Carbamylated low-density lipoprotein induces death of endothelial cells: A link to atherosclerosis in patients with kidney disease

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Carbamylated low-density lipoprotein induces death of endothelial cells: A link to atherosclerosis in patients with kidney disease.

Background. The presence of accelerated atherosclerosis in patients with kidney disease cannot be entirely explained by traditional cardiovascular risk factors. Exposure to urea, which is normally present in human blood plasma and elevated in patients with kidney disease, leads to the carbamylation of proteins. We postulated that low-density lipoprotein (LDL) carbamylated by urea has biologic effects relevant to atherosclerosis.

Methods. To produce carbamylated LDL (cLDL), human native LDL (nLDL) was chemically modified in vitro by exposure to potassium cyanate. Human coronary artery endothelial cells (HCAECs) and human coronary artery smooth muscle cells (CASMCs) were treated in vitro with cLDL or nLDL. Irreversible cell death was measured using the lactate dehydrogenase (LDH) assay, apoptosis was assessed by annexin V binding, and proliferation was determined using bromodeoxyuridine (BrdU) incorporation. Total plasma protein carbamylation and plasma cLDL were measured in hemodialysis patients using the homocitrulline assay and enzyme-linked immunosorbent assay (ELISA).

Results. Our studies demonstrated that cLDL but not nLDL induced dose-dependent vascular cell injuries relevant to atherosclerosis, which included the proliferation of vascular smooth muscle cells and endothelial cell death. Under light microscopy, endothelial cells treated with cLDL showed signs of morphologic alterations. The injury to endothelial cells measured by LDH release was time-dependent and correlated with the degree of LDL carbamylation. At least a part of the endothelial cell population treated with cLDL died by apoptosis. In patients with advanced renal disease on hemodialysis, total plasma protein carbamylation and plasma cLDL were several times higher than in control healthy individuals.

Key words: low-density lipoprotein, carbamylation, carbamylated LDL, kidney, end-stage renal disease, endothelial cells.

Conclusion. Collectively these data suggest the potential role of carbamylated LDL in accelerated atherosclerosis in patients with chronic renal disease and, possibly, in healthy individuals.

Chronic kidney disease is a worldwide medical and public health problem that is beginning to assume epidemic proportions. Chronic kidney disease, which can result from a wide variety of disorders (including diabetes, hypertension, and glomerulonephritis) is a common disorder affecting about 10% of the worldwide population [1–3]. A large body of evidence indicates that kidney disease is an independent risk factor for the development of cardiovascular disease [4]. Cardiovascular disease is a leading cause of death in patients with chronic renal insufficiency. After stratification for age, gender, race, and the presence or absence of diabetes, the cardiovascular mortality in patients with advanced kidney disease is 10 to 20 times greater than in the general population [4]. The increased prevalence of coronary artery disease in patients with kidney disease cannot be explained entirely by traditional cardiovascular risk factors.

Carbamylation is a protein modification induced by urea-derived cyanate normally present in human blood plasma and elevated in patients with chronic kidney disease [5]. We postulated that in patients with renal disease, carbamylation of low-density lipoprotein (LDL) is a nontraditional risk factor for cardiovascular disease. In order to obtain support for this hypothesis, we examined whether carbamylated LDL (cLDL) has biologic effects that are relevant to atherosclerosis. It is commonly accepted that endothelial cell injury is an initial event in atherosclerosis [6, 7]. Injured endothelial cells attract monocytes, which burrow beneath the endothelial cell layer and ingest modified LDL to form “foam cells” [8]. This process leads to the formation of atherosclerotic plaque (consisting of “foam cells” and macrophages covered by a fibrous cap) which protrudes into the vessel.
lumen over the proliferating vascular smooth muscle cells [8].

In the present study, we tested the atherosclerosis-prone effects of chemically cLDL: the ability to cause death of cultured human coronary artery endothelial cells (HCAECs) and induce proliferation of human vascular smooth muscle cells in vitro. In addition, we studied total plasma protein carbamylation and cLDL level in end-stage renal disease (ESRD) patients receiving hemodialysis in comparison to the control group of healthy individuals.

METHODS

LDL isoforms

Native human LDL (nLDL) was purchased from Fluka (Milwaukee, WI, USA). cLDL was prepared as described by Weisgraber, Innerarity, and Mahley [9]. Sterile potassium cyanate (KOCN) (Aldrich, Milwaukee, WI, USA) was added to the lipoprotein solution at 20 mg/mg LDL protein. The mixture was incubated at 35°C for 4 hours. KOCN was removed by excessive dialysis under sterile conditions at 4°C against 0.15 mol/L NaCl, 0.01% ethylenediaminetetraacetic acid (EDTA), pH 7.0, for 36 hours. About 5 mL of the LDL preparation was dialyzed against 5 L buffer, which was changed every 12 hours. The concentration of potassium cyanate after the first dialysis was 3.7 mmol/L and could not be detected after second or third dialyses using Dimension RxL analyzer (Dade Behring, Deerfield, IL, USA). At this concentration, KOCN did not have any cytotoxic effect on the endothelial cells. In addition, used buffer after the second or third dialyses did not have any effect in control experiments. cLDL was kept at 4°C, away from light and used within 2 weeks after preparation.

Lipoprotein electrophoresis

The electrophoretic mobility of nLDL and modified LDLs was determined by electrophoresis on 0.5% agarose gel in 50 mmol/L sodium barbital buffer, pH 8.6, as described by Noble [10]. After applying the samples (7 μg LDL protein per well), electrophoresis was run at 12 V/cm for 1 hour. The gel was fixed in ethanol-acetic acid-water mixture (60:10:30, vol:vol:vol) and stained in 0.2% Sudan Black B dissolved in 60% ethanol. The relative electrophoretic mobility (REM) was expressed as the ratio of the mobility of a modified LDL to that of the nLDL.

Assessment of carbamylation

A colorimetric method using diacetyl monoxime [11] was used to measure the degree of carbamylation in LDL preparations. Briefly, the LDL suspension (25 μg of protein) was digested in 50 μL phosphate-buffered saline (PBS), pH 7.4, 1% sodium dodecyl sulfate (SDS) with 2 μg proteinase K at 37°C for 2 hours. Then 250 μL of urea-nitrogen reagent (0.83 mol/L sulfuric acid, 1.13 mol/L orthophosphoric acid, 0.55 mmol/L thiosemicarbazide, and 2.6 mmol/L cadmium sulfate) and 50 μL diacetyl monoxime were added to the reaction mixture and the incubation continued at 97°C for 30 minutes. Precipitate was removed by centrifugation at 3500g for 10 minutes at room temperature. The supernatant (200 μL) was transferred into a 96-well plate and absorption was measured at 530 nm. A standard curve was generated using homocitrulline (e-amino-carbamyllysine, 0 to 30 nmol) (Advanced Asymmetrics, Millstadt, IL, USA). The results were expressed in nmol homocitrulline/mg LDL protein.

Assessment of oxidation

Oxidation of LDL was evaluated using the thiobarbituric acid reactive substances (TBARS) assay [12]. Freshly prepared 1,1,3,3-tetramethoxypropane, which yields malondialdehyde (MDA), was used as a standard. The results were expressed in nmol MDA/mg LDL protein.

Cell cultures

HCAECs and human coronary artery smooth muscle cells (CASMCs) were obtained from Clonetics (Walkersville, MD, USA) and used at passages between 4 and 8. HCAECs and CASMCs were maintained in endothelial growth medium microvasculature (EGM-MV) or smooth muscle growth medium-2 (SmGM-2) (Cambrex, Baltimore, MD, USA), respectively, supplemented with growth factors and 5% fetal bovine serum (FBS). Cells were grown in a humidified incubator (5% CO2, 37°C) and were treated with 50 to 400 μg/mL LDL isoforms in serum-free EGM-MV medium for 2 to 24 hours. Control cells were treated with PBS for the same period of time.

Cytotoxicity assays

After exposure to LDL isoforms, a lactate dehydrogenase (LDH) release assay kit (Promega, Madison, WI, USA) was used to measure cytotoxicity. The latter was expressed as the ratio of LDH released by treated cells into medium to the total LDH. Trypan blue exclusion was measured in combined attached and floating cells using the previously described procedure [13].

 Annexin V binding

HCAECs were treated with 200 μg/mL LDL isoforms for 16 hours in serum-free medium, and floating and attached cells were collected by centrifugation at 200g. Cell suspension was stained using ApoAlert Annexin
V Apoptosis Kit (Clontech, Palo Alto, CA, USA) and analyzed in a Becton Dickinson FACScan flow cytometer (San Jose, CA, USA).

**Bromodeoxyuridine (BrdU) assay**

The BrdU cell proliferation assay (Oncogene, Cambridge, MA, USA) was used to identify cells in the S phase of the cell cycle. CASMCs were seeded into a 96-well plate (3.5 × 10³ cells/well) and grown overnight in SmBM medium with 5% FBS. The cells were rinsed once with serum-free medium and then exposed to 0 to 200 µg/mL LDL isoforms in serum-free medium. One hour prior to the end of the experiment the BrdU label was added. Cells were fixed, denatured, and probed with anti-BrdU antibody as suggested by the manufacturer’s manual.

**Protein measurement**

Protein was measured using the BCA Protein Assay (Pierce, Rockford, IL, USA). Bovine serum albumin (BSA) was used as the standard.

**Human subjects**

Written informed consent was obtained from all patients and control subjects. The consent form and study design were approved by the University of Arkansas for Medical Sciences/Central Arkansas Veterans Healthcare System Institutional Review Board. Blood samples were obtained from 13 hemodialysis patients and 11 healthy subjects. In patients, the blood was collected immediately before dialysis. The groups were matched by age (48.2 ± 4.6 and 59.7 ± 3.9 in patients and control group, respectively) (P > 0.1) and gender (seven males and six females among patients, six males and five females in the control group) (P > 0.9) and had no significant difference by all used criteria, including body weight, smoking habit, diabetes, cardiovascular diseases, cholesterol, triglycerides, and others.

**Enzyme-linked immunosorbent assay (ELISA) detection of cLDL**

Antibody to cLDL was raised in rabbits and purified by affinity chromatography until they had no cross-reactivity with nLDL in ELISA or Western blotting (data not shown). Measurement of cLDL in fasting serum collected before dialysis using ELISA was performed according to the protocol described by Lorec et al [14], with the exception that anti-cLDL was used as primary antibody. Serum was used in a dilution 1:200. All measurements were performed in quadruplicating format.

![Fig. 1. Characterization of chemically prepared carbamylated low-density lipoprotein (cLDL). (A) Mobility of cLDL (visualized by a Sudan Black staining) in agarose gel is increased compared to native LDL (nLDL). (B) In vitro cLDL has increase portion of homocitrulline (carbamyl lysine), but is not oxidized as measured by the malondialdehyde (MDA) content.](image)

**Statistical analysis**

Statistical analysis was performed with analysis of variance (ANOVA) and Student t test. Results were expressed as mean ± standard error of the mean (SEM).

**RESULTS**

**Characterization of LDL isoforms**

During carbamylation, urea undergoes a spontaneous nonenzymatic transformation to cyanate, the active form of which, isocyanic acid, reacts with free nonprotonated amino groups of proteins. Carbamylation results in neutralization of the positive charge of the modified lysine and enhances the mobility of the LDL in agarose [5]. In our experiments, the electrophoretic mobility of cLDL was accelerated after carbamylation of nLDL with KOCN (Fig. 1A). The degree of carbamylation was assessed by measurement of homocitrulline, which indicated that after 6 hours of carbamylation, cLDL contained 221 ± 16 homocitrulline nmol/mg LDL protein, compared to 5 ± 1 nmol/mg protein in nLDL (Fig. 1B). Importantly, spontaneous oxidation did not occur during
carbamylation, as indicated by a low content of MDA in both nLDL (0.9 ± 0.3 nmol MDA/mg LDL protein) and in cLDL (0.7 ± 0.3 nmol MDA/mg LDL protein), thus providing us with the specific LDL isoforms for the experiments.

Assessment of cLDL cytotoxicity

To assess cytotoxicity of cLDL, cultured HCAECs were treated with 50 to 400 µg/mL LDL isoforms for up to 24 hours. Under light microscopy, cells treated with nLDL looked unaffected (Fig. 2). To the contrary, cells treated with cLDL showed signs of morphologic alterations and the presence of cellular debris. Many of the cells shrunk and detached from the plastic (Fig. 2).

LDH release assay was applied to assess irreversible cell injury. This assay showed that cLDL, but not nLDL, induced the increased release of LDH into the culture medium. At 200 µg/mL, cytotoxicity was 20 ± 2% with cLDL compared to 7 ± 1% induced by nLDL (Fig. 2).

To further characterize the cytotoxicity, cultured HCAECs were treated with 200 µg/mL LDL isoforms for a period of 2, 4, 8, 12, or 24 hours. The injury measured by LDH release was dose- and time-dependent, and correlated with the degree of LDL carbamylation (Fig. 3). Cytotoxicity measured using trypan blue exclusion in combined attached and floating cells was higher in the cell population treated with cLDL than with nLDL (9 ± 2% vs. 3 ± 2%, respectively). Total cell number was decreased 10% after 200 µg/mL cLDL treatment, while the same concentration of nLDL did not induce the reduction of cell number.

We also examined the ability of cLDL to induce apoptosis. For this we utilized the annexin V assay, which is based on the binding of annexin V to phosphatidylserine and provides a simple and accepted method to detect apoptosis at a very early stage. Fluorescence-activated cell sorting (FACS) analysis of cells stained with fluorescein-conjugated annexin V and propidium iodide was applied. It showed that endothelial cells exposed to cLDL revealed a higher percentage of apoptotic cells (annexin V+/propidium iodide−) than nLDL (24 ± 4% vs. 14 ± 3%) (P < 0.01) (Fig. 4). No significant change in the percentage of necrotic cells (annexin V−/propidium iodide+) plus annexin V+/propidium iodide+) was observed (35 ± 5% vs. 33 ± 4%) (P < 0.05).

Induction of vascular smooth muscle cell proliferation by cLDL

The proliferation of vascular smooth muscle cells is an important component of atherogenesis, contributing to narrowing of the vessel lumen [8]. We examined the ability of cLDL to induce proliferation of cultured human CASMCs utilizing BrdU incorporation into newly synthesized DNA. Our experiments demonstrated that although nLDL induced some CASMCs proliferation, cLDL induced proliferation of these cells several times higher at all used concentrations (Fig. 5). The effect of cLDL on CASMC proliferation was dose-dependent.

Measurement of cLDL in hemodialysis patients

As an approach to examine the relevance of LDL carbamylation to the increased incidence of atherosclerosis in patients with kidney disease, total plasma protein carbamylation was measured by homocitrulline assay and plasma cLDL was assayed by ELISA using antihuman cLDL. Our data showed that patients with advanced renal failure receiving hemodialysis treatment had significantly elevated level of protein carbamylation to 42 ± 4 nmol homocitrulline/mg protein (N = 13) above the control group level of 12 ± 3 nmol homocitrulline/mg protein (N = 11) (P < 0.01) (Fig. 6A). In good correlation with this observation, cLDL measured by ELISA was increased in hemodialysis patients to 0.352 ± 0.051 from the control level of 0.096 ± 0.030 (P < 0.001) (Fig. 6B).

DISCUSSION

Based on studies of oxidized LDL (oxLDL) and LDL with other modifications, it is commonly accepted that endothelial cell injury by modified LDLs is an initial event in atherosclerosis [6, 7]. Although different modified LDLs were found in uremic patients [15, 16], and oxLDL was shown to be elevated in uremic patients [17–19], no
mechanistic studies to establish a cause-effect relationship between oxLDL or another modified LDL and atherosclerosis during uremia were performed.

Urea is a normal component of human blood plasma where it undergoes spontaneous transformation to cyanate. With the decrease of renal function, there is an increased amount of urea, and cyanate acts as a uremic toxin through the carbamylation of proteins [5]. Cyanate can react reversibly with the free epsilon amino groups of lysine and N-terminal amino acids within proteins. The resulting in vivo carbamylation can change the structure of proteins [20, 21], and modify the activity of enzymes, cofactors, hormones, and antibodies [5, 22].

cLDL was shown to interact with cell surface receptors in human fibroblasts and prevent the binding of nLDL in human fibroblasts [9]. LDL isolated from uremic patients as well as chemically modified cLDL had a slower clearance from plasma in rabbits than LDL from normal subjects or nonmodified LDL [23, 24]. The effects of cLDL on endothelial cells have not been previously studied.

Our data indicate that human cLDL induces injury to endothelial cells in vitro, as measured both by LDH release (irreversible cell death) and by annexin V binding (apoptosis). We showed that human cLDL also induced proliferation of vascular smooth muscle cells, which is usually associated with a proatherosclerotic action of modified LDL [25].

The combination of in vitro studies demonstrating the ability of cLDL to have biologic effects relevant to atherosclerosis (endothelial cell injury and vascular smooth muscle proliferation), along with elevated cLDL in hemodialysis patients, suggests an important role of LDL carbamylation in atherosclerosis observed in ESRD patients.

It must be emphasized that although we have studied only patients with ESRD, it is very likely that patients with moderate renal insufficiency would also have increased cLDL. Because urea is present in normal plasma, it can be hypothesized that these data can be applied not only to renal patients but also to normal individuals. It is yet to be determined whether and how cLDL may be involved in the development of atherosclerosis by other mechanisms.
(e.g., endothelial cell dysfunction, expression of adhesion molecules, increase of monocyte adhesion to endothelial cells, increase of procoagulant activity). Further studies are required to identify the specific signaling pathway for apoptosis and receptor(s) for cLDL. Future attempts to prevent LDL carbamylation and/or reduce the effects of carbamylation may provide novel treatments for the prevention of atherosclerosis.

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