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● *Original Contribution*

NITRIC OXIDE-ENHANCED MOLECULAR IMAGING OF ATHEROMA USING VASCULAR CELLULAR ADHESION MOLECULE 1-TARGETED ECHOGENIC IMMUNOLIPOSOMES

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Abstract—The aim of this study was to determine whether pre-treatment with nitric oxide-loaded echogenic liposomes (NO-ELIP) plus ultrasound can improve highlighting by molecularly targeted (anti-vascular cell adhesion molecule 1 [VCAM-1]) ELIP of atheroma components. Atherosclerotic animals were treated with anti-VCAM-1-ELIP or immunoglobulin (IgG)-ELIP. Each group was selected at random to receive pre-treatment with standard ELIP plus ultrasound, NO-ELIP without ultrasound and NO-ELIP plus ultrasound. Intravascular ultrasound highlighting data for the same arterial segments were collected before and after treatment. Pre-treatment with NO-ELIP plus ultrasound resulted in a significant increase in acoustic enhancement by anti-VCAM-1-ELIP (21.3 ± 1.5% for gray-scale value, 53.9 ± 3.1% for radiofrequency data; $p < 0.001$ vs. IgG-ELIP, $p < 0.05$ vs. pre-treatment with standard ELIP plus ultrasound or NO-ELIP without ultrasound). NO-ELIP plus ultrasound can improve highlighting of atheroma by anti-VCAM-1 ELIP. This NO pre-treatment strategy may be useful in optimizing contrast agent delivery to the vascular wall for both diagnostic and therapeutic applications. (E-mail: Susan.T.Laing@uth.tmc.edu) **© 2015 World Federation for Ultrasound in Medicine & Biology.**

Key Words: **Molecular imaging, Atheroma, Early atherosclerosis, Vascular cell adhesion molecule, Contrast agent, Nitric oxide, Ultrasound, Contrast echocardiography.**

INTRODUCTION

Adhesion molecules such as vascular cell adhesion molecule 1 (VCAM-1) and intercellular adhesion molecule 1 (ICAM-1) play an important role in the recruitment of circulatory monocytes into atherosclerotic plaques and are considered early markers of atherosclerosis, that is, before the development of intimal xanthomas (Dansky et al. 2001; Huo and Ley 2001; Kasper et al. 1996; Misiakos et al. 2001; O'Brien et al. 1993; Van der Wal et al. 1992). Although persistent expression of ICAM-1 may be a more reliable marker for the inflammatory activity in the arterial wall, VCAM-1 expression is considered to be more specific for initiation of the atherosclerotic process (Cybulsky et al. 2001). Hence, molecular imaging techniques that evaluate VCAM-1 expression in the arterial wall may represent a potentially clinically relevant screening tool for unmasking early pathophysiologic changes in atherosclerotic development, and may predict future risk of developing severe obstructive atherosclerotic disease.

We and others have reported that VCAM-1 expression can be detected by ultrasound or magnetic resonance imaging targeted imaging probes (Hamilton et al. 2004; Kaufmann et al. 2007, 2010; Kelly et al. 2005; McAteer et al. 2008; Nahrendorf et al. 2006). We have found that our echogenic liposomes (ELIP) can be modified to target VCAM-1 and have reported highlighting of early atheroma in a Yucatan miniswine model of atherosclerosis (Hamilton et al. 2004). However, histologic examination of animal and human models of atherosclerosis has revealed that the expression of adhesion molecules is not restricted to the endothelium, but in fact spans the entire thickness of the neointima and the vasa vasorum (Broisat et al. 2007; Galkina and Ley 2007; Hamilton et al. 2004; Tanaka et al. 1993; Toursarkissian et al. 1997). Because of their size (ranging from 3–8 μm), most ultrasound contrast agents are considered to be true intravascular

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contrast agents that are only capable of interrogating biomarkers expressed on the luminal side of the endothelium. As a result, targeted ultrasound contrast agents may actually underestimate the true extent of adhesion molecule expression and, thus, underestimate atherosclerotic plaque burden.

We have developed two unique approaches in an attempt to solve this issue: (i) reducing the size of our ultrasound contrast agents, and (ii) incorporating vaso-active gases inside our ELIP to facilitate the entry of ELIP into the arterial wall *via* the vasa vasorum. Unlike other ultrasound contrast agents that are homogeneous in size and distribution and tend to be greater than 3 μm, our ELIP formulations vary in size from <100 nm to several micrometers, with bimodal medians of ~90 and 800 nm (Kopechek et al. 2011). Smaller sizes allow these contrast agents to penetrate all layers of the vascular bed, including the adventitia *via* the vasa vasorum.

Nitric oxide (NO) is a potent bio-active gas with a wide range of vaso-active properties (Fischer et al. 2004). Although delivery of NO to the arterial wall has several potential benefits, successful NO delivery to targeted tissues is challenging because of the presence of endogenous NO scavengers such as hemoglobin (Tsao et al. 1994), as well as poor tissue retention. Our laboratory has developed techniques for encapsulating NO into our ELIP, thus preventing its sequestration by hemoglobin in the bloodstream (Huang et al. 2009). By encapsulating NO into ELIP, we have also observed retention of NO in cultured endothelial cells (Huang et al. 2009). In animal models, we found that locally released NO from NO-containing ELIP (NO-ELIP) can facilitate the penetration of ICAM-1 antibody-conjugated ELIP into all layers of the arterial wall and improve highlighting of the atheroma (Kee et al. 2014; Kim et al. 2013).

In the present study, we hypothesized that ultrasound can trigger the release of NO from NO-ELIP and allow penetration of anti-VCAM-1 targeted ELIP into all layers of the arterial wall for more accurate identification and quantitation of VCAM-1 expression in an early/intermediate-stage and late-stage Yucatan miniswine atherosclerosis model. This novel approach may eventually allow the clinical use of diagnostic molecular imaging for staging of atherosclerotic disease.

METHODS

ELIP preparation

Preparation of standard ELIP. Standard ELIP were prepared by mixing the lipid components L-α-phosphatidylcholine (chicken egg), 1,2-dipalmitoy l-*sn*-glycero-3-phosphocholine, 1,2-dipalmitoyl-*sn*-glycero-3-[phosphor-rac-1-glycerol], 1,2-dipalmitoyl-*sn*-glycero-3-phosphoethanolamine and cholesterol at a 27:42:8:8:15 molar ratio in a round-bottom flask as a chloroform solution (Huang et al. 2002). The chloroform was evaporated under argon while spinning the flask submerged 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. The dry lipid film was rehydrated 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 crystal phase during hydration. Sonication was performed on the lipid mixture in a water bath for 5 min. Each aliquot of the suspension was placed in a −80°C freezer and lyophilized for 24–48 h. Each lyophilized dry cake was re-suspended in nanopure water immediately before treatment.

Preparation of NO-ELIP. Liposomes of the same composition as described above were prepared by our pressurization-under-freeze method (Huang et al. 2008). Briefly, the lipid film was dried and hydrated, and aliquots of the suspension (300 μL) were transferred to 2-mL borosilicate glass vials (12 × 32 mm). The glass vials were sealed using Teflon-coated silicon rubber septal screw caps. NO gas (5.4 mL STP) was washed and purified by passage through a saturated sodium hydroxide solution to eliminate nitrogen dioxide that could be created after reaction with oxygen. A mixture of NO and argon was injected into each vial through the septum and pressurized at 9 atm using a syringe (27G × 1/2-in. needle). The gas/liposome dispersion under pressure was incubated at room temperature for 30 min, frozen on dry ice for >30 min and stored in a −80°C freezer. For each treatment, the vial was transferred on dry ice, and the pressure was released by loosening the cap. The NO-ELIP suspension was thawed at room temperature and used for treatment.

Preparation of antibody-conjugated ELIP. For conjugation, standard ELIP were prepared as described above, and 1,2-dipalmitoyl-*sn*-glycero-3-phosphoethanolamine-*N*-[4-(*p*-maleimidophenyl) butyrate] (MPB-PE) was substituted for PE. For protein thiolation, 0.4 mg monoclonal anti-human/porcine VCAM-1 (Neomarkers clone 1 G11.B1) and 1.6 mg nonspecific mouse immunoglobulin G (IgG, Rockland Immunochem, Gilbertsville, PA, USA) 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 pH 5.5. Protein fractions were identified using a spectrophotometric technique (Genesys 10 uv, Thermo Electron, Milford, MA, USA) at a wavelength of 280 nm, pooled and

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