Quantification of Carbamylated LDL in Human Sera by a New Sandwich ELISA

Eugene O. Apostolov, Sudhir V. Shah, Ercan Ok, and Alexei G. Basnakian

Background: We previously suggested that increased carbamylated LDL (cLDL), a product of nonenzymatic modification of LDL in human serum by urea-derived cyanate, may cause cardiovascular complications in patients with chronic renal insufficiency. An assay for precise measurement of cLDL in serum was not previously available.

Methods: Polyclonal antibodies against human cLDL and nonmodified, native LDL (nLDL) were raised in rabbits and extensively purified by affinity chromatography. New sandwich ELISAs to measure cLDL and nLDL with use of these antibodies were developed. Serum concentrations of cLDL and nLDL were measured by the sandwich ELISAs in 41 patients with end-stage renal disease (ESRD) and 40 healthy controls.

Results: Both assays showed satisfactory reproducibility, linearity, and recovery. The assays could detect 2.7 mg/L cLDL with a linear detection range of 5–1000 mg/L and 5 mg/L nLDL with a linear detection range of 50–1000 mg/L. These measurements showed that patients with ESRD have significantly increased serum cLDL [281.5 (46.9) mg/L compared with 86.1 (29.7) mg/L in a control group; \( P < 0.001 \)]. There was no significant difference in nLDL concentrations between the groups.

Conclusions: These assays are a potentially valuable tool for cardiovascular research in renal patients and healthy individuals. The cLDL concentration appears to be the highest among all previously described modified LDL isoforms in both controls and ESRD patients.

Chronic renal failure in humans is associated with a severalfold increased risk of developing cardiovascular diseases from atherosclerosis (1–4). Different types of modified LDL have been shown to play a major role in the development of atherosclerosis and cardiovascular diseases. Endothelial cell damage by modified LDL is known to be strongly correlated with atherosclerosis development (5). The most studied modified LDL isoform is oxidized LDL (oxLDL), which has been shown to be a potential factor impacting endothelial cells by recruitment of monocytes/macrophages into vessel walls and accumulation of esterified cholesterol in the cells (6,7). Other LDL isoforms, such as glycated LDL (gLDL) and acetylated LDL (acLDL) have also been shown to participate in atherosclerosis progression (7).

Modification of LDL by carbamylation occurs in the presence of urea. Carbamylation is a spontaneous nonenzymatic modification of proteins and amino acids by urea-derived isocyanate, which is generally present in human serum and is increased in uremic patients (8). The carbamylation of several proteins has been reported recently (9,10) and is usually associated with a partial or complete loss of protein function. Several studies revealed that the protein component of the LDL particle, apolipoprotein B (ApoB), may be carbamylated at lysine or the terminal protein amino acids (8,11–13). The role of carbamylated LDL (cLDL) in atherosclerosis had not been studied, however, partly because of the lack of a reliable quantitative assay.

At present, cLDL can be quantified by colorimetric measurement of protein carbamylation in the LDL fraction after isolation by ultracentrifugation, but this is an expensive, complicated, and time-consuming procedure that requires considerable amounts of serum (14). On the other hand, measurements of other LDL isoforms by
immunoassays using specific antibodies are more precise, simpler, and usually can be performed in a clinical laboratory (15). The aim of the current study was to develop an immunoassay to measure cLDL in human serum. We produced specific anti-cLDL and anti-native LDL (nLDL) antibodies and developed the method based on sandwich ELISA. Using this method, we measured cLDL in controls and demonstrated its increase in patients with end-stage renal disease on hemodialysis.

**Materials and Methods**

**CHEMICALS AND BUFFERS**

Polyclonal anti-human ApoB (goat) antibody was purchased from Rockland. Secondary anti-rabbit IgG and anti-goat IgG antibodies conjugated with horseradish peroxidase (HRP) and the HRP Labeling Kit were obtained from Zymed. Bovine serum albumin (BSA; V fraction) was supplied by Fisher. nLDL from human serum and all other chemicals were purchased from Sigma unless stated otherwise. The buffers and solutions used regularly were as follows: phosphate-buffered saline [PBS; 10 mmol/L sodium phosphate buffer (pH 7.4) containing 140 mmol/L NaCl]; PBS-EDTA (PBS-E), PBS containing 200 μmol/L EDTA; blocking buffer, PBS containing 20 g/L BSA, 0.5 mL/L Tween 20, and 200 μmol/L EDTA; washing buffer (PBS-Tween), PBS containing 0.5 mL/L Tween 20; barbital buffer (Sigma), which was prepared as suggested by the manufacturer; and ELISA stop solution, 1 mol/L H2SO4.

**PREPARATION OF cLDL**

cLDL was prepared in vitro modification of nLDL as described by Weisgraber et al. (12). In short, nLDL (5 g/L protein) supplied in 0.15 mol/L NaCl–0.1 g/L EDTA was diluted to 3.3 g/L protein with sterile 0.3 mol/L sodium borate buffer (pH 8.0). Sterile potassium cyanate (Aldrich) was added to the lipoprotein solution at 20 g/g of LDL protein. The mixture was incubated at 35 °C for 4 h with gentle shaking every hour. Potassium cyanate was removed by extensive dialysis (1000-fold volume of dialysis buffer, repeated three times) against 0.15 mol/L NaCl–200 μmol/L EDTA (pH 7.0) for 36 h under sterile conditions at 4 °C. nLDL was dialyzed separately the same way. A colorimetric method using diacetyl monoxime was used to measure the degree of carbamylation in LDL preparations. The electrophoretic mobilities of nLDL and cLDL were determined by electrophoresis in 0.5% agarose gels containing 2 g/L BSA as suggested by Noble (16). The cLDL prepared as described above was characterized by a degree of carbamylation from 190 to 240 μmol homocitrulline/g of LDL protein and had a relative electrophoretic mobility in agarose of 2.0–2.5 compared with the mobility of nLDL. Oxidation of both LDL isoforms, measured by the thiobarbituric acid reactive substances assay (17–19), was <1 μmol malondialdehyde/g of protein and was not different between the isoforms. cLDL and nLDL stock solutions were prepared by adjustment to 1 g/L protein with PBS-E and were kept at 4 °C away from light and used within 2 weeks after preparation. If sediment appeared during storage, it was precipitated out, and only soluble fractions of the LDL isoforms were used for experiments. Other modified forms of LDL, such as oxLDL, acLDL, and gLDL, were used for antibody testing. These were prepared and dialyzed as described previously (20–22).

**PROTEIN MEASUREMENTS**

Protein concentrations were measured by the BCA protein assay (Pierce) with BSA used as the calibrator.

**PREPARATION OF POLYCLONAL ANTIBODIES**

Polyclonal antibodies against cLDL and nLDL were raised in rabbits. Immunization followed the standard protocol from Strategic Biosolution Company. Rabbits were immunized with 200 μg of either cLDL or nLDL in complete Freund’s adjuvant. Reimmunizations and test bleedings were performed twice with every lipoprotein isoform (200 μg of protein/injection), accomplished with noncomplete Freund’s adjuvant. Final bleeding and exsanguination were performed on days 70 to 75 after immunization was started. The obtained blood sera were kept frozen at −80 °C. Serum proteins were precipitated with saturated ammonium sulfate and then dialyzed against PBS-E three times over 24 h. The antibodies were purified by affinity chromatography using antigens immobilized on cyanogen bromide-activated Sepharose® 4B. The resin and columns were prepared according to the product manual. Briefly, serum proteins were passed through the column with the antigen used for immunization (homologous antigen). Absorbed antibody was eluted with 0.1 mol/L glycine-HCl (pH 2.6) buffer and monitored with a SHIMADZU UV-2100PC spectrophotometer (Shimadzu Corp.) at 280 nm. The resulting immunoglobulins were dialyzed against PBS-E and passed two to four times through an affinity column containing an immobilized heterologous antigen (nLDL column for anti-cLDL or cLDL column for anti-nLDL). After every chromatography, the specificity of purified antibodies was evaluated by Western blotting and indirect ELISA (see procedure below) with both antigens. The final antibody was concentrated in an Amicon-10 filter (Millipore), preserved with 1 g/L NaN3, and stored either for 1 year at 4 °C (working solution) or frozen at −20 °C (stock solution) without detected change of activity or specificity for pure antigens for at least 1 year.

**WESTERN BLOTTING**

For characterization of the obtained antibodies, we performed several forms of Western blotting: (a) dot-blot with pure cLDL and nLDL antigens; (b) Western blotting after nondenaturing electrophoresis in 0.5% agarose gels; and (c) Western blotting with cLDL, nLDL, and ApoB after sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) in 4–20% gradient gels. For dot-
blots, either cLDL or nLDL was applied on a nitrocellulose membrane (1 μg/dot) and dried. Proteins from both agarose and polyacrylamide gels were transferred to a nitrocellulose membrane in Novex transferring buffer (Invitrogen) at 40 V for 2.5–3 h. Membranes were incubated in the blocking solution overnight at 4 °C and then exposed to either anti-cLDL or anti-nLDL antibody (diluted 1:1000). Primary antibodies were detected by goat anti-rabbit IgG conjugated with HRP by use of the SuperSignal chemiluminescence reagent set (Pierce).

**INDIRECT ELISA**

Indirect ELISA was performed to determine the specificities and titers of antibodies as described by Coligan et al. (23). Briefly, the wells in a 96-well plate were precoated with either cLDL or nLDL solution (50 μL of 5 mg/L in PBS-E per well) overnight at 4 °C. The excess protein was washed out with PBS-Tween. The plates were blocked with blocking buffer, probed with the tested antibody for 2 h at room temperature (serial dilutions from 1:10 to 1:100 000 in blocking buffer), washed three times with PBS-Tween, and treated with a 1:20 000 dilution of goat anti-rabbit IgG-HRP conjugate. For detection, 3,3’,5,5’-tetramethylbenzidine was used as a substrate for 5–15 min under visual control. Stop solution was applied to terminate the reaction. Internal controls (calibrators) were applied if more than one plate was processed at one time. Absorbance was measured at 450 nm by the Synergy HT-I plate reader (Bio-Tek). The mean background of control wells was subtracted. All measurements were performed in quadruplicate.

**Table 1. Clinical and biochemical characteristics of control and patient groups.**

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Controls (n = 40)</th>
<th>Patients (n = 41)</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age, years</td>
<td>43.0 (8.9)</td>
<td>46.8 (1.8)</td>
<td>NS</td>
</tr>
<tr>
<td>Gender, F/M</td>
<td>24/16</td>
<td>22/19</td>
<td></td>
</tr>
<tr>
<td>Race (Caucasian), %</td>
<td>100</td>
<td>100</td>
<td>NS</td>
</tr>
<tr>
<td>Body weight, kg</td>
<td>61.8 (3.8)</td>
<td>58.3 (1.2)</td>
<td>&lt;0.005</td>
</tr>
<tr>
<td>Arterial hypertension</td>
<td>0</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>Smoking, %</td>
<td>30</td>
<td>17</td>
<td>NS</td>
</tr>
<tr>
<td>Cholesterol, mg/L</td>
<td>1887.0 (87.0)</td>
<td>1746.0 (64.0)</td>
<td>NS</td>
</tr>
<tr>
<td>Triglycerides, mg/L</td>
<td>1360.0 (230.0)</td>
<td>1822.0 (283.0)</td>
<td>NS</td>
</tr>
<tr>
<td>Albumin, g/L</td>
<td>41.0 (1.0)</td>
<td>38.0 (1.0)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Hematocrit, %</td>
<td>38.3 (0.3)</td>
<td>29.9 (0.7)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Creatinine, mg/L</td>
<td>8.7 (0.2)</td>
<td>93.0 (4.0)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Urea, mg/L</td>
<td>270.5 (17.0)</td>
<td>1330.0 (49.0)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>nLDL, mg/L</td>
<td>1420.0 (50.0)</td>
<td>1210.0 (60.0)</td>
<td>NS</td>
</tr>
<tr>
<td>cLDL, mg/L</td>
<td>86.1 (29.7)</td>
<td>281.5 (46.9)</td>
<td>&lt;0.001</td>
</tr>
</tbody>
</table>

*Parametric data are presented in actual values; nonparametric data are presented as 0/1 for no/yes.
* Mean (SE).
* NS, difference not significant by Student or/and Mann–Whitney U-test.
* Normal blood pressure in patients was maintained by volume control during hemodialysis and low-salt diet.

**PREPARATION OF ANTIBODY-HRP CONJUGATE**

To detect LDL particles by sandwich ELISA, we labeled anti-ApoB antibody with HRP according to the manual for the HRP Labeling Kit. Briefly, antibody was dialyzed against 0.1 mol/L NaHCO₃ (pH 9.5), concentrated to 2 g/L, and coupled to active HRP for 3 h at room temperature. The remaining HRP was blocked with 1 mol/L l-lysine. The labeled antibody was diazylated against PBS and preserved with the reagent provided in the HRP Labeling Kit.

**COLLECTION AND STORAGE OF SERA**

Written informed consent was obtained from all participants. The consent form and study design were approved by the Institutional Review Boards of Ege University Medical School and the University of Arkansas for Medical Sciences/Central Arkansas Veterans Healthcare System. Sera were obtained from venous blood samples from 81 individuals [40 controls and 41 patients with chronic renal failure (collected before hemodialysis)] at the Ege University Medical School hospital. Both groups were matched by gender and age and did not have significant differences of many characteristics except those related to uremia and dialysis (e.g., body weight, hematocrit, and serum creatinine, urea, and albumin concentrations; Table 1). Other indices in patients (e.g., cardiothoracic index, heart rate, systolic and diastolic blood pressures, and serum phosphate concentration) were within reference values. Blood samples from patients and healthy volunteers were collected in the morning into dry tubes without preservatives, allowed to clot at 37 °C for 30 min, and centrifuged at 2500 g for 10 min; the obtained sera were transported to the laboratory on wet ice and stored at 4 °C with 200 μmol/L EDTA, 0.05 g/L butylated hydroxytoluene.

**Fig. 1. Specificity of the anti-cLDL and anti-nLDL antibodies.**

(A), antibody specificity was measured before and after purification by indirect ELISA as described in the Materials and Methods. (B), Western dot-blot analysis of the purified antibodies.
ene, and 1 g/L sodium azide. At the same time, small aliquots of sera were frozen and stored at −80 °C.

**SANDWICH ELISA**

The sandwich ELISA was performed according to the general procedure described by Engvall and Perlman (24) as modified by Holvoet et al. (25). Polystyrene 96-well plates (Costar) were coated with 50 μL/well of either anti-cLDL or anti-nLDL antibody in PBS at 4 °C overnight. For each plate, control wells were coated with antibody vehicle. The plates were rinsed three times with washing solution and blocked with blocking solution at 37 °C for 2 h. Calibrators were prepared as described above. All patient sera were diluted 1:200 and 1:1000 in blocking buffer for the cLDL and nLDL measurement assays, respectively. The wells were filled with 80 μL of blocking buffer and 20 μL of diluted patient serum or calibrator and incubated for 2 h at 37 °C. The final dilutions for serum samples were 1:1000 and 1:5000 for the cLDL and nLDL assays, respectively. After being washed six times, the wells were incubated with anti-ApoB-HRP conjugate (1:1000 dilution; 50 μL/well) at room temperature for 2 h and washed as described above. Bound HRP conjugate was detected with 3,3′,5,5′-tetramethylbenzidine for 10 min (50 μL/well) at room temperature. The reaction was terminated with ELISA stop solution (50 μL/well), and the absorbance was measured at 450 nm within 30 min. The background of the wells that were not coated with an antibody was subtracted. All measurements were performed in quadruplicate. An internal standard with a known concentration of antigen was added to wells in every plate.

**STATISTICAL ANALYSES**

Results are reported as the mean (SE). Statistical analysis was performed with the ANOVA, the Student *t*-test, and the Mann–Whitney *U*-test. Linear regression analysis included the *F*-test, normality, and CV tests. Nonnumeric data were coded as 0/1 (no/yes), randomized, and subjected to a nonparametric analysis. Spearman rank correlation coefficients were computed to identify correlations that were significantly associated with differences among cLDL values. Significance was set at *P* < 0.05.
Results

Characterization of Anti-cLDL and Anti-nLDL Antibodies

Polyclonal antibodies against cLDL and nLDL were raised in rabbits and purified by affinity chromatography as described in the Materials and Methods. Characterization of the final antibodies showed that both antibodies had high specificities to their own antigen with no or minimal cross-reactivity to the heterologous antigen. The ratios of specificity measured by indirect solid-phase ELISA for anti-cLDL and anti-nLDL before purification were 10.6 (cLDL/nLDL) and 5.7 (nLDL/cLDL), respectively. After purification, the ratios were 234 and 255 for anti-cLDL and anti-nLDL, respectively (Fig. 1). Cross-reactivity of anti-cLDL antibody to gLDL, acLDL, and oxLDL, evaluated by indirect ELISA, was extremely low (ratios, 380 for cLDL/gLDL, 810 for cLDL/acLDL, and 2630 for cLDL/oxLDL).

We examined the specificity and cross-reactivity of anti-cLDL antibody to other possible antigens by agarose gel electrophoresis and Western blotting. The results suggested that nLDL, native VLDL, native HDL, and carbamylated HDL were not recognized by anti-cLDL (Fig. 2). The only major band observed in the blot was cLDL. A minor band was detected with carbamylated VLDL particles, perhaps resulting from carbamylation of ApoB, which has been previously described in the protein profile of VLDL (26). Control human serum samples subjected to the same Western blotting showed single main bands with electrophoretic mobilities similar to that for nLDL. SDS-PAGE of cLDL, nLDL, ApoB protein, and human sera followed by Western blotting confirmed that ApoB is the main target for anti-cLDL and anti-nLDL antibodies (Fig. 3). Subjected to the same Western blotting analysis, human serum samples showed the only major band with a mobility similar to that of ApoB. No cross-reactivity or interference was detected with globulins (~100–120 kDa), albumin (67 kDa), or other low-molecular-mass proteins (down to 8 kDa) with the anti-nLDL or anti-cLDL antibody.

An anti-ApoB-HRP conjugate, used as a detection antibody in the sandwich ELISA system, was able to detect both cLDL and nLDL (Fig. 4A). The epitope competition between anti-cLDL and ApoB-HRP was 5%, whereas the competition between anti-nLDL and ApoB-HRP was <2% (Fig. 4B).

Sandwich ELISA for cLDL and nLDL Quantification

For calibration of the ELISA, cLDL and nLDL calibration solutions were prepared as described earlier. Both systems showed a dose-dependent response for cLDL or nLDL measurements with calibrators and human sera (Fig. 5). Absorbance was predictable by the dilution factor (P < 0.05) with normality and CV tests passed.

To evaluate the amounts of cLDL and nLDL in serum, we used extensively chemically carbamylated LDL or nLDL as calibrators. Calibrators were applied in quadruplicate to every plate the same way as the samples. Calibration curves were established for cLDL and nLDL (Fig. 6). The intra- and interassay CVs of the ELISAs were
4.3–6.8% and 7.9–10% for the cLDL assay and 1.6–3.5% and 2.4–4.5% for the nLDL assay, respectively. The assay was linear for cLDL concentrations of 5–1000 mg/L and nLDL concentrations of 50–1000 mg/L, which were designated as the detection ranges for these assays. The detection limits were 2.7 and 5 mg/L for cLDL and nLDL, respectively. We studied analytical recovery by adding sufficient amounts of either cLDL or nLDL to human serum samples. The mean index showed recoveries of 99% and 98% of those predicted for the cLDL and nLDL assays, respectively (Table 2).

The preservatives and antioxidants used (EDTA, butylated hydroxytoluene, sodium azide) did not affect the measurement of cLDL or nLDL in human sera. According to our data, signal depression never exceeded 0.4% and was not significant. There was no substantial interference from hemoglobin, triglycerides, human albumin, human γ-globulins, EDTA, butylated hydroxytoluene, sodium azide, or Tween 20.

We also determined whether sample freezing affected the cLDL or nLDL measurements. A short single freezing-thawing cycle produced an equal absorbance increase at 450 nm of 1.7- to 2-fold in serum samples and control antigens for both cLDL and nLDL measurements. Mea-

Table 2. Analytical recoveries of cLDL and nLDL in the sandwich ELISAs.

<table>
<thead>
<tr>
<th>Amount added, mg/L</th>
<th>Serum 1 Measured, mg/L</th>
<th>Recovery, %</th>
<th>Serum 2 Measured, mg/L</th>
<th>Recovery, %</th>
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<tbody>
<tr>
<td>cLDL</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>None</td>
<td>340</td>
<td>99</td>
<td>39</td>
<td>98</td>
</tr>
<tr>
<td>5</td>
<td>311</td>
<td>90</td>
<td>43</td>
<td>98</td>
</tr>
<tr>
<td>10</td>
<td>353</td>
<td>101</td>
<td>47</td>
<td>96</td>
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<td>371</td>
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<td>97</td>
</tr>
<tr>
<td>100</td>
<td>473</td>
<td>107</td>
<td>141</td>
<td>101</td>
</tr>
<tr>
<td>Mean recovery</td>
<td></td>
<td>99</td>
<td>98</td>
<td></td>
</tr>
<tr>
<td>nLDL</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>None</td>
<td>980</td>
<td>99</td>
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<td>1000</td>
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<tr>
<td>Mean recovery</td>
<td></td>
<td>97</td>
<td>98</td>
<td></td>
</tr>
</tbody>
</table>

* The recoveries were tested in two human serum samples as described in the Materials and Methods.
We measured serum cLDL and nLDL in age- and gender-matched healthy control volunteers and uremic patients as described above. We observed no significant differences between these two groups for smoking habits, presence of diabetes mellitus or cardiovascular disease, blood pressure, or triglyceride or cholesterol concentrations. Patients had significantly lower body weights and increased serum creatinine concentrations compared with controls. The mean (SE) serum cLDL concentrations were 86.1 (29.7) mg/L (range, 0.4–905.0 mg/L) for the controls and 281.5 (46.9) mg/L (range, 4.0–966.0 mg/L) for the uremic patients. The nLDL concentration in serum did not vary as much as the cLDL concentration [1420.0 (50.0) mg/L (range, 890.0–2200.0 mg/L) for controls and 1210.0 (60.0) mg/L (range, 800.0–2000.0 mg/L) for patients; Fig. 7]. The difference in cLDL concentrations was significant (P < 0.001), whereas nLDL was not significantly different between these two groups.

Because cLDL is a part of the total LDL fraction, we analyzed whether the cLDL increase in patients reflects a possible difference in total LDL. We compared the cLDL/nLDL and cLDL/total cholesterol (TC) ratios in both groups and found that patients had significantly more cLDL per nLDL or TC unit. Correlation analysis revealed a moderate positive correlation in both groups between cLDL concentration and female gender (r = 0.52; P < 0.05) and a weak positive significant correlation between cLDL concentration and age (r = 0.28; P < 0.05). We compared the amount of nLDL in serum with the TC concentration measured by an enzymatic method; the results indicated a strong positive correlation between TC and nLDL (r = 0.95; P < 0.001).

**Discussion**

Uremia is known to be associated with the appearance of modified LDL. Gonen et al. (5) named the special type of LDL that appeared as a result of ApoB carbamylation in chronic renal failure patients “uremic LDL” because of the absorbance and binding-to-receptor properties differed from those of nLDL. Kraus and Kraus (8) described cLDL generation as a product of the chemical modification of nLDL by urea-derived isocyanate. An etiopathogenetic role of cLDL for atherosclerosis development in uremic patients was hypothesized by Horkko and coworkers (27, 28). Roxborough et al. (29) suggested the possible atherogenic activity of cLDL through intensified LDL oxidation, but they did not show that cLDL provokes atherosclerosis by a direct detrimental effect on endothelial cells. We have recently postulated and demonstrated in vitro that cLDL induces injury and the dysfunction of endothelial cells (30, 31). Our studies showed that endothelial cell damage by cLDL in vitro is dose-dependent. The quantity of serum cLDL may therefore be critical for atherosclerosis development.

There are several methods to characterize and measure different LDL isoforms. The classic methods are mainly based on gradient ultracentrifugation followed by LDL isolation and measurement of protein modification (32, 33). Protein carbamylation in serum can be assayed by a colorimetric method using diacetyl monoxime as a detector and homocitrulline as a calibrator (14). Alternatively, carbamylated proteins can be measured by HPLC...
(34–37). To maximize the specificity and preciseness of measurements, sandwich ELISA is usually a method of choice for assaying of proteins and lipoproteins in serum (38, 39). Antibody specificity is important in immunologic assays such as ELISAs (23). Modified LDLs have been shown to induce specific antibodies in vivo (40). For the purpose of measuring cLDL, we raised an antibody that specifically recognizes cLDL and developed a sandwich ELISA method to detect cLDL in human serum.

Although chemicals have not been found to seriously impact the assay, a single freeze-thaw cycle increased the plate reading. The same effect was described for the sandwich ELISA-based detection of oxLDL (15) and may perhaps be explained by LDL particle destruction followed by better antigen presentation to the antibody (41). However, evaluation of measurements of frozen-thawed samples may be possible with a calibrator that has been prepared and stored under the same conditions.

Our assay allowed the measurement of cLDL concentrations in human sera from uremic patients and healthy individuals. Both groups were matched for several variables, but the patients had increased creatinine concentrations and decreased body weights compared with the controls, which were considered as signs of chronic renal failure. Increased creatinine is a commonly used marker in uremia or chronic hemodialysis. The concentration of oxLDL was 10.8 (2.8) units/mL (15), or 0.5 (0.3) units/μg or LDL protein (32) (1 unit was equivalent to 1 μg of mild oxLDL). The cLDL concentration in our study was much higher [~86.0 (29.7) mg/L] than the reported concentrations of oxLDL or MDA-LDL. We demonstrated that cLDL in patients with chronic renal failure was increased 3.3-fold, to 281.5 (46.9) mg/L. The MDA-LDL was increased 2.5- to 5-fold to 15.8 (15.0) mg/L or 3.7 (2.0)% of the total LDL pool (~37 mg/L), and oxLDL was detected at an 8-fold higher concentration (32, 33, 50). Thus, our results showed that cLDL is most common LDL isoform in both healthy individuals and in uremic patients on hemodialysis. Taken together with our previous data on the cytotoxicity of cLDL to endothelial cells (30, 31), we can hypothesize that cLDL is an important proatherosclerotic LDL isoform in patients with end-stage renal disease and in humans in general.

In conclusion, this is the initial presentation of a simple, rapid, and effective method of cLDL detection in human serum. This method meets the requirements of a method that can be used for future studies of the impact of carbamylation on the lipoprotein profile and atherosclerosis development.

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