Association of monocyte expression of Toll-like receptor 4 and its related cytokines with coronary luminal stenosis

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Received 18 April 2013; revised 20 May 2013; accepted 30 May 2013

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ABSTRACT

Toll-like receptors are well-defined barriers in innate immunity. Among them hTLR4 on the surface of monocytes, plays a critical role in the formation of atherosclerotic plaques, plaque instability and arterial remodeling through production of inflammatory cytokines. This study was designed to examine the association of hTLR4 monocyte expression and response with the severity of coronary stenosis in patients with stable angina (SA). Blood samples were obtained from 39 patients with SA who were scheduled for a coronary angiography and from 28 healthy volunteers. The samples were collected before the procedure. Expression of hTLR4 on CD14+ monocytes and serum levels of TNF-α and IL-1β were measured using flowcytometry and ELISA techniques respectively. Percentage stenosis diameter was measured by comparing the area of coronary stenosis to an adjacent normal segment of the vessel. Compared with control group, patients showed upregulation of hTLR4+/CD14+ monocytes. Furthermore, patients with more severe coronary stenosis exhibited enhanced expression of hTLR4+/CD14+ monocytes (p < 0.01). This was paralleled by elevation in the serum levels of TNF-α (p < 0.05) and IL-1β. In addition, significant correlations were seen between percentage stenosis diameter and monocyte expression of hTLR4 as well as TNF-α. hTLR4 monocyct expression and related cytokines are positively associated percentage stenosis diameter. These results suggest that hTLR4 activity may be involved in progression of atherosclerosis.

Keywords: Atherosclerosis; Cytokines; Inflammation; hTLR4

1. INTRODUCTION

Atherosclerosis is the main cause of cardiovascular mortality. A significant body of evidence suggests that inflammation plays a key role in the formation of atherosclerotic plaques [1-3]. Toll like receptors (TLRs) are cell membrane receptors that are activated by different pathogen-associated molecular patterns (PAMPs) [4]. TLRs are widely considered as central element of innate immune system. Ten functional human TLRs (hTLR) have been identified so far [5]. Among them, hTLR4 is well recognized in cardiovascular disease particularly in the formation of atherosclerotic plaques [6,7]. It is documented that exogenous ligands like lipopolysaccharide (LPS) of bacteria, fungi and viruses can activate this receptor and cause immune responses [8]. Several experimental and clinical studies have suggested that there are anumber ofendogenousligands for hTLR4like fibrinogen, heat shock protein (HSP), minimally modified LDL, oxidized LDL, heparan sulfate and hyaluronan [9,10]. Activation of hTLR4 is followed by interaction with the adaptor protein, myeloid differen- 

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injured endothelial cells and may contribute to progres-
sion of atherosclerosis [16]. Angina pectoris represents
an imbalance between myocardial oxygen supply and
demand. Atherosclerotic disease of the coronary arteries,
which leads to narrowing of the vessels, is the funda-
mental cause of angina pectoris. Medications, life-style
modifications and revascularization techniques such as
coronary artery bypass graftsurgery (CABG) or percu-
taneous coronary interventions (PCI), including balloon
angioplasty and stents, are routine approaches to restor-
ing this imbalance. Despite being on maximal medical
therapy, some patients may suffer from angina and will
experience revascularization therapies [17,18]. In the
present study we evaluated the expression of hTLR4 on
peripheral CD14+ monocytes in patients with stable an-
gina who were on standard medications. Our goal was to
find an association between hTLR4 expression and related
inflammatory cytokines with degree of coronary stenosis.

2. MATERIALS AND METHODS

2.1. Study Patients

The ethical board of Tabriz University of Medical Scien-
ces approved the study and informed consent letter was
obtained from all participants. Forty-five patients with
diagnosis of coronary artery disease admitted to Shahid
Madani Heart Hospital, Tabriz, Iran were included in the
study. They were on standard treatments including rou-
tine medications, changing life style and weight reduc-
tion. They had complaints about various degrees of chest
pain and other symptoms of angina like premature ex-
haustion and breathlessness. The exclusion criteria were
as follows: autoimmune diseases (2 patients), inflam-
matory conditions (3 patients), advanced hepatic or renal
disease and malignant neoplastic diseases (1 patient) and
patients who receive glucocorticoids. None of the patien-
ts had valvular heart disease. Control group included 28
healthy volunteers who did not show symptoms of heart
diseases. Cardiovascular risk factors, medications, sex,
age and previous medical history were recorded by ques-
tionnaires. White blood cell count, cholesterol, glucose,
PT, PTT, BUN, creatinine, sodium and potassium were
measured immediately after admission. All angiography
procedures were performed according to the routine
protocols of hospital.

2.2. Analysis of Coronary Vessels

Shortly, a guide wire and a catheter were inserted into the
femoral artery. They freely passed up to the aorta. Pa-
tients routinely received 5000 units of heparin. Power-
 injection of a contrast media was used to assess left
ventricle function and coronary arteries. The severity of
coronary artery lesions was measured by comparing the
area of narrowing to an adjacent normal segmentas a
percentage reduction, and as a percentage reduction and
calculated in the projection which demonstrates the most
severe narrowing. This was done using Syngo software,
Siemens, Germany.

2.3. Blood Collection and Processing

A total 6 ml blood was drawn from patients with a 21-
gauge needle via antecubital venipuncture at time of
admission. 2 ml was kept in EDTA anticoagulant tubes
for flowcytometry and the rest for analysis of biomarkers.
4 ml of blood were centrifuged immediately (3000 × g
for 5 min) to obtain serum. Serums were kept in –80°C
for future analysis.

2.4. Flowcytometry Analysis

Briefly, cells were stained at 4°C for 30 minutes with
monoclonal antibodies for human CD14 (Abcam, UK)
conjugated with fluorescein isothiocyanate (FITC) and
hTLR4 (Abcam, UK) conjugated with phycoerythrin (PE).
FITC and PE-conjugated non-specific mouse IgG2a anti-
bodies were used for isotype controls (Abcam, UK).
Cells were washed and cell-associated fluorescence was
measured using a FACSCalibur flow cytometer (BD
Biosciences, US). Data were analyzed by CellQuest soft-
ware (BD Biosciences, US).

2.5. Measurement of TNF-α and IL-1β by ELISA

A sandwich enzyme-linked immunosorbent assay (ELI-
SA) was performed (Ray Bio, US). In short, 100 µl
serum was added to microtiter plates. The incubation
time was 2.5 hours at room temperature. After that, 100
µl prepared biothin antibody was added to each well and
incubated for 1 hour at room temperature. Then 100 µl
streptavadin solution was added and incubated for 45
minutes at room temperature. The intensity of the color
was measured at 450 nm by Stat Fax 2600 (Awareness
Technology, US) plate reader. Stat Fax 2100 (Awareness
Technology, US) was used for washing steps.

2.6. Statistical Analysis

Data are reported as mean ± SD. Mann-Whitney U test
was used to calculate differences between two indepen-
dent variables. Categorical variables were compared us-
ing one-way analysis of variance. The correlation be-
tween hTLR4 and degree of coronary stenosis were
determined using Pearson test. Probability value <0.05 was
considered to denote significant differences. All ana-
lyses were performed by SPSS 16.

3. RESULTS

3.1. Characteristics of Patients

Biological and clinical data of 2 groups are summarized
in Table 1. There were no significant differences in white blood cell count; medications including nitroglycerines, β blockers, statins, antiplatelet drugs, or ACE inhibitors and risk factors among the patients.

### 3.2. Coronary Artery Angiography

Percentage stenosis diameter was calculated by coronary angiography. Patients showed various stenoses in different coronary arteries (Figure 1).

#### 3.3. Assessment of hTLR4+/CD14+ Monocytes in Relation to Percentage Stenosis Diameter

Protein levels of hTLR4 on the surface of monocytes were studied by flowcytometry analysis (Figures 2(a)-(d)). A mean of 19.4% ± 1.9% of CD14+ monocytes in patients with less severe stenosis showed surface expression for hTLR4 but did not reach statistical significance. Compared with healthy controls, patients with more severe stenosis showed a significantly higher monocytic hTLR4 expression (15.1% ± 2.3% versus 29.3% ± 3.7%; \( p < 0.01 \)) (Figure 2(e)).

#### 3.4. Serum Levels of TNF-α in Relation to Percentage Stenosis Diameter

Circulating serum TNF-α levels were significantly lower in controls than in patients with more severe stenosis (11.1 ± 2.2 versus 21.5 ± 3.1 pg/ml; \( p < 0.05 \)) (Figure 3). A Similar relation was seen in patients with less severe stenosis, but it was not statistically significant.

### Table 1. Baseline characteristics and laboratory parameters of patients and control.

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>Control (n = 28)</th>
<th>Patients (n = 39)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age, years</td>
<td>41 ± 10</td>
<td>52 ± 8</td>
</tr>
<tr>
<td>Male</td>
<td>25 (89.2)</td>
<td>27 (69.2)</td>
</tr>
<tr>
<td>Hypertension</td>
<td>...</td>
<td>22 (56.4)</td>
</tr>
<tr>
<td>Diabetes</td>
<td>...</td>
<td>24 (61.5)</td>
</tr>
<tr>
<td>Hyperlipidemia</td>
<td>...</td>
<td>14 (35.8)</td>
</tr>
<tr>
<td>Familial history</td>
<td>...</td>
<td>7 (17.9)</td>
</tr>
<tr>
<td>Smoking</td>
<td>...</td>
<td>11 (28.2)</td>
</tr>
<tr>
<td>Medications</td>
<td>...</td>
<td></td>
</tr>
<tr>
<td>Nitrates</td>
<td>23 (58.9)</td>
<td></td>
</tr>
<tr>
<td>β Blockers</td>
<td>29 (74.3)</td>
<td></td>
</tr>
<tr>
<td>ACE1 inhibitors</td>
<td>10 (25.6)</td>
<td></td>
</tr>
<tr>
<td>A2 blockers</td>
<td>7 (17.9)</td>
<td></td>
</tr>
<tr>
<td>Calcium channel blockers</td>
<td>4 (10.2)</td>
<td></td>
</tr>
<tr>
<td>Statins</td>
<td>34 (87.1)</td>
<td></td>
</tr>
<tr>
<td>ASA2</td>
<td>31 (79.4)</td>
<td></td>
</tr>
</tbody>
</table>

Laboratory parameters

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Control</th>
<th>Patients</th>
</tr>
</thead>
<tbody>
<tr>
<td>C-reactive protein (mg/dl)</td>
<td>0.2 ± 0.1</td>
<td>0.5 ± 1.3</td>
</tr>
<tr>
<td>Cholesterol (mg/dl)</td>
<td>180 ± 36</td>
<td>192 ± 37</td>
</tr>
<tr>
<td>LDL (mg/dl)</td>
<td>121 ± 20</td>
<td>139 ± 21</td>
</tr>
<tr>
<td>HDL (mg/dl)</td>
<td>42 ± 8</td>
<td>40 ± 9</td>
</tr>
<tr>
<td>Glucose (mg/dl)</td>
<td>95 ± 11</td>
<td>99 ± 21</td>
</tr>
<tr>
<td>White blood cell count (U/L)</td>
<td>7.2 ± 0.3</td>
<td>7.2 ± 0.4</td>
</tr>
</tbody>
</table>

Data are shown in number (%) or mean ± SD. 1: angiotensin converting enzyme; 2: acetyl salicylic acid.

![Figure 1](https://example.com/figure1.png)  
**Figure 1.** Stenosis in the right coronary artery (a); Stenosis in the proximal left anterior descending artery (b).

![Figure 2](https://example.com/figure2.png)  
**Figure 2.** Gating monocyte population in the samples of controls and patients ((a) and (b)). Representative dot plot showing hTLR4+/CD14+ monocytes in controls and patients with stable angina ((c) and (d)). Data are shown as mean ± SD. Ten thousand cells were analyzed by flowcytometry in relation to percentage stenosis diameter (%) of the diseased vessel (e); \( p < 0.01 \) vs control.
3.5. Serum Levels of IL-1β in Relation to Percentage Stenosis Diameter

In comparison with controls, patients with more severe coronary occlusion exhibited higher levels of IL-1β (8.4 ± 2.4 versus 13.8 ± 1.8 pg/ml) (Figure 4).

3.6. Pearson Correlations

Strong correlation was seen between the frequency of circulating hTLR4+/CD14+ monocytes and the percentage of coronary luminal stenosis (r = 0.5 and p = 0.05) (Figure 5). A significant correlation was noted between the frequency of serum levels of TNF-α and coronary luminal stenosis (r = 0.40 and p = 0.05) (Figure 6). Despite positive association between serum levels of IL-1β and coronary stenosis, it failed to reach a significant correlation.

4. DISCUSSION

In this investigation we used flowcytometry and ELISA to describe an association between monocyte expression levels of hTLR4 and its related signaling events with percentage diameter stenosis of the coronary arteries. Patients with lower stenosis diameter expressed lower expression of hTLR4 as well as lower serum levels of both TNF-α and IL-1β. Notably, enhanced expression of hTLR4 was associated with elevations of IL-1β and TNF-α. Evidence is accumulating that outward arterial remodeling is associated with vulnerable plaque phenotype [19] and increase in hTLR4 expression [20]. Amelziane et al. [21] showed that polymorphism of Gly299 allele of the hTLR4 gene is associated with reduction in acute coronary events. It is believed that upregulation of hTLR4 contributes to activation of monocytes, whereas downregulation can suppress proinflammatory responses [22]. Elevated release of IL-1β and TNF-α in link with antigen presentation has been shown to activate T cells and cause differentiation into T-helper 1 cells [11,23], which may cause plaque instability [24]. Versteeg et al. [25] provided evidence that hTLR 4 response was associated with the percentage diameter stenosis and the number of the diseases vessels. In comparison with our work, they used a different method and focused on TNF-
α concentration. Akira and colleagues [26] showed that hTLR4 signaling pathway may have central role in the production of proinflammatory cytokines. Serum concentrations of TNF-α and mRNA expression of hTLR4 in peripheral monocytes are significantly increased after thrombolysis therapy [15]. Some animal studies proved that during 30 minutes of ischemia followed by reperfusion, elevated expression of hTLR4 mRNA was positively correlated with both TNF-α expression and its serum concentration [27]. Björkbacka et al. [28] found that that My d88-deficient mice showed a marked reduction in early stages of atherosclerosis. Moreover, Michelsen et al. showed that lack of TLR4 results in reduction of atherosclerosis in mice [29]. A study showed that direct inhibition of TLR4 could significantly reduce the detrimental effects of myocardial ischemia reperfusion [30]. Those findings were further supported by the point, that hTLR4 deficiency leads to alteration in lipid content of atherosclerotic plaque and reduces macrophage infiltration. Taken together, the findings of the present investigation may set a link between hTLR4 expression and proinflammatory cytokines with coronary stenosis. Outward arterial remodeling is thought to be the primary cause of atherosclerosis and restenosis [31]. In vitro studies showed that TLR4 is involved in arterial remodeling [32]. Additionally, it is believed that increase in serum levels of proinflammatory cytokines is associated with CAD [33,34]. We demonstrated that lower degree of stenosis leads to lower monocyte expression and response of hTLR4. It should be noted that hTLR4 involvement in coronary stenosis is not mechanistically understood. It is proposed that gradual infiltration of hTLR4+ monocytes in developing plaques and production of cytokines in concert with other important players can deteriorate atherosclerosis. Our prior study revealed a positive correlation between hTLR4 monocyte expression and serum levels TNF-α and IL-1β in patients with SA who underwent percutaneous coronary intervention [35]. Upregulation in hTLR4 and pro-inflammatory cytokines and increased arterial remodeling may impair vasodilatation, reduce coronary flow and thus contribute to facilitate ischemic damages [33,34]. It is noteworthy that inflammation plays its distinguished role not only inside the atherosclerotic plaques, but also in systemic blood circulation. The determining factor for this increase in systemic inflammatory responses remains unknown. However, these events may partially explain the refractoriness to medications in patients with chronic SA who need revascularization therapies. As plaque encroaches into the lumen, the coronary artery diameter decreases. Luminal narrowing of more than 60 percent may result in transient ischemia and angina. More importantly, there is a poor correlation between the severity of stenosis and its propensity to cause myocardial infarction or sudden cardiac death. Currently it is believed that inhibition of hTLR4 or hTLR4 related downstream activity can subsequently attenuate plaque formation or even plaque rupture [35,36]. Of note, formation of collateralar new vessel growth which is an important mechanism to increase blood flow to ischemic parts [37,38] is mediated by inflammatory cytokines [39,40]. The beneficial effects of hTLR4 in arteriogenesis were seen by Grote study [41]. Thus, a paradox exists in which enhanced expression of hTLR4 has positive role in arteriogenesis and increases the likelihood of events that results in plaque formation, instability and rupture. Future experimental and clinical studies should provide more perception about hTLR4 role in coronary artery disease. Methé’s study revealed that statins decreased monocyte expression of hTLR4 [42]. Quite recent data showed that simvastatin or the combination of simvastatin with ezetimibe reduced TLR4 expression and IL-6 and IL-1β production in monocytes of hypercholesterolemic patients [43]. Our studied patients did not show significant differences in the medications and despite being on statin treatment, patients with more severe stenosis exhibited higher expression of hTLR4, suggesting the role of hTLR4 in deterioration of ischemic heart disease. In conclusion, our study demonstrated that hTLR4 monocyte expression and pro-inflammatory cytokines are positively associated with the degree of coronary stenosis. These findings may emphasize the potential role of hTLR4 in CAD.

5. ACKNOWLEDGEMENTS

This study was a PhD project No.57 supported by a grant from vice chancellor for research of Tabriz University of Medical Sciences. The authors wish to thank the staff of Shahid Madani Heart Hospital. The authors declare that there are no conflicts of interests.

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