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Nitric Oxide-Enhanced Molecular Imaging of Atheroma using Vascular Cellular Adhesion Molecule-1 Targeted Echogenic Immunoliposomes

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Abstract

This study aimed to demonstrate whether pretreatment 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 randomized to receive *pretreatment* with standard ELIP plus ultrasound, NO-ELIP without ultrasound, or NO-ELIP plus ultrasound. Intravascular ultrasound highlighting data of the same arterial segments were collected before and after treatment. Pretreatment with NO-ELIP plus ultrasound demonstrated a significant increase in acoustic enhancement by anti-VCAM-1 ELIP ($21.3 \pm 1.5\%$ for gray scale value, $53.9 \pm 3.1\%$ for radiofrequency data; $p < 0.001$ vs. IgG-ELIP, $p < 0.05$ vs. pretreatment 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 pretreatment strategy may be useful for optimizing contrast agent delivery to the vascular wall for both diagnostic and therapeutic applications.

Keywords

Molecular Imaging; Atheroma; Early Atherosclerosis; Vascular Cell Adhesion Molecule; Contrast Agent; Nitric Oxide; Ultrasound; Contrast Echocardiography

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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, i.e., 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 the 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 demonstrated that VCAM-1 expression can be detected by ultrasound or magnetic resonance imaging targeted imaging probes (Hamilton et al. 2004; Kaufmann et al. 2007; Kaufmann et al. 2010; Kelly et al. 2005; McAteer et al. 2008; Nahrendorf et al. 2006). We have demonstrated that our echogenic liposomes (ELIP) can be modified to target VCAM-1 and have shown highlighting of early atheroma in a Yucatan miniswine model of atherosclerosis (Hamilton et al. 2004). However, histological examination of animal and human models of atherosclerosis have 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). Due to their size (ranging from 3 to 8 μm), most ultrasound contrast agents are considered to be true intravascular 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 to attempt to solve this issue, i.e., by a) reducing the size of our ultrasound contrast agents and by b) incorporating vasoactive 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 sizes 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 bioactive gas with a wide range of vasoactive 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 demonstrated retention of NO in cultured

endothelial cells (Huang et al. 2009). In animal models, we were able to demonstrate 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 current study, we hypothesize 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 atherosclerotic disease.

MATERIALS AND METHODS

ELIP Preparation

Preparation of standard ELIP—Standard ELIP were prepared by mixing 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) 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 consequent lipid film was placed under vacuum for 4 hours 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 minutes 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 minutes. Each aliquot of the suspension was placed in a -80°C freezer and lyophilized for 24–48 hours. Each lyophilized dry cake was resuspended in nanopure water immediately prior to treatment.

Preparation of NO-ELIP—Liposomes in the same composition as 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 μ l) were transferred to 2-ml borosilicate glass vials (12 $\times$ 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 passing through a saturated sodium hydroxide solution to eliminate nitrogen dioxide that could be created following 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 $\times$ 1/2" needle). The gas/liposome dispersion under pressure was incubated at room temperature for 30 minutes, frozen on dry ice for over 30 minutes, 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 1G11.B1) 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 minutes at $24\pm 1^{\circ}\text{C}$. 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 0.05M citrate-phosphate buffer at a pH of 5.5. Protein fractions were identified using a spectrophotometric technique (Genesys 10 uv, 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 25mM dithiothreitol (DTT) for 30 minutes at $24\pm 1^{\circ}\text{C}$. 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 0.1M phosphate buffer at a pH of 6.62) under argon overnight at $24\pm 1^{\circ}\text{C}$. 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 Lasche et al. (2003), and eluted with 0.02 M phosphate-buffered saline (PBS containing 90 mM sodium chloride to render the liposomes isotonic when reconstituted) 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-VCAM-1 ELIP were lyophilized with 0.1M D-mannitol.

The conjugation efficiency (CE) of 5 lots of anti-VCAM1-ELIP used for the experiments in this study, as determined by a quantitative immunoblot assay for liposomal IgG (Klegerman et al. 2002), ranged from 1.9 to 3.6 μg antibody/mg lipid (20% of total IgG conjugated) and 496 to 4,373 molecules antibody/liposome, respectively. Beckman Coulter Multisizer 4 analysis enabled determination of both ELIP number and size characteristics for ELIP $> 0.4 \mu\text{m}$ equivalent spherical diameter, which ranged from 470 to 910 nm; ELIP number ranged from 1.35×10^9 to 1.58×10^{10} liposomes/mg lipid.

Animal Model

All the animal studies were approved by the Animal Welfare Committee at the University of Texas Health Science Center at Houston. Atherosclerosis in Yucatan miniswine (total of 24 animals) was induced using balloon denudation and hyperlipidemic diet (Demos et al. 1999). Briefly, a hyperlipidemic diet containing 2% cholesterol and 15% lard was given to Yucatan miniswine. The endothelial denudation procedure was conducted in the animals after two weeks of hyperlipidemic diet. The animals were anesthetized using ketamine (35 mg/kg), xylazine (5 mg/kg), and 1–3% inhaled isoflurane. A 4F Fogarty catheter was introduced into the right carotid artery and advanced to the iliofemoral arteries under fluoroscopic guidance. Diluted iodixanol (Visipaque) solution was injected to help visualization of the inflation of the catheter's balloon tip under fluoroscopy, and the catheter was pulled back and forth twice to denude the endothelium of the arteries. The carotid artery was denuded using the same technique prior to removing the catheter, and the incision was sutured. All surgical procedures were performed under sterile conditions. The animals continued to be fed a hyperlipidemic diet for another 45 days (for early/intermediate stage of

atherosclerosis) or 105 days (for late stage of atherosclerosis), and imaging experiments were performed as described below.

Intravascular Ultrasound Imaging

The animals were anesthetized using the same methods described above. A 6F sheath was inserted into each femoral artery. Intravascular ultrasound (IVUS) imaging was performed using a 3.5F Eagle Eye Gold IVUS catheter (20 MHz, Volcano Corporation, San Diego, CA) connected to a Volcano s5i Imaging System (Volcano Corporation, San Diego, CA). The IVUS catheter was introduced through the sheath and advanced retrogradely across the injured region to obtain IVUS images of the iliofemoral or carotid arterial segments of interest. IVUS imaging of the arterial segments of interest was performed using an automatic catheter pullback device (constant speed of 0.5 mm/s), and the IVUS signal data were continuously recorded in digital format. IVUS data were collected at baseline and 5 minutes after ELIP treatment (See below). Instrument settings for gain and zoom levels in the IVUS system were set so that the luminal border and the arterial wall structure were visibly detectable at baseline for each artery. These settings remained constant throughout IVUS imaging. The animals were sacrificed after all the procedures were completed and the arteries were harvested for histopathologic analysis.

ELIP Treatments

The animals were randomized into two primary treatment groups: treatment with anti-VCAM-1 ELIP or control IgG-ELIP. Each treatment group comprised three subgroups: *pretreatment* with i) standard ELIP (i.e., air-containing ELIP only) plus ultrasound, ii) NO-ELIP without ultrasound, or iii) NO-ELIP plus ultrasound (n=3 arteries per sub-group) administered 5 minutes prior to IgG- or Ab-ELIP treatment. ELIP were administered through a catheter that was introduced retrogradely via the femoral arterial sheaths. For the groups that received pretreatment with NO-ELIP, a total of 1.0 mg of NO-ELIP lipid was injected in a total volume of 0.5 ml PBS. For all treatment groups, 5.0 mg of IgG-ELIP or anti-VCAM-1-ELIP was injected in 1.9–3.0 ml of PBS. IVUS data of the same arterial segments were collected before and after treatment. For the subgroups receiving ultrasound pretreatment, a 12-MHz vascular ultrasound transducer (HDI 5000, Philips Healthcare, Andover, MA) was placed over the iliofemoral or carotid artery in parallel to the direction of blood flow. Color Doppler ultrasound (Doppler frequency = 6 MHz, mechanical index = 0.4, pulse repetition frequency = 5 kHz) was applied to facilitate NO release from NO-ELIP (Smith et al 2007; Laing et al. 2012) during the entire 5 minutes prior to IgG- or Ab-ELIP administration.

Image Analysis and 3D Visualization of IVUS Data

IVUS signal data were utilized to reconstruct acoustic intensity data sets in the polar coordinate (radial vs. circumferential axes) (Kim et al. 2010). IVUS signal envelope data were collected at 1,024 data points per scan line. A total of 256 scan lines were collected along the radial direction per IVUS slice (Eagle Eye Gold catheter, Volcano Corporation, San Diego, CA). The acoustic intensity data sets in the polar coordinate were transformed to the Cartesian coordinate system for standard IVUS imaging (Kim et al. 2010). As the

imaged arterial segments of interest were relatively straight, it was assumed that the pullback direction of IVUS catheter was parallel to the longitudinal direction of the artery. Our graphical user interface (GUI)-based image processing system was utilized to interactively trace and segment the arterial structure for image analysis (Kim et al. 2010; Kim et al. 2013). In a blinded manner, borders of the endothelium/atheroma and the outer edge of the dense adventitia were manually segmented in each IVUS slice, and the acoustic enhancement within these arterial wall borders after each treatment was quantitated by both mean gray scale values (i.e. pixelated brightness data) and radio-frequency (RF) magnitude values (i.e. signal intensity data). Three-dimensional (3D) reconstruction was followed such that a series of segmented IVUS images of the artery were placed in a tomographic sequence along the longitudinal direction. Two types of 3D reconstruction were performed to facilitate the demonstration of acoustic enhancement following targeted ELIP treatment. First, 3D mapped arterial images were created to better visualize and compare the extent and distribution of the highlighted regions of interest across the arterial structure between treatment groups. Data were displayed with respect to the longitudinal direction (i.e. blood flow direction) and the circumferential direction of the artery. Averaged acoustic enhancement data (both gray scale and RF data) along the radial direction were utilized in the 3D mapped images. Secondly, we created volumetric 3D IVUS images of the arteries using our novel shape-based nonlinear interpolation method (Kim et al. 2010). A total of 90 IVUS slice images were utilized to construct an image stack to create volumetric 3D IVUS image data for each artery.

Histology

Immediately after the animals were euthanized, the arteries were harvested and cut into 5-mm segments. All segments were either snap-frozen or fixed in 4% formalin. The fixed arterial segments were embedded in paraffin, and cut into 5- μ m slices.

Immunohistochemical staining was performed with the same anti-VCAM-1 antibody used for ELIP conjugation (1:1000 dilution). A modified Movat's pentachrome staining was performed for the adjacent slices to demonstrate elastic tissue (black), fibrous tissue (blue-green), and cell cytoplasm (red).

Statistical Analysis

Data are reported as percent change in acoustic highlighting compared to baseline (n=3 arteries per group; 15 IVUS slices per artery). All statistical analyses were performed using SigmaStat (Systat Software Inc, Point Richmond, CA). For comparison of treatment groups, t-test or analysis of variance (ANOVA) was used for parametric data, and the Wilcoxon rank sum test or the Kruskal-Wallis analysis of variance of ranks and median test for nonparametric data. The Dunn's Method was utilized for pairwise differences between groups. Data are presented as mean and standard error. A p-value of 0.05 was considered to be statistically significant.

RESULTS

Figure 1 demonstrates a representative artery with early/intermediate atheroma and atheroma. Progression of atheroma development is clearly observed. Figure 2 shows

representative VCAM-1-stained images of an uninjured control artery, an early/intermediate atherosclerotic artery and a late atherosclerotic artery. There was expression of VCAM-1 in the neointima and the arterial wall including the adventitia in both early/intermediate and late atheroma. Immunohistochemistry of the arteries pretreated with or without NO-ELIP showed no differences in VCAM staining between the subgroups at baseline.

For the early/intermediate atherosclerotic stage animals, there was little acoustic enhancement with IgG-ELIP treatment compared to baseline ($p>0.05$) both in the gray scale and RF data sets (Figure 3). There was also no difference in highlighting among arteries treated with IgG-ELIP by pretreatment types (i.e., standard ELIP plus ultrasound, NO-ELIP without ultrasound, or NO-ELIP plus ultrasound; $p>0.05$; Figure 3 & 4). All the subsequent data analyses with IgG-ELIP treatment groups were therefore compiled as one control group.

Anti-VCAM-1 ELIP treatment demonstrated enhanced highlighting in atherosclerotic arteries on the IVUS image data compared to IgG-ELIP treatment regardless of the pretreatment subgroup ($p<0.001$; Figure. 3). However, there was a difference in highlighting observed among the different pretreatment subgroups when anti-VCAM-1 ELIP treatment was given. Pretreatment with standard ELIP plus ultrasound or NO-ELIP without ultrasound revealed a comparable enhancement of highlighting ($12.8 \pm 2.1\%$ vs. $13.5 \pm 1.3\%$ for gray scale values, $p>0.05$; $27.1 \pm 3.2\%$ vs. $32.6 \pm 3.1\%$ for RF data, $p>0.05$) compared to baseline ($p<0.001$ vs. IgG-ELIP; Figure. 3 & 4). On the other hand, there was a significant increase in acoustic enhancement when arteries were pretreated with *both* NO-ELIP and ultrasound activation ($21.3 \pm 1.5\%$ for gray scale value, $53.9 \pm 3.1\%$ for RF data; $p<0.001$ vs. IgG-ELIP, and $p<0.05$ vs. pretreatment with standard ELIP plus ultrasound or NO-ELIP without ultrasound; Figures 3 & 4).

For the late atherosclerotic animals, similar patterns of acoustic enhancement were found when compared with the early/intermediate atherosclerotic group. IgG-ELIP treatment demonstrated little enhancement of highlighting regardless of pretreatment type (Figure 5). Similarly all subsequent data analyses with IgG-ELIP treatment groups for the late atherosclerotic animal model were compiled as one control group. Treatment with anti-VCAM-1 ELIP similarly resulted in enhanced highlighting of the arteries when compared to IgG-ELIP treatment ($p<0.001$ vs. IgG-ELIP; Figure 5). However, we noted that the overall improvement in highlighting following anti-VCAM-1 ELIP treatment for the late atherosclerotic stage animals was less compared to the early/intermediate stage animals. For the groups pretreated with standard ELIP plus ultrasound followed by anti-VCAM-1 ELIP, there was a $7.7 \pm 0.8\%$ increase in gray scale values (vs. $12.8 \pm 2.1\%$ for the early/intermediate stage animals; $p=0.014$) while those pretreated with NO-ELIP without ultrasound resulted in a $10.7 \pm 1.0\%$ acoustic enhancement in gray scale values (vs. $13.5 \pm 1.3\%$ for the early/intermediate stage animals; $p>0.05$). These differences were further appreciated with RF data. There was an increase of $12.1 \pm 1.4\%$ and $19.7 \pm 1.8\%$ in RF signal values for pretreatment with standard ELIP plus ultrasound and NO-ELIP without ultrasound, respectively, compared to a $27.1 \pm 3.2\%$ and $32.6 \pm 3.1\%$ in RF data (for the early/intermediate stage animals; $p<0.05$ for both). Similar to the early/intermediate stage animal model, pretreatment with *both* NO-ELIP and ultrasound followed by anti-VCAM-1 ELIP resulted in

a further increase in highlighting of atheroma ($16.1 \pm 1.1\%$ and $36.4 \pm 2.2\%$, in gray scale and RF data respectively, $p < 0.001$ vs. IgG-ELIP, and $p < 0.05$ vs. pretreatment with standard ELIP plus ultrasound or NO-ELIP without ultrasound; Figure 5). However, the overall improvement in highlighting following anti-VCAM-1 ELIP treatment with pretreatment using *both* NO-ELIP and ultrasound was less in the late atherosclerotic model compared to the early/intermediate stage animals [vs. $21.3 \pm 1.5\%$ for gray scale value ($p = 0.039$) and $53.9 \pm 3.1\%$ for RF data ($p < 0.01$) in the early/intermediate stage animals].

Representative 3D images of the arteries treated with IgG-ELIP vs. those pretreated with NO-ELIP plus ultrasound activation followed by anti-VCAM-1 ELIP are demonstrated in Figure 6. These 3D images were created with respect to the longitudinal and radial directions of the artery on each axis, respectively. Correct registration of the regions of interest in the artery has been appropriately verified by well-defined landmarks of arterial bifurcation in the 3D mapped images at baseline and following treatment with anti-VCAM-1 ELIP. There was no significant acoustic enhancement across the arterial structure prior to and following IgG-ELIP treatment. Anti-VCAM-1 ELIP treatment with pretreatment of NO-ELIP plus ultrasound activation, however, clearly demonstrated enhanced highlighting across the entire arterial structure compared to baseline. Color-mapped RF data images provided improved demonstration and spatial localization of acoustic enhancement with anti-VCAM-1 ELIP treatment with a pretreatment strategy of NO-ELIP plus ultrasound activation.

Figure 7 demonstrates representative volumetric 3D IVUS images of the arteries before and after treatment. A shape-based nonlinear interpolation method was utilized to demonstrate not only realistic volumetric geometry of the arterial segment but also acoustic backscatter information within the arterial structure. IgG-ELIP treatment displayed similar highlighting in the volumetric 3D IVUS images at baseline and following treatment with the ELIP contrast agent. On the other hand, anti-VCAM-1 ELIP contrast injection after pretreatment with NO-ELIP plus ultrasound activation demonstrated significant highlighting over the whole outer surface of the arterial segment as well as within the arterial wall when compared to baseline.

DISCUSSION

We have previously shown that anti-ICAM-1-conjugated ELIP enhances IVUS reflectivity of early/intermediate stage atheroma (Kim et al. 2010). In a more recent study (Kim et al. 2013), we demonstrated further enhancement of highlighting by anti-ICAM-1 ELIP when arteries were pretreated with NO-ELIP plus ultrasound. In this study, we observed a very similar pattern of VCAM-1 highlighting using anti-VCAM-1 targeted ELIP in miniswine atheroma, which increased significantly after NO-ELIP plus US pretreatment. Our results are consistent with our prior work and further supports our hypothesis that NO-ELIP plus ultrasound results in increased permeability across the arterial wall, allowing greater penetration of targeted ELIP for enhanced highlighting of atheroma components. Although the gray scale image data can demonstrate the effect of ELIP highlighting of atheroma components, RF data provide further detailed information pertaining to intensity distribution across the arterial wall and RF data analysis is useful to more specifically and spatially

differentiate the highlighted regions of interest; this may allow improved molecular ultrasound imaging of atherosclerosis by targeted ELIP.

Although the increase of vascular permeability by NO has previously been reported (Brithaupt-Faloppa et al. 2009; Duran et al. 2010), it is still not known whether this effect is secondary to an increased rate of blood flow due to vessel dilation or whether endothelial intercellular junctions are directly affected. We have previously obtained evidence that the latter hypothesis may play a role by showing that NO-ELIP doubled the number of stem cells that can pass through endothelial cell monolayers on multiwell plate inserts (Klegerman et al. 2010). However both mechanisms likely play a role and further investigation is needed for confirmation. Nevertheless, this study supports our hypothesis that NO-loaded ELIP can be combined with the vascular permeability-enhancing effects of ultrasound to improve image enhancement using targeted ELIP.

Acoustic enhancement using VCAM-1 was higher in the early/intermediate atherosclerotic arteries than in the late atheroma model. This finding supports the observation by others that VCAM-1 expression decreases with atheroma progression (Iiyama et al. 1999). It is well known that the role of VCAM-1 in atheroma development is confined to monocyte recruitment during early stages of the disease (Cybulsky et al. 2001; Kaufmann et al. 2007; Nakashima et al. 1998), which would imply that this adhesion molecule may be downregulated during later stages of atheroma progression. Our findings are consistent with this observation, supporting the role of VCAM-1 as a marker of early atheroma formation. This is an important finding when evaluating atheroma markers. ICAM-1 tends to be a marker of general inflammation and is known to be consistently expressed in all tissues having even minimal inflammation. VCAM-1 on the other hand, is deemed to be a more specific marker of aggressive and atheroma-prone inflammation. As atheroma becomes more mature/stable, the expression of VCAM-1 tends to decrease. Thus our findings that anti-VCAM-1 ELIP have higher enhancement in the early/intermediate atheroma period may have implications for targeted VCAM-1 molecular imaging of atheroma as this strategy may identify targets requiring more aggressive treatment to minimize/stabilize atheroma progression.

One limitation of the study is that to obtain maximal atheroma enhancement, intraarterial injection of the ELIP was performed. This may limit day to day clinical applications of this pretreatment strategy. However, clinical translation of this intraarterial delivery strategy can be done routinely when coronary and peripheral arterial angiograms are performed. In this study, we did not quantitate VCAM-1 expression in the arteries as this was not the focus of our study. We also did not focus on identifying the ELIP distribution within the arterial wall and we are unable to quantitate the relative number of liposomes deposited on the vascular wall vs. the endothelium. Future experiments will address this question. Another limitation is that we chose to evaluate highlighting using one molecular targeting agent (anti-VCAM-1 ELIP) and did not evaluate highlighting using other atheroma components.

In summary, NO-loaded ELIP when combined with ultrasound activation can effectively improve endothelial permeability facilitating anti-VCAM-1 conjugated ELIP delivery to inflammatory components across the arterial wall. This NO pretreatment strategy may be useful for optimizing ELIP (or any other contrast agent) delivery to the vascular wall for

both diagnostic and therapeutic applications. Further investigation of this novel technique may yield important insights for the development of targeted, controlled-release formulations for tissue-specific localization and delivery of contrast agents, drugs, genes and therapeutic cells in general.

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Figure 1.
Representative early/intermediate atheroma (on the left) and late atheroma (on the right) in a Yucatan miniswine (Movat's staining: red=smooth muscle cell or endothelial cell, black=elastic fibers, blue-green=extracellular matrix).

Figure 2. Representative VCAM-1-stained images of an uninjured control artery, an early/intermediate atherosclerotic artery (60 days) and a late atherosclerotic artery (120 days).

Figure 3.
Acoustic enhancement of the atherosclerotic arteries following 60 days of hyperlipidemic diet utilizing either gray scale value or RF data with respect to baseline.

Figure 4.

Representative gray scale IVUS images and RF data of atherosclerotic arteries following image enhancement algorithms. Images from the 60 day atherosclerotic animals. (a) Standard ELIP/US + IgG-ELIP, (b) NO-ELIP + IgG-ELIP, (c) NO-ELIP/US + IgG-ELIP, (d) Standard ELIP/US + anti-VCAM-1 ELIP, (e) NO-ELIP + anti-VCAM-1 ELIP, (f) NO-ELIP/US + anti-VCAM-1 ELIP.

Figure 5.

Acoustic enhancement of the atherosclerotic arteries following 120 days of hyperlipidemic diet utilizing either gray scale value or RF data with respect to baseline.

Figure 6. Comparison of 3D mapped images of the arteries following (a) standard ELIP/US + IgG-ELIP vs. (b) NO-ELIP/US + anti-VCAM-1 ELIP.

(A) Standard ELIP/US + IgG-ELIP

(B) NO-ELIP/US + Anti-VCAM-1-ELIP

Figure 7.

Volumetric 3D IVUS images of the arteries following (a) standard ELIP/US + IgG-ELIP vs. (b) NO-ELIP/US + anti-VCAM-1 ELIP.