Ascorbic Acid Protects against Peroxidative Modification of Low-Density Lipoprotein, Maintaining Its Recognition by LDL Receptors

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(Received April 25, 2000)

Summary Peroxidatively modified low-density lipoprotein (LDL) may contribute to atherosclerotic processes; therefore, protecting LDL against peroxidation may thus reduce or retard the progression of atherosclerosis. We have evaluated the protective effects of ascorbic acid on copper-catalyzed LDL peroxidative modification. The protective effects of ascorbic acid on copper-catalyzed LDL peroxidative modification were examined by measurement of concentration of lipid hydroperoxides in LDL and by the provision of LDL cholesterol to peripheral blood lymphocytes via LDL receptor-mediated pathway. The measurement of concentration of lipid hydroperoxides in LDL showed that ascorbic acid inhibited peroxidative modification of LDL. Also, ascorbic acid preserved the ability of LDL to be recognized by LDL receptors in peripheral blood lymphocytes to the same extent as native LDL. These findings indicate that ascorbic acid may protect LDL against peroxidative modification, maintaining its ability to act as a ligand for LDL receptors in vivo.

Key Words ascorbic acid, peroxidized LDL, LDL receptor

It has been found that low-density lipoprotein (LDL), the major cholesterol transport lipoprotein in human plasma, is taken up by extracellular parenchymal cells such as cultured human fibroblasts and lymphocytes via a receptor-mediated pathway and degraded (1). Ascorbic acid (vitamin C), a water-soluble radical-scavenging antioxidant, protects LDL polyunsaturated fatty acids against peroxidation (2).

Cross-sectional epidemiological studies have revealed an inverse correlation between plasma ascorbic acid and the incidence of coronary heart disease, which may be related to direct inhibition of LDL peroxidation by ascorbic acid (3). Peroxidative modification of LDL alters the lipoprotein to the extent that it is no longer recognized by the LDL receptor in the extracellular parenchymal cells; the modified LDL is then taken up by macrophages via the scavenger receptor pathway, ultimately generating foam cells (4–9). The protection of LDL against peroxidation may thus reduce or retard the progression of atherosclerosis. LDL peroxidation in vitro can be inhibited by adding ascorbic acid to medium containing isolated LDL (2). Jialal et al. (10) reported that ascorbic acid prevented the uptake and degradation of peroxidatively modified LDL by the scavenger-receptor mechanism in cultured macrophages.

In this study, we investigated the protective effects of ascorbic acid on copper-catalyzed LDL peroxidative modification by measurement of the concentrations of lipid hydroperoxides in LDL and by observing whether ascorbic acid preserves the ability of LDL to be recognized by LDL receptors in peripheral blood lymphocytes to the same extent as native LDL.

MATERIALS AND METHODS

LDL isolation. LDL (density = 1.019–1.063 g/mL) was isolated from pooled serum of 7 healthy, fasting Japanese adults by sequential flotation according to the method of Havel et al. (11). The separated lipoprotein fractions were dialyzed against 3 L of phosphate buffered-saline (PBS) three times at 4°C. After dialysis, the protein concentration was determined with a Pierce BCA Protein Assay Kit using bovine serum albumin as the standard (12).

Preparation of samples
Native LDL: LDL isolated from serum was diluted to 1,000 μg/mL (protein concentration) with PBS and employed as the control.

Peroxidized LDL (ox-LDL): Isolated LDL was diluted to 1,000 μg/mL (protein concentration) with PBS and incubated with 12.5 μM CuSO4 at 37°C for peroxidation. After a 6-h incubation, EDTA (0.1 mM) was added and the preparation was refrigerated at 4°C to stop peroxidation. LDL was then dialyzed against 3 L of PBS three times to remove copper ions.

Ascorbic acid treated LDL: LDL was prepared in the same manner as peroxidized LDL, but incubated with 20 μg/mL of ascorbic acid in addition to 12.5 μM CuSO4. It was confirmed in former experiments that
these conditions are ideal for observation of the protective effects of ascorbic acid against copper-catalyzed LDL peroxidation (13).

**Assays for products of lipid peroxidation.** The concentration of lipid hydroperoxides in LDL was measured by the methylene-blue hemoglobin method using a Determiner LPO kit (Kyowa Medix, Tokyo, Japan) (14). Cumene hydroperoxide was used as a standard.

**Measurement of the binding capacity of LDL to LDL receptors of lymphocytes.** The method used to measure binding capacity is based on the observation that lymphocyte proliferation induced by phytohemagglutinin (PHA, mitogen) in culture is dependent on a source of cholesterol for synthesis of plasma membranes (15). When endogenous sterol synthesis is blocked by pravastatin and exogenous cholesterol is provided only as LDL, lymphocyte proliferation depends on its uptake. Therefore the binding capacity of LDL to LDL receptors can be estimated by analyzing lymphocyte proliferation when culture is performed under these conditions (16–18). Proliferation is assayed by measuring DNA synthesis (19). D,L-Mevalonic acid lactone was purchased from Sigma Chemical Co. (St. Louis, MO, USA). Pravastatin, a specific inhibitor of 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase, was provided by Sankyo (Tokyo) and dissolved in RPMI 1640 medium (Gibco Laboratories) at 0.3 mM. Peripheral blood was obtained from normolipidemic fasting adults and mononuclear cells were isolated by the method of Boyum (20) and suspended at 2.5 × 10⁵/mL in RPMI 1640 containing 10% (vol/vol) lipoprotein-depleted fetal calf serum (LDFCS, d>1.230 g/mL). LDFCS was prepared by removing lipoproteins from fetal calf serum by ultracentrifugation according to the method of Havel et al. (11). The cells were incubated in sterile microtiter wells (Becton Dickinson, Oxnard, CA, USA) with 0.025% PHA (DIFCO) as the stimulus. Cultures were also treated with or without 0.3 mM pravastatin in the absence or presence of one of three kinds of LDL, that is, native-LDL, ox-LDL, or ascorbic acid-treated LDL, at three different cholesterol concentrations (0, 0.25 and 1.0 mg/dL) at 37°C. After 72 h, ³H-thymidine (370 KBq/mL) was added and 18 h thereafter cells were harvested onto glass filter paper using a semiautomated microharvesting device (MASH II, Microbiological Associates). Lymphocyte DNA synthesis was assessed by measuring the incorporation of ³H-thymidine with a Beckman LS-5800 scintillation counter (Beckman Instruments, Fullerton, CA, USA). DNA synthesis was expressed as counts per minute (cpm). Percentage inhibition induced by pravastatin was calculated according to the following equation:

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\text{Percentage (\% inhibition) = } \frac{1 - (\text{cpm experimental/cpm control})}{100}
\]

Cpm experimental is the PHA-induced incorporation of ³H-thymidine in the presence of pravastatin and native-LDL, peroxidized LDL, ascorbic acid treated LDL or 10 mM mevalonate. Cpm control is the PHA-induced incorporation of ³H-thymidine in the absence of pravastatin and exogenous cholesterol or mevalonate.

**Data analysis.** Data presented are mean ± SD values (n=5). Comparisons of data between groups were made with the Mann-Whitney U-test and the Wilcoxon signed-rank test.

### RESULTS

The concentration of lipid hydroperoxides in peroxidized LDL (376.36±259.87 nmol/mL) was significantly higher than in native LDL (9.12±4.39 nmol/mL) or ascorbic acid treated LDL (7.08±1.93 nmol/mL), *p<0.005* (Table 1). In the absence of exogenous cholesterol, the % inhibition of lymphocyte DNA synthesis was 85.2±3.0%. The inhibitory effect of pravastatin was completely prevented by the addition of mevalonate, the product of the inhibited enzyme, HMG-CoA reductase. Suppression of the lymphocyte response was also prevented by native LDL cholesterol, % inhibition decreasing to 51.2±2.7% at 0.25 mg/dL and to 11.1±3.2% at 1.0 mg/dL. Similarly, when ascorbic acid-treated LDL was provided, the % inhibition decreased to 48.3±3.2% at 0.25 mg/dL and to 7.8±2.5% at 1.0 mg/dL. With peroxidized LDL, the % inhibition...
was 86.2±3.5% at 0.25 mg/dL and 86.1±3.3% at 1.0 mg/dL (Fig. 1).

DISCUSSION

Accumulating evidence supports the hypothesis that peroxidative modification of LDL is a key step in the genesis of atherosclerotic lesions (4), and the in vivo existence of peroxidized LDL is being well-documented (21–23). Peroxidative degradation of LDL lipids results in the extensive fragmentation of apoB to smaller peptides (24–26) and it has been proposed that these, together with the covalent binding of aldehydes, lead to a complete structural rearrangement of the protein, creating new epitopes that no longer bind to the LDL receptor (B/E receptor) but rather to the scavenger receptor of macrophages. Ascorbic acid is generally recognized as an important water-soluble antioxidant, having the ability to act at a proximal step in the peroxidation process, scavenging free radicals and protecting lipids from significant peroxidative attack (2). In the present study ascorbic acid protected low-density lipoprotein against peroxidation, as evidenced by the its lowered concentration of lipid hydroperoxide in the LDL (Table). Ascorbic acid preserved the ability of LDL to be recognized by LDL receptors in peripheral blood lymphocytes to the same extent as native LDL (Fig. 1).

It is known that peroxidized LDL has characteristics of cytotoxicity (27, 28). The result in this study suggest that peroxidized LDL cannot bind with LDL receptor without the possibility of cytotoxic effects, leading to a decrease of cell proliferation.

Epidemiologic studies have demonstrated an inverse relationship between the plasma level of ascorbic acid and the incidence of ischemic heart disease (3). The concentration of ascorbic acid used in this study is high but within the physiologic range (29). The data thus suggest that depletion of the ascorbic acid in plasma may dispose LDL to peroxidative modification in vivo, which will promote atherosclerosis.

In conclusion, our results suggest that ascorbic acid protects LDL against peroxidative modification, maintaining its ability to act as a ligand for LDL receptors in vivo.

Acknowledgment

Financial support for this study was provided by the Vitamin C Research Committee, Tokyo, Japan.

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