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Inhibitory Effects of Garlic Oil on Human Low Density Lipoprotein Oxidation

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ABSTRACT - Growing evidence indicates that oxidized low density lipoprotein (LDL) may promote atherogenesis. Therefore, inhibition of LDL oxidation may impede this process. The inhibitory effected on the susceptibility of human LDL to Cu^{2+} or macrophages induced oxidation was investigated by monitoring thiobarbituric acid reactive substances (TBARS). Organosulfur compounds of garlic oil contains diallyldisulfide, diallyltrisulfide, diallyltetrasulfide, and diallyl pentasulfide in order. Garlic oil inhibited LDL oxidation by Cu^{2+}, or macrophages in a dose dependently, with a 20~60 μg, as increased TBARS assay. Garlic oil, at 60 μM, almost completely inhibited macrophages induced increase in electrophoretic mobility of LDL. When compared with several other antioxidants, probucol showed highest ability, and then garlic oil showed a much higher ability than natural occurring antioxidants, α-tocopherol and ascorbic acid. The results suggested that garlic oil might play the inhibitory effects in the process of LDL oxidation.

Key words: Low density lipoprotein (LDL), garlic oil, macrophages

An increased concentration of low density lipoprotein (LDL) cholesterol is considered a key event in the early development of atherosclerosis[1]. An early event in atherogenesis is the accumulation of lipid-laden foam cells in the arterial intima, which can progress to fatty streaks and plaques. Most of foam cells are likely derived from resident tissue macrophages, which can lead to cellular cholesterol accumulation[2]. Oxidized LDL has entered the artery wall and then accumulated in foam cells would affect atherosclerotic progress[3]. Oxidative moditication of LDL, that alters physicochemical and biological properties of the particles, is thought to play a central role in atherogenesis[4,5].

Evidence in support of the oxidized LDL hypothesis also comes from studies using antioxidants[6,7]. If oxidized LDL is crucial to atherogenesis, the potential role of antioxidants in the prevention of the oxidative modification of LDL assumes great importance. LDL oxidation was used to test the effectiveness of antioxidants to slow atherosclerosis in animal models[8]. It was observed that antioxidants or drugs with antioxidant activity were consistently able to reduce the extent of atherosclerosis. The exact mechanism by which LDL undergoes oxidation in vivo is not clear yet but there is little doubt that it involves free radical peroxidation of LDL[9]. Since oxidized LDL seems to play a role in the development of atherosclerosis, prevention by antioxidants such as probucol[10,11], β-carotene[12], Vitamin E[13,14], vitamin C[15,16] and garlic[17] may be a therapeutic option.

The natural antioxidants discovered recently have been expected to replace the synthetic antioxidants which are widely used at present time. One such plant that has attracted much attention over the last 10 years, is garlic. Commercial and non commercial preparations of garlic are increasingly used as health supplements. Whereas the in vitro antioxidant properties of garlic are well recognized studies investigating the effect of garlic supplementation on the oxidation resistance of LDL[17,18]. Dietary garlic has been found to reduce atheroma formation[19~21]. Aged garlic extract is an aqueous ethanolic extract of garlic has been shown to process in vitro antioxidant properties[20,22].

Attention has been recently focused on the importance of the protective defense systems in living cells against damages caused by LDL[18].

Our interest has been focused on garlic oil which are known to contain high content of sulfur compound. With these in consideration, the aim of this study was to determine inhibitory effects of garlic oil on LDL oxidation induced by copper and to establish whether the garlic oil could terminate LDL oxidation once initiated.

Materials and Methods

Materials

Preparation of garlic oil

Garlic oil obtained from Fooddiet Co, Korea.

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Analysis of sulfur compound in garlic oil

The constituents of garlic oil was performed using HPLC. Garlic oil was qualitatively analysed by a method[23]. Eighty mg of the oil were diluted to 10 mL dimethyl sulfoxide. The sample was filtered through a glass fibre/0.45 μm hydrophilic filter cartridge into an autosampler vial and injected onto HPLC. For separation, HP-Lichrosopher RP-18e HPLC columns, (4 × 125 mm, 5 μm) were used. The eluent used for separation of the constituents was acetonitrile:water (70 : 30) for 10 min at flow rate 0.8 mL/min. The peaks were named by elution order comparison to those found by other[23].

Cell culture

Transformed mouse macrophage, J774 cells was obtained from American type culture collection (Rockville, MD, USA) was maintained in Ham's F-10 supplemented with 10% (v/v) foetal calf serum. $NaHCO_3$ (2 g/L). and 4 mM Herpes. pH 8.1. A series of antibiotics were included in rotation of the medium. The cells were cultured routinely in large dishes (90 mm diameter) in 10 mL of medium. Cultures were maintained in a humidified incubator at 37°C and the medium was changed every 48 h.

Pulmonary artery endothelial cells was obtained from American type culture collection (Rockville, MD, USA). Endothelial cells were grown in eagles minimum essential medium (EMEN) with 20% bovine calf serum and 200 μl/mL penicillin-streptomycin solution. All cells were incubated at 37°C in a 5% humidified CO_2 atmosphere for at least 3-4 days before use. Throughout the experiments, cell viability was always than 95% as determined by tryptophan blue exclution.

Lipoproteins

Human LDL was isolated through ultracentrifugation[24] and dialyzed extensively against 0.9% (w/v) NaCl and 0.004% (w/v) EDTA, pH 7.4. Prior to oxidation, LDL was dialyzed against phosphate-buffered saline, pH 7.4, to remove the EDTA.

Oxidation of LDL

Two different methods were used to examine the effect of novel compound in the oxidation of LDL. In the first method, LDL (100 protein/mL) was incubated in the presence of 5 μM $CuSO_4$ in phosphate buffered saline, pH 7.4 at 37 for 18 h[25]. In the second method, LDL (100 μg protein/mL) was incubated with J774 macrophages in Ham's F-10 culture medium for 24 h at 37°C. To examine the effect of antioxidant on LDL oxidation, sample was added to the LDL containing solutions at the beginning of the incubation period. The oxidation of LDL was stopped by adding EDTA (final concentration of 10 μM) and placing the lipoproteins on ice.

Assay of thiobarbituric acid-reactive substances (TBARS)

TBARS levels were determined spectrophotometically[26]. 1 mL of 20% trichloroacetic acid and 1 mL of 1% thiobarbituric acid containing EDTA were added to 0.1 mL aliquots of post-incubation mixture and tetramethoxypropane standards. Tubes were placed in a boiling water bath for 30 min. After cooling, tubes were centrifuged at 1,500 × g for 15 min. Absorbance of the supernatant was measured at 532 nm.

LDL gel electrophoresis

Electrophoresis of oxidized and native LDL was carried out on agarose gel in barbital buffer, pH 8.6. The agarose plates were then stained with Nile red. Result are expressed as relative electrophoretic mobilities compared to the migration of native LDL[27].

LDL oxidation by endothelial cells

Primary cultures of human umbilical vein endothelial cells were obtained from cord vein, after 15 min digestion by 0.2% collagenase solution [33]. Cells were plated into a 75 cm^2 tissue culture flask and allowed to grow to confluence in RPMI 1460 containing 20% foetal calf serum, 10 mL penicillin, 10 μg/mL streptomycin and 2 mM L-glutamine at 37°C in a humidified atmosphere of 95% air and 5% CO_2. Confluent human endothelial cell cultures in multiwell clusters (1.5 × 10 cells cm) were washed three times with serum-free medium, supplemented with 5 μM $CuSO_4$, and incubated with LDL (100 μg, protein/mL) in serum-free medium containing 1% human serum albumin. Before addition to endothelial cells LDL was loaded (30 min at 37°C) with vitamin E, garlic oil or their vehicles (DMSO or ethanol respectively), and sterilized by passage through 0.22 μm millipore filters. After 18 h incubation at 37°C the medium was aspirated, centrifuged to remove cell debris and processed for lipid peroxidation assay[28].

Determination of cellular protein

Cell protein was measured by Lowry method using bovine serum albumin as standard[29].

Statistics

Data in text and figures are mean ± SEM values[30]. Statistical analysis was performed as indicated in the figure legends; significant difference was accepted at $P < 0.05$.

Results and Discussion

Garlic oil analysis

Garlic oil contain different complex mixtures of organosulfur compounds. Allicin, the main sulfur-containing com-

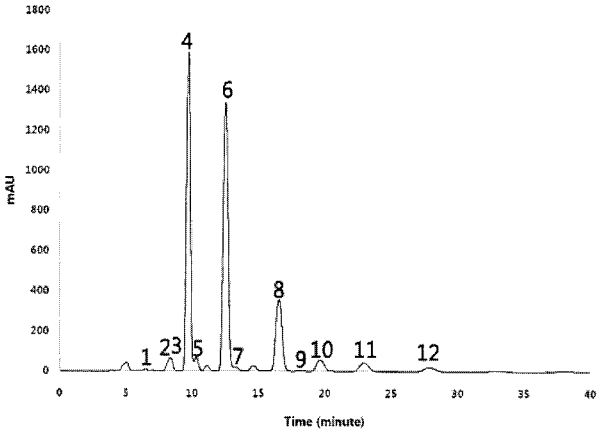

Fig. 1. HPLC chromatograms of garlic oil. When compared to quantitative HPLC analysis using varified standards, peak eluting 4, 6, 8, 10 and 11 corresponded to diallyldisufide, diallyltrisulfide, diallyltetrasulfide, diallylpentasulfide and allyl methyl hexasulfide, respectively.

pounds then converts to different organosulfur compounds depending on the temperature at which the conversion take place and other factors[31].

Volatile sulfur compounds in garlic oil are shown in Fig. 1. Three of the 12 separated volatile compounds, diallyldisulfide, diallyltrisulfide and diallyl tetrasulfide had the highest relative peak area and are expected to have varying degrees of antioxidative activity. The major antioxidative activity of LDL are expected to be in this group.

Inhibitory effect of garlic oil on LDL oxidation

The inhibitory antioxidative effect of garlic oil on the oxidation of LDL, as measured by the production of TBARS, was initially examined at various concentrations of garlic oil, As shown in Fig. 2, garlic oil demonstrated a concentration dependent inhibition of the production of oxidized after 6 and 24 h of incubation. At a concentration of 60 μg garlic oil, the oxidation of LDL was almost inhibited that observed in the absence of garlic oil. Although research supports the *in vivo* existence of oxidized LDL, the most persuasive data inhibited on the role of oxidized LDL. Atherogenesis derives from studies showing that antioxidants prevent atherosclerosis in animal models while some antioxidants such as BHA and BHT prevented atherosclerosis in anamals[8], but their side effects preclude their use in human subjects[11]. Oxidation of LDL in the presence of copper was maximal between 2 and 3 h of incubation; oxidation for 24 h of incubation was almost four fold greater than at 6 h[32]. This may reflect the level of endogenous antioxidants present in the LDL preparation, which may vary with individual donors. For example, vitamin E as dietary antioxidant protects against LDL oxidation, but LDL of donors from smokers were more susceptible to oxidation than that from non-smoker[32].

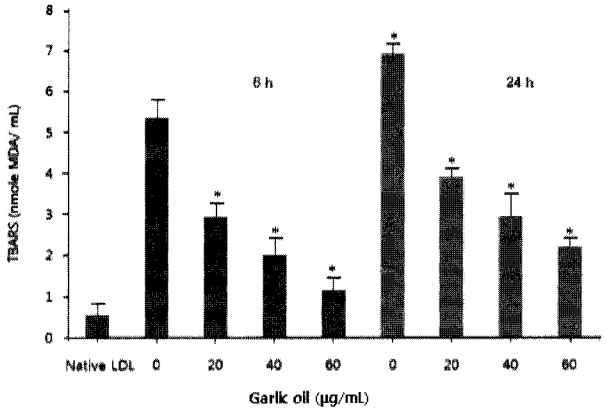

Fig. 2. Concentration-dependent inhibition of garlic oil on LDL oxidation mediated Cu^{2+}. LDL (100 μg protein/mL) was incubated for 6 and 24 h at 37°C in phosphate-buffered saline containing 5 μM $CuSO_4$ in the presence or absence of increasing concentration of garlic oil. Results are presented as means ± SEM of there to five independent experiments.

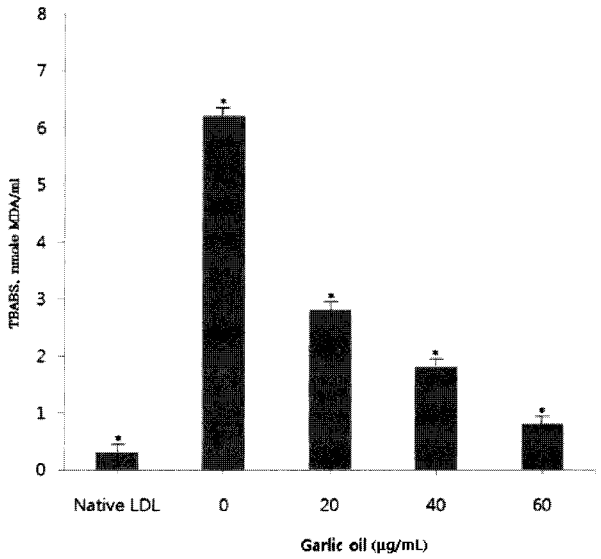

Fig. 3. Concentration-dependent inhibition of macrophage induced LDL oxidation by LDL (100 μg protein/dish) was incubated for 24 h with macrophages in Ham's F-10 in the presence of absence (control) of the garlic oil. Results are presented as mean ± SEM of three to five independent experiments.

As shown in Fig. 3, human LDL was oxidized by macrophages, in a time-dependent manner and the production of TBARS reached a plateau after 24 h of incubation. Therefore, all data relating to macrophages presented here were obtained following 24 h incubation. Increasing the number of macrophages per dish increased the production of TBARS. The oxidation of LDL by macrophages was almost completely inhibited in the presence of 60 μg garlic oil in the culture medium. Although the mechanism of oxidation is not known, LDL oxidation may involve cellular lipoxygenases. The

inhibition of macrophage induced oxidation by garlic oil is consistent with the role of lipoxygenase[33]. Macrophages on endocytose and degrade oxidatively modified LDL via scavenger receptors at a much greater rate than native LDL[33]. Many other cells types have since been shown to oxidize LDL *in vitro*, e.g., mouse peritoneal macrophages[29]. It was observed that protection by cell-induced LDL oxidation may be, in part, through its capacity to scavenge O_2^- radicals. However, inhibition of Cu^{2+}-mediated LDL oxidation was less marked than the oxidation by macrophages. The differences in garlic oil potency in the two systems might be due to the fact that oxidative modification is more complex than a simple free radical reaction. Moreover, the mechanism of LDL oxidation by transition metals still remains to be clarified.

To further examine this relationship, this study first investigated the affinity of garlic oil for the lipoprotein and the corresponding inhibitory effect. garlic oil inhibited to LDL oxidation but did not stop the oxidation of lipoprotein.

Comparison of inhibitory effects of garlic oil α-tocopherol and probucol

Fig. 4 shows inhibitory potencies of garlic oil, probucol and α-tocopherol on AAPH-induced lipid peroxidation of LDL. The LDL was then incubated with 2 mM AAPH and various concentrations of garlic oil (60 μg/mL), probucol (60 μg/mL) and α-tocopherol (60 μg/mL) and TBARS was measured at 24 h of incubation.

Under these experimental conditions, both garlic oil and α-tocopherol inhibited AAPH induced LDL oxidation at the concentration of 60 μM. Concentrations of garlic oil and α-tocopherol were inhibited at 60 μg/mL and 60 μg/mL, respectively. This experiment proposed an antioxidative effect on garlic oil, which has a hindered suffer containing compound. This study demonstrated that garlic oil exhibited an inhibitory effect on the oxidative modification of human plasma LDL, and that its inhibitory potency was higher than that of α-tocopherol. The hindered sulfur containing compound of garlic oil acts as a chain breaking antioxidant, whereas the major function of the sulfur structure is to retain the molecule in LDL. Although the mechanism of enhanced inhibitory effect of garlic oil is not clear, the sulfur group of garlic oil may effect the stability of the molecule and the affinity for LDL.

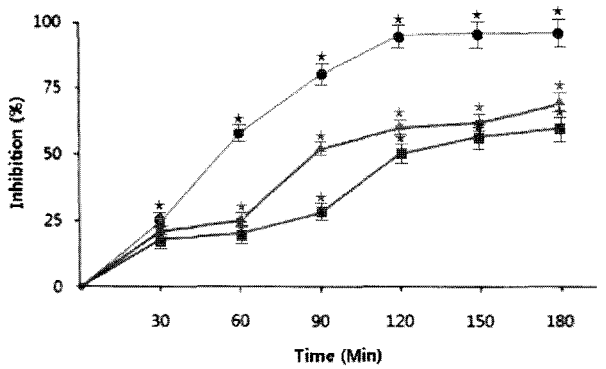

Fig. 4. Comparison of inhibition of LDL oxidation by garlic oil, α-tocopherol and probucol. LDL (100 μg protein / mL) was subjected to oxidation with 2 mM AAPH in PBS present (▲) of garlic oil (60 μg/mL) α-tocopherol (60 μg/mL) (■) and probucol (60 μg/mL) (●). TBARS contents were expressed as a percent of maximum content observed at 12 h of incubation with 2 mM AAPH and vihicle alone : Data are presented as the mean ± SD of 5 independent experiments.

In all cases, garlic oil inhibited LDL oxidation with the characteristic induction of a concentration-dependent lag time, similar to classic antioxidants.

Inhibitory effects of garlic oil on LDL oxidation by electrophoretic mobility

As shown in Table 1, the inhibitory effect of garlic oil on the electrophoretic mobility of LDL submitted to oxidative modification by macrophages. Garlic oil reduced the relative electrophoretic mobility of LDL at dose dependently. LDL oxidized by $CuSO_4$ displayed a greater electrophoretic mobility in agarose gels compared to native LDL. When LDL was incubated with various concentration of garlic oil. LDL (100 μg/mL) was incubated for 24 h in Hams F-10 medium in 35-min dishes containing macrophages in the presence of absence of garlic oil.

The effect of 24 h preincubation with garlic oil on the ability of macrophages to oxidized LDL was also investigated with garlic oil significantly reduced the ability of the cells to oxidized LDL in a dose-dependent fashion with concentration of 20~60 μg/mL.

The effect of garlic oil on the electrophoretic mobility of LDL submitted to oxidative modification by the macrophages is shown in Table 1. A markedly increase from 1.95 ± 0.02 mm in the electrophoretic mobility of control LDL and incubated with macrophages for 24 h indicated lipid peroxidation of LDL increased nagative charge in the LDL molecule.

Table 1. Effects of garlic oil as assessed by electrophoresis mobility

Incubation conditions	Relative electrophoretic mobility (mm)
Native LDL	1.0
LDL + cells + vehicle (control)	1.95 ± 0.02
LDL + cells + 20 μg/mL garlic oil	1.63 ± 0.02
LDL + cells + 40 μg/mL garlic oil	1.42 ± 0.01
LDL + cells + 60 μg/mL garlic oil	1.13 ± 0.01

LDL (100 μg/mL) was incubated for 24 h in Ham's F-10 medium 35 min dishes containing macrophages in the presence or absence of garlic oil. The electrophoretic mobility of LDL was determined in agarose gel as described in the text. Results are means ± SEM of three to five independent experiments.
The data are means of 3 experiments.

The oxidation of LDL by macrophages was almost inhibited in the presence of 60 μM per dish in culture medium. Although the mechanism of oxidation is not known, LDL oxidation may involve celluar lipoxgenase[33]. The result of the present study clearly demonstrate that garlic oil can markedly prevent a macrophage induced LDL oxidation. Although garlic extracts have been reported to inhibit copper catalyzed lipid peroxidation[20], the present study is, to our knowledge, the first demonstration of a inhibitory effect of garlic oil against LDL peroxidation by cell mediated mechanism.

Protective activity of garlic oil, ascorbic acid, α-tocopherol and probucol

The inhibitory effect towards LDL oxidation was compared to the activity exerted by the synthetic antioxidant, probucol and the physiological antioxidants, ascorbic acid and α-tocopherol. Inhibitory activity was studied in the LDL oxidation by copper, copper/hydrogen peroxide and by endothelial cells. Cumulative data, shown in Table 2, are based on analysis of MDA and hydroperoxide formation. For each data are obtained from concentration dependent inhibition experiments

The protective activity was probucol > garlic oil > α-tocopherol > ascorbic acid. The magnitude of antioxidant garlic oil was higher than α-tocopherol and ascorbic acid in order. In the endothelial cell-mediated LDL oxidation, Inhibition of MDA and hydroperoxide formation required similar between α-tocopherol and garlic oil. Probucol was higher, apart from the diene system where the difference is reduced to a factor. Finally, garlic oil is more potent than α-tocopherol, ascorbic acid by a factor ranging from 0.1 to 0.3 times, depending on the system.

To further support the physiological relevance of these results, garlic oil was compared to probucol, α-tocopherol and ascorbic acid, which can be considered reference antioxidants on biological system. α-Tocopherol, a normal constituent of LDL, is generally thought to function as a major lipid-soluble antioxidant, and ascorbic acid is considered the most important aqueous phase antioxidant in plasma[15]. Probucol was selected because most research into LDL oxidation has concentrated on the protection by this synthetic compound[8]. In all instances, garlic oil produced the most potent inhibition of LDL oxidation. The LDL oxidation is a complex mechanism involving initiator radicals generated by Fenton-like chemistry, and propagating carbon-oxygen radicals, like peroxy radicals. Thus, garlic oil should operated at two main levels, the initiation and propagation, of the lipid peroxidation. In addition, the superiority of garlic oil can be explained in terms of partitioning in the LDL lipids/phase and by better accessibility to sites of free radical attack. Garlic oil, as demonstrated in this study, strongly associates with LDL and is sufficiently oil in soluble enough to be able to intercept both the oxidation initiating radicals coming from the lipid phase and the lipid radicals generated during the chain reaction.

On the basis, the results suggest that garlic oil might play a regulatory role in the process of LDL oxidation, *in vivo*, which may change the metabolic fate of the particle, increasing its atherogenic potential.

Table 2. Relative inhibitory activity of garlic oil and the synthetic antioxidant, probucol and the natural occurring antioxidants, ascorbic acid and α-tocopherol

	Lipid peroxidation (Cu^{2+} / H_2O_2)		Endothelial cells mediated oxidation
	MDA	LOOH	
Garlic oil (60 μg/mL)	16.8	17.0	21.3
Probucol (60 μg/mL)	14.5	15.0	15.6
α-Tocopherol (60 μg/mL)	18.3	17.8	24.5
Ascorbic acid (60 μg/mL)	19.6	18.5	25.8

For each inhibitory data are obtained from concentration dependent inhibition experiments.

요 약

본 연구에서는 동맥경화의 원인으로 알려진 사람 low density lipoprotein (LDL)에 대한 garlic oil의 산화억제 효과에 대하여 실험하였다. Garlic oil을 HPLC로 분석한 결과 diallyl disulfide, diallyltrisulfide, diallyltetrasulfide and diallyl pentasulfide 등의 유황화합물이 많이 들어 있었다. 사람 LDL을 Cu^{2+} 및 macrophages 유도 oxidized LDL로 산화시킬 때 garlic oil을 20~60 μg/mL 범위에서 첨가하며 실험하였을 때 용량 의존형으로 억제되었고, garlic oil을 60 μg/mL 첨가하였을 때 LDL의 산화가 거의 억제되었으며, 산화 LDL의 전기영동에 의한 이동거리도 60 μg/mL 농도에서 거의 억제되었다. 그리고 garlic oil은 동맥의 내피세포에서도 용량 의존형으로 억제효과를 나타내었다. 또한 garlic oil은 α-tocopherol, ascorbic acid 보다 약간 높은 억제효과를 나타내었다. 이상의 결과에 따르면 garlic oil은 macrophage 및 내피세포에서 LDL의 항산화 효과가 있어 LDL의 산화 억제효과가 있는 것으로 나타났다.

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