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Oxidative modification of low-density lipoproteins and the outcome of renal allografts at 1½ years

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Oxidative modification of low-density lipoproteins and the outcome of renal allografts at 1½ years.

Background. Previous studies reported a significant association between hyperlipidemia of the recipient and chronic allograft nephropathy (CAN). However, the nature and the pathogenic mechanism of circulating lipid abnormalities in CAN remain unclear.

Methods. In a prospective study of 50 consecutive adult recipients of a cadaveric renal allograft, we investigated the impact of lipid abnormalities on the outcome of the graft at 1½ years. Besides morphometric analysis of implantation and protocol biopsies, clinical and biochemical variables were studied at three-month intervals. Plasma concentrations of oxidized low-density lipoprotein (OxLDL) were determined by means of enzyme-linked immunosorbent assay. Immunohistochemical staining for OxLDL and macrophages was performed on paired renal biopsies. Study end points were the fractional interstitial volume and the 24-hour creatinine clearance at 1½ years.

Results. High-density lipoprotein (HDL) cholesterol of the recipient ≤47 mg/dL was a risk factor for the functional (RR = 1.56; 95% CI, 0.978 to 2.497) and the morphological (RR = 2.75; 95% CI, 1.075 to 7.037) outcome of the graft, mainly in patients without acute rejection (RR = 2.03; 95% CI, 1.13 to 3.65, and RR = 4.67; 95% CI, 1.172 to 18.582, respectively). Interstitial accumulation of OxLDL was inversely associated with HDL cholesterol ($R = -0.476$, $P = 0.019$), and was associated with a higher density of tubulointerstitial macrophages ($R = 0.656$, $P = 0.001$) and a higher fractional interstitial volume at 1½ years ($P = 0.049$).

Conclusion. Decreased HDL cholesterol levels of the recipient adversely affect the outcome of renal allografts through the accumulation of OxLDL in the renal interstitium of the graft. Interstitial accumulation of OxLDL was associated with the presence of macrophages and the development of interstitial fibrosis.

Key words: oxidized LDL, kidney graft, transplantation, chronic allograft nephropathy, high density lipoprotein, end-stage renal disease.

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Hyperlipidemia occurs in 40 to 80% of renal allograft recipients [1, 2]. Several clinical studies provide evidence for an association between hypertriglyceridemia, low levels of high-density lipoprotein (HDL) cholesterol, and chronic allograft nephropathy (CAN) [3]. Strong circumstantial evidence substantiates that oxidized low-density lipoprotein (OxLDL) is more common in renal transplant recipients than in normal individuals [4–6]. The detrimental effects of OxLDL on glomerular and tubular epithelial cells have been well documented in experimental studies [7–16] and could be prevented by dietary antioxidants [17] and HDL [18].

Nevertheless, the exact role and the pathogenic mechanism of circulating lipid abnormalities and the contribution of OxLDL to the development of CAN are currently unknown [19].

In a prospective clinicopathologic study in 50 consecutive adult recipients of a cadaveric renal allograft, under a cyclosporine-based immunosuppressive regimen, we investigated the nature and the impact of lipid abnormalities on the functional and morphological outcome of renal allografts at 1½-years post-transplantation. In addition, the role of oxidative modification and lipid peroxidation of LDL for the development of CAN was assessed. Oxidative modification of LDL may occur via mechanisms that are either dependent or independent of lipid peroxidation: Malondialdehyde-modified LDL is generated in the absence of lipid peroxidation, while oxidized LDL is generated by peroxidation of lipids in LDL [20]. Plasma concentrations of malondialdehyde-modified and OxLDL were determined by means of an enzyme-linked immunosorbent assay (ELISA), while immunohistochemical staining for native, malondialdehyde-modified, and OxLDL was performed on implantation and protocol biopsies of the graft at 1½ years.

METHODS

Subjects

Fifty consecutive adult (age >18 years) recipients of a cadaveric renal allograft were included in this prospec-

tive study at our institution from January 1996 to December 1997. This study was approved by the local ethical committee. Informed consent was obtained from all patients. This study population, as well as the study design, was previously described [21].

Study design

In all patients, renal allograft biopsies were performed at implantation—before reperfusion—with a 16G Tru-Cut® Needle (Travenol, Baxter Healthcare Corp., Deerfield, IL, USA). Subsequent protocol biopsies at 18 months were similarly performed in 46 of these patients under computed tomography scan guidance. A protocol biopsy could not be performed in four patients (8%): Three patients were on anticoagulant therapy for imperative cardiovascular indications, and a fourth patient returned to dialysis because of chronic rejection. Clinical and biologic data were prospectively registered in an MS-Access 7.0 database at implantation, 6 weeks, and 3, 6, 9, 12, 15, and 18 months post-transplantation. The immunosuppressive regimen was based on cyclosporine and prednisolone in all patients, but in 36 out of 50 (72%) patients, either azathioprine or mycophenolate was added to the therapy at some time after transplantation. Antihypertensive drugs were used either in monotherapy (24% of the patients) or in combination treatment (76% of the patients). Calcium channel inhibitors were the most frequently used antihypertensives.

Morphology

All biopsies were fixed in Duboscq-Brazil and were embedded in paraffin. Four micrometer sections were stained with hematoxylin and eosin, periodic acid-Schiff (PAS), Masson trichrome. and silver methenamine. Assessment of specimen adequacy and histologic diagnosis were made by means of the Banff 97 criteria [22], in addition to the classic light microscopic histologic diagnosis and immunofluorescence studies for the detection of immune deposits. The biopsy samples included a mean number of 13 glomeruli and 4 arteries per sample.

Additional morphometric analysis was performed on Masson trichrome-stained slides [21] and included the following: (*1*) the extent of glomerulosclerosis, expressed as the proportion of obsolete glomeruli (percentage); (*2*) the fractional interstitial volume, measured at a magnification of ×100 on all available cortical sections by means of a Vidas 2.5 image analysis program and expressed as the proportion of the total cortical area consisting of interstitial space after deletion of glomeruli and arterial structures; and (*3*) the ratio of arterial intima:media area and arterial vessel wall:total area of all cortical arteries. This ratio was calculated from measurements on eight orthogonal points of all cortical arteries by means of the sector elliptic approach and was expressed as a percentage [23]. Thirty-one adequate paired biopsies were available for this morphometric analysis.

Immunohistochemical staining for oxidatively modified LDL

Additional implantation and protocol biopsy samples were retrieved and snap frozen in liquid N_2 within one hour in OCT compound and were kept stored at −85°C until processing. Six-micrometer sections were stained for native, malondialdehyde-modified, and oxidatively modified (OxLDL), by means of the avidin-biotin complex (ABC) method, with specific mouse monoclonal antibodies (L8A2, 1H11 [24], and Ox4E6 [25], respectively). The sections were fixed for 20 minutes in 4% paraformaldehyde in phosphate-buffered saline. After rinsing with distilled water, the sections were washed for five minutes in TSB (0.01 mol/L Tris-HCl, pH 7.4, in 0.9% NaCl) with 1% bovine serum albumin (BSA). After 30 minutes of incubation with normal horse serum 1/5 in TSB/BSA, the sections incubated overnight at room temperature with the respective antibodies (L8A2, 1/6000; 1H11, 1/8000; Ox4E6, 1/6000). Thereafter, endogenous peroxidase activity was blocked by incubation for 30 minutes in 0.03% H_2O_2 in TSB. In the next step, the sections incubated for 30 minutes with HoAM 1/200 in TSB/BSA and for one hour with ABC (Vector-kit, Vectastrain, Vector Laboratories, Inc., Burlingame, CA, USA) in TSB/BSA. After washing with TSB/BSA and rinsing with distilled water, the sections incubated for five minutes with 0.02 mol/L Na-acetate (pH 5.1) and subsequently for five minutes with amino ethyl carbazole [(AEC) 20 mg AEC diluted in 24 mL dimethyl sulfoxide (DMSO)], with an addition of 200 mL 0.02 mol/L AE buffer (pH 5.1) and 4 mL 0.3% H_2O_2. After rinsing with aq. dest., counterstaining was performed by incubation for three minutes with hemalun in aqueous solution.

Control staining, after omission of the primary antibody, was performed on frozen sections of five renal transplant biopsies. Additional control staining with an unrelated primary antibody, that is, a mouse monoclonal antibody directed against placental alkaline phosphatase (clone 7E8) [26], and staining after omission of the secondary horse anti-mouse antibody, was performed on frozen sections of six renal transplant biopsies.

The specificity of the immunohistochemical staining for native, malondialdehyde (MDA)-modified, and OxLDL was assessed by means of preabsorption of each monoclonal antibody with native, MDA-LDL, and OxLDL. Each immunostaining was performed on five renal transplant biopsies, as described previously in this article, before and after preincubation of L8A2, 1H11, and Ox4E6 with freshly prepared native LDL (13 μg/mL), MDA modified LDL (5 μg/mL), and OxLDL (9.4 and 18.8 μg/mL).

All sections were blinded to the reader and were subsequently semiquantitatively scored at magnification 25 × 10. Only biopsies including cortical material were

analyzed. On each section, the arterial, glomerular, tubular and interstitial compartments were separately scored for immunostaining by means of the following criteria: 0 = no staining; 1 = staining of less than 30% of the area; 2 = staining between 30 and 60% of the area; and 3 = staining more than 60% of the area. Because of sampling limitations, 24 paired (implantation and protocol) cortical biopsies were available for this analysis.

Immunohistochemical staining for macrophages

Immunostaining for macrophages was performed on 6 μm sections of cortical biopsies, after fixing in formol calcium solution and embedding in paraffin. According to the previously described ABC method, immunostaining was performed with a specific mouse monoclonal antibody, directed against human CD68 (Clone PG-M1; Dako®, Carpinteria, CA, USA), in addition to a nuclear counterstaining with methyl green. All sections were blinded to the reader. On each section, the number of macrophages was determined on 10 consecutive fields at magnification (40 × 10), for the arterial, glomerular, and tubulointerstitial compartments separately. Results are expressed as the mean (± SD) number of cells per field. Because of sampling limitations, 21 paired (implantation and protocol) cortical biopsies were available for this analysis.

Determination of the plasma concentration of malondialdehyde-modified and oxidized LDL

The plasma concentration of malondialdehyde-modified and OxLDL was determined in the last-included 36 patients and 30 healthy controls (8 males and 22 females, mean age of 36.1 ± 4.75 years) who were recruited among the medical and nursing staff of the hospital.

At least one blood sample was withdrawn in a CTAD tube (Beckton Dickinson, Mountain View, CA, USA) and centrifuged for 20 minutes at 4500 r.p.m. at 4°C after the addition of 50 μL of a solution, constituted of 7 mL tri-Nacitrate, 1 mL vitamin E (117 mmol/L), and 2 mL ethylenediaminetetraacetic acid (EDTA; 100 mg/mL). After the addition of 10 μL solution, consisting of 100 mmol/L butylated hydroxytoluene in ethanol ×100 diluted in tri-Nacitrate (3.13%), 1 mL centrifuged plasma was kept stored at −85°C in glass tubes until processing. The plasma concentration of MDA-modified and OxLDL was determined by means of an ELISA with 1H11 and Ox4E6, respectively, as previously described [24, 25].

Clinical and biological data

Data retrieved at implantation were age and gender of the donor, recipient age, cold ischemia time, and first and second warm ischemia time. Data retrieved post-transplantation were delayed graft function (defined as the need for dialysis in the first postoperative week), the number and time of occurrence of acute rejection episodes (73% biopsy-proven, 27% clinically suspected), the Banff score of acute rejections, and the weekly cytomegalovirus (CMV)-antigenemia (expressed as the number of cells/200,000 leukocytes, detected by means of immunofluorescence with a mouse monoclonal antibody reacting with the CMV pp65 structural protein; Biosof, Varilhes, France) for the first 12 postoperative weeks. Data retrieved once every three months included cyclosporine trough level (determined at 12 hours postintake on whole blood samples with the TDx fluorescence polarization immunoassay, Abbott Laboratories, North Chicago, IL, USA), serum creatinine level, 24-hour creatinine clearance normalized for body surface area, 24-hour proteinuria, body mass index, systolic and diastolic blood pressure values, and determinations of plasma concentration of total, HDL, LDL cholesterol, triglycerides, apolipoprotein A1 and B, as well as the calculated value of very low-density lipoprotein (VLDL) cholesterol.

The exposure of the recipient to CMV, the lipid and lipoprotein fractions, and cyclosporine was derived from the retrieved data by means of the summation of the mean exposure at successive observation intervals. The calculation of the mean exposure was based on the trapezoidal rule, that is, the multiplication of the mean of two consecutive measurements with the time interval (days). Each exposure was normalized per day by dividing the total exposure by the duration of observation (days).

Study end points

Two surrogate markers for long-term graft outcome were used as end points for this study and were identical to the end points defined in a previous study [21]. Morphological outcome was assessed by means of the fractional interstitial volume at 1½ years, while functional outcome was assessed by means of the 24-hour creatinine clearance (normalized for body surface area) at 1½ years.

Statistical analysis

All data presented are expressed as mean (± SD). The following statistical tests were used where appropriate: multivariate linear regression analysis (Table 2), the determination of relative risk (Fig. 1), *t* test for independent samples (Figs. 2, 6, and 7), the determination of the Spearman correlation coefficient for categorical variables (Tables 3–5), Wilcoxon's test for paired samples (Fig. 5), and the determination of the Pearson correlation coefficient for the association between the recipient's exposure to triglycerides and HDL cholesterol and plasma OxLDL. Since the association between two biological variables may be related to the order of magnitude of these variables, rather than to their individual values, we divided into quartiles the continuous variables HDL cholesterol (Table 3), density of interstitial macrophages (Tables 4 and 5), and fractional interstitial vol-

ume at 1½ years (Table 5), and subsequently determined the nonparametric Spearman correlation coefficient.

The predictive value of donor-related and post-transplant risk factors was determined in multivariate linear regression analysis of the dependent variables, that is, the fractional interstitial volume and the creatinine clearance at 1½ years. Variables tested in the predictive model were donor age, glomerulosclerosis at implantation, the fractional interstitial volume at implantation, the logarithmic transformed value of the ratio of arterial intima:media area and arterial wall:total area at implantation, as well as the cold ischemia time, the number and the Banff grade of acute rejection episodes, the proteinuria and the mean blood pressure of the recipient, the mean exposure of the recipient to triglycerides, HDL and LDL cholesterol, the mean exposure of the recipient to cyclosporine dose and trough level, and CMV infection. Goodness-of-fit of the predictive model was assessed by means of R square, the coefficient of determination. The predictive model yielding the highest R^2 for the multivariate regression analysis of both dependent variables was selected as the best fitting model. Regression analysis for the fractional interstitial volume at 1½ years was performed on all cases with adequate implantation and protocol biopsies ($N = 31$). Regression analysis for the creatinine clearance at 1½ years was performed on all cases with adequate implantation biopsies ($N = 41$).

The relative risk for interstitial fibrosis or decreased renal function at 1½ years was calculated as the ratio of the incidences of the adverse outcome in the group at risk and the group not at risk. Interstitial fibrosis was defined as the fractional interstitial volume above 30% (50th percentile of the fractional interstitial volume at 1½ years). Decreased renal function was defined as the measured creatinine clearance below 64 mL/min (50th percentile of the creatinine clearance at 1½ years). The cut-off value for the risk factor was defined as the 50th percentile of the mean exposure of the recipient to HDL cholesterol, that is, 47 mg/dL, and the 50th percentile of the mean exposure of the recipient to triglycerides, that is, 168 mg/dL. The relative risk for interstitial fibrosis was determined on all cases with adequate protocol biopsies ($N = 36$), while the relative risk for decreased renal function was calculated on all cases with available creatinine clearance at 1½ years ($N = 49$). All tests were performed with SPSS 9.0.

Table 1. Demographic data and risk factors of the total study cohort and the morphometrically studied patients

	Total study cohort ($N = 50$)	Morphometrically studied patients ($N = 31$)
Donor age *years*	41 ± 16	43 ± 17
Recipient age *years*	48 ± 14	49 ± 12
Sex of donor *male/female*	31/19	18/13
Cold ischemia time *hours*	20 ± 5	20 ± 6
2nd ischemia time *minutes*	31 ± 9	30 ± 9
Delayed graft function *N* (%)	16/50 (32%)	9/31 (29%)
Acute rejection		
Clinically suspected *N* (%)	3/50 (6%)	2/31 (6.5%)
Banff 1 *N* (%)	4/50 (8%)	2/31 (6.5%)
Banff 2 *N* (%)	7/50 (14%)	4/31 (13%)
CMV infection *N* (%)	20/50 (40%)	14/31 (45%)
Cyclosporine dose exposure *mg/kg*	3.5 ± 1.5	3.4 ± 1.1
Cyclosporine trough level exposure *ng/mL*	164 ± 57	165 ± 55
Mean arterial blood pressure *mm Hg*	101 ± 6	102 ± 6
Proteinuria *mg/24 h*	450 ± 872	346 ± 631
Recipient's exposure to total cholesterol *mg/dL*	221 ± 46.7	226 ± 31.2
Recipient's exposure to HDL cholesterol *mg/dL*	53.7 ± 17	49.4 ± 12
Recipient's exposure to triglycerides *mg/dL*	172 ± 72	185 ± 65
Creatinine clearance at 1½ years *mL/min*	62 ± 18	61 ± 15

Data are mean ± SD or number (%). Abbreviations are: CMV, cytomegalovirus; HDL, high-density lipoprotein.

RESULTS

Since the demographic data and risk factors were not statistically different in the total study cohort ($N = 50$) and the morphometrically studied patients ($N = 31$; Table 1), it can be assumed that the latter subpopulation is representative for the whole study cohort.

In a multivariate regression analysis, the occurrence of an acute rejection episode, logarithmic-transformed value of the ratio of arterial intima/media area at implantation, exposure of the recipient to HDL cholesterol, and diastolic blood pressure substantially predicted the fractional interstitial volume ($R^2 = 0.314$, $P = 0.028$) and the creatinine clearance ($R^2 = 0.310$, $P = 0.006$) at 1½ years (Table 2).

The relative risk of the recipient's exposure to HDL cholesterol ≤47 mg/dL was 2.75 (95% CI, 1.075 to 7.037) for interstitial fibrosis and 1.56 (95% CI, 0.978 to 2.497) for decreased renal function at 1½ years (Fig. 1A). The relative risk of the recipient's exposure to triglycerides ≥168 mg/dL was 2.26 (95% CI, 0.778 to 6.570) for interstitial fibrosis and 1.66 (95% CI, 1.018 to 2.700) for decreased renal function at 1½ years (Fig. 1B). Since the occurrence of at least one acute rejection episode adversely affected the fractional interstitial volume (FIV) at 1½ years (36.6 ± 13.4% vs. 29.0 ± 5.8%, $P = 0.036$; Fig. 2), the impact of the recipient's exposure to HDL cholesterol and triglycerides was re-evaluated in patients without any acute rejection episode.

In patients without an acute rejection episode(s), the relative risk of the recipient's exposure to HDL choles-

Table 2. Multivariate linear regression models of the fractional interstitial volume (FIV) and creatinine clearance at 1½ years

	A. FIV in protocol biopsies (R^2 = 0.314, P = 0.028)				B. Creatinine clearance (R^2 = 0.310), P = 0.006)			
Selected covariates	Coefficient	SE	Beta	95% CI	Coefficient	SE	Beta	95% CI
Acute rejection number	1.914	1.684	+0.188	−1.536–5.365	5.012	4.253	+0.172	−3.598–13.621
Log intima:media area	18.602	8.354	+0.356	1.489–35.716	−38.821	18.897	−0.285	−77.075–−0.566
HDL exposure *mg/dL/day*	−0.09654	0.066	−0.246	−0.232–0.039	0.560	0.161	+0.531	0.235–0.885
Diastolic blood pressure *mm Hg*	0.104	0.172	+0.099	−0.249–0.457	0.692	0.367	+0.273	−0.051–1.436

The magnitudes of the standardized beta coefficients indicate the relative strength of the association of, respectively, FIV (A) and creatinine clearance (B) with the respective baseline covariates. Abbreviations are: HDL, high density lipoprotein; SE, standard error; CI, confidence interval.
[a] Logarithm transformation applied to the ratio of arterial intima:media area in implantation biopsies

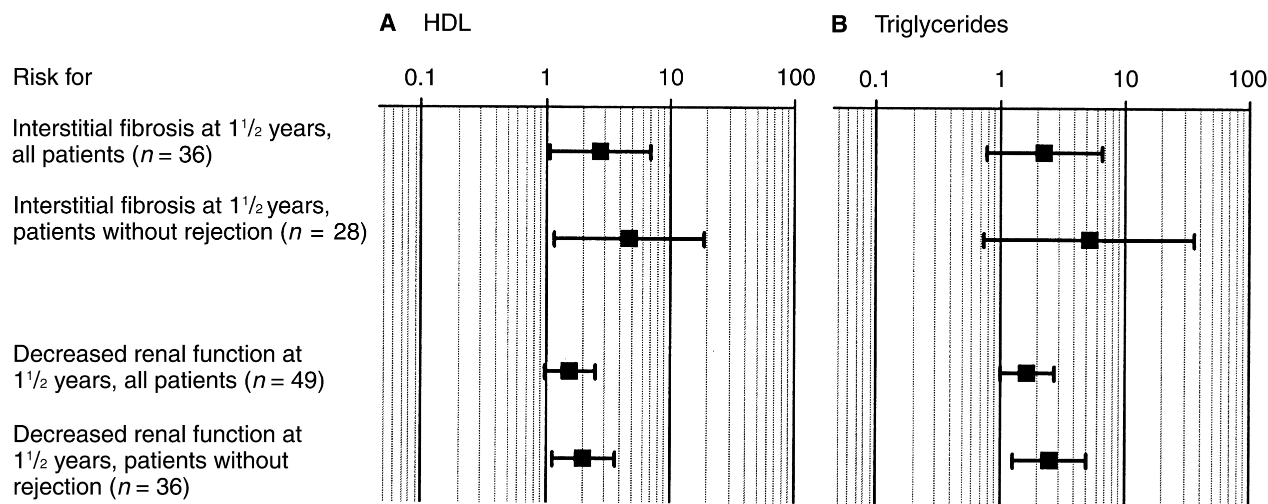

Fig. 1. Relative risk and 95% CI of the recipient's exposure to high-density lipoprotein (HDL) cholesterol ≤47 mg/dL (*A*) and triglycerides ≥168 mg/dL (*B*) for the development of interstitial fibrosis (FIV >30%) or decreased renal function (creatinine clearance <64 mL/min) at 1½ years in all recipients and in recipients without acute rejection.

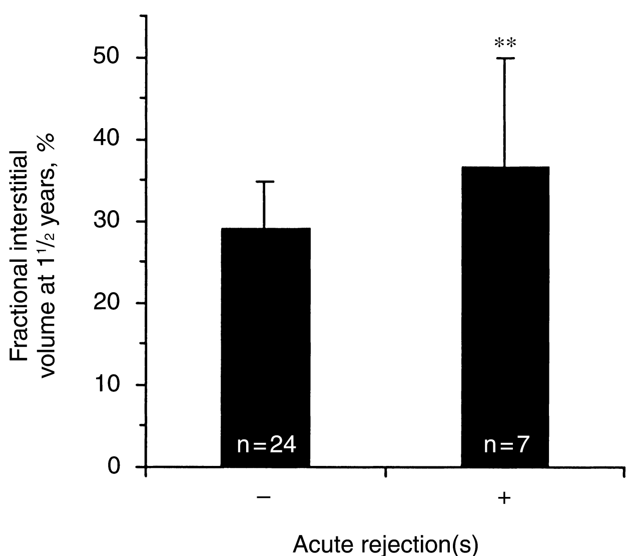

Fig. 2. Fractional interstitial volume (mean ± SD) in protocol biopsies of recipients without (−) and with at least one acute rejection episode (+) (P = 0.036).**

terol ≤47 mg/dL was 4.67 (95% CI, 1.172 to 18.582) for interstitial fibrosis and 2.03 (95% CI, 1.130 to 3.650) for decreased renal function at 1½ years (Fig. 1A). The relative risk of the recipient's exposure to triglycerides ≥168 mg/dL was 5.18 (95% CI, 0.747 to 35.863) for interstitial fibrosis and 2.5 (95% CI, 1.26 to 4.96) for decreased renal function at 1½ years in patients without an acute rejection episode(s) (Fig. 1B).

Immunohistochemical expression of L8A2 (specific for native LDL) and of Ox4E6 (specific for oxidized LDL) was clearly observed in four and, respectively, three out of the five biopsies used for the specificity assessment. Immunostaining of glomeruli, arteries, and interstitium for native and OxLDL cholesterol was specific. Indeed, the expression of L8A2 (Fig. 3A) was completely inhibited after preincubation with native LDL (Fig. 3B) in four out of four biopsies, and the expression of Ox4E6 (Fig. 3C) was completely inhibited after preincubation with OxLDL (Fig. 3D) in three out of three of the biopsies. Since the immunostaining for malondialdehyde-modified LDL was absent in three out of five biopsies and was only weakly positive in glomeruli and arteries in two remaining

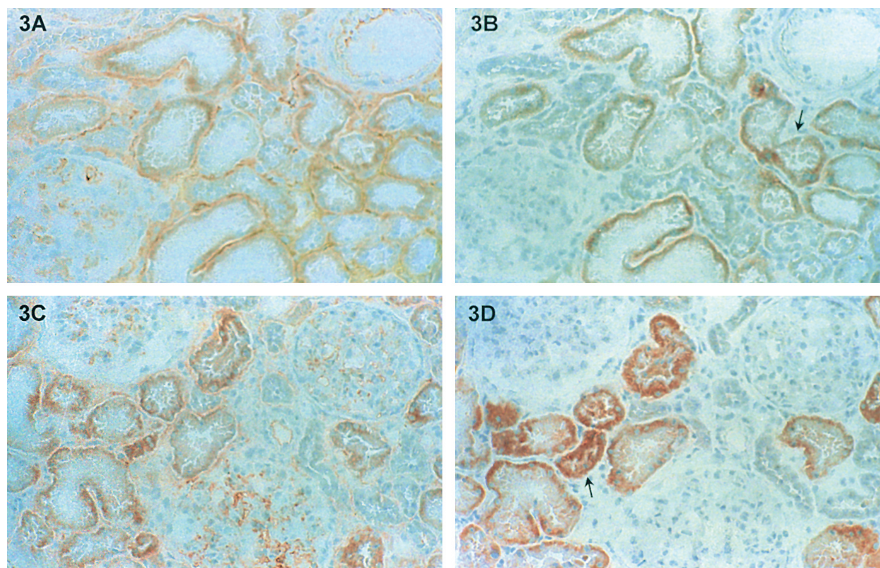

Fig. 3. Immunohistochemical expression of L8A2 and Ox4E6 before (*A*, L8A2; *C*, Ox4E6) and after preincubation with a competitive antigen (*B*, L8A2 + LDL; *D*, Ox4E6 + OxLDL) at a magnification of ×250. Glomerular, arterial, and interstitial immunostaining was inhibited after preincubation of L8A2 with native LDL (B vs. A) and after preincubation of Ox4E6 with OxLDL (D vs. C). Note the unspecific staining of tubuli in B and D (arrows), persisting after preincubation of the primary antibodies with the competitive antigen.

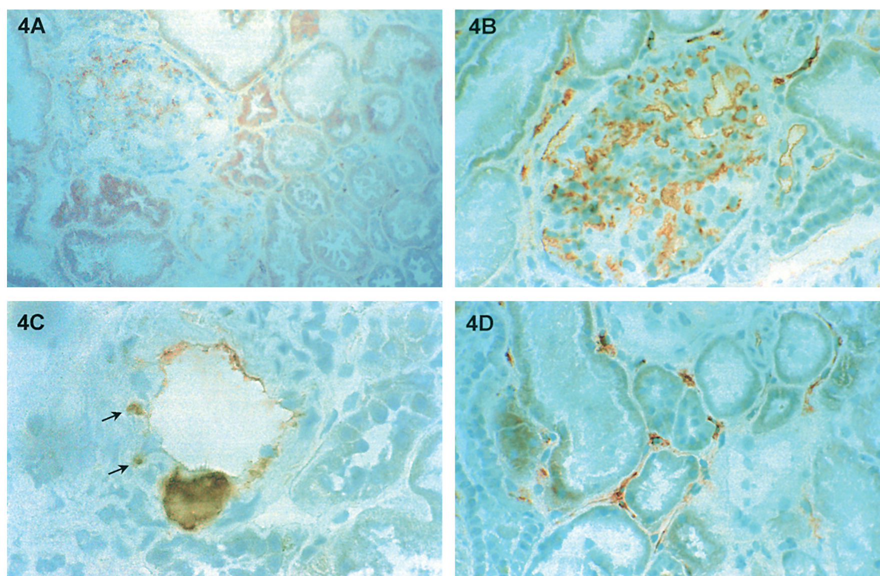

Fig. 4. Immunohistochemical expression of Ox4E6 in a renal allograft at 1½ years (*A*) at a magnification of ×250. Glomerular staining for OxLDL shows a mesangiocapillary pattern (*B*, magnification ×400). In arteries, immunostaining for OxLDL is mainly observed in endothelial cells and in isolated cells in the arterial wall (arrows; *C*, magnification ×800). Interstitial immunostaining for OxLDL is more diffuse (A and *D*, magnification ×400).

biopsies, no valid conclusions could be drawn about the specificity of this staining, after preincubation of 1H11 with malondialdehyde modified LDL. Moreover, the immunohistochemical staining for native and OxLDL was nonspecific in tubuli, since a positive staining of the tubuli was also observed in control stainings without a primary or secondary antibody or with an unrelated primary antibody (data not shown). Tubular immunostaining for native and OxLDL was not inhibited after preincubation with the respective antigens (Figs. 3 B, D).

Glomerular immunostaining for LDL and OxLDL was observed in a mesangiocapillary pattern (Fig. 4B). In arteries, endothelial cells and isolated cells in the arterial intima and media stained positive for LDL and OxLDL (Fig. 4C). In contrast, a more diffuse immunostaining for LDL and OxLDL was observed in the renal interstitium (Fig. 4 A, D).

Although immunostaining for LDL and OxLDL was already positive in some implantation biopsies (Fig. 5), a significantly increased immunostaining of the interstitial compartment could be observed for both native and OxLDL in protocol biopsies compared with implantation biopsies ($P = 0.003$ and $P = 0.004$, respectively, Wilcoxon's signed rank test; Fig. 5). In addition, immunostaining for OxLDL was also increased in glomeruli of protocol biopsies versus implantation biopsies ($P = 0.002$, Wilcoxon's signed rank test; Fig. 5).

Accumulation of OxLDL, that is, an increase of the semiquantitative score in protocol versus implantation biopsies, was observed in the renal interstitium of 15 out

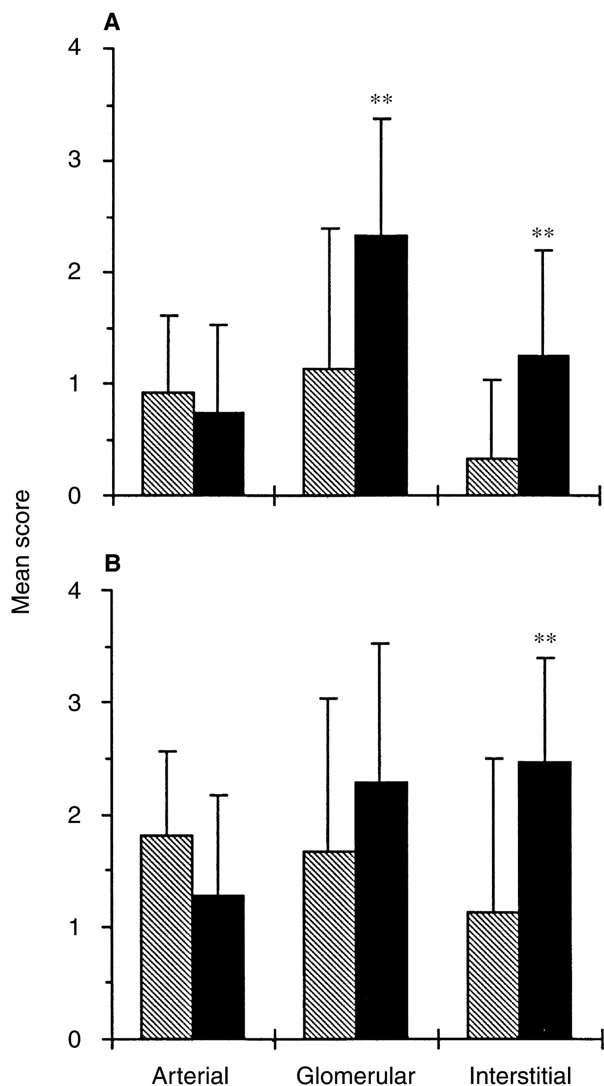

Fig. 5. Semiquantitative score of the immunohistochemical expression of Ox4E6 (*A*) and L8A2 (*B*) in the arterial, glomerular, and interstitial compartment of 24 paired implantation (▧) and protocol (■) biopsies. Data are mean ± SD; $^{**}P < 0.01$. The semiquantitative scores are: 0 = no staining; 1 = <30%; 2 = 30 to 60%; and 3 = > 60% staining of the area.

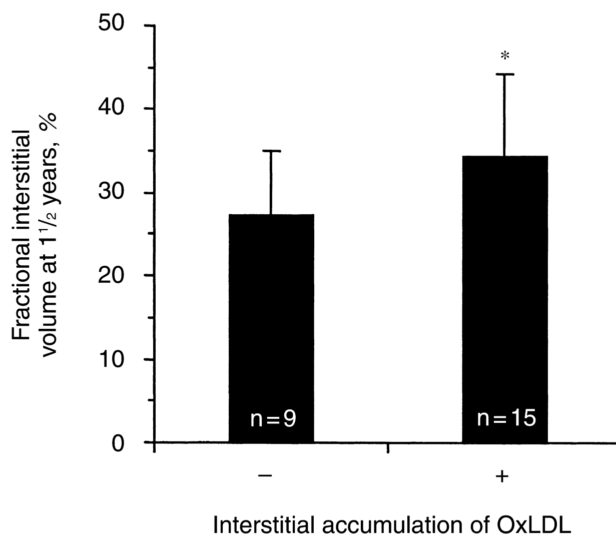

Fig. 6. Fractional interstitial volume at 1½ years in grafts with stable or decreased (−) interstitial expression and grafts with increased (+) interstitial expression of OxLDL (mean ± SD) in protocol biopsies versus implantation biopsies ($^{*}P = 0.049$).

Table 3. Inverse correlation between the recipient's exposure to HDL cholesterol (divided into quartiles), and the increase (positive values) or decrease (negative values) of the interstitial semiquantitative score of oxidized LDL in protocol vs. implantation biopsies

		Interstitial accumulation of OxLDL						
	Quartile	−2	−1	0	1	2	3	Total
HDL cholesterol *mg/dL*	<42				1	4	1	6
	≥42–<47		1	2	2	1		6
	≥47–<64			2	1	3		6
	≥64	1	1	2	1	1		6
Total		1	2	6	5	9	1	24

Data are by the Spearman correlation: R = −0.476; $P = 0.019$; $N = 24$.

Table 4. Correlation between the increase (positive values) or the decrease (negative values) of the interstitial semiquantitative score of OxLDL in protocol versus implantation biopsies, and the density of tubulointerstitial macrophages (divided into quartiles) at 1½ years

		Interstitial accumulation of OxLDL						
	Quartile	−2	−1	0	1	2	3	Total
Density of interstitial macrophages *cells/field*	<2	1	1	1	2			5
	≥2–<7		1	1	2	1		5
	≥7–<12			2		4		6
	≥12				1	3	1	5
Total		1	2	4	5	8	1	21

Data are by the Spearman correlation: R = 0.656; $P = 0.001$; $N = 21$.

of 24 paired biopsies and was associated with a higher FIV (35.45 ± 9.9 vs. 27.33 ± 7.8%, $P = 0.049$; Fig. 6). The accumulation of oxidized LDL in the renal interstitium was inversely associated with the recipient's exposure to HDL cholesterol (R = −0.476, $P = 0.019$, Spearman correlation; Table 3). The recipient's exposure to triglycerides tended to be associated with accumulation of OxLDL in the renal interstitium, although this trend was not significant (R = 0.336, $P = 0.105$, Spearman correlation).

Moreover, the accumulation of OxLDL in the renal interstitium was associated with a significantly higher density of macrophages in the tubulointerstitial compartment of renal biopsies at 1½ years (R = 0.656, $P = 0.001$, Spearman correlation; Table 4).

Furthermore, the density of macrophages in the tubulointerstitial compartment was significantly associated

Table 5. Correlation between the fractional interstitial volume (divided into quartiles) and the density of tubulointerstitial macrophages (divided into quartiles) in protocol biopsies at 1½ years

		Fractional interstitial volume %				
	Quartile	<27%	≥27%–<33%	≥33%–<37%	≥37%	Total
Density of interstitial macrophages *cells/field*	<2	2	1	2		5
	≥2–<7	2	2	1		5
	≥7–<12	2	2	1	1	6
	≥12		1		4	5
Total		6	6	4	5	21

Data are by the Spearman correlation: R = 0.481; P = 0.027; N = 21.

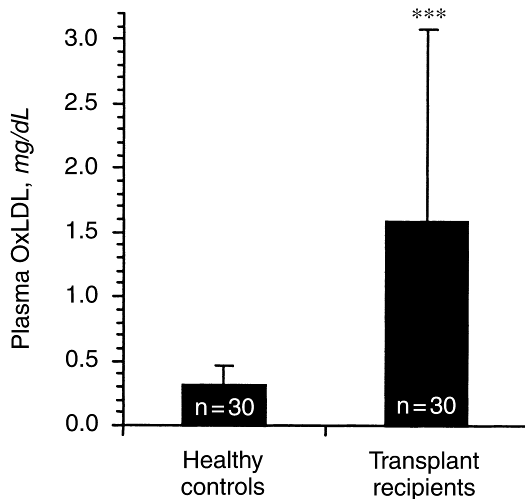

Fig. 7. Plasma concentration of OxLDL in healthy controls and transplant recipients (mean ± SD). ***$P < 0.001$.

with the extent of interstitial fibrosis at 1½ years (R = 0.481, P = 0.027, Spearman correlation; Table 5).

The plasma concentration of OxLDL was significantly higher in transplant recipients compared with healthy controls (1.58 ± 1.50 vs. 0.31 ± 0.16 mg/dL, $P < 0.001$; Fig. 7).

The plasma levels of OxLDL correlated with the recipient's exposure to triglycerides (R = 0.574, $P < 0.001$, Pearson correlation) and were inversely associated with HDL exposure (R = −0.347, P = 0.038, Pearson correlation). However, no correlation could be demonstrated between the plasma concentration of malondialdehyde-modified or OxLDL and the accumulation of OxLDL in the renal interstitium.

DISCUSSION

While several previous studies highlighted a significant association between hyperlipidemia of the recipient and the development of CAN, many questions remain unsolved. A primary controversy is the exact nature of the dyslipidemia associated with CAN [3]. Indeed, some authors found an association between LDL cholesterol and CAN [27, 28], while others reported a significant correlation between decreased HDL cholesterol, increased triglycerides, and CAN [29]. Equally controversial is whether dyslipidemia plays a causative role in the development of CAN or is merely an epiphenomenon of CAN [30]. In this regard, prospective randomized, controlled studies analyzing the impact of lipid-lowering drugs on the development of CAN are presently lacking. Finally, although a causative role of the oxidatively modified LDL for the progression of renal disease has been suggested [7, 31], the pathophysiologic mechanism by which hyperlipidemia of the recipient could induce the development of CAN is still not elucidated.

Despite the small number of patients included in this study, the prospective design with the inclusion of all known risk factors for graft failure and combination of morphological and functional end points enabled us to evaluate the role of hyperlipidemia in the genesis of CAN. To our knowledge, this is the first prospective study in renal transplant recipients with a documented morphometric analysis of baseline and protocol biopsies, as well as documented exposure to a variety of risk factors.

In this prospective clinicopathologic study, exposure of the recipient to HDL cholesterol levels below 47 mg/dL during the first 18 months post-transplantation was an independent predictor for the development of interstitial fibrosis and reduced renal function at 1½ years in multivariate regression analysis. This risk was even more pronounced in patients who did not sustain any acute rejection episode. Since the detrimental effect of an acute rejection episode on graft outcome has been repeatedly documented [32–34] and was recently confirmed in a large survey [35], as well as in our predictive model, the occurrence of an acute rejection episode is a major risk factor for graft outcome. However, according to our data, exposure of the recipient to lower HDL cholesterol levels is also a risk factor for graft outcome, particularly in patients who did not experience the adverse effects of an acute rejection episode.

Moreover, exposure of the recipient to triglycerides above 168 mg/dL also represented an increased risk for adverse graft outcome. These data are in line with previous studies [28, 29]. In contrast with others [27, 28],

however, we were unable to demonstrate any association between total, LDL, VLDL cholesterol exposure, and subsequent graft outcome.

Although a significant association does not necessarily imply a causal relationship, some arguments favor the hypothesis that low HDL levels and hypertriglyceridemia of the recipient may adversely affect the subsequent graft outcome. In our prospective study, the morphologic and functional alterations were preceded by the exposure. Observations in primary renal diseases reported a similar association between elevated triglycerides [36, 37] or lower HDL cholesterol [38] and the subsequent progression of renal disease. In a recent large study in 12,728 participants of the Atherosclerosis Risk in Communities Study, with baseline serum creatinine less than 2 mg/dL, low HDL cholesterol and elevated triglycerides were independent risk factors for the subsequent decline in renal function [39]. Moreover, experimental studies in the analbuminemic rat demonstrated the protective role of the reduction of triglycerides, for the subsequent development of glomerulosclerosis and proteinuria [40]. Finally, in heart transplant recipients, HDL cholesterol was the only lipid parameter significantly and inversely associated with the occurrence of new major coronary events [41], while OxLDL predicted transplant-associated coronary artery disease [42].

How could exposure of the recipient to low levels of HDL cholesterol and elevated triglycerides contribute to the deterioration of the renal allograft? Experimental and human studies strongly suggest that many biochemical and histologic features that accompany glomerulosclerosis are similar to those observed in the systemic vascular lesions of atherosclerosis [32]. The promoting role of oxidatively modified LDL in the formation of the arteriosclerotic lesion has been well documented [43, 44]. Several animal and in vitro experiments have similarly highlighted the effects of oxidatively modified LDL on glomerular and tubular epithelial cells [7–16], as well as the protective effects of dietary antioxidant [17], probucol [45], and HDL [18]. Two distinct mechanisms could contribute to the protective role of HDL in the formation of OxLDL [46–49]. Paraoxonase 1 [50] and platelet-activating factor acetylhydrolase [51], two HDL-associated enzymes, have been shown to inhibit LDL lipid peroxidation in vitro. In addition, HDL could act as a reservoir for lipid peroxides generated on LDL [46], thereby enhancing the hepatic uptake and catabolism of oxidized cholesteryl esters [52].

In the present study, immunostaining for LDL and OxLDL in renal allografts was observed in a mesangiocapillary pattern in glomeruli, in vascular endothelial cells, and in the interstitial space. Interestingly, a similar distribution pattern of LOX-1, an OxLDL receptor, was recently demonstrated in renal cortical tissue of Dahl salt-sensitive rats [53].

Interstitial immunostaining for OxLDL was increased in 15 out of 24 protocol biopsies compared with implantation biopsies in our study. This interstitial accumulation of OxLDL inversely correlated with the exposure of the recipient to HDL cholesterol and showed a trend for association with the recipient's exposure to triglycerides. Increased plasma levels of triglyceride and decreased levels of HDL cholesterol have been consistently correlated with the presence of small dense LDL, indicated as the LDL subclass pattern B [54, 55]. Ghanem et al found an increased prevalence of small dense LDL in renal transplant recipients that was associated with a higher plasma triglyceride and a lower HDL cholesterol level [4]. This LDL subclass pattern B exhibited an increased susceptibility to oxidative modification in vitro [4]. Similarly, Sutherland et al showed an increased susceptibility for oxidation of triglyceride-rich LDL in female renal transplant recipients [5]. In view of these data, the documented exposure of the recipient to lower HDL cholesterol and elevated triglycerides in our study potentially reflects the presence of small dense LDL. Since small dense LDL is more prone to oxidative modification than larger LDL [56, 57], the presence of small dense LDL could have contributed to the interstitial accumulation of OxLDL in recipients with lower HDL cholesterol and elevated triglyceride levels.

The density of macrophages in the tubulointerstitial compartment was significantly higher in renal allografts with interstitial accumulation of oxidized LDL at 1½ years and was associated with the extent of interstitial fibrosis at 1½ years. Several studies have demonstrated the chemotactic capacity of modified LDL for monocytes and macrophages, not only at the arterial level [58–60], but also in glomeruli [10–12].

The fibrogenic role of macrophages in glomerulosclerosis [61] and interstitial fibrosis has been well described [62]. According to current insights, macrophages release tissue inhibitor of metalloproteinase-1 (TIMP-1), a protease inhibitor [62–64], as well as fibrosis-promoting cytokines, such as transforming growth factor-β1 (TGF-β) [63].

Although the plasma concentration of OxLDL in transplant recipients was significantly higher than in healthy controls and was inversely correlated with HDL exposure, we were unable to demonstrate any correlation between this parameter and the accumulation of OxLDL in the renal interstitium. This observation suggests that the oxidative modification of LDL could occur in situ in the renal parenchyma and is less likely to result from the deposition of circulating OxLDL. Similar observations were made in an experimental model in normotensive obese rats [65].

In conclusion, decreased HDL cholesterol levels of the recipient adversely affect the morphologic and functional outcome of cadaveric renal allografts. Our data provide evidence that this may be due to accumulation

of oxidatively modified LDL in the renal interstitium of the graft. Interstitial accumulation of oxidatively modified LDL was associated with the presence of macrophages and the development of interstitial fibrosis. Prospective, randomized, and controlled studies, with pharmacologic intervention on HDL cholesterol and triglycerides, are needed to confirm our data.

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APPENDIX

Abbreviations used in this article are: ABC, avidin-biotin complex; BSA, bovine serum albumin; CAN, chronic allograft nephropathy; CI, confidence interval; CMV, cytomegalovirus; DMSO, dimethyl sulfoxide; FIV, fractional interstitial volume; HDL, high-density lipoprotein; LDL, low-density lipoprotein; MDA, malondialdehyde; OCT, 22-oxacalcitriol; OxLDL, oxidized low-density lipoprotein; RR, relative risk; VLDL, very low-density lipoprotein.

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