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Amelioration of atherosclerotic inflammation and plaques via endothelial adrenoceptor-targeted eNOS gene delivery using redox-sensitive polymer bearing L-arginine

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ABSTRACT

Endothelial dysfunction combined with inflammation leads to atherosclerosis. Endothelium-specific delivery of therapeutic agents at the cellular level—specifically *in vivo*—is still a difficult task for proper management of atherosclerosis. We designed a redox-sensitive poly(oligo-L-arginine) (rsPOLA) playing dual roles as an endothelium α-2 adrenoceptors(α-2ARs)-targeted gene carrier and as a substrate for endothelial nitric oxide synthase (eNOS). Overexpression of α-2ARs on atherosclerotic endothelial cells was confirmed and the eNOS/rsPOLA nanoplexes following systemic injection demonstrated to 1) enhance eNOS gene delivery into endothelial cells *via* α-2ARs/L-arginine specific binding, 2) increase intracellular level of nitric oxide, 3) suppress inflammatory response in endothelium and finally 4) reduce atherosclerotic plaque in a $Ldlr^{-/-}$ atherosclerotic mouse model. Among the tested nanoplexes [eNOS/rsPOLA, eNOS/{poly(oligo-D-arginine), rsPODA} and eNOS/(racemic mixture, rsRM)], eNOS/rsPOLA reduced atherosclerotic inflammation most effectively as we hypothesized. Current treatment strategy provides strong potential for further development of a gene therapeutic system to ameliorate inflammation and progressive atherosclerotic plaques.

1. Introduction

Endothelial dysfunction combined with inflammation collectively leads to a dynamic and advanced deterioration in health, called atherosclerosis. Rupture of atherosclerotic plaques typically results in myocardial infarctions and strokes [1]. Transformations in the vasculature system at the cellular level are key contributors to the initiation and progression of atherosclerosis. Endothelial cells initiate transformations by release of factors into the bloodstream, alteration of intercellular junctions, dynamic expression of specific receptors and adaptation of the immune response [2]. These effects mostly results in dysfunctional endothelium promoting diseases in the body [3]. Endothelial nitric-oxide synthase (eNOS) in endothelial cells is responsible for 'endothelium-dependent relaxation' of blood vessels by catalysing nitric oxide (NO) production from L-arginine to maintain vascular tone. NO is a critical communication molecule responsible for multiple biofunctions including inhibition of leukocyte chemotaxis, aggregation of platelets and smooth muscle cells, vasodilatation and re-endothelialization [4]. Therapeutic modulation of NO production is challenging because its effects must occur at the right time, location and amount. Due to the pleiotropic nature of NO, a variety of therapeutic agents that either increase NO signaling or enhance endogenous NO production have been studied in cardiovascular diseases. However, the therapeutic use of organic nitrates, such as nitroglycerin, is limited by poor pharmacokinetics and development of tolerance [5]. Hybrid drugs such as NO inhalers and polymers have been also explored for clinical use, but the short half-life of NO (~2–5 s), low bioavailability and potential of cardiovascular side effects distal from the site has led to 'failure of first-generation of NO-generating therapies'.

Site-specific delivery of therapeutic agents at the cellular level—specifically *in vivo*—is still a difficult task for proper management of atherosclerosis because only direct delivery into the endothelium can further enhance NO production. The eNOS gene delivery is an attractive option for the cure of endothelial dysfunction as it ensures sustained local NO production [6–8]. The determined Km value of L-arginine is 2.9 μM for eNOS-dependent NO production [9] and intracellular concentrations of L-arginine are approximately 0.8–2.0 mM, implying that cells preserve saturating levels of L-arginine as a precursor. However, external supplementation of L-arginine can enhance the NO generation by a phenomenon called the "L-arginine paradox" [10,11].A meta-analysis of clinical trials showed a decrease in systolic and diastolic pressure by ~5 mmHg and ~3 mmHg, respectively, with oral

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supplementation of L-arginine [12]. Another study showed that shear stress-mediated NO formation is dependent on the presence of extracellular L-arginine, which increase cGMP levels in a concentration dependent manner (EC50 = 123 μM) [13]. This confirms that substrate availability is the rate-limiting step of eNOS-mediated NO production, which produces reactive oxygen species in the absence of L-arginine. L-arginine contains a guanidinium group and thus exhibits a structural resemblance to ligands for imidazoline receptors (I-receptors) and alpha-2 adrenoceptors (α-2AR). Imidazoline and α-2ARs-mediated production of NO upon L-arginine supplementation provides a mechanistic explanation for the selective uptake of L-arginine by endothelial cells [14]. Endothelial cells express a number of receptors including adrenoceptors [14,15], adenosine A2 receptors and imidazoline receptors [16,17]. The stimulation of α-2ARs on endothelial cells has been demonstrated to indirectly induce dilation of coronary arteries *in vivo*, by the release of endothelium deriver release factor (EDRF), which counteracts the contractions of smooth muscle cells [18,19]. Recently, overexpression of α-2ARs was also reported in diabetes [20]. The integrity of the endothelium should be considered when interpreting vascular responses to α-2ARs agonists.

Here, we studied the overexpression of α-2ARs on endothelial cells in $Ldlr^{-/-}$ atherosclerotic *in vivo* models and it was hypothesized that overexpressed α-2ARs could be target receptors for redox-sensitive poly (oligo-L-arginine) (rsPOLA) delivering DNA into the endothelial cells and participating in NO production as a substrate pool for eNOS catalysis. For auxiliary boost of NO production, eNOS gene was selected as a therapeutic DNA. Furthermore, rsPOLA were compared with redox-sensitive poly(oligo-D-arginine) (rsPODA) and racemic mixture (rsRM) of rsPOLA and rsPODA. The rsPOLA and rsPODA were synthesized by oxidative polymerization [21] and then nanoplexes with the eNOS gene were prepared. Redox-sensitive eNOS/poly(oligo-arginines) nanoplexes were studied on enhancing NO production and lowering inflammatory cytokine levels, *i.e.* IL-1β, IL-6, TNF-α, MCP-1, and VCAM-1 for attenuation of atherosclerosis.

2. Materials and methods

2.1. Materials and reagents

Peptides C-D9R-C and C-L9R-C were purchased from Peptron Inc. (Daejeon, Korea). Branched polyethyleneimine (PEI Mw = 25,000) and Griess reagent (G4410) were purchased from Sigma Aldrich (Saint Louis, MO, USA). Endothelial nitric oxide synthase (eNOS) plasmid in pCMV vector (pcDNA 3.1) was obtained from Thermo Scientific (CA, USA). SVEC4-10 cells were purchased from ATTC (ATCC® CRL-2181™). Raw264.7 and HEK293 cells were acquired from the Korea cell bank. Dulbeco's Modified Eagle's Medium (DMEM), fetal bovine serum, penicillin and streptomycin were bought from Welgene (Seoul, Korea). ELISA kits for IL-1β, IL-6, TNF-α and MCP-1 were bought from eBioscience (San Diego, CA, USA) and the kit for VCAM-1 was from R & D Systems (Minneapolis, MN, USA). PE Annexin V kit was purchased from BD Bioscience (San Diego, CA, USA). Label for redox-sensitive poly(oligo-arginines), Alexa488, was purchased from Life Technologies (USA) and the label for DNA vector, Cy®5.5, was bought from Mirus (WI, USA).

2.2. Synthesis and characterization of redox-sensitive poly(oligo-arginines)

Both rsPOLA and rsPODA were prepared by oxidative polymerization of Cys-L9R-Cys and Cys-D9R-Cys in phosphate buffer solution containing 30% DMSO. The reaction was continued for 1 week and low molecular weight impurities were removed from resulting the polymers, rsPOLA and rsPODA, by dialysis (MWCO: 3500 Da). Molecular weights of peptides and polymers were measured by liquid chromatography/mass spectroscopy (LC-MS) in a time-dependent mode until they reached a plateau. First, peptides (C-L9R-C and C-D9R-C) and polymers (rsPOLA, rsPODA, and rsRM) were rinsed repeatedly with diethylether and dried under vacuum. The products were then purified by preparative reversed-phase HPLC (RP-HPLC) on a Shimadzu 5 μm Shiseido Capcell Pak C18 column (4.6 × 50 mm) by using gradient elution with the following eluents: A) 0.1% trifluro acetic acid (TFA) in water and B) 0.1% TFA in acetonitrile. After sample application, a linear gradient from 3 to 60% was generated over 60mins at room temperature with a flow rate of 1 ml/min. UV detection was performed at λ = 220 nm and the purity was investigated using analytical RP-HPLC on a Shiseido Capcell Pak C18 column (4.6 × 50 mm). The relative molecular weight of the peptides and polymers was determined mass spectrometry (HP 1100 series LC/MSD, Peptron, Daejon, Korea). The molecular weights of purified rsPOLA and rsPODA were determined as 20,610.3 Da and 21,697.3 Da, respectively. This represents a significant increase in molecular weight of resulting the redox-sensitive poly(oligo-arginines) over the original peptides (C-L9R-C and C-D9R-C) molecular weight of 1627.3 Da.

2.3. Characterization of nanoplexes

Morphologic characteristics of DNA condensed with redox-sensitive poly(oligo-arginines) were confirmed by energy filtered transmission microscopy (EF-TEM) (EM9120, Carl Zeiss Vision GmbH, Germany) in the Korea Basic Science Institute, Chuncheon Centre (Chuncheon, Korea). To prepare samples, 10 μg of DNA was condensed with PEI, rsPOLA, rsPODA and rsRM in deionized water. After incubation for 30 min, samples were dried at 50 °C on 10 × 10 TEM grids and coated with uranyl acetate for negative staining. Nanoplexes with different amounts of cationic redox-sensitive poly(oligo-arginines) including rsPOLA, rsPODA, rsRM and PEI were prepared with 1 μg of DNA in deionized water. After incubation for 30 min at room temperature, samples were electrophoresed on 0.8% (w/v) agarose gel (Lonza, ME, USA) in TBE buffer solution at 100 V for 20 min and the position of the DNA was examined using an imaging station. For the gel retardation assay in the presence of β-mercaptoethanol (β-ME), nanoplexes were incubated for 30 min at RT. After incubation β-ME was added to break the disulfide bonds between cysteines under gentle stirring for 1 h at 37 °C. The samples were electrophoresed as described above. DNA nanoplexes were prepared by mixing DNA with rsPOLA, rsPODA and rsRM in deionized water at w/w ratios of 1:1, 1:2, 1:3 and 1:4. To measure mean diameters and surface charges we performed dynamic light scattering using Zetasizer-Nano ZS (Malvern Instruments, Worcestershire, UK), after incubation for 30 min at RT. To observe nanoplexes behaviors under reductive environments, β-ME was added to samples and an equivalent volume of PBS was added to the control groups. To test whether rsPOLA, rsPODA and rsRM protects DNA in serum, naked DNA and DNA/rsPOLA, rsPODA and rsRM nanoplexes were incubated in 50% mouse serum for 2, 6, 18 and 24 h and their stability was observed using the imaging station.

2.4. Cell culture

SVEC4-10 cells were maintained in Dulbecco's Modified Eagle's Medium (DMEM) high glucose with 1.5 g $NaHCO_3$. Raw 264.7 and HEK 293 cells were maintained in high glucose DMEM supplemented with 10% fetal bovine serum (FBS) and antibiotics, 100 U/ml penicillin, and 100 mg/ml streptomycin.

2.5. Quantitative measurement and immunofluorescence assay of α-2ARs

To observe the expression of α-2ARs on endothelial cells (SVEC4-10), the mRNA level for α-2ARs was measured after TNF-α activation. Inactivated cells were used as negative control. A total of 1×10^6 cells was collected after centrifugation and resuspension in PBS. Total RNA was isolated from the cell suspension using a RNA easy tissue mini kit (Qiagene GmbH, Germany) according to the manufacturer's protocol.