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Inhibition of Chitin Sulfate on Human Low Density Lipoprotein(LDL) Oxidation by Macrophages

Beung Ho Ryu†, Seung Taek Yang, and Yoon Hee Moon

Department of Food Science and Biotechnology, Kyungsung University, Busan, 608-736, Korea

ABSTRACT – Growing evidence indicates that oxidized low density lipoprotein (LDL) may promote atherogenesis. Therefore, inhibition of LDL oxidation may impede this process. The effect of chitin sulfate on the susceptibility of human low density lipoprotein (LDL) to macrophages-induced oxidation was investigated by monitoring a thiobarbituric acid reactive substance (TBARS). Chitin sulfate inhibited LDL oxidation by macrophages in a dose dependent manner, with a 50~100 μM, as assessed by TBARS assay. Chitin sulfate, at 100 μM, almost completely inhibited the macrophage-induced increase in electrophoretic mobility of LDL. Also, chitin sulfate almost completely inhibit O_2^- at concentration of 100 μM. These observations suggest that chitin sulfate might be an effective in prevention of atherosclerosis.

Key words □ Low density lipoprotein (LDL), chitin sulfate, macrophages

An increased concentration of plasma low density lipoprotein(LDL) cholesterol is considered as a key event in the early development of atherosclerosis. According to the oxidative modification hypothesis, LDL initially accumulates in the extracellular subendothelial space of artery and through the action of resident vascular cells, is accumulated in macrophages that express abundant scavenger receptors for modified LDL[1]. The accumulating monocytes and macrophages stimulate further peroxidation of LDL[2]. This completely oxidized LDL is recognized by scavenger receptors on macrophages and internalized to foam so-called foam cells[3]. Oxidized LDL is also cytotoxic to vascular cells[4]. Thus promote the release of lipids and lysosomal enzymes into the intimal extracellular space and enhancing the progression of atherosclerosis lesions[5~8].

Evidence in support of the oxidized LDL hypothesis also comes from studies using antioxidants[9,10]. If oxidized LDL is crucial to atherogenesis, the potential role of antioxidants in the prevention of the oxidative modification of LDL assumes great importance. Therefore, inhibition of LDL oxidation has been suggested as a approach to impede atherogenesis. LDL carries several antioxidants, such as α-tocopherols and carotenoids, which protect them from oxidation. Dietary supplying of vitamin E inhibits LDL oxidation, and prevents oxidized LDL mediated vascular injury[11,12].

In this compartment, LDL may interact also with proteoglycans (PGs), forming complexes that are taken up avidly by macrophages[9].

A class of compounds has recently been identified that appears to work by either mimicking or reducing LDL oxidation which was found decrease lipid free radical peroxidation[13].

As experimental model, the present study was designed to assess the effect of chitin sulfate on the oxidation of human LDL by mouse macrophages, as estimated by measuring the formation of thiobarbituric acid reactive substances (TBARS) and electrophoretic mobility

Materials and Methods

Cell culture

Mouse resident peritoneal macorphages were obtained from male CD mice (20-30 g) (The University of Georgia, Altens, Georgia, USA). Ca^{2+}, Mg^{2+} -free phosphate-buffered saline (pH 7.4) was injected into the peritoneal cavity, then macrophages were collected by peritoneal lavage and pelleted by centrifugation (1000 rpm for 5 min). The cells were resuspended in Ham's F-10 cell medium containing 100 μg/ml of gentamicin, plated into 35-mm dishes at 4×10^6 cells/dish and allowed to adhere for 2 h in a 37°C humidified 5% CO_2 incubator. Non-

†Author to whom correspondence should be addressed.

adherent cells were removed with 2 washes of Ham's F-10 medium oxidation of LDL.

Lipoproteins

Human LDL was isolated through ultracentrifugation [14] 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

LDL (100 μg protein/mL) was incubated with macrophges 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[15].

Detection of conjugated dienes

The formation of conjugated dienes associated with oxidized LDL was measured by monitoring the absorption at 234 nm using a UV-VIS spectrophotometer[15]. Briefly, 1 mL LDL solution (100 μg LDL, protein/mL) in phosphate-buffered saline, pH 7.4, was incubated with 5 μM $CuSO_4$ at 37°C in both the presence and absence of samples, and the absorbance at 234 was measured every 30 min. The formation of conjugated dienes in control solutions containing antioxidant in the absence of LDL and 5 μM $CuSO_4$ was also determined.

Assay of thiobarbituric acid-reactive substances (TBARS)

TBARS levels were determined spectrophotometrically[16]. To 0.1 mL aliquots of post-incubation mixture and tetramethoxypropane standards were added 1 mL of 20% trichloroacetic acid and 1 mL of 1% thiobarbituric acid containing EDTA. 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

Electrophresis 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 with the migration of native LDL[17].

Measurement of superoxide (O_2^-) released from mouse macrophages

Superoxide (O_2^-) was measured as Yue, et al[18]. Briefy, macrophages (1×10^6 cells) were seeded in 96-well microtiter plate (Nunc, high affinity) and solutions containing PMA (10^{-7} M) and Cyto c (0.32 mM) were applied to each of three wells. Three wells received Cyto c and HEPES buffer to measure basal, unstimulated release of O_2^-. One well served as an assay blank which contained Cyto c, buffer and 700 unit SOD to confirm that Cyto c reduction was inhibitable by SOD. Changes in the optical density were measured intermittently on a MAX microplate reader at 550 nm. O_2^- release was calculated using the following conversion: nmol O_2^- = [(mean O.D. of three test wells) - (mean O.D. of three regent blank)] × 15.9.

Determination of cellular protein

Cell protein was measured using bovine serum albumin as a standard[19].

Statistics

Data in text and figures are mean ± S.E.M. values[20]. Statistical analysis was performed as indicated in the figure legends; significant difference was accepted at $P < 0.05$.

Results and Discussion

Antioxidative activity on the human LDL

Human LDL was oxidized by mouse macrophages in a time-dependent manner and the production of TBARS reached a plateau after 24 h of incubation (Fig. 1). Therefore, all data relation to macrophages presented here were obtained following 12~48 h incubation. The production of TBARS for 24 h in the presence (LDL+cells) or absence (LDL alone) of the macrophages were 4.8 ± 0.3 nmol and 0.8 ± 0.2 nmol MDA equivalent/mg protein (n=14), respectively. Increasing the number of macrophages per dish increased the production of TBARS.

As shown in Fig. 2, chitin sulfate in dose-dependently inhibited LDL oxidation by the macrophages with concentrations of 25, 50, 75, 100 μM. Chitin sulfate at the maximal concentration tested have an apparent effect on

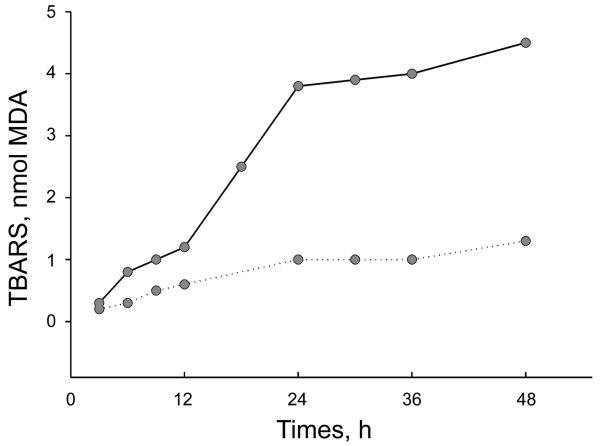

Fig. 1. Time courses of LDL oxidation by mouse macrophages
LDL(100 μg protein/dish) was incubated in Ham's F-10 medium at 37°C in the presence (3.8×10^6 cells, ●–●) or absence(LDL alone, ●--●) of macrophages for various time intervals. The LDL oxidation was estimated by the formation of TBARS and expressed in nmol equivalent MDA as described in Methods. The data are means of duplicate derminations from a representative experiment.

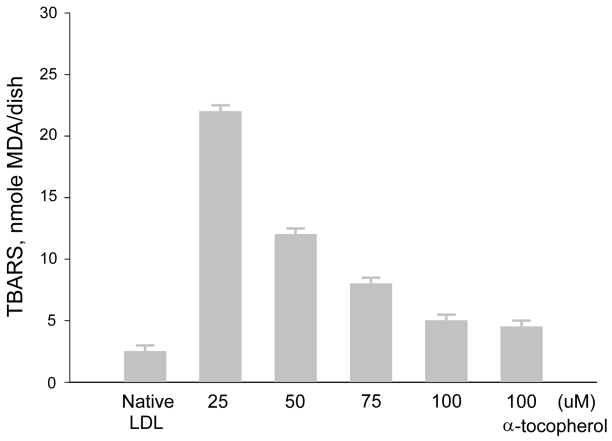

Fig. 2. Concentration-dependent inhibition of macrophage induced LDL oxidation.
LDL(100 μg protein/dish0 was incubated for 24 hr with macrophages in Ham's F-10 medium in the presence or absence(control) of the test agent. The LDL oxidation was estimated by the formation of TBARS and expressed in nmol equivalent MDA as described in Methods. TBARS produced in cell-free conditions(medium+LDL) was subtracted from dose of the dishes containing macrophages. Each point is the mean ± S.E.M. of 3-5 experiments done duplicate, while the data for α-tocopherol are the average of two experiments done duplicate.

the cells as determined by TBARS. α-Tocopherol inhibited LDL oxidation by the macrophages, but was similar with chitin sulfate. Increasing concentration of α-tocopherol (over 100 μM) resulted in an apparent effect on macrophages viability. Under the same condition of 100 μM concentration, the TBARS of chitin sulfate and α-tocopherol were 6.54 ± 0.12 and 5.26 ± 0.11 MDA nmole/dish, respectively.

Effects of chitin sulfate on the LDL oxidation by electrophoretic mobility

The effect of a 24 h preincubation with chitin sulfate on the ability of macrophages to oxidize LDL was also investigated. On the other hand, pretreatment of macrophages with chitin sulfate significantly reduced the ability of the cells to oxidize LDL in a dose-dependent fashion with concentration of 10~100 μM.

The effect of chitin sulfate on the electophoretic mobility of LDL submitted to oxidative modification by the macrophages is shown in Table 1. A marked increase from 1.86 ± 0.12 mm in the electophoretic mobility of control LDL and incubated with macrophages for 24 h indicated lipid peroxidation of LDL increased negative charge in the LDL molecule. Chitin sulfate reduces the relative electophoretic mobility in dose indepenetly, while α-tocopherol had no significant effect at the concentration indicated.

The oxidation of LDL by macrophages was inhibited in the presence of 10, 25, 50 or 100 μM per dish in the

Table 1. Effects of chitin sulfate on LDL oxidation as assessed by electrophoretic mobility

Incubation conditions	Relative eletrophoretic mobility	P
Native LDL	1.0	
LDL + cells (control)	1.86 ± 0.12	
LDL + cells + CS 10 μM	1.79 ± 0.03	
LDL + cells + CS 25 μM	1.57 ± 0.02	<0.05
LDL + cells + CS 50 μM	1.18 ± 0.03	<0.01
LDL + cells + CS 100 μM	1.08 ± 0.04	<0.01
LDL + cells + tocoperol 100 μM	1.20 ± 0.03	<0.01

LDL(100 μg/mL) was incubation for 24 h in Ham's F-10 medium in 35 mm dishes containing macrophages in the presence or determined in agarose gels as described in Methods. Result are mean ± S.E.M of 4-6 independent experiments. The data are means of 2~3 experiments.

culture medium. Although the mechanism of oxidation is not known, LDL oxidation may involve cellular lipoxygenase[21]. Macrophages endocytose and degrade oxidatively modified LDL via scavenger receptors at a much greater than native LDL, and this property was used to assess the protection afforded LDL by coincubation with chitin sulfate during incubation period. Many other cell types have since been shown to oxidize LDL *in vitro*, e.g., mouce peritoneal macrophages[22]. It was observed that inhibition by chitin sulfate on cells-induced LDL oxidation may be, in part, through its capacity to scavenger O_2 radicals.

The result of the present study clearly demonstrate that chitin sulfate can markedly prevent macrophage-induced LDL oxidation. Although proteoglycans have been reportd to inhibit iron-catalyzed lipid peroxidation[9], the present study is, to our knowledge, the first demonstration of a protective effect of a chitin sulfate against LDL peroxidation, by both a cell-mediated mechanism. The effective concentration of chitin sulfate for inhibition of LDL oxidation by macrophages was about 50~100 μM.

Effects of chitin sulfate on conjugated diene formation

Macrophages induced oxidation of human LDL were determined by the curves of diene, of a measurement of the LDL oxidative process.

In the absense of chitin sulfate, the increase of time in the reaction mixture coupled with a increase of the value of lag time, determined graphically by the intercept of the tangents to the slow and fast increase of the diene absorption. As shown in Fig. 3, chitin sulfate did not modify the maximum formation of conjugated dienes, but decrease significantly. When higher amounts of chitin sulfate were employed, the conjugated dienes formed were significantly lower in the presence of chitin sulfate than that of control. The Cu^{2+} concentration was coupled with decrease of conjugated diene formation, which an index of a propagation phase and depends only on the lipid composition of LDL. The presence of 50~100 μM chitin sulfate in the incubation mixture delay the reaching of high absorbance and this delay was higher at concentration of 100 μM.

Effects of chitin sulfate on superoxide released from macrophages

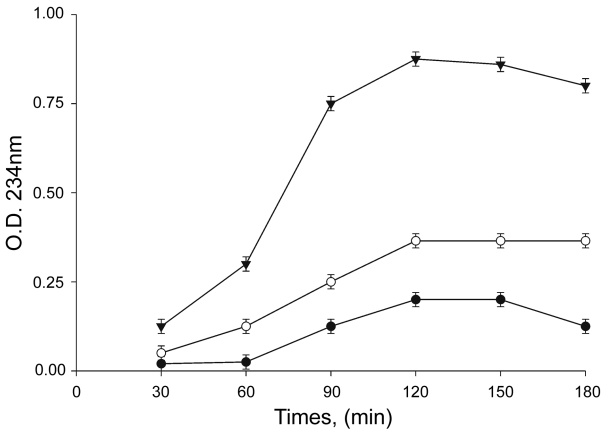

Fig. 3. Antioxidative effect of chitin sulfate on the formation of conjugated diene observed during the oxidation of LDL.

LDL(100 μg protein/mL) was incubated in the presence or abscence of 50 or 100 μM chitin sulfate. Oxidation was initiated by the addition of 5 μM $CuSO_4$. The formation of conjugated dienes was measured by LDL oxidation.
▼–▼: Control (LDL + 5 μM $CuSO_4$)
○–○: LDL + 5 μM $CuSO_4$ + 75 μM chitin sulfate
●–●: LDL + 5 μM $CuSO_4$ + 100 μM chitin sulfate
Results are presented as means ± S.E.M. of 3-5 independent experiments.

Phorbol myristate acetate (PMA, 0.1 μm) induced a time-dependent increase in O_2^- production by mouse macrophages and the production of O_2^- reached a plateau at 60 min (inset in Fig. 4). As such, all data were obtained at 60min incubation following stimulation. As a shown in Fig. 4, the production of O_2^- increased from a basal value of 0 to 1.78 ± 0.12 nmol. Chitin sulfate protected PMA-induced O_2^- production in a dose independent manner at concentration of 25 to 100 μM. It has been reported that O_2^- is an important oxygen radical in mediating LDL oxidation by smooth muscle cells[22] and human monocytes[23,24] and that O_2^- dismutase(SOD) inhibits oxidation by smooth muscle cells(17). Our previous study demostrated that flavonoids scavenges O_2^- in human endothelium cells[25]. In order to examine whether chitin sulfate inhibits LDL oxidation by inhibiting O_2^-, this studies, the effect of chitin sulfate on O_2^- released from macrophages. The results shows that chitin sulfate almost completely inhibits O_2^- at concentration of 100 uM. Chitin sulfate is likely to effect also the amount of macrophages available for the oxidation, as suggested by the marked increase of the constant in the presence of chitin sulfate. Under our experimental conditions, chitin

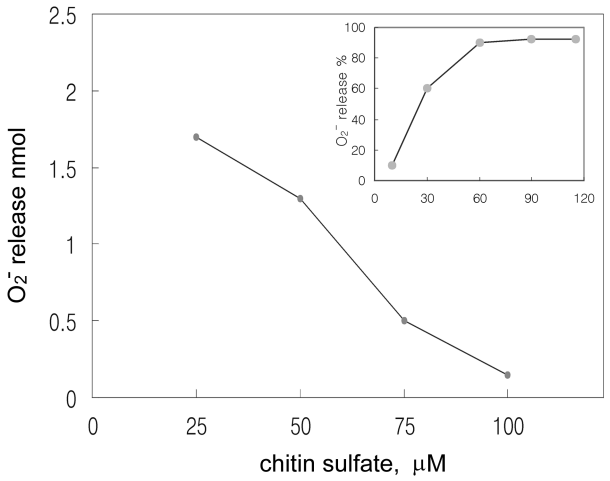

Fig. 4, Effect of chitin sulfate on PMA-induced O_2^- release from mouse macrophages.

Cells were treated with chitin sulfate for 20 min at 37°C and then activated by addition of PMA (0.1 μM). O_2^- formation was monitored at 550nm by measurement of ferricytochrome reduction and the incubation time was 60 min as described in Methods. Each point represents the mean ± S.E.M. (n=3-5). The inset is the time course of PMA (0.1 μM)-stimulated prodution of O_2^- from mouse macrophages. Each point is the average of duplicate samples.

sulfate could be inhibited on LDL oxidation which employed at a low concentration. This phenomenon could be explained on the basis of two different mechanisms: (1) a partial sequestration of free radical by the polyanion chain of chitin sulfate; (2) the possible changes in the ability of LDL to bind free radical, due to the structural modification of the particle following the interaction with chitin sulfate. Our study emphasizes that the antioxidant effect of chitin sulfate protects not only a free fatty acid, but also a biological at structure, such as human LDL against, cells induced oxidation.

This model of oxidation was demonstrated to produce LDL sharing many structural and functional properties with LDL oxidized by cells[25].

Moreover, it should be reminded that oxidized LDL are contained in aortic wall and increase in atherosclerotic plaques[26], where they may contribute to the development of the lession by oxidative stress. On the basis, the results suggest that chitin sulfate(and probably other glycoaminoglycans oceurring in natural sources). might play a inhibitory a role in the process of LDL oxidation *in vivo*, which may change the metabolic fate of the particle, increasing its atherogenic potential[6,22].

The present results that macrophages pretreated with chitin sulfate at 100 μM for 72 h showed a lower capability to oxidize LDL, suggest that chitin sulfate might enter and accumulate in the cells or the LDL particles, it is conceivable that an effective level of chitin sulfate for inhibition of LDL oxidation may be attainable *in vivo*. The observation that chitin sulfate markedly inhibits human LDL oxidation by macrophages suggest that chitin sulfate may have antiathrogenic effects. This novel action of chitin sulfate would increase in its therapeutic value as an LDL oxidation, since lipid peroxidation.

Acknowlegment

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국문요약

본 연구는 동맥경화의 원인으로 알려진 사람 oxidized low density lipoprotein (LDL) 에 대한 chitin sulfate의 산화억제 효과에 대하여 실험하였다. 사람 LDL을 쥐의 마크로파지 유도 oxidized LDL로 산화시킬 때 chitin sulfate를 첨가하여 TBARS와 전기영동에 의한 이동상 및 O_2 소거 기능을 확인하였다. 사람 LDL에 macrophage를 이용해서 LDL을 산화시킬 때 chitin sulfate를 50~100 μM 첨가하였을 때 억제 효과가 높았으며, chitin sulfate를 100 μM 첨가하였을 때 LDL의 산화가 거의 억제되었고, 전기영동에 의한 이동거리도 100 μM chitin sulfate에서 완전히 억제되었다. 그리고 phorbol myristate acetate를 처리한 마크로파아지 유도 활성 산소의 소거 효과는 chitin sulfate의 농도가 100 μM일 때 거의 소거하였다. 이상의 결과로 보아 chitin sulfate는 동맥경화의 예방에 효과적이라는 결론을 얻었다.

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