Increased Plasma Levels of the Soluble Form of Fas Ligand in Patients With Acute Myocardial Infarction and Unstable Angina Pectoris

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OBJECTIVES
To examine whether the Fas/Fas ligand system is involved in the pathogenesis of acute myocardial infarction (AMI), we measured the levels of the soluble form of the Fas ligand (sFasL) in the plasma of patients with AMI and stable or unstable angina pectoris (AP).

BACKGROUND
The Fas ligand (FasL) is rapidly cleaved off by a metalloproteinase from the cell membrane to become a soluble form as a cytokine. Fas is expressed in most cells, including cardiomyocytes, whereas FasL is mainly expressed in inflammatory cells such as macrophages, which are greatly accumulated in unstable plaque.

METHODS
Thirty patients with AMI, 10 patients with unstable AP, 10 patients with stable AP and 30 control subjects were enrolled in the present study.

RESULTS
Plasma sFasL levels were significantly elevated on hospital admission in patients with AMI and unstable AP, compared with control subjects. Time-course studies revealed that plasma sFasL levels rapidly decreased within 3 h and then increased again after percutaneous transluminal coronary angioplasty in patients with AMI, but not in patients with stable AP. Importantly, the sFasL levels were higher in the coronary sinus than in the circulation. In addition, in vitro studies showed that the expression of FasL messenger ribonucleic acid was upregulated in mononuclear cells isolated from patients with AMI and that hypoxia stimulated the release of sFasL from isolated mononuclear cells.

CONCLUSIONS
This demonstration of elevated levels of sFasL in patients with AMI and unstable AP suggests that activation of the Fas/FasL system may play a pathogenic role in AMI and acute coronary syndromes. (J Am Coll Cardiol 2002;39:585–90) © 2002 by the American College of Cardiology

Apoptosis is an important physiologic cell death mechanism that serves to remove damaged or unwanted cells. However, the dysregulation of apoptosis has been implicated in many human diseases, such as cancer, neurodegenerative diseases and ischemic heart diseases (1). Recent studies in pathology have shown that many cardiomyocytes die by apoptosis as well as necrosis during myocardial infarction (2,3). Although the molecular mechanism of this process is not clear, cardiomyocyte apoptosis can be induced by many stimuli, including hypoxia (4), ischemia/reperfusion (5), mechanical stretch (6), tumor necrosis factor-α (TNF-α) (7) and reactive oxygen species (8). Yaoita et al. (9) recently reported that pretreatment with the caspase inhibitor ZVAD-fmk resulted in a reduction in infarct size and an improvement of acute functional variables in a rat model of acute myocardial infarction (AMI). In addition, Daemen et al. (10) reported that at the time of reperfusion, administration of the anti-apoptotic agents insulin-like growth factor-1 and ZVAD-fmk prevented not only renal apoptosis but also inflammation, suggesting that the inhibition of apoptosis may be a useful therapeutic strategy for treatment in these ischemic animal models. However, it is not clear whether inhibition of apoptosis is beneficial in human AMI. To address this issue, it is necessary to define the key molecules that regulate cardiomyocyte apoptosis during AMI.

The Fas ligand (FasL; also called the CD95 or APO-1 ligand) is a cytokine that mediates apoptosis by binding to its receptor, Fas (also called CD95 or APO-1) through activating caspases (11). Fas is expressed almost ubiquitously in a variety of cells, including cardiomyocytes, whereas FasL is mainly expressed in natural killer cells, activated T cells and macrophages (12) as well as immune-privileged tissues of the eye and testes (13). The findings that cardiomyocytes die by apoptosis in association with upregulation of Fas under hypoxia (14) and that FasL is constitutively expressed in the myocardium (15) suggest that Fas-mediated cardiomyocyte apoptosis may be involved in the mechanism of AMI. On the other hand, Nelson et al. (16) recently reported that the FasL transgenic heart shows no myocardial apoptosis or necrosis but has proinflammatory consequences, resulting in cardiac hypertrophy and...
fibrosis, suggesting that FasL could mediate proinflammatory signals in cardiovascular diseases.

Importantly, FasL, a member of the TNF family, is rapidly cleaved off by a metalloproteinase from the membrane to become a soluble form (sFasL) as well as TNF-α (17). In human studies, sFasL has been detected in the serum of patients with myocarditis (18) and congestive heart failure (CHF) (19), in the cerebrospinal fluid after a severe head injury (20) and in the bronchoalveolar lavage fluid of patients with acute respiratory distress syndrome (21). In contrast, Nishigaki et al. (22) reported that plasma levels of soluble Fas, but not sFasL, were elevated in patients with CHF. To examine the possible participation of the Fas/FasL system in the pathogenesis of AMI, we measured the levels of plasma sFasL in patients with AMI without CHF. We found that plasma sFasL levels were significantly increased in the early phases of AMI and that the levels of sFasL were correlated with its severity.

METHODS

Subjects. We included 30 patients with AMI, 10 patients with unstable angina pectoris (AP) and 10 patients with stable AP. The average time from the onset of symptoms to hospital admission was 3.5 ± 0.4 h (range 1 to 12). All patients with unstable AP had experienced ischemic chest pain at rest within the preceding 48 h (i.e., Braunwald's class IIIIB [23]), but they had no evidence of myocardial necrosis by enzymatic criteria. Transient ST-T segment depression or T-wave inversion, or both, were present in all patients.

Study protocol. CORONARY ANGIOGRAPHY. In all of the 30 patients with AMI, coronary angiography was performed immediately after admission. One patient was treated with percutaneous transluminal coronary recanalization through intracoronary infusion of urokinase, and 29 patients were treated with direct percutaneous transluminal coronary angioplasty (PTCA). We excluded AMI patients with CHF, concomitant inflammatory diseases, cancer or other significant heart diseases. All patients gave written, informed consent to be included in the study, which was approved by the Ethics Committee on Human Research of Osaka University.

BLOOD SAMPLING AND ASSAY FOR sFasL. Blood sampling for measuring sFasL concentrations was taken from the peripheral vein on admission before PTCA or thrombolysis in all patients with AMI, and in five patients it was repeated at 3, 6, 12, 18 and 24 h after admission. In five patients with AMI, blood samples were drawn from the coronary sinus, using a 7F Swan-Ganz catheter, as well as from the peripheral vein, at the same time in the same patient. The samples were then centrifuged immediately at 3,000 rpm (4°C, 15 min) and stored at −80°C until assay. We measured the plasma levels of sFasL by an enzyme-linked immunosorbent assay (ELISA), as described previously (17).

MEASUREMENT OF HEMODYNAMIC VARIABLES. A Swan-Ganz catheter was inserted into the femoral vein immediately after admission, before coronary angiography, and was kept in place during the first two days in all patients. The pulmonary artery wedge pressure and cardiac output in all patients with AMI were measured by using a 7F Swan-Ganz thermodilution catheter (Baxter Healthcare Corp., Santa Ana, California), and the cardiac index was calculated as the cardiac output/body surface area ratio. Then left ventricular end-diastolic pressure was measured by using a 5F pigtail catheter, and left ventriculography was performed. The left ventricular ejection fraction was calculated using a centreline method.

MEASUREMENT OF Fasl mRNA LEVELS. Peripheral blood mononuclear cells (PBMCs) were obtained from heparinized blood by Ficoll-Paque Plus (Pharmacia LKB Biotechnology, Piscataway, New Jersey) gradient centrifugation. Total ribonucleic acid (RNA) was prepared using Isogen (Nippon Gene, Tokyo, Japan). One microgram of total RNA was reverse transcribed in a final volume of 10 μl with 200 U of Super Script II (Life Technologies, Tokyo, Japan) containing 0.5 mmol/l of deoxynucleotide triphosphate, 10 mmol/l of dithiothreitol and 100 ng of the antisense primer (5′-GCCGAAAAACGTCTGAGATT) for human FasL. After incubation at 42°C for 60 min, 1 μl of the reaction mixture was used for polymerase chain reaction (PCR), which was carried out with 2.5 U of Taq deoxyribonucleic acid (DNA) polymerase (Pharmacia) in 50 μl of reaction buffer containing 0.25 mmol/l of dNTP, 600 ng of each sense primer (5′-TGTTTACGCTTCATTACACTA) and antisense primer (5′-GTCCCTCATGTAGACGCTTGTG). The conditions for PCR were as follows: 94°C for 1 min; 55°C for 2 min; and 72°C for 3 min, for 25 cycles with a thermal cycler (Perkin Elmer, Norwalk, Connecticut). To amplify human G3PDH mRNA, 100 ng of random hexamers (Gibco, Tokyo, Japan) was used in complementary DNA synthesis, and 30 cycles of PCR (at 94°C for 1 min, 55°C for 2 min and 72°C for 3 min) were performed using a commercial primer set (Clontech, Palo Alto, California). The amplified products were subjected to electrophoresis on 1.2% agarose gels and visualized by ethidium bromide staining. The predicted size of the FasL fragment was 344 base pairs, and that of G3PDH was 983 base pairs.

### Abbreviations and Acronyms

- **AMI**: acute myocardial infarction
- **AP**: angina pectoris
- **CHF**: congestive heart failure
- **CK-MB**: creatine kinase-MB isoenzyme
- **ELISA**: enzyme-linked immunosorbent assay
- **mFasL**: membrane-bound Fas ligand
- **sFasL**: soluble Fas ligand
- **PBMCs**: peripheral blood mononuclear cells
- **PCR**: polymerase chain reaction
- **PTCA**: percutaneous transluminal coronary angioplasty
- **TNF-α**: tumor necrosis factor-α
HYPOXIA EXPERIMENTS. Isolated PBMCs cultured in serum-free Roswell Park Memorial Institute 1640 medium were exposed for the indicated times to the ambient oxygen concentration of 1% oxygen, 5% carbon dioxide, 94% nitrogen at 37°C (using a controlled incubator with carbon dioxide/oxygen monitoring and carbon dioxide/nitrogen gas sources). The sFasL levels in the supernatant were measured by ELISA, as described earlier.

Statistical analysis. All data are expressed as the mean value ± SD. A combination of analysis of variance, based on ranks, and a multiple comparisons procedure (i.e., the Bonferroni/Dunn procedure) was used. A p value <0.05 was considered significant.

RESULTS

Patient characteristics. Table 1 shows the clinical characteristics of the patients with stable AP, the patients with unstable AP, the patients with AMI and the control subjects. There were no significant differences in age or gender ratio among these four groups. There were significantly more hypertensive patients, smokers and diabetics in the three groups of patients with ischemic heart disease than in the control group.

Plasma sFasL levels on hospital admission. The plasma sFasL levels were elevated in patients with AMI and unstable AP on admission (3.5 ± 0.4 h after symptom onset), compared with the control subjects (Fig. 1). However, the sFasL levels were not elevated in patients with stable AP.

Correlation of plasma sFasL levels with clinical variables. Next, we examined the correlation of plasma sFasL levels with the maximal levels of creatine kinase MB isoenzyme (CK-MB), a marker of infarct size, and the hemodynamic variables within the first two days after admission. There was no significant correlation between the plasma sFasL levels on admission and the maximal levels of CK-MB or hemodynamic variables, such as the cardiac index (Fig. 2, A and B).

Time course changes of plasma sFasL in patients with AMI. To examine the physiologic significance of the measurement of plasma sFasL levels, we examined the time course of the changes of plasma sFasL levels in five patients with AMI after admission. As shown in Figure 3A, the plasma sFasL levels decreased rapidly within 3 h after admission. However, they increased again after PTCA and then returned to the basal level within 24 h. In contrast, the sFasL levels remained high at least within 24 h after admission in two patients with severe clinical complications such as re-occlusion and ventricular fibrillation (data not shown). Importantly, there was no increase in the levels of sFasL after PTCA in patients with stable AP.

Levels of sFasL in the coronary sinus. We measured the sFasL levels in blood samples obtained from the coronary sinus in five patients with AMI whose culprit lesions located at left anterior descending coronary artery, and we compared them with those obtained from the peripheral vein at the same time in the same patient. As shown in Figure 3B, the level of sFasL was significantly higher in the coronary sinus than in the peripheral vein, indicating that local concentrations of sFasL may be much higher in the culprit lesion.

Expression of FasL and release of sFasL in PBMCs. Recent pathologic studies have shown that unstable plaque from patients with acute coronary syndromes contains many more monocytes and macrophages, FasL-expressing cells, compared with stable plaque (24). To address the source of sFasL released in the circulation, we measured the levels of FasL mRNA in PBMCs isolated from three patients with AMI and the control subjects. As shown in Figure 4A, FasL mRNA expression in isolated PBMCs was significantly upregulated in a patient with AMI, compared with control subjects. However, this upregulation disappeared at 10 days after admission. Similar results were obtained in two other patients with AMI. In addition, in vitro cell culture studies showed that hypoxia (1% oxygen) significantly stimulated the release of sFasL from PBMCs isolated from patients with AMI (Fig. 4B). These findings indicate that one of the sources of sFasL could be circulating mononuclear cells.

Table 1. Patient Characteristics

<table>
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<th>Control Subjects (n = 30)</th>
<th>Patients With Stable AP (n = 10)</th>
<th>Patients With Unstable AP (n = 10)</th>
<th>Patients With AMI (n = 30)</th>
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<td>58 ± 10</td>
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Data are presented as the mean value ± SD or number of patients or control subjects.

AMI = acute myocardial infarction; AP = angina pectoris.
DISCUSSION

In the present study, we demonstrated for the first time that sFasL levels in the circulation were elevated at the early phase in patients with AMI and unstable AP, compared with control subjects.

Soluble FasL may serve as a local cytokine that induces cardiomyocyte apoptosis during AMI. The Fas ligand, a member of the growing TNF family, is synthesized as a type II membrane protein that induces apoptosis in Fas-expressing cells. The membrane-bound FasL (mFasL) can be cleaved by a metalloproteinase to become sFasL (17,25). Although both mFasL and secreted sFasL appear to be biologically active, the physiologic functions of sFasL have not been elucidated. Tanaka et al. (25) reported that sFasL inhibits Fas-mediated lymphocyte apoptosis. However, Brown and Savill (26) recently reported that phagocytosis triggers macrophage release of sFasL and induces apoptosis of bystander monocytes. Matute-Bello et al. (21) also reported that the bronchoalveolar lavage concentration of sFasL at the onset of acute respiratory distress syndrome was significantly higher in patients who died and that bronchoalveolar lavage from patients with acute respiratory distress syndrome induced apoptosis of distal lung epithelial cells. These observations suggest that high concentrations of sFasL may induce apoptosis in Fas-bearing cells in a paracrine pathway if these cells are sensitized to Fas-mediated apoptosis (27,28). Because hypoxia has been shown to upregulate Fas expression in cardiomyocytes and microglial cells (14,29), cardiomyocytes may be sensitized to Fas-mediated apoptosis during AMI. In addition, we have shown that sFasL levels in the coronary sinus were much higher than those in the circulation. Taken together, these findings suggest that high local concentrations of sFasL in the culprit lesion could facilitate cardiomyocyte apoptosis during AMI. However, there was no significant correlation between the plasma sFasL levels on admission and the maximal levels of CK-MB or hemodynamic variables, such as the cardiac index, in patients with AMI. Although the mechanism by which plasma sFasL levels are elevated in patients with AMI and unstable AP is not clear at present,

Figure 2. Correlation of plasma soluble Fas ligand (sFasL) levels with the maximal levels of creatine kinase-MB isoenzyme (CK-MB) (A) and with the cardiac index (B).

Figure 3. Time course of the changes in plasma soluble Fas ligand (sFasL) levels and the differences in sFasL levels between the coronary sinus and peripheral vein. For the time-course experiment, peripheral blood sampling was repeated at 3, 6, 12, 18 and 24 h after admission in five patients with acute myocardial infarction (AMI) (A). Blood samples were collected from the coronary sinus and the peripheral vein at the same time in the same patient with AMI (B). AP = angina pectoris; PTCA = percutaneous transluminal coronary angioplasty.
it should be noted that the levels of plasma sFasL changed rapidly after the onset of AMI (Fig. 3). Thus, it is possible that in some patients plasma sFasL concentrations may have already returned to the basal levels on admission, because the time from the onset of symptoms to admission ranged from 1 to 12 h. In addition, the levels of plasma sFasL could rapidly increase again in response to many forms of stress, including reperfusion (Fig. 3). Furthermore, the finding that sFasL levels were significantly higher in the coronary sinus than in the peripheral vein suggests that sFasL may serve mainly as a local cytokine in culprit lesions. Taken together, we assume that measuring the maximal levels of sFasL in the circulation, or the levels of sFasL in the coronary sinus, would be more appropriate ways of determining the correlation between the levels of sFasL and these clinical variables.

Soluble FasL may serve as a proinflammatory factor. It has been shown that FasL can induce biologic functions unrelated to cell death, such as the induction of proinflammatory effects (30,31). For example, Ottonello et al. (32) recently reported that sFasL, at concentrations incapable of inducing cell apoptosis, has potent chemotactic properties toward human neutrophilic polymorphonuclear leukocytes. Seino et al. (33) also reported that the chemotactic activity of sFasL against phagocytes is independent of the death domain-mediated apoptosis. In addition, Nelson et al. (16) reported that FasL expression in the heart is proinflammatory, but in contrast to other examples of FasL-mediated inflammation, the consequences appear to be mild, resulting in hypertrophy and interstitial changes rather than tissue destruction. Thus, it could be possible that sFasL may also serve as a proinflammatory factor in patients with AMI and unstable AP.

Mechanism for the elevation of plasma sFasL. The mechanism of an increase in the plasma levels of sFasL in patients with AMI and unstable AP is not clear at the present time. However, we demonstrated that FasL mRNA expression of the circulating mononuclear cells was upregulated in the early phase of AMI, and it was downregulated in the late phase, when plasma sFasL returned to the control levels. Thus, one of the sources of sFasL could be circulating mononuclear cells. Recent histologic studies have shown that inflammatory cells such as monocytes and macrophages are greatly accumulated in unstable plaque obtained from patients with AMI and unstable AP, compared with stable plaque (24). In the present study, the levels of sFasL in the coronary sinus were higher than those in the circulation. In addition, plasma sFasL levels increased again after PTCA in the patients with AMI, but not in the patients with stable AP. Furthermore, hypoxia stimulated the release of sFasL from mononuclear cells isolated from patients with AMI. Taken together, these observations suggest that mononuclear cells accumulated in unstable plaque may serve as a source of sFasL in patients with AMI. On the other hand, Wollert et al. (15) recently reported that FasL, as well as Fas, are constitutively expressed in the rat myocardium. Thus, hypoxia/reperfusion could stimulate the release of sFasL from the myocardium, as well as inflammatory cells. It has been proposed that expression of FasL by some tissues contributes to their immune privilege status by preventing the infiltration of inflammatory cells (13). Thus, FasL expression is downregulated by the conversion of mFasL to sFasL during hypoxia/reperfusion, it could promote the infiltration of inflammatory cells into the myocardium in patients with AMI and unstable AP. However, further studies are needed to clarify these points.

Conclusions. We have demonstrated that plasma sFasL levels are elevated in patients with AMI and unstable AP and that hypoxia stimulated the release of sFasL from mononuclear cells isolated from patients with AMI. These findings may provide further insights into the mechanism underlying the pathogenesis of AMI and acute coronary syndromes.
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