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Osteogenic Differentiation of Human Mesenchymal Stem Cells through Alginate-graft-Poly(Ethylene Glycol) Microsphere-Mediated Intracellular Growth Factor Delivery

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Abstract

The intracellular delivery of growth factors increases opportunities for controlling cell behavior and maintaining tissue homeostasis. Recently, VEGFA was reported to enhance osteogenic differentiation of mesenchymal stem cells (MSCs) through an intracrine mechanism, suggesting a new strategy to promote bone tissue formation in osteoporotic patients. The goal of this study was to design and fabricate ligand-conjugated alginate-graft-poly(ethylene glycol) microspheres for intracellular delivery and release of VEGFA in primary human MSCs to enhance osteogenic differentiation as a potential therapeutic. Three types of microspheres were synthesized and characterized by scanning electron microscopy, in vitro drug release kinetics, MSC uptake and internalization: alginate alone (Alg), alginate-graft-poly(ethylene glycol) (Alg-g-PEG) and alginate-graft-poly(ethylene glycol)-S-S-arginine-glycine-aspartic acid (Alg-g-RGD). Each of the different microsphere formulations successfully transported bioactive VEGFA into primary human MSCs within 48 h of culture, and significantly enhanced osteogenic differentiation compared to control treatments with empty microspheres (intracellular control) or non-encapsulated VEGFA (extracellular control). Adipogenic differentiation was not affected by the presence of VEGFA intracellularly or extracellularly. These results demonstrating the internalization of alginate-based

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microspheres and intracellular delivery of VEGFA support the efficacy of using this drug delivery and intracrine mechanism to control the fate of human MSCs and enhance osteogenic differentiation.

1. Introduction

Osteoporosis is defined by a reduction in the quantity and quality of bone that results in skeletal fragility [1]. As people age, bone resorption rates become unbalanced and bone resorption dominates over bone formation, which leads to reduced bone mass and altered bone architecture. Human bone marrow progenitor cells known as multipotent stromal cells or mesenchymal stem cells (MSCs) can differentiate into osteoblasts, chondrocytes and adipocytes and show promise for clinical bone repair [2]. A notable recent study with murine MSCs showed that intracellular as opposed to extracellular signaling of vascular endothelial growth factor A (VEGFA) had the ability to distinctly regulate MSC lineage commitment toward osteoblasts or adipocytes [3]. Liu *et al.* (2012) demonstrated that murine bone marrow stromal cells were more likely to differentiate into osteoblasts when MSC expressed VEGFA and possessed intracellular VEGFA, compared with no change in osteoblast differentiation following exposure of MSCs to extracellular VEGFA. The investigators proposed that VEGFA regulated differentiation through an intracrine mechanism unique to the intracellular form of VEGFA and different from the typical mechanism for secreted, extracellular VEGFA and signaling through cell surface receptors. For patients, the intracellular delivery of VEGFA into MSCs prior to cell therapy could be a potential approach to speed the repair of large or complex bone fractures or perhaps even to treat chronic skeletal diseases such as osteoporosis [4]. In this work, we developed and functionally tested a specialized drug delivery system designed to accomplish the controlled, intracellular delivery of VEGFA in MSCs.

Modern drug delivery systems are designed to maintain the structure and bioactivity of biomolecules and to release therapeutics in a controlled and predictable manner. Micro-encapsulation is one of the core technologies used in polymer drug delivery systems [5]. Polymeric particles or microspheres for controlled drug delivery applications are designed to provide uniform particle dimensions, shield the drug from the extracellular environment, and be biocompatible [6]. Alginate microspheres have attracted much attention for the development of controlled- and sustained-release drug delivery systems for proteins [7], cytokines [8] and cells [9, 10]. Alginate is a naturally occurring polysaccharide extracted from brown seaweed that is generally regarded as non-toxic. The fabrication of alginate microspheres is favorable for drug delivery due to the relatively mild yet rapid gelation process that omits the use of harsh chemicals to ensure stability of encapsulated biomolecules [11-14]. However, the application of alginate microspheres has been limited due to their relatively large diameters (10 – 100 μm) and rapid drug-release rates (< 24 h) [15]. In addition, the anionic nature of alginate interferes with the encapsulation and release of charged molecules such as polyelectrolytes. To overcome these limitations, alginate microspheres have been refined by varying molecular weight and concentration, stirring conditions, degree of crosslinking, and chemical modification of the alginate polymer to achieve sustained drug release [16, 17].

Poly(ethylene glycol) (PEG) is the most widely applied synthetic polymer in the emerging field of biomaterials for drug delivery. Recently, alginate has been modified with PEG for the fabrication of microcapsule coatings [18], mucoadhesive polymers [19, 20], self-assembling nanospheres [21, 22] and hybrid microspheres [23, 24]. The ability of PEG to influence the pharmacokinetic properties of drugs and drug carriers has been used to modify many different pharmaceutical compounds and components [25]. Biocompatibility and stealth behavior make PEG an ideal material to avoid opsonization and subsequent elimination by the reticuloendothelial system [26]. In addition, PEG-modified products are less immunogenic and antigenic; hemolysis and aggregation of erythrocytes may also decrease, as can the risk of embolism. However, the lack of a cell adhesion ligand limits its application in targeted drug delivery systems. To address this issue, we developed novel PEG-modified alginate copolymer microspheres with and without the surface conjugation of a bioactive cell adhesion ligand.

Proteins containing tri-peptide arginine-glycine-aspartate (RGD) attachment sites, along with the integrins serving as receptors for them, constitute a major recognition system for cell adhesion to the extracellular matrix (ECM) [27]. Consequently, researchers continue to utilize RGD in order to mimic cell adhesion proteins and bind to integrins [28], inhibit apoptosis, angiogenesis, and tumor formation [29], coat surfaces for use as biomaterials [30], and enhance drug delivery systems [31, 32], including microspheres [33]. In this study RGD was utilized as a model cell-recognition molecule to demonstrate the efficacy of immobilizing ligands onto microsphere surfaces. Our results demonstrate that the incorporation of an adhesion ligand onto the surface of alginate-graft-PEG (Alg-g-PEG) microspheres containing VEGFA provides a promising strategy to regulate osteoprogenitor cell differentiation and bone tissue homeostasis, in addition to providing efficacy for the use of surface ligands for cell-targeted therapeutic applications *in vivo*.

2. Materials and methods

2.1 Materials

Sodium alginate ($M_w = 65\text{--}75$ kg/mol, 60-70% guluronic acid residues) was generously donated by FMC BioPolymer. Cysteine-L-arginyl-glycyl-L-aspartic acid (CRGD) was purchased from Genscript. Amine-poly(ethylene glycol)-thiol ($\text{NH}_2\text{-PEG-SH}$, $M_w = 1000$ g/mol) and methyl-poly(ethylene glycol)-amine (mPEG- NH_2 , $M_w = 500$ g/mol) were purchased from Laysan Bio. N-ethyl-N'(3-dimethylaminopropyl) carbodiimide hydrochloric acid (EDC), N-hydroxysuccinimide (NHS), 2,2'-dithiodipyridine, methanol (MeOH, anhydrous, 99.8%), biology-grade mineral oil, Span 80, Tween 80, ethylenediaminetetraacetic acid (EDTA), dexamethasone, ascorbic acid, β -glycerol phosphate, hydrocortisone, isobutylmethylxanthine, indomethacin, deuterium oxide (D_2O), the *In Vitro* Toxicology Assay Kit (MTT-based), Hoechst 33342 and Alizarin red were purchased from Sigma-Aldrich. One molar hydrochloric acid (HCl) and 1 M sodium hydroxide were purchased from BDH ARISTAR®PLUS. Dichloromethane (DCM, 99.9%), sodium citrate, isopropanol, sodium chloride (NaCl), sodium acetate (NaAc), alpha-modified eagle medium (α -MEM, Hyclone), DyLight 550 Microscale Antibody Labeling Kit, and 20X phosphate buffered saline (PBS) were purchased from Fisher Scientific. Fetal

bovine serum (FBS) was purchased from Atlanta Biologics and screened for a lot that best supported growth of human MSCs. Penicillin, streptomycin and Trypsin EDTA were purchased from Corning Cellgro. AdipoRedTM was purchased from Lonza Inc. Human VEGF DuoSet and human osteoprotegerin ELISA Kits were purchased from R&D Systems. Oligo (dT) and dNTP mix were purchased from Promega Inc. A Roche High Pure RNA Isolation Kit was purchased from Life Science. Polymerase chain reaction (PCR) primers were purchased from Integrated DNA Technologies.

2.2 Alg-g-PEG-S-S-Pyridine copolymer synthesis

First, NH₂-PEG-SH (0.1 mg, 0.1 mM) was dissolved in 5 mL of de-gassed acetate buffer (0.1 N sodium acetate adjusted to pH 4.6 with acetic acid, 0.3 M sodium chloride, and 1 mM EDTA) into which a solution of 2,2'-dithiodipyridine (88.124 mg, 0.4 mM) dissolved in 10 mL of MeOH was added; the mixture was stirred at room temperature under a flowing N₂ atmosphere for 4 h. The MeOH was extracted three times with DCM and the sample was prepared for ¹H-NMR analysis [34].

Alginate was modified with the newly formed NH₂-PEG-S-S-pyridine using EDC and NHS chemistry. The COOH:EDC:NHS molar ratio remained consistent at 1:8:3.2 during the carbodiimide reactions, where COOH refers to the moles of alginate carboxyl groups. A 1% (w/v) alginate solution was adjusted to pH 5 with 1 N HCl. EDC was then added followed by NHS and the solution was mixed at room temperature for 30 min. NH₂-PEG-S-S-pyridine and mPEG-NH₂ (the control PEG graft chain) were added to separate alginate solutions, respectively; the target degrees of PEG modification of the alginate was 10 %. After 12 h of reacting at room temperature, the various alginate and PEG polymer solutions were placed into dialysis cassettes (MWCO 20 kDa, Pierce Biotechnology) and dialyzed against deionized (DI) water for 4 d; dialysis solution was changed every 12 h. The Alg-g-PEG-S-S-pyridine copolymer product was lyophilized and the powder was stored in a desiccator until use. PEG retention post-fabrication was verified using ¹³C-NMR (Bruker AVANCE III 500 MHz high-field NMR spectrometer) in D₂O [34]. As a control, Alg-g-PEG copolymers were synthesized with this approach using mPEG-NH₂. The polymer was dissolved in D₂O and the result was verified via ¹H-NMR (Bruker AVANCE III 500 MHz high-field NMR spectrometer).

2.3 Microsphere fabrication

To fabricate microspheres containing VEGFA, Alg-g-PEG-S-S-pyridine was dissolved in phosphate buffered saline (PBS) with pH pre-adjusted to 5.0 with 1 N HCl. Non-modified alginate (Alg) and Alg-g-PEG microspheres were fabricated using a 1% (w/v) polymer solution. VEGFA was added to the copolymer solution at a ratio of 10⁶:1 (copolymer:VEGFA). Microspheres without VEGFA were fabricated as blank controls. One mL of polymer/VEGFA solution was slowly added to 6.72 mL of biological-grade mineral oil containing 5% (v/v) Span 80 while mixing at 1200 rpm for 5 min at room temperature. Next, 400 µL of 30% (v/v) Tween 80 was added and the emulsion was mixed for an additional 5 min. Then, 5 mL of 1 M calcium chloride (CaCl₂) solution was added slowly. After 30 min of mixing, 3 mL of isopropanol was added to the emulsion and allowed to mix for 5 min, then was centrifuged at 400 rpm for 5 min to precipitate microspheres. The

microspheres were washed sequentially with isopropanol ($\times 2$) and DI water ($\times 2$), respectively, and centrifuged after each wash. The Alg-g-PEG-S-S-pyridine microspheres were formed in a similar fashion, however, the DI water wash was replaced with a 1% (w/v) CRGD solution ($\times 2$); the corresponding microspheres are here-after identified as Alg-g-RGD. Microspheres were flash frozen in liquid N_2 .

After lyophilization Alg, Alg-g-PEG and Alg-g-RGD microspheres were characterized by scanning electronic microscopy (SEM, JEOL 600; samples were sputter coated with 45 nm of gold). SEM micrographs of various magnifications were used to quantify microsphere diameters; images were analyzed using ImageJ (3 images per group).

2.4 Cytotoxicity assay

Human MSCs were isolated from bone marrow aspirates with an IRB-approved protocol. Human MSCs (passage 7) were seeded in 24-well tissue culture polystyrene (TCPS) plates at a density of 20,000 cells/well in 500 μ L/well of standard MSC growth medium (α -MEM, 10% FBS, 100 U/mL penicillin, 100 μ g/mL streptomycin) and allowed to adhere for 24 h. Cells were incubated in the presence of Alg, Alg-g-PEG, and Alg-g-RGD microspheres encapsulating VEGFA ($n = 3$ per group) at concentrations of 10, 50, 100, 500 μ g/mL at 37°C and 5% CO_2 . After 24 h of incubation, medium containing the microspheres was removed, and cells were rinsed two times in sterile PBS then analyzed using a MTT-based In Vitro Toxicology Assay Kit following the manufacturer's protocol. The optical density was measured at 570 nm using a BioTek plate reader. Background absorbance at 690 nm was subtracted from the measured absorbance. Absorbance values for the experimental and control samples were normalized to non-modified TCPS controls [35].

2.5 DyLight 550 labeling of human VEGFA

Recombinant human VEGFA was cloned, expressed in HEK 293 cells, and purified in the Spees laboratory [36]. 1 mg/mL of purified VEGFA in PBS was used for labeling. The DyLight Microscale Antibody Labeling Kit was used according to the manufacturer's instructions. Briefly, 100 μ L of 1 mg/mL protein was mixed with a commercial vial of DyLight 550 reagent and incubated for 60 min in the dark after gentle vortexing and a quick centrifugation step to mix the sample and dye. Spin columns provided by the kit were then placed into microfuge collection tubes and used for purification of protein from unbound dye. The labeling solution was mixed with purification resin and centrifuged in the spin column to purify labeled protein. The dye:protein ratio was determined based on methods described in the labeling kit.

2.6 VEGFA encapsulation and release

A known amount of lyophilized VEGFA-loaded Alg, Alg-g-PEG and Alg-g-RGD microspheres was dissolved by immersion in 3% (w/v) sodium citrate solution to dissolve the microspheres by displacing calcium ions [37]. The VEGFA concentration was measured with a DuoSet ELISA Development Kit. Briefly, standard series and sample solutions were added to a culture plate (100 μ L/well) pre-coated with capture antibody after washing in mild detergent (0.05% Tween 20 in PBS). After 2 h of blocking and incubation at room temperature, the biotinconjugated detection antibody was added and incubated for another 2

h. Then 100 μ L poly HRP conjugated streptavidin substrate was incubated in each well for 20 min after washing the detection antibody, followed by incubation with 100 μ L substrate (ABTS, Thermo Scientific.) in the dark at room temperature. The absorbance was measured at 450 nm with a BioRad microplate reader. The VEGFA concentration was determined with a standard curve, and the VEGFA encapsulation efficiency (mass of VEGFA encapsulated in the microspheres / mass of VEGFA added when forming the microspheres) was calculated.

A VEGFA release test was performed in a 48-well plate at 37°C. Six mg lyophilized VEGFA-loaded Alg, Alg-g-PEG and Alg-g-RGD microspheres was dissolved in 500 μ L of PBS (pH 7.4). At each time point (1, 2, 4, 8, 12, and 24 h, followed by collection each day for a total of 14 d) 100 μ L of the PBS was removed and another 100 μ L was added to maintain the total volume. The released VEGFA concentration was determined with the DuoSet ELISA Development Kit described above.

2.7 Human MSC intracellular delivery

Green fluorescent protein (GFP)-labeled primary human MSCs were generated by lentiviral transduction used to express the gene and enriched by selecting for GFP-positive cells by fluorescence activated cell sorting (FACS) [38, 39]. DyLight 550-labeled VEGFA (red fluorescence) was incorporated within Alg, Alg-g-PEG and Alg-g-RGD microspheres using the same above approach except the ratio of polymer to VEGFA was increased to 10^5 :1 to increase the intensity of the visualized signal. Microspheres (500 μ g/mL) were cultured with GFP-labeled primary human MSCs (100,000 cells, passage 4, green fluorescence) for 24 h in glass bottom culture dishes pre-coated with poly-d-lysine (MatTek Corporation). Confocal laser scanning (CLS) microscopy (Zeiss LSM 510 META) was used to visualize the microspheres by detecting directly through the plate. Z-stack images were formed with the use of AimImage Software.

2.8 Human MSC differentiation assays

2.8.1 Colorimetric osteogenesis and adipogenesis assays—Human MSCs (passage 4) were seeded in 12-well TCPS plates at a seeding density of 50,000 cells/well. Cells were cultured at 37°C and 5% CO₂ in standard MSC growth medium containing 100 μ g/mL of VEGFA-encapsulated microspheres (Alg, Alg-g-PEG or Alg-g-RGD) or blank microspheres not containing VEGFA. The experiments were done in triplicate. After 48 h, old medium was aspirated off and one plate was given osteogenic differentiation medium (growth medium including 1 nM dexamethasone, 50 μ g/ml ascorbic acid, 10 mM β -glycerol phosphate) and the other plate received adipogenic differentiation medium (growth medium including 0.5 μ M hydrocortisone, 0.5mM isobutylmethylxanthine, 60 μ M indomethacine). Cells that served as controls were cultured without the addition of microspheres (either with VEGFA or without VEGFA). Differentiation medium was changed every 3 d. After 14 d of culture in differentiation medium, cells were rinsed with sterile PBS and analyzed via an Alizarin Red osteogenic differentiation assay [40, 41] or an AdipoRedTM adipogenesis assay (Sigma).

In addition, six different concentrations of VEGFA ranging from 0 to 20 ng/ml (0, 0.01, 0.025, 0.05, 0.5, 20 ng/ml) were incubated with human MSCs for the control assay (extracellular VEGFA). The extracellular VEGFA differentiation experiment was conducted in 24-well TCPS plates with a seeding density of 30,000 cells/well. After 48 h, medium containing VEGFA was aspirated off and the plates were given either osteogenic or adipogenic differentiation medium. Differentiation medium was changed every 3 d. After 7 d of culture in differentiation medium, cells were rinsed with sterile PBS and analyzed via an Alizarin Red osteogenic differentiation assay [40, 41] or an AdipoRedTM adipogenic differentiation assay (Sigma).

2.8.2 Osteoprotegerin ELISA—Protein analysis was performed on blank (i.e., empty) and VEGFA-encapsulated Alg-g-PEG microsphere-treated hMSCs after 14 days of culture in osteogenic differentiation medium. To determine the concentration of osteoprotegerin in the supernatant of culture medium, an ELISA was performed with human osteoprotegerin ELISA DuoSet Kit according to the manufacturer's instruction.

2.8.3 Reverse transcription polymerase chain reaction (RT-PCR)—To quantitatively determine gene and protein information from differentiated MSCs, we selected on experimental microsphere group, Alg-g-PEG, to detect osteogenesis marker via RTPCR and ELISA. Primary human MSCs were exposed to VEGFA-encapsulated Alg-g-PEG microspheres, blank Alg-g-PEG microspheres or an empty control (no microspheres) for 48 h and then in osteogenic differentiation medium for 14 days. After differentiation, half of the cells were lysed to extract RNA; RNA extraction was performed using a Roche High Pure RNA Isolation Kit according to the manufacturer's instructions. Total RNA (0.25 µg) was reverse-transcribed in a 15 µl reaction using SuperScript[®] III CellsDirectTM cDNA Synthesis Kit (Invitrogen) according to the manufacturer's instructions. The PCR reactions were conducted using 25 µl PCR super mix (Invitrogen), 2 µl of each sample and 1 µl of each primer under the following conditions: 94 °C for 5 min, followed by 35 cycles of 3.5 minutes denaturation at 94 °C, 30 s annealing at 53 °C, and 60 s extension at 72 °C. The cycles were followed by a denaturation at 72 °C for 2 min. PCR products were separated using agarose gel with 1% ethidium bromide. Primers used included: 5'-ATGAGAGCCCTCACA CTCCTC-3' (sense) and 5'-CGTAGAAGCGCCGAT AGGC-3' (antisense) for osteocalcin; 5'-GATGACACTGCCACCTCTGA-3' (sense) and 5'-GACTGGCGGGGTGTA AGT AA-3' (antisense) for core-binding factor-α1 (RUNX2); 5'-GATGGATTCCAGTTC GAGTAT G-3' (sense) and 5'-GTTTGGGTTGCTTGCTG TTTG-3' (antisense) for collagen type I; 5'-GTCAGTGGTGGACCT GACCT-3' (sense) and 5'-AGGGGAGATTCACTG TGG TG-3' (antisense) for glyceraldehyde 3-phosphate dehydrogenase (GADPH) [42].

2.9 Statistical methods

All experiments were performed in triplicate; results are reported as mean ± standard deviation. Statistical analysis was performed on the cytotoxicity data and the differentiation data, respectively, using one-way ANOVA with Tukey multiple comparisons ($\alpha = 0.05$) via the SAS statistics program in the GLM procedure as the post-test to compare all of the groups. A $p < 0.05$ is considered significantly different.

3. Results

3.1 Synthesis of Alg-g-PEG and Alg-g-PEG-pyridine copolymers

The copolymer reactions for the chemical modification of alginate are shown in **Fig. 1**. To synthesize Alg-g-PEG-pyridine, the oligomer NH₂-PEG-S-S-pyridine was synthesized first. ¹H-NMR spectral analysis confirmed the presence of both PEG and pyridine in the final product (7.1 ppm, 7.5 ppm, 7.7 ppm, 7.9 ppm corresponding to the hydrogen on pyridine ring and 3.6 ppm corresponding to the hydrogen within the repeat group in PEG) when compared to the alginate and PEG controls (**Supplemental Fig. 1**). The large peak at 1.8 ppm corresponds to the excess acetate in the reaction system; this by-product does not interfere with the carbodiimide chemistry and can be removed through dialysis in the proceeding step of the copolymer reaction. Quantification of the reaction yield by peak integration determined approximately 70% reaction efficiency.

The natural polysaccharide alginate was grafted with a short chain PEG decorated with either pyridine or methyl end groups resulting in the copolymers Alg-g-PEG or Alg-g-PEG-pyridine. For the synthesis of Alg-g-PEG, ¹H-NMR spectral analysis confirmed the presence of both alginate and PEG functional groups in the final purified graft copolymers through the appearance of new peaks and peak shifts in the Alg-g-PEG spectrum (**Supplemental Fig. 2**); 3.36 ppm corresponds to the -CH₃ moiety of the PEG side chain and 4.31 ppm corresponds to the shift of hydrogen atoms due to the grafting of the PEG side chain when compared to the alginate and PEG controls. As stated in the methods, the theoretical degree of PEG modification was 10 molar % of alginate backbone. Quantification of PEG conjugation by peak integration of the ¹H NMR spectra was not successful due to extensive broadening and overlapping of peaks corresponding to alginate in the Alg-g-PEG spectra. The synthesis of Alg-g-PEG-pyridine was confirmed by ¹³C-NMR (**Supplemental Fig. 3**); 132.31 ppm, 136.05 ppm, 141.37 ppm, 143.53 ppm, 149.24 ppm correspond to carbon on the pyridine ring, 69.76 ppm corresponds to the carbon within the repeat unit of PEG, and 42.84 ppm corresponds to the carbon on the alginate. Verification of NH₂-PEG-S-S-pyridine by ¹³C-NMR confirmed that the reaction was successful.

3.2 Fabrication of Alg, Alg-g-PEG and Alg-g-RGD microspheres

Complete microsphere fabrication strategies are shown in **Fig. 2**. Alg, Alg-g-PEG and Alg-g-RGD microspheres were designed to encapsulate VEGFA without interfering with the bioactivity and electrostatically-condensed structure of VEGFA. The mild gelation method by the addition of CaCl₂ during an emulsion was successful in creating microspheres encapsulating VEGFA. The surface functionalization of microspheres through CRGD conjugation did not appear to affect the shape or yield of the microspheres. SEM photomicrographs (**Fig. 3A-F**) demonstrate that the microspheres (Alg, Alg-g-PEG and Alg-g-RGD) were spherical in shape with an average diameters and standard deviations of approximately 1.9 ± 1.0 μm, 0.5 ± 0.1 μm, and 1.1 ± 0.4 μm, respectively (**Fig. 3G**). VEGFA encapsulation efficiency values were 52, 22 and 35% respectively for the different microsphere groups; values represent an average of multiple batches within a group (n = 3).

3.3 Alg-g-PEG microspheres exhibit no cytotoxic effects

The viability of human MSCs in the presence of Alg, Alg-g-PEG and Alg-g-RGD microspheres containing no VEGFA was determined at increasing concentrations (10, 50, 100 and 500 $\mu\text{g/mL}$, **Fig. 3H**). The results demonstrate that Alg, Alg-g-PEG and Alg-g-RGD microspheres were non-toxic (cell viability > 90%) at concentrations up to 100 $\mu\text{g/mL}$. and cell viability was greater than 75% at a concentration as high as 500 $\mu\text{g/mL}$. A Tukey statistical test was performed to compare the experimental groups to the control groups ($n = 3$). No significant differences were seen between experimental microsphere groups at the same concentration ($p > 0.05$).

3.4 VEGFA *in vitro* release

VEGFA release from Alg, Alg-g-PEG and Alg-g-RGD microspheres are shown in **Fig. 4** as the total percent of VEGFA released, determined from cumulative VEGFA concentrations and encapsulation efficiencies. VEGFA encapsulation efficiency values were 52, 22 and 35% respectively for the different microsphere groups (**Fig. 3G**). After 48 h of release, the Alg, Alg-g-PEG and Alg-g-RGD microsphere groups released 573, 802, and 1509 pg of the encapsulated VEGFA. The percentages released for the microsphere groups were 17.7, 54.2 and 61.1 %, respectively (**Fig. 4A**). All of the microsphere groups sustained VEGFA release for 14 d (**Fig. 4B**). After 14 d of release, the percentages released for the microsphere groups were 43.8, 100, and 98.2 %, respectively. When comparing the 48 h release profile with the longer 14 d release profile, it is evident that a burst release within the first 12 h was followed by a gradual sustained release until the termination of the study.

3.5 Microsphere internalization

Internalization of Alg, Alg-g-PEG and Alg-g-RGD microspheres containing DyLight 550 (red fluorescent) labeled VEGFA was verified after examination of CLS micrographs. GFP human MSCs (green) successfully internalized DyLight-labeled VEGFA (red) encapsulated Alg, Alg-g-PEG and Alg-g-RGD microspheres (**Fig. 5**). The red microspheres were spread throughout the cytoplasm and appeared to surround the nucleus. The intensity of the red signal is a qualitative visualization of microsphere internalization rather than a quantitative measurement.

3.6 Human MSC differentiation

The UV absorbance of Alizarin red and fluorescent AdipoRed™ assays were used to quantify the osteogenic and adipogenic differentiation of human MSCs, respectively. The extent of differentiation was normalized to cell number by measuring the fluorescence intensity of Hoechst nuclear staining. As shown in **Fig. 6A**, incubation of human MSCs with microspheres containing VEGFA resulted in a significant increase in osteogenic differentiation when compared with non-modified TCPS controls. Statistical analysis of the data indicated a significant difference ($p < 0.04$) between the experimental groups (Alg, Alg-g-PEG and Alg-g-RGD) and the control group (cells cultured without the addition of VEGFA-encapsulated microspheres). The p-values for Alg, Alg-g-PEG and Alg-g-RGD microsphere groups compared to the control group were 0.0001, 0.0005 and 0.0330, respectively. For the adipogenesis differentiation assay, the Alg microsphere group showed

a significant enhancement compared to the control group ($p = 0.02$). For the other two groups (Alg-g-PEG and Alg-g-RGD), there were no significant differences for the adipogenesis assay with p -value of 0.28 and 0.57 respectively (**Fig. 6B**). The empty microspheres and pure VEGFA experimental groups did not exhibit any trends or significant differences for either adipogenic or osteogenic differentiation ($p > 0.06$) as shown in **Fig. 6C-F**. The significant differences between the experimental and control groups demonstrate that the VEGFA-encapsulated microspheres (Alg, Alg-g-PEG, Alg-g-RGD) were internalized by hMSCs and that VEGFA was delivered intracellularly, resulting in a functional output (osteogenic differentiation).

The concentration of osteoprotegerin produced by differentiated MSCs was quantified in each of the following groups: no microspheres (empty control), blank (*i.e.*, empty) Alg-g-PEG microspheres, and VEGFA-encapsulated Alg-g-PEG microspheres (**Fig. 7A**). Osteoprotegerin concentrations after 14 d of culture in osteogenic medium were 205, 189, and 780 pg/mL, respectively, for the control and experimental groups. Further analysis of these results indicate a significant increase in osteogenic differentiation of MSCs after incubation with VEGFA-encapsulated Alg-g-PEG microspheres for 48 h ($p < 0.0001$).

RT-PCR analysis 14 days after culture indicated osteogenic differentiation in all the 3 treatment groups. Cells in the VEGFA-encapsulated microsphere treatment group showed greater message for osteocalcin and type I collagen, while all the treatment groups contained message for RUNX2 confirming their osteogenic identity. GAPDH was used as a loading control.

4. Discussion

There is a growing demand for injectable acellular therapeutics for the enhanced mineralization of osteoporotic bone. This study provides the first evidence that Alg, Alg-g-PEG and Alg-g-RGD microspheres, fabricated from graft copolymers of alginate and amine-terminated PEG or alginate and pyridine-conjugated PEG, may be used for the controlled intracellular delivery of VEGFA into living human stem/progenitor cells for enhanced osteogenic differentiation. Through the use of a biodegradable natural polymer, mild gelation methods, chemical conjugation of PEG, and immobilization of the adhesion ligand RGD, the applicability of controlled drug delivery by means of microspheres increases. The Alg, Alg-g-PEG and Alg-g-RGD microspheres were uniform in shape, of a moderate size to allow for internalization into human MSCs, and proved adaptable to surface-modification [34, 35]. The fabrication was easy to perform and translate to engineering applications compared to other approaches which require higher spinning speeds and double emulsion processes.

VEGFA release from alginate-based microspheres was sustained over a period of 14 d, which is promising for targeted intracellular delivery. However, the intracellular delivery of VEGFA into human MSCs took place over a shorter time-span of 48 h. The non-selectivity of the microspheres in culture with MSCs allowed uptake to occur quickly, and this is also believed to be the case for the internalization of microspheres during the differentiation assay; the microsphere concentration and duration of co-culture were consistent among the

internalization and differentiation studies. The short time frame allowed for delivery also suggests that the VEGFA content of the internalized microspheres was high enough to significantly affect MSC differentiation. Drug diffusion may be hindered by PEGylation of the alginate; an effect that has been reported for Alg-g-PEG chains of moderate to high molecular weights [19]. The varying release kinetics with time suggest that the microsphere structure may be optimized further for controlled release applications [43]. Decreasing the variability in the diameter of the microspheres for all three experimental groups may result in different encapsulation efficiencies and/or release rates. However, microsphere size homogeneity and drug release profile optimization were outside the scope of this study and may be addressed through further investigations. Indeed, in regards to the MSC differentiation experiments, encapsulation efficiency was a more critical parameter compared to the *in vitro* extracellular release kinetics of the microspheres.

Multiple uptake pathways have been targeted as a means of delivering material intracellularly, namely endocytosis, in the case of cationic nanoparticles [44] or for larger micron-scale particles [45]. Due to the mild and quick gelation used to fabricate Alg, Alg-g-PEG and Alg-g-RGD microspheres, encapsulated drugs and proteins are better protected. Chemical conjugation of RGD onto PEG utilized disulfide bonds, which are covalent linkages arising from the oxidation of two sulfhydryl (SH) groups of cysteine connected to RGD (CRGD) and the SH terminal group on PEG (SH-PEG-NH₂). Disulfide bonds exist commonly in eukaryotic cell proteins with the function of fortifying the protein tertiary structure. Due to the reversibility and relative stability in plasma, the disulfide bond linkage becomes attractive in designing drug delivery systems. In addition, the physical properties and network structure of the calcium crosslinked alginate-based microspheres are readily changed after alterations in pH (such as in an endosome) or in the presence of calcium chelators. For the system described herein, it is hypothesized that the microspheres rapidly released VEGFA into the cytoplasm once inside the MSCs [46, 47] due to the disassembly of the microspheres.

RGD is widely utilized in tissue engineering studies and has been used in studies with alginate [48, 49]. In the current study RGD was used a model ligand which may be replaced with more relevant cell-recognition molecules for targeting MSCs *in vivo*. However, RGD has been shown to increase uptake of surface modified DNA complexes for intracellular delivery [50]. Although a limitation of the current study was in the collection of qualitative internalization results, future work will investigate RGD modified microspheres for enhanced MSC uptake and it may be combined with other cell-recognition motifs to control drug delivery for target cell populations.

Results of the differentiation colorimetric assays did not exhibit significant enhancement of the RGD or PEG modification between alginate microsphere groups in promoting osteoblast differentiation. Alg-g-PEG microspheres were chosen to further quantify the significance of the intracellular delivery method of VEGFA-encapsulated microspheres. MSCs in the VEGFA-encapsulated microsphere treatment group showed greater message for osteocalcin and type I collagen compared to the control groups. These results are promising and warrant further investigation into the intracrine activation mechanisms of growth factors for regulating MSC differentiation.

Although Alg-g-RGD microspheres resulted in the lowest Alizarin red absorbance, several reasons may account for this. The cells in the current study were only directed to differentiate for 14 d, meaning that the human MSCs cultured with Alg-g-RGD microspheres might still result in enhanced osteogenesis but more time is needed to demonstrate that result. Another possible explanation is RGD's influence on differentiation signaling. The research of Garcia *et al.* (2005) and Park *et al.* (2010) indicate a promotion effect of RGD in stem cell differentiation [33, 51]. In addition, it could also enhance chondrogenic differentiation [52]. However, other studies present findings in disagreement with the aforementioned work [53, 54]. The potential for Alg, Alg-g-PEG and Alg-g-RGD microspheres to deliver VEGFA intracellularly may be applied to investigate a wide array of differentiation lineages for MSCs and perhaps other progenitor cells.

Of special interest, our functional tests with VEGFA-bearing microspheres support an intracrine mechanism for VEGFA in human MSCs and are consistent with results reported by Liu *et al.* (2012) with retroviral expression of VEGFA in murine MSCs. We found that intracellular delivery of VEGFA via alginate-based microspheres resulted in up-regulated osteogenic differentiation by human MSCs, suggesting the effectiveness of our drug delivery system to control the intracrine mechanism that balances osteoblast and adipocyte activity. Extracellular VEGFA did not alter differentiation of human MSCs. To carefully evaluate the function of intracellular VEGFA in regard to specificity for bone formation, we used adipogenesis as an alternative differentiation out-put. MSCs from osteoporotic bones exhibit a higher adipogenic capacity compared to healthy MSC donors, which is why adipogenic differentiation was chosen as the alternative lineage [55]. Except for the Alg microsphere group, in which adipogenesis by MSCs was enhanced, there was not a significant difference in adipogenesis for MSCs incubated with the VEGFA-encapsulated Alg-g-PEG and Alg-g-RGD microspheres. Based on the enhanced adipogenesis we observed in the VEGFA-encapsulated Alg microspheres group, Alg-g-PEG and Alg-g-RGD microspheres may be better suited for controlled intracellular delivery to enhance osteogenesis *in vivo*. The investigation of various cell-recognition molecules (*i.e.* ligands, antibodies, *etc.*) may provide a viable option for targeting a specific cell population *in vivo* for enhanced mineralization of osteoporotic bone.

5. Conclusion

This study is the first to report the fabrication and surface-functionalization of Alg, Alg-g-PEG and Alg-g-RGD microspheres for the encapsulation and intracellular delivery of a bioactive growth factor, VEGFA. Alg, Alg-g-PEG and Alg-g-RGD microspheres containing VEGFA promoted osteogenic differentiation of human MSCs upon intracellular delivery after 48 h of incubation time. These significant results provide encouraging evidence for development of a systemic growth factor delivery system with potential to treat many debilitating diseases. Future work will involve manipulation of the surface ligand to enhance cell-targeted internalization of microspheres for intracellular growth factor delivery.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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A Synthesis of Alg-g-PEG **B Synthesis of Alg-g-PEG-S-S-pyridine** **Fig. 1.**

Chemical modification of alginate (Alg) with two different poly(ethylene glycol) (PEG) oligomers with methyl and pyridine end groups, respectively. The synthesis of Alg-g-PEG was conducted using carbodiimide chemistry at pH 5 and room temperature. To synthesize Alg-g-PEG-S-S-Pyridine, NH_2 -PEG-SH was reacted with 2,2'-dithiodipyridine in degassed acetate buffer at pH 4 and room temperature under N_2 flow. Next, the modified NH_2 -PEG-pyridine was conjugated to alginate using carbodiimide chemistry.

Fig. 2.

Schematic representation of microsphere fabrication techniques. Microspheres with or without VEGFA were prepared by premixing alginate or alginate-based copolymer solutions with VEGFA and creating a water/oil emulsion at room temperature in the presence of 1M calcium chloride. An additional surface modification step was performed on Alg-g-PEG-S-S Pyridine microspheres to chemically conjugate CRGD via disulfide bonds. For Alg-g-RGD microspheres, the conjugation of 2,2'-dithiodipyridine was used to exchange the thiol group on the cysteine-RGD (CRGD) after microsphere fabrication.

Fig. 3.

The shape, average diameter, VEGFA encapsulation efficiency and cytotoxicity of alginate-based microspheres were verified using scanning electron microscopy (SEM), an ELISA assay ($n = 3$), and an MTT-based *In Vitro* Toxicology assay. SEM micrographs representing (A,D) Alg, (B,E) Alg-g-PEG, and (C,F) Alg-g-RGD microspheres were fabricated without the addition of VEGFA through a water/oil emulsion; micrographs shown in A,B,C have a scale bar = 10 μm while micrographs in D,E,F have a scale bar = 1 μm. In preparation for imaging, microspheres were frozen by immersion in liquid N₂ and subsequently lyophilized; samples were sputter-coated with 45 nm of Au-Pb. (G) Microsphere diameters were determined using ImageJ analysis on SEM micrographs. (H) The effect of microsphere concentration (10, 50, 100, and 500 μg/mL) on primary human MSC viability after 24 h culture in standard MSC growth medium at 37°C and 5% CO₂ was determined. Experimental groups were normalized to non-modified confluent human MSCs cultured on tissue culture polystyrene. A Tukey statistical test was performed. No significant differences were seen between groups at the same concentration.

Fig. 4.

Quantitative release of VEGFA (%) was calculated using the encapsulation efficiencies of each sample group and the VEGFA concentration after each time point. Sample aliquots were collected and VEGFA concentration was determined using an ELISA assay (n = 3). (A) Cumulative VEGFA release (ng/mL) from alginate-based microspheres in PBS at pH 7.4 and 37°C over a sampling period of 48 h and (B) 14 d was determined.

Fig. 5. Confocal light microscopy images of GFP-labeled human MSCs (green) after 24 h of culture with DyLight 550 labeled-VEGFA-encapsulated alginate-based microspheres (red) at a concentration of 500 μg/mL in standard MSC growth medium. The Alg, Alg-g-PEG and Alg-g- RGD images all verify the internalization of microspheres, especially compared to the non- modified empty Alg microsphere control. The red intensity is a qualitative visualization of microspheres internalization rather than a quantitative measurement.

Fig. 6.

In vitro osteogenic (gray) and adipogenic (black) differentiation assay results of human MSCs after 14 days in culture with differentiation growth medium. (A) and (B) represent the VEGFA-encapsulated microspheres, (C) and (D) represent empty microspheres, and (E) and (F) represent extracellular delivery of pure VEGFA at different concentrations ranging from 0 to 20 ng/ml. A Tukey statistical test was performed to compare the experimental groups to the control groups ($n = 3$). ** shows a significant p-value less than 0.0005 and * shows p-value less than 0.04. For the control group, only standard MSC growth medium was added. The graphs illustrate a significant difference between VEGFA encapsulated Alg, Alg-g-PEG and Alg-g-RGD microsphere groups compared to the control group in the osteogenesis assay as well as for the VEGFA encapsulated Alg microsphere group in the adipogenesis group. No significant differences were seen ($p > 0.05$) between control and experimental

groups for the osteogenesis and adipogenesis assays when empty microspheres were used or VEGFA was delivered extracellularly.

Fig. 7.

In vitro osteogenic differentiation results. (A) protein results of human MSCs after 14 days in culture with differentiation growth medium. Sample aliquots were collected and osteoprotegerin concentration (pg/mL) in the supernatant medium was measured using an ELISA assay (n = 3). * indicates significantly higher result, $p < 0.0001$. (B) RT-PCR results verify production of osteocalcin and RUNX2 in MSCs after 14 days in culture with osteogenic differentiation growth medium. Collagen type I was upregulated for MSCs pre-treated with VEGFA-encapsulated Alg-g-PEG microspheres prior to differentiation. GAPDH was used as an internal control.