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Non-Invasive Imaging of Vulnerable Plaques by Molecular Targeting of Oxidized LDL With Tagged Oxidation-Specific Antibodies

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Abstract The concept of the ''vulnerable'' plaque has recently emerged to explain how quiescent atherosclerotic lesions evolve to cause clinical events. Vulnerable plaques are generally non-obstructive, asymptomatic lesions that may abruptly rupture and induce thrombotic occlusion leading to tissue ischemia and its attendant sequelae. They are responsible for over 50% of cases of sudden death and acute myocardial infarction. The lipid component of vulnerable plaques, which is abundant and highly enriched in oxidized low-density lipoprotein (OxLDL), strongly contributes to their propensity to rupture through physical, inflammatory, and thrombogenic properties. We hypothesized that OxLDL would serve as an ideal target to detect vulnerable plaques. In a series of experimental studies, we have shown that oxidation-specific antibodies (Ox-AB) specifically accumulate in vivo within lipid-rich atherosclerotic lesions but not normal arteries, provide a quantitative measure of the content of OxLDL, allow detection of atherosclerosis progression and regression in the context of enhanced or reduced OxLDL content and non-invasively image atherosclerotic lesions. Ox-AB may be tagged with appropriate labels for use in nuclear scintigraphy, magnetic resonance, or ultrasound imaging. Potential research and clinical applications include studying the natural history of atherosclerosis in animal models and humans, evaluating novel drug or genetic therapies on progression and regression of atherosclerosis, evaluating plaque stability, screening and serial follow-up of high-risk individuals, non-invasive imaging of vulnerable plaques, and assessing the clinical efficacy of new treatments of atherosclerosis. J. Cell. Biochem. Suppl. 39: 138–146, 2002.

Key words: atherosclerosis; lipoproteins; oxidation; plaque rupture; radionuclide imaging; radioisotopes

Since the invention of the stethoscope by René Laennec in 1816, X-rays by Wilhelm Roentgen in 1895, and the electrocardiogram by Willem Einthoven in 1902, physicians have sought more precise methods to diagnose cardiovascular disease (CVD). In parallel, scientists have sought to unravel fundamental insights of the pathogenesis of atherosclerosis. In the 20th century, the clinical diagnosis and treatment of CVD was significantly improved by invasive catheter-based angiographic techniques. However, although angiographic methods are currently indispensable in the diagnosis and treatment of patients with CVD, the ability to non-invasively visualize atherosclerotic lesions and predict their natural history has been a long-sought and elusive goal by the cardiovascular community. Recent advances suggest that in the 21st century complete non-invasive diagnosis of both structure and function of clinically relevant vessels will become a reality.

WHAT IS A VULNERABLE PLAQUE?

In the 1960s, Constantinides discovered that coronary atherosclerotic plaques in patients dying of acute myocardial infarction were generally non-obstructive, lipid-rich lesions with a

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disrupted fibrous cap encased with overlying occlusive thrombus [Constantinides, 1966]. These observations were under-appreciated clinically until 1980 when they were reaffirmed by DeWood et al. [1980] by performing coronary angiography during the acute phase of myocardial infarction. Subsequently, several groups showed that such rupture-prone plaques were composed histologically of a characteristic triad: a thin, collagen-deficient fibrous cap, abundant activated macrophages at the expanding shoulder regions of the plaque, and a large lipid pool [Davies et al., 1993]. The atheromatous lipid pool, occupying more than 40% of the total plaque volume [Davies et al., 1993], consists predominantly of oxidized lipids generated in situ from oxidation of plasma-derived LDL and from byproducts of necrotic and apoptotic macrophage/foam cells [Ylä-Herttuala et al., 1989; Carpenter et al., 1995; Piotrowski et al., 1996; Chang et al., 1999].

Detection of such vulnerable plaques is currently not clinically feasible. X-ray angiography is inadequate since it images only the vessel lumen and only indirectly the plaque as it impinges the lumen. In addition, outward "Glagovian" expansion of the media/adventitia preserves the lumen dimensions as the plaque is growing so that a significant amount of plaque ($>40\%$ of cross-sectional lumen area) may be present before it will cause a stenosis and be detected by angiography [Glagov et al., 1987]. Although non-invasive and invasive imaging modalities can detect these atherosclerotic lesions and even quantitate lumen dimensions and vessel size, they cannot reliably differentiate plaque characteristics nor predict plaque rupture [Nissen, 2002]. Thus, novel techniques are urgently needed to directly image the vessel wall and its contents in patients at increased risk prior to the onset of symptoms.

RATIONALE FOR OXIDIZED LOW-DENSITY LIPOPROTEIN (OXLDL) AS A MOLECULAR TARGET TO IMAGE VULNERABLE PLAQUES

Experimental evidence clearly supports the role of LDL oxidation in the initiation and progression of atherosclerosis [Tsimikas and Witztum, 2000]. Since plasma contains abundant antioxidants, oxidation is believed to be primarily initiated in the vessel wall and not in the circulation. After LDL enters and is retained in the subintimal space [Williams and Tabas, 1998], it is oxidatively modified by toxic metabolic products from vascular cells, first into minimally-modified LDL (mmLDL) [Navab et al., 1996] and then to fully oxidized LDL. Blood-derived monocytes are recruited into the vessel wall after products of mmLDL and OxLDL mediate upregulation of endothelial cell adhesion molecules. These monocytes differentiate into macrophages that take up OxLDL through an unregulated manner via scavenger receptors and become foam cells, the hallmark of early atherosclerotic lesions. Accumulation of foam cells and inflammatory extracellular lipids results in a vicious cycle of inflammation and induces progression and destabilization of atherosclerotic plaques. OxLDL also directly influences several other atherogenic mechanisms including upregulation of inflammatory genes and growth factors, endothelial dysfunction, platelet aggregation, thrombus formation, and plaque destabilization [reviewed in Tsimikas et al., 2001b].

Several direct and indirect lines of evidence have shown that OxLDL exists in vivo within atherosclerotic lesions of humans and animals: (1) monoclonal antibodies (Ox-AB) to oxidation-specific epitopes of OxLDL strongly immunostain OxLDL in animal and human arteries [Rosenfeld et al., 1990], (2) OxLDL can be extracted from human atherosclerotic plaques [Ylä-Herttuala et al., 1989], (3) autoantibodies to various epitopes of OxLDL are found in both lesions and plasma of animals and patients with atherosclerosis [Palinski and Witztum, 2000], (4) products of lipid peroxidation, such as isoprostanes, are found in atherosclerotic lesions and plasma [Praticó, 1999], (5) circulating OxLDL is found in plasma of patients with CVD and acute coronary syndromes [Holvoet et al., 1999; Ehara et al., 2001; Tsimikas et al., 2001b, 2003], and finally, (6) OxLDL can be imaged with radiolabeled Ox-AB in live animals [Tsimikas et al., 1999, 2000].

Several human studies have shown that oxidized lipids and their byproducts are present within these lipid-rich plaques in substantial quantities [Carpenter et al., 1995; Piotrowski et al., 1996; Felton et al., 1997]. In particular, Nishi et al. [2002] have recently shown that vulnerable carotid plaques from humans are greatly enriched in OxLDL and that the plasma levels of OxLDL correlated with plaque content of OxLDL. In addition, plasma levels of OxLDL have been shown to correlate with endothelial

dysfunction [Penny et al., 2001] and increased carotid intima-media thickness [Hulthe et al., 2001]. Conversely, in experimental studies of atherosclerosis regression, reduced OxLDL content (measured by Ox-AB MDA2, which detects malondialdehyde-lysine epitopes of OxLDL) and lower OxLDL autoantibody titers have been documented [Cyrus et al., 1999; Tsimikas et al., 2001a; Aikawa et al., 2002]. In humans pretreated with pravastatin to lower plasma cholesterol, significantly reduced OxLDL immunostaining has been noted (with Ox-AB NA59, which recognizes 4-hydroxynonenal epitopes of OxLDL [Rosenfeld et al., 1990]) in carotid plaques removed during carotid endarterectomy [Crisby et al., 2001].

Therefore, a large number of experimental and human studies suggest that OxLDL is intimately involved in the initiation, progression, and destabilization of lipid-rich plaques. Therefore, imaging of atherosclerotic lesions rich in Ox-LDL would be of potential value in detecting vulnerable plaques.

DEVELOPMENT OF TAGGED OXIDATION-SPECIFIC ANTIBODIES FOR IMAGING VULNERABLE PLAQUES

Agents used for detection of vulnerable plaques must have strong specificity for their target (oxidized lipids, macrophage/foam cells, fibrous caps, and other inflammatory targets) and optimal pharmacokinetics (rapid uptake in the target, fast blood clearance, and high target/blood ratio). In addition, they must provide an accurate assessment of the extent of disease, detect clinically relevant changes, and ultimately provide prognostic information.

When autologous LDL is oxidatively modified, breakdown of polyunsaturated fatty acids results in the generation of multiple highly reactive short chain aldehydes and ketones, which in turn bind to both to amino groups of phospholipids and apoB-100 on LDL. We have termed these neoepitopes "oxidation-specific" epitopes to signify the fact that they are derived from lipid peroxidation [Palinski et al., 1990]. These oxidation-specific epitopes are highly immunogenic and result in formation of OxLDL autoantibodies. Using murine OxLDL to immunize mice, a series of murine monoclonal antibodies to these model oxidation-specific epitopes of OxLDL were generated in Dr. Joseph Witztum's laboratory. These Ox-AB, such as MDA2 and NA59, recognize and selectively immunostain well-defined model oxidation-specific epitopes within foam cells and extracellular lipid deposits in atherosclerotic tissue [Rosenfeld et al., 1990; Tsimikas et al., 1999]. Subsequently, similar human Ox-AB were generated from patients with CVD and elevated autoantibody titers to OxLDL, as described below [Shaw et al., 2001].

IN VIVO PLAQUE UPTAKE AND SELECTIVITY OF ^{125}I-MDA2 FOR LIPID-RICH PLAQUES

^{125}I-MDA2 was injected intravenously into LDLR$^{-/-}$ and apoE$^{-/-}$ mice and Watanabe Heritable Hyperlipidemic (WHHL) rabbits to assess in vivo plaque uptake and selectivity for atherosclerotic lesions, and aortas prepared for lipid staining and autoradiography to assess ^{125}I-MDA2 accumulation, as previously described [Tsimikas et al., 1999]. Comparison of the sites of in vivo aortic ^{125}I-MDA2 accumulation with areas of lipid staining with Sudan IV, which detects neutral lipid by non-specifically dissolving within it, shows a striking co-localization of lipid staining and the presence of OxLDL, confirming that ^{125}I-MDA2 accumulates only in lipid-rich areas (Fig. 1). Aortic areas with no visible lipid have no visible ^{125}I-MDA2 accumulation. Quantitative assessment shows approximately a 20-fold greater uptake of ^{125}I-MDA2 within atherosclerotic aortas of WHHL rabbits versus normal aortas of New Zealand White (NZW) rabbits [Tsimikas et al., 1999]. Aortic immunostaining for OxLDL confirmed the increased presence of significant amounts of OxLDL within foam cells and in the extracellular lipid debris.

QUANTITATION OF ATHEROSCLEROSIS WITH ^{125}I-MDA2

An important aspect of imaging patients will be the ability to quantitate the extent of disease, to document the natural progression or regression of disease and to serially track changes following therapeutic interventions. We have shown in both hypercholesterolemic mice and rabbits that a significant correlation exists between the aortic uptake of ^{125}I-MDA2 and measures of global plaque burden, such as % atherosclerotic surface area and aortic weight ($r = 0.95$, $P < 0.001$). In fact, the aortic weight, which increases linearly as the lesions expand three-dimensionally, is a better measure of the

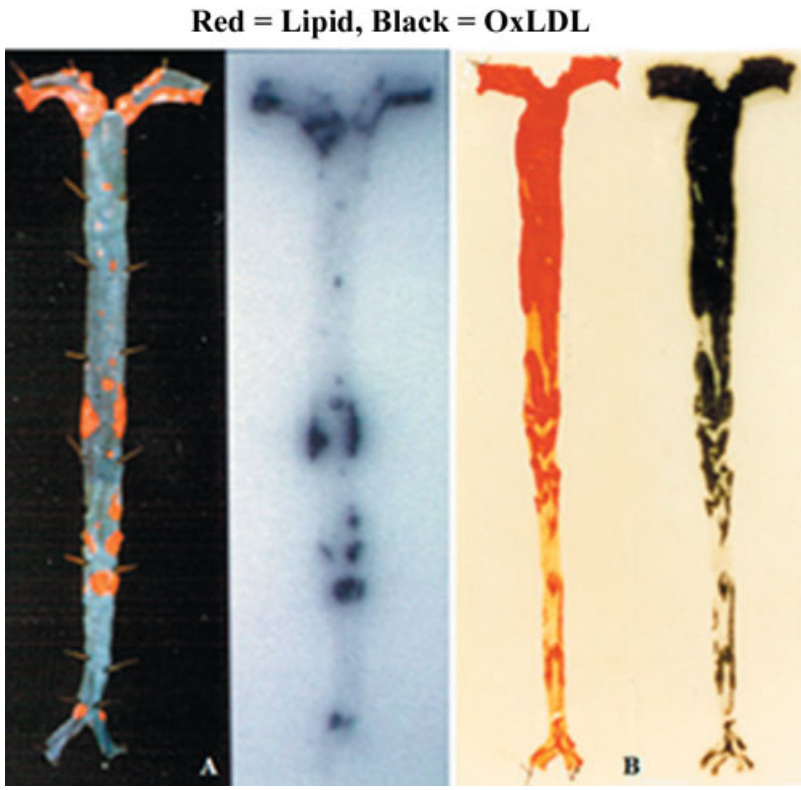

Fig. 1. En-face preparations of Sudan-stained aortas from an apoE$^{-/-}$ mouse (**A**) and WHHL rabbit (**B**) injected with 10 and 100 μCi ^{125}I-MDA2, respectively. Red color (**left panels**, respectively) signifies the presence of lipid within the atherosclerotic plaque and black color in the corresponding autoradiograph (**right panels**) signifies the presence of OxLDL where ^{125}I-MDA2 has accumulated. Reprinted with permission from the American Journal of Cardiology and the Journal of Nuclear Cardiology.

extent of atherosclerosis than surface area [Tsimikas et al., 2000].

ROLE OF ^{125}I-MDA2 IN DETECTING CHANGES IN OXLDL CONTENT AS MEASURES OF PROGRESSION AND REGRESSION OF ATHEROSCLEROTIC LESIONS

Several human coronary angiographic studies have shown that following dietary or lipid lowering therapies very small (and hemodynamically insignificant) improvements were noted in lumen dimensions [Superko and Krauss, 1994]. However, dramatic improvements were noted in clinical outcomes, which were loosely attributed to "plaque stabilization," although the underlying mechanisms were not established. Since OxLDL has been associated with multiple early events in atherogenesis, we hypothesized that depletion of plaque OxLDL would be an important early event of plaque regression. In a dietary progression/regression study in LDLR$^{-/-}$mice, we have shown that progression of atherosclerosis, measured by increases in % atherosclerotic surface area and aortic weight, correlates very well with the accumulation of ^{125}I-MDA2 plaque uptake after intravenous injection. This was documented quantitatively [Tsimikas et al., 2000] and an example can be seen in Figure 2A which shows photomicrographs of a Sudan stained aorta, the corresponding autoradiograph documenting excellent co-localization of lipid staining and ^{125}I-MDA2 uptake, and immunostaining for MDA–LDL showing extensive OxLDL content in the lesion. However, during a subsequent regression period, ^{125}I-MDA2 uptake actually diminished to a greater extent (measured quantitatively) than total lipid in areas where plaques had not fully regressed physically (Fig. 2B). Immunohistochemistry confirmed that almost complete depletion of plaque OxLDL occurred after 6 months of dietary intervention before significant physical regression

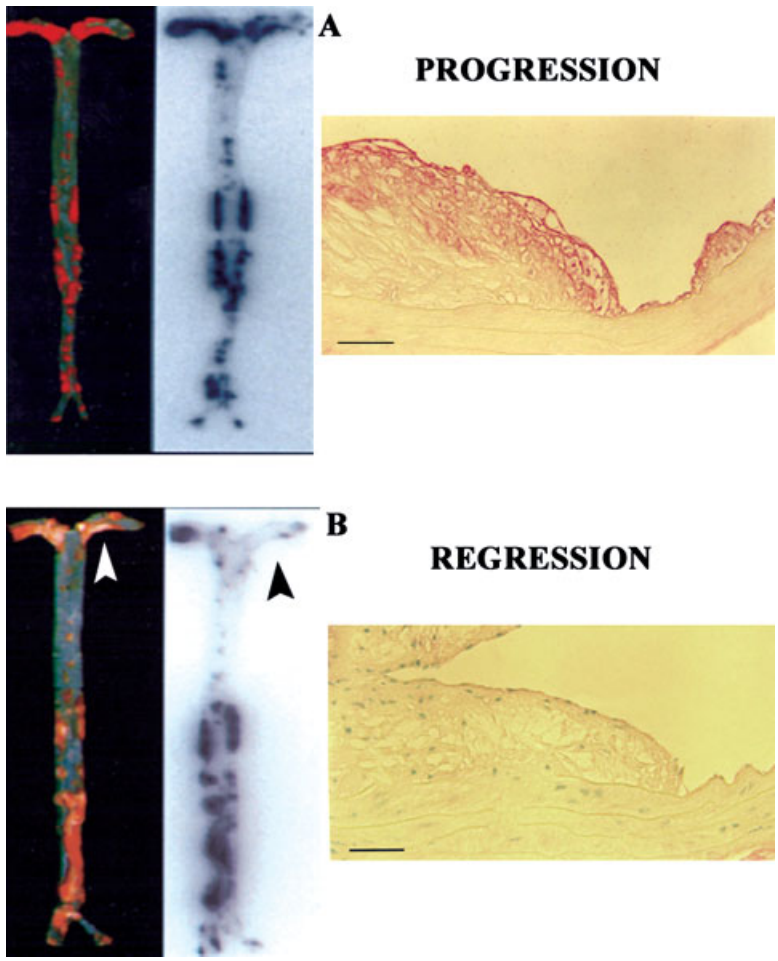

Fig. 2. En-face preparations of Sudan-stained aortas (**left**) and corresponding autoradiographs (**right**) from $LDLR^{-/-}$ mice injected with 10 μCi ^{125}I-MDA2. **A**: Aorta from the Progression group showing co-localization of lipid staining (**left panel**) and ^{125}I-MDA2 uptake (**right panel**). Corresponding immunohistochemical staining with guinea pig antiserum MAL2 shows extensive OxLDL staining (red) within the atherosclerotic lesions. **B**: Aorta from the Regression group showing diminished or absent ^{125}I-MDA2 uptake (arrowheads) in some areas of lipid staining, signifying the removal of OxLDL before complete plaque regression has occurred. Corresponding immunohistochemical staining shows a preferential depletion of OxLDL, even though cholesterol crystals are still present within the lesion. Bar = 100 μm. Reprinted with permission from the American Heart Association.

had occurred (Fig. 2B). Similar data showing preferential depletion of OxLDL, as opposed to other apoB-containing proteins, were recently published by Aikawa et al. [2002] in a rabbit atherosclerosis regression study.

These data provide novel and unique information about plaque regression and suggest that OxLDL depletion is an early mechanism of atherosclerosis regression and plaque stabilization. This may explain the findings in human angiographic regression studies that showed a lack of hemodynamically significant changes in coronary artery lumen dimensions but showed significant improvement in clinical outcomes. In addition, depletion of OxLDL from the vessel wall may explain the rapid clinical benefits (within 4 months) noted in humans treated with statins following presentation with acute coronary syndromes [Schwartz et al., 2001].

NON-INVASIVE GAMMA CAMERA SCINTIGRAPHY OF OXLDL-RICH LESIONS WITH ^{99M}TC-MDA2

In vivo imaging was performed with ^{99m}Tc-MDA2 as described previously [Tsimikas et al., 1999]. Atherosclerotic WHHL and non-atherosclerotic NZW rabbits were imaged supine with a planar gamma camera after intravenous injection of ^{99m}Tc-MDA2 (200 μg, 5 mCi). Figure 3 demonstrates that OxLDL-rich plaques are seen in the thoracic and abdominal

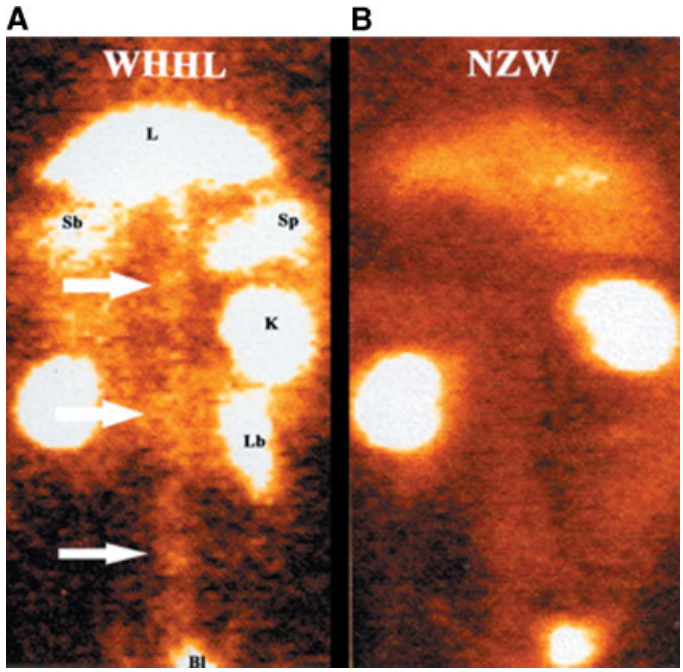

Fig. 3. In vivo planar gamma camera imaging with ^{99m}Tc-MDA2. **A**: Imaging in atherosclerotic WHHL rabbit shows selective uptake ^{99m}Tc-MDA2 in the aorta (arrows). **B**: Imaging in non-atherosclerotic New Zealand White (NZW) rabbit shows no aortic uptake. Interestingly, there is significantly more liver, spleen, and gut uptake of ^{99m}Tc-MDA2 in the WHHL rabbit where increased oxidation-specific epitopes are known to exist. L, liver; Sb, small bowel; Sp, spleen; K, kidney; Lb, large bowel; Bl, bladder. Modified and reprinted with permission from the Journal of Nuclear Cardiology.

aorta of the WHHL rabbit whereas no signal is noted in the normal NZW rabbits. The signal corresponded well to areas with particularly large Sudan stained lesions documented on post mortem Sudan staining. Further refinements in the imaging techniques using sophisticated SPECT or PET imaging are being evaluated to enhance the translation of this technique to human imaging.

HUMAN OXLDL AUTOANTIBODIES FOR IMAGING VULNERABLE PLAQUES

Human Ox-AB, particularly small fragments or peptides, have significant theoretical imaging advantages over murine Ox-AB, including improved pharmacokinetics and reduced immunological/allergic reactions, that would be particularly important in serial imaging to assess the natural history of lesion development or assess the impact of therapeutic interventions.

We generated a human Fab monoclonal autoantibody phage display combinatorial library to OxLDL derived from a patient with CVD and elevated OxLDL autoantibody titers [Shaw et al., 2001]. IK17, the first human Ox-AB, was selected by displaying the library on a filamentous phage surface and panning against MDA–LDL. IK-17 binds strongly to MDA–LDL as well as to copper-oxidized LDL and recognizes both the oxidized lipid moieties and the delipidated protein moiety of OxLDL but not native LDL. IK17 has the important biological property of inhibiting the uptake of OxLDL by macrophages and it also binds to apoptotic cells and likewise inhibits their phagocytosis by macrophages. IK17 immunostains human atherosclerotic lesions with a distinct pattern suggesting a unique predilection for the lipid-rich necrotic core, compared to other Ox-AB tested thus far which display specific but diffuse staining properties of all oxidation-specific epitopes in the entire lesion. This property may be valuable in detecting the lipid-rich, necrotic cores of vulnerable human atherosclerotic lesions. Similar to other Ox-AB, IK17 has also been shown to have selective in vivo uptake for atherosclerotic plaques and holds promise as an imaging agent in patients (Fig. 4).

FUTURE DIRECTIONS IN VULNERABLE PLAQUE DETECTION AND OXLDL IMAGING

Many efforts are currently underway to detect vulnerable plaques. The current state of the art consists primarily of invasive techniques,

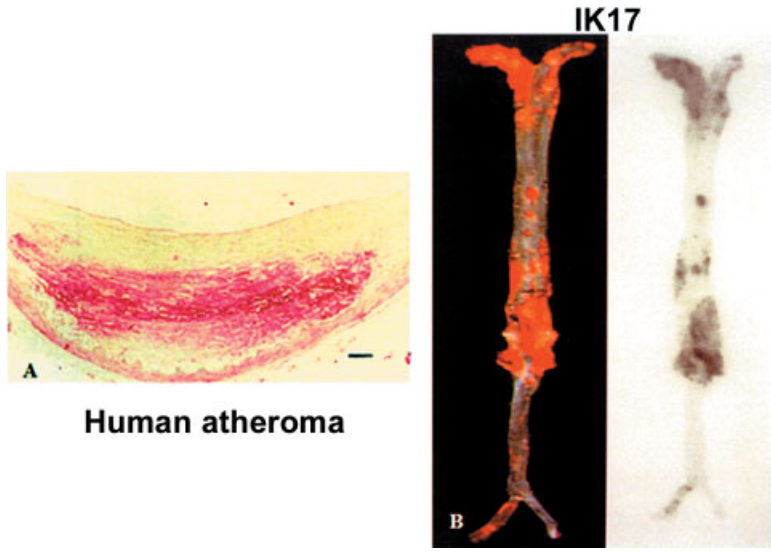

Fig. 4. **A**: Human atheroma immunostained with IK17 showing strong staining within the necrotic core which is highly enriched in oxidation-specific epitopes and OxLDL. **B**: En-face preparation of Sudan-stained aorta (**left**) and corresponding autoradiograph (**right**) of an $LDLR^{-/-}$ mouse intravenously injected with 10 μCi ^{125}I-IK17. Bar = 100 μm. Modified and reprinted with permission from the American Heart Association.

such as optical coherence tomography or intravascular ultrasound imaging, which detect anatomical structures, or thermography and infrared imaging which provide functional assessments such as temperature and pH changes that are a byproduct of plaque inflammation. Currently, these procedures can only be applied to patients already undergoing coronary angiography for another reason. Unless overwhelmingly strong predictive value is demonstrated with these techniques, it is unlikely that asymptomatic patients will consent to this type of invasive assessment. In addition, detecting vulnerable plaques may be a moving target, since human pathology studies have shown multiple episodes of rupture and healing of the same lesions [Virmani et al., 2000]. It is also likely that the concept of vulnerable plaque imaging and treatment will not be one of identifying "the" single vulnerable plaque, but rather the total burden of vulnerability of all plaques in the entire patient. Therefore, systemic therapeutic measures are likely to be more effective for less advanced lesions, in accordance with the diffuse nature of the atherosclerotic process, and focal invasive treatments reserved for more advanced high-risk lesions.

Non-invasive imaging of vulnerable plaques with human Ox-AB has the potential to conceptually change how we view subclinical atherosclerotic lesions. Early diagnosis will allow the physician to intervene on asymptomatic disease and potentially affect its natural history and improve clinical outcomes. We envision this technique to initially be tested in clinically relevant, easily accessible arteries, such as the carotid arteries, where recent data has shown that lipid-rich plaques are associated with increased risk of stroke [Zhao et al., 2001; Yuan et al., 2002]. In concert with the diffuse nature of the disease, long-term approaches may be development of whole body imaging techniques for OxLDL, similar to a bone scan, with quantitation of global disease burden. Once a baseline is established, interventions such as diet and drug therapies may be followed with repeated scanning to assess the progression or regression of disease and assess plaque stabilization. This approach of measuring global burden will need to be compared to direct imaging of OxLDL content in the coronary arteries, for example, to assess which approach is technically more feasible and as importantly, predictive of events. In that regard, all imaging techniques will need to prove their utility in accurately assessing risk and providing prognostic information in prospective studies.

Nuclear (SPECT, PET), magnetic resonance (Ox-AB contrast agents tagged with heavy metals), and ultrasound (Ox-AB coated echogenic liposomes and microbubbles) approaches are currently being developed to define the

optimal approach in human imaging. Development and application of these techniques in humans may allow us to non-invasively detect, quantify, and assess the stability/vulnerability of atherosclerotic lesions, screen high risk asymptomatic individuals, assess drug/diet efficacy, and rationally tailor therapies to those patients that will derive the greatest benefit. With further research and diligence, the goal of non-invasive detection of high-risk atherosclerotic lesions will hopefully become a reality in the near future.

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