Laboratory Research

Induction of macrophage inflammatory cytokines by Ox-LDL is ABCA1 dependent

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Objective The current study aimed to evaluate whether the induction of macrophage inflammatory cytokines by Ox-LDL is related to the expression of ABCA1 pathway. Methods After THP1/PMA macrophages were transfected with ABCA1 antisense oligonucleotides (100nmol/L) followed by treatment with Ox-LDL (30mg/L), the expressions of ABCA1, ICAM-1 and MCP-1 mRNA and protein were determined by real-time fluorescent quantitative RT-PCR, Western blot or ELISA. Results Ox-LDL induced expressions of ABCA1, ICAM-1, and MCP-1 at both mRNA and protein levels from THP1/PMA macrophages. Transfection with ABCA1 antisense oligonucleotides reduced ABCA1 mRNA levels after 3 and 6 hours and protein levels after 12 and 24 hours. The expression of ICAM-1 and MCP-1 induced by Ox-LDL was also decreased after inhibition of ABCA1 protein expression by ABCA1 antisense oligonucleotide decreased. Conclusion The induction of macrophage inflammatory cytokines by Ox-LDL is partially dependent on expression of ABCA1. Our studies disclose new functions of ABCA1 in macrophages (J Geriatr Cardiol 2010; 7:166-170).

Key words ATP-Binding cassette A1; THP1/PMA macrophage; inflammatory cytokine

Introduction

Atherosclerosis is a major pathogenesis of coronary artery disease (CAD) and stroke. Besides the traditional view that hyperlipidemia is a main cause of atherosclerosis, a lot of accumulating evidences suggests that atherosclerosis is also a chronic inflammatory disease.¹ Oxidized low-density lipoprotein (Ox-LDL) induces inflammatory responses in vasculature, such as monocyte/macrophages, and contributes to the development of atherosclerosis. ATP-binding cassette transporter A1 (ABCA1), a molecule using the hydrolysis of ATP as an energy source, stimulates the efflux of free cholesterol and phospholipids from macrophages and generates mature high density lipoprotein (HDL). Animal studies demonstrated that highly expressed ABCA1 inhibits the development of atherosclerosis. In contrast, deletion of ABCA1 expression promotes formation of atherosclerotic lesions in aortas. In humans, ABCA1 mutations result in a Tangier disease and familial hypoalphalipoproteinemia characterized by the significant reduction in plasma HDL cholesterol (HDL-C) levels and the early onset of CAD. ²–⁶ ABCA1 has great impact on the formation of macrophage/foam cells and development of atherosclerosis. However, it is not clear if ABCA1 influences the production of inflammatory cytokines induced by Ox-LDL in monocytes/macrophages. To test this hypothesis, we evaluated the effects of ABCA1 expression on the production of inflammatory cytokines. We observed that induction of inflammatory cytokines by Ox-LDL is blocked by inhibition of ABCA1 expression.

Methods

Preparation of Ox-LDL

Low-density lipoprotein (LDL) was isolated from healthy human serum by a density ultracentrifugation method as following description. Serum was separated by centrifugation from human blood without addition of an anticoagulant reagent. Density of the serum was adjusted to 1.30 g/ml by a potassium bromide solution. After ultracentrifugation for 5h at 50,00g and 4⁰C, the LDL was collected and followed by dialysis against 1xPBS for 48h at 4⁰C. Ox-LDL was prepared by incubation of LDL in 1xPBS solution containing 5 μmol/ml CuSO4 for 24h at 37⁰C and then dialyzed for another 24h in 1xPBS containing 0.2mmol/L EDTA. After filtration with a 0.22 m filter, Ox-LDL was stored at 4⁰C. The oxidative degree of Ox-LDL was analyzed by a thiobarbituric acid (TBA) reaction method.

Cell culture

To induce macrophage differentiation, 2 × 10⁴ THP1 monocytes/cm² in 9.6cm² were added with phorbol 12-
myristate 13-acetate (PMA) at a final concentration of 100 nmol/L, and continued incubation for 72 hours. After removal of PMA and washed by PBS, THP1/PMA macrophages were continued culture in completed RPMI 1640 medium for 24 hours. Cells were then switched into serum-free RPMI 1640 medium containing 2 mg/ml BSA for 12 hours before the initiation of treatment. The cells were pre-treated with or without anti-ABCA1 antisense oligonucleotide for 5 hours and then incubated with 30mg/L of Ox-LDL for the indicated time.

**Fluorescent quantitative reverse transcription-polymerase chain reaction**
RNAgents total RNA isolation system and RNA reverse transcription system were purchased from Promega (Madison, WI, USA). Analysis of mRNA expression by fluorescent quantitative reverse transcription-polymerase chain reaction (RT-PCR) was performed as follows. Total RNA was extracted from ~10^6 macrophages by phenol/chloroform/isoamyl alcohol. cDNA was synthesized by using the reverse transcription kit according to the instruction from manufacturer and with 1 μg total RNA, and followed by PCR with primers and reaction conditions listed in Table 1.

**Western blotting for ABCA1, ICAM-1 and MCP-1 protein**
After treatment, THP-1/PMA macrophages (10^6) were lysed in lysis buffer. The whole cellular lysate was extracted by centrifugation at 13,000 × g at 4°C. After determination of the content, 50g protein from each sample was loaded on and separated by a 6~15% sodium dodecyl sulfate-polyacrylamide gel electrophoresis. After electrophoresis, proteins were transferred onto a nitrocellulose membrane. After blocking, the membrane was incubated with a primary antibody overnight at 4°C followed by incubation with a horseradish peroxidase conjugated goat anti-rabbit IgG. The membrane was then incubated in a chemiluminescence solution followed by exposure to an X-Omat AR film (Amersham Biosciences Limited, USA).

Anti-ABCA1, ICAM-1 and MCP-1 polyclonal antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA) and used for Western blot analysis of these molecule protein levels. The enzyme-linked immunosorbent assay (ELISA) kits for determination of ICAM-1, MCP-1 and IL-1β were purchased from R&D system (Minneapolis, MN, USA).

**The sequence and synthesis of antisense oligonucleotide for ABCA1**
Anti-ABCA1 antisense oligonucleotide (sequence: 5’-CATGTTGTTCATAGGGTGGGTAGCTC-3’) were prepared essentially as instructed by the manufacturer (Aoke Biotechnology, Beijing, China). Medium was mixed with Lipofectamine reagent (Invitrogen, Carlsbad, CA) (5min), then was added with anti-ABCA1 antisense oligonucleotide. The complex was allowed to equilibrate for 30 min at room temperature, after which time they were immediately added to the cell cultures. The final concentration of anti-ABCA1 antisense oligonucleotide is 100nmol/L.

**Statistical analysis**
All data were collected from six repeatable experiments. Data were analyzed with SPSS (Version 13.0) and were presented as mean ± standard deviation (SD). Multiple comparisons among experimental groups were performed by one-way ANOVA analysis. Statistical significance was considered at p ≤ 0.05.

**Results**
Effect of Ox-LDL and ABCA1 antisense oligonucleotide on ABCA1, ICAM-1 and MCP-1 mRNA levels of THP1/PMA macrophage
To study the effects of Ox-LDL on expression of ABCA1, ICAM-1 and MCP-1 mRNA, THP1/PMA macrophages in serum-free medium were treated with 30 mg/L Ox-LDL for 3 and 6 hours respectively. Changes in mRNA levels of these molecules were determined by fluorescent quantitative RT-PCR (Figure 1). Compared with control group, Ox-LDL increased ABCA1 mRNA expression by 18% and 42% after 3 and 6 hours treatment, respectively, while the levels of

<table>
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<th>Table 1</th>
<th>RT-PCR reaction condition and PCR primer sequences</th>
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<td>RT reaction condition</td>
<td>42°C 60 min, 95°C 5 min, 4°C 5 min</td>
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<tr>
<td>PCR reaction condition</td>
<td>95°C 5 min; 96°C 20 s, 56°C 20 s, 72°C 20 s, 50 cycles; 72°C 5 min</td>
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<td>Sequences of primers for PCR and the length of PCR products (bp)</td>
<td>ABCA1 (201 bp): sense primer, 5’ GAT GGC AAT CAT GGT CAA TGG 3’; anti-sense primer, 5’ AGC TGG TAT TGT AGC ATG TTC CG 3’ ICAM-1 (185 bp): sense primer, 5’ CGA GGT GAC CGT GAA TGT GCT 3’; anti-sense primer, 5’ TGG CTT GTG TGT CGT TTC CG 3’ MCP-1 (186 bp): sense primer, 5’ TCA GCC AGA TGC AAT CAA TGC 3’; anti-sense primer5’, TCC TGA ACC CAC TTC TGC TTG 3’</td>
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ICAM-1 mRNA were increased by 19% and 48%, and the levels of MCP-1 mRNA were increased by 15% and 43%, respectively.

Antisense oligonucleotide for ABCA1 reduced expression ABCA1 mRNA (by 28 and 16% after 3 and 6 hours of transfection, respectively). Interestingly, the inhibition of ABCA1 expression also decreased the mRNA levels of ICAM-1 (by 32 and 18% after 3 and 6 hours of transfection, respectively) and MCP-1 mRNA (by 26% 13% after 3 and 6 hours of transfection, respectively).

**Effect of Ox-LDL and ABCA1 antisense oligonucleotide on expressions of ABCA1, ICAM-1, and MCP-1 by THP1/PMA macrophages**

To study if the changes in ABCA1, ICAM-1 and MCP-1 mRNA were associated with the change in protein levels of these molecules, the whole cellular proteins were extracted and used to determine ABCA1, ICAM-1 and MCP-1 protein levels by Western blot (Figure 2). Compared with control group, Ox-LDL induced ABCA1 protein by 59% and 29% after 12 and 24 hours treatment, respectively. ICAM-1 protein levels were increased by 68% and 53%, respectively while MCP-1 protein levels were increased by 57% and 48%, respectively, by Ox-LDL ($P<0.01$, Figure 2). These results suggested that Ox-LDL up-regulated expression of these molecules at both transcriptional and translational levels.

In contrast, transfection of antisense oligonucleotide for ABCA1 inhibited Ox-LDL induced expression of ABCA1, ICAM-1 and MCP-1 proteins (after 12 hours: 11%, 20% and 16% respectively; after 24 hours: 26%, 32% and 29% respectively; All $P<0.01$) (Figure 2). The protein levels of these molecules by ELISA method showed similar results (data not shown).

**Fig.1 Effects of Ox-LDL and antisense oligonucleotide for ABCA1 on mRNA expressions of ABCA1, ICAM-1 and MCP-1 in THP1/PMA macrophages.** THP1/PMA macrophages in serum-free medium were pre-treated with or without anti-ABCA1 antisense oligonucleotide (100nmol/L) for 5 hours and then incubated with 30mg/L of Ox-LDL for indicated time. The total RNA was extracted and subjected to fluorescent quantitative RT-PCR. The mRNA levels of ABCA1 (A), ICAM-1 (B) and MCP-1 (C) was determined as described previously. Cells cultured in medium alone were served as control. Data are expressed as mean ± SD. $n=6$ in each group at each time point. * $P<0.01$ vs. Ox-LDL group; # $P<0.01$ vs. control.
Discussion

In our study, we observed that Ox-LDL increased mRNA and protein levels of ABCA1, ICAM-1, and MCP-1. However, the induction of these molecules by Ox-LDL was blocked by the transfection of antisense oligonucleotide for ABCA1. Atherosclerosis is considered as a disorder of inflammation. Several inflammatory cytokines are involved in the development of atherosclerosis, particularly the cytokines are related to plasma lipid metabolism. Our results suggested that induction of macrophage inflammatory cytokines by Ox-LDL is partially dependent on expression of ABCA1. ABCA1 might be a pro-atherogenic factor, since when it was inhibited the expression of inflammatory cytokines by macrophages was decreased.

ABCA1 increases the reverse cholesterol transport and exerts its anti-atherosclerosis properties. Expression of ABCA1 can be up-regulated by Ox-LDL, acetylated-LDL, cyclic AMP and apoAI. The induction of ABCA1 expression by Ox-LDL might be a compensative mechanism to attenuate Ox-LDL induced atherosclerosis, even this protective effect is greatly overwhelmed by the strong pro-atherogenic effects of Ox-LDL. ABCA1-induced efflux of free cholesterol and phospholipids from peritoneal tissues is required for the synthesis of high density lipoprotein.

Although ABCA1 was considered as an anti-atherogenic factor in previously published literates, our study suggested that ABCA1 played a pro-atherogenic role in the pathogenesis of atherosclerosis. It has been reported an interplay between ABCA1 and inflammatory cytokines. For example, IL-1β and ABCA1 are able to influence expression of each other. Zhou et al observed that the IL-1β level in macrophages isolated from Tangier disease patients is lower than
that from healthy controls. Inhibition of ABCA1 by transfection of ABCA1 antisense oligonucleotides results in 30-50% decrease in expression of IL-1β. Similarly, down-regulation of ABCA1 expression by glibenclamide also reduces expression of IL-1β in mouse peritoneal macrophages and in human monocytes. Those data imply that decreased ABCA1 expression may decrease IL-1β secretion from macrophages. PD169316, a p38 MAPK inhibitor, has been reported to decrease ABCA1 expression in THP1 cells suggesting the role of p38 MAPK in regulation of ABCA1 expression.

Our finding adds some useful information for understanding the effect of ABCA1 on the pathogenesis of atherosclerosis. According to our data and others studies, we extrapolate that when ABCA1 increased the reverse cholesterol transport and exerted anti-atherosclerosis properties, it also induced inflammatory process and serve as a pro-atherosclerosis factor. These divergent effect shows that it exists complexity in ABCA1 contributing to atherosclerosis. The role of ABCA1 in the pathogenesis of atherosclerosis deserves further investigation.

In conclusion, the present study demonstrated that induction of macrophage inflammatory cytokines by Ox-LDL is partially dependent on expression of ABCA1. Our studies suggested that ABCA1 might be a pro-atherogenic factor on the level of inflammatory cytokines during the pathogenesis of atherosclerosis.

Acknowledgments

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References