Atherosclerosis and Lipoproteins

Reduced In Vivo Aortic Uptake of Radiolabeled Oxidation-Specific Antibodies Reflects Changes in Plaque Composition Consistent With Plaque Stabilization

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Objective—Labeled oxidation-specific antibodies (Ox-AB) detect, quantify, and noninvasively image lipid-rich atherosclerotic lesions. However, it is unknown whether Ox-AB detect plaque stabilization.

Methods and Results—The aortic uptake of intravenously injected \(^{125}\text{I}-\text{MDA2}\) (Ox-AB to malondialdehyde [MDA]–low-density lipoprotein [LDL]) was quantitated in: (1) LDL receptor\(^{-/-}\) mice with established atherosclerosis continued on Western diet (Progression) or switched to chow (Regression) or chow + vitamins E and C (Regression-VIT) for 6 months; and (2) Watanabe rabbits (3- to 57-months old) with naturally evolved atherosclerotic lesions. In mice, the Progression group had more extensive atherosclerosis, higher \(^{125}\text{I}-\text{MDA2}\) uptake, high concordance of Sudan (lipid)-staining and \(^{125}\text{I}-\text{MDA2}\) uptake, and stronger oxidized LDL (OxLDL) and macrophage immunostaining than both Regression groups. In contrast, the Regression groups showed Sudan-positive lesions with focally diminished \(^{125}\text{I}-\text{MDA2}\) uptake, which coincided with reduced OxLDL and macrophages but more smooth muscle cells (SMCs) and collagen. In rabbits, areas of increased \(^{125}\text{I}-\text{MDA2}\) uptake were associated with high Sudan concordance and strong immunostaining for OxLDL and macrophages. Interestingly, advanced lesions with focally diminished \(^{125}\text{I}-\text{MDA2}\) uptake showed stronger immunostaining for SMCs and collagen, particularly at the fibrous cap.

Conclusion—Ox-AB uptake is focally diminished in plaques displaying accepted features of plaque stability. Imaging techniques to detect the presence and depletion of OxLDL may be useful in assessing plaque stabilization. (Arterioscler Thromb Vasc Biol. 2004;24:2307-2312.)

Key Words: oxidation ■ lipoproteins ■ radionuclide ■ imaging ■ antibodies

Human atherosclerotic plaques susceptible to rupture or disruption contain thin, mechanically weak fibrous caps, an abundance of inflammatory cells, and a large quantity of extracellular lipid. The mechanisms of plaque rupture are not fully defined, but a key etiologic component is the underlying inflammatory milieu, which is significantly enhanced by oxidized lipids and multiple other triggering mechanisms. In vitro studies have demonstrated that oxidized low-density lipoprotein (OxLDL) is proinflammatory and upregulates the synthesis and release of collagen-degrading metalloproteinases from macrophages that amplify the underlying inflammatory state, leading to collagen degradation and plaque disruption. Studies in humans and animals have shown that OxLDL and oxidized phospholipids are present in significant amounts within atherosclerotic lesions and may contribute to plaque instability by inducing proinflammatory pathways. Several invasive and noninvasive techniques are being intensively evaluated for detection of such plaques. OxLDL and oxidation-specific epitopes, which are relatively accessible targets, particularly when present in the form of large extra-cellular deposits in the lipid core, are rational targets for imaging and may serve as markers of plaque vulnerability.

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We have shown previously that intravenously injected, radiolabeled oxidation-specific antibodies (Ox-AB), such as the murine monoclonal antibody (MAB) MDA2, which detects malondialdehyde (MDA)–lysine epitopes, have a strong and specific predilection for atherosclerotic lesions but not normal arteries. Aortic uptake of \(^{125}\text{I}-\text{MDA2}\) is enhanced in lipid-rich lesions and reduced in lesions of LDL receptor\(^{-/-}\) mice on dietary/antioxidant diets. \(^{99}\text{Tc}\)-labeled MDA2 noninvasively images atherosclerotic lesions in live animals. However, it is unknown whether plaque uptake of radiolabeled Ox-AB may reflect the changes that occur when plaques become more stable, a property that would potentially enhance the utility of this imaging approach in patients at risk for plaque rupture. The present study therefore assessed the rela-
tionship between the in vivo plaque uptake of $^{125}$I-MDA2 and immunohistologic features of plaque stability.

**Materials and Methods**

**Animal Models**

**Mice**

The experimental protocol used in this study was reported previously. Starting at 6 to 12 months of age, 43 LDLR$^{-/-}$ mice were fed 20% milk fat supplemented with 1.25% cholesterol for 6 months. After atherosclerosis was established ($\pm 25\%$ of the aortic surface area documented in a subgroup of 13 mice), the remaining mice were fed for an additional 6 months the atherogenic diet as above (Progression), normal mouse chow (Regression), or mouse chow supplemented with $1.0\%$ vitamin E and $0.05\%$ vitamin C added to the drinking water (Regression-VIT). One mouse died in the Progression and Regression-VIT groups before the end of the study, and 2 mice in the Regression group had poor tail vein injection and 1 inadequate aorta preparation. The final cholesterol levels in the Progression, Regression, and Regression-VIT groups were 1526$\pm$858, $334\pm56$, and $406\pm149$ mg/dL, respectively.

Mice were injected intravenously with $10\ \mu$Ci $^{125}$I-MDA2, and aortic uptake (percentage injected dose per gram aortic tissue [%ID/g]) was determined at 24 hours by placing the entire aorta in a gamma counter. Aortas were stained with Sudan IV staining, and $^{125}$I-MDA2 uptake (percentage injected dose per gram aortic tissue) was determined at 24 hours by placing the entire aorta in a gamma counter. Aortas were stained with Sudan IV staining, and computer-assisted determination of atherosclerosis surface area followed established protocols. After atherosclerosis quantitation, 3 aortas from each group that had lesions representative of the whole group were placed in autoradiography cassettes for 2 weeks to assess $^{125}$I-MDA2 distribution. Aortas used for autoradiography were not used for immunostaining. Cross-sectional atherosclerosis area measurement and comparative immunohistochemistry were performed in 3 mouse aortas (9 lesions per group) from each group with lesions in the arch, thoracic, and abdominal aorta, obtaining a sample from each aortic segment.

**Rabbits**

Nineteen Watanabe heritable hyperlipidemic (WHHL) rabbits aged 3 to 57 months on normal rabbit chow were studied to assess the “natural history” of atherosclerotic plaques and their relationship to $^{125}$I-MDA2 uptake. Cholesterol levels were not measured in experimental rabbits, but in a contemporaneous group of rabbits ($n=24$) of the same age and gender distribution, the mean total cholesterol levels were 699$\pm83$ mg/dL (range 626 to 964). Rabbits were intravenously injected with $100\ \mu$Ci $^{125}$I-MDA2, and $^{125}$I-MDA2 uptake, Sudan staining, and autoradiography (3- to 5-day exposure at $-4\ ^\circ$C) were performed as described previously. Aortas were visually inspected for concordance or discordance of plaque distribution (Sudan) and $^{125}$I-MDA2 uptake (corresponding autoradiographs). Those aortas with total concordance of Sudan staining and autoradiography signal were designated as Plaque=Ox-AB, and those with clearly visibly diminished $^{125}$I-MDA2 uptake in areas of Sudan-positive lesions were designated as Plaque$\neq$Ox-AB.

Immunohistochemical analysis to define histological differences in areas that had normal versus reduced $^{125}$I-MDA2 uptake was performed in 4 of 13 rabbits (12 lesions, 1 from each segment of the aorta of each rabbit) in the Plaque=Ox-AB and in 4 of 6 rabbits (15 lesions, 7 concordant and 8 discordant) in the Plaque$\neq$Ox-AB. These studies were approved by the animal subjects committee of the University of California, San Diego.

**Immunohistochemistry**

Serial 8-µm-thick sections of paraffin-embedded mouse and rabbit aortas were rehydrated. Three oxidation-specific epitopes were detected immunohistochemically: (1) MDA–lysine epitopes were detected with unlabeled murine MAB MDA2 in rabbit aortas and by guinea pig antiserum MAL2 in mouse aortas (1:500 dilution), (2) oxidized phospholipid epitopes were detected with MAB E06 (1:400 dilution), and (3) a unique oxidation-specific epitope common to MDA–lysine and copper-oxidized phospholipids and prevalent in necrotic cores was detected by biotinylated IK17, a human IgG Fab (1:100 dilution). Apolipoprotein B-100 (apoB-100) was detected by MAB MB–47 (1:500 dilution). Smooth muscle cells (SMCs) were detected by MAB 1A4 (Sigma), recognizing the $\alpha$-smooth muscle actin isoform (1:20 dilution). Rabbit macrophages were detected with MAB RAM-11 (1:500 dilution) and mouse macrophages with rat IgG2a clone BM8 (Acris, Inc.) at 0.5 $\mu$g/mL after antigen retrieval by enzymatic digestion with proteinase K. Collagen content was determined by Trichrome staining.

MABs MDA2, MAL2, E06, MB–47, and RAM-11 bound to tissue on slides were detected using appropriate secondary antibodies and an avidin-biotin–alkaline phosphatase system (Vector Laboratories).

**Quantitative Immunohistological Analysis in LDLR$^{-/-}$ Mice**

Quantitative immunostaining for oxidation-specific epitopes (MDA–lysine epitopes), SMCs, and macrophages was determined as the percentage of cells (primarily macrophages but also SMCs) stained by MDA2, 1A4, and clone BM8, respectively, divided by total number of all cells present in an entire lesion, which was identified by the presence of hematoxylin-counterstained nuclei. Quantitation of collagen staining was performed by choosing a same-color threshold for the specific bright blue color in all specimens. Collagen staining was expressed as percent immunopositive area divided by total plaque area. One experienced operator (M.T.), blinded to the treatment regimens, performed all determinations.

**Statistical Analysis**

Analysis of quantitative parameters in mice was performed with 1-way ANOVA with the post hoc Tukey–Kramer multiple comparison test. Differences between the 2 WHHL rabbit groups (Plaque=Ox-AB and Plaque$\neq$Ox-AB) were assessed by Student $t$ test. Correlation between $^{125}$I-MDA2 uptake and aortic plaque weight was assessed by linear regression analysis. $P<0.05$ was considered significant.

**Results**

**LDLR$^{-/-}$ Mouse Regression Study**

**Aortic Lesion Composition and $^{125}$I-MDA2 Uptake in LDLR$^{-/-}$ Mice**

En face preparations of Sudan-stained aortas and autoradiographs from the Progression group showed near-perfect concordance between plaque and $^{125}$I-MDA2 distribution (Figure 1A), whereas both regression groups showed distinct areas containing Sudan-positive lesions that did not yield autoradiography signal (Figure 1B and 1C, arrowheads), indicating diminished or absent $^{125}$I-MDA2 uptake. Immunohistochemical staining of representative lesions demonstrates intense OxLDL (Figure 2A, pink/purple) and macrophage (Figure 2B, black) staining throughout the lesion in the Progression group. SMCs were infrequent and primarily in the subendothelial area (Figure 2C, pink). Collagen was present in moderate amounts throughout the lesion (Figure 2D, light blue). In contrast, both regression groups (Figure 2G through 2J and 2M through 2P) showed significantly reduced OxLDL and macrophage immunostaining, but enhanced presence of SMCs, particularly at the subendothelial area and the base of the lesion, and collagen accumulation throughout the lesions. Immunostaining with Ox-AB IK17 (pink color) and E06 (brown color) also showed significantly stronger im-
munostaining for different and distinct oxidation-specific epitopes in lesions of the Progression group (Figure 2E and 2F) compared with those in the regression groups (Figure 2K, 2L, 2Q, and 2R).

**Quantitative Immunohistochemistry in LDLR−/− Mice**

The Progression group had greater cross-sectional lesion area than the Regression and Regression-VIT groups (398±140 μm² versus 129±70 and 61±29; P<0.05; Figure 3A). 125I-MDA2 uptake was significantly greater in the Progression group, even when corrected by the tissue weight (ie, a measure that should correct for differences in lesion sizes [3.6±1.3% ID/g versus 1.7±0.5 and 2.0±1.0; P<0.01; Figure 3B]). Consistent with the findings of immunostaining, quantitative immunohistochemistry indicated that the lesions of the Progression group had a significantly greater relative content of OxLDL (MDA–lysine epitope) compared with the Regression and Regression-VIT groups, respectively (83±6% of all cells stained positive for MDA–lysine versus 22±3% and 17±3%; P<0.01; Figure 3C). Similarly, the Progression group had more macrophage content than both Regression groups (71±5.3% versus 14.7±2.2% and 14.1±2.7%; P<0.01; Figure 3D). In contrast, plaques in the Progression group had significantly reduced percentage of SMCs (25±7% cells staining positive for α-actin versus 55±3% and 48±9%; P<0.05; Figure 3E) and collagen content (50±4% of total plaque area versus 60±4% and 68±4%; P<0.01; Figure 3F). No significant differences were noted between the Regression and Regression-VIT groups.

**WHHL Rabbit Study**

We examined the concordance of 125I-MDA2 uptake with extent of Sudan staining in 19 WHHL rabbits of varying age (mean age 30.9±16.7, range 3 to 57 months), thus providing an assessment of these parameters over a wide range of naturally progressing lesions. In 13 of 19 rabbits, we observed (visually) almost total concordance of plaque distribution indicated by Sudan staining and 125I-MDA2 uptake detected by autoradiography (Plaque=Ox-AB; Figure 4). In contrast, in 6 of 19 rabbits, there was discordance between Sudan-stained areas and 125I-MDA2 uptake (Plaque≠Ox-AB; Figure 5). In these discordant arteries, only 71% of the Sudan-positive atherosclerotic surface area was reflected by the autoradiography signal (Table I, available online at http://atvb.ahajournals.org), and overall aortic 125I-MDA2 uptake was diminished. The Plaque=Ox-AB group was significantly younger and had less overall atherosclerosis burden, measured by aortic weight and percent atherosclerotic surface area, but interestingly had 58% greater 125I-MDA2 uptake than the Plaque≠Ox-AB group.

A representative aorta from the Plaque≠Ox-AB group is shown in Figure 5. Sections were compared by immunohistochemistry from the right edge (arrow) of the divided aortic arch, where fairly strong autoradiography signal is noted, and from the left edge (arrowhead), which yielded diminished signal in the middle of the plaque. The staining pattern from the right arch showed diffuse OxLDL, macrophage, and apoB-100 staining but minimal SMC and moderate collagen staining (Figure 5A through 5E). In contrast, the similar-sized plaque from the left side showed significantly weaker OxLDL staining, and the macrophage and apoB-100 staining was present primarily in the deep, necrotic area of the plaque. Conversely, collagen and SMC staining abounded in the fibrous cap and toward the luminal side (Figure 5I and 5J). The discordant plaques from the Plaque≠Ox-AB group
to noninvasively image atherosclerosis with appropriate radiolabels. This study suggests that serial imaging with labeled Ox-AB may allow the detection of reductions in plaque content of OxLDL that coincide with features of plaque stability.

Several recent studies have shown that the presence of OxLDL in coronary and carotid plaques is associated with plaque vulnerability. Importantly, Nishi et al explicitly demonstrated that carotid plaques, derived from symptomatic patients undergoing carotid endarterectomy, displaying pathologically confirmed features of plaque instability such as increased plaque rupture, intraplaque hemorrhage, fibrous cap thinning, and macrophage infiltration, have a 25-fold higher content of OxLDL than stable plaques and 70-fold higher levels than plasma OxLDL levels. These studies are also in agreement with recent observations in patients showing that increased levels of OxLDL in plasma, including levels of OxLDL-E06 (LDL containing oxidized phospholipid epitopes, measured by the antibody E06) are strongly associated with clinical features of plaque instability. In addition, we have shown recently that Ox-LDL-E06 levels are markedly elevated immediately after percutaneous coronary intervention, which appears to emanate from mechanically disrupted plaques. During lipid-lowering therapy with atorvastatin and pravastatin, respectively, it has been shown that OxLDL levels are significantly reduced in plasma (measured as OxLDL-E06) and within carotid plaques (measured as OxLDL-NA59), respectively.

The underlying mechanisms involved in plaque regression or stabilization have not been well defined. Early animal studies of atherosclerosis regression have shown that cholesterol esters and macrophage foam cells are depleted within 6 months but that up to 2 years may be required to solubilize and deplete cholesterol crystals. Changes in vessel wall content of OxLDL during dietary-induced atherosclerosis regression have only recently been evaluated. It was demonstrated previously that Ox-AB uptake is enhanced in progressing atherosclerotic lesions but reduced in lesions undergoing dietary regression of LDLR mice. In this study, LDLR mice showed reduced 125I-MDA2 uptake in conjunction with reduction in several distinct oxidation-specific epitopes of OxLDL and reduced macrophage content but enhanced SMCs and collagen. In WHHL rabbits, there was evidence of thickened fibrous caps and redistribution of OxLDL and macrophage immunostaining to the deeper areas within lesions. In support of these findings, Aikawa et al demonstrated recently that dietary lipid lowering in hypercholesterolemic rabbits was associated with preferential depletion of OxLDL, measured by immunostaining with antibody MDA2, compared with LDL. Commensurate with these changes, there was a downregulation of metalloproteinases and increased collagen formation, which is thought to enhance matrix stability. Our current findings suggest that with appropriate development of imaging methods, such changes may be imaged in patients, which may ultimately have significant clinical utility.

In the LDLR−/− mouse study, evidence was provided for a transition to a “stable” atherosclerotic lesion phenotype in conjunction with reduced 125I-MDA2 uptake after robust consistency showed enhanced SMC and collagen content and had a fibrous cap, completely covering the lipid core, or at least areas particularly rich in SMCs and collagen adjacent to the lumen that resembled the fibrous cap of human lesions. In contrast, the relative paucity of SMCs and collagen in the respective areas of the plaque Ox-AB group was consistently seen in all sections examined from the concordant group.

Quantitatively, in the plaque Ox-AB group, the uptake of 125I-MDA2 (presented now as absolute 125I-MDA2 uptake [e.g., as percentage of total injected dose not normalized to aortic weight]) was strongly correlated with the aortic weight (R=0.93; P<0.001; Figure IA, available online at http://atvb.ahajournals.org). We have shown previously that aortic weight is a surrogate of plaque burden in LDLR−/− mice and WHHL rabbits. However, in the aortas of the 6 older rabbits in the plaque Ox-AB group, this relationship was no longer evident (R=0.20; P=0.70; Figure IB), consistent with significantly reduced 125I-MDA2 uptake (Table 1).

**Discussion**

The novel finding of this study is that aortic uptake of intravenously injected 125I-MDA2, a prototype Ox-AB, reflects changes in plaque composition that are consistent with established features plaque stabilization and progression in LDLR−/− mice.10 In this study, LDLR−/− mice showed reduced 125I-MDA2 uptake in conjunction with reduction in several distinct oxidation-specific epitopes of OxLDL and reduced macrophage content but enhanced SMCs and collagen. In WHHL rabbits, there was evidence of thickened fibrous caps and redistribution of OxLDL and macrophage immunostaining to the deeper areas within lesions. In support of these findings, Aikawa et al demonstrated recently that dietary lipid lowering in hypercholesterolemic rabbits was associated with preferential depletion of OxLDL, measured by immunostaining with antibody MDA2, compared with LDL. Commensurate with these changes, there was a downregulation of metalloproteinases and increased collagen formation, which is thought to enhance matrix stability.

Our current findings suggest that with appropriate development of imaging methods, such changes may be imaged in patients, which may ultimately have significant clinical utility.

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**Figure 3.** Quantitative analyses of lesion size (A), 125I-MDA2 uptake (as %ID/g; B), cell-associated OxLDL immunostaining (MDA-lysine epitope; C), macrophages (D), SMCs (E), and interstitial collagen (F) in LDLR−/− mice. The 125I-MDA2 uptake was determined in the entire aorta for all mice and the other parameters in 9 lesions per group. *P<0.05; **P<0.01; ***P<0.0001, all compared with Progression group.
reduction in plasma cholesterol levels. It should be noted that LDLR−/− mice do not commonly exhibit plaque rupture and thrombosis. However, increasing evidence from several groups indicates that apoE−/−, LDLR−/−, and scavenger receptor-B1 (SR-B1)−/−/apoE−/− mice can, in principle, develop rupture and deep erosion of lesions and even thrombotic occlusion in the innominate and coronary arteries and in the aorta.24 Although these animal models in general may not ideally reflect vulnerable plaques in humans, they do contain features of unstable plaques that have been documented in patients.25 Even in patients, although some studies have shown physical regression of lesions with statins,26 plaque stability has been inferred mainly from a significant reduction in clinical events, and no conclusive data have proven, thus far, that “plaque stabilization” (thicker fibrous caps and reduced oxidation, lipid, and macrophage content) occurs in humans.

In the WHHL rabbit study, focally reduced 125I-MDA2 uptake and increased histological markers of plaque stability were observed despite the fact that these rabbits were very hypercholesterolemic, even in the absence of conditions that normally lead to lesion regression. Rabbits with focally reduced 125I-MDA2 uptake were older and, interestingly, had increased plaque burden. Even among lesions of the same size, lesions yielding a weaker autoradiography signal appeared to have characteristics of more stable plaques, as indicated by increased collagen and SMCs and a redistribution of OxLDL and macrophages to the core. A parallel situation may occur in humans for whom progression of atheroma to complex atherosclerotic lesions may lead to a similar configuration of advancing plaques, which are more likely to result in fixed obstructive lesions and stable angina compared with smaller plaques that result in acute coronary syndromes.27 Interestingly, in pathological studies of human coronary arteries obtained after death from myocardial infarction,28,29 fibrous tissue is generally the major component of atherosclerotic plaques when viewed in total; although specific lesions that rupture are more enriched in inflammatory cells and lipid.1 In this study, the fact that many of the macrophages and OxLDL seemed to be redistributed to the deeper part of aortic plaques may suggest that these epitopes were inaccessible to the injected 125I-MDA2, a condition that may also favor prevention of thrombotic occlusion.

Interestingly, addition of vitamins E and C after switching to a chow diet in this very hypercholesterolemic model (mean cholesterol levels 1526 mg/dL) did not result in additional reductions in 125I-MDA2 uptake and vessel wall OxLDL or increased SMCs and collagen compared with chow alone. This suggests that reduction in the substrate for oxidation (LDL and other lipids) is a more powerful mediator of reduction in OxLDL levels than weak antioxidants such as vitamins E and C,30,31 at least for the short duration of this regression study (6 months). Similar results have been noted recently in human studies of vitamin E supplementation in high-risk patients or patients with documented cardiovascular disease.32

Limitations of this study include the fact that the content of OxLDL was detected by immunostaining and by 125I-MDA2 uptake but not directly measured by elution of apoB-100 from atherosclerotic lesions. However, we used the colocalization of 3 antibodies to distinct oxidation-specific epitopes and apoB-100 as a surrogate measure of OxLDL in plaques. Because many of these oxidation-specific epitopes are not limited exclusively to apoB-100 but are present on many...
nonlipid-associated matrix proteins anchored to the vessel wall, this type of assessment may more globally reflect the presence of oxidative stress. Second, cholesterol levels were not measured in the WHHH rabbits, which limited our ability to assess whether changes in cholesterol levels with aging affected plaque characteristics.

Translation of these findings to human imaging may provide a means to monitor changes in lesion characteristics. After plaques are identified and quantitated, plaque stabilization may be detected by absence of signal in a previously detected site. Potential clinical applications may include surveillance of plaque changes after treatment with novel agents for treatment or prevention of atherosclerosis, earlier and improved diagnosis of oxidation-rich plaques, particularly in high-risk asymptomatic individuals, and potentially deriving prognostic information if borne out by clinical trials.

Acknowledgments

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<th>Plaque=Ox-AB</th>
<th>Plaque≠Ox-AB</th>
<th>P-Value</th>
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<tr>
<td>Age (months)</td>
<td>24 ± 14</td>
<td>46 ± 11</td>
<td>0.006</td>
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<tr>
<td>Aortic weight (mg)</td>
<td>965 ± 408</td>
<td>1720 ± 452</td>
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<tr>
<td>$^{125}$I-MDA2 uptake (%ID/g)</td>
<td>0.066 ± 0.028</td>
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<td>0.005</td>
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<tr>
<td>Atherosclerosis (% surface area)</td>
<td>63 ± 29</td>
<td>90 ± 7</td>
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<td>Autoradiography area (% of Sudan-positive atherosclerotic area)</td>
<td>100</td>
<td>71 ± 13</td>
<td>&lt;0.001</td>
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