

Inhibitory Effect of Chlorogenic Acid on Low-Density Lipoprotein Oxidation Induced by Cu ion

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Abstract - Chlorogenic acid, formed of an ester of caffeic acid and quinic acid, which is naturally abundant in many plant species, was used as a model *O*-dihydroxy phenolic compound. In the previous study, we have reported that the isolated constituent from *Apocynum venetum* leaves has an inhibitory effect on Cu²⁺-induced oxidative modification of low-density lipoprotein (LDL). Among them, chlorogenic acid showed the most potent anti-LDL oxidative activity than other compounds. For the reason, we investigated the inhibitory effect of the chlorogenic acid on Cu²⁺-induced oxidative modification of LDL, monitored a lag time in the conjugated-diene formation and TBARS formation, and measured TNBS free amino acid group, and form cell formation *in vitro* system. The TBARS- and diene- formation were strongly inhibited by chlorogenic acid (0 ~ 100 µg / ml) with dose dependent manner. On the other hand, TNBS reactive lysine amino groups on LDL oxidation were protected by chlorogenic acid- treated cell group. Therefore, chlorogenic acid inhibited to cholesterol accumulation in the isolated peritoneal macrophage.

Key words - Chlorogenic acid, LDL oxidation, Foam cell formation, Atherosclerosis

Introduction

The oxidative modification of low-density lipoprotein (LDL) may play an important role in contributing to the development of atherosclerosis (Shaikh et al 1988; Steinberg et al 1989; Witztum et al 1991). Oxidatively modified LDL is a potent ligand for scavenger receptors on macrophages and thus contributes to the generation of macrophage-derived foam cells, the hallmark of early atherosclerotic fatty streak lesions (Parthasarathy et al 1992). Many additional mechanisms by which oxidized LDL may contribute to atherosclerosis have been identified (Leake et al 1993). The oxidative modification hypothesis of atherosclerosis is supported by numerous in vivo findings, e.g., the presence of epitopes of oxidatively modified LDL in atherosclerotic lesions (Witatum et al 1991) and elevated titers of circulating auto-antibodies against oxidized LDL in patients with carotid atherosclerosis (Steinbrecher et al 1990). The formation of atherogenic oxidized LDL (Ox-LDL) can be inhibited by

dietary antioxidants such as vitamin C and E, carotenoids and phenolic compounds (Sato et al 1990; Lavy et al 1993). Some lipid lowering drugs (namely statins) have also been shown to reduce the susceptibility of LDL to oxidation in hyperlipidaemic patients (Hoffman et al 1992; Kleinveld et al 1993; Hussein et al 1997).

Chlorogenic acid, formed of an ester of caffeic acid and quinic acid, which is naturally abundant in many plant species, was used as a *O*-dihydroxy phenolic compound. The presence of chlorogenic acid in plants has been linked with defense mechanisms against insects and fugal and bacteria pathogens *in vitro* (Friedman 1997).

In this paper, we represent the inhibitory effect of the chlorogenic acid on Cu²⁺-induced oxidative modification of LDL, monitored a lag time in the conjugated-diene formation and TBARS formation, and measured TNBS free amino acid group.

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Materials and Methods

Isolation of compounds

Apocynum venetum leaves (Luobuma) which had been roasted twice were extracted with hot water at 70°C for 3 h. After filtration, the solution was evaporated under reduced pressure to give a residue. The residue was extracted with MeOH to give MeOH-soluble and insoluble fractions. Column chromatography of the MeOH-soluble fraction on Sephadex LH-20 with 10% ~ 70% MeOH afforded four fractions. Fraction 2 was subjected to reversed phase column chromatography (MeOH-CHCl₃, 1:9) to give chlorogenic acid and its analogues. Fraction 3 was also separated with Sephadex LH-20 (20% ~ 40% MeOH) to give three catechin derivatives and a flavonoid mixture. High-performance liquid chromatography [Shimadzu LC-9A system: column, ODS AP 302 YMC; mobile phase, MeOH:H₂O:AcOH (30: 100:5)] of the flavonoid mixture led to isolation of hyperoside and isoquercitrin. These compounds were identified by comparing their ¹H-NMR, ¹³C-NMR, mass and IR spectra with those of authentic samples.

Cell culture and treatment

Macrophages were harvested from the peritoneal fluid of male BALB/c strain mice (6 weeks old), 4 days after intraperitoneal injection of 2 ml of 3% thioglycollate medium. The cells were maintained in DMEM containing 100 U / ml penicillin and 100 µg / ml streptomycin with 10% FCS in 24 well tissue culture plates. After confluence has been reached, macrophages were plated at 1 x 10⁶ cells/well in 6 well tissue culture plates containing poly-L-lysine-coated coverslips. The macrophages were allowed to adhere by incubation for 4 h under routine conditions. Adherent macrophages were washed three times with DMEM and cultured for a further 72 h in DMEM containing 200 U / ml penicillin and 200 µg / ml streptomycin supplemented with 20% FCS, LDL (150 µg protein / ml) or 20 µM CuSO₄ and / or chlorogenic acid.

Oxidative modification of LDL

Human LDL (Sigma Co., St Louis, USA) was dialysed twice against ca. 100 volumes of 0.15 M NaCl (pH, 7.4) for 48 h at 4°C. After filtration through a 0.45 µM filter, the

protein content of LDL was determined as described by Lowry *et al.* 1951), using serum albumin as the protein standard. LDL was adjusted with 0.15 M NaCl (pH. 7.4) to a concentration of 0.15 mg protein/mL, and incubated with 10 µM CuSO₄ at 37°C for 6 h. Chlorogenic acid from *A. venetum* leaves were dissolved in 0.15 M NaCl by sonication in an ultrasonic bath for 10 min at 25°C followed by filtration through a 0.45 µM filter.

Determination of TBARS

The TBARS level was fluorometrically determined; 1.5 mL of 0.67% thiobarbituric acid (TBA) and 1.5 mL of 20% trichloroacetic acid (TCA) were added to a 0.2 mL aliquot of post incubation mixture and standard of tetramethoxypropane (10 nmol). Tubes were placed in a boiling water bath for 45 min. After cooling, tubes were centrifuged at 1000 x g for 15 min. The fluorescence intensity of the supernatant was measured with a fluorospectrometer (Shimadzu RF-550, Kyoto, Japan) with excitation at 515 nm and emission at 553 nm. The values were expressed as nmol of malondialdehyde equivalents.

Determination of conjugated diene formation

LDL (0.05 mg protein/mL) was incubated at 37°C in the presence of CuSO₄ (final concentration, 10 µM), and the conjugated-diene formation was continuously monitored as the change in absorbance at 234 nm with a Shimadzu (Kyoto, Japan) UV 2200 spectrophotometer equipped with a CPS-240A thermostat cell positioner. Chlorogenic acid (1 ~ 100 µg / mL) was added to the solution. Before starting the LDL oxidation, the solution in a cuvette was stirred for 20 s. The absorbance was recorded at 10 min intervals for 700 min. The end of the lag time was defined as an intercept of the straight lines derived from the lag phase and the propagation phase (Esterbauer *et al.*, 1989).

Determination of protein modified in LDL

Reaction amino group in protein were estimated with trinitrobenzenesulfonic acid using valine as the standard. The 50 microgram (protein) of LDL was mixed with 1 ml of 4% NaHCO₃ (pH 8.4) and 50 µl of 0.1% trinitrobenzenesulfonic acid. After incubation for 1 h at 37°C, 100 µl of 1 N HCl and

100 μ l of 10% sodium dodecyl sulfate were added and the absorbance at 340 nm was measured. TNBS reactivity in the Ox-LDL was expressed as the percentage of that measured in unoxidized LDL.

Determination of cholesterol content

The total and free cholesterol contents were determined using commercial assay kits (Cholesterol E-Test Wako obtained from Wako Pure Chemical Industries, Ltd., Osaka, Japan). The esterified cholesterol content was calculated from the differences between total cholesterol and free cholesterol values.

Statistical analysis

The results are represented as mean \pm SE of the number (n) of experiments. Dunnett's test was used for comparisons among groups. *P* values less than 0.05 were considered significant.

Results

Oxidative modification of LDL

The degree of LDL oxidation was measured by monitoring of TBARS production. Fig. 1 showed TBARS production upon incubation of LDL with CuSO₄ in the presence of increasing concentrations of chlorogenic acid. The TBARS concentration was significantly and dose-dependently reduced

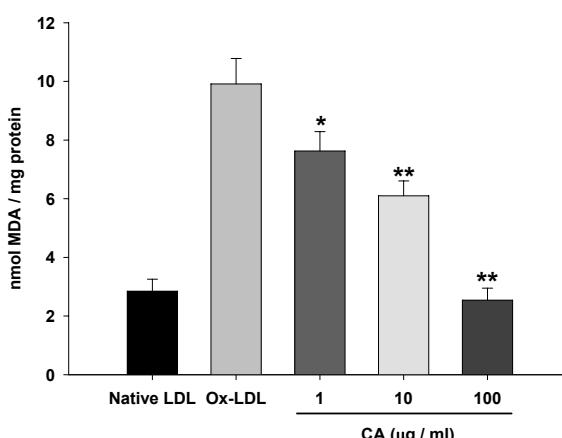


Fig. 1. Effects of chlorogenic acid on TBARS formation in the Cu²⁺-induced LDL oxidation Statistical significances : *p<0.05, **p<0.01 vs. Ox-LDL value.

by chlorogenic acid treatment, presenting from 9.89 to 2.67 nmol / mg LDL (*P* < 0.01). The TBARS level in native LDL was maintained at 3 nmol / mg protein, whereas in Ox-LDL, it was significantly high to about 3.3.times of the native LDL value.

Conjugated diene formation

The ability of chlorogenic acid to inhibit the formation of Cu²⁺-induced conjugated dienes was directly compared to Ox-LDL level. The results are summarized in Table 1 and Fig. 2. Fig. 2 shows the kinetic profile of conjugate diene production upon incubation of LDL with CuSO₄ in the presence of increasing concentration of chlorogenic acid. A dose dependent inhibition of lipid oxidation is apparently increased in the lag time for conjugate diene production with 182 min in the Ox-LDL level and 315 min in the 100 μ g / ml chlorogenic acid. The lag time for conjugate diene production is considered a very sensitive indicator of antioxidant effect *in vitro*.

Table 1. Effect of chlorogenic acid on lag tame in conjugated diene formation.

Concentration (μ g / ml)	Lag time (min)
0	182
1	315
10	374
100	468

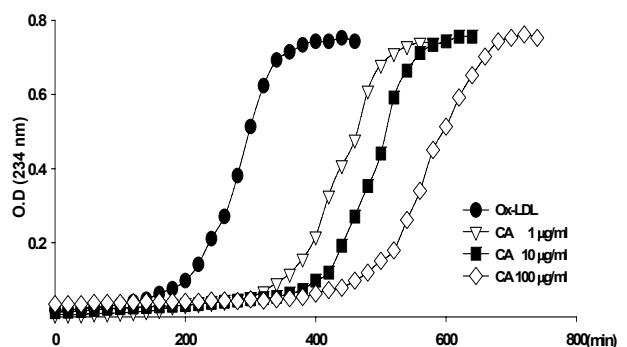


Fig. 2. Effects of chlorogenic acid on conjugated diene formation. The LDL solution (0.05 mg protein/ml) was supplement with 0(●), 0.1(▽), 10(■) and 100(◇) μ g/ml chlorogenic acid prior to addition of 10 μ M CuSO₄.

Protein modification of LDL

We also assessed the effect of chlorogenic acid on TNBS reactivity. The concentration of amino groups was determined by reference to a valine standard. Incubation of the LDL oxidation with CuSO₄ substantially reduced TNBS reactivity compared with native LDL to 53.3 after 1 h. Chlorogenic acid treatment significantly protected the Cu²⁺-induced decline in TNBS reactivity to 57.3% at 10 µg / ml CA and 116.2% at 100 µg / ml chlorogenic acid.

Cholesterol content in macrophage

Ox-LDL has been proposed to be the atherogenic particle responsible for the free- and esterified-cholesterol stores in the macrophages. The present studies the ability of mildly Ox-LDL to induce free- and esterified-cholesterol accumulation in mouse peritoneal macrophages. At concentrations used in this study, chlorogenic acid did not show cytoto-

xicity according to the results of the MTT assay (data not shown). In mouse macrophages, the level of total-cholesterol was 37.9% more in Ox- LDL treated cells compared with native LDL cells. When the cells were incubated with Ox-LDL in the presence of the chlorogenic acid (100 µg / ml), the present esterified-cholesterol decreased by 64.6% in comparison to incubation with Ox-LDL alone. As shown in Fig. 4, the content of total-cholesterol and esterified-cholesterol in the macrophages were dose-dependently reduced by treatment with chlorogenic acid (1 ~ 100 µg / ml). On the other hand, the level of free-cholesterol was dose-dependently increased.

Discussion

LDL lipid peroxidation is considered to be essential in the pathogenesis of atherosclerosis (Chisolm et al 2000; Weber et al 2000). Although data concerning the mechanisms by which lipid peroxidation occurs in vivo are scarce, several lines of evidence suggest that some endogenous and exogenous compounds with antioxidant activity could have some beneficial effects in the prevention of the disease (Fuhrman et al 2001, Craig et al 1999). Accordingly, animal and human studies suggest that these compounds may have some preventive effect against the development of clinical coronary heart disease. Many plant phenols and flavonoids may be important dietary antioxidants and it has been speculated that in particular procyanidins and quercetin in red wine or in the Mediterranean diet could explain the ‘French paradox’ (Giugliano et al 2000).

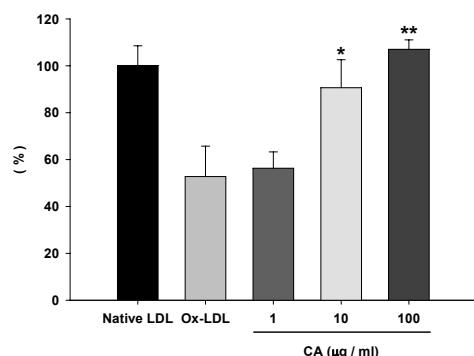


Fig. 3. Effects of chlorogenic acid on reactive lysine amino groups Statistical significances : *p < 0.05, **p < 0.01 vs. Ox-LDL value.

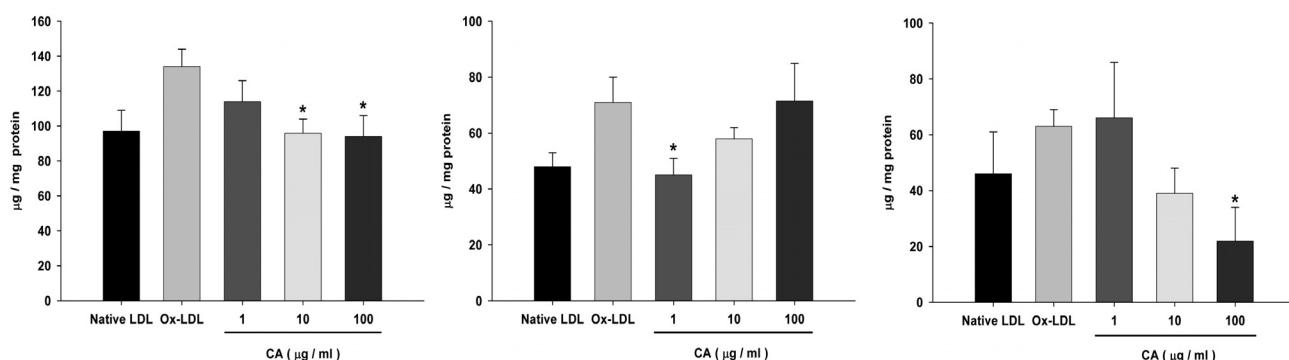


Fig. 4. Effects of chlorogenic acid on cholesterol accumulation in mouse peritoneal macrophages. (A) ; total cholesterol, (B) ; free cholesterol, (C) ; esterified cholesterol Statistical significances : *p < 0.05 vs. Ox-LDL value.

In this study, we set out to demonstrate the antioxidant properties of one herbal preparation widely used in complementary and alternative medicine in vast areas of the world. Oxidative modification of LDL is a key process for the recognition of LDL by the scavenger receptors on macrophages. In the previous study, we have reported that chlorogenic acid showed the most potent anti-LDL oxidative activity than other compounds (Kim et al 2000). Chlorogenic acid had been reported anti-genotoxicity, anti-complementary action, anti-carcinogenesis, and anti-mutagenicity, and antioxidant properties (Tanaka *et al.*, 1990; Kono *et al.*, 1997; Ejzemberg *et al.*, 1999; Jiang *et al.*, 2000b). Indeed, the compound was demonstrated to display strong superoxideanion and peroxy radical scavenging activities on pathological diseases and biological damages (Jiang *et al.*, 2000a).

Our data showed that chlorogenic acid has stronger inhibitory activity against the LDL oxidation. LDL was oxidized by incubation with Cu²⁺ ions. The lag time of this reaction, which indicates the intrinsic antioxidant activity of the LDL particles, was prolonged by chlorogenic acid treatment (table 1 and figure 2). Chlorogenic acid also reduced the concentration of the TBARS that results from lipid peroxidation (figure 1). Copper ions react with preformed lipid hydroperoxides in LDL, resulting in the production of lipid alkoxyl radicals that can initiate lipid peroxidation.

In the present study, incubation of the LDL oxidation with CuSO₄ decreased TNBS reactivity (figure 3). This observation indicates that Cu²⁺-induced LDL oxidation results in a loss of reactive lysine amino groups on LDL, thereby increasing the negative charge of LDL. Chlorogenic acid treatment reduced this decrease in TNBS reactivity, suggesting that chlorogenic acid may prevent the derivatization of LDL. Furthermore, the CA-treated LDL particles may be resistant to oxidation and, therefore, less likely to be taken up by macrophages, leading to a decreased risk of coronary heart disease.

The accumulation of cholesterol in macrophages is an important feature of atherosclerosis (Jerome *et al* 1985, Fowler *et al* 1980, Jerome *et al* 1984). It has been proposed that ox-LDL is the atherogenic particle responsible for the buildup of cholesterol in lesions (Steinberg *et al* 1989). Consistent with this concept, ox-LDL has not only been

found in atherosclerotic lesions, but is avidly taken up via nonregulated scavenger receptors (Steinberg *et al* 1989, Yla-Herttuala *et al* 1989).

These studies demonstrate that mildly ox-LDL is effective at inducing foam cell formation in mouse macrophages (figure 4). This suggests that the high cholesterol content of mildly oxLDL contributes to its ability to induce significant cholesterol accumulation. In the cellular cholesterol concentrations, when chlorogenic acid was added to the culture medium, these changes were less conspicuous. In particular, accumulation of esterified-cholesterol was decreased to a degree comparable to that observed in the presence of native LDL, showing the anti-atheroscloric effect of chlorogenic acid at the cellular level.

In summary, chlorogenic acid on Cu²⁺-induced LDL oxidation was significantly inhibited to TBARS-, and conjugated diene formation in cell free system. IC₅₀ value of chlorogenic acid was 1.9 μM in TBARS-formation. Furthermore, chlorogenic acid treatment significantly reduced the TBARS-formation and TNBS reactivity in macrophage-mediated oxidation system. From the result of cell-mediated oxidation system, chlorogenic acid reduced the accumulation of cholesterol profiles in macrophages.

From these results, we suggest that chlorogenic acid increase LDL resistance to oxidation, decrease the consumption of endogenous antioxidants, and may be of benefit in the prevention of atherosclerosis and cardiovascular disease.

Acknowledgment

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