# Lymphocyte Cytotoxicity of oxLDL in Patients with Atherosclerosis

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## ABSTRACT

**Background:** Atherosclerosis, a chronic inflammatory disease of the vessel wall is characterized by local and systemic immune responses to a variety of antigens. Oxidized low-density lipoprotein (oxLDL) is considered as an important determining factor in the pathogenesis of atherosclerosis. **Objective:** The purpose of this study was to investigate the degree of peripheral blood mononuclear cells (PBMC) vulnerability to in vitro oxLDL-induced cytotoxicity from atherosclerotic patients in comparison to healthy individuals. **Methods:** Thirty patients with atherosclerotic lesions, confirmed by angiography, and 30 matched healthy individuals were investigated. PBMC was prepared from individuals' blood samples which were further stimulated with low dose (1 μg/mL) and high dose (50 μg/mL) of extensively oxidized LDL. MTT assay was utilized to measure cell viability and proliferation. Stimulation index (SI) was calculated as mean ratio of optical density (OD) of the stimulated cells divided by OD of untreated cells. **Results:** Low dose oxLDL treatment caused no significant proliferative or cytotoxic effect in the control group; however, similar treatment caused significant cytotoxic effect in the patient group compared to the controls ($p=0.026$). High dose oxLDL treatment induced more significant cytotoxicity in the patient compared to the control group ($p=0.006$). Comparison of the SI between the two groups of patients and controls showed significantly lower index by either the low ($p=0.03$) or the high dose ($p<0.001$) oxLDL in the patients compared to the controls. **Conclusions:** PBMC from patients with atherosclerosis showed increased susceptibility to oxLDL-induced cytotoxicity. Our results imply that prolonged exposure to elevated levels of circulating oxLDL could weaken the cellular defense mechanisms by progressive depletion of the pool of antiapoptotic proteins, rendering the cells more vulnerable to oxLDL-induced cell death.

**Keywords: Atherosclerosis, Cytotoxicity, oxLDL, Peripheral Blood Mononuclear Cells**

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## INTRODUCTION

Atherosclerosis is a chronic inflammatory disease of the vessel walls characterized by involvement of both innate and adaptive immunity in lesion formation and disease progression (1,2). Oxidized low-density lipoproteins (oxLDL), encompassing a variety of oxidatively modified lipids and proteins, are believed to play a central role in atherogenesis (3). OxLDL provokes defective endothelium-mediated vasodilation, induces local chemokine production, endothelial adhesion molecule expression, monocyte recruitment to the arterial wall and subsequent foam cell formation. It also promotes smooth muscle cell migration and proliferation associated with increased extracellular matrix synthesis, and subsequently shifts the haemostatic mechanisms in favor of thrombosis (4).

OxLDL-induced cytotoxicity in a variety of cell types, including monocytes (5), macrophages (6), lymphocytes (7), endothelial cells (8), and vascular smooth muscle cells (9), is potentially involved in necrotic core formation, endothelial cell lining defect and plaque rupture with subsequent thrombus formation (3). The toxic lipid constituents of oxidized lipoproteins, including a variety of oxysterols, have been demonstrated to increase intracellular reactive oxygen species (ROS) generation, induce structural modifications of cell proteins, and alter a variety of proapoptotic signaling pathways and gene expression (10). Oxysterols trigger both mitochondrial (intrinsic) and death receptor-dependent (extrinsic) apoptotic pathways in various cell types; however, the respective roles of individual compounds remain to be elucidated (11,12).

In early atheromatous lesions, increased macrophage apoptosis and efficient clearance of apoptotic cells, a process known as efferocytosis, have been shown to slow the progression of atherosclerosis (13-15), possibly through prevention of post-apoptotic secondary necrosis and induction of transforming growth factor (TGF)-β, IL-10, and other mediators of inflammation resolution (16). In contrast, defective efferocytosis has been demonstrated in advanced atheromatous lesions, which contributes enormously to necrotic core formation and increased plaque vulnerability (17). Several hypotheses have been proposed to account for defective efferocytosis (16), including poor display of efferocytosis ligands on the apoptotic cells, defective expression or function of bridging molecules or efferocytosis receptors, and altered subpopulations of phagocytes (18). In addition, extensive macrophage apoptosis may limit the pool of competent efferocytes and overwhelms the phagocytic capacity of the remaining macrophages (16).

The cytotoxic effect of oxLDL on normal human peripheral blood mononuclear cells (PBMC) has been demonstrated in vitro (5,19-21). Given the paucity of similar studies in patients with atherosclerosis, the purpose of the present study was to investigate the degree of PBMC vulnerability to in vitro oxLDL-induced cytotoxicity from atherosclerotic patients in comparison to healthy individuals.

## MATERIALS AND METHODS

**Subjects.** Thirty patients (25 male and 5 female; mean age of 53.2 ± 7.8 years, ranging from 40 to 64 years) with diagnosis of coronary artery disease (CAD), who referred to the Division of Cardiology in the teaching hospitals of Tehran University of Medical

Sciences, were enrolled in this study. All these patients had significant atherosclerotic lesions confirmed by angiography, defined as luminal stenosis ≥50% in at least one major coronary artery branch. Subjects with active infections or autoimmune diseases were excluded from the study. Thirty healthy sex- (25 male and 5 female) and age-matched (mean age of 51.0 ± 6.8 years, ranging from 40 to 62 years) individuals with no history of CAD were also enrolled. The study was approved by the Ethics Committee of Tehran University of Medical Sciences and Health Services. Written informed consent was obtained from all subjects prior to blood collection.

**Cell preparation.** 10 mL of fasting venous blood sample was drawn from all subjects and collected in sodium citrate tubes. Prior to PBMC separation, the blood was centrifuged at 400 × g for 10 minutes at room temperature to remove the plasma, and sufficient quantity of sterile phosphate-buffered saline (PBS) was added to bring the blood to its original volume. Subsequently, the blood was diluted with an equal volume of PBS and was carefully layered onto half the volume of Ficoll-Histoprep (BAG Health Care GmbH, Germany), using plastic pipettes. Centrifugation was carried out at 800 × g for 30 minutes at room temperature. The layer of mononuclear cells at the interface was removed using a sterile disposable Pasteur pipette, diluted with PBS, and centrifuged at 250 × g for 10 minutes at room temperature. The washing step was repeated twice. Cell viability and cell counts were assessed by Trypan blue exclusion method. Mononuclear cells were resuspended in culture medium (RPMI-1640 supplemented with 10% heat inactivated fetal calf serum; Gibco, Invitrogen, UK), diluted to $1 \times 10^6$ cells per mL, and plated on 96-well flat-bottomed microtiter plates at a density of $1.5 \times 10^5$ cells per well. Cultured cells were stimulated with a low dose (1 μg/mL) and a high dose (50 μg/mL) of extensively oxidized LDL and incubated at 37˚C under a 5% $CO_2$ environment for 66 hours.

**Preparation of oxidized LDL.** 500 μg/mL (100 μg/mL protein) of low density lipoprotein (LDL; Sigma, USA) was incubated with 10 μM copper sulfate ($CuSO_4$; Aldrich, USA) overnight at 37˚C in order to prepare extensively oxidized LDL (22). Oxidation was continuously monitored by measuring the kinetics of conjugated diene formation at 234 nm over a period of 8 hours (23). Oxidation reaction was terminated by cooling and addition of ethylenediaminetetraacetic acid (EDTA). The degree of LDL oxidation was determined by measuring thiobarbituric acid reactive substances (TBARS) (24). Exposure of LDL to10 μM $CuSO_4$ resulted in an increase in the amount of TBARS in the LDL preparation from 2.1 to 42 nano mole MDA eq/mg LDL protein. OxLDL preparation was kept in 2-8˚C and used within one week.

**Colorimetric MTT assay.** In order to determine cell proliferation and cytotoxicity, the number of metabolically active cells was measured using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay (25). Following the incubation period of 66 hours, 0.5 mg/mL MTT dye was added and the plates were incubated at 37˚C under a 5% $CO_2$ environment for 4 hours. The formazan water insoluble precipitate was extracted and dissolved in 200 μL dimethyl sulfoxide (DMSO). Subsequently, the absorbance (optical density, OD) was determined at 550 nm using micro ELISA plate reader (ELX 800, BioTek, USA). Stimulation index (SI) was calculated as the mean ratio of the OD of the stimulated cells divided by that of the non-stimulated cells.

**Statistical analysis.** Statistical analysis was performed using the Statistical Package for the Social Sciences (SPSS) software, version 16.0. Comparisons of OD between untreated and stimulated cells were made using paired-samples t-test. Comparisons of

the results between two groups of patients and controls were done using independent samples t-test. P value of less than 0.05 was considered statistically significant.

## RESULTS

**Stimulation with low dose oxLDL.** Although stimulation of PBMC with low dose oxLDL did not cause any significant proliferative or cytotoxic effect in the control group (0.82 ± 0.14 *vs.* 0.84 ± 0.16, p=0.45), similar treatment caused significant cytotoxic effect on the PBMC of the patient group (0.91 ± 0.16 *vs.* 0.87 ± 0.17, (p=0.026) (Figure 1). Comparison of SI between patients and controls revealed a significantly lower index in the patient group (p=0.03, Table 1).

**Table 1. Comparison of the stimulation index after stimulating the mononuclear cells with different doses of the stimulation**.

| | Stimulation index | | P value |
|---|---|---|---|
| **Stimulant** | **Patients** | **Controls** | |
| Low dose oxLDL | 0.95±0.14 | 1.04 ± 0.19 | 0.032 |
| High dose oxLDL | 0.63±0.16 | 0.85 ± 0.20 | <0.001 |
| P value | <0.001 | <0.001 | <0.001 |

**Stimulation with high dose oxLDL.** Increased cytotoxicity was found when the cells were treated with a high dose of oxLDL (p<0.001) in both the patients and the controls (Figure 1).

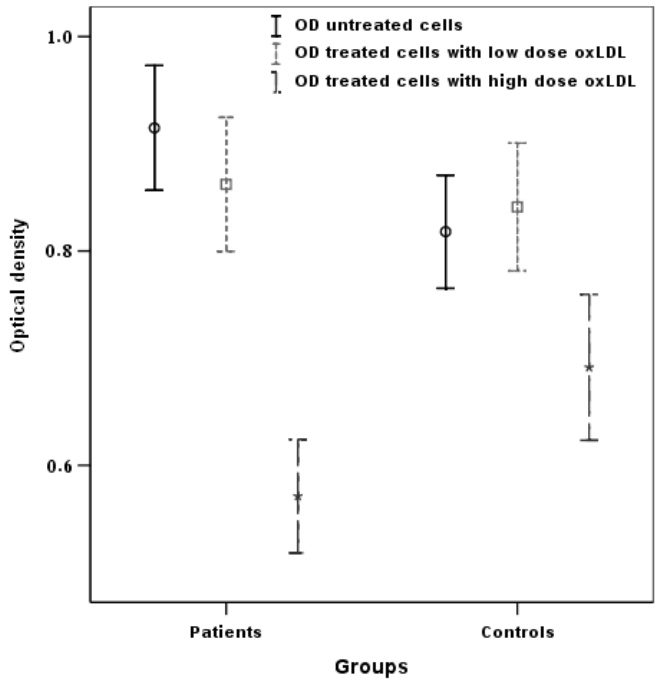


**Figure 1.** Comparison of optical density (OD) between unstimulated and stimulated cells treated with either the low or high dose of oxLDL in both the patients and controls.

After stimulating with a high dose of oxLDL, the mean OD was 0.57 ± 0.14 in the patient group, which was significantly lower than the value of 0.69 ± 0.18 observed in the control group ($p=0.006$). The high dose of oxLDL caused a significantly more cytotoxicity, and therefore decreased the SI in the patient group ($p<0.001$, Table 1). Comparison of the stimulation with the high and low dose of oxLDL revealed significantly higher inhibitory effect of the high dose of oxLDL in both the patient and control groups ($p<0.001$, Table 1).

## DISCUSSION

In the present study, higher cytotoxicity of oxLDL in cultured PBMC from atherosclerotic patients was found compared to the healthy individuals. In response to the low dose of oxLDL, no significant proliferative or cytotoxic effect was detected in the PBMCs obtained from the healthy subjects, whereas a similar treatment caused a significant cytotoxic effect in the patient group. Increased cytotoxicity was detected in cultured cells of both groups treated with the high dose of oxLDL, an effect which was more prominent in the patient group. Over the past decade, oxLDL-induced cell death, including both necrosis and apoptosis, has been demonstrated repeatedly in healthy individuals (5-9,19-21).

OxLDL has been shown to induce apoptosis in unstimulated human monocytes freshly isolated from the peripheral blood of healthy individuals via ROS generation, mitochondrial Bax translocation with mitochondrial membrane potential disruption, cytosolic *cytochrome c* release and subsequent caspase-9 and -3 activation (5). In addition, oxLDL significantly inhibits PBMC proliferative responses to different mitogens, antigens, and human recombinant IL-2 (19,20), which was counterbalanced by the addition of *N*-acetylcysteine, a free radical scavenger and glutathione precursor (19). The results of the present study point to the increased vulnerability of PBMCs from atherosclerotic patients to OxLDL-induced cytotoxicity in vitro. One plausible explanation is that prolonged exposure to elevated levels of circulating oxLDL would down regulate the expression of the antiapoptotic members of the Bcl-2 family of proteins (26-28) and the inhibitor of apoptosis proteins (IAPs) (29,30) in PBMCs from atherosclerotic patients. The latter specifically binds to and inhibits active caspases and is negatively regulated by IAP-binding proteins, including Omi and Smac/DIABLO (31).

It seems that the cellular response induced by oxLDL depends on the degree of oxidation, duration of exposure, and its concentration (6). A 24-hour exposure to 100 μg/mL of the lightly oxidized LDL induced macrophage activation and proliferation, whereas at the same concentration, the extensively oxidized LDL induced cell death. In contrast, cells incubated for 4-6 hours showed a proliferative response to 100 μg/mL of the extensively oxidized LDL. Furthermore, 200 μg/mL of oxLDL caused cell death regardless of the degree of oxidation (6). In the present study, cultured PBMC from atherosclerotic patients treated with 1 μg/mL of oxLDL for 66 hours showed increased cell death. Therefore, comparing the results of differently designed in vitro studies using oxidatively modified LDL may not be conclusive due to the variations in the mode and degree of oxidation, dose, and exposure time.

In our study, cultured PBMC from atherosclerotic patients showed increased vulnerability to oxLDL-induced cytotoxicity in comparison to healthy individuals. Hyperlipidemia, ongoing systemic inflammation, and the resultant increase in circulating oxLDL concentration could be hypothetically involved in increased oxLDL-induced cytotoxicity. Although continuous therapy with lipid lowering agents and antioxidants is proven to be beneficial in patients with atherosclerosis, in order to intervene more effectively, further studies to uncover the underlying cellular and molecular mechanisms with potential therapeutic targeting would be of immense value.

## ACKNOWLEDGMENTS

This research has been supported by Tehran University of Medical Sciences and Health Services grant (86-04-27-6617). The authors are very grateful for all colleagues in the Department of Nutrition and Biochemistry, School of Public Health, Tehran University of Medical Sciences for their kind helps and advices in the laboratory. We would like to thank all the patients and control subjects for their kind collaboration in this study.

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