Ascorbic Acid Augments Cytotoxicity Induced by Oxidized Low-Density Lipoprotein

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Although ascorbic acid (ASA) is known as a water-soluble antioxidant, we found that it accelerated the cytotoxicity induced by oxidized low-density lipoprotein (OxLDL) in vitro. This suggests that ASA may enhance the oxidation of LDL to augment the atherogenic activities of OxLDL under certain conditions. Thus, this study was designed to investigate the underlying mechanism that ASA enhances OxLDL-induced cytotoxicity. ASA enhanced the cytotoxicity of macrophage cell line (J774) induced by OxLDL in a dose-dependent manner, whose effect was more apparent in high glucose concentration in the medium. The ASA-induced enhancement in cytotoxicity was inhibited by the presence of lipid-soluble antioxidants, such as α-tocopherol and probucol, suggesting that the pro-cytotoxic effect by ASA is likely due to its pro-oxidant property. We also investigated the effects of ASA at different time points on the Cu2+-mediated oxidation of LDL. ASA decreased the rate of conjugated dienes formation when added at the early phase of oxidation, whereas it increased when added at the late phase of oxidation. These data suggest that ASA may act as a pro-oxidant under the condition of extensive LDL oxidation. To prevent oxidation stress, ASA would be better used together with lipid-soluble antioxidants for antioxidant therapies. J Atheroscler Thromb, 2003; 1: 7-12.

Key words: Ascorbic acid, Oxidized LDL, Cytotoxicity, Antioxidant therapies

Introduction

It is generally accepted that the oxidation of low-density lipoprotein (LDL) plays an important role in the onset and development of atherosclerosis (1). Oxidized low-density lipoprotein (OxLDL) has various atherogenic effects (1-4). Most importantly, it is readily taken up by macrophages, thereby leading to the formation of lipid-laden foam cells, the hallmark of early atherosclerotic lesions.

Other atherogenic properties of OxLDL include cytotoxicity, the induction of monocyte chemotactic protein-1 in vascular endothelial and smooth muscle cells, the enhancement of leukocyte adhesion to the vascular endothelium, and the inhibition of nitric oxide-mediated vasodilation. Since the oxidation of LDL may facilitate the progress of atherosclerosis, the inhibition of this process with an antioxidant therapy is a promising strategy to prevent and/or retard the onset and progression of atherosclerosis (5).

We previously hypothesized that a metabolite of gemfibrozil, M1, may have antioxidant properties because of its hydroxylated phenol ring, 5-(4-hydroxy-2,5-dimethyl-phenoxy)-2,2-dimethyl pentanoic acid. In fact, M1 dose-dependently inhibited the oxidative modification of LDL,
and diminished the cytotoxicity induced by OxLDL (6). Since ascorbic acid (ASA) is a major water-soluble antioxidant in human blood plasma, we speculate that ASA may also have the same effect as M1. In fact, ASA in physiological concentrations inhibits LDL oxidation induced by macrophages or copper ions, and functions to preserve endogenous lipid-soluble antioxidants (7-8). It has also been reported that ASA regenerates the antioxidant effects of probucol (9) and α-tocopherol (10) consumed by free radicals. Retsky et al. (11) have reported two different mechanisms by which ASA may protect LDL against oxidative modification; 1) ASA may scavenge free radicals in the aqueous phase, and 2) dehydro-L-ascorbic acid, the oxidation product of ASA, or its decomposition products may modify LDL, thereby leading to decreased copper (Cu2+) binding to the LDL particle and increased resistance to Cu2+-mediated oxidation.

However, we found that ASA paradoxically accelerated the cytotoxicity induced by OxLDL. Therefore, this study was designed to elucidate the underlying mechanism of such a paradoxical effect of ASA.

Materials and Methods

Materials

RPMI 1640 medium, fetal calf serum, penicillin-streptomycin, and L-glutamine were obtained from GIBCO (Grand Island, NY). Ascorbic acid (ASA) and α-tocopherol were purchased from Sigma Chemical (St Louis, MO). Probucol was supplied by Daiichi Pharmaceutical (Tokyo, Japan).

Preparation of lipoproteins and OxLDL

LDL (d = 1.019 to 1.063 g/ml) was separated from pooling plasma drawn in fasting condition of normolipidemic subjects by ultracentrifugation using a vertical rotor (RP67-VF; Hitachi, Tokyo, Japan) according to the method of Chung et al. (12). Centrifugation was performed at 65,000 rpm for 90 min at 4°C. After isolation, the LDL was extensively dialyzed for 20 hrs against phosphate-buffered saline ([PBS] pH 7.4).

OxLDL used for cytotoxicity was prepared by dialyzing the LDL against at least 100 vol dialysate (0.01 M Tris and 0.15 M NaCl, pH7.4) including 30 µM CuSO4.(5) at 4°C for 16 hrs. After adding 1mM EDTA to stop the oxidation, the OxLDL was stored at 4°C until the cytotoxicity studies (6). In our preliminary experiments, we have found TBARS levels of 16 to 30 nM MDA/mg LDL protein and LPO levels of 230 to 500 nM/mg LDL protein for the OxLDL prepared by this method.

The protein concentration was measured by the method of Lowry et al. (13) using bovine serum albumin as a standard.

Cytotoxicity assay

J774 macrophages were used for the cytotoxicity studies. The cells were seeded at a density of 5 × 10⁴ cells/ml in 96-well plates in RPMI 1640 medium containing 5% lipoprotein-deficient fetal bovine serum. Media containing different concentrations of glucose [200 and 400 mg/dl (11.1 and 22.2 mM)] were used. The cells were incubated with OxLDL and the reagents (ASA, α-tocopherol, probucol) at 37°C, under 5% CO2 for 48 hrs. After incubation, the cytotoxicity induced by OxLDL was determined by 3-[4,5-dimethyl-thiazol-2-yl]-2,5-diphenyl tetrazolium bromide (MTT) assay according to the method of Mosmann (14). Briefly, stock MTT solution was added to the plates at the indicated times and incubated at 37°C for 3 hrs, followed by the addition of dimethyl sulfoxide and thoroughly mixed. After further incubation for 25 min at 37°C, the absorbance was measured on a micro-enzyme-linked immunosorbent assay reader (MPR-A4i; Tosoh, Tokyo, Japan). Cell viability was measured by the MTT method and expressed as absorbance or the percentage to that of native LDL. Each experiment was performed in duplicate.

Kinetics of Cu²⁺-mediated LDL oxidation

The kinetics of Cu²⁺-mediated LDL oxidation was measured continuously by monitoring the change of absorbance at 234 nm on a spectrophotometer (Shimazu UV-160A, Kyoto, Japan) with 6 cuvette positions, according to the method of Esterbauer et al. (15). Briefly, the oxidation of LDL (100 µg protein /ml in PBS, pH 7.4) mediated by Cu²⁺ (final concentration, 5 µM) was monitored at 37°C for 5 hrs. The time-course shows three consecutive phases consisting of lag-, propagation- and decomposition-phase. During a lag- and a propagation -phase, conjugated dienes (CD) are formed mildly and rapidly, respectively. After CD formation reached the peak (Tmax), it gradually decreases during a decomposition phase. To investigate the effects of ASA added at different time points on LDL oxidation in a cell-free system, ASA was added to samples with or without α-tocopherol (20 µM) or probucol (3 µM) at 50 min after the Tmax and 120 min after the Tmax.

We also measured the kinetics of the oxidation of minimally OxLDL mediated by Cu²⁺. To investigate whether ASA acts as a pro-oxidant or an antioxidant in this system, ASA (80 µM) was added either at the start of the assay or at the Tmax.

Statistical Analysis

Results are expressed as the mean ± SD. All data were analyzed by the Student’s unpaired 2-tailed t test.

Results

Effect of ASA on Cytotoxicity Induced by OxLDL

The effect of ASA on the cytotoxicity to J774 macrophages induced by OxLDL is shown in Fig.1. ASA enhanced
Ascorbic Acid Augments Cytotoxicity

Ascorbic acid augments cytotoxicity in a dose-dependent manner (5-50 µM). This effect was more evident in the medium containing higher concentration of glucose [400 mg/dl (22.2 mM)] than lower concentration [200 mg/dl (11.1 mM)], although there was no significant difference (p > 0.05).

Table 1 shows the effect of ASA on OxLDL-induced cytotoxicity in the absence or the presence of lipid-soluble antioxidants (α-tocopherol and probucol). Both α-tocopherol (40 µM) and probucol (40 µM) significantly reduced the pro-cytotoxic effect induced by ASA (20 µM).

**Table 1.** Effect of ASA on OxLDL-induced cytotoxicity with or without lipid-soluble antioxidants

<table>
<thead>
<tr>
<th>A. α-Tocopherol</th>
<th>Cell viability</th>
<th>Absorbance (OD570-630 nm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Native LDL</td>
<td>0.767 ± 0.058</td>
<td>100</td>
</tr>
<tr>
<td>OxLDL</td>
<td>0.392 ± 0.139</td>
<td>51.1</td>
</tr>
<tr>
<td>OxLDL + ASA (20 µM)</td>
<td>0.231 ± 0.175**</td>
<td>30.1</td>
</tr>
<tr>
<td>OxLDL + ASA (20 µM) + α-tocopherol (40 µM)</td>
<td>0.502 ± 0.113***#</td>
<td>65.4</td>
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</tbody>
</table>

<table>
<thead>
<tr>
<th>B. Probucol</th>
<th>Cell viability</th>
<th>Absorbance (OD570-630 nm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Native LDL</td>
<td>0.731 ± 0.046</td>
<td>100</td>
</tr>
<tr>
<td>OxLDL</td>
<td>0.412 ± 0.104</td>
<td>56.4</td>
</tr>
<tr>
<td>OxLDL + ASA (20 µM)</td>
<td>0.265 ± 0.149*</td>
<td>36.3</td>
</tr>
<tr>
<td>OxLDL + ASA (20 µM) + probucol (40 µM)</td>
<td>0.589 ± 0.114****#</td>
<td>80.6</td>
</tr>
</tbody>
</table>

**Discussion**

This study demonstrates that ASA enhances OxLDL-induced cytotoxicity, which is inhibited by lipid-soluble antioxidants. Both α-tocopherol and probucol significantly inhibited the cytotoxicity enhanced by ASA, suggesting that the augmentative effect of ASA on OxLDL-induced cytotoxicity is due to its pro-oxidant property. We also investigated whether a high glucose condition could af-

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*J774 macrophages were incubated with OxLDL and (a) α-tocopherol (40 µM) or (b) probucol (40 µM) for 48 hrs at 37°C with or without ASA (20 µM). The glucose concentration in the medium was 200mg/dl. Results are expressed as the mean ± SD (n = 6). *p < 0.05, **p < 0.001, ***p < 0.0001 vs OxLDL. #p < 0.005, ##p < 0.0001 vs OxLDL+ASA.
ffect ASA's effect on cytotoxicity induced by OxLDL, because it has been reported that elevated levels of glucose in the diabetic state increase LDL lipid peroxidation (16, 17) and cholesterol ester synthesis in macrophages (18). The cytotoxicity was more apparent in the medium containing 400 mg/dl (22.2 mM) glucose than 200 mg/dl (11.1 mM), although the difference did not reach statistical significance. Under such high glucose conditions, increasing lipid hydroperoxides may enhance oxidative stress, which contributes to the augmentation of cytotoxicity.

This study also shows that the effect of ASA on CD formation in LDL depends on the time when it is added, depending on the oxidative state of LDL. ASA inhibited the rate of CD formation when added shortly after the Tmax. In contrast, ASA increased it when added at the late phase of CD formation, suggesting that ASA could act as a pro-oxidant under advanced oxidative conditions.

It has been reported that ASA may act as a pro-oxidant under certain conditions in vitro (19-21). Hunt et al. (19) have shown that ASA can both inhibit and promote the oxidation of native LDL was initiated by the addition of 5 µM Cu²⁺ and measured continuously by monitoring the change in 234 nm diene absorption. (A) In the first sample, only 20 µM ASA was added at 50 min after the Tmax (a) (— —), and in the second sample, only 20 µM α-tocopherol was added at point a (———). In the third sample, 20 µM α-tocopherol was added at point a and 20 µM ASA was also added at 120 min after the Tmax (b) (—— ). (B) In the first sample, only 20 µM ASA was added at 50 min after the Tmax (a) (— —), and in the second sample, only 3 µM probucol was added at point a (———). In the third sample, 3 µM probucol was added at point a and 20 µM ASA was also added at 120 min after the Tmax (b) (—— ). Data are representative of three experiments.

Fig. 2. Effects of ASA added at the early and the late phase of CD formation with or without lipid-soluble antioxidants. The oxidation of native LDL was initiated by the addition of 5 µM Cu²⁺ and measured continuously by monitoring the change in 234 nm diene absorption. (A) In the first sample, only 20 µM ASA was added at 50 min after the Tmax (a) (— —), and in the second sample, only 20 µM α-tocopherol was added at point a (———). In the third sample, 20 µM α-tocopherol was added at point a and 20 µM ASA was also added at 120 min after the Tmax (b) (—— ). (B) In the first sample, only 20 µM ASA was added at 50 min after the Tmax (a) (— —), and in the second sample, only 3 µM probucol was added at point a (———). In the third sample, 3 µM probucol was added at point a and 20 µM ASA was also added at 120 min after the Tmax (b) (—— ). Data are representative of three experiments.

Fig. 3. Effect of ASA added at different time points on the Cu²⁺-mediated oxidation of minimally OxLDL. Oxidation of minimally OxLDL was initiated by the addition of 5 µM Cu²⁺ and measured continuously by monitoring the change in 234 nm diene absorption. Eighty µM ASA was added at the starting point (a) and Tmax (b). Data are representative of three experiments.
formation of ceroid in a model system, depending on its concentration. When trace levels of transition metals are present, ASA in low concentrations can increase ceroid formation in macrophages. Stait et al. (20) have reported that ASA can either increase or decrease the oxidative modification of LDL by macrophages, depending on the time required for the LDL preparation; ASA inhibited the macrophage-mediated oxidation of freshly prepared LDL, whereas it had no effect on mildly OxLDL produced by autoxidation during storage in the refrigerator for a few months. The modification of mildly OxLDL by ASA increased TBARS levels in the medium, whose effect was completely inhibited by the antioxidant, butylated hydroxytoluene.

Although the concentrations of several antioxidants in the extracellular fluid, such as proteins, ascorbic acid and uric acid, are sufficient to inhibit LDL oxidation, LDL remains to be oxidized in vivo conditions. While the mechanism of LDL oxidation in vivo is poorly understood, it appears likely that the antioxidants may be constantly consumed and depleted on the arterial walls exposed to a variety of insults such as shear stress, injury, and inflammation (2). Our data suggest that some antioxidants such as ASA may even act as pro-oxidants under certain conditions. In fact, not all epidemiological studies have shown that ASA is a protective agent against cardiovascular disease. For example, Stampfer et al. (22) and Rimm et al. (23) have shown that high vitamin C intake was not associated with a reduced risk of coronary heart disease, while vitamin E supplement reduced the risk. In another study by Podmore et al. (24), vitamin C supplement caused a pro-oxidant effect in vivo. After daily vitamin C supplement (500mg) for 6 weeks, the oxidative damage to lymphocytes as assessed by 8-oxoadenine, a marker of modified DNA bases, increased. These data suggest that ASA may accelerate the in vivo oxidative process of LDL under certain conditions. Further studies will be needed to clarify where and how ASA accelerates and/or inhibits LDL oxidation in vivo.

In conclusion, this study shows that ASA increases cytotoxicity to macrophages induced by extensive OxLDL, whose effect is inhibited in the presence of α-tocopherol or probucol, suggesting that its pro-oxidant effect is observed under a severe oxidative stress. Therefore, ASA would be better used in combination with lipid-soluble antioxidants (25) to prevent oxidative stress.

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References


(14) Mosmann T: Rapid colorimetric assay for cellular growth and survival: application to proliferation and


(20) Stait SE and Leake DS: Ascorbic acid either increase or decrease low-density lipoprotein modification. FEBS Lett, 341: 263-267, 1994


