Oxidized Low Density Lipoproteins in Patients With Transplant-Associated Coronary Artery Disease

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Abstract—The monoclonal antibody 4E6–based ELISA was used to quantify levels of oxidized LDL in plasma of 65 control subjects, 47 patients transplanted for dilated cardiomyopathy (DCM), and 60 patients transplanted for coronary artery disease (CAD). Levels of oxidized LDL were 0.68±0.039 mg/dL (mean±SEM), 1.27±0.14 mg/dL ($P<.001$ versus control), and 1.73±0.13 mg/dL ($P<.001$ versus control and <0.01 versus DCM), respectively. Levels of oxidized LDL were significantly lower in transplanted patients with angiographically normal coronary arteries (grade 0, 1.16±0.053 mg/dL; n=79) than in patients with mild (grade 1, 2.13±0.30 mg/dL; n=18; $P<.001$ versus grade 0) or severe (grade 2, 3.18±0.45 mg/dL; n=10; $P<.001$ versus grade 0 and $P<.05$ versus grade 1) coronary artery stenosis. Logistic regression analysis identified three parameters that were significantly and independently correlated with posttransplant CAD: plasma levels of oxidized LDL ($P=.0001$), length of follow-up ($P=.0008$), and donor age ($P=.047$). Thus, the present study demonstrates that plasma levels of oxidized LDL correlate with the extent of CAD in heart transplant patients and suggests that elevated levels of oxidized LDL may be a marker for CAD. **(*Arterioscler Thromb Vasc Biol*. 1998;18:100-107.)**

Key Words: oxidized LDL ■ coronary artery disease ■ transplantation

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The development of CAD after cardiac transplantation is a leading cause of graft failure in recipients who survive the first year after surgery.[1,2] This posttransplant arteriopathy is characterized by rapid development,[3,4] concentric narrowing of smaller coronary arteries,[5,6] and the lack of correlation with known atherogenic risk factors.[1–6] Understanding the etiology of accelerated atherosclerosis might allow identification of patients at risk for this complication.

It has been suggested that ischemic injury of the heart during the peritransplant period significantly contributes to the development of accelerated atherosclerosis in heart transplant patients.[7] A correlation between LDL oxidation and atherogenesis was first suggested by experiments showing that oxidized LDL mediated injury to endothelial cells (reviewed in Reference 8) and was further supported by studies showing a protective effect of antioxidants against progression of atherosclerosis.[9] The study of the correlation between oxidized LDL and atherosclerosis has, however, been hampered by the lack of a sensitive and specific assay for oxidized LDL in plasma. In the present study we have used an ELISA that is highly specific for oxidized LDL.[10] The ELISA was used to study the association between plasma levels of oxidized LDL and CAD.

Methods

Patients

The posttransplant study group contained 47 patients transplanted for DCM and 60 patients treated for CAD. The clinical characteristics of these patients are summarized in Table 1. At the time of blood sampling, between 12 and 84 months after surgery, all patients were in a stable cardiac condition without evidence of acute rejection. Adequate information about the smoking habits was available for 92 of the 107 patients (16 smokers and 76 nonsmokers). There was no adequate information about smoking habits of donors. Rejection was graded by the histopathologic classification of the International Society for Heart and Lung Transplantation. Rejections ≥ grade 3A were treated. Maintenance immunosuppression consisted of triple-drug therapy including cyclosporin, azathioprine, and prednisone. Rejection episodes were treated with high-dose intravenous corticosteroids and steroid-resistant rejection with a course of OKT3, a murine anti-CD3 mAb.[11] Cytomegalovirus infection was defined as seroconversion of a seronegative recipient or a fourfold rise in titer postoperatively in a previously cytomegalovirus-positive recipient.

Blood samples of 65 nonsmoking control subjects (31 men/34 women; age, 52±1.3 years) without a history of atherosclerotic cardiovascular disease were obtained. The control subjects were matched for age and levels of LDL cholesterol. They were selected from the laboratory and clinical staff (n=27; age, 54±2.0 years; 15 men/12 women) and from a hospital control population without a history of atherosclerotic cardiovascular disease (n=38; age, 52±2.2 years; 16 men/22 women).

Coronary Angiography

Routine annual coronary angiograms were available for all posttransplant patients at the time of blood sampling. CAD was assessed by two angiographers (J.V.C. and J.V.H.) who where unaware of the oxidized LDL levels and was 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, ≥50% stenosis of primary or secondary branches, or distal involvement with impaired left ventricular function. It is well known that angiography systematically underestimates the extent of coronary intimal thickening in cardiac transplant recipients.[12] This study therefore does not attempt to accurately quantify the extent of coronary

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Selected Abbreviations and Acronyms

apo = apolipoprotein
CAD = coronary artery disease
DCM = dilated cardiomyopathy
HLA = major histocompatibility complex class I
HLA-DR = major histocompatibility complex class II
mAb = monoclonal antibody
MDA = malondialdehyde

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 histopathologic findings and prognosis.[13,14] The study was approved by the Institutional Review Board, and the study subjects provided informed consent.

Blood Sampling

Venous blood samples from fasting patients and control subjects were collected on 0.1 vol of 1 mol/L citrate, containing 1 mmol/L EDTA, 20 μmol/L vitamin E, 10 μmol/L BHT, 20 μmol/L dipyridamole, and 15 mmol/L theophylline to prevent in vitro LDL oxidation and platelet activation. Blood samples were centrifuged at 3000*g* for 15 minutes at room temperature within 1 hour of collection and stored at −20°C until the assays were performed.[10]

Lipoproteins: Isolation and Modification

LDLs were isolated from pooled plasma of fasting normolipidemic donors by density gradient ultracentrifugation.[15] Standard preparations of MDA-modified and copper-oxidized LDL were prepared as described elsewhere[16,17] and were used as assay control subjects.[10] Characterization of modified LDL involved measurement of thiobarbituric acid–reactive substances, determination of electrophoretic mobility on 1% agarose gels, quantification of cholesterol and fatty acids by high-performance liquid chromatography on a Nova-Pak C18 reversed-phase column (Waters Associates), and quantification of proteins by Lowry assay and of phospholipids by enzymatic assay (Biomérieux).[18] Oxidized LDLs were isolated from the purified LDL fractions of patients by fast flow chromatography on a mono-Q-Sepharose column.[18] The apo B-100 molecules of in vitro MDA-modified LDL and of copper-oxidized LDL contained on average 244 and 210 substituted lysines (from a total of 356), respectively.[17–19] Whereas the extent of lysine substitution in in vitro MDA-modified LDL and copper-oxidized LDL is very similar, the lipid moiety in in vitro MDA-modified LDL is not oxidized. We have demonstrated that the specificity of the mAb E6 depends on the extent of protein modification only.[10] All lipoprotein concentrations were therefore expressed in terms of protein.

Assays

An mAb 4E6–based ELISA was used for the quantification of oxidized LDL in plasma.[10] 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 (copper-oxidized LDL added as a competing ligand at a final concentration ranging between 50 and 500 ng/mL) were mixed and incubated for 30 minutes at room temperature. Then 200-μL aliquots of the mixtures were added to coated wells. Samples were incubated

TABLE 1. Clinical Data of Heart Transplant Patients

	Heart Transplant Patients		
	DCM	Ischemic Heart Disease	
Characteristic	(n=47)	(n=60)	*P*
Age of recipient, y	54±1.6	55±0.95	NS*
Sex of recipient, M/F	41/6	53/7	NS
Age of donor, y	29±1.5	29±1.4	NS†
Sex of donor, M/F	31/16	44/16	NS*
Length of follow-up, mo	39±3.1	50±2.7	.008†
Duration of ischemia, min	130±7.0	140±5.3	NS†
No. of HLA mismatches			
DR	1.5±0.09	1.4±0.08	NS†
B+DR	3.1±0.13	3.0±0.13	NS†
No. of rejection episodes	0.38±0.13	0.25±0.06	NS†
Cytomegalovirus infection	26	43	NS*
Hypertension	37	53	NS*
Diabetes	4	3	NS*
CAD			
Grade 0	39	40	NS*
Grade 1	5	13	NS*
Grade 2	3	7	NS*
Lipid-lowering drugs	17	39	.004*
Statins	13	33	.006*
Fibrates	4	6	NS*
Calcium channel blockers	31	47	NS*

Data represent mean±SEM or number of patients. NS, not significant.
*Value of *P* determined by χ^2 test.
†Value of *P* determined by Dunnett's multiple comparison test.

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TABLE 2. Laboratory Data of Control Subjects and Heart Transplant Patients

Characteristics	Control Subjects (n=65)	Heart Transplant Patients: DCM (n=47)	*P* vs Control	Heart Transplant Patients: CAD (n=60)	*P* vs Control	*P* vs DCM
Serum triglyderides	130±7.5	130±8.3	NS	140±7.0	NS	NS
HDL cholesterol	48±2.7	54±2.5	NS	49±1.9	NS	NS
LDL cholesterol	105±5.4	100±4.4	NS	110±3.3	NS	NS
Oxidized LDL	0.68±0.039	1.27±0.14	<.001	1.73±0.13	<.001	<.01

Data represent mean±SEM and are expressed in milligrams per deciliter. Values of *P* are determined by Dunnett's multiple comparison test. NS, not significant. To convert values for serum triglycerides to millimoles per liter, multiply by 0.011. To convert values for serum cholesterol to millimoles per liter, multiply by 0.026.

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. The peroxidase reaction was performed as described earlier,[12] and the absorbance (A) was read at 492 nm. Control subjects without competing ligand and blanks without antibody were included routinely. The percentage inhibition of binding of mAb 4E6 to the immobilized ligand was calculated as (A_{492nm} control−A_{492nm} sample)/(A_{492nm} control−A_{492nm} blank), and standard curves were obtained by plotting the percentage inhibition versus the concentration of competing ligand.

The C_{50} values, ie, concentrations that are required to obtain 50% of antibody binding, were 25 mg/dL for native and acetylated LDL; 1, 0.1, and 0.025 mg/dL for MDA-modified LDL with 60, 90, and at least 120 aldehyde-substituted lysines per apo B-100 molecule, respectively; and 0.025 mg/dL for copper-oxidized LDL. A 50-fold higher molar concentration of aldehyde-substituted lysines in MDA-modified albumin was required to obtain a similar extent of inhibition of antibody binding compared with MDA-modified LDL, whereas up to 1000-fold higher molar concentrations of MDA-modified lysine did not affect antibody binding. The lower limit of detection was 0.020 mg/dL of standard preparations of MDA-modified and in vitro oxidized LDL in undiluted human plasma. When copper-oxidized LDLs were added to human plasma at a final concentration of 0.25 and 2 mg/dL, respectively, recoveries were 95 and 105%, respectively.

Titers of autoimmune antibodies against oxidized LDL were measured as described earlier.[10,18] Immobilized antigens for this assay included native LDL prepared from pooled human plasma (protected against in vitro oxidation as described above), copper-oxidized and MDA-modified LDL, and human serum albumin. The plasma samples were diluted 40-fold and incubated for 2 hours at room temperature in the coated microtiter plates. The wells were then washed and incubated for 1 hour with horseradish peroxidase–conjugated goat IgG raised against human immunoglobulins (both IgG and IgM) and washed again. The peroxidase reaction was then performed as described above. The antibody titers for native LDL were expressed as the ratios of the absorbance in wells coated with native LDL versus wells coated with serum albumin. The antibody titers for copper-oxidized LDL and for MDA-modified LDL were expressed as the ratios of the absorbance in wells coated with copper-oxidized LDL and MDA-modified LDL, respectively, versus wells coated with native LDL.

Cholesterol and triglycerides were measured by enzymatic methods (Boehringer Mannheim). Typing of HLA-B and HLA-DR antigens was performed by the microlymphocytotoxicity technique.

Statistical Analysis

Control subjects and patients were compared by ANOVA followed by the nonparametric Mann-Whitney *U* test or Dunnett's multiple comparison test on logarithmically transformed values, with the Instat V2.05a statistical program (GraphPad Software). Nonquantitative parameters were compared by χ^2 analysis. Oxidized LDL levels measured in three plasma samples obtained from the same patient on 3 consecutive days as well as levels measured in three aliquots of the same plasma sample on 3 different days were compared in the Friedman nonparametric repeated measures test. Logistic regression analysis, using SAS software (SAS Institute, Inc.), was performed to evaluate the correlation between angiographically assessed coronary artery stenosis (as the dependent variable) and plasma levels of oxidized LDL; age and sex of recipients; age and sex of donors; pretransplant history of ischemic heart disease or DCM; duration of ischemia; length of follow-up; number of rejections; number of HLA mismatches; frequency of cytomegalovirus infection, hypertension (antihypertensive treatment), or diabetes; treatment with lipid-lowering drugs (statins or fibrates); and serum levels of LDL cholesterol, HDL cholesterol and triglycerides as independent variables. Values of $P<.05$ were considered to indicate statistical significance.

Results

The association between oxidized LDL and coronary artery stenosis was evaluated in 47 patients transplanted for DCM and in 60 patients treated for CAD. Analysis of clinical data for the two groups of heart transplant patients (Table 1) revealed no significant differences in age and sex of the recipients, age and sex of donors, duration of ischemia of the donor heart, number of rejection episodes, number of HLA mismatches, frequency of cytomegalovirus infections, hypertension, or diabetes, and grade of coronary artery stenosis. Patients transplanted for CAD were followed up longer and more frequently received lipid-lowering drugs (Table 1). Analysis of the laboratory data (Table 2) revealed no significant differences in serum levels of LDL cholesterol, HDL cholesterol, and triglycerides between groups of patients or between patients and control subjects. However, significant differences in levels of oxidized LDL were observed. Mean plasma levels of oxidized LDL were 0.68±0.039 mg/dL in control subjects, 1.27±0.14 mg/dL in DCM patients ($P<.001$ versus control subjects), and 1.73±0.13 mg/dL in CAD patients ($P<.001$ versus control subjects and <0.01 versus DCM patients) (Table 2).

Blood samples were stored for <1 week before oxidized LDL analysis. Levels of oxidized LDL were not different in samples that were stored for 24 hours to 10 months after collection, and up to four thawing and freezing cycles did not cause an increase of oxidized LDL levels. These findings indicated that the addition of EDTA, antioxidants, and antiplatelet agents adequately prevented the in vitro oxidation of LDL. In a subset of 87 consecutive plasma samples, levels of oxidized LDL were measured in three separate aliquots on 3 different days. The levels were 1.38±0.074, 1.48±0.101, and

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TABLE 3. Laboratory Data of Heart Transplant Patients Without and With Coronary Artery Stenosis

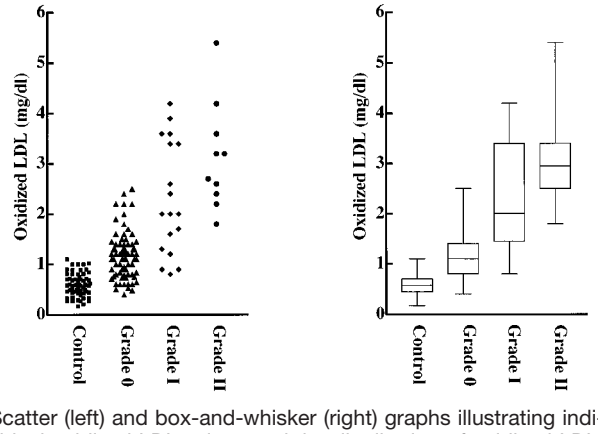
	Stenosis					
Characteristic	Grade 0 (n=79)	Grade 1 (n=18)	*P* vs Grade 0	Grade 2 (n=10)	*P* vs Grade 0	*P* vs Grade 1
Serum triglycerides	130±4.4	130±11	NS	100±4.8	NS	NS
HDL cholesterol	49±2.3	54±5.4	NS	50±2.5	NS	NS
LDL cholesterol	120±4.1	110±9.6	NS	110±5.3	NS	NS
Oxidized LDL	1.16±0.053	2.13±0.30	<.001	3.18±0.45	<.001	<.05

Data represent mean±SEM and are expressed in milligrams per deciliter. NS, not significant.
*Values of *P* determined by χ^2 test.
†Values of *P* determined by Dunnett's multiple comparison test.

1.46±0.090 mg/dL, respectively. Friedman nonparametric repeated measures test revealed no significant differences between levels measured in different aliquots of the same sample. The interassay coefficient of variation was 9.6%.

The day-to-day variability of the oxidized LDL levels has been investigated in an independent study group of 33 nontransplanted ischemic heart disease patients with recurrent acute myocardial infarction. Levels of oxidized LDL in plasma samples obtained on 3 consecutive days were 2.84±0.30, 3.04±0.28, and 3.2±0.30 mg/dL, respectively. Friedman nonparametric repeated measures test revealed no significant differences between levels measured in subsequent plasma samples.

Mean oxidized LDL levels were 1.16±0.053 mg/dL (n=79) in posttransplant samples of patients with angiographically normal coronary arteries (grade 0), 2.13±0.30 mg/dL in patients with grade 1 coronary artery stenosis (n=18; *P*<.001 versus grade 0), and 3.18±0.45 mg/dL in patients with grade 2 coronary artery stenosis (n=10; *P*<.001 versus grade 0 and *P*<.05 versus grade 1) (Table 3). The Figure represents scatter and box and whisker graphs illustrating individual oxidized LDL values and the distribution of oxidized LDL levels in control subjects and patients with grade 0, grade 1, and grade 2 posttransplant coronary artery stenosis. Serum levels of LDL cholesterol, triglycerides, and HDL cholesterol were very similar in patients with higher grade of coronary artery stenosis (Table 3), and oxidized LDL levels did not correlate with LDL cholesterol levels (data not shown). Levels of oxidized LDL in plasma samples of patients transplanted for DCM or CAD, with the same grade of coronary artery stenosis, were similar: 1.00±0.066 and 1.32±0.073 mg/dL for grade 0 patients and 2.63±0.60 and 2.53±0.29 mg/dL for patients with higher grade of coronary artery stenosis. The number of patients with elevated levels of oxidized LDL (ie, >mean levels of control subjects+2 SD) were 43 (of 60) in the subpopulation of transplanted CAD patients and 21 (of 47) in the subpopulation of transplanted DCM patients (*P*=.0057). Of 79 patients with angiographically normal coronary arteries, 42 had elevated levels of oxidized LDL. Elevated levels were detected in 12 (of 18) patients with grade 1 and in all patients with grade 2 stenosis (*P*=.0046 for trend).



Scatter (left) and box-and-whisker (right) graphs illustrating individual oxidized LDL values and the distribution of oxidized LDL levels in control subjects and patients with grade 0, grade 1, and grade 2 posttransplant coronary artery stenosis. Whiskers extend from minimum to maximum values.

Titers of autoimmune antibodies reacting with native LDL were ≈1. Titers of autoimmune antibodies reacting with oxidized LDL were 8.30±0.23 in control subjects, 7.81±0.38 in patients with angiographically normal coronary arteries, and 7.88±0.88 in patients with grade 1 and grade 2 stenosis. Titers of autoimmune antibodies reacting with MDA-modified LDL were very similar. Those data suggested that the higher levels of oxidized LDL in the transplantation patients did not result from lower antibody titers.

Logistic regression analysis (Table 4) identified 3 parameters that were significantly and independently correlated with posttransplant coronary artery stenosis: levels of oxidized LDL, length of follow-up, and donor age. In contrast, pretransplant history of DCM or of ischemic heart disease; age and sex of recipients; sex of donors; duration of ischemia of the donor heart; extent of HLA mismatch; frequency of rejection, hypertension, or diabetes; and serum levels of LDL cholesterol, HDL cholesterol, and triglycerides in recipients did not significantly contribute to the individual variations in extent of coronary artery stenosis (Table 4). Serum levels of LDL cholesterol, HDL cholesterol, and triglycerides in patients were similar to those in control subjects (Table 2), so that higher grade of coronary artery stenosis was unlikely to depend on those variables in this study group. Of 107 transplant patients, 56 received lipid lowering drugs (46 with statins and 10 with fibrates) (Table 2), but the treatment with these drugs was not correlated with the incidence of angiographic graft vasculopathy (Table 4). Of 107 patients, 75 were treated with calcium channel blockers. The plasma levels of oxidized LDL in those

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TABLE 4. Logistic Regression Analysis of the Relation Between Clinical-Laboratory Data and Extent of Coronary Artery Stenosis in Heart Transplant Patients

Independent Variable	χ^2 Value	P
Oxidized LDL	18	.0001
Length of follow-up	11	.0008
Age of donor	3.9	.047
Age of recipient	0.12	.73
Sex of recipient	1.8	.18
Sex of donor	0.025	.88
History of pretransplant DCM (n=47) or ischemic heart disease (n=60)	0.0018	.97
Duration of ischemia	0.25	.62
No. of HLA mismatches	1.6	.20
No. of rejection episodes	3.0	.081
Cytomegalovirus infection	0.17	.47
Hypertension	1.9	.16
Diabetes	0.0016	.97
Treatment with lipid-lowering drugs		
Statins	1.1	.30
Fibrates	0.12	.73
Treatment with calcium channel blockers	0.16	.49
Serum triglycerides	0.18	.67
Serum HDL cholesterol	0.25	.61
Serum LDL cholesterol	0.044	.83

The data set contained 107 patients. Original cardiac disease was DCM in 47 and ischemic heart disease in 60 patients. Coronary artery stenosis was assessed angiographically. All quantitative parameters were transformed logarithmically to obtain a normal distribution for linear regression. Values of χ^2 were obtained after adjustment for all other variables.

patients (1.53±0.11 mg/dL) were very similar to those in nontreated patients (1.74±0.14 mg/dL), and treatment with these drugs was not correlated with the extent of coronary artery stenosis.

LDL fractions were isolated from the plasma of 10 patients with the highest extent of coronary artery stenosis by gel filtration on a Superose 6HR 10/30 column, as described previously.[19] Of the immunoreactive material, 80±4% (mean±SD) was recovered in the LDL fractions. No immunoreactive material migrated in the serum albumin position. The inhibition curves obtained with the respective LDL fractions were parallel to those obtained with in vitro copper-oxidized or MDA-modified standard LDL preparations. Oxidized LDL was isolated from isolated LDL fractions of those patients by ion-exchange chromatography on a mono-Q-Sepharose column[19] with a recovery of 75%. The oxidized LDL fraction isolated from the plasma of those patients was characterized by a 1.3-fold higher electrophoretic mobility on agarose gels, a 75% reduction of the arachidonic levels, and a 80% reduction of the linoleic acid levels. The fraction of aldehyde-substitution of lysine residues was 37% of that in standard preparations of MDA-modified LDL, indicating that about 90 lysine residues in the apo B-100 moiety of the isolated oxidized LDL were substituted. The inhibition curves obtained with oxidized LDL isolated from the plasma of the patients were parallel to these obtained with oxidized LDL that was obtained by in vitro oxidation of LDL that had been isolated from the plasma of control subjects.

A subset of 46 heart transplant patients had a normal coronary angiogram 3 years before the present study. In 12 of those patients, CAD had developed during the 3-year follow-up period. There were no differences in age and sex of recipients; age and sex of donors; duration of ischemia; extent of HLA mismatch; frequency of cytomegalovirus infections, hypertension, and diabetes (Table 5); nor serum levels of triglycerides, HDL cholesterol, and LDL cholesterol (Table 5) between patients who had developed CAD and those who had not. However, levels of oxidized LDL were significantly elevated in patients with development of CAD (Table 5). Logistic regression analysis revealed that plasma levels of oxidized LDL (χ^2 value=7.1; P=.0076) and age of donor (χ^2 value=4.4; P=.035) correlated with the development of CAD in those patients.

Discussion

The present study demonstrates that plasma levels of oxidized LDL are significantly elevated in ischemic heart disease patients and in heart transplant patients, both in patients transplanted for DCM and those treated for ischemic heart disease, and that plasma levels of oxidized LDL are significantly higher in patients who develop posttransplant coronary artery stenosis. The finding that plasma levels of oxidized LDL correlated with the extent of coronary artery stenosis and also with its development suggests that oxidized LDL may be a marker for patients at increased risk of posttransplant CAD.

It has been suggested that posttransplant atherosclerosis results from a "response to injury" of the endothelium.[20] The extent of ischemic injury in endomyocardial biopsies was indeed found to be a strong predictor of the development of accelerated atherosclerosis.[7] Endothelial injury may be induced by cellular delayed-type hypersensitivity immune responses elicited by class II histocompatibility (HLA) antigens on coronary artery endothelium,[21–25] by cytomegalovirus infection,[26,27] by cyclosporin,[28] and by oxidized LDL[29] that may act synergistically with cyclosporin.[30] In the present study, the extent of histoincompatibility between pairs of donors and recipients, and the number of episodes of rejection or cytomegalovirus infection did not correlate with the grade of coronary artery stenosis, whereas oxidized LDL levels were significantly and independently correlated with posttransplant CAD. The observed association between the age of the donor and the occurrence of CAD is in agreement with previous findings that coronary atherosclerosis in the donor heart predisposes to accelerated posttransplant coronary artery stenosis.[31]

In vitro data suggest that oxidized LDL may be linked to atherogenesis by a sequence of events (reviewed in References 32 to 34). Endothelial cells exposed to oxidized LDL secrete adhesion molecules, chemoattractant proteins, and colony-stimulating factors that enhance the infiltration, proliferation, and accumulation of monocytes/macrophages in the arterial wall. Uptake of oxidized LDL by infiltrated macrophages may result in the generation of foam cells that produce oxygen radicals and thus further contribute to the oxidation of LDL. It

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TABLE 5. Clinical and Laboratory Data of Heart Transplant Patients Without and With Progression of Coronary Artery Stenosis During a 3-Year Follow-up Period

Characteristic	Heart Transplant Patients		
	Without Progression (n=34)	With Progression (n=12)	*P*
Age of recipient, y	58±1.4	60±1.4	NS*
Sex of recipient, M/F	21/13	11/1	NS†
Age of donor, y	25±1.3	32±3.8	NS*
Sex of donor, M/F	27/7	10/2	NS†
Duration of ischemia, min	130±6.7	140±11	NS*
No. of HLA mismatches			
DR	1.2±0.13	1.5±0.15	NS*
B+DR	2.8±0.21	3.2±0.24	NS*
Cytomegalovirus infection	24	11	NS†
Hypertension	21	10	NS†
Diabetes	1	2	NS†
Serum triglycerides	130±8.6	150±14	NS
HDL cholesterol	50±2.7	49±4.9	NS
LDL cholesterol	110±3.6	105±8.7	NS
Oxidized LDL	1.20±0.069	2.61±0.33	.0005

Data represent mean±SEM or number of patients. NS, not significant.
*Values of *P* determined by Dunnett's multiple comparison test.
†Values of *P* determined by χ^2 analysis.

has been demonstrated that oxidized LDL inhibits the migration of aortic endothelial cells in vitro, suggesting that oxidized LDL may limit the healing response of the endothelium after injury, and that basic fibroblast growth factor reverses the atherosclerosis associated impairment of human coronary angiogenesis-like responses in vitro.[35,36] Oxidized LDL may also contribute to rapidly progressing coronary atherosclerosis by inducing platelet adhesion, by decreasing the anticoagulant and fibrinolytic capacities of activated endothelium, and by impairing vasodilation and inducing shear stress.[32–34]

Increased intracellular levels of ferritin[37] or of α-tocopherol analogues[38] decreased the extent of endothelial injury elicited by oxidized LDL in vitro, whereas antioxidants protect against progression of atherosclerosis in experimental animals (reviewed in Reference 9). These observations suggest that inhibition of oxidation of LDL or of the effects of oxidized LDL on arterial cells may reduce the risk for rapidly progressing atherosclerosis in heart transplant patients, but such effects would need to be confirmed in prospective clinical trials. It has been suggested that in the general population, trials with antioxidants may require very long observation periods (>5 years) for a beneficial effect to become apparent.[9] The heart transplant population may provide us more rapidly with an answer as to the value of antioxidant therapy, inasmuch as posttransplant coronary artery stenosis could serve as a paradigm for an accelerated form of CAD in general. In a preliminary study we found that levels of oxidized LDL were very similar in patients with ischemic heart disease, as evidenced by recurrent acute myocardial infarction, and in patients with posttransplant CAD, suggesting that oxidized LDL may indeed be a marker for CAD.

Previously it has been demonstrated that after injection of small amounts of radiolabeled, extensively oxidized LDL in rats it was cleared rapidly by means of scavenger receptors on Kupffer cells.[39] On the basis of those data, it was hypothesized that oxidized LDL would not circulate in human blood. However, it has recently been demonstrated that the half-life of Tc^{99m}-labeled, extensively oxidized LDL in humans was only one third shorter than that of native LDL.[40] Those data are in agreement with the hypothesis that small amounts of oxidized LDL could circulate in human blood. The moderate extent of lysine substitution, on average 90 lysine residues per apo B-100 molecule, in oxidized LDL in the plasma of transplant patients may result in a delayed clearance compared with that of extensively oxidized LDL. The high affinity of mAb 4E6 for apo B-100 with at least 60 aldehyde-substituted lysines per molecule leads to a very high sensitivity of the ELISA, allowing the detection of amounts of oxidized apo B-100 that represent only 0.03% of the total amounts of apo B-100 (typically 80 mg/dL) and thus of the detection of oxidized LDL in human plasma. The finding that plasma levels of oxidized LDL were also elevated in nontransplanted ischemic heart disease patients suggests that elevated levels of oxidized LDL in transplant patients are not solely due to impaired clearance of oxidized LDL in association with transplantation.

It is difficult to identify the source of the oxidized LDL in these patients. Recently we have demonstrated that the progression of coronary atherosclerosis in hypercholesterolemic rabbits is associated with increased accumulation of oxidized LDL both in the coronary lesions and in the plasma. It was

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concluded that the significant correlation between plasma levels of LDL and the amounts of oxidized LDL in the plasma and the lesions of those rabbits may indicate (1) that the oxidized LDLs in the lesions are directly derived from the plasma oxidized LDL that infiltrate in the arterial wall, or (2) that both the lesion amounts and the plasma levels of oxidized LDL reflect similar oxidative stresses in both the arterial wall and plasma, or (3) that there is backdiffusion of oxidized LDL generated in the arterial wall in the blood.[41] Previously we have demonstrated that acute myocardial infarction is associated with an increase of plasma levels of MDA-modified LDL, suggesting that leakage from arterial lesions may be a source of oxidatively modified LDL in the plasma.[18] The finding that the oxidized LDL levels in the patients did not correlate with the plasma LDL cholesterol levels further suggests that the oxidation of the LDL does not occur in the blood but rather in the arterial wall. The present data are thus in agreement with a continuous release of moderately oxidized LDL in the blood that is not cleared very rapidly from the circulation.

Thus, results of the present study demonstrate that plasma levels of oxidized LDL correlate with the extent of coronary artery stenosis in heart transplant patients and suggest that elevated levels of oxidized LDL may be a marker for CAD. Prospective studies are required to establish a causal role of oxidized LDL in the development of CAD.

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References

1. Billingham ME. Cardiac transplant atherosclerosis. *Transplant Proc.* 1987; 19(suppl 5):19–25.
2. Gao SZ, Alderman EL, Schroeder JS, Silverman JF, Hunt SA. Accelerated coronary vascular disease in the heart transplant patient: coronary arteriographic findings. *J Am Coll Cardiol.* 1988;12:334–340.
3. Schroeder JS, Hunt SA. Chest pain in heart transplant recipients. *N Engl J Med.* 1991;324:1805–1807.
4. Bajaj S, Shah A, Crandall C, Ibrahim H, Vetrovec G, Disciascio G, Guerraty A, Salter DR, Mohanty PK. Coronary collateral circulation in the transplanted heart. *Circulation.* 1993;88(suppl II):II-263-II-269.
5. Johnson DE, Gao SZ, Schroeder JS, De Campli WM, Billingham ME. The spectrum of coronary artery pathologic findings in human cardiac allografts. *J Heart Transplant.* 1989;8:349–359.
6. Ip JH, Fuster V, Badimon L, Badimon J, Taubman MB, Chesebro JH. Syndromes of accelerated atherosclerosis: role of vascular injury and smooth muscle cell proliferation. *J Am Coll Cardiol.* 1990;15:1667–1687.
7. Gaudin PB, Rayburn BK, Hutchins GM, Kasper EK, Baughman KL, Goodman SN, Lecks LE, Baumgartner WA, Hruban RH. Peritransplant injury to the myocardium associated with the development of accelerated arteriosclerosis in heart transplant recipients. *Am J Surg Pathol.* 1994;18: 338–346.
8. Penn MS, Chisolm GM. Oxidised lipoproteins, altered cell function and atherosclerosis. *Atherosclerosis.* 1994;108:S21–S29.
9. Steinberg D. Clinical trials of antioxidants in atherosclerosis: are we doing the right thing? *Lancet.* 1995;346:36–38.
10. Holvoet P, Donck J, Landeloos M, Brouwers E, Luijtens K, Arnout J, Lesaffre E, Vanrenterghem Y, Collen D. Correlation between oxidized low density lipoproteins and von Willebrand factor in chronic renal failure. *Thromb Haemost.* 1996;76:663–669.
11. Kung PC, Goldstein G, Reinherz EL, Schlossman SF. Monoclonal antibodies defining distinctive human T cell surface antigens. *Science.* 1979; 206:347–349.
12. St Goar FG, Pinto FJ, Alderman EL, Valantine HA, Schroeder JS, Gao SZ, Stinson EB, Popp RL. Intracardiac ultrasound in cardiac transplant recipients: in vivo evidence of "angiographically silent" intimal thickening. *Circulation.* 1992;85:979–987.
13. Johnson DA, Alderman EL, Schroeder JS, Gao SZ, Hunt S, DeCampli WM, Stinson E, Billingham M. Transplant coronary artery disease: histopathologic correlations with angiographic morphology. *J Am Coll Cardiol.* 1991;17:449–457.
14. Constanzo MR, Naftel DC, Pritzker MR, Hellman JK, Boehmer JP, Brozena SC, Dee WD, Ventura HO, Kirklin JK, Bourge RC, Miller LW for the Cardiac Transplant Research Data Base (CTRD). Heart transplant coronary artery disease detected by angiography: a multi-institutional study. *J Heart Lung Transplant.* 1996;15:S39. Abstract.
15. Havel RJ, Eder HA, Bragdon JH. The distribution and chemical composition of ultracentrifugally separated lipoproteins in human sera. *J Clin Invest.* 1995;34:1345–1353.
16. Haberland MD, Fogelman AM, Edwards PA. Specificity of receptor-mediated recognition of malondialdehyde-modified low density lipoproteins. *Proc Natl Acad Sci U S A.* 1982;79:1712–1716.
17. Steinbrecher UP. Oxidation of low density lipoprotein results in derivatization of lysine residues of apolipoprotein B by lipid peroxide decomposition products. *J Biol Chem.* 1987;262:3603–3608.
18. Holvoet P, Perez G, Zhao Z, Brouwers E, Bernar H, Collen D. Malondialdehyde-modified low density lipoproteins in patients with atherosclerotic disease. *J Clin Invest.* 1995;95:2611–2619.
19. Sparrow CP, Partharasathy S, Leake DS, Witztum JL, Steinberg D. Enzymatic modification of low density lipoprotein by purified lipoxygenase plus phospholipase-A_2 mimic cell-mediated oxidative modification. *J Lipid Res.* 1988;29:745–753.
20. Fish RD, Nabel EG, Selwyn AP, Ludmer PL, Mudge GH, Kirshenbaum JM, Schoen FJ, Alexander RW, Ganz P. Responses of coronary arteries of cardiac transplant patients to acetylcholine. *J Clin Invest.* 1988;81:21–31.
21. Libby P, Salomon RN, Payne DD, Schoen FJ, Pober JS. Functions of vascular wall cells related to development of transplantation-associated coronary arteriosclerosis. *Transplant Proc.* 1989;21:3677–3684.
22. Hruban RH, Beschorner WE, Baumgartner WA, Augustine SM, Ren H, Reitz BA, Hutchins GM. Accelerated arteriosclerosis in heart transplant recipients is associated with a T-lymphocyte-mediated endothelialitis. *Am J Pathol.* 1990;137:871–882.
23. Rose EA, Smith CR, Petrossian GA, Barr ML, Reemtsma K. Humoral immune responses after cardiac transplantation: correlation with fatal rejection and graft atherosclerosis. *Surgery.* 1989;106:203–208.
24. Tanaka H, Sukhova GK, Swanson SJ, Cybulsky MI, Schoen FJ, Libby P. Endothelial and smooth muscle cells express leukocyte adhesion molecules heterogeneously during acute rejection of rabbit cardiac allografts. *Am J Pathol.* 1994;144:938–951.
25. Crisp SJ, Dunn MJ, Rose ML, Barbir M, Yacoub MH. Antiendothelial antibodies after heart transplantation: the accelerating factor in transplant-associated coronary artery disease? *J Heart Lung Transplant.* 1994;13:81–92.
26. Grattan MT, Moreno-Cabral CE, Starnes VA, Oyer PE, Stinson EB, Shumway NE. Cytomegalovirus infection is associated with cardiac allograft rejection and atherosclerosis. *JAMA.* 1989;261:3561–3566.
27. Koskinen P, Lemstrom K, Bruggeman C, Lautenschlager I, Hayry P. Acute cytomegalovirus infection induces a subendothelial inflammation (endothelialitis) in the allograft vascular wall: a possible linkage with enhanced allograft arteriosclerosis. *Am J Pathol.* 1994;144:41–50.
28. Cartier R, Dagenais F, Hollmann C, Cambron H, Buluran J. Chronic exposure to cyclosporin affects endothelial and smooth muscle reactivity in the rat aorta. *Ann Thorac Surg.* 1994;58:789–794.
29. Chin JH, Azhar S, Hoffman BB. Inactivation of endothelial derived relaxing factor by oxidized lipoproteins. *J Clin Invest.* 1992;89:10–18.
30. Galle J, Schollmeyer P, Wanner C. Cyclosporin and oxidised low density lipoproteins synergistically potentiate vasoconstriction: influence of the endothelium. *Eur Heart J.* 1993;14(suppl):111–117.
31. Tuzcu EM, Hobbs RE, Rincon G, Bott-Silverman C, De Franco AC, Robinson K, McCarthy PM, Stewart RW, Guyer S, Nissen SE. Occult and frequent transmission of atherosclerotic coronary disease with cardiac transplantation. Insights from intravascular ultrasound. *Circulation.* 1995;91: 1706–1713.

32. Steinberg D, Witztum JL. Lipoproteins and atherogenesis: current concepts. *JAMA*. 1990;264:3047–3052.
33. Ross R. The pathogenesis of atherosclerosis: a perspective for the 1990s. *Nature*. 1993;362:801–809.
34. Holvoet P, Collen D. Oxidized lipoproteins in atherosclerosis and thrombosis. *FASEB J*. 1994;8:1279–1284.
35. Murugesan G, Chisolm GM, Fox PL. Oxidized low density lipoprotein inhibits the migration of aortic endothelial cells in vitro. *J Cell Biol*. 1993;120:1011–1019.
36. Chen CH, Nguyen HH, Weilbaecher D, Luo S, Gotto AM Jr, Henry PD. Basic growth factor reverses atherosclerotic impairment of human coronary angiogenesis-like responses in vitro. *Atherosclerosis*. 1995;116:261–268.
37. Juckett MB, Balla J, Balla G, Jessurun J, Jacob HS, Vercellotti GM. Ferritin protects endothelial cells from oxidised low density lipoprotein in vitro. *Am J Pathol*. 1995;147:782–789.
38. Mabile L, Fitoussi G, Periquet B, Schmitt A, Salvayre R, Negre-Salvayre A. Alpha-Tocopherol and trolox block the early intracellular events (TBARS and calcium rises) elicited by oxidised low density lipoproteins in cultured endothelial cells. *Free Radic Biol Med*. 1995;19:177–187.
39. Van Berkel TJ, De Rijke YB, Kruijt JK. Different fate of oxidatively modified low density lipoprotein and acetylated low density lipoprotein in rats: recognition by various scavenger receptors on Kupffer and endothelial liver cells. *J Biol Chem*. 1991;266:2282–2289.
40. Iuliano L, Signore A, Vallabajosula S, Colavita AR, Camastra C, Ronga G, Alessandri C, Sbarigia E, Fiorani P, Violi F. Preparation and biodistribution of ^{99m}Tc labelled oxidized LDL in man. *Atherosclerosis*. 1996;126:131–141.
41. Holvoet P, Collen D. βVLDL hypercholesterolemia relative to LDL hypercholesterolemia is associated with higher levels of oxidized lipoproteins and a more rapid progression of coronary atherosclerosis in rabbits. *Arterioscler Thromb Vasc Biol*. 1997;17:2376–2382.

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