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Multifunctional nanoparticles for doxycycline delivery towards localized elastic matrix stabilization and regenerative repair

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ABSTRACT

Abdominal aortic aneurysms (AAAs) are abnormal expansions of the aortic wall, typically characterized by chronic up-regulation of matrix metalloproteases (MMPs)-2 and -9. These MMPs degrade elastin and elastic matrix within the aortic wall, leading to a progressive loss of elasticity of the abdominal aorta as the condition progresses. Doxycycline (DOX) is a tetracycline-based antibiotic which has shown significant promise in delaying and slowing the growth of AAAs in both clinical studies and animal models. However, it has been found to inhibit elastic matrix deposition by vascular cells at dosages in the μg ml^{-1} range, which is typically observed in the circulation, in addition to systemic side effects, following oral dosage. In this paper, we describe the development of DOX-loaded poly(lactic-*co*-glycolic acid) (PLGA) nanoparticles for localized, controlled and sustained DOX delivery towards AAA therapy. Further, we demonstrate that surface functionalization of these nanoparticles with cationic amphiphiles not only imparts them with a positive charge for potentially enhanced aortic uptake, but also enables enhanced elastin binding via hydrophobic interactions, as well as up-regulating activity of the elastin crosslinking enzyme lysyl oxidase. In addition to the DOX released from the nanoparticles being effective in inhibiting MMP-2 production and activity, we also demonstrate that surface functionalization of the nanoparticles cationic amphiphiles may also play a role in MMP-2 inhibition via (i) electrostatic interactions with negatively charged residues in the active-site of MMP-2 or (ii) steric blockade of the active site on account of the presence of two dodecyl chains in the DMAB molecule. Thus, in addition to enhanced aortic uptake and retention illustrated in studies by other groups, we have demonstrated that cationic functionalization of PLGA nanoparticles enhances elastogenic outcomes by targeted binding to elastin, as well as their potential to inhibit elastolysis. These results establish their multifunctionality as a localized delivery system for AAA therapy. Overall, this delivery system has the potential to enhance regenerative outcomes at sites of proteolytic matrix disruption/degradation by enabling targeted, controlled and long-term release of therapeutic agents.

1. Introduction

Abdominal aortic aneurysm (AAA) is a disease condition that primarily afflicts older humans (males > 65 years of age) [1]. It involves localized dilatation of the abdominal aorta due to progressive elastic matrix loss, leading to weakening of the vessel wall and potentially culminating in fatal rupture [2]. More than 90% of the detected human AAAs are small (maximal diameter < 5.5 cm) [3], and grow very slowly (~10% per year) [4] over 5 or more years. During this period, AAAs are typically monitored passively via ultrasound until they attain a critical size (diameter of 5.5 cm), beyond which they are prone to rupture. At this point they are treated via surgical or minimally invasive methods, which may frequently be unsuitable for senior patients. Presently there are no non-surgical AAA treatment strategies applicable during this 5 year window of opportunity to treat patients with small, asymptomatic AAAs, or those high-risk patients who may not withstand surgery.

AAA growth has been linked to progressive elastolysis in the aortic wall [5,6]. This is mediated by matrix metalloproteinases (MMPs), especially MMP-2 and -9, which are overexpressed by inflammatory cells recruited to the AAA site following initial causative injury. MMP-9, in particular, has been shown to play a critical role in AAA growth in experimental animal models of AAA, in that targeted deletion of the MMP-9 gene has been shown to suppress AAA development [7]. On this basis, pharmacological inhibition of MMP production and/or activity is a potent strategy for inhibiting elastic matrix degradation to attenuate or even suppress AAA growth.

Doxycycline (DOX), a tetracycline derivative, is known to non-specifically inhibit a variety of MMP-types via direct coordination with the catalytic site, and by transcriptional inhibition of MMP mRNA [8–10]. It has been shown to be useful in delaying the

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growth of induced AAAs in animal models [7,11–15], as well as in clinical studies in human AAAs [16–18]. Further, DOX has also been shown to directly inhibit MMP-2 and -9 activity or pro-MMP activation in some cultured cell types [19], resulting in loss of their enzymatic activity [20].

To date, treatment of AAAs in humans and animal models has been achieved via the oral route. However, oral administration of DOX has been shown to lead to dose-dependent systemic side effects, such as gastrointestinal tract disturbances, dental discoloration and cutaneous photosensitivity in some patients [16]. Moreover, since MMPs are also involved in remodeling of healthy tissues, their system-wide inhibition can have an adverse impact. Hence, it is imperative to deliver DOX for effective AAA treatment to the aortic wall in a localized and sustained manner, to avoid or minimize any systemic side effects associated with its oral or parenteral administration.

Recent studies have reported on localized periaortic delivery of DOX via implanted micropump systems [11,21], and have demonstrated the efficacy of DOX delivered in this manner in suppressing the development of experimental AAAs at doses 100-fold lower than that achievable by systemic DOX administration [11]. While these studies suggest that localized DOX delivery over a limited time can also inhibit AAA dilatation, due at least in part to its inhibition of matrix elastolysis, the side effects of long-term implantation of microosmotic pumps is unknown, but are likely adverse.

As an alternative, in this study we investigated a localized DOX delivery approach for treating AAAs via its controlled, long-term release from poly(lactic-*co*-glycolic acid) nanoparticles (PLGA NPs). We specifically sought to (i) examine the release of DOX from the NPs as a function of DOX loading and NP concentration, and (ii) analyze the impact of DOX released from NPs on elastic matrix deposition and MMP inhibition in aneurysmal smooth muscle cell (SMC) cultures. The latter aspect is particularly relevant since DOX has been implicated in inhibition of matrix synthesis in a variety of cell types [22], including vascular SMCs [23], and thus can interfere with new elastic matrix deposition or accumulation in AAA tissue. Additionally, cationic amphiphiles, which are used to impart a positive surface charge on NPs to enhance their aortic uptake and retention [24], are also known to have been found to bind elastin and stimulate activity of lysyl oxidase (LOX) [25,26], the enzyme that mediates crosslinking of elastin precursors into a mature matrix. Based on this, we evaluate the effects of surface modification of NPs with cationic amphiphiles from the standpoint of targeted binding to elastin when delivered to AAAs, and enhancement of LOX activity. This work will not only guide localized, controlled delivery of DOX within AAAs, or more generally at sites of tissue proteolytic disease from the standpoint of inhibiting MMPs, but also enhance regenerative elastic matrix repair towards slowing, arresting and even regressing tissue disruption.

2. Materials and methods

2.1. Isolation and culture of SMCs from elastase perfusion-induced rat AAAs

All animal procedures were conducted with the approval of the Institutional Animal Care and Use Committee at the Cleveland Clinic (ARC # 2010-0299). The clinic's animal facility is AAALAC-approved and has animal assurance (#A3145-01). Aneurysmal rat aortic SMCs (EaRASMCs) were isolated from adult male Sprague–Dawley rats ($n = 3$) at 14 days post-AAA induction via elastase infusion, as described in an earlier study by our group [27]. The aortae were cut open longitudinally and the intimal layer scraped off gently with a scalpel. The medial layer was then dissected from the underlying adventitial layer, chopped into ~0.5 mm long slices and washed twice with warm, sterile phosphate-buffered saline (PBS). These were pooled and enzymatically digested in DMEM-F12 cell culture medium (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, centrifuged (400*g*, 5 min) and cultured for over 2 weeks in T-75 flasks. The cells were cultured in DMEM-F12 medium (Invitrogen) supplemented with 10 vol.% fetal bovine serum (FBS; PAA Laboratories, Etobicoke, Ontario) and 1 vol.% penicillin–streptomycin (PenStrep; Thermo Fisher, South Logan, UT). The primary EaRASMCs obtained from these tissue explants were propagated over 2 weeks, and passaged when they attained confluence.

Primary healthy rat aortic smooth muscle cells (RASMCs) were similarly isolated from aortae excised from healthy adult Sprague–Dawley rats ($n = 3$), and propagated for cell culture experiments as described in previous studies by our group [28,29].

2.2. Determination of appropriate PLGA NP size for delivery to SMC cultures

An acceptable PLGA NP size based on their relative exclusion by cells in the extracellular space was determined by incubating fluorescent PLGA NPs (FITC–PLGA; Fluorophorex™, Phosphorex, Inc., Fall River, MA) of three different sizes (100, 200 and 500 nm hydrodynamic diameter) with cultured EaRASMCs. Briefly, the EaRASMCs were seeded at 1.5×10^4 cells $well^{-1}$ in sterile, two-well Permanox® chamber slides (Nalge Nunc International, Rochester, NY) and cultured in DMEM-F12 cell culture medium supplemented with 2 vol.% FBS and 1 vol.% PenStrep. The FITC–PLGA NPs were added to the EaRASMCs at a concentration of 0.2 mg ml^{-1} and incubated at 37 °C for 48 h. The cells were then fixed with 4 vol.% formaldehyde, labeled with the lipophilic membrane stain DiI (Invitrogen, Carlsbad, CA), and then mounted with Vectashield containing the nuclear dye 4′,6-diamidino-2-phenyindole (DAPI; Vector Laboratories, Burlingame, CA). Imaging to visualize NP uptake or exclusion was performed using a spectral laser scanning confocal microscope (Leica TCS SP2, Leica Microsystems, Inc., Buffalo Grove, IL). To visualize and confirm the intracellular presence or extracellular exclusion of NPs, z-stack maximum projections (overlays) were created from images acquired at 1 μm intervals across the thickness of the cell layers using Leica LAS AF software. Outcomes were deduced from a minimum of 10–12 images per NP size.

2.3. Formulation of DOX-loaded PLGA NPs

Poly(DL-lactic-co-glycolic acid) nanoparticles (PLGA; 50:50 lactide:glycolide; inherent viscosity 0.95–1.20 dl g^{-1} in hexafluoroisopropanol; Durect Corporation, Birmingham, AL) loaded with doxycycline hyclate (DOX; Sigma–Aldrich, St. Louis, MO) were prepared by a double emulsion solvent evaporation technique [24,30,31]. Briefly, PLGA was dissolved in chloroform (Fisher Scientific, Fair Lawn, NJ) at a concentration of 2.5–3.0% w/v. An aqueous DOX solution at three different loadings (2, 5 and 10 wt.% ratios of DOX:PLGA) was emulsified into the PLGA solution using a probe sonicator (Q500; QSonica LLC, Newtown, CT) for 30 s on ice, at an amplitude setting of 20%. The water-in-oil emulsion thus formed, was further emulsified into an aqueous solution of 1% w/v didodecyldimethylammonium bromide (DMAB; Sigma–Aldrich) using the probe sonicator for 30 s on ice at 20% amplitude setting, to form a water-in-oil-in-water emulsion. This second emulsion was stirred for 16 h at room temperature, then desiccated for 1 h under a vacuum to remove any residual chloroform. The NPs formed were recovered by ultracentrifugation at 35,000 rpm (Beckman L-80, Beckman Instruments, Inc., Palo Alto, CA). The NPs were washed twice with nanopure water to remove residual DMAB and