Increased Plasma Oxidized Phospholipid:Apolipoprotein B-100 Ratio With Concomitant Depletion of Oxidized Phospholipids From Atherosclerotic Lesions After Dietary Lipid-Lowering

A Potential Biomarker of Early Atherosclerosis Regression

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Background—Oxidized phospholipids (OxPL) are pro-inflammatory. We evaluated whether changes in plasma levels of OxPL associated with apolipoprotein B-100 (apoB-100) reflect changes in OxPL content in atherosclerotic plaques during dietary-induced atherosclerosis progression and regression.

Methods and Results—OxPL content was measured in plasma and immunohistochemically in aortic plaques with antibody E06 in cynomolgus monkeys and New Zealand White rabbits at baseline, after a high-fat/high-cholesterol diet and after reversion to normal chow. The OxPL/apoB ratio, representing the content of OxPL on individual apoB-100 particles, and Total apoB-OxPL (OxPL/apoB multiplied by plasma apoB levels), reflecting the OxPL content on all apoB-100 particles, were measured. Total apoB-OxPL plasma levels increased 3-fold ($P<0.0001$) during hypercholesterolemia and decreased $\approx75\%$ ($P<0.0001$) during reversion to normocholesterolemia. In contrast, OxPL/apoB levels decreased significantly ($P<0.0001$) during hypercholesterolemia and increased significantly ($P=0.0002$) during reversion to normocholesterolemia. Immunostaining revealed that during atherosclerosis progression OxPL co-localized with apoB-100, whereas during regression OxPL virtually disappeared.

Conclusion—In the setting of overall reduction of plasma OxPL levels after dietary lipid-lowering, increases in the OxPL/apoB ratio reflect reduced content of OxPL in atherosclerotic plaques. These data suggest that changes in the OxPL/apoB ratio may reflect early atherosclerosis regression. (Arterioscler Thromb Vasc Biol. 2007;27:175-181.)

Key Words: antibodies ▪ atherosclerosis ▪ lipoproteins ▪ oxidation ▪ phospholipids

Initial experimental studies evaluating the progression and regression of atherosclerosis focused on physical measures, such as atherosclerosis surface area in the aorta. Recent studies expanded this type of analysis to encompass morphological features related to mechanisms of plaque stabilization, which occur before decreases in geometric plaque dimensions. Observations that both diet and statin therapy markedly reduced clinical events engendered much of this work when it was realized that angiographically determined measures of lumen dimensions proved insensitive in providing mechanistic explanations. Despite abundant animal data, evidence of atherosclerosis regression in humans has only recently been documented with intravascular ultrasound. However, less invasive measures that reflect vessel wall pathophysiology could provide useful tools to assess atherosclerosis regression and plaque stabilization in humans.

Oxidized lipids, particularly oxidized phospholipids (OxPL), accumulate in atherosclerotic lesions and can be measured in plasma on apolipoprotein B-100 (apoB) particles with antibody E06 and expressed as the ratio of OxPL/apoB. The OxPL/apoB ratio independently predicts angiographically determined coronary artery disease (CAD), the presence and progression of carotid and femoral atherosclerosis in unselected patients from the general community, and reflects acute coronary syndromes. In addition, the OxPL/apoB ratio increases acutely after iatrogenic plaque disruption during acute coronary syndromes. The OxPL/apoB ratio increases acutely after iatrogenic plaque disruption during...
percutaneous coronary intervention,8 suggesting release of OxPL from atherosclerotic plaques.

Interestingly, increases in the OxPL/apoB ratio were also documented in patients treated with atorvastatin9 and pravastatin,10 and even in healthy subjects on low-fat diets where the plasma level of apoB-100 remained unchanged.11 Although such increases in the OxPL/apoB ratio remain unexplained, we previously hypothesized that mobilization of OxPL may occur from atherosclerotic lesions, sites of inflammation, or even via transfer from other lipoproteins to OxPL acceptors, such as Lp(a), which strongly binds OxPL.5–10,12

This study evaluated the hypothesis that a change in the plasma OxPL/apoB ratio reflects a change in the vessel wall content of OxPL during atherosclerosis progression or regression after dietary intervention in cynomolgus monkeys and New Zealand White (NZW) rabbits.

Methods

Cynomolgus Monkeys

The study used 43 adult male cynomolgus monkeys (Macaca fascicularis) consisting of a Progression group (N = 16), a Regression group (n = 19), and a group of normolipidemic monkeys (n = 8) that served as normal controls, primarily for immunostaining, as previously reported.13–15 In the Progression Study, 16 adult Cynomolgus monkeys ingested a normal monkey chow diet for 4 months (baseline time point) followed by an atherogenic diet (0.7% cholesterol and 43% fat) for 17.2 ± 1.5 months (progression time point).14 The Regression Study consisted of 19 adult male Cynomolgus monkeys fed the same atherogenic diet as mentioned for a mean of 45 ± 1 months. Ten monkeys were euthanized at this time point (termed pre-regression) and the remaining 9 monkeys were placed on a normal diet for an additional 8 months (termed regression).

Blood samples and femoral artery segments were obtained at each time point for measurement of OxPL/apoB levels and immunohistochemistry, as previously described.13,15

NZW Rabbits

The experimental protocol for this part of the study was previously described.16,17 Briefly, after balloon injury to produce experimental atherosclerotic lesions, 30 rabbits ingested an atherogenic diet (0.3% cholesterol and 4.7% coconut oil) for 4 months. At this time point, 15 rabbits were euthanized (Baseline group). Five rabbits were continued on the atherogenic diet (High group) and the remaining 10 rabbits (Low group) reverted to normal rabbit chow for an additional 16 months.

OxLDL-E06 Levels

Chemiluminescence enzyme-linked immunosorbent assay was used to measure the content of oxidized phospholipids (OxPL) per apoB-100 particle (OxPL/apoB) using the murine monoclonal antibody E06, which binds the phosphocholine head group of oxidized but not native phospholipids.5,18 Microtiter wells were coated with the murine monoclonal antibody MB47 (5 μg/mL) to bind apoB-100, a 1:50 dilution of plasma was added followed by biotinylated E06, and the data presented as relative light units (RLU) per 100 millilongseconds.9 In parallel plates, to obtain the amount of apoB-100 captured in each plate, we used murine monoclonal antibody MB24 in the same format, instead of E06, which binds a separate apoB-100 epitope.19 We previously documented in ~1500 human samples that under these conditions, an equal and minute fraction of apoB-100 is captured in all plates, irrespective of the plasma apoB-100 level, thereby normalizing the OxPL content to apoB-100.6 The capture antibody MB47 binds to a highly conserved epitope of apoB-100 (equally well on low-density lipoprotein [LDL], very-low-density lipoprotein, intermediate-density lipoprotein, or Lp(a)) on or near the receptor binding domain of apoB-100.19,20

OxLDL-E06 data are presented in 2 ways: (1) as the OxPL/apoB ratio, derived by dividing the OxPL (E06) RLU by the apoB RLU (MB24) RLU; and (2) as Total apoB-OxPL, derived by multiplying the plasma OxPL/apoB value by the plasma apoB-100 levels, measured independently by routine methods as noted. The Total apoB-OxPL measure reflects the OxPL content on all apoB-100–containing particles in plasma. These assays do not detect OxPL that are not associated with apoB-100.

Measurement of Plasma ApoB-100 and Lp(a) Levels

In the cynomolgus monkeys, apoB-100 and Lp(a) levels (mg/dL) were measured by a commercial kit (Diasorin, Inc) and Lp(a) levels were additionally measured with monoclonal antibody LPA4.8 NZW rabbits do not have Lp(a) and there was insufficient plasma to measure apoB-100 levels, thus Total apoB-OxPL levels in NZW rabbits could not be determined.

Immunohistochemistry

Cynomolgus Monkeys

Immunostaining of NZW femoral arteries (8 μm-thick paraffin-embedded sections) for OxPL was performed with IgM E06 (1:400 dilution), for malondialdehyde (MDA)-lysine epitopes with murine monoclonal antibody IgG MDA2 (1:400) and for apoB-100 with murine monoclonal antibody IgG MB47 (1:500 dilution) with corresponding secondary antibodies and an avidin-biotin-alkaline phosphatase system.21

NZW Rabbits

Immunostaining of NZW frozen sections was performed by 2 independent laboratories (Torzewski and Aikawa/Libby Laboratories) using 2 different methods. The first method treated slides with 3% H2O2 to block endogenous peroxidase activity. To block non-specific conjugation, slides were then incubated with 5% normal rabbit serum. Antibody E06 (1:500) was added for 1 hour at room temperature and slides were incubated first with biotin-conjugated secondary anti-mouse antibody for 30 minutes at room temperature and then with avidin-biotin-peroxidase reagent for 45 minutes at room temperature. The reaction products were revealed by immersing the slides in diaminobenzidine tetrachloride to give a brown reaction product. In the second method, immunohistochemistry on fresh-frozen sections (6-μm) used a standard method using the avidin-biotinylated enzyme complex. After brief fixation with acetone and blocking with 4% normal horse serum, the mouse monoclonal antibodies E06 (1:75) and MB47 (1:50) were applied to the slides and incubated for 60 minutes at room temperature. After washing in phosphate-buffered saline, sections were incubated with a biotinylated horse antibody against mouse IgM or IgG (BA-2001, Vector), respectively, for 30 minutes at room temperature, followed by streptavidin-alkaline phosphatase complex (Vector). Fast Red (Sigma) containing levamisole then revealed alkaline phosphatase activity. Counterstain was performed using hematoxylin.

Sections from each experiment were also stained without primary antibody as a negative control. None of these sections showed any staining (not shown).

Statistical Analysis

Comparisons between cynomolgus monkey groups were performed with Student paired t test and between the 3 NZW rabbit groups by analysis of variance (ANOVA) with Bonferroni post hoc test. P < 0.05 was considered significant. Data are presented as means ± standard deviation.

Results

Cynomolgus Monkey Study

Progression Study

The mean total cholesterol and apoB-100 levels at the baseline time point were 120 ± 17.1 mg/dL and 22.0 ± 7.3
mg/dL, respectively. At the end of the study (17.2 \pm 1.5 months), the mean total cholesterol and apoB levels were 521 \pm 158 mg/dL and 115 \pm 44 mg/dL, respectively (P < 0.0001 for both, comparing baseline versus progression time points).

The Total apoB-OxPL (ie, OxPL/apoB \times apoB) plasma levels were significantly higher at the progression time point compared with the baseline time point (12.4 \pm 5.3 mg/dL versus 3.5 \pm 1.0 mg/dL, P < 0.0001; Figure 1A). In contrast, the OxPL/apoB ratio (ie, OxPL content in individual apoB-100 particles) decreased 31% at the progression time point compared with the baseline time point (0.112 \pm 0.030 versus 0.162 \pm 0.029, P < 0.0001; Figure 1B). A trend toward decreased Lp(a) levels was noted in the progression compared with the baseline time point (25.3 \pm 13.0 versus 32 \pm 19.5 mg/dL, P = 0.077; Figure 1C).

**Regression Study**

The mean total cholesterol and apoB-100 levels at the pre-regression time point were 418 \pm 85 mg/dL and 115 \pm 20 mg/dL, respectively. At the regression time point, they were 64.1 \pm 12.9 mg/dL and 18.4 \pm 4.8 mg/dL, respectively (P < 0.0001 for both, comparing pre-regression versus regression time points).

The Total apoB-OxPL plasma levels decreased significantly at the regression time point compared with the pre-regression time point (4.0 \pm 0.8 mg/dL versus 17.4 \pm 4.2 mg/dL, P < 0.0001; Figure 2A). Interestingly, the OxPL/apoB ratio increased 47% at the regression time point

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**Figure 1.** Total apoB-OxPL (A), OxPL/apoB (B), and Lp(a) (C) levels in the Progression study (n = 16) of cynomolgus monkeys.

**Figure 2.** Total apoB-OxPL (A), OxPL/apoB (B), and Lp(a) (C) levels in the Regression study (n = 9) of cynomolgus monkeys.
(0.220±0.043 versus 0.150±0.022, P=0.0002; Figure 2B). Lp(a) levels did not differ (34.2±30.2 versus 30.6±15.9 mg/dL, P=0.60; Figure 2C).

**Immunohistochemistry**

Figure 3 shows representative examples of immunostaining at each time point. Immunostaining of nonatherosclerotic femoral arteries with E06 (OxPL), MB47 (apoB-100), and MDA2 (MDA-lysine) did not reveal any specific staining (Figure 3A, 3D, 3G). In the pre-regression time point, strong immunostaining (red color) was noted for apoB-100, OxPL, and MDA-lysine. In contrast, in the regression time point, immunostaining did not reveal presence of OxPL or MDA-lysine, and there was markedly reduced presence of apoB-100 (Figure 3C, 3I, 3F, respectively).

**Assessment of the Relationship Between the OxPL/apoB Ratio and Lp(a) in Cynomolgus Monkeys**

Prior human studies showed a very high correlation between OxPL/apoB and Lp(a) (r=0.85 to 0.90) and that Lp(a) binds >90% of OxPL in plasma. Cynomolgus monkeys have circulating Lp(a) and many cynomolgus monkeys from both the progression and regression studies had elevated levels (supplemental Figure I, available online at http://atvb.ahajournals.org). For comparison, a human sample with an Lp(a) level of ~100 mg/dL is included. Interestingly, in contrast to the Lp(a) levels, all of the OxPL/apoB values were quite low (supplemental Figure IA), despite ingestion of a high-fat diet, and no correlation was noted between Lp(a) and OxPL/apoB combined from all time points (R=−0.187, P=0.19) (supplemental Figure IB) or at any individual time point (data not shown). There was also no correlation between Total apoB-OxPL and Lp(a) (R=−0.045, P=0.76).

**NZW Rabbit Study**

**OxPL/apoB Levels and Immunostaining**

Compared with the Baseline (4 months of high-cholesterol diet) group, OxPL/apoB levels remained unchanged in the High-cholesterol group (High) but increased 94% in the Regression group (Low) [OxPL/apoB ratio:0.051±0.009 (Baseline), 0.043±0.010 (High), and 0.099±0.059 (Low), P=0.0004 by ANOVA, Figure 4, upper panel]. The lower panel of Figure 4 shows a representative example of immunostaining (brown) with the avidin-biotin-peroxidase method. Qualitatively, immunostaining for OxPL (E06) increased in the High group compared with the Low group. Interestingly, in the Low group the luminal surface of the atheroma exhibited markedly reduced immunostaining for OxPL (arrow, lower panel Figure 4), as confirmed by immunostaining of a different rabbit aorta with a different method (streptavidin-alkaline phosphatase) (Figure 5). In the Baseline and High groups, OxPL immunostaining co-localized with apoB-100 immunostaining. The lesions in the Low group showed negligible immunostaining for OxPL, whereas some apoB-100 persisted, albeit at markedly reduced extent.

**Discussion**

Originally, we developed the OxPL/apoB measure as an indicator of minimally oxidized LDL in plasma that might reflect the overall content of circulating OxLDL. We surmised that the OxPL/apoB ratio would increase during hypercholesterolemia and atherosclerosis progression and decrease during atherosclerosis regression. Counterintuitively, we have found the opposite, in both animals and humans. As demonstrated here in two animal models of atherosclerosis, the OxPL/apoB ratio increased 50% to 100% in plasma in the setting of lesion regression, concomitant with reduced presence of OxPL in atherosclerotic lesions. This
observation suggests that efflux of OxPL from arterial lesions into plasma occurs preferentially early during atherosclerosis regression, even more extensively than depletion of apoB-100 (Figures 3 through 5) or physical plaque regression. This is consistent with an earlier report showing that MDA-lysine epitopes, detected by antibody MDA2, were depleted more extensively than apoB-100 in the same NZW rabbits studied in this report.17 This study also provides mechanistic support for a qualitatively similar increase in the OxPL/apoB ratio in humans in response to low-fat diets (median 27%11 and 29.3%10 increase), atorvastatin (median 9.5%9 increase) and pravastatin therapy (median 48.7%10 increase). Indeed, the current study suggests that preferential depletion of oxidized phospholipids from atherosclerotic lesions, compared with apoB-100, may contribute to the reduction in inflammatory markers and early clinical benefits with aggressive lipid-lowering therapies.9,22,23

Many earlier studies documented mechanisms of atherosclerosis regression and plaque stabilization in animal models. Initial studies showed that atherosclerosis regression in monkeys occurs at least 6 months after low-fat/low-cholesterol diets.1,24 More recently, Aikawa and Libby25–27 described a number of potential mechanisms leading to both plaque stabilization in animal models, as well as plaque rupture in human autopsy studies.16,17,28 In addition, the recently published Asteroid trial18 showed evidence of reduced atheroma volume (ie, regression) in humans after 24 months of rosuvastatin therapy using intracoronary ultrasound. It would be of interest to determine if changes in the OxPL/apoB ratio correlate with changes in atheroma volume in this study. However, despite anatomic assessment of plaque regression, the underlying changes in plaque composition remain largely unexplored in patients undergoing aggressive lipid lowering therapy. Therefore, additional non-invasive methods of assessing plaque stabilization in humans, particularly at early phases, will likely provide significant clinical benefit in managing patients with cardiovascular disease.

Although many diverse biomarkers currently in development aim to detect cardiovascular disease,29 none can measure plaque stabilization. Because OxPL participate in many seminal events during the initiation and progression of atherosclerosis, they have considerable attraction as potential biomarkers.30 For example, a recent study from our laboratory showed that switching LDLR−/− mice with pre-existing atherosclerosis to a low-fat/low-cholesterol diet resulted in almost complete depletion of OxLDL from the vessel wall, despite the persistence of plaque components such as cholesterol crystals.21 OxLDL content was determined directly by immunostaining with 3 unique antibodies (E06, MDA2, and IK17)21 binding distinct oxidation-specific epitopes and indirectly by showing reduced plaque uptake of 125I-MDA2, used for detecting and imaging MDA-lysine epitopes in athero-
sclerotic lesions. In addition, there was evidence of a change related to plaque stabilization with reduced macrophage content and enhanced smooth muscle cell and collagen content. Additionally, Aikawa et al. showed similar findings of augmented features of plaque stabilization in the same NZW rabbits evaluated in this study. The current study therefore expands on those observations by showing an association between increased plasma OxPL/apoB ratio and OxLDL depletion from atherosclerotic lesions.

In the cynomolgus monkey study, increased Total apoB-OxPL and decreased OxPL/apoB levels after a high-fat/high-cholesterol diet may result from increased numbers of apoB-100 particles relative to the number of OxPL epitopes present in plasma after a strong atherogenic dietary stimulus. To explain this, we propose that there was enhanced deposition of OxPL in atherosclerotic lesions, as documented by immunostaining with E06, resulting in a lower OxPL/apoB ratio. After monkeys developed atherosclerosis and were placed on normal chow, the number of apoB-100 particles in plasma decreased concurrently as the number of OxPL epitopes were being mobilized from the artery wall, and potentially from other peripheral sources, thus yielding more OxPL per apoB particle, ie, increased OxPL/apoB. The concomitant reduction in OxPL immunostaining in atherosclerotic lesions supports the hypothesis that OxPL egress from the vessel wall during dietary lipid lowering and atherosclerosis regression. Other possibilities to explain the depletion of OxPL include in situ clearance and degradation by macrophages in the vessel wall and transfer of OxPL to plasma apoB-100 particles from other sites of inflammation, the reticuloendothelial system or other lipoproteins. It is also possible that upregulation of LDL receptors during lipid-lowering preferentially removes LDL particles compared with LDL particles enriched in OxPL. The data also provide a potential mechanism for similar observations noted in humans in response to different statins and low-fat diets. Future experimental studies will be required to further evaluate these findings in studies of atherosclerosis regression. In addition, prospective studies in humans need to be performed to assess whether the change in the OxPL/apoB predicts clinical outcomes.

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Disclosures
The University of California holds patents/patent applications in the names of Drs Tsimikas and Witztum for uses of oxidation-specific antibodies. Dr Witztum was a consultant to Atherogenics, Inc.

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Figure I. Determination of Lp(a) levels and OxPL/apoB ratio (A, upper panels) in cynomolgous monkey plasma samples and in one human control with elevated Lp(a) levels (~100 mg/dl). Spearman correlations analysis of Lp(a) levels and OxPL/apoB ratio in cynomolgous monkey plasma (B).