

Oxidized Low Density Lipoprotein Is a Prognostic Marker of Transplant-Associated Coronary Artery Disease

Paul Holvoet, Johan Van Cleemput, Désiré Collen, Johan Vanhaecke

Abstract—Retrospective studies identified oxidized low density lipoprotein (LDL) in the blood as a diagnostic marker of coronary artery disease (CAD). This prospective study sought to determine the prognostic value of oxidized LDL for CAD in cardiac transplant patients. Oxidized LDL was measured in 99 cardiac transplant patients with normal coronary angiograms at baseline and was measured again after a median follow-up of 2 years at the time of a second angiogram. Twenty-one patients developed angiographically detectable cardiac transplant vasculopathy (cases), and 78 individuals did not (controls). Cases had significantly higher baseline plasma levels of oxidized LDL than did controls: 1.18 ± 0.70 versus 0.57 ± 0.20 mg/dL (mean \pm SD, $P < 0.0001$). The increase of oxidized LDL at the end of the follow-up was significantly higher in cases than in controls: 0.75 ± 0.73 mg/dL versus 0.14 ± 0.27 mg/dL ($P < 0.0001$). Baseline levels of oxidized LDL predicted cardiac transplant vasculopathy ($\chi^2 = 16$, $P < 0.0001$) independent of pretransplant ischemic cardiomyopathy, time after transplantation, age, and serum levels of LDL and high density lipoprotein cholesterol. The development of transplant CAD was associated with a further increase of plasma levels of oxidized LDL ($\chi^2 = 14$, $P = 0.0002$). Oxidized LDL is a prognostic marker of transplant CAD. (*Arterioscler Thromb Vasc Biol.* 2000;20:698-702.)

Key Words: lipoproteins ■ transplantation ■ coronary artery disease ■ prognosis

An association between LDL oxidation and atherogenesis was first suggested by experiments showing that oxidized LDL caused injury to endothelial cells (reviewed in Reference 1) and was further supported by studies in animal models showing a protective effect of antioxidants against the progression of atherosclerosis (reviewed in Reference 2). In retrospective studies, we have demonstrated an association between angiographically detected coronary artery disease (CAD) and plasma levels of oxidized LDL.^{3,4} However, the retrospective data did not allow us to conclude whether the increase of plasma levels of oxidized LDL was a consequence of the development of CAD or whether oxidized LDL was an independent risk factor of transplant CAD. The aim of the present prospective study was to assess the prognostic value of oxidized LDL for CAD. Because similar increases of oxidized LDL were observed in patients with cardiac transplant vasculopathy and in patients with ischemic heart disease and because transplant vasculopathy progresses rapidly, the role of oxidized LDL in the development of CAD was first assessed in cardiac transplant patients. Therefore, oxidized LDL was measured in 99 cardiac transplant patients with normal coronary angiograms at baseline and was measured again after a median follow-up of 2 years at the time of a second angiogram.

Methods

Study Design

The cohort of the present prospective study constituted 99 of 105 potentially recruitable cardiac transplant patients: patients transplanted at least 1 year before collection of the baseline blood sample (between December 1993 and May 1996), with a normal baseline coronary angiogram and a follow-up angiogram at least 2 years later. The baseline angiogram was obtained at a median of 36 months (12 to 84 months) after transplantation. The median of follow-up was 24 (range 24 to 36) months.

All coronary angiograms were assessed by 2 angiographers (J.V.C. and J.V.), who were unaware of the oxidized LDL levels. Angiograms were visually graded as follows: grade 0, normal coronary arteries; grade 1, minor abnormalities with $< 50\%$ stenosis of primary or secondary branches and normal left ventricular function; and grade 2, $\geq 50\%$ stenosis of primary or secondary branches or distal involvement with impaired left ventricular function. Seventy-eight patients still had angiographically normal coronary arteries (controls), whereas 21 showed angiographically detectable atherosclerotic lesions in the coronary arteries (cases). All cases had grade 1 stenosis. It is well known that angiography systematically underestimates the extent of coronary intimal thickening in cardiac transplant recipients.⁵ Therefore, the present study does not attempt to accurately quantify the extent of coronary artery stenosis. Rather, the subdivision in groups defined above relies on angiographic data that are easily distinguishable and that have been shown to correlate with histopathological findings and prognosis.^{6,7} Blood samples for analysis of oxidized LDL were collected at the time of angiography and were analyzed blindly. For 6 of 105 eligible

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TABLE 1. Characteristics of Cases and Controls

	Controls (n=78)	Cases (n=21)	P
Age, y	53±13	60±7.4	0.025
Sex, male/female	68/10	18/3	0.97
Smokers, n	11	2	0.30
Pretransplant history, ischemic/nonischemic heart disease	33/45	13/8	0.11
Time after transplantation at baseline, mo	36 (12–84)	36 (24–72)	0.025
Follow-up, mo	24 (24–36)	24 (24–36)	0.83
Hypertension, yes/no	56/23	14/7	0.71
Diabetes, yes/no	4/74	2/19	0.45
Peripheral vascular disease, yes/no	5/73	1/20	0.78
Cytomegalovirus infection, yes/no	51/27	12/9	0.49
Rejections, yes/no	17/61	4/17	0.78
Statins, yes/no	23/55	11/10	0.049
Fibrates, yes/no	7/71	1/20	0.53
Calcium channel blockers, yes/no	52/26	11/10	0.23
Total cholesterol, mg/dL	190±32	187±35	0.57
LDL cholesterol, mg/dL	120±31	110±31	0.88
HDL cholesterol, mg/dL	51±17	46±18	0.038
Triglycerides, mg/dL	129±58	149±63	0.15
MDA-modified LDL at baseline, mg/dL	0.39±0.14	0.38±0.14	0.66
MDA-modified LDL at 2 y, mg/dL	0.39±0.20	0.37±0.14	0.88
Oxidized LDL at baseline, mg/dL	0.57±0.20	1.18±0.70	<0.0001
Oxidized LDL at 2-y follow-up, mg/dL	0.70±0.31	1.93±0.95	<0.0001
Increase of oxidized LDL, mg/dL	0.14±0.27	0.75±0.73	<0.0001

Values are mean±SD, median and intervals, or number of patients. Continuous parameters were compared by nonparametric Mann-Whitney *U* test, whereas discontinuous parameters were compared by χ^2 analysis.

patients, no blood sample was collected at the time of the second angiography; therefore, these patients were not included.

Maintenance immunosuppression consisted of triple-drug therapy, including cyclosporin, azathioprine, and prednisone. Rejection episodes were treated with high-dose intravenous corticosteroids. Cytomegalovirus infection was defined as seroconversion of a seronegative recipient or a 4-fold rise in titer after surgery in a previously cytomegalovirus-positive recipient. The Institutional Review Board approved the study, and the patients provided informed consent.

Blood sampling

Venous blood samples were collected in 0.1 vol of 0.1 mol/L citrate containing 1 mmol/L EDTA, 20 μ mol/L vitamin E, 10 μ mol/L butylated hydroxytoluene, 20 μ mol/L dipyridamole, and 15 mmol/L theophylline. Blood samples were centrifuged at 3000g for 15 minutes at room temperature within 1 hour after collection and stored at -30°C until the assays were performed. Under these conditions, in vitro LDL oxidation and platelet activation are adequately inhibited.⁸

Assays

A monoclonal antibody (mAb-4E6)-based ELISA was used for the quantification of oxidized LDL in plasma.^{3,4,8} Standard oxidized LDL and plasma samples were diluted in PBS containing antioxidants and antiplatelet agents as described above. Equal volumes of diluted purified mAb-4E6 solution (final concentration 7.5 ng/mL) and of diluted standard solution were mixed and incubated for 30 minutes at room temperature. Then 200- μ L aliquots of the mixtures were added to the oxidized LDL-coated wells. Samples were incubated for 2 hours at room temperature. After they were washed,

the wells were incubated for 1 hour with horseradish peroxidase-conjugated rabbit IgG raised against mouse immunoglobulins and washed again. Thereafter, the peroxidase reaction was performed as described earlier,⁸ and the absorbance (*A*) was read at 492 nm. Control samples and blanks, without antibody, were included routinely. The percentage inhibition of binding of mAb-4E6 to the immobilized ligand was calculated as $(A_{492\text{ nm control}} - A_{492\text{ nm sample}}) / (A_{492\text{ nm control}} - A_{492\text{ nm blank}})$, and standard curves were obtained by plotting the percentage inhibition versus the concentration of competing ligand. When copper-oxidized LDL was added to human plasma at a final concentration of 0.25 and 2.0 mg/dL, respectively, recoveries were 95% and 105%, respectively. The intra-assay and interassay coefficients of variation were 10% and 12%, respectively.

mAb-4E6 is directed against a conformational epitope in the apoB-100 moiety of LDL that is generated as a consequence of substitution of lysine residues of apoB-100 with aldehydes. These aldehydes may be produced by peroxidation of lipids of LDL, resulting in the generation of oxidized LDL. Aldehydes that are released by endothelial cells under oxidative stress or by activated platelets may also induce the oxidative modification of apoB-100 in the absence of peroxidation of lipids of LDL. Previously, we have been referring to this type of oxidatively modified LDL as to malondialdehyde (MDA)-modified LDL. The C_{50} values, ie, concentrations that are required to obtain 50% inhibition of antibody binding in the ELISA, are 25 mg/dL for native LDL, 0.025 mg/dL for MDA-modified LDL with at least 60 aldehyde-substituted lysines per apoB-100, and 0.025 mg/dL for oxidized LDL. An ELISA based on the monoclonal antibody mAb-1H11, which has an affinity for MDA-modified LDL similar to that of mAb-4E6 but a 500-fold lower affinity for oxidized LDL, has been used to measure specifi-

cally plasma levels of MDA-modified LDL.⁴ Total and HDL cholesterol and triglyceride levels were measured by enzymatic methods (Boehringer-Mannheim). LDL cholesterol levels were calculated with the Friedewald formula.

Statistical Analysis

Continuous parameters were compared by nonparametric Mann-Whitney *U* test, and discontinuous parameters were compared by χ^2 analysis. Logistic regression analysis was performed to evaluate the correlation between transplant CAD (response) and age and sex of the recipients; smoking; time after transplantation; length of follow-up; pretransplant history of ischemic or nonischemic heart disease; frequency of hypertension, diabetes, cytomegalovirus infection, and rejections; treatment with lipid-lowering drugs (statins or fibrates) and calcium channel blockers; levels of total, HDL, and LDL cholesterol and of triglycerides; and levels of oxidized LDL. Linear and nonlinear regression analyses were performed to evaluate the correlation between levels of oxidized LDL and these different parameters. Logarithmically transformed values of oxidized LDL and of serum cholesterol were used for statistical analysis. All analyses were performed with the use of the S-plus program (version 4.5, Mathsoft). A value of $P < 0.05$ was considered to be significant.

Results

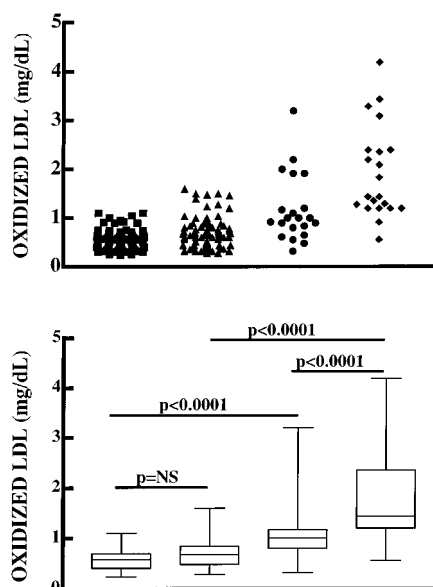
Ninety-nine cardiac transplant patients with normal baseline coronary angiograms were included in the present prospective study. During a 2-year follow-up, 21 patients developed cardiac transplant vasculopathy (cases) and 78 did not (controls). The incidence of cardiac transplant vasculopathy was thus 21.2 or 9.1% per year.

Compared with controls, cases were older, were treated more frequently with statins, and had significantly lower HDL cholesterol levels. Baseline blood samples of cases were collected somewhat later after transplantation (Table 1). Cases and controls did not differ in sex (male-to-female ratios); smoking habits; pretransplant history of ischemic or nonischemic heart disease; length of follow-up; frequency of hypertension, diabetes, and peripheral vascular disease; occurrence of cytomegalovirus infection and rejection; or treatment with fibrates or calcium channel blockers. Serum levels of total cholesterol, LDL cholesterol, and triglycerides in cases and controls were similar (Table 1).

Baseline plasma levels of oxidized LDL were 1.18 ± 0.70 mg/dL (mean \pm SD) in cases and 0.57 ± 0.20 mg/dL in controls ($P < 0.0001$, Figure and Table 1). Baseline levels of MDA-modified LDL were similar in cases and in controls: 0.38 ± 0.14 and 0.39 ± 0.20 mg/dL, respectively (Table 1). At the end of a 2-year follow-up, plasma levels of oxidized LDL were 2.8-fold higher ($P < 0.0001$) in cases than in controls (Table 1 and Figure). During the follow-up, plasma levels of oxidized LDL increased 5.4-fold more in cases than in controls ($P < 0.0001$, Table 1).

At a cutoff value of 1 mg/dL, exceeding the 95th percentile of distribution in individuals without CAD,⁴ 10 of 21 cases had increased levels of oxidized LDL in baseline samples compared with 4 of 78 controls (odds ratio 17 [95% CI 4.49 to 63], $P < 0.0001$). At 2-year follow-up, 19 of 21 cases and 11 of 78 controls had increased plasma levels of oxidized LDL (odds ratio 22 [95% CI 5.43 to 88], $P < 0.0001$).

Baseline levels of oxidized LDL correlated with original ischemic heart disease ($P = 0.017$) and with LDL cholesterol levels ($P = 0.014$) (Table 2). There was no correlation with age; sex; time after transplantation; occurrence of hypertension, diabetes, and peripheral vascular disease; occurrence of



Scatter and box-and-whisker plots of the distribution of baseline levels of oxidized LDL and of levels of oxidized LDL after a median follow-up of 2 years in controls ($n = 78$) and cases ($n = 21$). Symbols are as follows: controls at baseline (■), controls at 2 years (▲), cases at baseline (●), and cases at 2 years (◆). NS indicates not significant.

cytomegalovirus infection or rejection; treatment with statins, fibrates, or calcium channel blockers; or serum levels of HDL and triglycerides. Smoking was not associated with higher levels of oxidized LDL, possibly because of the rather low incidence of smoking in our study population.

Development of transplant CAD correlated most strongly with plasma levels of oxidized LDL ($P < 0.0001$ in univariate

TABLE 2. Relation of Baseline Levels of Oxidized LDL and Posttransplant CAD With Possible Covariates

Covariate	Oxidized LDL		CAD	
	r^2	P	χ^2	P
Age	0.0057	0.46	4.8	0.029
Sex	0.0095	0.78	0.03	0.86
Pretransplant history	0.055	0.017	2.49	0.11
Time after transplantation	0.0025	0.68	4.60	0.033
Hypertension	0.00019	0.89	0.12	0.73
Diabetes	0.0013	0.72	0.54	0.46
Peripheral vascular disease	0.0020	0.86	0.08	0.78
Cytomegalovirus infection	0.0012	0.73	0.48	0.49
Rejections	0.0015	0.71	0.050	0.94
Statins	0.011	0.31	3.70	0.054
Fibrates	0.0019	0.66	0.38	0.54
Calcium channel blockers	0.014	0.24	1.44	0.23
Total cholesterol	0.010	0.66	0.16	0.69
LDL cholesterol	0.059	0.014	1.83	0.18
HDL cholesterol	-0.014	0.24	-5.8	0.016
Triglycerides	0.011	0.88	1.05	0.30
Baseline oxidized LDL	16	<0.0001

Relation of baseline levels of oxidized LDL with covariates was assessed by linear regression. The relation of transplant CAD with covariates was assessed by logistic regression.

logistic regression), significantly with age ($P=0.029$) and time after transplantation ($P=0.033$), and inversely with plasma levels of HDL cholesterol ($P=0.016$) (Table 2). There was no association between transplant CAD and sex; occurrence of hypertension, diabetes, and peripheral vascular disease; occurrence of cytomegalovirus infection or rejection; treatment with fibrates or calcium channel blockers; or serum levels of LDL cholesterol and triglycerides (Table 2). Treatment with statins was not associated with a lower incidence of transplant CAD independent of their cholesterol-lowering effect (Table 2).

Multivariate logistic regression analysis showed that baseline plasma levels of oxidized LDL predicted the development of cardiac transplant vasculopathy ($\chi^2=16$, $P<0.0001$) independent of pretransplant ischemic cardiomyopathy, time after transplantation, age, and serum levels of LDL and HDL cholesterol. Baseline levels of oxidized LDL ($r^2=0.42$, $P<0.001$) and the development of cardiac transplant vasculopathy ($r^2=0.81$, $P=0.001$) had a significant effect on levels of oxidized LDL at the time of the second angiogram. Multivariate logistic regression analysis, allowing correction for baseline levels of oxidized LDL, revealed that the development of transplant CAD was associated with a significant increase of plasma levels of oxidized LDL ($\chi^2=14$, $P=0.001$).

Discussion

The present study shows that the baseline level of oxidized LDL is an independent predictor of the development of transplant CAD that was associated with a further increase of plasma levels of oxidized LDL. These data suggest that oxidized LDL is a prognostic marker of transplant CAD.

It has been suggested that cardiac transplant vasculopathy results from a "response to injury" of the endothelium.^{9,10} This injury may be induced by cellular immune responses elicited by class II histocompatibility antigens on coronary artery endothelium,¹¹⁻¹⁵ by cytomegalovirus infection,^{16,17} by cyclosporin,¹⁸ and by oxidized LDL¹⁹ that may act synergistically with cyclosporin.²⁰ Our previous retrospective study³ showed an association between the extent of posttransplant coronary stenosis and plasma levels of oxidized LDL independent of the other possible inducers of endothelial injury. The present prospective study shows that oxidized LDL is a risk factor for transplant CAD. The incidence of transplant CAD (9.1% per year) in the present study was comparable with previously reported data,²¹ suggesting that the present study population is representative.

In the present study, cytomegalovirus infection and rejection were not correlated with the development of CAD. This finding was not unexpected, because in the previous retrospective study, no relation was found between the extent of coronary artery stenosis in heart transplant patients and cytomegalovirus infection or the number of rejections.³ There was also no correlation with serum levels of LDL cholesterol, again in agreement with previous retrospective data.³ The lack of correlation may be due to the frequent treatment with statins of patients included in the study population, resulting in similar levels of LDL cholesterol in controls and in cases and also in patients with original ischemic and nonischemic heart disease. An inverse relation was observed between HDL cholesterol levels and risk of transplant CAD that was

independent of a correlation between HDL cholesterol and oxidized LDL levels. However, we cannot exclude the possibility that the protein composition of HDL, such as the ratio of apoA-I to apoA-II or the level of antioxidative enzymes, such as paraoxonase, determines to a higher extent the oxidation of LDL than the cholesterol content of HDL. Previously, an association has been demonstrated between levels of Lp(a) and development of accelerated CAD after heart transplantation.²² This association may be due to an interaction of Lp(a) with known risk factors such as the ratio of total to HDL cholesterol.²³ However, the interaction of Lp(a) with oxidized LDL remains to be investigated.

The presence of coronary atherosclerotic lesions was assessed angiographically, and baseline angiograms did not show coronary abnormalities. However, coronary atherosclerosis might have been detected with more sensitive methods, such as intravascular ultrasound. It is indeed well known that angiography systematically underestimates the extent of coronary intimal thickening in cardiac transplant recipients.⁵⁻⁷ Nevertheless, the observed increase in oxidized LDL in patients with angiographically detected progression of coronary atherosclerosis suggests that oxidation of LDL is associated with coronary atherogenesis. All cases had grade 1 coronary stenosis: minor abnormalities with <50% stenosis of primary or secondary branches and normal left ventricular function. Thus, the relation between the oxidation of LDL and the early steps in the development of transplant CAD was studied independently of the time after transplantation. It remains to be investigated whether there is a relation between oxidized LDL and the further progression of transplant CAD.

In our previous retrospective study in heart transplant patients,³ we determined the titers of autoimmune antibodies against oxidized LDL. The titers were 7.81 ± 0.38 for patients with angiographically normal coronary arteries and 7.88 ± 0.88 for patients with coronary artery stenosis. Titers of autoimmune antibodies against MDA-modified LDL were very similar. Those data demonstrated that differences in levels of oxidized LDL in heart transplant patients were not due to differences in titers of autoimmune antibodies. In the present study, we did not determine plasma concentrations of antioxidant vitamins and provitamins. However, we recently started an intervention study in which the effects of vitamin E, vitamin C, β -carotene, zinc, and selenium on the levels of oxidized LDL in heart transplant patients are investigated.

Recently, the oxidation of LDL in the arterial wall was found to correlate with the progression of coronary atherosclerosis in hypercholesterolemic rabbits and miniature pigs. Plasma levels of oxidized LDL correlated with the amounts of oxidized LDL in the lesions and with the extent of coronary atherosclerosis but not with plasma levels of LDL cholesterol.^{24,25} These data suggest that circulating oxidized LDL is released from the atherosclerotic arterial wall in the blood rather than generated in the blood.

Previously, we have demonstrated that plasma levels of oxidized LDL are very similar in patients with acute coronary syndromes and in patients with stable CAD.⁴ These data suggest that the increase of plasma oxidized LDL is due to a continuous back diffusion of oxidized LDL in the blood rather than a sudden burst in release/production due to plaque instability associated with oxidative stress in endothelial cells and platelet adhesion/aggregation.⁴ However, plasma levels

of MDA-modified LDL were significantly higher in patients with acute coronary syndromes than in patients with stable CAD,⁴ suggesting that MDA-modified LDL, in contrast with oxidized LDL, is not released continuously from atherosclerotic plaques but is generated in unstable plaque. In the present study, plasma levels of MDA-modified LDL were very similar in baseline samples and in samples obtained at the 2-year follow-up from controls and cases, suggesting that the increase of oxidatively modified LDL in cases was not due to acute events.

Heart transplant patients were selected because rapidly progressing coronary atherosclerosis is a leading cause of graft failure in recipients who survive the first year.^{26,27} From the present study, one cannot extrapolate the findings to conclude that oxidized LDL is a risk factor of ischemic cardiomyopathy. However, recent retrospective studies showed increases of oxidized LDL in patients with ischemic cardiomyopathy that were similar to those in patients with cardiac transplant vasculopathy.⁴

In conclusion, the present data provide the first prospective evidence for a relation of the oxidation of LDL with coronary atherosclerosis in humans. Intervention studies are needed to assess the causal role of oxidized LDL in the development of CAD.

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