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Nitric oxide improves molecular imaging of inflammatory atheroma using targeted echogenic immunoliposomes

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ABSTRACT

Objective: This study aimed to demonstrate whether pretreatment with nitric oxide (NO) loaded into echogenic immunoliposomes (ELIP) plus ultrasound, applied before injection of molecularly targeted ELIP can promote penetration of the targeted contrast agent and improve visualization of atheroma components.
Methods: ELIP were prepared using the pressurization-freeze method. Atherosclerosis was induced in Yucatan miniswine by balloon denudation and a hyperlipidemic diet. The animals were randomized to receive anti-intercellular adhesion molecule-1 (ICAM-1) ELIP or immunoglobulin (IgG)-ELIP, and were subdivided to receive pretreatment with standard ELIP plus ultrasound, NO-loaded ELIP, or NO-loaded ELIP plus ultrasound. Intravascular ultrasound (IVUS) data were collected before and after treatment.
Results: Pretreatment with standard ELIP plus ultrasound or NO-loaded ELIP without ultrasound resulted in 9.2 ± 0.7% and 9.2 ± 0.8% increase in mean gray scale values, respectively, compared to baseline ($p < 0.001$ vs. control). Pretreatment with NO-loaded ELIP plus ultrasound activation resulted in a *further* increase in highlighting with a change in mean gray scale value to 14.7 ± 1.0% compared to baseline ($p < 0.001$ vs. control). These differences were best appreciated when acoustic backscatter data values (RF signal) were used [22.7 ± 2.0% and 22.4 ± 2.2% increase in RF signals for pretreatment with standard ELIP plus ultrasound and NO-loaded ELIP without ultrasound respectively ($p < 0.001$ vs. control), and 40.0 ± 2.9% increase in RF signal for pretreatment with NO-loaded ELIP plus ultrasound ($p < 0.001$ vs. control)].
Conclusion: NO-loaded ELIP plus ultrasound activation can facilitate anti-ICAM-1 conjugated ELIP delivery to inflammatory components in the arterial wall. This NO pretreatment strategy has potential to improve targeted molecular imaging of atheroma for eventual true tailored and personalized management of cardiovascular diseases.

1. Introduction

Progression of atheroma to a vulnerable morphology, resulting in plaque rupture and activation of the extrinsic coagulation pathway, has been implicated in acute myocardial infarction and ischemic stroke [1,2]. Inflammation is thought to be a central mechanism for progression of atheroma from a stable lesion to a vulnerable plaque and its consequent clinical events. Much work has been done to develop molecularly targeted contrast agents for various imaging modalities [3–6], in order to identify atheroma and atheroma components with the eventual goal of identifying an atheroma's potential to progress to a vulnerable or otherwise dangerous pathological state. However, to date, there remains no clinically useful diagnostic methodology for staging atherosclerotic disease [7].

We have developed echogenic immunoliposomes (ELIP) for targeted diagnostic and ultrasound-sensitive therapeutic controlled release applications [8–10]. The former application includes proof-of-principle staging of atheroma progression by B-mode intravascular ultrasound (IVUS) imaging of atheroma in the

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carotid and iliofemoral arteries using ELIP targeted to adhesion molecules in an early and mid-stage miniswine atherosclerosis model [11,12]. We have also found that both continuous wave and pulsed wave Doppler ultrasound enhances the penetration of drugs and stem cells into all layers of the arterial wall, whether released from or associated with ELIP [13–15].

Nitric oxide (NO) is a well-known vasodilator that also increases vascular wall permeability in animal models [16,17]. We have encapsulated NO into ELIP, producing a bioactive gas delivery vehicle characterized by a bimodal diffusive release profile and the ability to release its gas load instantaneously by application of either continuous wave or pulsed wave Doppler ultrasound [18–20]. This formulation was shown to inhibit atheroma progression in a rabbit model of neointimal hyperplasia [19] and to enhance the passage of ELIP-bound stem cells through an endothelial cell layer in vitro [21].

We hypothesize that administration of NO-loaded ELIP with applied ultrasound before injection of molecularly targeted ELIP will promote penetration of targeted ELIP into the arterial wall and improve visualization of atheroma components by combining the vascular wall permeability-enhancing mechanisms of ultrasound and NO. For these experiments, we utilized IVUS as an imaging modality and intercellular adhesion molecule-1 (ICAM-1) targeted ELIP to identify early and mid-stage inflammatory components of atheromas.

2. Materials and methods

2.1. ELIP preparation

Preparation of standard ELIP: A 27:42:8:8:15 M ratio of the lipid components L-α-phosphatidylcholine (chicken egg; EPC), 1,2-dipalmitoyl-sn-glycero-3-phosphocholine (DPPC), 1,2-dipalmitoyl-sn-glycero-3-[phosphor-rac-1-glycerol] (DPPG), 1,2-dipalmitoyl-sn-glycero-3-phosphoethanolamine (DPPE), and cholesterol (CH) were mixed in a round-bottom flask as chloroform solutions. The chloroform was then removed by evaporation under argon, while the flask was rotated in a 50 °C water bath. The resulting lipid film was placed under vacuum for 4 h at ≤100 mTorr pressure for complete removal of the solvent, followed by rehydration of the dry lipid film with 0.32 M mannitol to a concentration of 10 mg lipid/ml. The hydrated lipid was incubated at 55 °C for 30 min to ensure that all lipids were in the liquid phase during hydration. The mixture was then sonicated in a water bath for 5 min. Aliquots of the suspension were frozen at −80 °C and lyophilized for 24–48 h. Each lyophilized dry cake was resuspended with the original volume of nanopure water immediately before use.

Preparation of NO-loaded ELIP: Liposomes of the above composition were prepared according to a previously developed pressurization-freeze method [22]. Briefly, after drying and hydrating the lipid film, 300-μl aliquots of the suspension were transferred to 2-ml borosilicate glass vials (12 × 32 mm), which were then sealed with Teflon-coated silicon rubber septal screw caps. Nitric oxide (5.4 ml STP), washed and purified by passage through a saturated sodium hydroxide solution in order to remove nitrogen dioxide produced by contaminating oxygen, or a mixture of NO and argon was introduced into each vial through the septum and pressurized to 9 atm using a syringe fitted with a 27G × 1/2″ needle. The pressurized gas/liposome dispersion was incubated for 30 min at room temperature, followed by freezing on dry ice for ≥30 min. Vials were stored at −80 °C. Prior to use, the pressure was released by loosening the caps immediately after removal from storage, followed by thawing of the NO-loaded ELIP suspension at room temperature.

Preparation of antibody-conjugated ELIP: For conjugation, standard ELIP were prepared as described above, substituting 1,2-dipalmitoyl-sn-glycero-3-phosphoethanolamine-N-[4-(p-maleimidophenyl) butyrate] (MPB-PE) for PE. For protein thiolation, 0.4 mg monoclonal anti-human/porcine ICAM-1 (Neomarkers clone 15.2) and 1.6 mg nonspecific mouse Immunoglobulin G (IgG; Rockland Immunochem., Inc., Gilbertsville, PA) or 2 mg IgG alone were reacted with 3-(2-pyridyldithiolpropionic acid)-N-hydroxysuccinimide ester (SPDP) at a SPDP:IgG protein molar ratio of 15:1 for 30 min at 24 ± 1 °C. Protein was separated from unreacted SPDP by gel chromatography on a 50 ml Sephadex G-50 column (Sigma–Aldrich, St. Louis, MO, USA) equilibrated with 0.05 M citrate-phosphate buffer at a pH of 5.5. Protein fractions were identified using a spectrophotometric technique (Genesys 10uv, Thermo Electron Corp., Milford, MA) at a wavelength of 280 nm, pooled and concentrated to ≤ 2 ml using Centricon YM-10 centrifugal filter units (Millipore, Billerica, MA, USA). The SPDP-activated protein was reduced in 25 mM dithiothreitol (DTT) for 30 min at 24 ± 1 °C. The thiolated protein was isolated using a Sephadex G-50 column, equilibrated and eluted with pH 6.7 citrate-phosphate buffer. The thiolated protein was reacted with reconstituted MPB-ELIP (10 mg lipid/ml 0.1 M phosphate buffer at a pH of 6.62) under argon overnight at 24 ± 1 °C. Rh-ELIP were separated from free protein and low molecular weight products by gel filtration on a 20-ml Sepharose CL-4B column (Sigma–Aldrich) that had been pre-saturated with unconjugated, unlabeled ELIP according to the method of Lasch et al. [23], and eluted with 0.02 M phosphate-buffered saline (PBS) at a pH of 7.4. Liposome-containing fractions were identified by optical absorbance at a wavelength of 440 nm prior to elution of free IgG. The IgG- or anti-ICAM-1-ELIP were lyophilized with 0.1 M D-mannitol.

2.2. Animal model

All animal experiments were approved by the Animal Welfare Committee at the University of Texas Health Science Center at Houston. Atherosclerosis was induced in Yucatan Miniswine with a standard procedure of combined balloon denudation and a hyperlipidemic diet [24]. Briefly, Yucatan miniswine was given a hyperlipidemic diet containing 2% cholesterol and 15% lard. After two weeks, the animals underwent the denudation procedures. Under sterile conditions, the animals were anesthetized with ketamine (35 mg/kg), xylazine (5 mg/kg) and 1%–3% inhaled isoflurane. Under fluoroscopic guidance, a 4F Fogarty catheter was inserted into the right carotid artery and advanced to the iliofemoral arteries. The balloon tip of the 4F Fogarty catheter was inflated with diluted iodixanol (Visipaque) solution to aid in its visualization under fluoroscopy and the catheter pulled back and forth twice to denude the arterial endothelium. Similarly, the carotid artery was also denuded before the catheter was removed and incision was sutured. The animal continued to receive a hyperlipidemic diet for another 45 days before undergoing the imaging experiments described below.

2.3. Imaging procedure

The animals were similarly anesthetized as described above. A 6 Fr sheath was inserted into both femoral arteries. For intravascular ultrasound (IVUS) imaging, a 3.5F Eagle Eye Gold IVUS catheter (20 MHz, Volcano Corporation, San Diego, CA) was utilized with a Volcano s5i Imaging System (Volcano Corporation, San Diego, CA). The IVUS catheter was inserted through the arterial sheath via the left or right femoral arterial sheath and advanced retrogradely past the region of injury to image the