes is not as wide as on hydrogel surfaces with most cells smaller than 400 μm^2 .

Figure 9. Circularity of 32D cells in PEG hydrogel and TCPS wells

32D cells on samples with no RGDS present retained a round shape. 32D cells on bioactive hydrogels were less circular indicating spreading. 32D cells on TCPS coated plates were rounder and less spread than cells on hydrogels with covalently immobilized SCF or SDF1 α . Black bars are hydrogel wells while grey bars are TCPS wells. Bars that do not share a common letter are significantly different. $n=105$ (0 $\mu\text{g}/\text{cm}^2$ RGDS), 185 (2.5 $\mu\text{g}/\text{cm}^2$ RGDS), 2454 (25 $\mu\text{g}/\text{cm}^2$ RGDS), 699 (250 $\mu\text{g}/\text{cm}^2$ RGDS), 497 (25 $\mu\text{g}/\text{cm}^2$ RGDS+SCF), 458 (25 $\mu\text{g}/\text{cm}^2$ RGDS+SDF1 α), 482 (1 $\mu\text{g}/\text{cm}^2$ FN), 861 (1 $\mu\text{g}/\text{cm}^2$ FN+SCF), 617 (1 $\mu\text{g}/\text{cm}^2$ FN+SDF1 α), 625 (SCF), 388 (SDF1 α) ($p < 0.05$).