Association of anti-oxidized LDL and candidate genes with severity of coronary stenosis in the Women’s Ischemia Syndrome Evaluation study

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Abstract  Atherosclerosis is the major cause of coronary artery disease (CAD), and oxidized LDL (oxLDL) is believed to play a key role in the initiation of the atherosclerotic process. Recent studies show that inflammation and autoimmune reactions are also relevant in atherosclerosis. In this study, we examined the association of antibodies against oxLDL (anti-oxLDL) with the severity of CAD in 558 Women’s Ischemia Syndrome Evaluation (WISE) study samples (465 whites; 93 blacks) determined by coronary stenosis (<20%, 20%–49%, >50% stenosis). We also examined the relationship of anti-oxLDL with serum lipid levels and nine candidate genes including APOE, APOH, APOA5, LPL, LRP1, HL, CETP, PON1, and OLR1. IgM anti-oxLDL levels were significantly higher in the >20% stenosis group than in the ≤20% stenosis group in whites (0.69 ± 0.02 vs. 0.64 ± 0.01, respectively; \( P = 0.02 \)). IgM anti-oxLDL levels correlated significantly with total cholesterol (\( r^2 = 0.01; P = 0.03 \)) and LDL cholesterol (\( r^2 = 0.017; P = 0.004 \)) in whites. Multiple regression analysis revealed a suggestive association of LPL/S447X single-nucleotide polymorphism (SNP) with both IgG anti-oxLDL (\( P = 0.02 \)) and IgM anti-oxLDL (\( P = 0.07 \)), as well as between IgM anti-oxLDL and the OLR1/3′UTR SNP (\( P = 0.020 \)). Our data suggest that higher IgM anti-oxLDL levels may provide protection against coronary stenosis and that genetic variation in some candidate genes are determinants of anti-oxLDL levels.—Chen, Q., S. E. Reis, C. Kammerer, W. Craig, D. M. McNamara, R. Holubkov, B. L. Sharaf, G. Sopko, D. F. Pauly, C. N. B. Merz, and M. Ilyas Kamboh for the WISE study group.

Supplementary key words  anti-oxLDL antibodies • genetics • low density lipoprotein

Coronary artery disease (CAD) is a multifactorial chronic disease caused by atherosclerosis. Although the initiation and progression of atherosclerosis depends largely on genetic factors and lifestyle factors, the underlying cellular and molecular mechanism remains unclear. Accumulating data suggest that oxidized low-density lipoprotein (oxLDL) plays an important role in the development and progression of atherosclerosis (1, 2). OxLDL, generated by the action of reactive oxygen species, is taken up by macrophages, which develop into foam cells. Autoantibodies against oxLDL (anti-oxLDL) are found in both atherosclerotic lesions and plasma (3), and thus, the state of oxidative stress might be measured by serum oxLDL antibody levels (4). The serum levels of anti-oxLDL have been reported to predict the progression of carotid and coronary atherosclerosis (5–8). Published studies assessing the relationship between anti-oxLDL antibodies

Abbreviations:  anti-oxLDL, autoantibodies against oxLDL; CAD, coronary artery disease; MDA-LDL, malondialdehyde-modified LDL; oxLDL, oxidized low-density lipoprotein; SNP, single-nucleotide polymorphism; UTR, untranslated region.

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and atherosclerosis severity by different methods have yielded inconsistent results (6, 9–12) partly because the two antibodies of oxLDL (IgM and IgG) have different mechanistic functions in the atherosclerosis pathway. While IgM antibodies inhibit macrophage uptake of oxLDL (13, 14), oxLDL and IgM immune complexes induce accumulation of macrophages (14). Genetic factors may also affect the association of oxLDL levels with atherosclerosis. While a number of candidate genes have been associated with CAD risk (15), their possible associations with oxLDL parameters have not been studied extensively.

The present study was designed to (1) investigate the separate associations of IgM and IgG anti-oxLDL with stenosis severity in the well-characterized cohort of Women’s Ischemic Syndrome Evaluation (WISE) study, and to (2) examine the association between anti-oxLDL levels and genetic variation in selected candidate genes, including the apolipoprotein E (APOE), apolipoprotein H (APOH), apolipoprotein 5 (APOA5), lipoprotein lipase (LPL), low-density lipoprotein receptor related protein-1 (LRP1), hepatic lipase (HL), cholesteryl ester transfer protein (CETP), paraoxonase (PON1 and PON2), and oxLDL receptor 1 (OLR1) genes. These candidate genes are actively involved in the lipoprotein metabolism pathway, and thus variations in these genes might have significant impact on the levels of anti-oxLDL.

MATERIALS AND METHODS

Subjects

Study subjects were collected as part of the WISE study. Detailed information on the study has been described elsewhere (16, 17). Briefly, female patients were recruited during their clinical examination at one of the four clinical centers (University of Alabama at Birmingham; Allegheny University of the Health Sciences at Pittsburgh; University of Florida at Gainesville; and University of Pittsburgh). Recruitment criteria included (1) ≥18 years of age; (2) presence of chest pain or other symptoms suggestive of myocardial ischemia; (3) clinically indicated coronary angiography; and (4) the ability to give informed written consent. Major exclusion criteria were: pregnancy, cardiomyopathy, contraindications to provocative diagnostic testing, New York Heart Association class IV congestive heart failure, recent myocardial infarction, signficant valvular or congenital heart disease, and recent coronary angioplasty or coronary bypass surgery.

As previously described (18), patients were divided into three groups based on their angiographic CAD severity. Patients with <20% stenosis in all coronary arteries were labeled the normal group, having minimal stenosis (227 whites, 48 blacks); women with ≥1 stenosis of between 20% and 49% were considered to have mild stenosis (150 whites, 27 blacks); and women with ≥1 stenosis of ≥50% were considered to have severe stenosis (206 whites, 45 blacks). In addition, we also analyzed a measure of overall CAD severity using a score that was developed to account for severity of stenosis, adjusting for partial and complete collaterals, and lesion location (18). Coefficients of variation for the angiographic measurements ranged from 3.8%–6.3% (18).

Table 1 presents a comparison of lipid profile and other parameters among the three stenoses groups in the WISE sample. Informed consent was obtained from each subject, and the study was approved by the Institutional Review Board.

IgG and IgM anti-oxLDL measurement

Sera from 465 white and 93 black women in the WISE study were available to measure IgG and IgM anti-oxLDL. IgG and IgM autoantibodies against native and malondialdehyde-modified LDL (MDA-LDL) were assayed in sera by ELISA, as described elsewhere (19, 20), with the modification that sodium azide was omitted from the wash buffer. Data were calculated as the difference in antibody binding between MDA-LDL and native LDL, and then expressed as a percentage of the value of the plasma pool.

Genotyping

The gene fragments containing each genetic polymorphism of a selected candidate gene were amplified by PCR, followed by restriction enzyme digestion for genotyping screening. Detailed methods are described elsewhere for the SNPs APOA-5/T−1131C (21), APOE E2/E3/E4 polymorphism (22), APOH/V247L and W316S (23), CETP/Tag1B (24), HL/C−514T (25), OLR1/5′ untranslated region (UTR) C/T (19), LPL/S447X and HindIII G/T (26), LRP1/A216V (27), and PON1/Q192R, L55M and PON2/S311C (16).

Statistical analysis

All continuous variables, including anti-oxLDL and lipid levels, were tested for distribution normality prior to analysis. To reduce the non-normality, in particular, total cholesterol and LDL cholesterol were transformed using a square root transformation, and triglycerides and HDL cholesterol were transformed using a natural logarithm transformation. All outliers (+4 standard deviations) were removed prior to statistical analyses, and 0–6 values were removed for each variable. All analyses were performed separately for whites and blacks.

Stepwise linear regression analysis was used to identify significant covariates for IgG anti-oxLDL, and IgM anti-oxLDL (assuming an overall 10% level of significance). The potential covariates considered included age, body mass index, statins, drug use history, history of using other lipid-lowering agents, smoking, alcohol use, family history of CAD, history of hypertension, history of diabetes, menopause, and serum lipid levels. Due to the small number of blacks (n = 34), the possible association between the antibody levels and genetic variations was tested in whites only. Based on power calculations, with a sample size of 465 individuals, we would have 80% power at a P level of <0.05 to detect genotypic mean differences of ≥0.07 for IgG or IgM anti-oxLDL levels.

Pearson’s correlation coefficients were calculated to determine significant relationships between the adjusted anti-oxLDL variables and lipid levels. All analyses were performed using R version 2.0.1 software (R Foundation for Statistical Computing, Vienna, Austria).

RESULTS

Correlations between the measurements of anti-oxLDL antibodies and various covariates

Table 2 presents the pairwise correlations (r and P values) between the measurements of anti-oxLDL antibodies and all potential covariates available. Each covariate was examined for its association separately. In whites, the IgM antibody levels were positively correlated with total (r = 0.01, P = 0.03) and LDL (r² = 0.017, P = 0.004) cholesterol, and cigarette smoking (r = 0.014, P = 0.007), and negatively correlated with age (r = 0.012, P = 0.02). White women with diabetes also had lower IgG antibody levels...
than nondiabetic women ($P = 0.04$). Among black women, only cigarette smoking was found to be significantly associated with IgG anti-oxLDL ($r = 0.044, P = 0.04$). These significant covariates were included in the subsequent general linear regression analysis models to test the association between anti-oxLDL antibody levels and CAD severity (measured categorically as CAD stenosis groups and by a severity score), as well as the association between the antibody levels and genotypic variations.

### Table 2: Correlation between anti-oxLDL measures and various potential covariates in the WISE sample

| Covariate                      | White women (n = 465) | Black women (n = 93) |
|-------------------------------|-----------------------|----------------------|
|                                | IgG       | IgM     | r       | P value | IgG       | IgM     | r       | P value |
| Potential covariates          |           |         |         |         |           |         |         |         |
| Total cholesterol             | 0.02      | 0.06    | 0.10    | 0.03    | 0.09      | 0.36    | 0.07    | 0.48    |
| Triglycerides                 | 0.01      | 0.81    | 0.03    | 0.48    | 0.13      | 0.21    | 0.06    | 0.57    |
| HDL cholesterol               | 0.04      | 0.43    | 0.007   | 0.88    | 0.04      | 0.69    | 0.05    | 0.66    |
| LDL cholesterol               | 0.03      | 0.53    | 0.13    | 0.06    | 0.07      | 0.59    | 0.09    | 0.96    |
| Statins drug use              | 0.07      | 0.12    | 0.0099  | 0.98    | 0.02      | 0.86    | 0.03    | 0.79    |
| Other lipid lowering agents   | 0.03      | 0.55    | 0.0001  | 0.75    | 0.14      | 0.18    | 0.15    | 0.15    |
| Cigarette smoking            | 0.05      | 0.26    | 0.12    | 0.007   | 0.21      | 0.04    | 0.19    | 0.05    |
| Alcohol use                   | 0.06      | 0.20    | 0.0001  | 0.46    | 0.02      | 0.87    | 0.009   | 0.95    |
| Family history of CAD        | 0.02      | 0.65    | 0.0008  | 0.86    | 0.01      | 0.92    | 0.05    | 0.10    |
| History of hypertension      | 0.01      | 0.74    | 0.0005  | 0.90    | 0.06      | 0.50    | 0.02    | 0.81    |
| History of diabetes          | 0.09      | 0.04    | 0.06    | 0.17    | 0.14      | 0.18    | 0.15    | 0.15    |
| Body mass index              | 0.004     | 0.93    | 0.02    | 0.67    | 0.13      | 0.22    | 0.10    | 0.35    |
| Age                           | 0.009     | 0.85    | 0.04    | 0.35    | 0.09      | 0.40    | 0.15    | 0.14    |
| Menopause                     | 0.005     | 0.91    | 0.04    | 0.35    | 0.09      | 0.40    | 0.15    | 0.14    |
cholesterol levels, IgM anti-oxLDL antibody levels remained slightly but significantly higher in the <20% stenosis group than in the >20% stenosis groups (0.69 ± 0.02 vs. 0.64 ± 0.02, respectively; \( P = 0.03 \)). After adjusting for history of diabetes, no significant association was found between IgG anti-oxLDL levels and stenosis severity. Finally, no significant association was observed between IgM or IgG anti-oxLDL level and severity of stenosis in black subjects. In contrast, we found no significant relationship between the angiographic severity score and IgM or IgG anti-oxLDL antibody levels (\( P = 0.41 \) and 0.88, respectively).

**Association between the anti-oxLDL antibody levels and candidate genes**

The results of association analyses between adjusted anti-oxLDL antibody levels and various candidate gene polymorphisms are summarized in Table 4. A significant association (\( P = 0.02 \)) was observed for IgM anti-oxLDL levels and **OLR1** genotypes. The **OLR1/3′UTR single-nucleotide polymorphism (SNP)** showed gene dosage effects on the IgM antibody levels, with the lowest value in the TT genotype (mean = 0.64 ± 0.02), the highest value in the CC genotype (mean = 0.65 ± 0.02), and an intermediate value in the TC genotype (mean = 0.71 ± 0.02). As the XX genotype of the **LPL/S447X SNP** was uncommon among our subjects (\( n = 5 \)), we combined XX and SX genotypes to compare with the SS wild-type genotype. The **LPL/S447X SNP** showed significant association with IgG anti-oxLDL (\( P = 0.02 \)) and borderline association with IgM anti-oxLDL (\( P = 0.07 \)) (Table 4). While 447X allele carriers had higher IgM antibody levels than SS homozygotes (0.72 ± 0.03 and 0.65 ± 0.01, respectively), the reverse trend was observed for the IgG antibody level (0.71 ± 0.02 vs. 0.93 ± 0.01). Association analyses were also carried out to determine whether these polymorphisms were significantly correlated with stenosis severity; however, no significant results were discovered (data not shown).

**DISCUSSION**

It has been suggested that progression of atherosclerosis is modified by an immune reaction triggered by different immunogens (3, 28–32), with oxLDL as the major immunogen for such reaction (3, 31). In animal studies, immunization with modified LDL results in an increased titer of antibodies against MDA-LDL and suppression of atherosclerosis (33). Following the initial report of a significant association between anti-oxLDL antibodies and the progression of carotid intima-media thickness in 30 healthy Finnish men (7), subsequent studies have shown inconsistent associations between anti-oxLDL antibodies and cardiovascular disease or related risk factors, most probably due to methodological variations in the anti-oxLDL assay (34). The novel contribution of the present study is the examination of the impact of genetic variation in candidate genes on both IgM and IgG anti-oxLDL antibody levels while testing the association between anti-oxLDL antibodies and CAD severity in women. The WISE study participants are well characterized with detailed angiographic and ischemic assessment, allowing patient categorization by the severity of stenosis into <20% stenosis, 20%–49% stenosis, and >50% stenosis groups.

Age and gender are two major physiological factors related to the individuals’ anti-oxLDL levels in the general population (34–36). Since only women are included in our study, gender-specific effects on anti-oxLDL antibodies were not evaluated. However, we found that age is inversely correlated with IgM anti-oxLDL levels (\( r = -0.11, \ P = 0.02 \)), indicating that women of a younger age have higher levels of IgM antibody than older women. Consistent with this finding, Tinahones et al. (35) reported that the levels of anti-oxLDL antibodies were significantly higher in women 16–35 years old, with a significant decrease after 36 years old. Lower levels of anti-oxLDL antibodies have also been reported in elderly persons with high cardiovascular risk factors (37). In addition to the inverse relationship with age, we also found in whites a significant association between smoking and higher levels of both IgM and IgG anti-oxLDL, which is consistent with earlier reports (38). Given that higher levels of IgM anti-oxLDL are associated with less severe stenosis, this relationship with smoking appears to be counterintuitive; it may, however, reflect the coexistence of additional immunologic processes involving anti-oxLDL. For example, cigarette smoke increases leukocyte, platelet, and monocyte adhesion to endothelial cells and platelet aggregation, which might expand a local inflammatory response (38).

The major finding in this study is the inverse association between IgM anti-oxLDL and the severity of steno-

**TABLE 3. Mean anti-oxLDL antibody levels among coronary stenosis groups**

| Antibody            | <20%     | 20–49%   | >50%     | \( P \)  |
|---------------------|----------|----------|----------|---------|
| White women         |          |          |          |         |
| IgG anti-oxLDL      | 0.65 ± 0.01 | 0.64 ± 0.02 | 0.65 ± 0.02 | 0.84    |
| IgM anti-oxLDL      | 0.69 ± 0.02 | 0.64 ± 0.02 | 0.64 ± 0.02 | 0.02    |
| Black women         |          |          |          |         |
| IgG anti-oxLDL      | 0.69 ± 0.03 | 0.65 ± 0.03 | 0.75 ± 0.04 | 0.15    |
| IgM anti-oxLDL      | 0.65 ± 0.03 | 0.61 ± 0.05 | 0.70 ± 0.04 | 0.41    |

Data show adjusted means ± standard deviations (SD) of anti-oxLDL antibody levels (%M-L) among coronary stenosis groups, where %M-L is the difference in antibody binding between MDA-LDL and native LDL expressed as a percentage of the value of the plasma pool.

ANOVA \( P \) values adjusting for total and LDL cholesterol, history of smoking, and age.
oxLDL antibodies appear to be protective in atherosclerosis suggests a new immunologic paradigm that the immune recognition of oxidation-specific epitopes contributes to physiologic homeostasis of lipoproteins that have undergone oxidative changes that could possibly slow down atherosclerosis (50). Due to the complexity of the underlying mechanism, further molecular and cellular level characterizations of IgM anti-oxLDL antibodies are needed. In addition to the observed association between anti-oxLDL antibodies and severity of coronary stenosis, it would also be useful to determine the association between oxLDL levels and degree of stenosis. Although oxLDL levels were not available in this study, recently Tsikinas et al. (51) reported a significant association of oxidized phospholipids on apolipoprotein B-100 particles and Lp(a) with an increased risk of CAD events in a prospective case-control study, indicating the value of oxidation-specific biomarkers in CAD.

In our study, we also investigated the impact of 13 polymorphisms in 10 candidate genes on anti-oxLDL levels. The chosen candidate genes, including APOE, APOH, APOA5, LPL, LRPI, HL, CETP, PON1, PON2, and OLRI are involved in lipid metabolism, and genetic variations in these genes have been found to be associated with CAD and/or plasma lipid profile (52–57).

| Genetic polymorphism | IgG % M-L | IgM % M-L |
|-----------------------|-----------|-----------|
| APOE/V447L            | 0.96      | 0.12      |
| PON2/S311C            | 0.21      | 0.66      |
| PON1/Q192R            | 0.22      | 0.44      |
| PON1/L35M             | 0.23      | 0.17      |
| APOE/E2/E3/E4         | 0.85      | 0.14      |
| APOH/W316S            | 0.31      | 0.41      |
| OLRI/3′UTR C/T        | 0.58      | 0.02      |
| LRPI/A216V            | 0.62      | 0.85      |
| LPL/S447X             | 0.02      | 0.07      |
| LPL/HindIII G/T       | 0.58      | 0.69      |
| CETP/TaqI B           | 0.34      | 0.14      |
| HL/C–SN14T            | 0.37      | 0.62      |
| APOA5/T–1131C         | 0.30      | 0.87      |

% M-L is the difference in antibody binding between MDA-LDL and native LDL expressed as a percentage of the value of the plasma pool.

polymorphisms in 10 candidate genes on anti-oxLDL levels. The chosen candidate genes, including APOE, APOH, APOA5, LPL, LRPI, HL, CETP, PON1, PON2, and OLRI are involved in lipid metabolism, and genetic variations in these genes have been found to be associated with CAD and/or plasma lipid profile (52–57). We found one significant association with anti-IgG, and one with anti-IgM. The LPL/S447X polymorphism significantly correlated with anti-IgG (P = 0.02) and had borderline significant association with anti-IgM (P = 0.07). The unadjusted anti-IgG levels were higher in the wild-type SS genotype than in X allele carriers (0.93 ± 0.01 vs. 0.71 ± 0.02). On the other hand, X allele carriers had higher anti-IgM levels than SS homozygotes (0.72 ± 0.03 vs. 0.65 ± 0.01, respectively). The OLRI/3′UTR polymorphism was significantly associated with anti-IgM. There was a gene-dosage effect such that the highest levels were observed in CC homozygotes (mean, 0.71 ± 0.02), intermediate in CT heterozygotes (mean, 0.65 ± 0.02), and lowest in TT homozygotes (mean, 0.64 ± 0.02). The OLRI gene is a receptor for oxLDL, and it could affect the risk of CAD through its direct effect on the metabolism of oxLDL. Previously we have shown that the OLRI/3′UTR C allele, which is associated with higher anti-IgM levels, is protective against coronary stenosis (19). LPL has both anti-atherogenic and proatherogenic roles (58). The majority of the total LPL is located in the capillary endothelium where it hydrolyzes triglycerides and thus acts as an anti-atherogenic. However, a small fraction of LPL is also located in the arterial wall where it can act as a proatherogenic by retaining LDL in arterial intima and binding to oxLDL. A number of studies have suggested that the LPL/447X allele is associated with a favorable lipid profile and lower CAD risk (59). In this study, the 447X allele carriers had higher anti-IgM levels, which are protective against coronary stenosis. However, additional studies in independent samples are needed to confirm these genetic association data because of the modest observed P values, which will not stand if corrected for multiple testing.
CONCLUSION

In conclusion, our study shows that levels of IgM anti-oxLDL are significantly lower in patients with severe CAD than in patients with minimal stenosis. These data, in conjunction with previous studies, suggest that IgM anti-oxLDL may be protective in atherosclerosis. Our data also suggest that genetic variations in selected genes associated with atherosclerosis risk can influence serum IgM or IgG anti-oxLDL levels.

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