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Enhanced survival and engraftment of transplanted stem cells using growth factor sequestering hydrogels

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ABSTRACT

We have generated a bioinspired tunable system of hyaluronic acid (HyA)-based hydrogels for Matrix-Assisted Cell Transplantation (MACT). With this material, we have independently evaluated matrix parameters such as adhesion peptide density, mechanical properties, and growth factor sequestering capacity, to engineer an environment that imbues donor cells with a milieu that promotes survival and engraftment with host tissues after transplantation. Using a versatile population of Sca-1$^+$/CD45$^-$ cardiac progenitor cells (CPCs), we demonstrated that the addition of heparin in the HyA hydrogels was necessary to coordinate the presentation of TGFβ1 and to support the trophic functions of the CPCs via endothelial cell differentiation and vascular like tubular network formation. Presentation of exogenous TGFβ1 by binding with heparin improved differentiated CPC function by sequestering additional endogenously-produced angiogenic factors. Finally, we demonstrated that TGFβ1 and heparin-containing HyA hydrogels can promote CPC survival when implanted subcutaneously into murine hind-limbs and encouraged their participation in the ensuing neovascular response, which included blood vessels that had anastomosed with the host's blood vessels.

1. Introduction

Over the past decade, stem cell transplantation therapy has started to fulfill its long held promise as a means of promoting functional regeneration of tissues that have been damaged by injury and disease. As new cell therapies have translated into clinical studies, modest results have been reported for regeneration in several tissue types, including cardiac [1,2], skeletal muscle [3], and liver [4]. These studies pointed out several technological challenges regarding relevant cell transplantation parameters that must be optimized before these treatment options become widely available. One pressing problem is that immediately after transplantation into damaged tissue, donor cells encounter a harsh environment with substantial death-promoting stimuli (e.g., hypoxia, reactive oxygen species, etc.), and the vast majority (>90%) of donor cells are lost to necrosis and/or apoptosis within hours to days after transplantation [5,6]. Based on the poor cell survival rate after transplantation, there has been limited evidence that the donor cells can engraft and functionally integrate with the damaged tissue to participate directly to regeneration processes [7–9]. Instead, recent evidence indicates that paracrine signaling of the transplanted cells is the major contributor to any significant tissue regeneration observed [10–12].

One strategy for improving the survival of transplanted stem or progenitor cells, termed Matrix-Assisted Cell Transplantation (MACT), is to engineer a materials-based environment that promotes pro-survival paracrine signaling immediately after

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transplantation, and subsequently stimulates mechanisms of cell engraftment with the host tissues [13–19]. Matrigel™ was initially proposed as a material for MACT, but heterogeneous batch-to-batch material composition, quality control and biological sourcing issues, and the inability to engineer compositional changes have substantially impeded its clinical translation. Therefore, recent advances in this field have focused on transplanting cells with other natural or synthetic matrices [20–25]. Most work has focused on naturally occurring biopolymers such as collagen, alginate, fibrin, chitosan, and hyaluronic acid (HyA), with alginate and fibrin modestly attenuating the negative remodeling process. Ideally a material for MACT would exploit not only the pro-survival potential of naturally occurring biopolymers, but also allow for a wide design space to modulate parameters that are relevant in a pro-healing native extracellular matrix, including the ability to harness endogenously synthesized growth factors by sequestering them in the matrix.

To overcome the limitations that are currently associated with MACT, we have developed hyaluronic acid (HyA) -based hydrogels that were generated using a tunable method of synthesis. HyA was selected as the primary component of this structural matrix since it is biocompatible, biodegradable, non-immunogenic, and plays a critical role in fostering tissue development and repair [26]. Our method of synthesis enabled *independent* control over the hydrogel mechanical properties and biological features, including: (1) the density of peptide sequences for cell attachment via binding to integrin receptors; (2) matrix modulus; (3) the cell-mediated degradation kinetics by selective the MMPs [21]; and, (4) sequestration of exogenously added or endogenously synthesized growth factors via heparin conjugated within the hydrogel. Previously reported materials for MACT have not simultaneously explored the effect of all these matrix parameters on transplanted cell survival and engraftment.

Regarding the use of heparin, it is well known that soluble growth factors have their effect on cells for limited time due to their poor stability, soluble presentation, and short half-life *in vivo*. A number of groups have demonstrated that heparin can sequester and release exogenously added growth factors that ultimately improve wound healing and tissue regeneration [27–32]. Therefore, thiolated heparin macromers were incorporated into HyA based hydrogels for solid-phase presentation and prolonged retention of growth factors that were either added exogenously or endogenously produced by the entrained cells.

Using this easily tunable hydrogel system, the objective our study was to demonstrate how a suitable material for MACT can support donor cell survival during transplantation and encourage donor cell integration with the host tissue. In this study, we focused on murine cardiac progenitor cells (CPCs), a pluripotent population of GFP^+ Sca-1^+/$CD45^-$ cells that contribute to cardiac regeneration, at least in part by undergoing neovascular differentiation that is characteristic of endothelial cells [10,33]. With this versatile cell type, we examined how the biochemical and mechanical parameters of the HyA hydrogels influenced: (1) CPC survival, proliferation, and differentiation *in vitro*; and, (2) CPC survival and functional integration via neovascularization *in vivo*.

2. Materials & methods

2.1. Materials

Hyaluronic acid (HyA, sodium salt, 1.0 MDa and 500 kDa) was generously donated by Lifecore Biomedical (Chaska, MN). Adipic dihydrazide (ADH), 1-ethyl-3-[3-(dimethylamino)propyl] carbodiimide (EDC), sodium hydroxide (NaOH), hydrochloric acid (HCl) and 1-hydroxybenzotriazole (HOBt) were purchased from Aldrich (Milwaukee, WI). Dimethyl sulfoxide (DMSO), N-Acryloxysuccinimide (NAS), acetone, ethanol were obtained from Fisher Scientific (Waltham, MA). Paraformaldehyde (16% in H_2O) was obtained from Electron Microscopy Sciences (Hartfield, PA). Calcein was purchased from BD Biosciences (Pasadena, CA). The MMP-degradable crosslinker peptide (CQPQGLAKC) and the 15 amino-acid adhesion peptide (CGGNGEPRGDTYRAY), bsp-RGD(15), were synthesized by American Peptide (Sunnyvale, CA). Dialysis membranes (10,000 MWCO, SpectraPor Biotech CE) were purchased from Spectrum Laboratories (Rancho Dominguez, CA). All chemicals were used as received. All cell culture reagents were purchased from Invitrogen (Carlsbad, CA). 1× Dulbecco's phosphate buffered saline (DPBS) was purchased from Invitrogen.

2.2. Synthesis of AcHyA hydrogel components

Functionalization of HyA with acrylate groups using a two-step synthesis method and functionalization of heparin with a thiol group were performed as follows (Supplementary Fig. S1):

2.2.1. Acrylation of HyA

A HyA derivative carrying hydrazide groups (HyA-ADH) was synthesized using a previously reported method [34]. Specifically, 30 molar excess of ADH was added to HyA in deionized (DI) water (100 mL, 3 mg/ml). Solution pH was adjusted to 6.8 using 0.1 M NaOH and 0.1 M HCl. EDC (3 mmol) and HOBt (3 mmol) were dissolved separately in DMSO/water (1/1 volume ratio, 3 mL) and added to the HyA solution sequentially. The solution was allowed to react for 24 h, and the pH was maintained at 6.8 for at least the first 6 h. After 24 h, the solution pH was adjusted to 7.0 and exhaustively dialyzed against DI water. Then, NaCl was added to produce a 5% (w/v) solution, and HyA-ADH was precipitated in 100% ethanol. The precipitate was redissolved in H_2O and dialyzed again to remove the salt. Subsequently, NAS (700 mg) was reacted to the HyA-ADH solution (300 mg, 100 mL DI water) to generate acrylate groups on the HyA [22]. The product was then lyophilized for 3 days to obtain acrylated HyA (AcHyA). Using a previously described analysis [35], proton (^1H) NMR confirmed that ~28% of the available carboxyl groups were conjugated with acrylate groups on the final acrylated HyA product (AcHyA; Supplementary Fig. S2).

2.2.2. Synthesis of thiolated heparin (heparin-SH)

Heparin-SH synthesis was adapted from a previous report [36]. Heparin (50 mg) was dissolved in DI water at a concentration of 5 mg/mL and reacted with an excess amount of cystamine in the presence of EDC and HOBt at pH 6.8 for 5 h at room temperature. Next, the reaction solution was exhaustively dialyzed using a dialysis cassette to remove all small molecules not attached to heparin, and then the reaction product was lyophilized. After that, a 10-fold molar (moles per COOH of heparin) excess of tris (2-carboxyethyl) phosphine (TCEP) was added to reduce the oxidized disulfide groups in order to reduce any disulfide bonds that had formed between thiol groups. This solution was allowed to react for 3 h at pH 7.5 and then adjusted to pH 5.0 by the addition of 1.0 N HCl. The acidified solution was dialyzed against dilute HCl (pH 5.0) containing 100 mM NaCl, followed by dialysis against dilute HCl at pH 5.0. Then heparin-SH was lyophilized for 3 days, and the percentage of conjugation of thiol groups on the final product (heparin-SH) was determined by colorimetric Ellman assay.

2.3. Synthesis of HyA hydrogels

Prior to making HyA hydrogels, AcHyA-RGD derivative was synthesized by reacting CGGNGEPRGDTYRAY (**bsp-RGD(15)**) (10 mg) with AcHyA solution (25 mg, 10 mL DI water) at room temperature. AcHyA (13.3 mg/mL), AcHyA-RGD (20 mg/mL), and heparin-SH (0.013 mg/mL) were dissolved in 0.3 mL of triethanolamine-buffer (TEOA; 0.3 M, pH 8), and incubated for 15 min at 37 °C. HyA hydrogels were generated by *in situ* crosslinking of the HyA precursors with the MMP-13-cleavable peptide sequence CQPQGLAKC (50 μL TEOA buffer) [21,37]. Viscoelastic properties of the hydrogel were determined by an oscillatory rheometer (MCR 302 Modular Compact Rheometer; ***Anton Paar***, Ashland, VA) with a parallel plate geometry (25 mm diameter) under 10% constant strain and frequency ranging from 0.1 Hz to 10 Hz.

2.4. Incorporation of TGF-β1 and measurement of retention kinetics

Hydrogel macromers of AcHyA (13.3 mg/mL), AcHyA-RGD (20 mg/mL), and heparin-SH (0.013 mg/mL) were dissolved at various ratios (Supplementary Table S1) in 0.3 mL of triethanolamine-buffer (TEOA; 0.3 M, pH 8) and incubated for 15 min at 37 °C. Then, TGFβ1 (350 ng, Cell Signaling Technology, Danvers, MA) was mixed in the solution of HyA derivatives and incubated for another 15 min at 37 °C. To determine the release kinetics, TGFβ1 containing HyA hydrogels were transferred to cell culture inserts (Millipore Corporation, Billerica, MN) and TGFβ1 was allowed to release into 400 μL of cell culture media per well. At predetermined time points over the course of 3 weeks, the supernatant was withdrawn and fresh media was replenished, and the mass of TGFβ1 in each supernatant was determined with sandwich ELISA kits (RayBiotech, Inc, Norcross GA) (Supplementary Fig. S5a,b). Retention of TGFβ1 was calculated by the subtraction of released TGFβ1 from the calculated initial loading amount of TGFβ1 (Supplementary Fig. S5a). Similarly, retention kinetic of bovine serum albumin (BSA) from HyA hydrogels (3 wt.% with 100% crosslinked without heparin) was measured (Supplementary Fig. S5b).