Biomaterials 67 (2015) 297–307

Contents lists available at ScienceDirect

Biomaterials

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

The use of covalently immobilized stem cell factor to selectively affect hematopoietic stem cell activity within a gelatin hydrogel

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ARTICLE INFO

Article history:
Received 10 February 2015
Received in revised form
20 July 2015
Accepted 22 July 2015
Available online 23 July 2015

Keywords:
Bone marrow
Stem cell
Cell activation
Hydrogel
Growth factor

ABSTRACT

Hematopoietic stem cells (HSCs) are a rare stem cell population found primarily in the bone marrow and responsible for the production of the body's full complement of blood and immune cells. Used clinically to treat a range of hematopoietic disorders, there is a significant need to identify approaches to selectively expand their numbers *ex vivo*. Here we describe a methacrylamide-functionalized gelatin (GelMA) hydrogel for *in vitro* culture of primary murine HSCs. Stem cell factor (SCF) is a critical biomolecular component of native HSC niches *in vivo* and is used in large dosages in cell culture media for HSC expansion *in vitro*. We report a photochemistry based approach to covalently immobilize SCF within GelMA hydrogels via acrylate-functionalized polyethylene glycol (PEG) tethers. PEG-functionalized SCF retains the native bioactivity of SCF but can be stably incorporated and retained within the GelMA hydrogel over 7 days. Freshly-isolated murine HSCs cultured in GelMA hydrogels containing covalently-immobilized SCF showed reduced proliferation and improved selectivity for maintaining primitive HSCs. Comparatively, soluble SCF within the GelMA hydrogel network induced increased proliferation of differentiating hematopoietic cells. We used a microfluidic templating approach to create GelMA hydrogels containing gradients of immobilized SCF that locally direct HSC response. Together, we report a biomaterial platform to examine the effect of the local presentation of soluble vs. matrix-immobilized biomolecular signals on HSC expansion and lineage specification. This approach may be a critical component of a biomaterial-based artificial bone marrow to provide the correct sequence of niche signals to grow HSCs in the laboratory.

1. Introduction

Hematopoietic stem cells (HSCs) are of increasing interest in the field of regenerative medicine due to their role in the production and regulation of the body's blood and immune system. Dysregulations in HSC function can lead to severe blood-related disorders and cancers such as leukemia and lymphoma. These pathologies are typically treated clinically via HSC transplantation where whole donor bone marrow, or in some cases an enriched HSC fraction, is infused into the patient after myeloablative therapy to reconstitute the compromised marrow [1,2]. Clinical complications associated with both insufficient numbers of isolated donor cells or low homing and engraftment post-transplant require approaches to selectively expand primitive HSC fractions [3,4]. Bone marrow niches, defined by constellation of neighboring niche cells, the extracellular matrix (ECM), and biomolecules, are believed to significantly affect HSC quiescence, differentiation, migration and hematopoietic lineage specification [5–10]. Mimicking the niche as a coordinated entity of action requires understanding HSC fate decisions in response to multiplexed cell, biophysical, and biomolecular signals. Therefore our mission is to generate an instructive biomaterial able to selectively promote HSC quiescence and self-renewal over differentiation for targeted clinical applications.

While the biomolecular environment within the marrow is complex, a number of factors have been identified as critical regulators of HSC fate. *In vivo* studies that de-functionalize niches by removing cell or matrix constituents have provided insight regarding niche anatomical localization [11–13], putative niche cells [11,13–17] and niche-regulated signaling pathways (*e.g.*,

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http://dx.doi.org/10.1016/j.biomaterials.2015.07.042
0142-9612/

Jagged-1/Notch, CXCL12/CXCR4) [8,18–28]. Recently, biophysical signals (matrix mechanical properties, ligand presentation) have been suggested to directly impact HSC lineage specification and downstream myeloid differentiation [29–31]. The rarity of primitive HSCs within the marrow (<0.005% of marrow) [32] makes it difficult to study HSC-niche interactions *in vivo*. As such, efforts have turned towards developing two and three-dimensional (3D) biomaterials for HSC expansion or directed differentiation [33–36]. Biomaterial-based approaches that functionalize a synthetic niche with defined sequences of cues may provide significant insight regarding the coordinated impact of the niche environment on HSC fate [37]. To meet clinical-scale needs, efforts here concentrate on fully three-dimensional biomaterials.

Biomolecules play a deterministic role in HSC fate decision. Stem cell factor (SCF) is known to be actively involved in HSC survival and maintenance in the niche [38,39]. While a critical component of HSC culture media, SCF remains active when substrate bound [5,38–40]. Evidence from *in vivo* studies has implicated niche cells with membrane-bound SCF as being particularly significant in HSC lodging [39]; however it remains unclear whether that effect was due directly due to immobilized SCF or indirectly via other signaling mechanisms. Previous efforts have demonstrated the use of SCF-functionalized 2D substrates to promote expansion of hematopoietic progenitor cell lines [41]. Notably, *Cooper–White* et al., expanded M-07e human myeloid leukemia cells using physisorbed SCF on tissue culture plastic [38]. Work by *West* et al., used covalently immobilized SCF on RGD-functionalized PEG surfaces to expand an IL-3-dependent murine myeloid cell line (32D) [42]. While instructive, these efforts were proof-of-concept experiments on 2D substrates using immortalized cell lines, motivating work here that seeks to develop fully 3D biomaterials for selective culture of primary HSCs. Further, light-based immobilization methods have also been recently described for covalently immobilizing biomolecules within 3D biomaterial networks [43–45], suggesting potential to generate patterns of immobilized biomolecules. However, prior to examining the effect of complex 3D patterns of SCF, here we first explored the effect of bulk immobilization of SCF within a 3D biomaterial on the quiescence vs. expansion of primary murine HSCs. Additionally, the bone marrow is a complex, heterogeneous tissue containing gradients in cellular, biomolecular, extracellular matrix, and mechanical properties [29,46–48]. This suggests a need to create biomaterials able to mimic multiple distinct niches as well as the gradients linking them. Microfluidic mixing based approaches have previously been shown to generate protein gradients on two-dimensional (2D) substrates [34,49–51]. Recently, our group described a microfluidic templating tool to create overlapping patterns of HSCs and niche cells across a single 3D hydrogel [34]. Therefore, we explored the use of this microfluidic templating approach to examine the effect of gradients of matrix-immobilized SCF on primary HSCs.

Here we describe an approach to covalently incorporate SCF within a methacrylamide-functionalized gelatin (GelMA) hydrogel for *in vitro* culture of primary murine HSCs. GelMA hydrogel can be UV-immobilized, contains natural ligands (Fn motif RGD), and retains MMP-sensitive degradation sites [52,53]. We hypothesized that the methacrylamide groups used to crosslink the GelMA hydrogel were also potential sites for covalently incorporating SCF within the matrix. Further, we hypothesized that the mode of SCF presentation (soluble vs. matrix bound) would significantly affect the balance between HSC differentiation and expansion. The primary goal of this project is to demonstrate the selectivity of soluble vs. matrix-immobilized SCF on the proliferation and differentiation of primary murine HSCs. We also describe the use of microfluidic templating to create patterns of immobilized SCF across a single GelMA hydrogel to spatially control HSC response.

2. Materials and methods

2.1. Synthesis of methacrylamide-functionalized gelatin macromer

Methacrylamide-functionalized gelatin was synthesized as described previously [54] using 10% (w/v) gelatin (Type A, 300 bloom from porcine skin) and 20% (v/v) methacrylic anhydride (MA) (Sigma–Aldrich, St. Louis, MO) in phosphate buffered saline (PBS) (Gibco, Grand Island, NY). Following reaction, the GelMA was washed, dialyzed (12,000–14,000 M.W, Fisherbrand, Pittsburgh, PA), then lyophilized. The amount of MA added was chosen to create a GelMA macromer with 85% degree of MA functionalization, as previously verified via ^{1}H NMR [55].

2.2. Synthesis of photoinitiator

The lithium acylphosphinate (LAP) photoinitiator was synthesized as described by *Anseth* et al. [56]. Equimolar 2,4,6-trimethylbenzoyl chloride (Sigma Aldrich) was added to dimethyl phenylphosphonite (Sigma Aldrich) at room temperature and under argon. Fourfold excess lithium bromide in 2-butanone (Sigma–Aldrich) was added then heated to form a solid precipitate. The mixture was cooled to ambient temperature then filtered and washed with 2-butanone to remove unreacted lithium bromide. Excess solvent was removed by vacuum.

2.3. Pegylated stem cell factor (PEG-SCF)

3500 MW Acrylate PEG-NHS ester (JenKem Technology USA, Allen, TX) was reacted at room temperature with recombinant murine SCF (PeproTech, Rocky Hill, NJ) at a molar ratio of 24:1 (PEG-NHS: SCF) in PBS at pH 8.0 [57]. Unreacted PEG-NHS was removed using a 10K MW Pierce Concentrator PES (Thermo Scientific, Waltham, MA). The concentration of PEG-SCF was determined via ELISA (R&D Systems, Minneapolis, MN). Conjugation was confirmed via Western blot using a 15% Tris–HCl precast polyacrylamide gel (primary: rabbit polyclonal SCF; secondary: goat polyclonal anti-rabbit IgG conjugated to horseradish peroxidase). Detection was via SuperSignal West Pico Chemiluminescent Substrate kit (Thermo Scientific) imaged via ImageQuant LAS 4000 (General Electric). PEG-SCF was suspended in PBS (pH 7.4) with 0.1% BSA, aliquoted and stored at −80 °C for further use.

2.4. GelMA hydrogel synthesis

Hydrogels were generated from a solution of 5% (w/v) GelMA macromer and 0.1% (w/v) LAP photoinitiator in PBS. For PEG-SCF conjugated hydrogels, PEG-SCF was added to the pre-polymer solution at 100 or 400 ng/mL. Hydrogels were formed in an ABS plastic mold (7 mm dia. x 500 μm thick well) custom printed in our lab (Replicator 2x, Makerbot, Brooklyn, NY). 19 μl GelMA solution was pipeted into the mold sandwiched between glass slides then exposed to 10 mW/cm^2 UV light (365 nm) for 20 s [54]. Resulting hydrogels were removed from the mold and kept hydrated in PBS (pH 7.4) or StemSpan SFEM media (StemCell technologies, Vancouver, Canada) in a 5% CO_2 incubator at 37 °C for further culture and analysis.

2.5. Fluorescence imaging of PEG-SCF retention within the hydrogel

SCF retention was determined via fluorescent analysis of GelMA hydrogels containing either photopolymerizable PEG-SCF (100 ng/mL), non-functionalized soluble SCF (100 ng/ml) or no SCF (No SCF). The gels were placed in PBS in the incubator (37 °C, 5% CO_2) for 7 days. At days 2, 4 and 7, specimens were embedded in Tissue-Tek