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● *Original Contribution*

NITRIC OXIDE PRETREATMENT ENHANCES ATHEROMA COMPONENT HIGHLIGHTING *IN VIVO* WITH INTERCELLULAR ADHESION MOLECULE-1-TARGETED ECHOGENIC LIPOSOMES

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Abstract—We present an ultrasound technique for the detection of inflammatory changes in developing atheromas. We used contrast-enhanced ultrasound imaging with (i) microbubbles targeted to intercellular adhesion molecule-1 (ICAM-1), a molecule of adhesion involved in inflammatory processes in lesions of atheromas in New Zealand White rabbits, and (ii) pretreatment with nitric oxide-loaded microbubbles and ultrasound activation at the site of the endothelium to enhance the permeability of the arterial wall and the penetration of ICAM-1-targeted microbubbles. This procedure increases acoustic enhancement 1.2-fold. Pretreatment with nitric oxide-loaded echogenic liposomes and ultrasound activation can potentially facilitate the subsequent penetration of targeted echogenic liposomes into the arterial wall, thus allowing improved detection of inflammatory changes in developing atheromas. (E-mail: patrick.kee@uth.tmc.edu)

Key Words: **Echogenic liposomes, Intravascular ultrasound imaging, Nitric oxide, Atherosclerosis, Molecular imaging.**

INTRODUCTION

Adhesion molecules, such as vascular cell adhesion molecule-1 (VCAM-1) and intercellular adhesion molecule-1 (ICAM-1), are expressed on the endothelium of developing atheromas and play an important role in the recruitment of circulatory monocytes into plaques (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). Adhesion molecules are divided into three subgroups: selectins, integrins and members of the immunoglobulin superfamily of molecules (Dustin et al. 1988). Two subtypes of ICAM coexist: ICAM-1, which is inducible, and ICAM-2, which is constitutively and inducibly expressed on endothelial cells (Staunton et al. 1989).

During atherogenesis, endothelial cells are activated and express adhesion molecules such as ICAM-1 and VCAM-1 at high levels (Boyle 2005; Schwartz et al. 2003). These molecules facilitate leukocyte adherence and transmigration into the intima of the arterial wall and induce inflammation. Molecular imaging of adhesion molecule expression has correlated with the severity of diet-modulated vascular inflammation in late-stage apoE$^{-/-}$ mice (Kaufmann et al. 2007). The temporal expression of adhesion molecules was carefully studied in a rabbit model of balloon injury to the aorta (Tanaka et al. 1993). Ten days after the injury, expression of both ICAM-1 and VCAM-1 occurred initially in the neointimal endothelium and extended to the neo-intimal smooth muscle cells. By day 30, VCAM-1 expression was reduced, but ICAM-1 expression persisted beyond that time. The increased levels of adhesion molecules are thought to be related to the increased expression of monocyte chemotactic protein-1 and keratinocyte chemoattractant, which, in turn, encode inflammatory cytokines (Tanaka et al. 1993). Although transient VCAM-1 expression is considered more specific for the initiation of atherosclerosis (Cybulsky et al. 2001), the persistent expression of ICAM-1 may be a more reliable marker for molecular imaging of the inflammatory activity in the arterial wall. Apart from mechanical injury to the aorta, thrombosis by bead embolization and external ligation also resulted in an increase in ICAM-1 expression in the vascular media (Toursarkissian et al. 1997).

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Our laboratory has developed techniques to target adhesion molecule expression with echogenic liposomes (ELIP). A number of formulations for molecular imaging and drug and gene delivery have been developed using the intrinsically echogenic liposomal technology (Alkan-Onyuksel et al. 1996; Buchanan et al. 2008; Hamilton et al. 2002b; Huang et al. 2002b). The encapsulation of air into these liposomal formulations results in a small, traversable contrast agent that is suitable for ultrasound image enhancement and controlled release of therapeutic agents, while being stable in the circulation for a prolonged period (Buchanan et al. 2008). Intrinsically, ELIP compare favorably with commercially available microbubbles in terms of *in vivo* stability and targeting capabilities, as well as drug and gene delivery capacities (Demos et al. 1999; Huang et al. 2002a, 2002b). The conjugation of antibodies against a variety of adhesion molecules, tissue factors and thrombotic markers on the liposomal surface of ELIP has proven very effective in identifying developing atheromas with transvascular and intravascular ultrasound (Hamilton et al. 2004).

Most ultrasound contrast agents are considered intravascular contrast agents that lack the capability to penetrate the arterial wall. Unlike other ultrasound contrast agents that are homogeneous in size and distribution and tend to be larger than 3 μm, our ELIP formulations vary in size from $<$100 nm to several micrometers, with bimodal medians of $\sim$90 and 800 nm resulting from the formation of ELIP from lyophilized lipids during rehydration (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.

In agreement with previous studies using rabbit models of balloon injury (Tanaka et al. 1993), thrombosis (Toursarkissian et al. 1997) and low-density lipoprotein receptor deficiency (Broisat et al. 2007), as well as histologic examination of human atherosclerosis (Galkina and Ley 2007; O'Brien et al. 1993), we have observed that adhesion molecule expression is not restricted to the endothelial surface, but spans the entire thickness of the neo-intima and the vasa vasorum (Hamilton et al. 2004). As ICAM-1 expression in association with developing atherosclerosis is not restricted to the endothelial surface of the neo-intima, but also involves the upper medial layer (O'Brien et al. 1993) and the microvessels of the vasa vasorum (Galkina and Ley 2007), it is likely that identification of ICAM-1 expression within the deeper neo-intimal layers may provide better assessment of chronic arterial wall inflammation, allowing discrimination of transient inflammatory states such as infection. However, effective penetration of microbubbles or ELIP into the neo-intima and the vasa vasorum requires novel techniques to allow transmigration of the contrast agent through the endothelium, which motivates our development of a new class of ELIP for such an application.

Nitric oxide (NO) is a potent bio-active gas with a wide range of vaso-active properties (Fischer et al. 2004). The desirable characteristics of NO in modulating the development of various vascular diseases have prompted the development of various NO precursors, synthetic NO promoters such as L-arginine, endothelial NO synthase gene, NO donors and NO gas. One of the most popular modes of NO gas delivery clinically is *via* inhalation (Miller and Megson 2007; Radomski et al. 1987; Tsao et al. 1994). Although the delivery of NO to the arterial wall has a number of 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). Our laboratory has developed techniques for encapsulating NO (Huang et al. 2009) and other bio-active gases (Britton et al. 2010) into ELIP, resulting in agents that are suitable for both ultrasound imaging and ultrasound-mediated bio-active gas delivery. Our NO-loaded ELIP (NO-ELIP) have been found to release NO into the arterial wall effectively without NO degradation by scavenging hemoglobin in the bloodstream (Huang et al. 2009). Nitric oxide induces increased vascular permeability in guinea pig conjunctiva (Meijer et al. 1995).

There is accumulating evidence indicating that NO signaling and gap junction communication are interdependent and may be mediated by such key components as endothelial nitric oxide synthase, connexins and caveolin-1 (Looft-Wilson et al. 2012). Furthermore, we have refined antibody conjugation techniques in NO-loaded ELIP (NO-ELIP) to preserve antibody immunoreactivity and ultrasound-mediated bio-active gas delivery (Klegerman et al. 2010). For the first time, it is possible to target the inflamed endothelium for effective ultrasound-triggered local release of NO to facilitate penetration of ELIP into the arterial wall. In the present study, to standardize the delivery of anti-ICAM-1 antibody-conjugated ELIP (anti-ICAM-1 ELIP), NO-ELIP were administered separately before administration of anti-ICAM-1 ELIP.

We hypothesize that a vaso-active agent such as NO may improve the permeability of the vascular bed and allow penetration of anti-ICAM-1 ELIP into all layers of the arterial wall for better quantitation of ICAM-1 expression.

METHODS

ELIP preparation

The preparation of ELIP, anti-ICAM-conjugated ELIP and nitric oxide-loaded ELIP was described previously (Hamilton et al. 2002a; Huang et al. 2009; Lanza 2000).