The *in vitro* Effect of Oxidized LDL and PHA on Proliferation and Gene Expression of Regulatory T Cells in Patients with Atherosclerosis

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ABSTRACT

Atherosclerosis is a chronic inflammatory condition that affects the arterial wall. Oxidized low-density lipoprotein (ox-LDL) seems to have an important role in atherosclerotic plaque formation.

This study was performed to investigate the effects of ox-LDL as well as PHA on proliferation and gene expression of peripheral blood mononuclear cells (PBMCs) in patients with atherosclerosis compared to healthy controls. Proliferation of PBMCs was assessed by BrdU assay, while gene expression was assessed by real-time PCR.

Both PHA and ox-LDL significantly induced proliferation of PBMCs of patients and controls. PBMCs from controls showed significantly higher proliferation when stimulated with ox-LDL compared to patients. Expression of TGF-β was significantly lower in PBMCs from patients compared to healthy controls ($p < 0.001$). Following simulation with PHA, TGF-β and Foxp3 gene expression levels in patients and controls were significantly decreased ($p < 0.001$). Expression of Foxp3 in PBMCs treated with ox-LDL was significantly decreased in patients and controls.

Decreased expression of TGF-β and Foxp3 genes after ox-LDL stimulation may be due to more sensitivity of Treg cells than effector T cells to ox-LDL. Presence of ox-LDL within atheroma could be associated with the diminished population of Treg cells in the atherosclerotic patients.

Keywords: Atherosclerosis; Gene expression; Ox-LDL; Peripheral blood mononuclear cells; T regulatory cells

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INTRODUCTION

Atherosclerosis is a chronic inflammatory disease leading to thickening of artery walls. It seems that different immune cells, particularly T lymphocytes, involve in initiation and progression of atherosclerosis,[1] while several immune alterations have been reported in the Atherosclerotic patients.[2,3] There are some evidences showing T-cell chemotactic effects for oxidized low-density lipoprotein (Ox-LDL), at the beginning of atherosclerotic plaque formation.[4,5] Ox-LDL facilitates the adhesion of T cells and monocytes to the endothelium of vessels and thought to be involved in the pathogenesis of atherosclerosis.[6] Macrophages can be transformed into foam cells by ox-LDL and this conversion is characteristic of the early fatty streak.[7] After oxidation of LDL, toxic oxidized lipids, such as lipid peroxides, oxysterols and aldehydes can change cell proteins.

This modification demonstrates oxidative stress, lipid peroxidation and alters various signaling pathways and gene expression. These conditions may cause stimulation of intense, delayed and sustained calcium peak that displays either apoptosis or necrosis processes. Atherosclerotic areas that have suffered from apoptosis is potentially involved in endothelial cell lining defects, necrotic core formation and plaque rupture or erosion that may stimulate atherothrombotic events.[8]

T regulatory (Treg) cells are a subset of CD4+ T cells that express Foxp3 as transcription factor[9] and play a pivotal role in the regulation of T cell-mediated immune responses through suppression of T cell proliferation and by releasing anti-inflammatory cytokines such as interleukin (IL)-10 and transforming growth factor (TGF)-β [10]. It seems that Treg cells can actively participate in inhibition of development and progression of atherosclerosis.[1] Considering the hypothesis that atherosclerotic plaque inflammation relates to autoimmunity, inhibitory role of Treg cells in atherosclerosis can be explained.[11] The protective role of CD4+CD25+Foxp3+ Treg cells in development of plaque in atherosclerosis has been shown in previous studies.[12-14] Treg cells have the potential suppressive effects on the *in situ* inflammatory response.[11] Animal studies showed that the increase in the number of Treg cells in atherosclerosis prone mice is associated with decreased size of atherosclerotic lesions,[12,14] while altered status of CD4+CD25+ Treg cells in the peripheral blood of patients with acute coronary syndromes have been reported.[15]

This study was performed to investigate effects of PHA and ox-LDL on FoxP3 and TGF-β gene expression and cell proliferation of patients with atherosclerosis.

PATIENTS AND METHODS

Subjects

Thirty one atherosclerotic patients (16 men and 15 women, mean age of 52.75±7.1, ranging from 38 to 69 years) and 15 healthy controls (8 men and 7 women, mean age of 55.42±7.5, ranging from 39 to 62 years) were enrolled in this study. Patients and controls were matched for age and sex. Presence of atherosclerosis in patients had been confirmed by angiography by cardiologists. All patients had stable angina and were on aspirin and statin treatment. None of the patients or healthy controls had any disease that affects the immune system such as asthma, active viral disease, rheumatoid arthritis, systemic lupus erythematosus, multiple sclerosis, type 1 diabetes and inflammatory bowel disease and none of them was under any treatment such as corticosteroids or immunosuppressive therapy.

This study was approved by the Ethics Committee of Tehran University of Medical Sciences. All participants signed the written informed consent form before enrollment in this study.

Cell Isolation and Culture

Heparinized fasting peripheral blood was collected from all patients and controls. The plasma was separated, aliquoted and stored at -80 ℃ for further analysis. PBMC was purified using Ficoll-Histoprep (Sigma, USA) using standard procedures. Viability of separated cells was checked and was over 90%.

PBMCs were suspended in RPMI 1640 medium (Gibco, Invitrogen, UK) supplemented with 10% heat-inactivated fetal calf serum (Gibco, Invitrogen, UK) and 100U/ml penicillin/streptomycin (Sigma, USA) at concentration of 1×10^6 cells per milliliter. Cells were cultured in 24-well culture plates at a density of 1×10^6 cells per well in the presence of T cell activator, phytohaemagglutinin, (PHA; Sigma, USA) at final concentration of 10µg/mL or 2µg/mL specific antigen, ox-LDL (Biomedical Technologies Inc, USA). PBMCs were incubated in 5% CO_2 at 37°C for 66 hours.

Supernatants of cell culture were harvested and frozen at -80°C for other assays and subsequently all of the settled cells in the plates were collected for RNA extraction.

Cell Proliferation BrdU Assay

Proliferation test was carried out using colorimetric bromo-deoxyuridine (BrdU) assay (Roche, Basel, Switzerland). After a period of the incubating cells for 66 hours, 15 µl/well of BrdU labeling solution was added and re-incubated the cells for additional 4 hours at 37°C, and then plate was centrifuged at 300×g for 10 minutes. The supernatant was dsicarded by flicking off and then cells was dried by hair-dryer airflow. 200µl FixDenat was added to all wells and incubated at room temperature for 30 minutes. Following incubation, FixDenat solution was removed thoroughly by flicking off and tapping. 100µl/well anti-BrdU-POD working solution was added and incubated for 90 minutes at room temperature. Antibody conjugate was removed by flicking off and rinsing wells three times with 200µl/well washing solution (PBS, 1×).

Washing solution was removed by tapping and then 100µl/well substrate solution was added and incubated at room temperature until color development was sufficient for photometric detection (5-30 min). Following emergence of color, 25µl $1MH_2SO_4$ to each well was added and the plate was incubated for approximately 1 minute on the shaker at 300rpm.

Within 5 minutes after adding the stop solution, absorbance of the samples in an Enzyme-linked immunosorbent assay (ELISA) reader at 450nm was measured. Stimulation index (SI) was obtained from division of mean ratio of the stimulated cells optical density (OD) to that of the cells without stimulation.

RNA Extraction and cDNA Synthesize

Cytoplasmic RNA was extracted and purified using RNAeasy Plus mini kit (Qiagen, Valencia, CA) according to the manufacturer's protocol. Quantity and purity of extracted RNA was checked by Nanodrop spectrophotometer (NanoDrop Technologies, USA). A ratio of A 260/280 ranging 1.9 to 2.1 was considered as acceptable. Single strand cDNA was synthesized using QuantiTect Reverse Transcription Kit (Qiagen, Valencia, CA).

Real-time PCR for Gene Expression

Real-time polymerase chain reaction (PCR) was carried out in Step One system (Applied Biosystem, Foster city, CA, USA) with SYBR green detection method. PCR primers for TGF-β and Foxp3 genes and β-actin gene (as housekeeping) were designed by Primer express 3 software (Applied Biosystem, Foster city, CA, USA) and purchased from Metabion (Table 1).

PCR reactions were briefly as follows: PCR proceeded in special optical tubes in 48- reaction plates (MicroAmp Optical, ABI) with 20 µl reaction mixture containing 10µl Power SYBR® Green PCR Master Mix *(*Applied Biosystem, Foster city, CA, USA*),* 7µl DEPC treated water, 0.5µl forward primer, 0.5µl reverse primer and 2µl cDNA as template. The wells were sealed with optical adhesive film *(*Applied Biosystem, Foster city, CA, USA*),* and the plate was centrifuged for a few seconds at high speed. Amplification conditions were performed using the standard two-step run protocol; step 1:10s at 95°C, step 2: 40 cycles of 15s at 95°C plus 1s at 60°C. After completion of amplification cycles, melt curve was generated to verify if a single gene product had been amplified.

For each gene, mRNA expression level was normalized to the level of β-actin.

Statistical Analysis

Comparisons between two groups of patients and controls were analyzed by Independent-sample T Test. Comparison of gene expression between stimulated cells and non-stimulated cells were done using paired-samples t-test. *p* value of less than 0.05 was considered statistically significant. Analyses were performed by SPSS 18 (SPSS, Inc., Chicago, IL).

RESULTS

Stimulation with PHA and Ox-LDL

PHA significantly simulated PBMCs in both the patients (OD: 2.03±0.43 *vs.* 0.88±0.16, *p*<0.001) and

Table 1. Sequence of primers

Gene	Sequence
TGF-β	F: 5'- CTCTCCGACCTGCCACAGA-3'
	R: 5'-AACCTAGATGGGCGCGATCT-3'
FOXP3	F: 5'-GCAAAGTTGTTTTTGATACGTGACA-3'
	R: 5'-AGGCTTGGTGAAGTGGACTGA-3'
B-actin	F: 5'-CCTGGCACCCAGCACAAT-3'
	R: 5'-GCCGATCCACACGGAGTACT-3'

control group (OD: 2.28±0.34 *vs.* 1.01±0.24, *p*<0.001). Stimulation of PBMCs with ox-LDL also led to significant proliferations of both groups of patients (OD: 0.96±0.30 *vs.* 0.88±0.16, *p*=0.049) and controls (OD: 1.26±0.33 *vs.* 1.01±0.24, *p*<0.001) (Figure 1).

Stimulation of PBMCs with ox-LDL significantly induced higher proliferation in the controls than the patients, while SI of the patient group was significantly lower than controls (*p*=0.02, Table 2).

Table 2. Comparison of optical density (OD) and stimulation index (SI) of cells stimulated with PHA and ox-LDL and without stimulation in atherosclerotic patient and healthy control groups

Topics	Optical density		*p*-value
	Patients	Controls	
Non-stimulated cells	0.88±0.16	1.01±0.24	0.119
Stimulated with PHA	2.03±0.43	2.28±0.34	0.52
Stimulated with Ox-LDL	0.96±0.30	1.26±0.33	0.015*
SI PHA/non-stimulated	2.34±0.51	2.44±0.85	0.705
SI ox-LDL/non-stimulated	1.09±0.28	1.25±0.13	0.020*

SI: Stimulation index, * statistically significant value

Table 3. Effect of PHA and ox-LDL stimulation on FoxP3 and TGF-β gene expression in atherosclerotic patients and healthy controls

Groups	Gene	Stimulant			*p*-value
		PHA	ox-LDL	non-stimulated	
Patients	Foxp3 (delta Ct)	15.56±2.56		10.65±1.87	<0.001*
			13.07±1.70	10.65±1.87	<0.001*
	TGF-β (delta Ct)	13.10±1.51		5.33±0.78	<0.001*
			5.04±0.88	5.33±0.78	0.061
Controls	Foxp3 (deltaCt)	14.24±1.46		10.93±1.38	<0.001*
			12.99±1.03	10.93±1.38	0.003*
	TGF-β (delta Ct)	13.88±0.85		6.16±1.51	<0.001*
			5.00±0.71	6.16±1.51	0.044*

* Statistically significant value
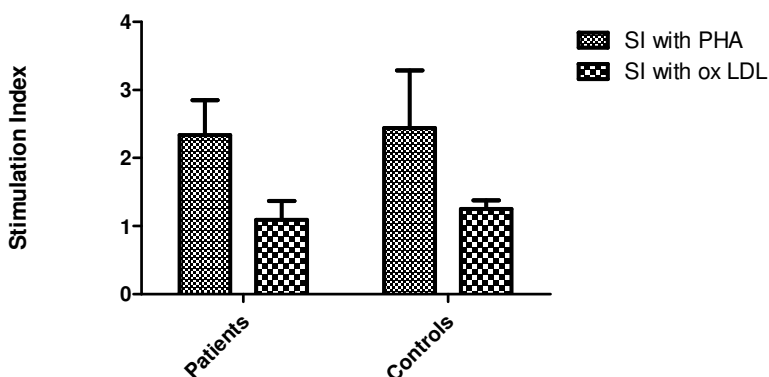
Figure 1. Proliferation of stimulated and non-stimulated PBMCs of atherosclerotic patients and healthy controls after PHA or ox-LDL stimulation
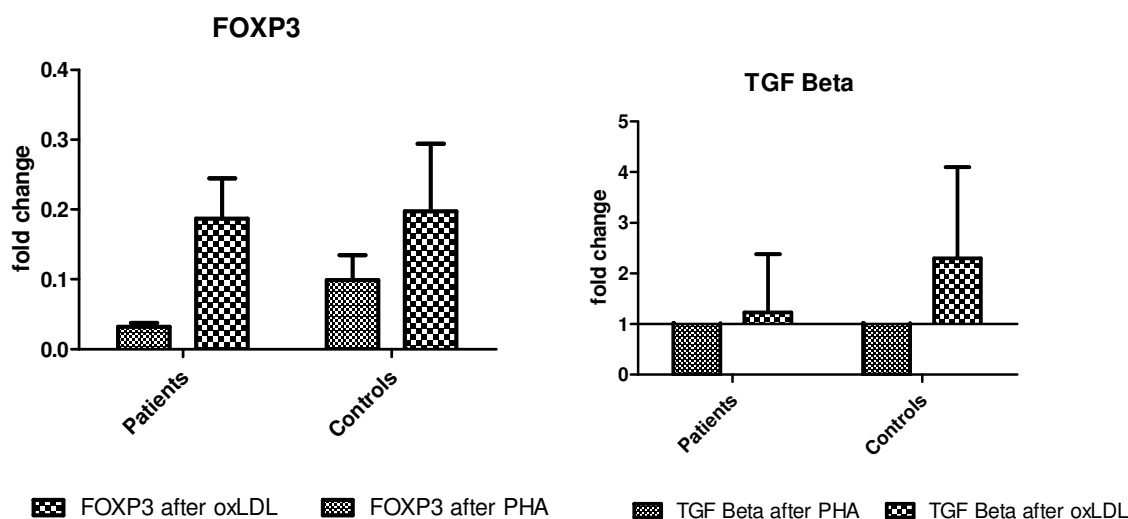
Figure 2. FoxP3 and TGF-β gene expression of fresh PBMCs obtained from atherosclerotic patients and healthy controls after PHA or ox-LDL stimulation

Foxp3 and TGF-β -Expression in Fresh PBMCs

Foxp3 and TGF-β expression levels in the patients were lower than this value in healthy controls. However, this difference was only statistically significant for TGF-β (*p*<0.001) (Figure 2).

Foxp3 and TGF-β Gene Expressions in Stimulated Cells

Following simulation with PHA, TGF-β gene expression levels in patients (Ct: 13.10±1.51 *vs.* 5.33±0.78, *p*<0.001) and controls (Ct: 13.88±0.85 *vs.* 6.16±1.51, *p*<0.001) were significantly decreased; Foxp3 gene expression was similarly decreased in both patients and control groups when stimulated with PHA (*p*<0.001). Foxp3 gene expression of in PBMCs treated with ox-LDL was also significantly decreased in patients and controls (Table 3).

DISCUSSION

This study showed lower proliferative response of PBMCs to ox-LDL in atherosclerosis patients compared to the controls. Recent evidence suggests that DNA synthesis in PBMCs exposed to 1µg/ml ox-LDL increased five folds. Although the best concentration of ox-LDL for stimulation seems to be 1-5µg/ml,[16] a recent study showed that 1µg/ml ox-LDL could not significantly proliferate PBMCs of patients with atherosclerosis and healthy controls.[17]

Monocytes and macrophages probably may begin activation of T cells via releasing IL-1β in the presence of ox-LDL.[16] The stimulatory effects of ox-LDL proceeds via monocyte-dependent processes, not through a direct effect on T cell.[16] Chloroquine displays a greatly specific inhibitory effect on large antigen presenting function of macrophages,[18] while low concentration of chloroquine can inhibit stimulatory effect of ox-LDL and have no effect on con A-stimulated DNA synthesis. This finding supports antigen processing role by monocytes/macrophages in stimulatory effect of ox-LDL on T cells.[16]

Previous studies showed that when CD4^{+} T cells derived from the spleen of ApoE-KO mice were incubated with 1µg/ml ox-LDL, the numbers of Treg cells significantly reduced the numbers of CD4^{+}CD25^{-} cells; as well as ox-LDL time-dependent downregulated Foxp3 expression were also significantly decreased..[14] In another study on human, Treg cells, numbers of Treg cells significantly decreased compared to negligible reduction in numbers of CD4^{+}CD25^{-} cells, when these cells were stimulated with 1µg/ml ox-LDL.[15]

In this study, we found that Foxp3 expression decreased in both patients and controls, when PBMCs were simulated with ox-LDL. As noted, we also found that PBMCs stimulated with ox-LDL were proliferated in patients and controls. One explanation

for these results could be that Treg cells probably are more sensitive than effector T cells to ox-LDL;[15] therefore as PBMCs are mixture of effector and Treg cells, increased proliferation is related to increase in effector T cells rather than Treg cell populations. Surprisingly, we found that TGF-β gene expression after treatment with ox-LDL increased in the both groups; albeit this increase was significant only in controls.

Results of this study revealed that Foxp3 and TGF-β gene expression levels in initiation of study were lower in patients compared to healthy controls. Probably due to the presence of ox-LDL in atheromatous lesions and low concentration in serum, population of Treg cells in the atherosclerotic patients and inside human plaques are diminished. [11] In patients with unstable angina, cytokines that demonstrate signature of Treg cells are diminished.[19,20] The imbalance in effecter T cells and Treg cells could be a possible mechanism of atherosclerosis [21] and it is not unexpected that Foxp3 and TGF-β expression levels in patients with atherosclerosis are less than healthy people. In agreement with results of this study, decreased levels of Treg cells in the patients with acute coronary syndromes were previously reported. [15] Treg cells are key regulators of system [22] and mutations in Foxp3, trigger several autoimmune syndromes. [23] Treg cells through the production and secretion of some cytokines including TGF-β and IL-10 modulate the activity and proliferation rate of the effecter T lymphocytes [22,24,25] Any factor that can reverse this balance in favor of Treg cells can be effective in prevention or attenuation of atherosclerosis. Further experimental investigations with more focus on effect of ox-LDL stimulation on gene expression are needed.

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