Defective T-cell Proliferation and IL-2 Production in a Subgroup of Patients with Coronary Artery Disease

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ABSTRACT

Atherosclerosis is a chronic inflammatory disease of the arterial wall characterized by innate and adaptive immune responses to a variety of microbial and self-antigens. Given the crucial role of adaptive immunity in the pathogenesis of atherosclerosis, this study was performed to investigate the proliferative response of peripheral blood mononuclear cells (PBMC) and interleukin (IL)-2 production in patients with coronary artery disease (CAD).

In this study, 25 patients with chronic stable CAD and 25 healthy individuals were investigated. The PBMCs were separated and stimulated with phytohaemagglutinin (PHA). MTT assay was performed to measure cell viability and proliferation. IL-2 concentrations in cell culture supernatants were determined by Enzyme-Linked Immunosorbent Assay.

PHA-stimulated cells revealed a significantly increased optical density (OD) in both groups of patients ($p=0.004$) and controls ($p<0.001$). However, the patient group showed a significantly lower Stimulation index (SI) ($p=0.001$). Upon in vitro stimulation with PHA, IL-2 levels were significantly increased in both groups of patients and controls ($p<0.001$). However, IL-2 concentrations were significantly lower in the patient group ($p=0.018$). Six patients showed defective IL-2 production, whereas similar finding was not observed in the normal control subjects ($p=0.022$). PBMCs from patients with coronary artery disease showed defective PHA-induced mitogenesis and IL-2 production.

Considering the autoimmune nature of atherosclerosis, decreased IL-2 production may potentially enhance the atherogenic process, leading to spontaneous activation of autoreactive T lymphocytes.

Key words: Atherosclerosis; Interleukin-2; Peripheral blood mononuclear cells; Proliferation
INTRODUCTION

Atherosclerosis is a chronic inflammatory disease of the arterial wall characterized by progressive lipid accumulation, inflammatory cell infiltrates, cell death, and fibrosis.\(^1,2\) Inflammation is now recognized as a major contributor to atheromatous plaque formation and thrombogenesis, where both innate and adaptive immunity have been implicated in the atherogenic process.\(^3,4\) Low-density lipoprotein (LDL) accumulates in the arterial intima where it is subject to oxidative or enzymatic modification.\(^5\) Oxidized LDL, encompassing a variety of oxidatively modified lipids and proteins, induce the endothelial cell expression of leukocyte adhesion molecules,\(^6,7\) which in turn promote the recruitment of monocytes and T cells into the arterial intima in response to locally produced chemokines.\(^8,9\) The infiltrated monocytes differentiate into macrophages characterized by abundant expression of pattern-recognition receptors, including scavenger receptors and Toll-like receptors.\(^5\) The former mediate the uptake and clearance of modified lipoproteins and thereby promoting foam cell formation, whereas the latter bind to a variety of ligands, instigating the production of pro-inflammatory mediators and induction of antigen-specific immune responses, thus linking the innate and adaptive immunity.\(^10\)

Atherosclerosis-related antigens arising from the arterial intima, including both microbial and self-antigens, are processed by dendritic cells and presented to naive T cells in the draining regional lymph nodes.\(^11\) Entering the blood, previously activated T cells bind to cell surface adhesion molecules and reside in the shoulder region of the atheromatous plaque. Subsequently, major histocompatibility complex (MHC) class II-restricted antigen presentation within the atherosclerotic lesion triggers a predominantly T-helper 1 (Th1) cell response characterized by secretion of interferon (IFN)-\(\gamma\), which further amplifies the inflammatory response.\(^12\) In line with the above statement, CD\(_4^+\) T cell populations within the atheromatous plaque are mostly CD45RO-expressing memory and/or effector T cells,\(^13\) which are capable of responding only to a limited number of antigens, suggesting oligoclonal, rather than polyclonal, expansion of the plaque-residing T lymphocytes.\(^14\)

Interleukin (IL)-2, formerly recognized as a potent T cell growth factor, exerts multiple effects on a variety of cell types, the most prominent of which is T lymphocyte.\(^15\) Upon T cell receptor (TCR) engagement, de novo synthesis of IL-2 is followed by the expression of a high affinity IL-2 receptor (IL-2R), thus permitting rapid and selective expansion of antigen-specific CD\(_4^+\) and CD\(_8^+\) T cells in an autocrine manner.\(^15\) The majority of IL-2 is derived from activated CD\(_4^+\) T cells, of which the Th1 subset produces the most.\(^16\) Given its profound impact on determining the magnitude and duration of primary and long-term memory T cell responses, IL-2 may potentially enhance atherogenesis. Intraperitoneal injection of IL-2 enhances atherosclerotic process in ApoE\(^{-/-}\) mice fed with atherogenic diet, whereas anti-IL-2 antibody exerts a profound antiatherogenic effect.\(^17\) In addition, increased circulating levels of IL-2 have been demonstrated in patients with ischemic heart disease,\(^18,19\) particularly in patients with acute myocardial infarction (AMI) and unstable angina (UA).\(^19\) \(^99m\)Tc-radiolabelled IL-2 has been demonstrated to accumulate in vulnerable carotid plaques, showing a significant correlation with the amount of IL-2R\(^+\) cells as measured by immunohistopathology of carotid endarterectomy specimens.\(^20\)

Given the compelling evidence on the prominent role of T cells in the pathogenesis of atherosclerosis and the proatherogenic effect of IL-2, the purpose of the present study was to investigate the proliferative response of peripheral blood mononuclear cells (PBMC) to polyclonal stimulus phytohaemagglutinin (PHA), which predominantly stimulates T lymphocytes,\(^21\) and the degree of IL-2 production in patients with chronic stable coronary artery disease.

PATIENTS AND METHODS

Participants

In the present study, 25 patients with atherosclerosis (confirmed by coronary angiography), who had been referred to the Division of Cardiology of Tehran University of Medical Sciences affiliated teaching hospitals, Tehran, Iran, were investigated. All patients enrolled were diagnosed with chronic stable coronary artery disease, who had no history of acute coronary events within 2 months prior to the study entry. Twenty five healthy age- and sex-matched individuals with no history of coronary artery disease were also enrolled. Subjects with active infections or autoimmune diseases were excluded from the study. Written informed consent was obtained from all subjects. This study was
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approved by the Ethics Committee of Tehran University of Medical Sciences and Health Services.

Cell Culture
Approximately 10 mL blood was obtained from the subjects in sodium citrate tubes. The whole blood was diluted 1:2 with sterile phosphate-buffered saline (PBS). The diluted blood was carefully layered onto half the volume of Ficoll-Histoprep (BAG Health Care GmbH, Germany). The sample was centrifuged and the interface layer containing the mononuclear cells was collected. After washing and centrifugation, the cells were resuspended in culture medium (RPMI-1640 supplemented with 10% heat inactivated fetal calf serum; Gibco, Invitrogen, UK), diluted to 1 × 10^6 cells per mL, and plated on 96-well flat-bottomed microtiter plates at a density of 1.5 × 10^5 cells per well. Cultured cells were left either untreated or stimulated with 10 µg/mL phytohaemagglutinin (PHA; Sigma, USA). Next, the cells were incubated at 37°C in a 5% CO₂ incubator for 66 hours. Subsequently, cell culture supernatants were harvested and frozen for cytokine assay.

Colorimetric MTT Assay
Cell proliferation was assessed by 3-(4,5-dimethlythiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. Following 66 hours incubation period, 0.5 mg/mL MTT dye was added and the plates were incubated again for 4 hours. The formazan precipitates were extracted and dissolved in 200 µL dimethyl sulfoxide (DMSO). Subsequently, optical density (OD) was determined at 550 nm using micro ELISA plate reader. Stimulation index (SI) was calculated as mean ratio of OD of the stimulated cells divided by OD of untreated cells.

Cytokine Assay
IL-2 concentrations in cell culture supernatants were determined using commercially available Enzyme-Linked Immunosorbent Assay (ELISA; BD Biosciences, San Diego, CA). Subsequently, the OD was determined at 450 nm using micro ELISA plate reader. Cytokine concentrations were extrapolated from the standard curve generated using recombinant IL-2. The results were expressed as pg/mL.

Statistical Analysis
Comparisons of the results of proliferative responses between two groups of patients and controls were done using Independent-Samples T Test, whilst comparison of data in each group was done by Paired-Samples T Test. As IL-2 production was not normally distributed, comparison of data in each group was performed using Wilcoxon Signed Ranks Test, whereas Mann-Whitney U Test was done to compare the results between two groups of patients and controls. Receiver Operating Characteristic (ROC) curve was used to sensitively and specifically identify the ratio of IL-2 production in PHA-stimulated cells to untreated cells in subjects with poor PBMC proliferative response. An optimal cut-off point was chosen as the sensitivity plus specificity were maximized. This level was used arbitrarily as a cut-off point to identify subjects with defective IL-2 production. P-value of less than 0.05 was considered to be significant.

RESULTS
Proliferative Response to Mitogen
In vitro stimulation with PHA led to significantly increased OD in both groups of patients (0.88±0.14 vs. 1.03±0.29, p=0.004) and controls (0.81±0.15 vs. 1.13±0.21, p<0.001), as judged by MTT assay (Figure 1). However, the SI of the cells from patients was significantly lower than the controls (1.16±0.25 vs. 1.41±0.25, p=0.001).

II-2 Production
The concentrations of IL-2 in the culture supernatant of PHA-stimulated PBMCs were significantly increased in both groups of patients and controls (p<0.001) when compared to untreated cells (Figure 2). However, stimulated production of IL-2 in the patient group was significantly lower than the control group [median 1200.0 (range: 20-6500) pg/mL in the patients vs. median 2800.0 (range: 425-8200) pg/mL in the controls, p=0.018].

Subjects with Defective Proliferative Response to Mitogen
The PBMCs of 9 individuals from the patient group (36.0%) were not proliferated in response to PHA (SI ≤1), whereas none of the control subjects were poor-responders (p=0.002). According to the ROC curve, the cut-off of 11.5 was selected with a sensitivity of more than 90%. Considering this cut-off point, 6 patients showed defective IL-2 production, while none of the control subjects had defects in IL-2 production (p=0.022).
Figure 1. Error bar graph based on optical density of the untreated and PHA-stimulated cells in the patient and control groups.

Figure 2. Box plot graph based on IL-2 production (pg/mL) in supernatants of untreated and PHA-stimulated cells in the patient and control groups.
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Table 1. Comparisons of risk factors associated with atherosclerosis between two groups of patients with normal response and defective response to mitogen

<table>
<thead>
<tr>
<th>Risk factors</th>
<th>Normal-responders</th>
<th>Poor-responders</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sex (Male/Female)</td>
<td>13/3</td>
<td>7/2</td>
<td>0.61**</td>
</tr>
<tr>
<td>Age (years)</td>
<td>53.9±7.1</td>
<td>61.4±11.6</td>
<td>0.10*</td>
</tr>
<tr>
<td>Diabetes mellitus</td>
<td>8 (50.0%)</td>
<td>2 (22.2%)</td>
<td>0.18**</td>
</tr>
<tr>
<td>Hypertension</td>
<td>8 (50.0%)</td>
<td>7 (77.8%)</td>
<td>0.18**</td>
</tr>
<tr>
<td>Abdominal obesity</td>
<td>10 (62.5%)</td>
<td>8 (88.9%)</td>
<td>0.35**</td>
</tr>
<tr>
<td>Hypertriglyceridemia</td>
<td>8 (50%)</td>
<td>5 (55.6%)</td>
<td>0.56**</td>
</tr>
<tr>
<td>Hypercholesterolemia</td>
<td>6 (37.5%)</td>
<td>1 (11.1%)</td>
<td>0.17**</td>
</tr>
<tr>
<td>Body mass index (kg/m²)</td>
<td>29.9±4.1</td>
<td>29.4±2.9</td>
<td>0.74*</td>
</tr>
<tr>
<td>Triglyceride (mg/dL)</td>
<td>182.1±75.1</td>
<td>181.1±91.1</td>
<td>0.98*</td>
</tr>
<tr>
<td>Cholesterol (mg/dL)</td>
<td>174.3±46.2</td>
<td>175.7±37.5</td>
<td>0.94*</td>
</tr>
<tr>
<td>High density lipoprotein (mg/dL)</td>
<td>45.8±7.3</td>
<td>50.1±21.3</td>
<td>0.57*</td>
</tr>
<tr>
<td>Low density lipoprotein (mg/dL)</td>
<td>93.1±31.2</td>
<td>90.9±21.7</td>
<td>0.84*</td>
</tr>
</tbody>
</table>

* Independent-Samples T Test  ** Chi-Square Test

Risk factors associated with atherosclerosis are also compared between two groups of patients based on their proliferative response to mitogen that did not show any significant difference (Table 1).

**DISCUSSION**

The results of the present study point to the poor PBMC proliferative response to PHA and defective IL-2 production in a subset of patients with chronic stable coronary artery disease in comparison with the healthy controls. Defective PHA-induced mitogenesis and IL-2 production in patients with atherosclerosis, similar to a number of inflammatory autoimmune diseases, might be depicted as part of a more generalized dysregulation of T-cell homeostasis.

Current evidence supports an autoimmune mechanism in the pathogenesis of atherosclerosis. Oxidized LDL, heat shock proteins (HSPs), and β2 glycoprotein I (β2GPI) have been identified as the culprit autoantigens in the development and progression of atherosclerosis. IL-2 exerts multitudinous, and seemingly paradoxical, effects on T-cell biology. One of the most important biological activities of IL-2 is to prevent autoimmunity acting primarily through the promotion of Fas-mediated cell death, development and maintenance of CD4⁺CD25⁺ regulatory T (Treg) cells, and suppression of inflammatory T-helper 17 (Th17) cell development. The transient nature of IL-2 production in the absence of continued antigenic stimulation limits IL-2-mediated T cell proliferation during an immune response. Upon IL-2 binding, the IL-2 receptor complex is internalized and degraded via a clathrin-independent/IL-2R-dependent endocytic pathway. Therefore, IL-2 consumption, rather than a feedback inhibition of IL-2 production, results in cytokine deprivation apoptosis. More important, IL-2 induces Fas-mediated T cell apoptosis secondary to enhanced FasL expression on activated T lymphocytes. IL-2 is not required to activate the Treg-specific transcription factor forkhead box protein 3 (Foxp3) in the thymus. On the contrary, IL-2 increases, whereas anti-IL-2 antibody decreases, Foxp3 expression in wild type peripheral Treg cells.

Indeed, IL-2 is more crucial for the maintenance of Foxp3 expression in peripheral Treg cells, rather than its induction in thymic Treg cells. IL-2-induced Foxp3 expression restricts IL-2 gene expression in the presence of continued TCR stimulation, thus regulating its own levels via a Foxp3-mediated negative feedback loop. Recently, the suppressive effects of CD4⁺CD25⁺ Foxp3⁺ cells has been attributed to passive consumption of IL-2 rather than to active suppression of TCR-mediated cytokine production or the resultant proliferation of effector T cells. Their ability to express the high affinity IL-2R, but not IL-2, induces cytokine deprivation-mediated apoptosis of effector T cells.
through internalization and degradation of IL-2. In addition, IL-2 inhibits the development of inflammatory Th17 cells via STAT5 signaling. IL-17, the prototype cytokine of the Th17 effector subset, is considered to be the key mediator of tissue inflammation in several inflammatory autoimmune diseases.

There is increasing evidence of defective T cell activation and/or impaired proliferation in patients with inflammatory autoimmune diseases, including primary biliary cirrhosis, ulcerative colitis, Crohn's disease, systemic lupus erythematosus, and autoimmune thrombocytopenic purpura. Considering the autoimmune nature of atherosclerosis, decreased IL-2 production secondary to defective T cell proliferative response or deficient IL-2 responsiveness of T lymphocytes may exacerbate the inflammatory process. Recently, Treg cells have been demonstrated to inhibit the development and progression of atherosclerosis by production of anti-inflammatory cytokines including IL-10 and transforming growth factor (TGF)-β. It has been postulated that in the absence of IL-2, and consequently Treg cells, the requirement for IL-2 in effector and/or memory T cell activation is reduced, leading to spontaneous activation of autoreactive T lymphocytes.

Hence, it seems justified to consider IL-2 as a multifaceted mediator in the pathogenesis of atherosclerosis: IL-2 is considered to be a proatherogenic inflammatory mediator, while its decreased level may potentially enhance atherogenesis through increased Th1 and Th17 and decreased Treg responses. Therefore, it could be hypothesized that the subset of patients with chronic stable coronary artery disease, in whom the PBMC proliferative response to PHA and the subsequent IL-2 production is decreased, are at increased risk of disease progression.

Given the crucial role of adaptive immunity in the pathogenesis of atherosclerosis, it is of immense importance to determine the dual role of IL-2 in the development and progression of atherosclerosis, and perhaps, identify those patients who might be at increased risk of developing vulnerable atheromatous plaques.

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