Atherosclerosis and Lipoproteins

Rapid Communication

Association of the Toll-Like Receptor 4 Gene Asp299Gly Polymorphism With Acute Coronary Events

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Objective—Atherosclerosis is a chronic inflammatory disease of the blood vessels. Toll-like receptor 4 (TLR4) is a transmembrane receptor that is involved in mediating inflammatory responses to bacterial endotoxin and other ligands. The aim of this study was to search for an association between a common functional polymorphism of TLR4—Asp299Gly—and acute coronary syndrome.

Methods and Results—We conducted a case-control study of 183 patients with acute coronary syndromes and 216 controls. We screened the TLR4 gene for the Asp299Gly polymorphism using a 5’ fluorogenic assay. The 299Gly allele was associated with a decreased risk of acute coronary events independently of standard coronary risk factors. The adjusted odds ratio associated with this allele was 0.41 (95% CI, 0.18 to 0.95; \(P=0.037\)). In controls, TLR4 heterozygosity was also associated with a significant decrease in plasma fibrinogen and soluble vascular cellular adhesion molecule-1 levels (\(P<0.01\)).

Conclusions—These results, which must be confirmed by a prospective longitudinal study, provide evidence of an association between the Asp299Gly polymorphism of the human TLR4 receptor and acute coronary syndromes. They confirm the previously reported involvement of TLR4 in carotid and femoral artery atherosclerosis. (Arterioscler Thromb Vasc Biol. 2003;23:e61-e64.)

Key Words: TLR4 ■ acute coronary events ■ genetics

Atherosclerosis, a chronic inflammatory disease of the blood vessels, is characterized by macrophage accumulation within the intima and by intimal hyperplasia. Within this microenvironment, macrophages express a variety of genes, including genes encoding proinflammatory cytokines, adhesion molecules, and tissue factor, that are regulated by the nuclear factor (NF)-κB system.1 Recent data suggest that microorganisms such as Chlamydia pneumoniae and Helicobacter pylori, which are commonly found in plaque, may aggravate atherosclerosis.

Human toll-like receptor 4 (TLR4), a transmembrane glycoprotein, is constitutively expressed by monocytes and endothelial cells; its expression is markedly increased in human atherosclerotic lesions, notably on macrophages and endothelial cells.2,3 Recently, TLR4 was identified as the signaling receptor for lipopolysaccharide (LPS),4 a Gram-negative bacterial wall component. TLR4 also interacts with heat shock proteins,5 fibronectin, fibrinogen,6 and minimally modified LDL.7 TLR4 ligation activates NF-κB, resulting in the expression of several inflammatory genes and proliferation of vascular smooth muscle cells. A common adenine to guanine substitution, 896 nucleotides downstream of the transcription start site (+896), results in the replacement of an aspartic acid residue by a glycine at amino acid position 299 (Asp299Gly). This missense polymorphism alters the extracellular domain of TLR4, attenuates the TLR4 signaling pathway, diminishes the inflammatory response to Gram-negative pathogens, and is associated with a diminished airway response to inhaled LPS in healthy volunteers.8 Recently, Kiechl and colleagues9,10 reported that the Asp299Gly polymorphism was associated with a decreased risk of carotid and femoral artery atherosclerosis.

To evaluate the possible role of the Asp299Gly polymorphism in the onset of acute coronary events, we con-
ducted a case-control study of European subjects with and without a history of myocardial infarction (MI) or unstable angina (UA).

Methods

Subjects
The patients and control subjects gave their written informed consent to the study, which was approved by the Pitié-Salpêtrière ethics committee. The subjects were all European, white, and younger than 65 years of age. The case patients were 183 subjects who had been admitted to Bichat hospital coronary care unit with a diagnosis of acute MI or UA. MI and UA were diagnosed according to previously reported criteria. Among patients with UA, those with angiography-proven ≥50% diameter stenosis were selected. Two hundred sixteen healthy control subjects were recruited among hospital employees and blood donors. Plasma fibrinogen and vascular cellular adhesion molecule (VCAM)-1 levels were analyzed according to the TLR4 genotype in a subset of 63 controls and 65 patients who consented to the procedure; the patients were tested 3 to 9 months after the acute coronary event.

Asp299Gly Polymorphism Screening Method
Genomic DNA was prepared from peripheral blood cells using standard procedures. Genotyping was performed using a validated 5′ nuclease assay (TaqMan allelic discrimination test) as described by Read et al using the Rotor Gene instrument (Corbett Research). The probes and primers were purchased from Eurogentec.

Fibrinogen and VCAM-1 Assays
Blood was collected into 0.129 mol/L trisodium citrate after a 12-hour fast. Soluble VCAM-1 was assayed with an enzyme-linked immunosorbent assay (R&D Systems), and fibrinogen was assayed with the Fibri-Prest kit (Diagnostica Stago).

Statistical Analysis
Univariate and multiple logistic regression analyses were performed to determine whether the Asp299Gly polymorphism was associated with the risk of acute coronary events after accounting for age, sex, and standard coronary risk factors (smoking, hypertension, diabetes, hypercholesterolemia, and obesity). Crude and adjusted odds ratios (ORs) are reported with their 95% CIs. Plasma levels of fibrinogen and soluble VCAM-1 were analyzed according to the TLR4 genotype by using the Mann-Whitney test. The results are expressed as mean±SEM. Statistical analyses were performed using SAS v8.2 software (SAS Institute Inc).

Results

Characteristics of the Study Population
The 183 cases (143 patients with MI and 40 patients with UA) were well-matched with the 216 controls in terms of age and sex. As expected, risk factors for coronary heart disease (including smoking, hypercholesterolemia, diabetes mellitus, hypertension, and obesity) were significantly more frequent among the patients than the controls (Table 1).

Arg299Gly Genotype Distribution
No deviation from Hardy-Weinberg equilibrium was observed for the Asp299Gly polymorphism in cases and controls. The frequency of the Gly allele was higher in the controls than in the cases (6.9 versus 3.8%, P=0.054) (Table 2). The frequency of the variant genotypes (Asp/Gly+Gly/Gly) was 13.3% in the controls and 7.7% in the cases. The OR, adjusted for age, sex, and standard coronary risk factors, was 0.41 (95% CI, 0.18 to 0.95; P=0.037). This showed that the Gly allele was associated with a decreased risk of acute coronary events and that this effect was independent of standard coronary risk factors.

Fibrinogen and VCAM-1 Levels According to the TLR4 Genotype
The relationship between the Asp299Gly polymorphism and plasma levels of 2 markers of inflammation was analyzed in detail in 65 cases and 63 controls (Table 3). The cases and controls had a similar age (data not shown). In controls, the presence of the rare Gly allele was associated with significantly lower levels of fibrinogen (P<0.001) and VCAM-1 (P<0.01) compared with the common Asp/Asp genotype. A similar decrease was observed in the cases but did not reach significance.

Discussion
These results show that the TLR4 Gly299 allele is associated with a reduced risk of acute coronary events independently of standard coronary risk factors. Plasma fibrinogen and soluble VCAM-1 levels were also lower in Gly299 heteroygotes. These results are in keeping with those obtained by Kiechl et al, who observed a smaller common carotid artery intima-media thickness in subjects carrying the Gly299 allele. The same subjects also had lower plasma levels of proinflammatory cytokines, soluble adhesion molecules, and acute-phase reactants. Our study thus confirms and extends the findings of this initial study, showing that TLR4 is involved in acute coronary events. Interestingly, in another recent study of symptomatic men with coronary artery disease, Gly299 allele carriers benefited significantly more than other subjects from pravastatin treatment. We did not collect follow-up data and could not thus evaluate this effect. Notably, the frequency of the Gly299 allele in our control population (6.9%) was similar to that reported by Read et al (5.9%) in 879 white northern English blood donors.

| TABLE 1. General Characteristics of Patients and Controls and Selected Risk Factors for Coronary Heart Disease |
|---------------------------------------------------------------|---|---|---|
| | Patients, | Controls, |
| | n=183 | n=216 |
| **Age, y** | | |
| Mean±SD | 48.2±7.3 | 48.7±6.7 |
| Range | 27–64 | 28–65 |
| Male sex | | |
| | 87.4 (160) | 89.4 (193) |
| Smoking* | | |
| | 74.3 (130/175) | 34.1† (73/215) |
| Hypertension‡ | | |
| | 23.1 (42/182) | 10.7† (23/215) |
| Hypercholesterolemia‡ | | |
| | 30.4 (55/181) | 7.9† (17/215) |
| Diabetes§ | | |
| | 12.6 (23/182) | 0.9† (2/215) |
| Body mass index≥30 kg/m² | | |
| | 17.1 (31/181) | 8.8† (19/215) |

Values are percentages, with the number of patients in parentheses.
*Smokers were defined as all subjects who reported having smoked regularly at any time.
†P<0.05.
‡Subjects were classified as hypertensive, hypercholesterolemic, or diabetic if they reported that they were currently prescribed drugs for one of these conditions.
As underlined in a recent review, acute coronary events are the clinical manifestation of the chronic development of coronary artery atheroma, the final pathologic process consisting of plaque rupture and coronary thrombosis. Atherosclerosis is now considered to be a chronic inflammatory disease of the arterial wall, and there is convincing evidence that *Chlamydia pneumoniae*, which is frequently found in human atheroma, can trigger arterial inflammation. TLR4 is the main LPS receptor and also recognizes fibrinogen, fibronectin, chlamydial heat-shock protein 60, and minimally oxidized LDL. TLR4 ligation results in NF-κB activation and upregulation of multiple genes whose products, including VCAM-1, tissue factor, and cytokines, may be involved in the atherosclerotic process.

The TLR4 Asp299Gly polymorphism is associated with receptor dysfunction, impaired LPS signaling, and a subnormal inflammatory response. Like epithelial cells from mutated subjects, THP-1 cells transfected with the Gly299 allele do not respond normally to LPS stimulation. Interestingly, in a recent prospective population-based survey of the epidemiology and pathogenesis of atherosclerosis, this common TLR4 polymorphism was associated with a reduced intima-media thickness, supporting a role of innate immunity in atherogenesis. We studied the influence of the polymorphism on fibrinogen and VCAM-1 levels in a small subgroup of cases and controls. As previously observed by Kiechl et al., we found significantly reduced levels of both fibrinogen and VCAM-1 in control subjects carrying the mutated genotype, pointing to a subnormal TLR4-mediated response to and VCAM-1 in control subjects carrying the mutated genotype.

### TABLE 2. Genotype and Allele Frequencies of the Asp299Gly Polymorphism of the TLR4 Receptor in Patients and Controls

<table>
<thead>
<tr>
<th>Genotypes</th>
<th>Patients, % (n)</th>
<th>Controls, % (n)</th>
<th>OR* (95% CI)</th>
<th>OR† (95% CI)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Asp/Asp</td>
<td>92.3 (169)</td>
<td>86.7 (187)</td>
<td>0.53 (0.27–1.04)</td>
<td>0.41 (0.18–0.95)</td>
</tr>
<tr>
<td>Asp/Gly</td>
<td>7.7 (14)</td>
<td>12.3 (28)</td>
<td>P=0.06</td>
<td>P=0.037</td>
</tr>
<tr>
<td>Gly/Gly</td>
<td>0</td>
<td>0.5 (1)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Frequency of the Gly allele, %</td>
<td>3.8</td>
<td>6.9†‡</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* Crude OR associated with the Asp/Gly+Gly/Gly vs the Asp/Asp genotype.
†OR associated with the Asp/Gly+Gly/Gly vs the Asp/Asp genotype after adjustment for age, sex, and standard cardiovascular risk factors. 
‡P=0.054.

(n=4), this effect was not significant in the cases. In this study, the mean fibrinogen and VCAM-1 levels according to the TLR4 genotype are different from those reported by Kiechl et al. Several factors may explain these differences, notably the assay methods (not clearly stated in the study by Kiechl et al.). Moreover, our population was younger (by about 10 years on average) and contained a higher proportion of men (80% versus 50%). Finally, in the study by Kiechl et al., the values of fibrinogen and VCAM-1 are given separately for heterozygotes according to the presence or absence of a second TLR4 polymorphism (Thr399Ile) that was not searched for in our study; however, the frequency of this second polymorphism is low (1%) and is unlikely to interfere strongly with the results. Circulating levels of fibrinogen, an acute-phase reactant, are predictive of atherosclerosis. They are largely regulated by interleukin-6, an LPS-dependent cytokine, the synthesis of which is mediated by NF-κB activation. In addition, soluble VCAM-1 levels have been shown to correlate with VCAM-1 mRNA expression within atherosclerotic plaque and with the extent of human atherosclerosis quantified by angiography.

Interestingly, proinflammatory cytokines, which are present within atherosclerotic lesions, induce VCAM-1 expression via the NF-κB pathway. Moreover, minimally modified LDL possesses many proatherogenic properties, including the induction of monocyte adhesion to endothelial cells. The activation of TLR4 mediated by its ligands may represent a new link in the chain of events leading to atherosclerosis. In conclusion, this study shows that the Gly299 allele of the TLR4 gene is associated with a decreased risk of acute coronary events in European white subjects, an effect that might involve decreased levels of inflammatory mediators.

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