Oxidized Low Density Lipoproteins Stimulate Eicosanoid Synthesis in Mesangial Cells

Katsumi Matoba,* Mitsuru Ozaki, Yoichi Yamada, Masatoshi Mune, and Susumu Yukawa

Third Department of Internal Medicine, Wakayama Medical College, Wakayama, Japan

Background: Recent evidence suggests that abnormalities of lipid metabolism, such as hypercholesterolemia, may contribute to the progression of renal disease. Mesangial cells are important producers of eicosanoids, which play a critical role in glomerular hemodynamics and inflammation in the kidney.

Methods: We investigated the cytotoxic effect of oxidized low-density lipoprotein (LDL) and its effect on arachidonate metabolism in cultured rat mesangial cells. The effects of the antioxidant probucol on these interactions were also examined.

Results: Cell-induced LDL oxidation was demonstrated in the presence of trace amounts of copper ions. Oxidized LDL was more cytotoxic to mesangial cells than native LDL, and stimulated cells to produce more eicosanoids, including prostaglandin E₂ and thromboxane B₂, by elevation of phospholipase A₂ activity and enhanced availability of arachidonic acid. This effect of oxidized LDL was attenuated by incubation of mesangial cells with probucol.

Conclusion: Lipid peroxidation of cell membranes exposed to oxidized LDL may contribute to oxidant injury by leading to activation of phospholipase A₂. These results suggest that peroxidative modification of LDL may play a role in the progression of glomerular injury by stimulating arachidonate metabolism, as in the progression of atherosclerosis.


Key words: oxidized LDL, arachidonate metabolism, phospholipase A₂, cytotoxicity

Hypercholesterolemia has been regarded as a complication of nephrotic syndrome. Recent evidence suggests that abnormalities of lipid metabolism, particularly hypercholesterolemia, contribute to the progression of renal disease. Studies using cholesterol-fed animal models of renal disease have indicated that hypercholesterolemia promotes the glomerulosclerotic process, and supports the hypothesis of lipid nephrotoxicity advocated by Moorhead et al. Oxidation of low-density lipoproteins (LDL) contributes to enhanced thromboxane production and to renal vasoconstriction in cholesterol-fed animals, since the antioxidant drug, probucol, corrects the hemodynamic abnormalities usually associated with a high-cholesterol diet. The development of atherosclerotic lesions has been linked to local accumulation of LDL and its local oxidation.

Oxidized LDL (Ox-LDL) has been demonstrated in atherosclerotic lesions, and it suggested that Ox-LDL is a naturally occurring modified form of LDL associated with the atherosclerotic process. Apolipoprotein B (apo B) deposition in the glomerular mesangium has been observed in glomerular diseases with severe tissue injury, providing further evidence that apo B-rich lipoproteins, particularly LDL, accumulate in the mesangium. Ox-LDL has been identified immunologically in glomeruli in an experimental focal glomerulosclerosis model, suggesting that Ox-LDL may affect mesangial cells and macrophages, and play a role in glomerular injury.

A mechanism of glomerular injury due to hypercholesterolemia has been proposed by Neugarten and Schlondorff. Excess LDL infiltrates the glomeruli and binds with the mesangial cells or matrix components. In the presence of inflammatory glomerular injury, free radicals generated by mesangial cells and macrophages oxidatively modify the LDL. Ox-LDL is incorporated into mesangial cells, where it exerts a cytotoxic effect, increases matrix production, and stimulates the release of cytokines and inflammatory mediators such as eicosanoids, promoting the glomerulosclerotic process.
Mesangial cells are important producers of eicosanoids, which play a critical role in glomerular hemodynamics and inflammation in the kidney.\textsuperscript{13} We investigated the cytotoxic effect of Ox-LDL and its effect on arachidonate metabolism in cultured rat mesangial cells, postulating that oxidation of LDL might influence its interactions with mesangial cells. We also examined the effects of probucol on these interactions.

**MATERIALS AND METHODS**

**Mesangial Cell Culture**

Mesangial cells were obtained from primary cultures of glomeruli isolated from rat renal cortex.\textsuperscript{14} The kidneys of 150 g male Wistar rats were removed. The renal cortex was finely minced and sieved to isolate the glomeruli, which were suspended in RPMI-1640 medium (Nissui Pharmaceuticals, Tokyo, Japan) supplemented with 20% fetal bovine serum (FBS) (Gibco Laboratories, Grand Island, NY, USA). Primary cultures were incubated at 37°C in a humidified atmosphere of 5% carbon dioxide (CO\textsubscript{2}) and 95% air to obtain mesangial cells. The cultures were maintained in RPMI-1640 medium supplemented with 20% FBS, 15 mmol/L (N-[2-hydroxyethyl]-piperazine-N\textsuperscript{-2-ethanesulfonic acid]) (HEPES) (Sigma Chemical, St. Louis, MO, USA), insulin (1 µg/mL) (Sigma), penicillin (100 U/mL) and streptomycin (100 µg/mL) (Gibco).

Once confluent (21 days after glomerular isolation), the mesangial cells were subcultured at 2-week intervals by treatment with 0.02% ethylenediaminetetra-acetic acid (EDTA) and 0.125% trypsin. Cells suspended at a density of 2 x 10\textsuperscript{4} cells/mL were seeded into 6-well culture plates (Corning, Corning, NY, USA) for subculture. Mesangial cells were identified morphologically and by detection of actin using indirect-immunofluorescent staining.\textsuperscript{14} Cells were tested for viability in medium containing D-valine substituted for L-valine (inhibitory to fibroblasts) and for sensitivity to puromycin (100 µg/mL). Cultured mesangial cells at the fourth to tenth passage were used in the experiments.

**LDL Preparation**

LDL (density range, 1.019 to 1.063 g/mL) was isolated from healthy human plasma by the ultracentrifugation method of Havel et al.\textsuperscript{15} The isolated LDL was dialyzed at 4°C for 24 hours against PBS (calcium- and magnesium-free phosphate-buffered saline) (Nissui) containing 1 mmol/L EDTA and 1% penicillin/streptomycin, pH 7.4. The LDL was stored at 4°C in the dark and used within 2 weeks.

Ox-LDL was prepared by suspending the LDL at a protein content of 5.0 mg in 2 mL of PBS containing 5 µmol/L cupric sulfate (CuSO\textsubscript{4}), and incubating at 37°C for 40 hours. The protein content of the LDL was quantified using the method of Lowry et al.\textsuperscript{16} The lipopolysaccharide content in the native LDL and Ox-LDL was less than 10 pg/mg LDL protein as measured by the limulus lysate assay. Oxidative modification of the LDL was evaluated by changes in electrophoretic mobility on agarose gels and analysis of thiobarbituric acid reactive substance (TBARS) content of samples. For agarose gel electrophoresis of lipoproteins, one microliter of LDL was applied to an agarose gel film (Corning), electrophoresed for 45 minutes at ambient temperature, and stained with Fat Red 7B stain (Sigma).

Lipid peroxide formation was estimated as TBARS using a lipoperoxide-test kit (Wako Chemicals, Osaka, Japan) according to the methods of Heinecke et al.\textsuperscript{17} and Yagi.\textsuperscript{18} One hundred micrograms of LDL suspended in 1 mL of PBS was mixed with 1 mL of 20% trichloroacetic acid and 1 mL of TBA agent, and heated at 95°C for 60 minutes. The mixture was cooled in a water bath and shaken vigorously with 3 mL of n-butanol. After centrifugation at 4000 g for 10 minutes, the upper n-butanol layer was removed and the fluorescence was measured on a fluorescence spectrophotometer with excitation at 515 nm and emission at 535 nm. Tetramethoxypropane was used as a standard. Lipid peroxide content was expressed as nanomoles per liter malondialdehyde (MDA) equivalents.

Prior to the experiment, native LDL or Ox-LDL was dialyzed against EDTA-free PBS containing 1% penicillin/streptomycin for 16 hours and filtered through a 0.45 µm filter (Toyo, Tokyo, Japan).

**Cytotoxicity Assessment**

Confluent mesangial cell cultures were incubated with various concentrations (10 to 200 µg/mL) of native LDL or Ox-LDL at 37°C in 5% CO\textsubscript{2} for 24 hours. Cytotoxic effect was assessed by a 3-(4,5-dimethylthiazolyl)2,5-diphenyl tetrazolium bromide (MTT) assay,\textsuperscript{19} or by an assay of lactate dehydrogenase (LDH) released into the medium.

Mesangial cells were seeded in 96-well plates at 2 x 10\textsuperscript{3} cells/100 µL per well with confluent growth after 1 week. The culture medium was then removed, and the cells were preincubated in serum-free RPMI-1640 medium for 72 hours. The medium was replaced by 100 µL of medium containing native LDL or Ox-LDL, and the cultures were incubated for 24 hours. Ten microliters of MTT agent (Chemicon International, Temecula, CA, USA) was added to each well and the plate was incubated for an additional 4 hours. The MTT formazan pigment formed was dissolved in 100 µL of isopropanol/0.04 N hydrochloric acid (HCl) and the optical density at 570 to 630 nm was measured using an enzyme-linked immunosorbent assay (ELISA) plate reader.