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Nanoparticles for localized delivery of hyaluronan oligomers towards regenerative repair of elastic matrix

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ABSTRACT

Abdominal aortic aneurysms (AAAs) are rupture-prone progressive dilations of the infrarenal aorta due to a loss of elastic matrix that lead to weakening of the aortic wall. Therapies to coax biomimetic regenerative repair of the elastic matrix by resident, diseased vascular cells may thus be useful to slow, arrest or regress AAA growth. Hyaluronan oligomers (HA-o) have been shown to induce elastic matrix synthesis by healthy and aneurysmal rat aortic smooth muscle cells (SMCs) in vitro but only via exogenous dosing, which potentially has side-effects and limitations to in vivo delivery towards therapy. In this paper, we describe the development of HA-o loaded poly(lactide-co-glycolide) nanoparticles (NPs) for targeted, controlled and sustained delivery of HA-o towards the elastogenic induction of aneurysmal rat aortic SMCs. These NPs were able to deliver HA-o over an extended period (>30 days) at previously determined elastogenic doses (0.2–20 μg ml^{-1}). HA-o released from the NPs led to dose-dependent increases in elastic matrix synthesis, and the recruitment and activity of lysyl oxidase, the enzyme which cross-links elastin precursor molecules into mature fibers/matrix. Therefore, we were able to successfully develop a nanoparticle-based system for controlled and sustained HA-o delivery for the in vitro elastogenic induction of aneurysmal rat aortic smooth muscle cells.

1. Introduction

Abdominal aortic aneurysms (AAAs) are rupture-prone localized expansions of the aortic wall, which account for over 16,000 deaths a year in the USA [1]. Screening and detection of early-stage AAAs in recognized high-risk patients are now possible using high-resolution ultrasound, magnetic resonance imaging (MRI) and computed tomography (CT) techniques [2]. Post-diagnosis, AAAs are typically monitored passively every 6 months during the slow growth phase, until they attain a critical size (>5.5 cm in diameter) or their growth rate exceeds 0.5 cm per year, at which stage surgical intervention is mandated [3]. However, since surgery is unsuitable for many patients and often has high complication rates, a non-surgical AAA treatment, applicable during the slow growth period, is desirable [4]. In this context, we seek to develop a matrix regenerative therapy for AAAs. Important to such an approach is the need to stimulate regeneration of elastin and elastic matrix, which, unlike collagen, the other major structural component of aortic tissues, is auto-regenerated rather poorly in adult tissues.

Elastin is a very important component of connective tissue as it determines the elasticity and resilience, long-range deformability and passive recoil of the aorta [5]. Auto-regeneration of elastic matrix post-disruption as mentioned is problematic since adult and especially diseased cells are very deficient in elastin synthesis [1]. Accordingly, a significant elastogenic stimulus needs to be provided to coax aneurysmal cells to be able to regenerate disrupted elastic matrix towards stabilizing and potentially arresting AAA growth. Importantly, for net accumulation of de novo synthesized elastic matrix at the AAA site to be achieved, matrix metalloproteinases (MMPs), proteolytic enzymes chronically expressed at the AAA site, must also be attenuated concurrently.

Prior studies in our laboratory [5] have demonstrated that the pro-elastogenic effects of hyaluronan (HA), a matrix glycosaminoglycan (GAG), depend on its molecular weight (MW) or chain length. Oligomeric forms of HA (HA-o), particularly mixtures of HA 4mers and 6mers, have been shown to be capable of providing a significant stimulus for precursor synthesis and elastic matrix assembly [6,7]. We have previously shown HA-o to induce elastin regeneration by cultured aneurysmal rat aortic smooth muscle cells (SMCs), which are very elastogenically deficient [8]. To ensure that HA-o is delivered in a localized, predictable and sustained manner to the site of tissue elastolysis, in this study we investigate the ability of HA-o to be delivered from nanoparticles (NPs) and the effect of surface modification of the NPs on aspects of elastic ma-

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trix assembly and proteolysis in vitro within aneurysmal rat aortic smooth muscle cell (EaRASMC) cultures.

2. Methods and materials

2.1. Formulation of HA-loaded PLGA NPs

Poly(DL-lactide-co-glycolide) (PLGA; 85:15 lactide/glycolide; inherent viscosity 0.55–0.75 dl g^{-1} in hexafluoroisopropanol; Durect Corporation, Birmingham, AL) NPs were synthesized, described in a previous paper from our group [9], using a well-established double emulsion solvent evaporation method [10–12]. The NPs were loaded with a mixture of HA oligomers (HA-o) containing 75 wt.% of 4mers and 25 wt.% 6mers, prepared as described our previous paper [7]. Briefly, PLGA (50 mg) was dissolved in 2 ml of organic solvent, dichloromethane (DCM) or chloroform, depending on the surfactant used. HA-o at desired loadings (3, 5 and 8 wt.% ratios of HA-o:PLGA) was emulsified with the organic solvent by sonication at 20% amplitude. The aqueous phase consisted of 12 ml of nanopure water, containing polyvinyl alcohol (PVA) or didodecyldimethylammonium bromide (DMAB) as the surfactant at a concentration of 0.25 wt./vol.%. Chloroform was used as a solvent with DMAB and DCM with PVA. The organic phase was added to the aqueous phase and sonicated, then left stirring overnight to evaporate the organic phase. Samples were desiccated for 1 h under vacuum prior to NP separation by ultracentrifugation at 35,000 rpm for 30 min (Beckman L-80, Beckman Instruments Inc., Palo Alto, CA). After separation, the NPs were further washed twice with nanopure water, sonicated and centrifuged at 30,000 rpm for 30 min prior to lyophilization (48 h) to generate NPs in a sterile dry powder form. The supernatants from the separation and wash steps were analyzed to calculate HA-o loading efficiency within the NPs.

2.2. Determination of size and surface charge of HA-o loaded PLGA nanoparticles

The mean hydrodynamic diameters of the NPs were determined by dynamic light scattering and NP surface charges or ζ-potentials were estimated with phase analysis light scattering, as described earlier [9]. Both measurements were performed on a commercial particle-sizing system (PSS/NICOMP 380/ZLS, Particle Sizing Systems, Santa Barbara, CA).

2.3. Isolation of healthy and aneurysmal rat aortic SMCs

Animal procedures were all conducted with approval of the Institutional Animal Care and Use Committee (IACUC) at the Cleveland Clinic. The animal facility is AAALAC-approved and has animal assurance (#A3145-01; expires 04/30/15). EaRASMCs were isolated from AAAs generated in adult male Sprague–Dawley rats ($n = 3$) via elastase infusion, as previously described by our laboratory [8,13], at 14 days post-induction. Briefly, the AAA tissue was harvested, cut longitudinally and the intimal layer was scraped off; the medial layer was then dissected from the adventitial layer and chopped into ~0.5 mm slices and rinsed twice in 37 °C phosphate–buffered saline (PBS). The tissue pieces were enzymatically digested in Dulbecco's modified Eagle's medium (DMEM-F12; Invitrogen, Carlsbad, CA) containing 125 U mg^{-1} collagenase (Worthington Biochemicals, Lakewood, NJ) and 3 U mg^{-1} elastase (Worthington Biochemicals) for 30 min at 37 °C. These were then centrifuged at 400*g* for 5 min and cultured in T-75 flasks for ~2 weeks in DMEM-F12 medium (Invitrogen) supplemented with 10 vol.% fetal bovine serum (FBS; PAA Laboratories Inc., Etobicoke, Ontario) and 1 vol.% penicillin–streptomycin (PenStrep; ThermoFisher, South Logan, UT). The isolated primary EaRASMCs were propagated for 2 weeks and passaged when they reached confluence.

2.4. Cytotoxicity of PLGA NPs and evidence of infiltration or exclusion of NPs by cells

The dose-dependent cytotoxic effects of the PLGA NPs generated using DMAB and PVA as stabilizers were quantified using a LIVE/DEAD® viability assay (Invitrogen). According to the manufacturer, this assay has been validated by several laboratories as being a faster, safer and more sensitive method to indicate cytotoxicity compared to other methods. Additionally, the assay has been used to quantify cell death via apoptosis [14,15] and cell-mediated cytotoxicity and necrosis [16,17]. Passage 2 EaRASMCs were seeded at a concentration of 7.5×10^4 cells per well in sterile 6-well plates (area = 9.6 cm^2) and cultured in DMEM-F12 cell medium supplemented with 5 vol.% FBS and 1 vol.% PenStrep for 48 h. PLGA NPs prepared with either PVA or DMAB were added to the cells at different concentrations (0, 0.2, 0.5 and 1 mg ml^{-1}) and the cells further cultured for 7 days. Thereafter, the cells were assayed and imaged on a fluorescent microscope (IX51, Olympus America, Center Valley, PA), with five random regions being visualized per replicate culture.

2.5. HA-o loading efficiency and release from PLGA NPs

A hyaluronan carbazole assay was used to detect and quantify HA-o release from NPs. The assay is based on the established modified Bitter–Muir carbazole method [18]. To determine the loading efficiency of HA-o within the NPs, supernatant saved from their synthesis was filtered in a 3 kDa filter centrifuge tube (Amicon, Billerica, MA) and assayed for HA content. The release study was carried out using a double chamber 3 kDa filter centrifuge tube, which was loaded with 8 ml PBS in the receiver side and 3 ml of NP suspension in PBS on the donor side; 0.5 ml aliquots were removed from the receiver chamber and replaced with fresh PBS at each time point (days 0, 1, 4, 7, 11, 14, 17, 21, 26 and 31). The tubes were incubated on a shaker in a 37 °C chamber to simulate physiological conditions and keep the nanoparticles in suspension. Each 0.5 ml sample was assayed for HA-o content and compared with known standards (4 and 6mer mixture of known concentrations) in order to quantify the extended release profiles of HA-o from the NPs. Based on a known volume (0.5 ml) of sample removed and the HA-o concentration measured within, the (a) amount of HA-o released since the previous sampling event and (b) cumulative release of HA-o up to that time point were calculated using the following formulae: $X_{n+1} = X_{n+1} - X_n \times (10.5/11)$ and $X_{\text{total}} = X_1 + X_2 + \ldots X_i$, where X represents the amount of HA-o detected in the sampled volume and n represents the sampling event itself. The loading efficiency was calculated based on the unencapsulated HA detected in the supernatant using the formula $100 \times$ [(loaded HA-unencapsulated HA)/loaded HA].

2.6. Cell culture with nanoparticles

Passage 2 EaRASMCs were seeded at 6×10^4 cells per well in 6-well plates and 3×10^4 cells per well in 2-well chamber slides (area = 4.2 cm^2) and cultured for 72 h in DMEM-F12 medium supplemented with 10 vol.% FBS and 1 vol.% PenStrep. After this period, the medium was aspirated and replaced with an NP suspension prepared in fresh medium containing 5 vol.% FBS and 1 vol.% PS. These suspensions contained NPs loaded with either 0 (control), 3, 5 or 8 wt.% HA-o provided at a concentration of 0.5 mg ml^{-1} ($n = 6$ cultures per HA-o loading). EaRASMCs cultured with no NPs served as additional treatment controls. The cells were

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