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Localized SDF-1 α Delivery Increases Pro-Healing Bone Marrow-Derived Cells in the Supraspinatus Muscle Following Severe Rotator Cuff Injury

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Abstract

To examine how the chemotactic agent stromal cell-derived factor-1 α (SDF-1 α) modulates the unique cellular milieu within rotator cuff muscle following tendon injury, we developed an injectable, heparin-based microparticle platform to locally present SDF-1 α within the supraspinatus muscle following severe rotator cuff injury. SDF-1 α loaded, degradable, N-desulfated heparin-based microparticles were fabricated, injected into a rat model of severe rotator cuff injury, and were retained for up to 7 days at the site. The resultant inflammatory cell and mesenchymal stem cell populations were analyzed compared to uninjured contralateral controls and, after 7 days, the fold-change in anti-inflammatory, M2-like macrophages (CD11b⁺CD68⁺CD163⁺, 4.3X fold-change) and mesenchymal stem cells (CD29⁺CD44⁺CD90⁺, 3.0X, respectively) was significantly greater in muscles treated with SDF-1 α loaded microparticles than unloaded microparticles or injury alone. Our results indicate that SDF-1 α loaded microparticles may be a novel approach to shift the cellular composition within the supraspinatus muscle and create a more pro-regenerative milieu, which may provide a platform to improve muscle repair following rotator cuff injury in the future.

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Graphical abstract

Keywords

stromal cell-derived factor-1α; heparin; microparticles; mesenchymal stem cells; macrophages

1. Introduction

The chemotactic protein stromal cell-derived factor-1alpha (SDF-1α), primarily through its G protein-coupled receptor, CXC chemokine receptor type 4 (CXCR4), has previously shown to attract a myriad of cell populations including immune cells such as monocytes [1,2] and lymphocytes [2,3], stem cells such as hematopoietic [4] and mesenchymal stem cells (MSCs) [5], and progenitor cell populations [6,7], among others [8,9]. Previously in our laboratory, we subcutaneously implanted SDF-1α loaded hydrogels into a murine dorsal skinfold window chamber model to enable the visualization of cell recruitment and vascular remodeling. After 2-3 days following implantation, more bone marrow-derived cells [10] and significantly more anti-inflammatory monocytes were detected near the SDF-1α loaded hydrogels [10,11] compared to unloaded gels. Moreover, after 7 days significantly more anti-inflammatory, M2-like macrophages, were observed near SDF-1α loaded gels compared to unloaded controls [11]. Thus, as SDF-1α can recruit certain potentially pro-regenerative cell populations, including MSCs and anti-inflammatory monocytes and macrophages, SDF-1α delivery may be an effective approach to modulate the cellular milieu during disease states involving tissue degeneration.

Despite these prior findings, SDF-1α treatment has yet to be explored in the unique context of muscle degeneration following rotator cuff tear. Though rotator cuff tear begins primarily as a tendon injury, significant muscle degeneration often accompanies rotator cuff tears, which can include muscle atrophy as well as fatty and fibrous infiltration into the muscle [12,13]. To fully understand the degenerative changes in muscle following rotator cuff tear, several laboratories have investigated the cellular milieu within rotator cuff muscles following injury [14–17]. In our work, significantly more mononuclear phagocytes, monocytes, and M2 macrophages, among others, were detected in the supraspinatus muscle 7 days following transection of two rotator cuff tendons in mice [17]. Furthermore, using a rat model with a similar tendon transection approach, it was found that macrophages were specifically observed near areas of fat accumulation [14] and, in patients with chronic full-thickness tears, lipid-laden macrophages were observed surrounding muscle fibers [16]. Collectively, these results suggest that specific inflammatory cell populations may play a role in muscle degeneration following rotator cuff tear, and further investigation and manipulation of these cell populations through the delivery of SDF-1α may enable a better understanding of the muscle degeneration observed as well as potential treatment strategies following rotator cuff tear.

For the facile delivery of SDF-1 α to muscle following rotator cuff injury, we have developed a degradable, injectable microparticle platform. First, our laboratory and others have developed biomaterials containing heparin, a highly sulfated glycosaminoglycan (GAG) that can bind and interact with a myriad of proteins to maintain or enhance protein bioactivity, including SDF-1 α [10,18–21]. Second, as natively sulfated heparin possesses potent anti-coagulant properties and may present safety issues *in vivo*, we have incorporated N-desulfated heparin (Hep^{-N}) within our biomaterials, which exhibits diminished anti-coagulant properties while maintaining the ability to bind protein and protect protein bioactivity [18,22]. Lastly, though heparin and heparin derivatives have been successfully incorporated within bulk hydrogels for SDF-1 α delivery [7,10,11,23], we and others have developed heparin-based microparticles (MPs) [18,24–26] as an injectable protein delivery method without exposure to free radicals that are required for *in situ* radically-polymerized hydrogels [27–29]. Furthermore, building on our previous work [19], we have incorporated dithiothreitol (DTT) within the MPs to vary the rate of hydrolytic degradation [30] and ultimately allow for more complete release of protein over time. In the present study, we have developed SDF-1 α loaded 10 wt% Hep^{-N} MPs comprised of Hep^{-N} methacrylamide, poly (ethylene glycol) diacrylate (PEGDA), and DTT and injected them into the supraspinatus muscle immediately following rotator cuff tendon transection and denervation in rats. The tendon transection and denervation model utilized in these studies recapitulates many hallmarks of muscle degeneration exhibited in the human condition of rotator cuff tear in as little as 3 weeks following injury [14,31,32] and thus provides an excellent platform to investigate and modulate the cellular milieu via SDF-1 α treatment. Specifically, after 3 and 7 days, the supraspinatus muscles were harvested and analyzed for changes in cellular composition by quantifying the number of myeloid cells, macrophages, macrophage subpopulations and MSCs present via flow cytometry. We hypothesized that significantly more pro-regenerative bone marrow-derived cells would be observed in muscle treated with SDF-1 α loaded Hep^{-N} MPs than untreated muscle after rotator cuff tendon transection.

2. Materials and Methods

2.1. Heparin modifications

N-desulfated heparin (Hep^{-N}) was prepared as described previously [18,33–35]. Briefly, heparin sodium salt (Hep) from porcine intestinal mucosa (Sigma) was dissolved at 10 mg/mL in dH₂O and passed through Dowex 50WX4 resin (mesh size 100-200, Sigma). Pyridine was added drop-wise to the heparin until the solution reached pH 6 and the solution was placed on a rotatory evaporator (Buchi) to remove excess dH₂O and pyridine. Heparin pyridinium salt solution was frozen in liquid nitrogen, lyophilized, and then dissolved at 1 mg/mL in 9:1 v/v dimethyl sulfoxide (DMSO)/dH₂O at 50°C for 2 hours. Following the reaction, the Hep^{-N} was cooled on ice and precipitated with 95% ethanol saturated with sodium acetate, then collected by centrifugation. The resulting material was dissolved in dH₂O, dialyzed for 3 days, lyophilized, frozen in liquid nitrogen, and stored at –20°C.

For methacrylamide (MAm) functionalization, 1.1 mM Hep^{-N}, 83.0 mM N-hydroxysulfosuccinimide (sulfo-NHS, Sigma), 101.0 mM N-(3-Aminopropyl) methacrylamide hydrochloride (APMAm, Polysciences Inc.), and 156.0 mM (N-3-

Dimethylaminopropyl)-N'-ethylcarbodiimide hydrochloride (EDC, Sigma) were dissolved in 10 mL phosphate buffer saline (PBS, Teknova) solution. After stirring on ice for 6 hours, the Hep^{-N}MAM was dialyzed for 2 days, lyophilized, frozen in liquid nitrogen, and stored at -20°C.

To fluorescently label Hep^{-N}MAM, Hep^{-N}MAM was dissolved at 10 mg/mL in 0.1 M Na₂HPO₄ solution at pH 6. Next, 10 mM EDC and 5.7 μM AlexaFluor633 hydrazide (AF633, Invitrogen) were added and the reaction proceeded in the dark for 90 mins at RT. AF633 Hep^{-N}MAM was dialyzed for 2 days, frozen in liquid nitrogen, lyophilized, and stored at -20°C.

2.2. Poly (ethylene glycol) diacrylate synthesis

Poly (ethylene glycol) (PEG, 3.4 kDa, Sigma) was reacted with acryloyl chloride (AcCl, Sigma) in an 8:1 AcCl to PEG molar ratio in dichloromethane (DCM) solution [30]. Triethylamine (TEA, Sigma) was added drop-wise in a 1:1 TEA to AcCl molar ratio as a catalyst to yield linear PEG-diacrylate (PEGDA).

2.3. Proton nuclear magnetic resonance

Proton nuclear magnetic resonance (¹H NMR) was performed to determine the degree of PEGDA and Hep^{-N}MAM functionalization, whereby each material was dissolved at 10 mg/mL in deuterated H₂O (Sigma), run on a Bruker Avance III spectrometer at 400 Hz, and analyzed using iNMR software [33,36].

2.4. Strong anion exchange high performance liquid chromatography

Strong anion exchange high performance liquid chromatography (SAX-HPLC) was performed at the University of Georgia Complex Carbohydrate Research Center (CCRC) to determine the disaccharide composition of Hep and Hep^{-N}. Hep and Hep^{-N} were dissolved at 12.5 mg/mL in a heparinase mixture of 0.5 U/mL heparinases I, II, and III for 24 hours at 37°C. The reaction was then quenched by heating the mixture for 2 mins at 100°C.

SAX-HPLC was carried out on an Agilent system using a Waters Spherisorb analytical column (4.6×250 mm; 5 μm particle size) at 25°C. Analytes were detected by their UV absorbance at 232 nm using a buffer system consisting of 2.5 mM sodium phosphate (Na₃PO₄) and pH 3.5, which was gradually transitioned from 0 to 1.2 M NaCl. The flow rate was 1.0 mL/min and detection was performed by post-column derivatization and fluorescence detection. Commercial standard disaccharides (Dextra Laboratories) were used for identification of each disaccharide based on elution time and calibration.

2.5. Size exclusion chromatography

Size exclusion high performance liquid chromatography (SEC-HPLC) was performed at the University of Georgia CCRC to analyze the average molecular weight and desulfation characteristics of Hep and Hep^{-N}. Hep and Hep^{-N} were dissolved at 20 mg/mL in a 0.5 M lithium nitrate buffer. Separations were carried out using two TSKGel G2000SWXL columns (7.8 mm ID × 30 cm), connected in series, on an Agilent 1200 LC instrument using refractive index detection and a sample flow rate and injection volume of 0.6 mL/min and 10

μL , respectively. Cirrus GPC software was used to construct 3rd order polynomial standard curves with the molecular weights and elution times of USP enoxaparin sodium molecular weight calibrants and broad standard USP heparin molecular weight calibrants. Weight molecular weights were calculated in Cirrus using the raw chromatograms exported from the SEC-HPLC instrument software.

2.6. Microparticle fabrication

10 wt% Hep^{-N} microparticles (MPs) were fabricated via water-and-oil emulsion. First, 50.0 mg PEGDA and 0.92-1.85 mg dithiothreitol (DTT, 20-40 mM, Sigma) were added to 273 μL 10 wt% bovine serum albumin (BSA, Thermo) PBS solution, consistent with previous studies in our laboratory [10,11], and incubated at $\sim$ pH 7 and 37°C for 30 mins to allow for Michael Type addition between PEGDA and DTT. Next, 5.6 mg Hep^{-N}MAM was added and the aqueous phase was incubated for an additional 30 mins. For fluorescently tagged Hep^{-N} MPs (AF633 Hep^{-N} MPs), a 1:1 ratio of AF633 Hep^{-N}MAM to Hep^{-N}MAM was used and for non-degradable MPs, no DTT was added during fabrication.

After 27 μL of 0.05 wt% Irgacure 2959 photo initiator (Ciba) was added, the aqueous phase described above was added drop-wise to an oil phase of 5 mL mineral oil (Amresco) with 3.0-3.2 μL Span80 (TCI) and allowed to homogenize at 4000 RPM (Polytron PT 3100, Kinematica) for 5 mins. The amount of Span80 in the oil phase was varied to ensure that MPs with different DTT concentrations were maintained at the same average diameter. The water-and-oil emulsion was nitrogen purged for 1 min then placed into a petri dish under UV ($\sim$ 15 mW/cm²) for 10 mins to allow for free radical polymerization between PEGDA and Hep^{-N}MAM. Finally, the MP solution was added to 35 mL dH₂O, centrifuged at 4000 RPM for 5 mins, and the oil phase was removed. MPs were washed once more with dH₂O, then pipetted through 40 μm cell strainers to remove most MPs under 40 μm in diameter.

Once fabricated, MPs were sterilized in 70% ethanol on rotary at 4°C for 30 mins, followed by 3 30-min washes in sterile PBS. MPs were imaged via phase microscopy and size distribution was measured using ImageJ software. MPs were stored in sterile PBS at 4°C and used within 2 weeks of fabrication.

2.7. *In vitro* SDF-1 α loading and release from microparticles

To load SDF-1 α onto MPs, 1.0-1.2 μg sterile human SDF-1 α (R&D Systems) was added to 0.6 mg MPs in 50 μL 0.1 wt% sterile BSA solution. SDF-1 α and MPs were incubated for 2 hours at 4°C, after which time MPs were rinsed by adding an additional 450 μL 0.1 wt% sterile BSA solution. The MPs were centrifuged for 3 mins at 10,000 RCF and 495 μL supernatant was removed.

For *in vitro* SDF-1 α release studies, the removed supernatant was replaced with 495 μL fresh 0.1 wt% sterile BSA solution and samples were incubated at 37°C. MPs were centrifuged and 495 μL supernatant was removed and replaced 3 hours, 1, 3, 7, 10 and 15 days following SDF-1 α loading. SDF-1 α protein levels were quantified with a human SDF-1 α ELISA kit (R&D Systems) using the manufacturer's protocol, except for standard curves which were made with recombinant human SDF-1 α rather than the provided standard. To ensure equivalent cumulative SDF-1 α release for each *in vivo* study, an *in vitro*

SDF-1 α release study from MPs was conducted prior to each individual surgery; n = 3-5 per release study.

2.8. *In vivo* fluorescently-tagged microparticle injection and imaging

Non-degradable (0 mM DTT) and degradable (35 mM DTT) AF633 Hep^N MPs were suspended in 120 μ L sterile dH₂O (4.3 mg MPs) and subsequently loaded into sterile syringes with 20-gauge 1.5 in. hypodermic needles (BD Precision Glide). Immediately following tendon transection and denervation, the MPs were injected into the supraspinatus muscle located posterior to the scapula. Uninjured contralateral supraspinatus muscles were not injected with MPs and served as negative controls. After 3 and 7 days, supraspinatus muscles were dissected from the scapula, sliced in half length-wise, stained with a 1:1000 dilution Hoechst cellular stain (Thermo) in PBS for 5 mins, and remained unfixed for imaging. Muscles were whole-mounted and single fluorescent images were obtained using a Zeiss LSM 700 confocal microscope with a 20 $\times$ objective to visualize AF633 Hep^N MPs (red) within the muscle tissue (blue); n = 2 animals per group per time point.

2.9. Rotator cuff injury model

Rotator cuff injury was induced using a similar method to previously established protocols [31]. Male Sprague-Dawley rats (250-300 g initial weight and 8-10 weeks old) were used in accordance with protocols approved by the Georgia Institute of Technology Institutional Animal Care and Use Committee. Prior to surgery animals were anesthetized by 5% isoflurane (Isothesia), followed by 2-3% isoflurane during surgery and were administered sustained release buprenorphine as an analgesic. The left chest and arm were shaved, wiped with alcohol/chlorhexidine, and a ~2 cm incision was made through the skin and deltoid, parallel to and just below the clavicle. To induce injury, a ~5 mm portion of the suprascapular nerve was resected and, after orienting the humerus to expose the supraspinatus and infraspinatus tendon insertions, both tendons were sharply transected. The deltoid and skin were closed using Vicryl 4-0 absorbable sutures (Ethicon) and wound clips, respectively. The right rotator cuff of each animal served as an internal uninjured contralateral control.

2.10. *In vivo* SDF-1 α loaded microparticle injection

For *in vivo* SDF-1 α loaded MP delivery, we based our dosage of SDF-1 α on previous results in mouse models whereby 15-20 ng SDF-1 α released from 10 wt% Hep^N hydrogels over ~7 days resulted in significant cell recruitment after 7 days [10]. As male Sprague Dawley rats used in our studies were approximately 10X the weight of the mice used in previous studies, we used a dosage of ~155 ng SDF-1 α released from 0.6 mg 10 wt% Hep^N MPs. For *in vivo* injection, MPs were loaded with SDF-1 α as described above but after centrifugation, MPs were resuspended in a total volume of 120 μ L sterile 0.1 wt% BSA solution. MPs were then loaded into sterile syringes with 20-gauge 1.5 in. hypodermic needles and injected into the supraspinatus muscle immediately following tendon transection and denervation. Unloaded MPs were prepared in the same way, except that the maximum concentration of MPs that could be delivered in 120 μ L was used (4.3 mg, 36 mg/mL).

2.11. Flow cytometry

For flow cytometry experiments, groups included SDF-1 α loaded MPs (0.6 mg MPs which released ~155 ng SDF-1 α *in vitro*), unloaded MPs (4.3 mg MPs), and injury only (no SDF-1 α or MPs). Supraspinatus muscles were harvested 3 and 7 days following injury and treatment, digested with collagenase 1A (Sigma) for 45 mins at 37° C, and passed through a 40 μ m cell strainer (Corning). One-half of each sample was stained with the inflammatory cell panel that included FITC-conjugated anti-CD11b (AbD Serotec), PE-conjugated anti-CD163 (BioRad), and APC-conjugated anti-CD68 (BioRad) and the other one-half was stained with the MSC panel that included PE-conjugated anti-CD29 (BioLegend), APC-conjugated anti-CD44 (BioLegend), and BV421-conjugated anti-CD90 (BioLegend). Samples were stained for 30 mins with the appropriate antibodies and fixed in 2% PFA for 20 mins, then analyzed using a FACS-AriaIIIu flow cytometer (BD Biosciences). Inflammatory cells were identified as CD11b+ myeloid cells, CD11b+CD68+ macrophages, and CD163 was used to differentiate M2-like (CD11b+CD68+CD163+) from M1-like macrophages (CD11b+CD68+CD163-) [37,38]. MSCs were identified as triple positive for CD29, CD44, and CD90 [39]; n = 4-9 animals per group per time point. For data analysis, each cell population was first calculated as a percentage of single cells:

$$\% \text{ of single cells} = \frac{\# \text{ of cells in subpopulation}}{\# \text{ of single cells}}$$

Then, each % of single cell value was divided by the % of single cells in the uninjured contralateral control of the same animal:

$$\text{Fold-change over contralateral control} = \frac{\% \text{ of single cells in experimental group}}{\% \text{ of single cells in contralateral control}}$$

2.12. Vascular staining of whole-mounted supraspinatus muscle

After 7 days following injury and treatment, supraspinatus muscles were fixed in 4% PFA for 30 mins at RT, rinsed in PBS, permeabilized in 0.2% saponin (Sigma) PBS solution for 24 hours at 4°C, and blocked in 10% BSA solution for 24 hours at 4°C. For vascular staining, muscles were incubated in 5 μ g/mL anti-mouse/rat CD31/PECAM-1 primary antibody (R&D Systems) in a 1.0% BSA, 0.3% Triton X-100 (Amresco), 0.01% sodium azide solution (incubation buffer) overnight at 4°C, followed by 4 30-min washes in 0.2% saponin solution. Muscles were then stained in a 1:200 dilution of NL557-conjugated anti-goat IgG secondary antibody (R&D Systems) in incubation buffer for 4 hours at RT, then washed in 0.2% saponin solution and PBS twice each for 30 mins. As a negative control, samples were stained using the same protocol but with polyclonal goat IgG isotype control (R&D Systems) in place of the primary antibody. Finally, muscles were incubated in a 1:1000 dilution of Hoechst cellular stain in PBS for 5 mins. Muscles were whole-mounted and single fluorescent images were obtained using a Zeiss LSM 700 confocal microscopy with a 10 $\times$ objective to visualize vasculature (green) within muscle tissue (blue); n = 2 animals per group.

2.13. Statistical analysis

All data are presented as mean $\pm$ standard deviation. One-way analysis of variance (ANOVA) and Tukey's post hoc multiple comparison test with a significance value set at p 0.05 were used to identify significant differences. Statistical analysis was performed with Prism software.

3. Results

3.1. Materials characterization

Using ^1H NMR analysis, PEGDA was determined to be ~55% functionalized and Hep^{-N} methacrylamide was determined to be 22-28% functionalized [36]. SAX-HPLC analysis was also used to assess Hep and Hep^{-N} disaccharide composition. Disaccharide elution patterns showed evidence of N-desulfation when comparing Hep^{-N} against Hep samples (Table S1). Finally, using SEC-HPLC, the weight average molecular weight was determined to be ~13.5 kDa for Hep^{-N} compared to ~18.4 kDa for Hep.

3.2. Microparticle fabrication

Degradable 10 wt% Hep^{-N} MPs were found to be 62 ± 65 μm in diameter (Figure 1B and S1). Additionally, by varying DTT concentration within MPs, MP degradation ranged between 30 days for 20 mM DTT to 8 days for 40 mM DTT (Figure S2). MPs used in all subsequent studies contained 35 mM DTT, which degraded within 16 days *in vitro* (Figure S2).

3.3. *In vitro* SDF-1 α loading and release from microparticles

For all MP batches, 370 ± 50 ng was loaded onto MPs and 155 ± 10 ng SDF-1 α was released from MPs over ~24 hours *in vitro* (Figure 1C). Using the ~155 ng dose of SDF-1 α , a small *in vivo* pilot study was conducted and results indicated that ~155 ng SDF-1 α induced significant cell recruitment compared to injury alone. Once ~155 ng SDF-1 α was determined to be an effective dose, we ensured that ~155 ng SDF-1 α was released from all subsequent batches of MPs by varying the initial mass of SDF-1 α added to each batch of MPs between 1.0-1.2 μg SDF-1 α and by conducting an *in vitro* release study prior to each surgery. The pilot study and additional data was combined and is shown in Figure 3 and 4.

3.4. *In vivo* injection of microparticles

Intact, non-degradable AF633 Hep^{-N} MPs (red) were visible within the supraspinatus muscle (blue) 3 and 7 days following injection (Figure 2Bi-ii), whereas degradable AF633 Hep^{-N} MPs were present at day 3 but no longer detectable by day 7 (Figure 2Biii-iv).

3.5. Inflammatory cell analysis

Supraspinatus muscles were analyzed for inflammatory cell infiltration via flow cytometry at days 3 and 7. Background fluorescence from antibody binding to inflammatory cell Fc receptors was tested by incubating separate samples with Fc blocker (CD16/32, BioLegend), and no significant differences were observed with any inflammatory cell population between

Fc blocked and non-Fc blocked samples (Figure S6). Therefore, Fc blocking was not used for the experiments and data presented in Figure 3.

For unloaded MPs, there was small but significant elevation in total myeloid cells ($1.8 \pm 0.6X$ compared to contralateral control) and macrophages ($1.7 \pm 0.6X$) compared to the uninjured contralateral controls after 3 days (Figure 3A-B). For all other groups, there were no significant differences in myeloid cells (1.3 - $2.1X$), macrophages (1.2 - $1.9X$), or macrophage subpopulations (1.1 - $2.6X$) between the experimental groups and the uninjured contralateral controls after 3 days (Figure 3A-D). There were also no differences between any of the experimental groups including SDF-1 α loaded MPs, unloaded MPs, and injury only at the day 3 time point.

In contrast, 7 days following injury, significantly more M2-like macrophages were observed in muscle treated with SDF-1 α loaded MPs than uninjured controls ($4.3 \pm 1.9X$), unloaded MPs ($1.4 \pm 0.4X$) and injury alone ($2.2 \pm 1.2X$, Figure 3H). There were also no significant differences in M1-like macrophages between groups (Figure 3G). Notably, significantly more total myeloid cells (2.0 - $2.3X$) and macrophages (1.6 - $2.1X$) were detected in each experimental group compared to the respective uninjured contralateral controls (Figure 3E-F), though experimental groups were not significantly different from each other.

3.6. Mesenchymal stem cell analysis

Similar to the trends seen in inflammatory cell analysis, there were no significant differences in MSC recruitment between any experimental group or between each experimental group and the uninjured contralateral controls at day 3 (1.1 - $1.4X$, Figure 4A). However, by day 7 there were significantly more MSCs in both the SDF-1 α loaded MP group ($3.0 \pm 0.8X$) and the unloaded MP group ($1.7 \pm 0.6X$) compared to their respective uninjured contralateral controls, and significantly more MSCs were recruited to the SDF-1 α loaded MP group than unloaded MPs and injury alone (Figure 4B).

3.7. Vascular staining of whole-mounted supraspinatus muscle

Compared to uninjured contralateral controls that had little CD31+ vascular staining (Figure 5A), CD31+ vascular staining was present in the injury only control (Figure 5B).

Furthermore, CD31+ vascular looping, a product of rapid angiogenesis whereby vessels elongate and form loops of vasculature [40], also appeared to be present in muscles treated with SDF-1 α loaded MPs (Figure 5C). No staining was observed in samples prepared with isotype controls (data not shown).

4. Discussion

Despite the well-documented ability for SDF-1 α to induce chemotaxis of pro-regenerative cell populations, SDF-1 α treatment has yet to be explored in the unique cellular context of muscle degeneration following rotator cuff tear. Furthermore, as current standard-of-care reattachment surgery for rotator cuff tear largely neglects the muscle tissue, leading to further degeneration and an increased likelihood of re-tear [12,13], SDF-1 α may be a promising strategy to recruit pro-regenerative cell populations to the degenerating muscle for improved rotator cuff repair prognoses in the future. We therefore developed injectable 10

wt% Hep^{-N} MPs capable of releasing bioactive SDF-1 α and subsequently degrading within 1-2 weeks (Figure 1A-B) to enable further investigation and manipulation of cell populations within the rotator cuff muscle.

By incorporating varying concentrations of DTT within the MP crosslinking network, it is thought that the thioether group established between PEGDA and DTT increases the atomic charge of the carbonyl carbon within the PEGDA molecule, thereby increasing its reactivity with water and ultimately resulting in ester hydrolysis [19,41]. Thus, DTT concentrations of 20-40 mM, all of which were previously shown to be non-toxic in *in vitro* studies [42], were incorporated within 10 wt% Hep^{-N} MPs and resulted in degradation within 8 to 30 days *in vitro* (Figure S2). Furthermore, while non-degradable MPs (0 mM DTT) remained intact over 7 days *in vivo*, degradable MPs (35 mM DTT) were no longer observable 7 days following injection in the supraspinatus muscle (Figure 2). Though we observed that MPs with 35 mM DTT degraded within 16 days *in vitro*, MP degradation rate can be concentration-dependent (data not shown) which may account for the differences observed between our *in vitro* and *in vivo* results. Furthermore, upon activation following injury or biomaterial implantation, cells including macrophages may secrete acids which reduce pH and accelerate ester hydrolysis [43], reactive oxygen intermediates which promote oxidation-mediated cleavage of the PEG ether backbone [44], and enzymes including esterases which catalyze the cleavage of PEGDA ester bonds [45], all which may have contributed to the accelerated MP degradation observed *in vivo*. Ultimately, 10 wt% Hep^{-N} MPs with 35 mM DTT degraded within 16 days *in vitro* and 7 days *in vivo*, and these particles were utilized in all subsequent *in vivo* studies to enable release of SDF-1 α .

Next, we assessed *in vitro* and *in vivo* SDF-1 α release from the Hep^{-N}-based MPs. In our system, though MP degradation occurred over 1-2 weeks *in vitro*, most of the ~155 ng SDF-1 α released from MPs within 3 days *in vitro* (Figure 1C). *In vivo*, fluorescently labelled SDF-1 α was observed for at least 3 days following SDF-1 α loaded MP injection, whereas little to no SDF-1 α signal was observed after 3 days following soluble SDF-1 α injection (Figure S4), which may indicate that MPs can retain SDF-1 α at the site of injury for a longer period of time than soluble SDF-1 α alone. It is important to note, however, that since the dose of MPs required for the *in vivo* tracking study was significantly higher due to sensitivity of the instrument, direct comparison with *in vitro* studies is not possible. Overall, this general release profile is similar to many other SDF-1 α hydrogel and MP delivery systems, whereby more than 60% of all SDF-1 α released over the time course of the experiment occurred within the first 1-3 days in all but one of these systems [5,7,9,21,23,46]. However, to improve the release kinetics of SDF-1 α in future studies, one method may be to adjust the heparin-SDF-1 α molar ratio, as increasing the heparin-to-protein molar ratio has previously been shown to reduce the release rate of basic fibroblast growth factor (bFGF) in a theoretical model of bFGF and heparin-containing biomaterials [47].

To assess the ability for SDF-1 α loaded MPs to modulate the cellular milieu within the supraspinatus muscle, we first assessed how injury alone affected the muscle and its inflammatory cell and MSC populations. First, a significant decrease in supraspinatus muscle weight was observed after 6 weeks and a significant increase in fibrous infiltration

was observed after only 3 weeks, both of which mimic symptoms of the human condition and indicate that significant muscle degeneration was induced within our animal model (Figure S3) [12,13]. Concurrently, we assessed changes in the cellular milieu following injury and while no significant differences in the inflammatory cell populations were observed at day 3, significantly more myeloid cells and macrophages were observed in the muscle from injured rotator cuffs compared to uninjured controls by day 7 (Figure 3E-F). Similar findings were also observed in our previous characterization of rotator cuff injury in mice, where significantly more mononuclear phagocytes, monocytes, macrophages, and dendritic cells were observed in the muscle from injured rotator cuffs compared to uninjured controls after 7 days [17]. Similar to injury alone, significantly more myeloid cells and total macrophages were also recruited following unloaded MP treatment at day 3 and day 7 (Figure 3A-B, E-F), and in no case was the unloaded MP group significantly different from the injury only group, which indicates that unloaded MPs did not elicit an additional inflammatory response. Overall, tendon transection and denervation resulted in significant total myeloid cell and macrophage recruitment after 7 days, and this characterization provided a baseline to further interrogate effect of SDF-1 α release on the supraspinatus cellular milieu.

In this injury model, after SDF-1 α loaded MP treatment, little change to inflammatory cell populations was observed after 3 days and, while significantly more total myeloid cells and macrophages were detected compared to uninjured controls after 7 days, this cell recruitment was not significantly different from unloaded MPs or injury alone (Figure 3). In analyzing macrophage subpopulations, however, significantly more M2-like macrophages were present at day 7 in muscle treated with SDF-1 α loaded MPs than all other groups, including uninjured, injured, and unloaded MPs (Figure 3H). To understand the timing of cell recruitment observed, it is important to note that while much of the SDF-1 α was released over 1-3 days, additional time may be required for a significant cellular response to be observed. For example, monocyte migration to sites of muscle injury typically peaks 7 days following injury [48] and monocyte to macrophage differentiation can require up to 10 days depending on the microenvironment [49,50]. Furthermore, our present findings parallel our previous work using a mouse backpack model, whereby significantly more M2-like macrophages were located near a subcutaneously implanted SDF-1 α loaded hydrogel after 7 days compared to unloaded hydrogels [11]. Finally, as our *in vivo* study of SDF-1 α release from MPs indicates that a small percentage of SDF-1 α may remain near the site of injection for at least 3 days, it is possible that this residual SDF-1 α observed at later time points may have contributed to the significant differences in cell recruitment observed at day 7.

The selective recruitment of M2 macrophages in this system may be explained by the fact that studies have found that as monocytes underwent *in vitro* macrophage differentiation, CXCR4 (the primary SDF-1 α receptor) gene expression increased 10-fold after 7 days [51], and in our recent work, anti-inflammatory monocytes exhibited higher CXCR4 surface expression than inflammatory monocytes [10]. In the same study, SDF-1 α loaded 10 wt% Hep^N hydrogels enabled a shift in monocyte composition, whereby significantly more anti-inflammatory monocytes and fewer pro-inflammatory monocytes were present compared to unloaded gels. Thus, it is possible that while SDF-1 α release was unable to significantly affect the broader, more heterogeneous population of leukocytes, SDF-1 α loaded MP

treatment could lead to significant enrichment of high CXCR4-expressing M2-like macrophages.

In the context of muscle regeneration, it is important to note that M1 and M2 macrophages, which exist on a spectrum between pro and anti-inflammatory phenotypes, possess unique and critical functions to muscle repair. While M1 macrophages have been shown to infiltrate quickly following muscle injury and act to phagocytose cellular debris and secrete pro-inflammatory proteins [37,52], M2 macrophages secrete anti-inflammatory proteins [37,53] and enhance the proliferation and differentiation of satellite cells [54], a muscle-derived stem cell subpopulation and the primary contributor to the reparative phase of muscle regeneration [55]. Thus, while significantly more anti-inflammatory, M2-like macrophages were observed in the muscle following SDF-1 α loaded MPs treatment, additional studies are required to fully elucidate the optimal balance between M1 and M2 macrophages for muscle regeneration following rotator cuff injury.

SDF-1 α loaded MPs also recruited significantly more MSCs compared to unloaded MPs, injury only, and uninjured contralateral controls 7 days following treatment (Figure 5). As MSCs have been previously utilized as a potential therapeutic in a variety of muscle injury types, several studies have investigated the potential mechanisms by which MSCs exhibit their muscle-regenerating capacity. Among these studies, some have shown that MSCs undergo myogenic differentiation *in vivo* [56], while others suggest that the MSC protein secretion profile may be primarily responsible for the regenerative effects of MSCs [57]. Specifically, MSCs possess many immunomodulatory functions, such as secreting proteins that mediate macrophage recruitment, mitigating the inflammatory response to injury, and promoting the polarization of pro-inflammatory, M1 macrophages into a more anti-inflammatory, M2 macrophage phenotype [58]. This immunomodulatory relationship is reciprocal as well, as macrophages have been shown to regulate MSC differentiation and promote MSC secretion of anti-inflammatory proteins such as TNF- α stimulated gene-6 (TSG-6) and pro-angiogenic proteins such as angiopoietin-1 [58,59]. Thus, while SDF-1 α loaded MPs may have initiated the enrichment of M2-like macrophages and the recruitment MSCs, it is possible that the reciprocal and synergistic relationship between these cell populations will enable additional modulation of the cellular milieu toward a more pro-regenerative microenvironment.

Both MSCs and M2 macrophages have previously shown to facilitate vascular remodeling and, in addition, it is well known that SDF-1 α can recruit other pro-angiogenic cells including HSCs and smooth muscle cell progenitors [60] which can lead to collateralization, sprouting, looping, and splitting of new microvasculature [40,61,62]. In our studies, whole-mount imaging of the supraspinatus muscle 7 days following injury suggested that while CD31+ vascular staining was present in injured muscle, CD31+ vascular looping appeared present in the SDF-1 α loaded MP group (Figure 5). As vascular looping is known to be a step of mature vessel formation, which can in turn support the regeneration of injured tissue [63,64] including muscle [65], the recruitment of pro-regenerative and pro-angiogenic cells to the supraspinatus muscle via SDF-1 α loaded MPs may ultimately improve muscle healing in the context of rotator cuff injury.

5. Conclusion

In this manuscript, we developed a heparin-based microparticle platform to recruit pro-regenerative cells to the supraspinatus muscle following severe rotator cuff injury. To locally present SDF-1 α , degradable 10 wt% Hep^{-N} microparticles were fabricated and subsequently injected within the supraspinatus muscle. After 7 days, significantly more anti-inflammatory, M2-like macrophages (4.3X increase compared to no injury) and MSCs (3.0X increase compared to no injury) were detected in muscle treated with SDF-1 α loaded MPs compared to unloaded MPs or injury alone. While no prior studies have investigated the use of chemotactic therapeutics such as SDF-1 α to treat muscle following rotator cuff injury, our results indicate that SDF-1 α loaded MPs can shift the cellular composition of the supraspinatus muscle, which may provide a platform to improve muscle repair after rotator cuff injury in the future.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Summary

Following rotator cuff injury, significant muscle degeneration is common and can increase the likelihood of re-tear following surgical treatment. Therefore, we aimed to establish a more pro-healing microenvironment within the muscle following rotator cuff injury by developing an injectable, degradable biomaterial system to deliver stromal cell-derived factor-1alpha (SDF-1 α), a protein known to attract pro-healing cell populations. After 7 days, a 4.3x increase in anti-inflammatory, M2-like macrophages (CD11b+CD68+CD163+) and a 3.0x increase in mesenchymal stem cells (CD29+CD44+CD90+) was observed in muscles treated with our SDF-1 α -loaded biomaterial, suggesting that our biomaterial system may be a method to shift the cellular composition and create a more pro-regenerative microenvironment within muscle after rotator cuff injury.

Future work statement

Future work will investigate the ability for SDF-1 α loaded microparticles, which were shown in this work to recruit anti-inflammatory, M2-like macrophages and mesenchymal stem cells to the supraspinatus muscle following rotator cuff injury, to reduce muscle degeneration and improve muscle function after tendon tear.

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Figure 1. Degradable 10 wt% N-desulfated heparin microparticles released SDF-1α over 24 hours *in vitro*

(A) Microparticles were fabricated with 10 wt% N-desulfated heparin methacrylamide, 90 wt% linear poly (ethylene glycol) diacrylate and 35 mM dithiothreitol (DTT). (B) Phase image analysis indicated that microparticles were $\sim 62 \pm 65 \mu\text{m}$ in diameter; black arrows indicate microparticles; scale bar is 100 μm . (C) Microparticles released $\sim 155 \text{ ng}$ SDF-1α over ~ 3 days *in vitro*; $n = 3-5 \pm \text{SD}$.

Figure 2. Injected 10 wt% Hep^N microparticles were retained within the supraspinatus muscle for up to 7 days

(A) Experimental design: AlexaFluor633 tagged Hep^N was crosslinked within 10 wt% Hep^N microparticles and injected into the supraspinatus muscle immediately following injury, then tracked at day 3 and 7 via confocal microscopy. (Bi-ii) Non-degradable microparticles (red) remained within the muscle (nuclei in blue) for at least 7 days while (Biii-iv) degradable microparticles appeared to have degraded by day 7; white arrow heads indicate microparticles; scale bar is 100 μm.

Figure 3. SDF-1 α loaded microparticles recruited significantly more M2-like macrophages 7 days following injury and treatment

(A-D) No significant increase in cell recruitment was observed after 3 days except in (A) total myeloid cells and (B) total macrophages after treatment with unloaded microparticles compared to uninjured controls. (E-F) By day 7, significantly more total myeloid cells and macrophages were recruited to all experimental groups compared to uninjured controls. (G) While there were no differences in M1-like macrophage recruitment between any of the experimental groups, (H) SDF-1 α loaded microparticles recruited significantly more M2-like macrophages than all other groups. #Significantly greater than contralateral control at that time point; *Significantly different; $p \leq 0.05$; $n = 4-9 \pm SD$.

Figure 4. SDF-1 α loaded microparticles recruited significantly more mesenchymal stem cells 7 days following injury and treatment

(A) No significant increase in mesenchymal stem cell (MSC, CD29+CD44+CD90+) recruitment was observed after 3 days but (B) by day 7, significantly more MSCs were recruited to the SDF-1 α loaded microparticle group than all other groups. #Significantly greater than contralateral control; *Significantly different; $p \leq 0.05$; $n = 4-11 \pm SD$.

Figure 5. Vascular looping was observed in SDF-1 α loaded MP treated supraspinatus muscle Supraspinatus muscles treated with SDF-1 α loaded MPs (C) appear to exhibit CD31+ vascular looping 7 days following injury and treatment compared to uninjured (A) or injured (B) controls; blue = Hoechst stained nuclei, green = CD31+ vasculature; white arrows indicate vascular loops; scale bar is 50 μ m; n = 2.