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PEG Hydrogel Containing Calcium-Releasing Particles and Mesenchymal Stromal Cells Promote Vessel Maturation

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Abstract

The use of human mesenchymal stromal cells (hMSC) for treating diseased tissues with poor vascularization has received significant attention, but low cell survival has hampered its translation to the clinic. Bioglasses and glass-ceramics have also been suggested as therapeutic agents for stimulating angiogenesis in soft tissues, but these effects need further evaluation *in vivo*. In this study, calcium-releasing particles and hMSC were combined within a hydrogel to examine their vasculogenic potential *in vitro* and *in vivo*. The particles provided sustained calcium release and showed proangiogenic stimulation in a chorioallantoic membrane (CAM) assay. The number of hMSC encapsulated in a degradable RGD-functionalized PEG hydrogel containing particles remained constant over time and IGF-1 release was increased. When implanted in the epididymal fat pad of immunocompromised mice, this composite material improved cell survival and stimulated vessel formation and maturation. Thus, the combination of hMSC and calcium-releasing glass-ceramics represents a new strategy to achieve vessel stabilization, a key factor in the revascularization of ischemic tissues.

Keywords

calcium; glass-ceramic particles; vascularization; hMSC; hydrogel

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1. Introduction

High incidence disorders such as peripheral artery disease (PAD), myocardial infarction and chronic wounds are characterized by restricted blood supply to the tissue, which impairs its repair and enhances its degeneration [1,2]. In adults, blood vessel formation occurs through three different mechanisms: arteriogenesis, angiogenesis and vasculogenesis. Arteriogenesis is the process of structural enlargement and remodeling of pre-existing small arterioles into larger vessels [3]. Angiogenesis arises from the sprouting of endothelial tubes from pre-existing capillaries through the activation, proliferation, migration, and differentiation of endothelial cells (ECs) [4]. Vasculogenesis is the formation of new blood vessels by homing and assembling of circulating endothelial progenitor cells (EPCs) into capillaries in the tissue [5–7]. However, since these endogenous mechanisms are generally not enough to restore blood supply in ischemic tissues, different therapies are being investigated. These include administration of angiogenic proteins, delivery of cells, or use of non-biological biomaterials [8,9].

Protein therapy through the administration of angiogenic growth factors, either individually or as a cocktail [8], has been one of the most explored strategies, including delivery of vascular endothelial growth factor (VEGF) and fibroblast growth factors (FGFs) [8]. Whereas this strategy has been successful in promoting new capillary formation, these vessels are often dysfunctional and regress upon loss of the vasculogenic stimulus [10]. In addition, translation of vasculogenic proteins faces several challenges, including excessive costs of production, poor stability and short half-life of the protein, and difficulties in delivering safe and effective doses [11]. Protein administration in the diseased tissues through gene therapy has improved vascularization but safety and regulatory concerns remain in the treatment with genetic manipulations [12].

Cell therapy has emerged as a promising strategy to promote revascularization because cells can function as release systems of a complex mixture of signaling factors in a controlled and sustained manner [13]. Indeed, human mesenchymal stromal cells (hMSC) have attracted significant attention as they can promote neovascularization [14,15]. The angiogenic potential of hMSC arises mainly from the myriad of angiogenic proteins that they release, which can stimulate ECs [16–23]. In addition, hMSC contribute to the maturation and stabilization of EC of newly formed vessels by acting as mural cells [18,24]. However, when administered either intravascularly [25,26] or directly into the tissue [27,28], MSC survival is very low.

To improve cell retention and function in the implanted site, biomaterials have been explored as delivery systems. Among them, hydrogels are particularly promising because they enable cell encapsulation and their mechanical properties can resemble native tissues [29–32]. Hydrogels are cross-linked hydrophilic polymers of natural and/or synthetic sources. The main advantage of synthetic polymers, such as poly(ethylene glycol) (PEG), over natural polymers is the greater control of the hydrogel's composition and properties [33]. In addition, synthetic hydrogels can be functionalized to promote cell adhesion, migration and scaffold degradation [33].

Interestingly, recent studies have shown that bioglasses and glass-ceramic materials can trigger angiogenic effects [9]. The application of these materials in regenerative medicine has extended from hard tissue (i.e. bone and teeth) to soft tissue and wound healing [34]. Ionic release of these materials can stimulate angiogenesis both *in vivo* and *in vitro* [9]. Calcium is one of the ions reported to stimulate the formation of vessels [35–38]. In fact, calcium, calcium-releasing bioglasses and glass-ceramics promote angiogenesis *in vitro* by increasing proliferation and tube formation in EPCs and human umbilical endothelial cells (HUVEC) [35,36]. In addition, composite materials of calcium-releasing bioglasses and polymeric matrices such as poly(lactic acid) and poly(caprolactone) promote blood vessel formation *in vivo* [37,38]. Nevertheless, the combined vasculogenic effect of bioactive glasses/glass-ceramics with hMSC in a hydrogel system for soft tissue applications has not been examined.

In the present study, we analyzed the vasculogenic potential of a new composite biomaterial composed of a cell-adhesive biodegradable hydrogel containing bone marrow-derived hMSC and calcium-releasing glass-ceramic microparticles. In addition to assessing the vasculogenic potential of calcium-phosphate glass-ceramic particles, we studied whether the incorporation of the particles in the hydrogel alters the mechanical properties of the hydrogel, stimulates the release of vasculogenic factors by hMSC, and impacts cell survival and vascularization in an *in vivo* model.

2. Materials and methods

2.1 Calcium-phosphate particles synthesis and characterization

Glass-ceramic particles with composition $\text{CaO}:\text{P}_2\text{O}_5:\text{Na}_2\text{O}:\text{TiO}_2$ in a 44.5:44.5:3:8 molar ratio (respectively), referred to as GC8, were prepared by controlled hydrolysis sol-gel method under an inert atmosphere. Chemicals utilized in the sol-gel fabrication process included phosphorous pentoxide (P_2O_5 , 99.99+%, Sigma-Aldrich), metallic calcium (Ca^{2+} , 99%, Sigma-Aldrich), metallic sodium (Na^+ , 99%, Panreac), titanium tetraisopropoxide ($\text{Ti}(\text{OCH}(\text{CH}_3)_2)_4$, 97%, Alfa Aesar), absolute ethanol (EtOH , 99.99% Sigma-Aldrich), anhydrous 2-methoxyethanol ($\text{C}_3\text{H}_8\text{O}_2$, 99.8%, Sigma-Aldrich), 2-propanol (99.7%, Sigma-Aldrich), hydrochloric acid (HCl , 37%, SigmaAldrich) and MilliQ water.

Calcium and sodium 2-methoxyethoxides precursor solutions were prepared by refluxing metallic calcium and sodium respectively in anhydrous 2-methoxyethanol at 124 °C for 24 h. Phosphorus ethoxide precursor solution was prepared by refluxing phosphorous pentoxide in absolute ethanol at 78 °C for 24 h. Titanium alkoxides precursor solution was prepared by diluting titanium tetraisopropoxide in absolute ethanol.

The sol-gel gel process started with the addition of the calcium, sodium and titanium precursors in a balloon maintained under an inert and dry atmosphere in an ice bath. After 1 h of vigorous stirring to ensure solution homogeneity, the phosphorous solution was added at a controlled rate of 2.5 mL/h with an infusion pump. Acid catalyst with a molar ratio of 1:60:0.3:15 ($\text{Ti}:\text{H}_2\text{O}:\text{HCl}:\text{2-propanol}$) was then added at a controlled flux of 1.0 mL/h. This mixture was transferred into a sealed vial and aged for 18 h at RT and 72 h at 80 °C. Vials were then opened, and samples were dried via heating from 80 °C to 120 °C through a slow

ramp (2 h) and treated at 120 °C for 24 h in a Nabertherm® oven (LV 9/11/P330). The dry sample was heated to 540 °C by a slow ramp (3 °C/min) followed by treatment at said temperature for 5 h. Finally, the resulting powder was mashed in a planetary ball miller (PM 100, Retsch®) and manually filtered through a 40 µm porous filter to ensure the micro/nano-metric scale of the final material.

SEM images were obtained from uncoated GC8 particles mounted on a silicon wafer (Nova Nano SEM-230; FEI Co.) at 20 kV, and material composition analysis was carried out on samples coated with an ultra-thin layer of carbon on a FESEM (J-7100F, Jeol) containing the Inca 250 EDS microanalysis system (Oxford Instruments) at 5 kV. Z-potential (ζ) measurements were carried out on a Zetasizer Nano ZS (Malvern Instruments) and size measurement was performed in a LS Particle Size Analyzer through laser diffraction (LS 13 320, Beckman Coulter). For these analyses, the micrometric powder sample was dispersed in absolute ethanol to avoid particle dissolution.

2.2 Preparation of the PEG-MAL hydrogel/calcium-phosphate particle composite

Hydrogels were cast as previously described [39,40]. Briefly, PEG-maleimide (PEG-MAL) four-arm macromers (20 kDa MW, Laysan Bio) were prefunctionalized with the peptide GRGDSPC containing the cell adhesive site RGD (Aaptec) for 15 min at 37 °C. At this point, cells and/or particles were added and mixed to disperse homogeneously. The mixture was crosslinked with the addition of the protease degradable peptide GCRDVPMRGGDRCG (VPM) (Aaptec) at 1:2 molar ratio of VPM peptide to available MAL groups. The final concentration of PEG-MAL and RGD peptide was 5% (w/v) and 1.0 mM, respectively. Hydrogels were cast on plastic paraffin film (Parafilm M®, Bemis NA) under sterile conditions, with previously sterilized reagents, and were incubated for 15 min at 37 °C to ensure complete crosslinking before transferring them into media. Sterile-filtered PBS supplemented with 20 mM HEPES with pH adjusted to 7.4 was used as buffering solution to dissolve the reagents.

2.3 Calcium release

Precast 30 µL hydrogels containing 1% (w/v) particles and 0.5 mg of particles without hydrogel were introduced in microtubes and incubated in 500 µL of either cell culture media (CCM) or MilliQ water with 10 mM HEPES (pH 7.4). The lid of the microtubes containing CCM was perforated with a needle to allow gas exchange and all samples were incubated at 37 °C in a humidified atmosphere containing 5% (v/v) CO₂. At different time points for a period of 13 days, 100 µL of media was replaced to measure pH and calcium concentration. pH was measured with a Laquatwin pHmeter (B-712, Horiba) while calcium released in the media was assessed using the quantitative colorimetric method 0-cresolphthalein complexone (Sigma-Aldrich) [41]. Absorbance readings were determined at 570 nm on the Infinite M200pro microplate reader (Tecan). Cumulative release was quantified taking into account the calcium moles removed at each time point and values were normalized to the total weight of particles per sample.

2.4 Mass swelling ratio

Hydrogels were formed as previously described and were allowed to completely swell in di-H₂O for 24 h at 37 °C. Gels were removed from solution and excess water was eliminated from the surface of hydrogels with filter paper prior to weighing. Then, swollen hydrogels were snap-frozen in liquid N₂ and lyophilized followed by dry mass measurement. Five replicates of 30 µL were used per condition. The mass swelling ratio is reported as the ratio of swollen mass to dry mass.

2.5 Rheological properties

Storage (G') and loss (G'') moduli of hydrogels were assessed by dynamic oscillatory strain and frequency sweeps on a **Discovery HR-2** rheometer (TA Instruments) with a 8 mm diameter, flat geometry (Plate SST 8mm Smart-Swap, TA Instruments). Since hydrogel surfaces were required to be flat for the measurement, 25 µL hydrogels were casted in 4 mm diameter molds of PDMS. Once crosslinked, hydrogels were allowed to swell in PBS for 24 h. For the measurement, hydrogels were loaded between the flat platen and the Peltier plate and the measuring system was lowered until the axial force detected was 0.02 N. To determine the viscoelastic range of the hydrogel, strain amplitude sweeps were performed at an angular frequency (ω) of 10 rad s⁻¹. After determining that 1% was a suitable strain, oscillatory frequency sweeps were used to quantify the storage and loss moduli (ω = 0.25–10 rad s⁻¹). Collagen hydrogels of 3.5 mg/mL (OptiCol™ Rat Type I Acid Soluble Collagen, Cell Guidance Systems) were used as an inter-experimental control. Five replicates were used per condition.

2.6 Cell culture and encapsulation

Human bone marrow-derived MSC provided by the Texas A&M Health Science Center College of Medicine Institute for Regenerative Medicine at Scott & White (NIH Grant P40RR017447) were used without further characterization. Following the provider's protocol, cells were expanded at low seeding densities (150 cells/cm²) in CCM composed of Minimum Essential Medium Alpha (αMEM, Invitrogen) supplemented with 2 mM L-glutamine (Invitrogen), 100 U/mL penicillin (Invitrogen), 100 U/mL streptomycin (Invitrogen), and 16% Hyclone fetal bovine serum (FBS; GE Healthcare). Cells were maintained at 37 °C in an atmosphere of 5% CO₂ and were used up to passage 5. During expansion, media was refreshed every 3 days.

Cells were encapsulated as previously described at a concentration of 3.5×10⁶ cells/mL of gel, in 20 µL hydrogels. Free-floating cell-containing hydrogels were cultured in 1 mL CCM in 24 well plates and media was replaced every 3 days.

2.7 In vitro cell survival within hydrogels

A Calcein assay was used to stain live cells and observe them within hydrogels after 24 h and 72 h of cell encapsulation. To avoid particle autofluorescence in the green and red fluorescent channels Calcein Deep Red acetate-TM (ATT Biorequest) was used following the manufacturer's instructions. Briefly, after being washed with Dulbecco's phosphate-buffered saline (DPBS; Invitrogen), cell-containing hydrogels were incubated for 1 h at

37 °C in 5% (v/v) CO₂ with 1 mL of non-supplemented α MEM containing 7.5 μ M calcein reagent and 2.5 mM probenecid (Sigma-Aldrich). After 30 min of incubation, two drops of a solution of Hoechst 33342 (NucBlue® Live ReadyProbes® Reagent, Life Technologies) were added to stain the nuclei of all cells. After incubation, hydrogels were washed with DPBS and cells were observed in media containing serumfree α MEM and 2.5 mM probenecid. Three replicates were used per condition and a z-stack of 400 μ m was acquired with a Leica TCS SP5 confocal laser scanning microscope (Leica Micro-systems) at three random spots per replicate. Images were processed with the ImageJ 1.51h software to obtain Z-stack projections of the outer 200 μ m thick section and a more inner section of 200 μ m thick.

2.8 Cell numbers within hydrogels

Cell number was assessed at 1, 3 and 7 days of cell encapsulation through the detection of lactate dehydrogenase (LDH) activity of the cell lysate contained in the hydrogels. Briefly, hydrogels were washed with DPBS Ca²⁺ Mg²⁺ (Invitrogen) and incubated at 37 °C for 1 h in Eppendorf microtubes containing 50 μ L of 3 mg/mL of collagenase type II (Invitrogen) for complete gel degradation. Thereafter, cells were lysed by adding 400 μ L Mammalian Protein Extraction Reagent (M-PER; Thermo Fisher) followed by incubation for 30 min in ice. Samples were sonicated with an ultrasonic processor (UP50H, Hielscher) in cold to ensure complete cell lysis and centrifuged at 10,000 rpm for 10 min at 4 °C. Supernatant was used with the LDH quantification kit (Roche) following the manufacturer's instructions and cell concentration was calculated through a calibration curve made with cell lysates of known cell concentration. Absorbance was read at 490 nm with an Infinite M200 PRO multimode plate reader instrument (Tecan). The experiment was carried out three times using triplicates per condition.

2.9 Chick chorioallantoic membrane assay (CAM)

An *ex ovo* CAM assay was performed as previously described [42,43]. Briefly, fertilized chicken eggs (Granjas Gibert SA) were incubated for 3 days in a humidified incubator at 37 °C. The entire egg content was then carefully transferred into a Petri dish (430167, Corning) and incubated for another 6 days. On embryonic day E9, sterile methylcellulose disks containing or without GC8 particles were carefully placed on the CAM. For each experimental condition, six specimens were used and 4 disks were placed on each membrane. The disks were prepared previously to the implantation day by drying 50 μ L drops of a solution containing 1.5% (w/v) (hydroxypropyl)methyl cellulose (Sigma-Aldrich) in MilliQ water with or without homogeneously distributed GC8 particles on a Teflon surface [44]. After 3 more days of incubation (E12), embryos were humanely sacrificed by decapitation and CAM was immediately fixed with a neutral buffered 10% formalin solution (Sigma-Aldrich) for 30 min. Finally, the membranes containing the disks were excised and images were acquired with an Olympus MVX10 Macroscope. Angiogenesis stimulation was quantitatively measured by counting the vessels converging towards the disks using ImageJ. A minimum of 10 samples were analyzed per condition.

2.10 Expression of vasculogenic proteins in vitro

CCM conditioned for 3 days by cells encapsulated in the hydrogels was used to quantify VEGF, IL-6, IFN- γ , TGF- β 1 and IGF-1 concentrations by commercial sandwich enzyme-linked immunosorbent assay (ELISA) kits (R&D systems) following the manufacturer's instructions. Conditioned media was centrifuged at 2,000 rpm at 4 °C for 10 min and the supernatant was stored at -80 °C until needed. For all experiments, a blank sample was included to subtract the protein background present in non-conditioned media. Absorbance was determined at 455 nm with a multimode microplate reader (Infinite M200 PRO, Tecan) setting wavelength correction to 540 nm. The results were normalized to the cell number concentration in the hydrogel quantified with the LDH detection kit as previously described. Six replicates were used per condition.

2.11 hMSC transduction with luciferase lentivirus

When hMSC reached 60–70% confluence, cells were transduced with lentivirus encoding for luciferase/tdtomato (pLenti-UbC-RFLuc-tdtomato, Targeting Systems, MOI 5–20) in complete media containing 100 μ g/mL protamine sulfate [45]. 24 hours after initial infection, media was replaced with fresh complete media. Six days after initial infection, transduction efficiency was measured by tdtomato expression by flow cytometry.

2.12 Implantation into mice and cell tracking

All animal experiments were performed with the approval of the Georgia Tech Animal Care and Use Committee (IACUC) under the supervision a research veterinarian and within the guidelines of the Guide for the Care and Use of Laboratory Animals. Precast hydrogels containing luciferase-expressing hMSC were implanted in the epididymal fat pad (EFP) of 8–12 week old immunocompromised B6.129S7-Rag1^{tm1Mom}/J male mice (Jackson Laboratory). Three conditions were examined: hydrogels without particles, hydrogels with 0.5% (w/v) GC8 particles and a positive control hydrogel containing 10 μ g/mL VEGF. Six animals were used per condition. Animals were anesthetized in a chamber with 5% isoflurane and maintained at 2% isoflurane during surgery. A midline incision in the abdominal wall was performed, and each EFP was exposed on sterile gauze pre-wet with sterile saline for hydrogel implantation. One hydrogel (30 μ L) was implanted in each EFP by wrapping the EFP tissue around it. To ensure hydrogel retention in site, a small suture of non-degradable thread (Ethicon) was applied to the proximal site of the tissue. The interior abdominal layer was closed with degradable suture while the exterior layer was sealed with wound clips. During surgical preparation, one dose of sustained-release buprenorphine (1 mg/kg) was administered intraperitoneally (IP) during surgical preparation to provide 72 continuous hours of pain relief.

At selected time points following implantation (0, 2, 4, 7, 13 days), anesthetized mice were IP injected with 150 mg/kg body weight of beetle luciferin (Promega) diluted in PBS and bioluminescence was acquired 15 min post-injection on an IVIS SpectrumCT imaging system. The signal detected was quantified by gating a region of interest (ROI) around the periphery of the implant and subtracting the average background counts in the surrounding tissue from the average total counts in the implant, as background intensity varied between animals. The final averaged count was normalized by the signal on day 0 of each animal.

2.13 Vessel labeling and quantification

Prior to euthanasia, animals were injected intravenously with Dylight 488-labeled tomato lectin (Vector Laboratories) to label functional vasculature [40]. After lectin was allowed to circulate for 15 min, animals were humanely euthanized with carbon dioxide and were perfused intracardially with 10 mL of saline through a 23-gauge cannula inserted into the left atrium. Blood and saline exited through the cut vena cava. EFPs were removed and fixed in 10% buffered formalin for 24 h at RT. Z-stack images were acquired of the fluorescent vasculature with a Nikon C2-Confocal Module connected to a Nikon Eclipse Ti inverted microscope with a 488 nm laser (Melles-Griot) and 525/50 filter. Images were processed with ImageJ for vasculature quantification. From each image, three different sites were analyzed and at least six fat pads of each condition were used for the quantification.

2.14 Immunostaining

For immunostaining analyses, fixed EFPs were dehydrated, embedded in paraffin and cut into 8- μ m thick sections. These sections were deparaffinized in xylene and ethanol, subjected to antigen retrieval by heating the samples at 95 °C for 20 min in sodium citrate buffer (10 mM sodium citrate, 0.05% Tween 20, pH 6.0) and permeabilized with 0.1% Triton X-100 (Sigma-Aldrich) in Tris-buffered saline (TBS) for 30 min. After blocking the tissue samples with 1% bovine serum albumin and 0.025% Triton X-100 for 2 h, sections were stained with primary antibodies against α -smooth muscle actin (α -SMA; 1:500; Sigma-Aldrich) at 4 °C overnight. Sections were then incubated with biotinylated secondary antibody goat anti-mouse (1:500; Abcam) for 1 h at RT. Following a 10 min incubation of streptavidin-peroxidase (Abcam), samples were exposed to DAB solution (Abcam) for 3 min and rinsed thoroughly with water. Specimens were counterstained with a 1/10 hematoxylin dilution for 1 min and mounted for imaging with a Nikon Eclipse E600 microscope (Nikon Instruments Inc.). The tissue area surrounding the hydrogel was used to quantify positively stained vessels, lumen area and thickness of vessels. Sections of five different fat pads of each condition were analyzed.

2.15 Statistics

Data are expressed as mean $\pm$ standard deviation (SD). Statistical analyses were performed using one-way ANOVA with Tukey's test for post hoc comparisons using GraphPad Prism software. A p-value of 0.05 was considered significant.

3. Results

3.1 GC8 particle characterization

GC8 particles were synthesized and characterized. SEM images showed high polydispersion of particle size (Fig. 1A) confirmed by size quantification through laser diffraction (Fig. 1C). Although average particle size was $\sim$ 9 μ m (Fig. 1C), SEM images showed that micrometric GC8 particles were structured in subunits of approximately 200 nm (Fig. 1B). Nanoparticle sintering might have occurred during the thermal treatment applied in the synthesis process. Atomic composition analysis by EDS showed that the GC8 particles had equivalent composition as the theoretical value (Fig. 1D), an indication that the particle composition is

properly controlled in the synthesis. Finally, Z-potential analysis indicated a negative value of -15.75 mV (Fig. 1D).

Since extracellular calcium can stimulate biological responses on cells, calcium released from the particles was measured in two different solutions (Fig. 2B). Calcium release was sustained for 13 days in both media, although release in CCM was significantly lower than in buffered MilliQ water. In addition, pH was measured from the samples and no changes were detected compared to the media without particles (data not shown). Thus, GC8 degradation does not contribute to changes in pH in the media.

3.2 Calcium release and mechanical properties of hydrogels with GC8

GC8 particles were embedded within PEG-MAL hydrogels as explained in Fig. 2A. The PEG-MAL hydrogel has been used in previous studies [39,40,46] and contains the cell adhesive peptide RGD and the protease-degradable VPM crosslinker, which serves as substrate of many proteases including matrix metalloproteinases-2 (MMP2) and MMP9. Gel crosslinking occurred in less than 10 min, allowing for uniform particle distribution within the hydrogel.

Calcium release from GC8 encapsulated in PEG-MAL hydrogels was quantified as before, and release was again higher in MilliQ water compared to CCM (Fig. 2B). In addition, calcium release was also higher for GC8 particles embedded in the hydrogels compared to free particles (Fig. 2B).

We next measured the equilibrium mass swelling ratio and viscoelastic properties of hydrogels without particles or with 0.5% or 1% (w/v) GC8 content. Fig. 2C shows that the equilibrium mass swelling ratio is significantly higher in empty hydrogels than in hydrogels with particles, indicating that the particles decrease the hydrogel capacity to absorb water and, therefore, present a denser network. The viscoelastic properties of the hydrogel were modified in the presence of 1% (w/v) GC8 particles but there were no differences between the 0.5% GC8 formulation and control (Fig. 2D). The storage modulus for the 1% GC8 formulation was approximately 50% lower than that for empty gels.

3.3 hMSC survival and growth within PEG-MAL hydrogels in vitro

hMSC survival and morphology within the hydrogels was studied by calcein staining of live cells on day 1 and 3 post-encapsulation (Fig. 3A). A far red calcein molecule was used to avoid GC8 autofluorescence in the red and green channel. Cell nuclei were stained in blue with Hoechst® 33342. Cell behavior was similar for all the conditions tested, with hMSC forming networks and showing high viability and spreading in the outer zones (~ 200 μ m), but lower survival and spreading in the interior of the gel. On day 1, hMSC showed a spread morphology and on day 3 most of the cells were organized in a complex network structure. In general, calcein signal was much lower in the interior. However, nuclei distribution changed from a homogeneous dispersion on day 1 to a more clustered organization on day 3, which implies that cell reorganization is also taking place in the interior of the hydrogel.

Cell numbers within the hydrogels were determined on day 1, 3 and 7 (Fig. 3B). The initial cell number seeded per hydrogel was 80,000 cells, and 24 h after encapsulation cell survival

was 80–100% for all conditions tested. Cell number remained constant throughout the 7 days in culture, except for the 1% GC8 formulation for which cell number decreased significantly by day 7.

3.4 Proangiogenic effect of GC8 particles on CAM model

The proangiogenic effect of GC8 particles was tested on the CAM model. This extraembryonic vascularized membrane that facilitates gas exchange during chick embryogenesis is a widely used system to test the pro- and antiangiogenic properties of substances and materials [47], including bioglasses [48,49]. The experiment was performed *ex ovo* to avoid possible interferences of the calcium from the egg shell and to test more replicates per embryo. Methylcellulose disks were used as support materials for the particles in the assay as previously reported [44,50–53]. Disks were placed on the CAM on E9 (Fig. 4A) and fixed, excised and imaged on E12 (Fig. 4B). Analysis of angiogenic stimulation found increased vascularization in the conditions with GC8 (Fig. 4C). No differences were detected between 0.5% and 1% GC8 content. This result suggests that the GC8 particles can stimulate angiogenesis in an *in vivo* context.

3.5 Release of angiogenic factors by hMSC encapsulated in GC8-containing hydrogels

We examined whether the presence of GC8 particles within the hydrogels stimulates encapsulated hMSC to release angiogenic factors. Because we did not see any effects in the CAM assay between 0.5% and 1% GC8 particles and there are no differences in viscoelastic properties between 0.5% GC8 and empty gels, we compared 0.5% GC8-containing gels to empty hydrogels. A protein array was performed to screen for differences in expressed proteins (Supplementary Fig. 1) and selected VEGF, IL-6, IFN- γ , TGF- β 1 and IGF-1 to be quantitatively evaluated by ELISA. As shown in Fig. 5, hMSC released VEGF, IL-6, TGF- β 1 and IGF-1, but IFN- γ was not detected in any of the conditions. Furthermore, a significant increase in IGF-1 secretion was detected in the hydrogels containing GC8 particles compared to empty hydrogels.

3.6 Enhanced hMSC survival in GC8-hydrogels implanted in the EFP

The vasculogenic properties of the PEG-MAL-GC8 system containing hMSC were next tested following implantation in the EFP of immunocompromised mice, in which hydrogels containing 0.5% GC8 particles were compared to empty hydrogels and hydrogel delivering VEGF. Avoiding or decreasing cell death associated with *in vivo* implantation is a major issue in tissue engineering [54]. To track cell survival post-transplantation, hMSC were transduced to constitutively express luciferase and transplanted cells were monitored longitudinally by bioluminescence imaging (Fig. 6A–6B). Bioluminescence signal decreased over time to background levels for all groups. However, higher signal was detected for hMSC implanted in hydrogels containing GC8 particles compared to hMSC in control hydrogels (Fig. 6B). This result indicates that GC8-containing hydrogel supported enhanced hMSC survival at early time points compared to control gels.

3.7 Analysis of vascularization at the implantation site

We evaluated vascularization in the implant site at 2 weeks post-implantation. Prior to euthanasia, functional vasculature in the mice was labeled by an intravenous injection of fluorescent lectin. Microscopic imaging of the excised fat pads (Fig. 7A) made possible the quantification of branch and junction density, average branch length and vessel diameter (Fig. 7B–7E). Significant differences were detected only in vessel diameter, suggesting the development of a more mature vasculature in the EFP with the GC8 hydrogel compared to the empty hydrogel control (Fig. 7E).

Sections of the tissue were immunostained for α -SMA and positively stained vessels surrounding the area of implantation of the hydrogel were counted (Fig. 8A). Hydrogels delivering VEGF and hydrogels containing GC8 particles showed significant higher vessel density and thicker wall vessels than the control empty hydrogel (Fig. 8B). In addition, the condition with 0.5% GC8 contained a greater number of vessels with bigger lumen area (Fig. 8C) and significantly thicker wall vessels than the VEGF condition (Fig. 8D). Taken together, these results indicate the presence of a denser and more mature vasculature for GC8-containing hydrogels with hMSC compared to control hydrogels containing hMSC.

4. Discussion

Cell therapy using hMSC for the treatment of pathologies with poor vascularization has received considerable interest, as demonstrated by recent clinical trials [55–58]. However, several issues need to be improved to achieve clinical success, such as sustained cell survival that allows a controlled and sustained release of signaling factors in the implanted site [59] and stabilization of the newly formed vessels [60]. Regarding their angiogenic properties, the use of bioglasses and glass-ceramics for soft tissue regeneration has also been suggested recently [9,34,61,62], but further research needs to be performed to show their angiogenic effects *in vivo* and on hMSC. In this study, we explored the vasculogenic potential of calcium-releasing glass-ceramic particles and their combination in a degradable hydrogel containing hMSC for soft tissue regeneration. The particles alone fostered angiogenesis in the CAM model, showing enhanced angiogenesis at the site of implantation. When particles were encapsulated in the hydrogel containing hMSC, cell number remained stable *in vitro*, and improved survival was observed for acute time points *in vivo* when compared to empty hydrogels. In addition, the calcium-releasing particles increased hMSC secretion of IGF-1, an important vasculogenic factor. Finally, hydrogels containing GC8 particles and hMSC stimulated increased and more mature vascularization compared to hydrogels containing hMSC.

Many studies have pointed out the need of an optimal dose in order to achieve bioactive stimulation with bioglasses and glass-ceramics [63–65], but normally fail in reporting the release profile of bioactive ions over time. For this reason, we measured the release of calcium from GC8 particles for several days. Also, since the type of media can influence degradation rate of bioglasses/glass-ceramics, we used CCM and buffered MilliQ water [66–68]. Despite achieving a sustained release for several days, calcium release was slower in CCM. This slower release could have been caused by the organic content of CCM, which has been reported to interfere in the degradation of bioglasses [68]. Alternatively,

precipitation of a layer of calcium phosphate on the surface of the particle could also have contributed to the reduced release in CCM [69]. These differences in calcium release highlight the importance in the selection of media to study bioglass/glass-ceramic degradation.

The glass-ceramic particles used in this study stimulated angiogenesis in the CAM model, and we attribute this effect to calcium released from the particles. Extracellular calcium concentrations of 10 mM were reported to stimulate migration and tube formation of EPCs [36]. Although our material did not reach this concentration in bulk solution, it is possible that the concentration sensed locally by the cells is higher than the one measured. Other studies have shown that the ionic release of calcium-phosphate ceramics can stimulate the release of angiogenic factors from fibroblasts [35], HUVEC [35], and EPCs [70] *in vitro*, so we speculate that the observed angiogenic effect in the CAM model might have been stimulated by the release of angiogenic factors from the cells present in the membrane.

Incorporation of glass-ceramic particles in hydrogels alters several physical properties that can modify the biological performance of the material. The degradation profile of the particles, as illustrated by the measurement of the calcium release, was improved by increasing the release rate and becoming more sustained, specially in CCM. We posit that particle degradation is faster within hydrogels as these are separated from each other whereas free particles aggregate likely influencing local release rates. Both particle concentrations tested reduced the swelling capacity of the hydrogel, which can correlate with smaller pore size and denser network. Also, the condition with higher particle concentration tested (1%) reduced the storage modulus of the material. We attribute the reductions in storage modulus to the high content of micrometric particles in this hydrogel that disrupts the hydrogel network reducing the ability of the hydrogel to support a load.

Incorporation of GC8 particles within hydrogels also increased the secretion of vasculogenic factors by encapsulated hMSC, supporting the conception that the degradation product of these biomaterials can stimulate cells to release factors [35,71]. More specifically, increased levels of IGF-1 were detected in media conditioned by hMSC encapsulated in hydrogels with glass-ceramic particles compared to media from hMSC encapsulated in control gels. IGF-1 stimulates angiogenesis *in vitro* and *in vivo* [72–74]. Su et al. [72] showed induced EC migration and capillary formation in an aorta ring assay, and Jacobo et al. [73] recently demonstrated a role of this factor in stabilizing neovessels. In addition, sustained IGF-1 expression through gene delivery improves cardiac function in a myocardial infarction model [74]. Extracellular calcium had been shown to increase IGF-1 expression in other cell types, such as EPCs and osteoblasts [36,75], contributing to EPC maturation [36,70].

Cell survival within the hydrogel is of great relevance for their application into clinics. We used hMSC expressing luciferase to track cell survival following cell transplantation within different hydrogel formulations in the murine EFP. Bioluminescence signal decreased over time to background levels for all groups. However, higher signal was detected for hMSC implanted in hydrogels containing GC8 particles compared to hMSC in control hydrogels. This result indicates that GC8-containing hydrogel supported enhanced hMSC survival at early time points compared to control gels.

We evaluated the effect of GC8 glass-ceramic particles on vascularization using two models: an *ex ovo* CAM assay and an *in vivo* study in the murine EFP. Both models agreed in finding increased vascularization in hydrogels containing glass-ceramic particles compared to control gels. In addition, the murine EFP where hMSC-containing hydrogels were implanted showed enhanced vessel maturation in hydrogels containing the particles compared to empty and VEGF-releasing hydrogels. Other studies had reported angiogenic properties for bioglasses and glass-ceramics [35,37,38,76], but to our knowledge, this is the first report demonstrating that these materials stimulate functional vasculature in mammalian soft tissues. Due to the complexity of the signaling pathways involved in the formation of stable vasculature, it is challenging to hypothesize what specific factor(s) might have promoted these effects. Improved cell survival in the condition with particles might have allowed the release of more angiogenic factors such as VEGF, TGF- β and IGF-1 by hMSC, which have been associated with improved vessel stabilization [73,77]. In addition, implanted hMSC may have contributed as mural cells [24]. Nevertheless, the contributing role of the glass-ceramic particles should be further studied in order to unravel the specific pathway by which they can stimulate vessel maturation. Overall, the material here presented made by the combination of a functionalized degradable PEG hydrogel, hMSC and calcium-phosphate glass-ceramic microparticles shows high vasculogenic potential in an *in vivo* context, as it permitted a more sustained cellular survival and stimulated increased vessel formation and maturation in the tissue.

5. Conclusions

We present a composite construct consisting of hMSC and calcium-releasing microparticles within a functionalized synthetic hydrogel system that enhances blood vessel formation and maturation *in vivo*. The presence of the glass-ceramic particles seems to stimulate these effects by improving implanted cell survival and angiogenic factor release. This study shed light into the potential of bioglasses/glass-ceramics to improve cell therapy for soft tissue regeneration.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. GC8 particle characterization. (A) (B) GC8 images with SEM, (C) distribution of particle size measured with laser diffraction, (D) table showing atomic composition of GC8 measured with EDS and Z-potential value.

Figure 2. PEG-MAL hydrogels containing GC8 particles. (A) 4-arm PEG-MAL macromer is functionalized with the cell adhesive peptide RGD. Cells and GC8 particles are added and the hydrogel is crosslinked via reaction with VPM peptide. (B) Cumulative calcium release in two different solutions (buffered MilliQ water or CCM) for free GC8 or GC8 particles encapsulated in hydrogels. (C) Effect of GC8 particle concentration (w/v) on the equilibrium swelling ratio. (D) Storage (G') or loss (G'') moduli for PEG-MAL hydrogels with or without different GC8 concentrations. A crosslinked type I collagen hydrogel is shown for reference. * $p < 0.05$.

Figure 3.

In vitro hMSC viability and spreading within hydrogels. (A) Fluorescent image stacks of 200 μ m of the outer and inner part of the hydrogels showing staining of the cytoplasm of live cells with calcein (green) and cell nuclei with Hoechst (blue) on day 1 and 3 after cell encapsulation. Scale bar: 100 μ m. (B) Quantification of cell number within the hydrogels on day 1, 3 and 7 post-encapsulation. Data represent the mean of three experiments with the S.D. * $p < 0.05$.

Figure 4.

Angiogenic effects of the GC8 particles on the CAM of chick embryos. (A) Methyl cellulose disks containing GC8 particles were applied on the CAM on E9. (B) Representatives images of the disks on the CAM after fixation and excision on E12. Scale bar 2 mm. (C) Quantification of the number of vessels converging towards the material normalized by the perimeter of the area selected performed through image processing. A minimum of 10 samples were used per condition and data is expressed as mean $\pm$ S.D. * $p < 0.05$ (vs. control). ** $p < 0.01$ (vs. control).

Figure 5. ELISA quantification of angiogenic factors VEGF, IL-6, TGF-β1 and IGF-1 secreted by hMSC in PEG-MAL hydrogels without particles or with 0.5% (w/v) GC8 particles. Blank values were subtracted and concentrations were normalized to the total number of cells contained in the hydrogel. Data represent the mean of six replicates $\pm$ S.D. *** $p < 0.001$.

Figure 6.

In vivo tracking of transplanted hMSC. (A) Representative images from IVIS scanning of the bioluminescence signal of hMSC in the hydrogels implanted in the EFP after luciferin injection on day 0, 2 and 7 post-implantation. The conditions used included a control without particles, a hydrogel with 0.5% (w/v) content and a positive control containing 10 μ g/mL VEGF. (B) Bioluminescence signal was determined by quantifying the average counts per unit area within a ROI and values were normalizing to day 0. The plot shows the mean of the six animals used for each condition at every time point (dot). The error bars are the S.D. Lines are sigmoid curves fitted to all the samples per condition. *** p <0.001 (vs. control and VEGF). * p <0.05 (vs. control).

Figure 7.

Analysis of the lectin-stained vasculature of excised EFP. (A) Fluorescent images of the EFP were acquired and processed (Supplementary Figure 2) for quantification of (B) total number of branches per area, (C) total number of junctions per area, (D) average branch length, and (E) measurement of vessel width for each condition. Data is expressed as the mean $\pm$ S.D of at least six fat pads per condition, from which three random areas were processed. * $p < 0.05$ (vs. control). ** $p < 0.01$ (vs. control). Scale bar: 200 μ m.