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Non-viral DNA delivery from porous hyaluronic acid hydrogels in mice

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ABSTRACT

The lack of vascularization within tissue-engineered constructs remains the primary cause of construct failure following implantation. Porous constructs have been successful in allowing for vessel infiltration without requiring extensive matrix degradation. We hypothesized that the rate and maturity of infiltrating vessels could be enhanced by complementing the open pore structure with the added delivery of DNA encoding for angiogenic growth factors. Both 100 and 60 μm porous and non-porous hyaluronic acid hydrogels loaded with pro-angiogenic (pVEGF) or reporter (pGFPluc) plasmid nanoparticles were used to study the effects of pore size and DNA delivery on angiogenesis in a mouse subcutaneous implant model. GFP-expressing transfected cells were found inside all control hydrogels over the course of the study, although transfection levels peaked by week 3 for 100 and 60 μm porous hydrogels. Transfection in non-porous hydrogels continued to increase over time corresponding with continued surface degradation. pVEGF transfection levels were not high enough to enhance angiogenesis by increasing vessel density, maturity, or size, although by 6 weeks for all pore size hydrogels more hydrogel implants were positive for vascularization when pVEGF polyplexes were incorporated compared to control hydrogels. Pore size was found to be the dominant factor in determining the angiogenic response with 60 μm porous hydrogels having more vessels/area present than 100 μm porous hydrogels at the initial onset of angiogenesis at 3 weeks. The results of this study show promise for the use of polyplex loaded porous hydrogels to transfect infiltrating cells in vivo and guide tissue regeneration and repair.

1. Introduction

Vascularization of tissue engineering constructs remains the primary reason for construct failure in vivo [1,2]. Without the rapid infiltration of blood vessels, diffusion alone is insufficient to sustain migrating endogenous or exogenously implanted cells more than 150–200 μm from the construct surface. Diffusion limitations then dictate the overall size and function of the implant, limiting their applicability in vivo to small injuries and defects [1,3]. Thus, the promotion of angiogenesis (i.e. the formation of new vessels from pre-existing vessels) is essential for tissue engineering construct success. Efforts have already been made to promote angiogenesis within implanted hydrogels for soft tissue repair and regeneration through smart hydrogel design, hydrogel materials, and incorporation of pro-angiogenic growth factors and genes.

Over the past ten years, a major emphasis has been placed on macroscopic biomaterial design to help promote biomaterial vascularization. Patterning technologies, such as micro-contact printing, micro-molding, photolithography, micromachining, and laser-guided writing, have been used to form functional vascular structures inside biomaterials [4]. Although these patterning technologies allow for precise control over structure, issues with mass production has so far limited their clinical use. Alternatively, micro-scale interconnected pores produced through salt-leaching [5–7], gas foaming [8–10], lyophilization [11–14], and sphere templating [15–18] have shown to be effective in allowing for cellular migration in vitro [13,19,20] and tissue integration and, subsequent, enhanced scaffold vascularization in vivo [6,16]. Chiu et al. demonstrated that increasing pore size from 25 to 150 μm in synthetic PEG hydrogels increased overall cellular infiltration and collagen deposition, as well as vascular infiltration from the surrounding tissue into the pores of the PEG hydrogel [6]. Similarly Madden et al. incorporated both spherical pores as well as channels into poly(2-hydroxyethyl methacrylate-co-methacrylic acid) hydrogels which were shown to significantly enhance neovascularization 4 weeks after myocardial implantation [16]. However, they demonstrated that scaffold architecture influenced macrophage polarity and that an intermediate pore size of 30–40 μm lead to increased neovascularization as a result of a shift in

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macrophages in the M1 pro-inflammatory phase to macrophages in the M2 pro-healing (anti-inflammatory) stage. In all of these reports, pore size was found to play a crucial role in the rate of angiogenesis and the size and maturity of the formed vessels.

The type of natural (i.e. collagen, alginate, chitosan, hyaluronic acid (HA)) or synthetic (i.e. poly(ethylene glycol), poly(ethylene oxide), poly(vinyl alcohol), poly(acrylic acid), polypeptides) polymer used for hydrogel preparation is an important factor in determining cell–material interactions, mechanical properties, fluid permeability and, subsequently, promotion of angiogenesis [2,11,21,22]. While a synthetic polymer, such as PEG, can be biochemically inert, natural polymers possess intrinsic qualities which can play a role in signaling to surrounding cells. HA, an anionic, non-sulfated glycosaminoglycan and major component of the ECM, has gained popularity as a biomaterial for tissue engineering and regeneration due to its high biocompatibility and low immunogenicity [23–26]. Moreover, degraded fragments of HA or HA oligomers are known to promote angiogenesis and up-regulate MMP expression [27–29]. HA specifically interacts with cell surface receptors, such as CD44, RHAMM (receptor for HA mediated motility) and ICAM-1 (intercellular adhesion molecule 1), and contributes to tissue hydrodynamics, cell proliferation and migration [30,31]. Using mild chemistries the HA backbone can be modified to contain functional groups, such as thiols, acrylates or amines, which can be further used as crosslinking sites to form hydrogels [25,32–34]. As a result, several studies have demonstrated that HA-based hydrogels are good candidates for culturing stem cells [35–38]. Semi-synthetic hyaluronic acid (HA) hydrogels which are degradable by hyaluronidases as well as matrix metalloproteinases (MMPs) via MMP-degradable peptide crosslinkers have previously been developed for culturing mouse mesenchymal stem cells in 3-dimensions [39,40]. MMPs are normally expressed during tissue remodeling and are up-regulated during wound healing, microenvironment remodeling, and in diseased states and can, therefore, serve as triggers for bioactive signal delivery.

The effective local delivery of angiogenic factors, including vascular endothelial growth factor (VEGF) and platelet derived growth factor (PDGF), are necessary to promote blood vessel formation. While peptides and growth factors can be easily incorporated within these hydrogels, rapid degradation by proteases generally limits their effectiveness in long-term cell culture. For tissue regeneration, localized gene delivery can promote the expression of tissue inductive factors to guide tissue formation. Poly(ethylene imine) (PEI) is the most widely utilized cationic polymer for non-viral gene delivery; it is able to condense DNA through electrostatic interactions between the positively charged amines on the PEI and the negatively charged phosphates on the DNA, forming nanoparticles (polyplexes) in the range of 50–200 nm [41–43]. PEI has been successfully used in vivo to deliver DNA or siRNA to the brain [44,45], lungs [46–50], abdomen [51], liver [52], and tumors [53–55]. DNA/PEI polyplexes have also been incorporated into fibrin [17,56], alginate [57], gelatin [58], and other natural polymer based hydrogels [59]. Researchers have likewise utilized enzymatically degradable synthetic polymer scaffolds, which release their payload upon cellular infiltration [60–62]. Since then MMP-degradable hyaluronic acid hydrogels have also been used to encapsulate DNA/poly(PEI) polyplexes as a means of non-viral gene delivery to stem cells [63]. We found that as the matrix degraded through cell-secreted proteases, the cells were transfected with the polyplexes encountered during their migration. DNA polyplexes can also be loaded at therapeutically relevant concentrations ($\geq$1 μg/μL) using a caged nanoparticle encapsulation process (CnE) inside a variety of hydrogel scaffolds without particle aggregation [64,65]. This approach utilizes neutral saccharides (sucrose) and polysaccharides (agarose) to protect the polyplexes from inactivation and aggregation during lyophilization and hydrogel formation, respectively.

Since the emergence of non-viral gene delivery from hydrogel scaffolds, emphasis has been placed on complementing gene transfer with matrix design to enhance transfection efficiency. Micron-sized pores is one key factor which has shown to increase both viral [66] and non-viral [8,9,17] gene transfer by increasing the available surface area for cells to degrade the biomaterial and release the encapsulated signal. We previously investigated gene transfer to mouse mesenchymal stem cells (mMSCs) seeded within porous hyaluronic acid hydrogel scaffolds in vitro [67]. Using the CnE process to incorporate DNA/PEI polyplexes, porous hydrogels allowed for sustained transfection and transgene expression of incorporated mMSCs in various pore size hydrogels. For all investigated pore sizes transgene expression was sustained for up to ten days. We anticipated that the presence of an open pore structure would increase the rate of vascularization through enhanced cellular infiltration into the gel and that the added delivery of DNA encoding for angiogenic growth factors would result in long lasting angiogenic signals. Here we describe the results when this hydrogel system was used to deliver DNA in vivo in a mouse subcutaneous implant model.

2. Materials and methods

2.1. Materials

Peptides Ac-GCRDGPQGIWGQDRCG-NH2 (HS-MMP-SH) and Ac-GCGYGRGDSPG-NH2 (RGD) were purchased from Genscript (Piscataway, NJ). Sodium hyaluronan (HA) was a gift from Genzyme Corporation (60 kDa, Cambridge, MA). Linear poly(ethylene imine) (PEI, 25 kDa) was purchased from Polysciences (Warrington, PA). Vectors expressing mammalian GFP-firefly luciferase (pGFPluc) and human VEGF-165 (pVEGF) were obtained from New England Biolabs (Ipswich, MA) and expanded using a Giga Prep kit from Qiagen following the manufacturer's protocol. All other chemicals were purchased from Fisher Scientific (Pittsburgh, PA) unless otherwise noted.

2.2. Hyaluronic acid modification

Sodium hyaluronan was modified to contain acrylate functionalities. Briefly, hyaluronic acid (2.00 g, 60 kDa, 5.28 mmol carboxylic acids) was reacted with 36.77 g (211.07 mmol) adipic acid dihydrazide (ADH) at pH 4.75 in the presence of 4.00 g (20.84 mmol) 1-ethyl-3-[3-dimethylaminopropyl] carbodiimide hydrochloride (EDC) overnight and purified through dialysis (8000 MWCO) in a 100 mM–0 mM salt gradient for 1 day followed by dialysis in DI water for 4–5 days. The purified intermediate (HA-ADH) was lyophilized and stored at −20 °C until used. Approximately 54% of the carboxyl groups were modified with ADH, which was determined using ^{1}H NMR (D_2O) by taking the ratio of peaks at $\delta = 1.6$ and 2.3 corresponding to the 8 hydrogens of the methylene groups on the ADH to the singlet peak of the acetyl methyl protons in HA ($\delta = 1.88$). All of the modified HA-ADH was reacted with N-Acryloxysuccinimide (NHS-Ac) (4.46 g, 26.38 mmol) in HEPES buffer (10 mM HEPES, 150 mM NaCl, 10 mM EDTA, pH 7.2) overnight and purified through dialysis in a 100 mM–0 mM salt gradient for 1 day followed by dialysis in DI water for 3–4 days before lyophilization. The degree of acrylation was determined to be ~12% using 1H NMR (D_2O) by taking the ratio of the multiplet peak at $\delta = 6.2$ corresponding to the cis and trans acrylate hydrogens to the singlet peak of the acetyl methyl protons in HA ($\delta = 1.88$).

2.3. Polyplex lyophilization

For CnE, plasmid DNA (250 μg) and L-PEI (228.3 μg, N/P = 7) were mixed in 3.5 mL water in the presence of 35 mg (0.10 mmol) of sucrose (Ultra pure, MP Biomedicals, Santa Ana, CA) and incubated at room temperature for 15 min. Low-melting point agarose (1.0 mg, UltraPure™ Agarose, *Tm* = 34.5–37.5 °C, Invitrogen, Grand Islands, NY) in 1.5 mL water was added before lyophilization. Each aliquot was intended for a 100 μL hydrogel. For smaller hydrogel volumes, both sucrose and agarose were scaled down proportionally.

2.4. Design template using PMMA microspheres

Microsphere templates for porous hydrogels were prepared using dry PMMA microspheres (27–33, 53–63, and 90–106 μm, Cospheric, Santa Barbara, CA). Approximately 20 mg of microspheres (1.19 mg/μL) were mixed with DI water for a final concentration of 20 mg per 100 μL. Then 100 μL of the microsphere solution was pipetted into each well in a glass-bottom silicon well mold (wells = 6 mm × 2 mm, D × H). The microspheres were then allowed to dry and pack (by naturally settling) over 3–4 h at 37 °C. The glass-bottom silicon wells were then placed into an oven and the microspheres were sintered for 22 h at 150 °C.