Expansion of Circulating Toll-Like Receptor 4–Positive Monocytes in Patients With Acute Coronary Syndrome

Heiko Methe, MD; Jong-Oh Kim; Sieglinde Kofler, MD; Michael Weis, MD; Michael Nabauer, MD; Joerg Koglin, MD

Background—Atherosclerosis is an inflammatory disease in which monocytes and macrophages have been suggested to play an essential role. The underlying signaling mechanisms are unknown thus far. We hypothesized that the human isoform of Toll-like receptor (hTLR)-4 is involved in monocyte activation of patients with accelerated forms of atherosclerosis.

Methods and Results—Expression of hTLR4 on circulating monocytes from 30 controls, 20 patients with stable angina (SA), 40 patients with unstable angina (UA), and 28 patients with acute myocardial infarction (AMI) was compared with the use of flow-cytometry and reverse transcription–polymerase chain reaction. Regulation of interleukin (IL)-12 and heat shock protein (HSP)60 inhibited CHO cell activation.

Conclusions—UA and AMI are associated with enhanced expression and signaling events downstream of hTLR4 in circulating monocytes. These observations suggest hTLR4 activation as a signaling mechanism in immune-mediated progression of atherosclerosis.

Key Words: coronary disease • monocytes • immune system • receptors, cell surface

Atherosclerosis resembles many features of a chronic inflammatory disease.1 Atherosclerotic lesions are enriched for macrophages and T cells that play important roles in early (innate) and advanced (acquired) immune responses. In general, the immune system responds effectively to danger signals by close interplay between innate and adaptive immune recognition systems. Progression of atherosclerosis has been associated with clonal expansion of differentiated T cells as a feature common to all adaptive immune responses.2 Efficent priming of adaptive immune responses requires not only presentation of antigen but also the induction of accessory signals (costimulators and cytokines) on antigen-presenting cells. The precise signaling mechanisms responsible for activation of these cells in the pathogenesis of atherosclerosis remain ill defined. Recently, Toll-like receptors (TLRs) have been identified as key recognition components of the innate immune system in mammals.3,4 Of 11 different human TLR family members, TLR4 is the best characterized. Several exogenous and endogenous ligands such as lipopolysaccharide (LPS),5,6 fibrinogen,7 minimally modified LDL,8 and heat shock protein (HSP)60 have been identified. Enhanced monocyte/macrophage expression of costimulatory molecules (such as B7-1 and B7-2) and proinflammatory cytokines (such as interleukin [IL]-1β, IL-6, IL-12, and tumor necrosis factor-α [TNF-α]) have been demonstrated as downstream effects of TLR activation.3,10

Although the importance of TLRs in antimicrobial responses is well established, their role in atherosclerotic disease processes as early triggers of immune-mediated initiation or progression is not well understood. Immunohistochemical staining of murine and human atherosclerotic tissue revealed prominent expression of TLR4, especially at the lipid-rich, macrophage-infiltrated shoulder region of plaques.11 Edfeldt et al12 provided evidence that TLR-expressing cells in the vessel wall are activated. Furthermore, TLR4 has been suggested to play a role in outward remodeling,13 and an enhanced in vitro response of monocytes to LPS has been demonstrated in patients with recurrent unsta-
Results

Table 1. Demographic Characteristics and Biological Parameters of Patients and Control Subjects

<table>
<thead>
<tr>
<th>Parameter</th>
<th>SA (n=20)</th>
<th>UA (n=40)</th>
<th>AMI (n=28)</th>
<th>Control (n=30)</th>
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<td>63 (63±10)</td>
<td>62 (60±16)</td>
<td>57 (59±13)</td>
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<tr>
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<td>14 (70)</td>
<td>29 (73)</td>
<td>17 (61)</td>
<td>19 (63)</td>
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<td>Hypertension</td>
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<td>19 (48)</td>
<td>13 (46)</td>
<td>...</td>
</tr>
<tr>
<td>Smoking</td>
<td>8 (40)</td>
<td>15 (38)</td>
<td>12 (43)</td>
<td>...</td>
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<tr>
<td>Cholesterol, mg/dL</td>
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<td>186 (184±29)</td>
<td>194 (192±35)</td>
<td>179 (168±36)*</td>
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<tr>
<td>LDL cholesterol, mg/dL</td>
<td>136 (145±31)</td>
<td>132 (134±24)</td>
<td>138 (139±28)</td>
<td>123 (122±21)*</td>
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<td>Lipoprotein(a), mg/dL</td>
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<td>31 (29±21)</td>
<td>36 (30±30)*</td>
<td>30 (23±27)</td>
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<td>Glucose, mg/dL</td>
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<td>105 (110±19)</td>
<td>98 (91±27)</td>
<td>96 (98±12)</td>
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<td>25 (63)</td>
<td>19 (68)</td>
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<td>17 (43)</td>
<td>11 (39)</td>
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<td>Laboratory parameters on admission</td>
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<td>C-reactive protein, mg/dL</td>
<td>0.3 (0.3±0.1)</td>
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<td>0.7 (0.2±1.2)</td>
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<td>7.4 (7.4±0.6)</td>
<td>7.2 (7.4±0.9)</td>
<td>7.5 (7.6±0.6)</td>
<td>7.3 (7.4±0.4)</td>
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<tr>
<td>CK, U/L</td>
<td>27 (25±15)</td>
<td>34 (34±13)</td>
<td>593 (600±218)*</td>
<td>28 (34±19)</td>
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<td>Troponin I, ng/mL</td>
<td>ND</td>
<td>4.8 (4.5±2.7)</td>
<td>104 (102±29)*</td>
<td>ND</td>
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</tbody>
</table>

Data are n (%) or mean (median±SD). ND indicates not detectable.

*P<0.05, †P<0.0001, ‡P<0.0001, AMI vs UA.

Discussion

The purpose of this study was to compare the expression of the human isoform of TLR4 (hTLR4) on circulating CD14+ monocytes in patients with various clinical stages of atherosclerosis (patients with stable angina [SA], UA, and AMI) to better understand the association of hTLR4 with the progression and deterioration of atherosclerosis. Changes in expression patterns for hTLR4 were correlated with changes in monocyte-specific expression of the costimulatory molecule B7-1 as well as IL-12 as typical hTLR4 downstream effects.

In trying to prove and identify a serum factor that might be responsible for the hypothesized atherosclerosis-associated activation of hTLR4, stably transfected Chinese hamster ovary (CHO)–TLR4+ cells were incubated with serum from patients with various clinical stages of atherosclerosis.

Methods

Study Patients
This investigation was performed with approval by the institutional ethics committee. Informed consent was obtained from all subjects. Eighty-eight of 143 consecutive patients admitted to our hospital with a diagnosis of coronary artery disease (CAD) were included in the study. Four groups of subjects were studied (Table 1). Blood samples were obtained from all patients in the recumbent position with a 21-gauge needle via antecubital venepuncture as soon as possible after admission. Concentrations of C-reactive protein, white blood cell count, cholesterol, glucose, troponin I, and creatine kinase (CK) were measured according to routine protocols.

Group I included 20 patients with clinical evidence of Canadian Cardiovascular Society class II and III SA and at least 1 coronary artery stenosis detected at angiography (>50% reduction of lumen diameter). Group II included 40 patients admitted to the Coronary Care Unit of our hospital with a diagnosis of UA characterized as class II or III within the Braunwald classification. All patients with UA had experienced ischemic chest pain at rest within the preceding 6 hours but no evidence of myocardial infarction by ST elevation or significant rise in CK or CK-MB (34±13 U/L). Troponin I was detected in 18 of 40 patients but in all was <8 ng/mL (average, 4.8±2.7 ng/mL). All UA patients exhibited transient ST-T-segment depression and/or T-wave inversion. Group III included 28 patients with an AMI who presented within 6 hours of the onset of pain. Identification of AMI followed the recent consensus document of the American Heart Association and the American College of Cardiology. All patients showed elevated ST segments and a characteristic pattern of myocardial serum enzymes in which the total CK was at least twice the upper reference limit for CK accompanied by a rise in CK-MB mass (593±218 U/L) and troponin I (104±29 ng/mL). All patients underwent coronary angiography during hospitalization.

Exclusion criteria were as follows: previous myocardial infarction within 6 months (13 patients); admission >6 hours from onset of symptoms for group II and III (21 patients); inflammatory conditions likely to be associated with an acute phase response (8 patients); autoimmune disease (5 patients); and neoplastic disease (8 patients). None of the included patients had advanced liver disease, renal failure, or valvular heart disease.

Group IV included 30 healthy volunteers with no clinical signs of CAD and without coronary risk factors. Controls were recruited from the staff of our hospital and from visitors. All of these controls had normal ECG and echocardiogram and no evidence of atherosclerosis by carotid artery sonography.
Isolation of Peripheral Blood Mononuclear Cells, mRNA Isolation, and Semiquantitative Reverse Transcription-Polymerase Chain Reaction

Flow Cytometry Analysis

Cells were incubated with hTLR4 antibody (mouse anti-human monoclonal antibody, clone HTA125, IgG2a, HyCult Biotechnology, Uden, Netherlands) and CD14-PE antibody (mouse anti-human BD7-1, Bio-Rad Laboratories, Munich, Germany) for 12 hours and stained with a monoclonal antibody, clone HTA125, IgG2a, HyCult Biotechnology, Cells were incubated with hTLR4 antibody (mouse anti-human monoclonal antibody, clone HTA125, IgG2a, HyCult Biotechnology, Uden, Netherlands) and CD14-PE antibody (mouse anti-human BD7-1, Bio-Rad Laboratories, Munich, Germany) for 12 hours and stained with a monoclonal antibody, clone HTA125, IgG2a, HyCult Biotechnology, Cells were incubated with hTLR4 antibody (mouse anti-human monoclonal antibody, clone HTA125, IgG2a, HyCult Biotechnology, Uden, Netherlands) and CD14-PE antibody (mouse anti-human BD7-1, Bio-Rad Laboratories, Munich, Germany) for 12 hours and stained with a monoclonal antibody, clone HTA125, IgG2a, HyCult Biotechnology, Cells were incubated with hTLR4 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centrifugation, and IL-12 concentration was quantified by ELISA (R&D Systems; detection limit <0.5 pg/mL). Supernatants for each individual were stored at −70°C and measured at the same time by the same ELISA to avoid variations of assay conditions.

**Statistical Analysis**

Data are presented as mean and median±SD (mean; median±SD) or number (percentage). Continuous variables between 2 groups were compared by Wilcoxon test and between ≥2 groups by Kruskal-Wallis test. Categorical variables were compared by the χ² test. Comparisons between B7-1 and IL-12 expression levels and frequencies of hTLR4+/CD14⁺ monocytes were performed with the use of Spearman’s ρ rank correlation test. Statistical analysis was performed with JMP statistical software (SAS Institute Inc). A probability value <0.05 was considered statistically significant.

**Results**

**Characteristics of Patients**

Demographic and clinical data for patients in the 3 groups and healthy controls are summarized in Table 1. There were no significant differences in white blood cell count; C-reactive protein; use of home medications including aspirin, β-blockers, 3-hydroxy-3-methylglutaryl coenzyme A reductase inhibitors, or ACE inhibitors; or risk factors except HDL cholesterol and lipoprotein(a) levels among patients with SA, UA, or AMI. UA and AMI patients were treated with standard antianginal medications, intravenous heparin, cilostazol, and the SA group. Patients with SA, UA, or AMI were treated with standard antianginal medications, intravenous heparin, cilostazol, and the SA group. Patients with SA, UA, or AMI were treated with standard antianginal medications, intravenous heparin, cilostazol, and the SA group.

**Assessment of Frequencies of hTLR4-Expressing CD14⁺ Monocytes**

Protein levels of hTLR4 on the surface of peripheral circulating CD14⁺ monocytes were quantified by flow cytometry analysis. A mean of 30.4% (32.4±6.3%) of peripheral circulating CD14⁺ monocytes in normal controls and of 32.7% (32.5±5.4%) in patients with SA showed surface expression for hTLR4 compared with 77.9% (77.3±9.9%) in patients with UA and 78.3% (76.7±14.0%) in patients with AMI. The frequencies of hTLR4+/CD14⁺ monocytes were significantly higher in patients with UA and AMI than in SA patients (P<0.0001; Figure 1) and AMI patients compared with patients with SA and healthy controls (P<0.0001 vs control and SA).

**Assessment of hTLR4 and Myd88 Transcript Levels**

mRNA expression by reverse transcription (RT)–PCR followed protein detection by FACS analysis. Transcript levels for hTLR4 were significantly higher in the UA (1.78; 1.83±0.26 relative units [RU]; P<0.0001) and the AMI groups (1.82; 1.83±0.33 RU; P<0.0001) than in controls (0.92; 0.93±0.11 RU) and in SA patients (0.96; 0.96±0.19 RU; Figure 2). There were no significant differences between the UA and AMI groups (P=0.46).

Similar results could be collected for the gene expression of the adaptor protein Myd88. UA and AMI patients had significantly increased transcript levels for Myd88 (0.96; 0.97±0.07 RU and 1.06; 1.04±0.24 RU) compared with controls (0.37; 0.42±0.18 RU; P<0.0001) and SA patients (0.42; 0.44±0.13 RU; P<0.0001; Figure 2). AMI patients had significantly higher Myd88 mRNA transcript levels than UA patients (P<0.05).

**Serum HSP60**

Circulating serum HSP60 levels were significantly lower in controls than in patients with SA (46; 21±55 versus 263; 275±96 ng/mL; P<0.0001), whereas only 12 of the controls showed any circulating HSP60. Patients with UA (1243; 1075±403 ng/mL; P<0.0001 versus SA) and with AMI (2342; 2298±687 ng/mL; P<0.0001; Figure 3) exhibited significantly higher levels of circulating HSP60 than controls and the SA group.

**Serum HSP60 Activates CHO Cells**

On the basis of enhanced expression of TLR4 in patients with acute coronary syndromes (ACS), we hypothesized the presence of circulating ligands for TLR4 in this clinical setting. Therefore, we evaluated whether circulating HSP60 could
activate TLR4-transfected CHO cells by challenging them with serum from patients. Sera from 2 patients with HSP60 serum levels in the highest tertile, from 4 patients with HSP60 serum levels in the middle tertile, and from 2 patients with HSP60 serum levels in the lowest tertile of all HSP60 measurements generated in each group were used. Western blot of different serum samples with the applied HSP60 antibody revealed its specificity (data not shown). As shown in Figure 4, serum from patients with ACS induced a significant CD25 expression on CHO cells that was 77% for the UA group and 79% for the AMI group compared with the positive control (1 μg/mL LPS). In comparison, serum from controls showed almost no CD25 expression ability (P<0.0001 versus UA and AMI), and challenging CHO cells with serum from SA patients yielded only a weak response (P<0.001 versus control; P<0.0001 versus UA and AMI). Coincubation of cells with serum and anti-HSP60 nearly rescinded CD25 expression (SA 10%, UA 10%, AMI 15% compared with the LPS response; P<0.0001; Figure 4), whereas coincubation with LPS and anti-HSP60 had no effect on CD25 expression by CHO cells. In addition, coincubation with an irrelevant antibody failed to inhibit the effect of serum or recombinant HSP60 on CD25 expression, respectively (data not shown).

### Upregulation of B7-1 and IL-12 in Patients With UA and AMI

To investigate expression patterns of TLR4-dependent molecules in LPS-stimulated monocytes, we assessed B7-1 expression via FACS analysis and IL-12 secretion via ELISA. Whereas in controls and in SA patients only few CD14+ monocytes stained positive for B7-1 (control: 2.4%; 1.4±2.3%; SA: 3.4%; 4.2±2.5%), frequencies of B7-1+/CD14+ monocytes were significantly higher in the UA (27.3%; 24.3±14.4%) and AMI groups (22.6%; 21.0±11.1%; P<0.0001 versus control and SA; Figure 5A). Compared with controls and SA patients, IL-12 secretion from LPS-treated monocytes was significantly higher in patients with UA and AMI (control: 2.1; 2.1±0.3 pg/mL; SA: 2.2; 2.0±0.5 pg/mL; UA: 35.5; 35.4±7.8 pg/mL; AMI: 31.8; 32.4±7.7 pg/mL; P<0.0002 versus control and SA; Figure 5B).

A strong correlation was noted between the frequency of peripheral circulating hTLR4+/CD14+ monocytes and expression levels of B7-1 (r=0.71; P<0.0001) and secretion of IL-12 (r=0.76; P<0.0001) across all patient groups (data not shown).

Comparable patterns could be seen on mRNA transcript levels with upregulation of B7-1 and IL-12 mRNA transcript levels in patients with UA and AMI (Figure 6).

### Discussion

In this study we used flow cytometry analysis, RT-PCR, and ELISA to characterize expression levels of hTLR4 and hTLR4 downstream signaling events in circulating monocytes from patients with different stages of CAD. To the best of our knowledge, this is the first study to show enhanced expression of hTLR4 on circulating monocytes in patients with ACS. Enhanced expression of hTLR4 in patients with ACS was associated with elevations of IL-12 and B7-1 expression. Moreover, using a CHO cell line transfected with expression plasmids for TLR4 and CD14, we found circulating HSP60 as a potential endogenous ligand activating TLR4 in this clinical setting.

Evidence is accumulating that TLR4 and its endogenous ligand HSP60 are important players in the initiation and acceleration of atherosclerotic disease. Animal experiments as well as human studies point to a combination of HSP60-specific humoral and cellular immune reaction in this disease. Quite recently, Zal et al proved the presence of HSP60-reactive T cells in patients with ACS, and Buono et al reported in a mouse model that T cell reactivity to HSP60 as well as development of atherosclerosis depends on co-

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**Figure 3.** Levels of circulating HSP60 measured by ELISA are presented as box plots. AMI and UA patients had higher HSP60 serum levels than SA patients or healthy controls. *P<0.0001 vs control; †P<0.0001 vs SA; ‡P<0.0001 vs UA.

**Figure 4.** Serum from patients with ACS activates the NF-κB signaling pathway through TLR4. TLR4+ CHO cells were incubated with sera from controls and from patients with SA, UA, and AMI or with LPS with or without anti-HSP60. CD25 expression was determined by flow cytometry. Results are expressed as percentage of fold increase in median fluorescence intensity compared with LPS-treated CHO cells and displayed as box plots. *P<0.001 vs SA-serum; †P<0.0001 vs UA and AMI serum; ‡P<0.0001 vs LPS+anti-HSP60.
stimulatory signaling (B7-1 and B7-2) provided by antigen-presenting cells. Furthermore, outward arterial remodeling, which is believed to be associated with a vulnerable plaque phenotype, has been linked with an increase in TLR4 expression and mRNA levels of endogenous ligands (HSP60 and extra domain A of fibronectin). This might further indicate that no exogenous ligands are necessary for atherogenesis, as Wright et al demonstrated in a murine model. However, it should be recognized that the knowledge about HSP60 as a potential endogenous ligand of TLR4 has been generated in in vitro and artificial in vivo experiments; as revealed in recent publications, it might be possible that the ligand preparations used were contaminated with LPS and/or other microbial components.

It has been shown that upregulation of TLR4 contributes to sensitization of monocytes, whereas downregulation decreases inducibility of a proinflammatory response. Elevated expression of IL-12 and B7-1, as typical downstream effects of TLR4 activation, in concert with antigen presentation has been shown to activate T cells and drive their differentiation into T-helper 1 cells, for which a role in increasing plaque instability has been shown (H. Methe, MD, unpublished data, 2004). Furthermore, upregulation of hTLR4 expression in patients with ACS was paralleled by enhanced Myd88 transcript levels in circulating monocytes. Myd88, which is an intracellular adaptor protein mediating TLR signal transduction, is subject to regulation by inflammatory stimuli in monocytes. However, Michelsen et al showed that atherosclerosis-prone hypercholesterolemic mice that also harbor a null mutation in either the adaptor molecule TLR4 or MyD88 exhibit reduced aortic atherosclerosis, plaque lipid content, and plaque macrophage infiltration. Moreover, MyD88 deficiency led to decreased levels of circulating IL-12. However, the role of innate immune mechanisms in advanced atherosclerotic lesions (plaque rupture, thrombus formation, or myocardial infarction) needs further analysis.

Taken together, the findings of the present study may directly link activation of TLR4 by circulating HSP60 with clinical instability of CAD. This coherence had been already supposed by Pasterkamp et al because endogenous TLR4 ligands, HSP60, and extra domain A of fibronectin have been observed in arthritic and oncological specimens in which matrix turnover is an important feature. Because we only studied the effect of circulating HSP60 on activation of TLR4-transfected CHO cells, we cannot exclude that other
endogenous ligands for TLR4 (eg, minimally modified LDL, extra domain A of fibronectin8,11,40) may play a role in the activation of innate immune mechanisms in the progression and deterioration of CAD.

Currently it is not well characterized how antiatherosclerotic pharmacotherapy may influence hTLR4 expression patterns and downstream signaling. To the best of our knowledge, besides an effect of statins on hTLR4 expression and signaling (H. Methe, MD, unpublished data, 2005),41 no results have been published with regard to an impact of ACE inhibitors, β-blockers, aspirin, or clopidogrel on hTLR4 signaling. Importantly, patients in the present study did not show significant differences in antiatherosclerotic pharmacotherapy. The high frequency of hTLR4+/CD14+ monocytes in patients with ACS, despite being on statin therapy, emphasizes even more the potential role of hTLR4 in progression and deterioration of CAD.

In conclusion, our study demonstrated that patients with UA and AMI exhibit enhanced monocytic expression of hTLR4 and signaling events downstream of hTLR4. In agreement with other reports, circulating HSP60 may be an endogenous ligand for hTLR4 in ACS. Therefore, hTLR4 may provide a link between innate and adaptive immunity, thereby enhancing the cellular immune response to antigens such as HSP60 in patients with ACS.

Acknowledgments

This study was supported in part by a grant from the Förderprogramm für Forschung und Lehre, University of Munich, to H. Methe. We thank Dr Douglas T. Golenbock, University of Massachusetts Medical School, Worcester, for providing us with the CHO cell lines. We are grateful to Vera Tolbert for her expert technical assistance and Dr Mark Vangel, Massachusetts Institute of Technology Clinical Research Center, for his valuable help.

References


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Circulation. 2005;111:2654-2661; originally published online May 9, 2005; doi: 10.1161/CIRCULATIONAHA.104.498865
Circulation is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 0009-7322. Online ISSN: 1524-4539

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