Expression Levels of Toll-Like Receptor Genes in Coronary Atherosclerotic Lesions of Patients With Acute Coronary Syndrome or Stable Angina Pectoris

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Background: The differences between acute coronary syndrome (ACS) and stable angina pectoris (SAP) in Toll-like receptor (TLR) expression levels in coronary plaques are not well known. TLR gene expression levels were examined, not only in peripheral blood mononuclear cells (PBMCs), but also in coronary plaques in ACS and SAP patients.

Methods and Results: TLR gene expression levels were examined in PBMCs using real-time RT-PCR in 27 ACS patients, 45 SAP patients and 28 control subjects. TLR2 and TLR4 expression levels in the PBMCs were significantly higher in the ACS group than in the SAP group. TLR9 expression levels were not significantly different among the 3 groups. TLR gene expression levels were also measured in directional coronary atherectomy (DCA) samples from 9 ACS and 14 SAP patients. The TLR2 expression levels in the DCA samples did not significantly differ between the 2 groups. The TLR4 expression levels were significantly higher in the ACS group than in the SAP group.

Conclusions: The results suggest that TLR4 signaling could be more associated with plaque destabilization than with plaque progression. TLR4 expression control may be a novel target for ACS treatment. (Circ J 2009; 73: 1479 – 1484)

Key Words: Acute coronary syndrome; Directional coronary atherectomy; Immune system; Toll-like receptor

Evidence has been accumulating that TLRs are important in both the initiation and the progression of atherosclerotic diseases. Meth et al demonstrated that TLR4 gene expression levels in circulating monocytes and signaling events downstream of TLR4 were significantly enhanced in ACS patients. Edfeldt et al indicated that TLR1, TLR2, and TLR4 expression levels were markedly enhanced in endothelial cells and in macrophages involved with carotid atherosclerotic plaques. Recently, Pryshchep et al reported that each vessel in the macrovascular tree exhibits a distinct TLR profile. Although many studies of TLRs have been performed, TLR profiles in human coronary arteries have not been reported, and differences in the TLR expression levels in coronary plaques of patients with ACS and those with stable angina pectoris (SAP) are not well known. TLR2, TLR4 and TLR9 have been suggested as recognizing C. pneumonia, cytomegalovirus, herpes simplex virus, heat shock protein 60 (HSP60) and oxidized low-density lipoprotein (LDL), are considered as candidate TLR ligands in atherosclerotic plaques.

Methods

Study Population

We obtained blood samples from 100 inpatients of Kumamoto University Hospital after they gave written informed consent. Patients who withdrew their informed consent were excluded. The study was in agreement with the guidelines approved by the Ethics Committee of Kumamoto University Graduate School of Medical Sciences.
The patients were divided into 3 groups: 27 ACS patients; 45 SAP patients; 28 control subjects. The ACS group consisted of 21 acute myocardial infarction (AMI) patients and 6 unstable angina pectoris (UAP) patients (Braunwald’s class II or III). All the SAP patients had 1 or more stenotic coronary arteries with more than 75% stenosis after nitroglycerin administration. The control subjects had either angiographically normal coronary arteries or presented no clinical signs of atherosclerotic diseases. Exclusion criteria were inflammatory conditions likely to be associated with acute phase responses, neoplastic diseases, advanced liver diseases, and autoimmune diseases.

All the ACS patients underwent coronary angiography and percutaneous coronary intervention (PCI). The blood samples from the ACS patients were obtained before PCI, and those from the SAP patients and control subjects were obtained before diagnostic cardiac catheterization.

In order to corroborate our results, we performed an additional study using tissue samples from coronary atherosclerotic lesions in 23 inpatients who underwent directional coronary atherectomy (DCA) in Kumamoto Central Hospital: 9 ACS patients and 14 SAP patients. We measured TLR2, TLR4, and TLR9 expression levels in the DCA samples. Written informed consent was given by these subjects and the study was in agreement with the guidelines approved by the Ethics Committee of the institution.

Separation of PBMCs and DCA Samples and Extraction of Total RNA

We layered a maximum of 10 ml of whole blood with 0.38% sodium citric acid over 4 ml of Ficoll-Paque (Pharmacia Biotech, Uppsala, Sweden), and then centrifuged for 15 min at 3,000 rpm at room temperature. We aspirated the mononuclear cells located at the plasma-Ficoll-Paque interface and centrifuged this aspirated solution for 15 min at 15,000 rpm at room temperature. We next extracted the total RNA of the PBMCs, using an RNeasy Mini Kit (Qiagen, GmbH Hilden, Germany). The DCA samples were stored at −80°C until total RNA was extracted from the homogenized frozen tissues using an RNeasy Mini Kit (Qiagen).

Quantitative Real-Time Reverse Transcription (RT)-Polymerase Chain Reaction (PCR) Analysis

The samples of total RNA (500 ng) were reverse transcribed using a High Capacity DNA Archive Kit (Applied Biosystems, Foster City, CA, USA). Human TLR2, TLR4, and gyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA levels were quantified using a 2-step real-time RT-PCR; the reaction mixture (10 μl) contained 250 ng of cDNA template, a set of primers, a related probe, and a TaqMan Universal Master Mix (Applied Biosystems). The primers and probe sets for GAPDH (Hs99999905) were purchased from TaqMan® Gene Expression Assays (Applied Biosystems).

The context sequence of the GAPDH gene was 5′-GGC CCT GGT CAC CAG GGC TGC TTT T. In TLR2, the sequences of the forward primer, reverse primer, and the probe were 5′-TTG TGC CCA TTA TTC TTA CCA C-3′, 5′-TGG CAT TGT CCA GTT CTT CA-3′, and 5′-FAM-CTT TCA ACT GTG AGT TGT GGG-MGB-3′, respectively. The respective sequences in TLR4 were 5′-CAG AAC TGC AGG TGC TGG ATT-3′, 5′-TGA TAT GCC CCA TCT TCA ATT G-3′, and 5′-FAM-CAG GTG TGA AAT CC-MGB-3′ and in TLR9 they were 5′-GGG AGC TAC TAG GCT GGT ATA AAA ATC T-3′, 5′-CGC TGC GGC AGA AAC C-3′, and 5′-FAM-AGT GTG AAT

Table 1. Patient Characteristics: PBMC Sample Analyses

<table>
<thead>
<tr>
<th></th>
<th>Control (n=28)</th>
<th>SAP (n=45)</th>
<th>ACS (n=27)</th>
<th>P value (among 3 groups)</th>
<th>P value (between SAP and ACS groups)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (years)</td>
<td>67±11</td>
<td>70±11</td>
<td>71±11</td>
<td>0.277</td>
<td>0.452</td>
</tr>
<tr>
<td>M/F</td>
<td>14/14</td>
<td>31/14</td>
<td>21/6</td>
<td>0.037</td>
<td>0.830</td>
</tr>
<tr>
<td>Smoking</td>
<td>12 (43%)</td>
<td>22 (49%)</td>
<td>16 (59%)</td>
<td>0.468</td>
<td>0.394</td>
</tr>
<tr>
<td>Alcohol</td>
<td>15 (54%)</td>
<td>28 (62%)</td>
<td>15 (56%)</td>
<td>0.733</td>
<td>0.577</td>
</tr>
<tr>
<td>Hypertension</td>
<td>18 (64%)</td>
<td>27 (60%)</td>
<td>13 (48%)</td>
<td>0.449</td>
<td>0.327</td>
</tr>
<tr>
<td>DM</td>
<td>7 (25%)</td>
<td>24 (53%)</td>
<td>14 (52%)</td>
<td>0.043</td>
<td>0.903</td>
</tr>
<tr>
<td>Dyslipidemia</td>
<td>17 (61%)</td>
<td>31 (69%)</td>
<td>15 (56%)</td>
<td>0.503</td>
<td>0.254</td>
</tr>
<tr>
<td>WBC</td>
<td>5.33±1.245</td>
<td>6.22±1.806</td>
<td>8.83±3.993</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>CRP</td>
<td>0.15±0.14</td>
<td>0.39±0.59</td>
<td>0.89±2.41</td>
<td>0.045</td>
<td>0.327</td>
</tr>
<tr>
<td>TC</td>
<td>193±28</td>
<td>180±41</td>
<td>173±33</td>
<td>0.036</td>
<td>0.660</td>
</tr>
<tr>
<td>LDL-cholesterol</td>
<td>119±27</td>
<td>107±38</td>
<td>107±27</td>
<td>0.036</td>
<td>0.375</td>
</tr>
<tr>
<td>HDL-cholesterol</td>
<td>55±15</td>
<td>55±25</td>
<td>48±19</td>
<td>0.156</td>
<td>0.235</td>
</tr>
<tr>
<td>Triglycerides</td>
<td>125±63</td>
<td>118±47</td>
<td>119±47</td>
<td>0.996</td>
<td>0.924</td>
</tr>
<tr>
<td>Blood glucose</td>
<td>99±21</td>
<td>112±35</td>
<td>13±68</td>
<td>0.004</td>
<td>0.032</td>
</tr>
<tr>
<td>No. of stenotic vessels</td>
<td>1</td>
<td>0</td>
<td>24</td>
<td>&lt;0.001</td>
<td>0.773</td>
</tr>
<tr>
<td>2</td>
<td>0</td>
<td>9</td>
<td>4</td>
<td>0.773</td>
<td>0.924</td>
</tr>
<tr>
<td>3</td>
<td>0</td>
<td>12</td>
<td>9</td>
<td>0.924</td>
<td>0.924</td>
</tr>
</tbody>
</table>

Values are mean±SD or n (%).

PBMC, peripheral blood mononuclear cell; SAP, stable angina pectoris; ACS, acute coronary syndrome; DM, diabetes mellitus; WBC, white blood cell; CRP, C-reactive protein; TC, total cholesterol; LDL, low-density lipoprotein; HDL, high-density lipoprotein; ACEI, angiotensin-converting enzyme inhibitors; ARB, angiotensin II receptor blockers.
Real-time PCR was performed according to the manufacturer’s prescribed protocol using an ABI PRISM 7900 Sequence Detector (Applied Biosystems). Standard curves for TLR2, TLR4, and TLR9 mRNA levels were generated using dilutions of samples of cDNA prepared from PBMCs. Expression levels of TLR2, TLR4, TLR9 and GAPDH were quantified with the critical threshold cycle, which is inversely proportional to the logarithm of the initial number of template molecules. We determined the gene expression levels as follows: TLR2 mRNA level/GAPDH mRNA level, TLR4 mRNA level/GAPDH mRNA level, and TLR9 mRNA level/GAPDH mRNA level.

### Statistical Analysis
Continuous variables are expressed as mean±SD or median, 10–90 percentile. The comparisons of continuous data among the 3 patient groups were performed using 1-way ANOVA followed by Scheffé’s test. The Mann-Whitney U-test and Kruskal-Wallis test were used to compare discrete variables. A probability value <0.05 was considered statistically significant.

### Results

#### Clinical Characteristics of the Patients
In the PBMC samples analyses, we compared the following clinical characteristics among the 3 groups: age, gender, smoking, alcohol, hypertension (HT), diabetes mellitus (DM), dyslipidemia, white blood cell (WBC) count, levels of C-reactive protein (CRP), total cholesterol (TC), LDL-cholesterol, high-density lipoprotein (HDL)-cholesterol, triglycerides and blood glucose, the number of stenotic vessels, and usage of aspirin, statins, angiotensin-converting enzyme inhibitors (ACEI)/angiotensin II receptor blockers (ARB), \(\beta\)-blockers, calcium antagonists, and nitrate (Table 1). There were significant differences among the 3 groups for the following: gender, DM, WBC count, levels of CRP, TC, LDL-cholesterol and blood glucose, the number of stenotic vessels, and aspirin and nitrate usage. There were significant differences between the ACS and SAP groups for WBC count and blood glucose level.

In the DCA samples analyses, we compared the following clinical characteristics between the 2 groups: age, gender, smoking, alcohol, HT, DM, dyslipidemia, WBC count, levels of CRP, TC, LDL-cholesterol, HDL-cholesterol, triglycerides and blood glucose, the number of stenotic vessels, and usage of aspirin, statins, ACEI/ARB, \(\beta\)-blockers, calcium...
antagonists and nitrate (Table 2). There were no significant differences in these parameters.

**TLR2, TLR4 and TLR9 Expression Levels in PBMCs**

The TLR2 expression level in PBMCs from the ACS, SAP and Control groups was 4.8±2.8, 3.5±1.4 and 3.3±2.1, respectively (Figure 1). The TLR2 expression level was significantly higher in the PBMCs from the ACS group compared with the other 2 groups. There were no significant differences between the SAP and Control groups.

The TLR4 expression level in PBMCs from the ACS, SAP and Control groups was 12.7±11, 7.5±7.0 and 8.0±6.0, respectively (Figure 2). The TLR4 expression level was significantly higher in the PBMCs from the ACS group compared with the SAP group. There were no significant differences between the SAP and Control groups.

The TLR9 expression level in PBMCs from the ACS, SAP and Control groups was 2.0±2.0, 2.7±2.7 and, 5.2±7.5, respectively (Figure 3). The TLR9 expression level was not significantly different among the 3 groups.

The ACS patients included 21 AMI patients and 6 UAP patients. The TLR2 expression level in PBMCs from the AMI and UAP groups was 2.0±2.0, 2.7±2.7 and, 5.2±7.5, respectively (Figure 1). The respective TLR4 and TLR9 expression levels were 11.6±10 and 16.8±15, and 1.9±2.1 and 2.3±1.7. There were no significant differences in gene expression levels between the AMI and UAP groups.

In the comparison of clinical characteristics in the PBMC samples analyses, there were significant differences in the WBC count and blood glucose level, so we evaluated...
whether these parameters affected the TLR2 and TLR4 expression levels in PBMCs from the Control group. The gene expression levels in the PBMCs did not correlate with either parameter.

**TLR2, TLR4 and TLR9 Expression Levels in the DCA Samples**
The TLR2 expression level in the DCA samples from the ACS and SAP groups was 0.26±0.18 and 0.17±0.18, respectively (Figure 4), showing no significant difference between groups. The respective TLR4 expression levels were 2.81±2.05 and 1.13±1.46 (Figure 5), significantly higher in the ACS group than in the SAP Group. The TLR9 expression level could not be detected in the DCA samples of either the ACS or SAP group.

**Discussion**
We demonstrated TLR2, TLR4 and TLR9 expression levels in both PBMCs and coronary plaques of ACS and SAP patients. The TLR2 and TLR4 expression levels in PBMCs were significantly higher in the ACS group than in the SAP group. There were no significant differences between the SAP and Control groups for TLR2 and TLR4 expression levels in PBMCs. The TLR9 expression level in PBMCs did not significantly differ among the 3 patient groups. We also found that TLR2 and TLR4 appear to be expressed in coronary plaques, but not TLR9. Moreover, we found higher TLR4 expression levels in the coronary plaques of ACS patients compared with SAP patients. There were no significant differences between ACS and SAP patients in TLR2 expression levels in coronary plaques. The TLR9 expression levels could not be detected in coronary plaques from either the ACS or SAP group.

As regards TLR gene expression levels in peripheral blood, Methe et al previously indicated that TLR4 expression levels were enhanced in monocytes among PBMCs from ACS patients. The increased TLR2 and TLR4 expression levels in PBMCs in our study could also be derived from monocytes in the circulating blood. These results could support the theory that TLR2 and TLR4 are associated with atherosclerosis.

Concerning TLR gene expression in atherosclerotic lesions, Edfeldt et al previously demonstrated that in carotid plaques the TLR1, TLR2, and TLR4 expression levels were markedly enhanced, and that TLR expression was derived from endothelial cells and macrophages. With regard to the cell types that express TLR genes in atherosclerotic plaques, the present study was unable to immunohistochemically localize TLR expression in the coronary plaques because we used homogenized frozen tissues obtained from DCA. DCs, macrophages, neutrophils, B cells, endothelial cells, vascular smooth muscle cells and adventitial fibroblasts could all contribute to the TLR expression in atherosclerotic lesions. Taking account of the procedure of DCA, which involves scraping plaque tissue from the arterial intima, we could assume that the samples would mainly contain endothelial cells, immune cells and extracellular matrix. According to a previous report, among the immune cells most TLR expression probably derives from macrophages.

Recently, Pryshchep et al reported that each vessel in the macrovascular tree exhibits a distinct TLR profile and their report further suggests that coronary arteries may have different TLR expression patterns to those observed in the carotid arteries. In the present study, TLR2 and TLR4 appeared to be expressed in the coronary plaques, but not TLR9. Our results could also support the theory that expression of TLR2 and TLR4 is associated with atherosclerosis.

Xu et al indicated that TLR4 expression in human macrophages was upregulated by oxidized LDL and Faure et al demonstrated that bacterial lipopolysaccharide and IFN-gamma induced TLR2 and TLR4 expression in human endothelial cells. In the present study, enhanced TLR2 and TLR4 expression in coronary plaques suggests that their signal pathways are activated and upregulated by some ligands in the plaques, which would support the theory that TLR2 and TLR4 ligands such as C. pneumonia, cytomegalovirus, herpes simplex virus, HSV-1 and oxidized LDL could be involved in atherosclerosis. Moreover, we found higher TLR4 expression levels in the coronary plaques of ACS patients compared with those of SAP patients, which could imply that the TLR4 signal pathway induces not only plaque progression, but also plaque destabilization.

In conclusion, our findings indicate that increased TLR4 expression levels in coronary plaques are strongly associated with ACS. These results suggest that TLR4 expression may be associated more with plaque destabilization than with plaque progression, and that control of TLR4 expression could be a novel target in the treatment of ACS patients.

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