Oxidized Low-Density Lipoproteins Activate CD4+ T Cell Apoptosis in Patients with End-Stage Renal Disease through Fas Engagement

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Oxidized LDL (oxLDL) are cytotoxic to vascular cells, but their possible action on T cells from patients with ESRD has not been evaluated. oxLDL concentrations were measured and compared in patients who were on long-term hemodialysis (HD), in patients who had ESRD and were on continuous ambulatory peritoneal dialysis, in nondialyzed patients with chronic kidney disease, and in age- and gender-matched control subjects. In parallel, the proliferative capacity of CD69+/CD4+ T cells and their rate of apoptosis, IL-2 expression, and intracellular expression of Bcl-2 and Bax were determined in vitro. The oxLDL concentrations were significantly higher in HD patients (all \( P < 0.001 \)). Upon phytohemagglutinin stimulation, CD69+/CD4+ T cells from HD patients proliferated significantly less than those from the other patients' group (both \( P < 0.001 \)). oxLDL but not the native LDL were led to CD69+/CD4+ T cells' program cell death in a dose- and time-dependent manner through Fas pathway (\( P = 0.001 \)). Cell surface Fas expression was followed by DNA fragmentation when CD69+/CD4+ T cells from HD patients or control subjects were cultured with oxLDL (200 \( \mu \)g/ml; \( 1 \pm 3 \% \) versus \( 3 \pm 2 \% \); \( P = 0.001 \)). In the presence of oxLDL, CD69+/CD4+ T cells from HD patients expressed significantly lower IL-2 levels, which strongly correlated with a decrease in the ant apoptotic Bcl-2 and conversely with an increase in the proapoptotic Bax expression. In conclusion, these data suggest that, in HD patients, exposure of activated CD4+ T cells to oxLDL leads to Fas-mediated apoptosis in association with inhibition of IL-2 expression. Subsequently, this may favor activation of mitochondrial-dependent apoptotic pathways, leading to activated CD4+ T cell dysfunction.


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Materials and Methods

Patients and Control Subjects
Investigations were carried out in 30 patients who had ESRD and were undergoing either chronic HD (\( n = 15 \)) or continuous ambulatory...
peritoneal dialysis (CAPD; \(n = 15\)) for at least 6 mo before the study, in 15 patients with chronic kidney disease (CKD) stage 4 to 5 (mean \(\pm SD\) GFR 15.2 \(\pm\) 4.6 ml/min per 1.73 m\(^2\)), and in 15 normotensive healthy subjects with normal kidney function (GFR >90 ml/min per 1.73 m\(^2\)) (12). The control subjects did not have BP-lowering agent, lipid-lowering agent, or aspirin. They were not known to have cardiovascular disease. No dialysis procedure was modified in dialyzed patients. Chronic HD patients had been dialyzed using the same membrane and had no significant residual renal function, as described previously (1,8). In PD patients, renal failure was due to glomerulonephritis in four cases, interstitial nephritis in two, hypertensive nephropathy in seven, and IgA nephropathy in two. All of the PD patients were performing four 2:1 exchanges a day using the Baxter TwinBag system (Baxter, Deerfield, IL). Dwell times generally were 4 to 6 h during the day and 8 h overnight. The glucose concentration ranged from 1.36 to 3.86%. PD patients had a mean \(\pm SD\) GFR of 10.7 \(\pm\) 2.3 ml/min per 1.73 m\(^2\). The dosage of dialysis regimens (equilibrated Kt/V [Kt/V]) and the normalized protein catabolic rate (g/kg per d) remained constant before and during the study. Intravenous iron therapy was administered according to the National Kidney Foundation Kidney Disease Outcomes Quality Initiative (K/DOQI) guidelines (13). Every HD and PD patient received vitamin B and C supplements. No chronic dialysis patient was on sevelamer.

The study groups were age and gender matched. eGFR was assessed according to the modified Modification of Diet in Renal Disease formula (14). Only nonsmokers were enrolled in the study. Patients with recent (<3 mo) major trauma, surgery, myocardial infarction, coronary revascularization (coronary angioplasty or bypass surgery), or stroke were excluded from the study. The other exclusion criteria were diabetes, the presence of an acute or chronic inflammatory process, infection, malnutrition (determined by subjective global assessment), use of immunosuppressive drugs, or evidence of malignancy. All patients and control subjects were vaccinated with tetanus and recombinant hepatitis B antigens. They were negative for circulating hepatitis B antigen, HIV, and hepatitis C antibody (Ab), and HIV. They had no active liver disease. No patient was nephrectomized. Arterial blood pH had to be between 7.38 and 7.42. No patient had received a blood transfusion in the 6 mo before the study. Informed consent was obtained from all patients and control subjects according to the Declaration of Helsinki. The study protocol was approved by the local institutional review board.

Lipid Determination
Blood for measurement of total cholesterol (TC) and triglyceride (TG) concentrations was collected in serum tubes; blood for HDL analysis was collected in EDTA-coated vacutainer tubes. Samples for lipid analysis were centrifuged at 1400 \(\times\) g for 10 min at room temperature, immediately frozen, and stored at -70°C for subsequent analysis. TC, TG, and HDL concentrations were determined as described previously (15,16). LDL concentrations were calculated using the Friedewald formula (LDL = TC - HDL - TG/5) (17).

ELISA for plasma oxLDL Determination
Plasma LDL concentrations were measured using a mAb-4E6–based ELISA (product no. 10-1158-01; Mercodia, Uppsala, Sweden). This Ab is directed against a conformational epitope in the apoB-100 moiety of LDL that is generated as a consequence of substitution of at least 60 lysine residues of apoB-100 with aldehydes. Venous blood samples from all patients and control subjects were obtained. The LDL fraction was separated from blood plasma before the ELISA procedure to minimize potential interferences with other plasma constituents, such as VLDL, anti-VLDL autoantibodies, and anti-phospholipid antibodies. oxLDL were measured in ELISA as described previously (18). In each ELISA plate, various concentrations of standard oxLDL, which was prepared by incubating LDL with 5 \(\mu\)mol/L CuSO\(_4\) at 37°C for 3 h, were run simultaneously to determine a standard curve.

Cell Phenotypic Analysis
Cell analysis from patients and control subjects was performed using the EPICS XL-MCL flow cytometer (Coulter Instrument, Hialeah, FL). To distinguish between T cell subpopulations, we used fluorochrome-conjugated anti-CD3, anti-CD4, anti-CD8, anticyCD69 (Aimunotech, Berkeley, CA). Combinations of murine mAb that conjugated directly to FITC, phycoerythrin, or phycoerythrin cyanine 5 were used according to the manufacturer’s instructions (Aimunotech) (19). The flow cytometer was calibrated with flow-count beads, and results were analyzed with System II software (both from Beckman-Coulter Instrumental, Miami, FL). Lymphocyte gating in general was performed by the software based on forward scatter, size scatter, and CD4 staining characteristics but also manually when indicated. Results were performed on a minimum of 1 \(\times\) 10\(^6\) cells and expressed as a percentage of CD4\(^+\) T cells.

LDL Isolation and Oxidation
LDL (density 1.019 to 1.063) were isolated from pooled fresh human sera of sequential ultrafiltration as described previously (20). LDL were dialyzed against 150 mM NaCl that contained 0.3 mM EDTA, sterilized by filtration (0.2-\(\mu\)m Millipore membrane), and stored at 4°C under nitrogen until use (up to 2 wk).

Macrophages were isolated from HD patients and control subjects using density gradient ultracentrifugation (21). The level of oxidization was measured by two methods: (1) Increased mobility in agarose gel (1.5-fold higher \(R_\text{f}\) versus native LDL) and (2) the thiobarbituric acid-reactive substances (TBARS) method.

Under the standard conditions, oxLDL contained 5.2 \(\pm\) 0.7 nmol TBARS/mg apolipoprotein B (apoB; versus 0.5 \(\pm\) 0.1 for native LDL). When native LDL were incubated with activated CD4\(^+\) T cells for 72 h, the oxidation level increased from 0.6 \(\pm\) 0.2 nmol TBARS/mg apoB at \(t = 0\) to 1.3 \(\pm\) 0.2 at \(t = 72\) h. The changes that were observed with oxLDL were approximately the same.

The extent of lipid peroxidation was estimated as malondialdehyde or 4-hydroxynonenal content by a colorimetric commercial kit (LPO 586; Bioxytech, Bonneuil sur Marne, France), resulting in a mean value of 68.2 \(\pm\) 9.4, 50.8 \(\pm\) 6.2, and 32.2 \(\pm\) 7.4 nmol malondialdehyde–4-hydroxynonenal/mg LDL protein in HD patients, patients with CKD, and control subjects, respectively. The oxLDL concentration that was used in our experiments (200 \(\mu\)g/mL) was equivalent to that reported in human plasma from HD patients, PD patients, patients with CKD, and healthy subjects (22,23).

CD4\(^+\) T Cell Preparation and Culture Conditions
Peripheral blood mononuclear cells (PBMC) were isolated by standard techniques using centrifugation through a Ficoll-Hypaque gradient (density = 1.077 g/L at 300 \(\times\) g; Pharmacia LKB, Uppsala, Sweden). PBMC were incubated on plastic tissue culture plates at 37°C for 1 h to allow monocytes to adhere. Nonadherent cells were aspirated. T lymphocytes then were passed over human T cell enrichment columns (R&D Systems, Minneapolis, MN) by use of high-affinity negative selection. The column-passed cells contained >98% CD3\(^+\) T cells, as
assessed by immunofluorescence using anti-CD3 mAb (Immunotech). Contamination with other cells was <2%.

CD4+ T cells were positively selected as described previously using CD4 MicroBeads (Miltenyi Biotech, Auburn, CA) whereby magnetically retained CD4+ T cells were eluted in the positively selected cell fraction as indicated by the manufacturer (24). The mean ± SD CD3+/CD4+ cell purity was 98.2 ± 0.5%. They then were positively selected using CD69 MicroBeads (Miltenyi Biotech) to obtain CD69+/CD4+ T cells. For exclusion of possible artifacts, dead cells were removed before labeling using Ficoll-Hypaque density gradient centrifugation (Pharmacia LKB, Uppsala, Sweden). All CD69+/CD4+ T cell cultures showed a purity that exceeded 97%. During experiments, CD69+/CD4+ T cells were incubated for up to 72 h in RPMI 1640 alone with or without LDL or oxLDL before washing in warm sterile PBS to remove the oxidant before analysis.

For analysis of the potential effect of culture medium, CD4+ T cells also were examined in vitro after incubation in RPMI-1640 that contained 10% human uremic (HD, PD, or CKD patients where mentioned) or normal (control subjects) serum and stimulated with phytohemagglutinin (PHA) as described next. Cells were counted by flow cytometry and resuspended in culture medium.

In Vitro CD69+/CD4+ T Cell Stimulation and Proliferation Assays

The CD69+/CD4+ T cells were stimulated in vitro in the presence of PHA (Murex, HA 16; Wellcome, Dartford, UK). In these assays, 1 ¥ 10^6 CD69+/CD4+ T cells from patients’ groups and control subjects were stimulated with 10 µg/ml purified PHA. The CD69+/CD4+ T cell stimulation also was analyzed in the presence of various concentrations of LDL or oxLDL (concentrations expressed as µg/ml apoprotein) and co-incubated with PHA.

Proliferation was measured by the standard [3H]thymidine uptake assay. The CD69+/CD4+ T cells were incubated with different reagents from 24 to 72 h at 37°C, and 1 µCi of [3H]thymidine (Ci = 37 Ci/Bq) was added to each well for the last 16 h. Cells were harvested on glass-fiber filters, and the amount of incorporated [3H]thymidine was measured in the supernatant plus the pellet multiplied by 100. Qualitative DNA fragmentation analysis into nucleosomal bands was detected by agarose gel electrophoresis as described previously (26).

Western Blot Analysis

Protein expression was determined using Western blots by standard methods. Briefly, protein was extracted from CD69+/CD4+ T cell cultures with RIPA buffer (50 mmol/L Tris-HCl, 150 mmol/L NaCl, 1% NP-40, 0.5% sodium deoxycholate, 1% SDS, 100 mmol/L PMSF, and protease inhibitor cocktail tablets). Protein concentrations were determined using Bio-Rad protein assay kit (Bio-Rad, Hercules, CA) in which protein absorbance is measured using spectrophotometry at 595 nm. Immunodetection was performed by enhanced chemiluminescence (ECL; Pharmacia, Piscataway, NJ) using Hyperfilm ECL (Amersham, Life Sciences, Arlington Heights, IL). Densitometric analysis was performed using an IS 5000 Digital Imaging System and Alphalager software (Alpha Innotech Corp., San Leandro, CA). The net intensity of each band of interest was measured in each of three separate Western blots. The ratio of the intensity of the detected band in HD, PD, and CKD patients was calculated relative to that of control subjects. In some experiments, the blots were stripped and rebotted with an Ab against tubulin to confirm equal loading.

Pro-Induced Apoptosis Inhibition Assay

CD69+/CD4+ T cells were cultured in the presence or absence of a Fas inhibitor that is known to block induction of apoptosis: The BMS140 mAb against Fas (anti-Fas Ab; 3 µg/ml; BenderMed System, Vienna, Austria). Cell viability and apoptosis were assessed as described previously.

IL-2 and sIL-2R Detection

Resting CD69+/CD4+ T cells from the patients’ groups and the control subjects were kept in culture medium as negative controls, and stimulated CD69+/CD4+ T cells were cultured with 10 µg/ml purified PHA (Murex, HA 16; Wellcome) for 20 h. Supernatants from cell cultures were harvested at 20 h. When needed, cells were co-cultured with PHA and LDL or oxLDL from 24 to 72 h. For intracellular IL-2 expression, CD69+/CD4+ T cells were incubated with saturating concentration of directly conjugated anti–IL-2 mAb FITC (Pharmingen, Becton Dickinson, Basel, Switzerland) and analyzed by flow cytometry. The amount of IL-2 in the supernatant was assayed in triplicate using a standard ELISA according to the manufacturer’s instructions (Pharmingen, Becton Dickinson). Absorbance was measured on a Bio-Rad microplate reader 450 and 570 nm, and concentrations were determined by comparison with a standard curve. Blockade of IL-2R was performed using 2.5-µg/ml blocking mAb (MAB1020 [α], MAB224 [β], MAB2841 [γ], R&D Systems) where appropriate. Supernatants of CD69+/CD4+ T cell cultures were assayed for the presence of sIL-2R by sandwich enzyme-linked immunoassay kit according to the manufac-
Lipid and oxLDL Concentrations

Table 2 shows the results of the measurements of the plasma lipid parameters and oxLDL concentration. T levels were significantly lower in HD and PD patients than in PD patients and patients with CKD (P < 0.05). In 20% of the HD patients, LDL levels >2.59 mmol/L (C < 100 mg/dl) were observed, compared with 54% in PD patients and 47% in patients with CKD (P = 0.001). The oxLDL concentrations were significantly higher in HD patients than in the other patients’ groups and the control subjects (P < 0.001); even though the HD patients were chronically taking a lipophilic hydroxymethylglutaryl-CoA reductase inhibitor, an angiotensin-converting enzyme inhibitor and/or an angiotensin receptor blocker, and/or vitamin B complex and C supplements.

expression of CD69 on CD4+ T cells

Absolute total lymphocyte numbers in each group showed no difference (data not shown). However, as indicated in Figure 1A, resting and PHA-stimulated CD4+ T cells from HD patients showed significantly higher expression of cell surface early activation markers (i.e., CD69+) than PD patients, patients with CKD, and control subjects (in both experimental conditions, P < 0.01). Furthermore, CD4+ T cells that were from control subjects and cultured with 10% human uremic serum from HD patients showed a higher percentage of CD69+ T cells (59 ± 9%) than CD4+ T cells that were cultured with 10% human uremic serum from PD patients (55 ± 4%) or patients with CKD after PHA stimulation (50 ± 6%; P = 0.01; Figure 1B).

PHA-stimulated CD69+/CD4+ T Cell Proliferation Response

PHA-stimulated CD69+/CD4+ T cells from HD patients expressed significantly lower proliferation response (2905 ± 202 cpm) than PD patients (4538 ± 317 cpm; P = 0.001), patients with CKD (7682 ± 485 cpm; P < 0.001), and control subjects (5558 ± 534 cpm; P < 0.001) as shown in Figure 2A. When cultured with 10% uremic serum from HD patients (C + HD), PHA-stimulated CD69+/CD4+ T cells from control subjects showed significantly less capacity to proliferate (Figure 2B). However, PHA-stimulated CD69+/CD4+ T cells that were cultured with 10% uremic serum from patients with CKD (C + CKD) showed comparable proliferation to control cells that were cultured in 10% heat-inactivated FCS (Control [C]). Furthermore, for studying the effect of native LDL and oxLDL on T cell proliferation, cells were incubated simultaneously with PHA and LDL or oxLDL. After 72 h of exposure, 200 µg/ml LDL (C + LDL 200) did not modify CD69+/CD4+ T cell proliferation compared with Control [C], whereas oxLDL already inhibited the proliferation at 100 µg/ml (C + oxLDL 100; P = 0.001). The effect of 200 µg/ml oxLDL in this way was close to that obtained with C + HD (Figure 2B). We then investigated various concentrations of oxLDL in CD69+/CD4+ T cell culture. T cells that were cultured in human serum were used for comparison. The inhibition that was induced by oxLDL was dosage and time dependent (data not shown).
Table 1. Baseline characteristics of the study groups of patients and control subjects

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>HD Patients (n = 15)</th>
<th>PD Patients (n = 15)</th>
<th>Patients with CKD (n = 15)</th>
<th>Control Subjects (n = 5)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (yr)</td>
<td>51 ± 5</td>
<td>52 ± 7</td>
<td>50 ± 4</td>
<td>52 ± 4</td>
</tr>
<tr>
<td>Men (n [%])</td>
<td>8 (53)</td>
<td>8 (53)</td>
<td>8 (53)</td>
<td>8 (53)</td>
</tr>
<tr>
<td>BMI (kg/m²)</td>
<td>24.7 ± 3.1</td>
<td>26.2 ± 5.3</td>
<td>23.3 ± 4.2</td>
<td>24.9 ± 7.7</td>
</tr>
<tr>
<td>Waist circumference (cm)</td>
<td>89 ± 4.2</td>
<td>91 ± 4.4</td>
<td>80 ± 6.4</td>
<td>88 ± 3.8</td>
</tr>
<tr>
<td>SBP (mmHg)</td>
<td>151 ± 9d</td>
<td>136 ± 8d</td>
<td>147 ± 5d</td>
<td>15 ± 6</td>
</tr>
<tr>
<td>DBP (mmHg)</td>
<td>91 ± 7d</td>
<td>83 ± 8d</td>
<td>86 ± 5d</td>
<td>77 ± 3</td>
</tr>
<tr>
<td>GFR (ml/min per 1.73 m²)</td>
<td>4.2 ± 2.6d</td>
<td>10.7 ± 2.1</td>
<td>15.2 ± 4.6</td>
<td>98.3 ± 3.8</td>
</tr>
<tr>
<td>CRP (mg/L)</td>
<td>12 ± 4d</td>
<td>14 ±</td>
<td>10 ± 3d</td>
<td>7 ± 2</td>
</tr>
<tr>
<td>Fibrinogen (g/L)</td>
<td>3.57 ± 0.23d</td>
<td>3.41 ± 0.15d</td>
<td>3.64 ± 0.34d</td>
<td>2.78 ± 0.29</td>
</tr>
<tr>
<td>Albumin (g/L)</td>
<td>38 ± 5</td>
<td>36 ± 7</td>
<td>20 ± 4</td>
<td>40 ± 5</td>
</tr>
<tr>
<td>Hb (g/L)</td>
<td>128 ± 6d</td>
<td>12 ± 8d</td>
<td>137 ± 5d</td>
<td>145 ± 8</td>
</tr>
<tr>
<td>Serum iron (mmol/L)</td>
<td>24 ± 3</td>
<td>2 ± 5</td>
<td>22 ± 3</td>
<td>22 ± 5</td>
</tr>
<tr>
<td>Serum uric acid (mmol/L)</td>
<td>420 ± 64</td>
<td>497 ± 63d</td>
<td>483 ± 47d</td>
<td>384 ± 49</td>
</tr>
<tr>
<td>iPTH (ng/L)</td>
<td>229 ± 58d</td>
<td>157 ± 62</td>
<td>118 ± 8d</td>
<td>43 ± 12</td>
</tr>
<tr>
<td>Homocysteine (mmol/L)</td>
<td>15 ± 4d</td>
<td>11 ± 4</td>
<td>12 ± 3</td>
<td>10 ± 3</td>
</tr>
<tr>
<td>EPO dosage (IU/kg per wk)</td>
<td>138 ± 18e</td>
<td>120 ± 13e</td>
<td>62 ± 1</td>
<td>10 ± 3</td>
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<tr>
<td>Statin</td>
<td>15 (100)</td>
<td>15 (100)</td>
<td>15 (100)</td>
<td>15 (100)</td>
</tr>
<tr>
<td>ACE inhibitor</td>
<td>10 (67)</td>
<td>12 (80)</td>
<td>7 (47)</td>
<td>7 (47)</td>
</tr>
<tr>
<td>ARB</td>
<td>5 (32)</td>
<td>7 (47)</td>
<td>2 (13)</td>
<td>2 (13)</td>
</tr>
<tr>
<td>β blocker</td>
<td>7 (47)</td>
<td>8 (53)</td>
<td>12 (80)</td>
<td>12 (80)</td>
</tr>
<tr>
<td>Calcium antagonist</td>
<td>7.47</td>
<td>6 (40)</td>
<td>10 (67)</td>
<td>10 (67)</td>
</tr>
<tr>
<td>Aspirin</td>
<td>12 (80)</td>
<td>11 (75)</td>
<td>15 (100)</td>
<td>15 (100)</td>
</tr>
<tr>
<td>Duration of HD (mo)</td>
<td>25 (10 to 35)</td>
<td>22 (15 to 30)</td>
<td>22 (15 to 30)</td>
<td>22 (15 to 30)</td>
</tr>
<tr>
<td>eKt/Vc</td>
<td>12.4 ± 0.07</td>
<td>7.41 ± 0.14d</td>
<td>6.4 ± 0.07</td>
<td>6.4 ± 0.07</td>
</tr>
<tr>
<td>nPCRc</td>
<td>1.36 ± 0.08</td>
<td>1.20 ± 0.15</td>
<td>1.14 ± 0.08</td>
<td>1.14 ± 0.08</td>
</tr>
</tbody>
</table>

aData are means ± SD, number (percentage), or median (range). ACE, angiotensin-converting enzyme; ARB, angiotensin II receptor blocker; BMI, body mass index; CKD, chronic kidney disease; CRP, C-reactive protein; DBP, diastolic BP; EPO, erythropoietin; Hb, hemoglobin; HD, hemodialysis; iPTH, intact parathyroid hormone; nPCR, normalized protein catabolic rate; SBP, systolic BP.

bNo medication.

cSee Materials and Methods for details.

dWeekly Kt/V.

Table 2. Plasma concentrations of lipids, lipoproteins, and oxLDL, in the study groups of patients and control subjects

<table>
<thead>
<tr>
<th>Parameter</th>
<th>HD Patients (n = 15)</th>
<th>PD Patients (n = 15)</th>
<th>Patients with CKD (n = 15)</th>
<th>Control Subjects (n = 5)</th>
</tr>
</thead>
<tbody>
<tr>
<td>TC (mmol/L)</td>
<td>5.17 ± 0.56d</td>
<td>5.68 ± 0.63b</td>
<td>5.57 ± 0.91c</td>
<td>4.58 ± 0.31</td>
</tr>
<tr>
<td>HDL (mmol/L)</td>
<td>0.16 ± 0.16e</td>
<td>0.86 ± 0.21c</td>
<td>1.08 ± 0.17b</td>
<td>1.87 ± 0.28</td>
</tr>
<tr>
<td>LDL (mmol/L)</td>
<td>2.71 ± 0.38c</td>
<td>3.95 ± 0.44e</td>
<td>4.08 ± 0.81c</td>
<td>2.46 ± 0.11</td>
</tr>
<tr>
<td>TG (mmol/L)</td>
<td>2.39 ± 0.15c</td>
<td>2.88 ± 0.38e</td>
<td>1.99 ± 0.42c</td>
<td>3.12 ± 0.24</td>
</tr>
<tr>
<td>oxLDL (U/L)</td>
<td>90.1 ± 5.9d</td>
<td>77.3 ± 6.3c,d</td>
<td>67.9 ± 2.4b</td>
<td>33.4 ± 5.3</td>
</tr>
</tbody>
</table>

aData are means ± SD. To convert cholesterol in mmol/L to mg/dl, divide by 0.02586; TG in mmol/L to mg/dl, divide by 0.0113.
bMyo, oxLDL, oxidized LDL; cTotal cholesterol; TG, triglycerides.

<refunasable marcador>
Figure 1. (A) Frequency of CD69+/CD4+ T cells in hemodialysis (HD) patients (n = 15; ), peritoneal dialysis (PD) patients (n = 15; ), patients with chronic kidney disease (CKD; n = 15; ), and control subjects (n = 15; ). CD69+/CD4+ T cells were cultured in vitro as resting cells and after phytohemagglutinin (PHA) stimulation. Data are means ± SEM of three independent experiments. (B) CD4+ T cells from control subjects (n = 15) were examined after incubation in 10% human uremic serum (five sera from HD patients [ ], five sera from PD patients [ ] or five sera from patients with CKD[ ]) as resting cells and after PHA stimulation. ANOVA. Data are means ± SEM of three independent experiments.

Effect of oxLDL on CD69+/CD4+ T Cell Viability and Apoptosis

The first signs of oxLDL toxicity were evident in the PHA-stimulated CD69+/CD4+ T cell culture at 10 μg/ml oxLDL, culminating in a 25% loss of viability at 200 μg/ml oxLDL (data not shown). In contrast, native LDL had a minor effect on the viability of PHA-stimulated CD69+/CD4+ T cells from control subjects. PHA-stimulated CD69+/CD4+ T cells from control subjects showed comparable viability when cultured with 10% uremic serum from HD patients (88 ± 6%; P > 0.05) to those that were cultured in the presence of 200 μg/ml oxLDL alone. However, CD69+/CD4+ T cells from HD patients in culture medium without oxLDL showed major destruction (68 ± 5% cell viability). This suggests that in vivo, oxLDL sensitizes CD4+ T cells, which, once stimulated, lose their capacity to proliferate and enter apoptosis.

Figure 3A shows that a significantly higher percentage of PHA-stimulated CD69+/CD4+ T cells from HD patients expressed Fas (31 ± 3%) compared with CD69+/CD4+ T cells from the other patients’ groups and the control subjects (P < 0.001). The higher percentage of Fas-expressing CD69+/CD4+ T cells was confirmed by Western blot (Figure 3B), and enhanced apoptosis was demonstrated directly by DNA analysis after culture and PHA stimulation (Figure 3C). In all experimental conditions, the amount of DNA fragmentation of Fas+/CD69+/CD4+ T cells was significantly higher in HD patients compared with PD patients and patients with CKD (P = 0.005) and control subjects (P = 0.001). Consistently, in control subjects, oxLDL caused a significant and concentration-dependent increase in the percentage of PHA-stimulated Fas+/CD69+/CD4+ T cells (Figure 3D). In contrast, the same concentration of native LDL had a minor effect (Figure
For further evaluation of the effect of oxLDL on PHA-stimulated CD69\(^{+}\)/CD4\(^{+}\) T cell Fas expression, CD69\(^{+}\)/CD4\(^{+}\) T cells from control subjects were incubated with 10% uremic serum from HD patients. The percentage of Fas\(^{+}\)/CD69\(^{+}\)/CD4\(^{+}\) T cells was comparable to the percentage of cells that were cultured in the presence of 200 \(\mu\)g/ml oxLDL (NS), but it was lower than those from HD patients \((P = 0.001)\). Taken together, these data strongly argued for induction of CD69\(^{+}\)/CD4\(^{+}\) T cell apoptosis by oxLDL exposure.

Figure 3. (A) Percentage of PHA-stimulated CD69\(^{+}\)/CD4\(^{+}\) T cell apoptosis determined by Fas (CD95) expression in HD patients (n = 15), PD patients (n = 15), patients with CKD (n = 15), and control subjects (n = 15) analyzed after 72 h of culture. Data are means ± SEM of three independent experiments. *P < 0.001, **P = 0.01 versus CD69\(^{+}\)/CD4\(^{+}\) T cell apoptosis from control subjects. (B) Representative chemoluminescent autoradiography for Fas in CD69\(^{+}\)/CD4\(^{+}\) T cells from HD patients (n = 15), PD patients (n = 15), patients with CKD (n = 15), and control subjects (n = 15). Values that were obtained by densitometric analysis of autoradiography for Fas were expressed relative to the control subjects’ values. Data are means ± SEM of three independent experiments. *P < 0.001, **P = 0.01 versus control subjects. (C) Agarose gel analysis of CD69\(^{+}\)/CD4\(^{+}\) T cell DNA fragmentation. Lanes represent T cell DNA isolated from an HD patient, a PD patient, a patient with CKD, and a control subject. (D) Percentage of PHA-stimulated Fas\(^{+}\)/(CD95\(^{+}\))/CD69\(^{+}\)/CD4\(^{+}\) T cells from control subjects (n = 5) in the presence of LDL or oxLDL. Percentage of PHA-stimulated Fas\(^{+}\)/(CD95\(^{+}\))/CD69\(^{+}\)/CD4\(^{+}\) T cells from control subjects (n = 5) in the presence of 10% uremic serum (HD patients) and from HD patients in culture medium (n = 5) also was analyzed for comparison. Data are means ± SEM of three independent experiments. *P < 0.001, **P = 0.01 versus LDL-treated T cells for the same concentration of lipoproteins; ***P = NS; **P = 0.001 versus oxLDL-treated T cells (200 \(\mu\)g/ml). (E) Fas expression on PHA-stimulated CD69\(^{+}\)/CD4\(^{+}\) T cells from a control subject under experimental conditions of apoptosis induction by LDL and oxLDL. Apoptotic cells induced by either LDL (200 \(\mu\)g/ml) or oxLDL (200 \(\mu\)g/ml) were analyzed by flow cytometry using Fas (CD95)-FITC after 48 h of culture (1 \(\times\) 10\(^6\) cells/ml). Cells were incubated in culture medium alone as control (gray line), with LDL (left) or oxLDL (right). Data are means of Fas-positive CD69\(^{+}\)/CD4\(^{+}\) T cells of three independent experiments (SD <15%).
**OxLDL Effect on Fas-Dependent CD69+/CD4+ T Cell Apoptosis**

For further investigation of the role of Fas in oxLDL-induced CD69+/CD4+ T cell apoptosis, PHA-stimulated CD69+/CD4+ T cells from HD patients were cultured in the presence of anti-Fas mAb. In our experimental conditions, the protection that was conferred by anti-Fas mAb toward oxLDL-induced Fas+/CD69+ T cell apoptosis led to a significant 15% reduction in DNA fragmentation that was caused by oxLDL (Figure 4A). Moreover, Fas expression in the presence of oxLDL and anti-Fas mAb was reduced significantly as shown in Figure 4B. However, the proapoptotic capacity of oxLDL alone was insufficient to explain the high percentage of CD69+ T cells that entered apoptosis in HD patients. We therefore postulated a complementary effect of IL-2 in CD69+ T cell apoptosis.

**IL-2 Levels and sIL-2R Release**

*In vitro* IL-2 levels that were released by PHA-stimulated CD69+/CD4+ T cells from HD patients released significantly less IL-2 (300 ± 24 pg/ml) than those from PD patients (56 ± 53 pg/ml; *P* = 0.01), patients with CKD (805 ± 74 pg/ml; *P* = 0.001), and control subjects (822 ± 69 pg/ml; *P* = 0.001; Figure 5A). After PHA stimulation, sIL-2R levels in CD69+/CD4+ T cell culture supernatants were significantly higher in HD patients compared with patients with CKD (*P* = 0.01) and control subjects (*P* = 0.005). However, after PHA stimulation, the increment in sIL-2R was much more substantial in patients with CKD and control subjects than in HD patients. Of note, *in vitro*, sIL-2R levels from patients with CKD were significantly higher than in control subjects (*P* = 0.01). sIL-2R levels remained stable over time in unstimulated CD69+/CD4+ T cell culture.

As shown in Figure 5B, PHA-stimulated CD69+/CD4+ T cells that were from HD patients and incubated with oxLDL produced significantly less IL-2 than those of control subjects (48 ± 1) after activation. No increase in IL-2 expression was observed in the presence of oxLDL. In contrast, incubation with incremental dosages of oxLDL induced a significant increase of IL-2 levels in CD69+/CD4+ T cell culture supernatants from control subjects. oxLDL induced a dosedependent increase of sIL-2R in CD69+/CD4+ T cell culture supernatants from both HD patients and control subjects. Copper-oxidized LDL, characterized by a mild oxidative stress, indicates that the inhibition of IL-2 expression was specific to activated CD4+ T cells from HD patients. For examination of the effect of oxLDL on IL-2 synthesis by CD69+/CD4+ T cells from HD patients, the intracytoplasmic IL-2 level was analyzed by cytofluorometry. In PHA-stimulated cells, sIL-2+/CD69+/CD4+ T cells were not detectable before 12 h by flow cytometry, and the intracytoplasmic IL-2 levels remained lower than those in CD69+/CD4+ T cells from control subjects (data not shown) (1).

**Bcl-2 and Bax Expression in CD69+ T Cells**

As shown in Table 3, Bcl-2 expression of PHA-stimulated CD69+/CD4+ T cells in HD patients was significantly lower than in PD patients, patients with CKD, and control subjects (*P* = 0.001). In contrast, Bax levels were significantly elevated in HD patients compared with patients with CKD (*P* = 0.001) and control subjects (*P* = 0.005). However, after PHA stimulation, the increment of Bcl-2 and lower Bax levels (Figure 6A).
Bax might be regulated, in part, by IL-2 expression in PHA-stimulated CD69^+ T cells through a Fas-mediated mechanism. Furthermore, in HD patients, oxLDL concentrations seem to contribute to lower IL-2 levels that are released by activated CD69^+/CD4^+ T cells. Interestingly, IL-2 levels nicely correlate with Bcl-2 expression, suggesting a CD69^+/CD4^+ T cell susceptibility to mitochondria-dependent apoptosis pathways.

Modification of LDL may involve the protein and/or the lipid moiety of apoB-apoE. oxLDL, apoB, may be post-translationally glycosylated or desialylated or may react with products of lipid peroxidation (39). In our study, oxLDL concentrations were significantly higher in ESRD patients compared with PD patients, patients with CKD, and control subjects, suggesting a difference in degree of apoB oxidation in patients with ESRD and especially in chronic HD patients (32). Indeed, HD sessions may modify atherosclerotic plaque composition favor plaque disruption, allowing the rise of oxLDL, because oxLDL were documented previously to be enriched in atherosclerotic lesions from HD patients (33). Another possibility may be a consequence of elevated oxidative stress levels and reduced antioxidation molecules secondary to uremia and the HD procedure itself (30,32,33). In patients with ESRD, low molecular weight components such as uric acid, which is present in high concentration in the circulation, may play a critical role in LDL oxidation. Once the lipid hydroperoxide levels within the LDL particle reach a certain threshold, uric acid has the potential to accelerate further peroxidation (34). These observations support the hypothesis that, in HD patients, who are known to present more extended and more severe disrupted plaques, oxLDL are found in the circulation, where they can bind to T cells (30,32,33).

The question thus arises as to the effect of oxLDL on T cell responses. We have addressed this point using oxLDL that were generated by copper oxidation. oxLDL that are generated under these conditions display a linear relationship between the concentration oxLDL (10 to 200 μg/ml) and the Fas-mediated CD69^+/CD4^+ T cell apoptosis. This suggests that not only the extent of oxidation but also the oxLDL concentration may be a contributing factor in CD4^+ T cell dysfunction in HD patients. The possibility that the dosage-dependent apoptosis that was observed in the presence of oxLDL was due to endotoxin contamination seems very unlikely, because no significant amounts of endotoxins were found in oxLDL preparation (≤3 pg/mg oxLDL protein in the test samples). Finally, our results clearly show that the noxious effect of oxLDL is due to a direct effect on T cells and does not require the presence of monocytes.

The results of FACS analysis suggest that, in activated CD4^+ T cells from the patients’ groups and, in particular, from HD patients, oxLDL induce Fas expression, an early-phase marker of cell apoptosis. The evaluation of intracellular Fas synthesis and DNA fragmentation confirms Fas-mediated apoptosis in CD69^+/CD4^+ T cells in response to oxLDL. In contrast, oxLDL do not induce cell necrosis as observed by propidium iodine staining.

**Discussion**

Our previous findings suggested that in chronic HD patients, a significant proportion of activated T cells ultimately did not proliferate but became apoptotic (8). In agreement with previous studies using endothelial or T cells (28,29), our data show that in HD patients, oxLDL induce apoptosis of activated CD69^+ T cells through a Fas-mediated mechanism. Furthermore, in HD patients, enhanced oxLDL concentrations seem to contribute to lower IL-2 levels that are released by activated CD69^+/CD4^+ T cells. Interestingly, IL-2 levels nicely correlate with Bcl-2 expression, suggesting a CD69^+/CD4^+ T cell susceptibility to mitochondria-dependent apoptosis pathways.

**Figure 5. (A) IL-2** (□), and soluble IL-2 receptor (sIL-2R; □□□□□) levels released by CD69^+/CD4^+ T cells in HD patients (n = 15), PD patients (n = 15), patients with CKD (n = 15), and control subjects (n = 15) after PHA stimulation. Correction of values was done on the basis of changes in plasma albumin concentration. Data are means ± SEM of three independent experiments. *P = 0.001; **P = 0.001 (ANOVA). (B) IL-2 and sIL-2R levels released by PHA-stimulated CD69^+/CD4^+ T cells from HD patients (n = 5); IL-2, sIL-2R; and control subjects (n = 5); IL-2, sIL-2R in response to varying dosages of oxLDL. Levels were measured after 48 h. Data are mean ± SEM of three independent experiments.
Overexpression of Fas sensitizes cells to Fas-induced apoptosis, suggesting that increased clustering of Fas on the plasma membrane results in a stronger ability to recruit procaspase-8, which would overcome the sequestering of procaspase-8 by Bcl-2 and could influence the inhibitory function of Bcl-2 or Bcl-xL on Fas-induced apoptosis. Moreover, our experiments with blocking antibodies to Fas suggest that mildly oxidized LDL act mainly by upregulating expression of Fas. Activation of the Fas pathway results in the oligomerization of Fas, in the recruitment of Fas-associated death domain (FADD) and of FADD homologues such as IL-1β-converting enzyme-like protease (FLICE), which activates caspases. The observation that the FLICE inhibitory protein is downregulated by oxLDL further supports the involvement of the Fas pathway in oxLDL-induced apoptosis (38). However, the mechanisms that are involved in Fas expression in response to oxLDL remain to be elucidated. Alcouffe et al. (29) demonstrated that mildly oxidized LDL stimulate Fas expression in PHA-stimulated T cells and their subsequent apoptosis by signaling pathways that involve reactive oxygen species production as well as extracellular signal–regulated kinase and c-Jun N-terminal kinase activation. In such conditions, it is conceivable that interaction of circulating Fas+–activated CD4+ T cells with the vascular wall may even lead to programmed cell death of endothelial cells. Furthermore, the CD4+ T cell apoptosis that is mediated by upregulation of Fas might locally dysregulate the adaptive immune system, facilitating the development of a proatherogenic chronic inflammation.

T cell activation via CD69 usually results in upregulation of cytokines such as IL-2, which exerts unique regulatory effects by controlling CD4+ T cell activation and apoptosis. We found that although the number of CD69+ T cells is significantly higher in HD patients than in control subjects, their proliferative capacity remained low and was associated with high apoptosis rate.

The mechanisms that relate IL-2 expression to T cell apoptosis still are unclear. On the one hand, IL-2 may provide qualitatively or quantitatively distinct signals that trigger T cell apoptosis instead of proliferation. Indeed, one of the major signaling pathways that are mediated by IL-2 is the upregulation of antiapoptotic proteins, including Bcl-2 and Bcl-xL. The low IL-2 levels that were encountered in cultured, activated CD4+ T cells from HD patients may explain the high susceptibility of these cells to become apoptotic in the absence of stimulatory agents such as oxLDL (via Fas pathway). Inhibition of IL-2 synthesis during T cell activation has been shown to downregulate Bcl-2 expression and to inactivate Bcl-2 through a phosphatase (39, 40). In this system, IL-2 deprivation upon oxLDL exposure might result in a gradual disappearance of Bcl-2 that is responsible at least in part, for the higher Fas-mediated apoptotic rate of activated CD4+ T cells in HD patients. Furthermore, proapoptotic Bax was hyperexpressed simultaneously contributing to their apoptosis. Because the precise molecular nature of the defective endogenous IL-2 function is unclear, it is tempting to speculate that the aberrant protein turnover and loss of cell-cycle control that were observed with chronically elevated levels of oxLDL in turn may contribute to IL-2 dysfunction through a yet-undefined uremic oxidative stress–related mechanism.

Our findings may have diagnostic and therapeutic implications. The presence of high rate of Fas+/CD69+/CD4+ T cell apoptosis in patients with ESRD raises the possibility of using such determinations as noninvasive markers for T cell immunodeficiency and for atherosclerosis-mediated microinflammation. Although it would be logical to expect antioxidants to be protective, no effect of anti-oxidation drugs such as HMG-CoA reductase inhibitors was seen in the patients’ groups. Nevertheless, it is worth noting that our study was not designed to evaluate such an effect. However, oxidative stress and other proinflammatory cytokines may represent promising targets for therapeutic strategies to modulate CD4+ T cell immunity and to slow progression of atherosclerosis in HD patients. This provides a rationale to maximize the biocompatibility of the dialysis procedure, that is, selection of nonactivating materials, use of ultrapure dialysis fluid, and, still theoretical, high-flux dialysis to remove oxidative stress.

**Conclusion**

Taken together, our in vitro experiments provide new insights into potential oxLDL-mediated CD4+ T cell dysfunction in patients with ESRD. Our results underline the role of apoptosis control in the pathogenesis of the CD4+ T cell dysfunction in HD patients. These results also define the harmful influence of oxLDL on these cells by increasing Fas-mediated apoptosis. Furthermore, the experimentally documented IL-2 dysregulation may have strong potential to perturb cell-cycle control (1). Indeed, the lower membrane expression of IL-2Ra (CD25) in patients with ESRD may be explained not only by the mild increment of IL-2Ra mRNA synthesis in stimulated CD69+/CD4+ T cells but also by increased shedding of IL-2Ra (1). Furthermore, these results could not rule out the possibility that other oxidative stress may induce Fas upregulation in activated CD4+ T cells and that other mechanisms may be involved in Fas+/CD69+/CD4+ T cell apoptosis in patients.
with ESRD. Further studies using oxLDL and \textit{in vitro} T cell lines are needed to understand better the role that is played by the oxidative stress on T cell function.

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**Disclosures**

None.

**References**


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NOTICE OF RETRACTIONS

At the request of the authors and after careful internal review, the following articles published in the *Journal of the American Society of Nephrology* have been retracted:


Concerns were raised over possible image manipulation and the provenance and integrity of the underlying data for both studies. The corresponding author, Dr. Pascale Meier, takes full and sole responsibility for the inability to present raw data supporting the published results. Coauthors involved in this study were unaware of any misconduct.