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● *Original Contribution*

ULTRASOUND MOLECULAR IMAGING OF ATHEROSCLEROSIS USING SMALL-PEPTIDE TARGETING LIGANDS AGAINST ENDOTHELIAL MARKERS OF INFLAMMATION AND OXIDATIVE STRESS

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Abstract—The aim of this study was to evaluate a panel of endothelium-targeted microbubble (MB) ultrasound contrast agents bearing small peptide ligands as a human-ready approach for molecular imaging of markers of high-risk atherosclerotic plaque. Small peptide ligands with established affinity for human P-selectin, VCAM-1, LOX-1 and von Willebrand factor (VWF) were conjugated to the surface of lipid-stabilized MBs. Contrast-enhanced ultrasound (CEUS) molecular imaging of the thoracic aorta was performed in wild-type and gene-targeted mice with advanced atherosclerosis (DKO). Histology was performed on carotid endarterectomy samples from patients undergoing surgery for unstable atherosclerosis to assess target expression in humans. In DKO mice, CEUS signal for all four targeted MBs was significantly higher than that for control MBs, and was three to sevenfold higher than in wild-type mice, with the highest signal achieved for VCAM-1 and VWF. All molecular targets were present on the patient plaque surface but expression was greatest for VCAM-1 and VWF. We conclude that ultrasound contrast agents bearing small peptide ligands feasible for human use can be targeted against endothelial cell adhesion molecules for inflammatory cells and platelets for imaging advanced atherosclerotic disease. (E-mail: linderj@ohsu.edu)

Key Words: **Atherosclerosis, Contrast-enhanced ultrasound, Microbubbles, Molecular Imaging.**

INTRODUCTION

The pathophysiology of atherosclerosis commonly involves the slow development of arterial plaque over decades, which can culminate in acute atherothrombotic complications. Although drug therapy for primary prevention of atherosclerosis is established (Chrispin et al. 2013), treatment gaps remain, some of which could be addressed by better methods for risk assessment. For example, some of the potent anti-atherosclerotic therapies that are currently being tested in clinical trials will require a strict selection process to guide their use based on their projected costs and side effect profiles (Back and Hansson 2015; Charo and Taub 2011). Accordingly, the ability to identify individuals at a very early age who are at exceptionally high risk for accelerated disease over the ensuing decades is an important goal. Yet, existing paradigms for estimating 10-y risk are heavily influenced by age, leading to underestimation of lifetime risk in young individuals (Jorstad et al. 2016). In those with known disease, it may be important to identify those with greatest vulnerability for atherothrombotic events.

Improvements in biomarker-based risk stratification are possible by non-invasive imaging of the biologic processes that govern plaque growth and instability. Contrast-enhanced ultrasound (CEUS) molecular imaging relies on imaging inflammatory and prothrombotic events that occur at the vascular–blood pool interface (Davidson et al. 2012; Hamilton et al. 2004; Kaufmann et al. 2010; Li et al. 2010; Shim et al. 2015; Villanueva et al. 2007; Wang et al. 2012). In this study, we evaluated a panel of targeted microbubble ultrasound contrast agents bearing covalently conjugated

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Table 1. Small molecule ligands for targeting

Target	Ligand	Species
VCAM-1	HGRANLRILARY-COOH	Human, Mouse
VWF	RVVCEYVFGRGAVCS (cyclic)	Human, Mouse
P-selectin	LVSVLDLEPLDAAWL	Mouse
LOX-1	LSIPPKA	Human

LOX-1 = lectin-like oxidized low-density lipoprotein receptor-1; VCAM-1 = vascular cell adhesion molecule-1; VWF = Von Willebrand factor.

small molecular ligands, thereby making them appropriate for potential human use, with respect to their ability to detect inflamed plaque with molecular imaging. Agents targeted to endothelial cell adhesion molecules (P-selectin, vascular cell adhesion molecule-1 [VCAM-1]), von Willebrand factor (VWF) and oxidized low-density lipoprotein (LDL) cholesterol receptor-1 (LOX-1) were tested in a murine model of accelerated atherosclerosis. In addition, we characterized the expression and location of each of these targets in human carotid endarterectomy samples.

METHODS

Synthesis of peptide ligands and peptide–phospholipid conjugates

Small peptide ligands that have been reported to have high affinity for preferably human extracellular portions of VCAM-1 (Dimastromatteo et al. 2013), VWF (Ulrichts et al. 2001), P-selectin (Molenaar et al. 2003) and Lox-1 (White et al. 2001) were identified (Table 1) and synthesized (Lifetein, Franklin Township, NJ, USA). The peptides were of at least 95% purity as assessed by high-performance liquid chromatography analysis and positive ion electrospray mass spectroscopy. The peptide ligands were conjugated to a DSPE-PEG2000 moiety either by method 1—first reaction of the peptide with suberic acid bis-*N*-hydroxysuccinimide (NHS) ester to provide the peptide suberoyl-mono-NHS ester, followed by reaction of that with distearoyl-*sn*-glycerophosphatidylethanolamine-polyethyleneglycol-2000-NH_2 (DSPE-PEG2000-NH_2) or method 2—coupling of the amino terminus of the peptide to DSPE-PEG2000-C(=O)-NHS. Though method 1 was employed without incident for numerous peptides (Pillai et al. 2010), it did not provide the expected product for certain peptides used in this study. Hence, method 2 was employed in cases in which the peptide suberoyl-mono-NHS ester was not formed cleanly or was difficult to isolate from remaining excess suberic acid bis-NHS ester. Positive ion electrospray mass spectroscopy of the DSPE-PEG2000-peptide conjugates gave values for doubly, triply or quaternary charged ions consistent with the expected structure.

Targeted microbubble preparation

Microbubbles with a decafluorobutane gas core and a bifunctional lipid moiety for ligand conjugation were prepared. A 37.5-mg mixture composed of DPPC (90 mol%), DPPE-MPEG-2000 (9 mol%) and the targeted phospholipid-PEG-2000-linker-peptide conjugate (1 mol %) was added to stirred propylene glycol (5 mL) at 50 °C–65 °C and stirred until dissolved. This solution was added in several aliquots to a solution of 45 mL of phosphate-buffered saline containing 5% glycerol by volume at 50 °C–65 °C and stirred. The solution was transferred to a vial, the headspace of which was purged with nitrogen, and the vial was stoppered and crimp capped. The solution was allowed to cool to ambient temperature and then stored at 4 °C. The chilled solution was aliquoted in 1.5-mL portions into 2-mL nominal-capacity serum vials followed by application of light vacuum and refilling with perfluorobutane gas. Vials were stored at 4 °C until use, whereupon microbubbles were formed by agitation (Vial Mix, Lantheus, North Billerica, MA, USA) for 45 s. Control microbubbles were prepared that lacked the bifunctional linker-peptide conjugate. For studies requiring fluorescence labeling, either dioctadecyloxacarbocyanine perchlorate (DiO) or dioctadecyl tetramethylindocarbocyanine perchlorate (DiI) was added to the lipid suspension before activation. Microbubble size distribution and concentration were measured by electozone sensing (Multisizer III, Beckman Coulter, Brea, CA, USA).

Animal model of atherosclerosis

The study was approved by the Animal Care and Use Committee of the Oregon Health & Science University. A double knockout (DKO) murine model (Powell-Braxton et al. 1998) of atherosclerosis that has gene-targeted deletion of both the LDL receptor and the apolipoprotein-B mRNA editing enzyme catalytic polypeptide 1 (Apobec-1) was studied at 40 wk of age when plaque is present over approximately 25% of the aortic surface area, average cross-sectional plaque area in the ascending aorta is 0.5 to 1.0×10^5 μm^2, and approximately 25% of the cross-sectional plaque area is composed of macrophage-rich lipid core (Kaufmann et al. 2010; Liu et al. 2013). Endothelial expression of VCAM-1, P-selectin and VWF has been previously established in these animals (Kaufmann et al. 2010; Liu et al. 2013; McCarty et al. 2010; Shim et al. 2015). Age-matched wild-type C57Bl/6 mice were used as controls. Mice were anesthetized with inhaled isoflurane (1.5%–2.0%). The jugular vein was cannulated for intravenous administration of contrast agents.

In vivo *molecular imaging*

Contrast-enhanced ultrasound molecular imaging was performed in 20 DKO and 10 control mice. Among the