Unstable Angina

Pericardial Fluid from Patients With Unstable Angina Induces Vascular Endothelial Cell Apoptosis

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OBJECTIVES
The purpose of this study was to investigate whether pericardial fluid from patients with unstable angina (UA) would modulate vascular endothelial cell survival.

BACKGROUND
Apoptosis of vascular endothelial cells promotes the coagulation process, playing an important role in the formation of coronary arterial thrombi. However, little is known about the mechanisms of vascular endothelial cell death in acute coronary syndrome. We hypothesized that factors inducing apoptosis are produced by the ischemic heart and accumulated in high concentrations in pericardial fluid.

METHODS
Pericardial fluid was obtained during coronary artery bypass surgery from patients with UA (group A, \( n = 8 \)) and those with stable angina (group B, \( n = 23 \)). A survival assay of F2 cells from a mouse vascular endothelial cell line was performed in the presence of 10% pericardial fluid from each patient.

RESULTS
Pericardial fluid levels of vascular endothelial growth factor were significantly higher in group A than in group B, indicating that group A had more ischemic insults than group B. Pericardial fluid from group A, but not from group B, markedly induced F2 cell death (cell survival relative to fetal bovine serum; group A: 33 ± 26% vs. group B: 91 ± 22%, \( p < 0.01 \)). Cell death was associated with internucleosomal DNA fragmentation, a hallmark of apoptosis. Fractionation of pericardial fluid using a Centricon C-100 demonstrated that apoptosis-inducible activities exist in the Centricon C-100 retentates but not in the filtrates.

CONCLUSIONS
Factors that induce vascular endothelial cell apoptosis are secreted into the pericardial space from the hearts of patients with UA. These factors are large complexes or unknown new proteins larger than 100 kDa. (J Am Coll Cardiol 2000;35:1785–90) © 2000 by the American College of Cardiology

Fissuring or rupture of atherosclerotic plaque and subsequent formation of mural or occlusive thrombi in coronary arteries are fundamental to the development of the acute coronary syndrome (ACS), as has been shown in pathological studies on patients who died suddenly or shortly after an episode of UA or acute myocardial infarction (1–3). Detection of endothelial cells in blood from patients with acute coronary heart disease suggests substantial endothelial cell death in these patients (4). Because endothelial cells play a pivotal role in preventing the initiation and propagation of the coagulation process, death of these cells triggers coagulation by exposure of the subendothelial matrix or tissue factor expressed on extravascular cells. In addition, Bombeli et al. (5) demonstrated that apoptotic vascular endothelial cells themselves become procoagulant. Thus, it is likely that endothelial cell death plays an important causal role in the formation of coronary arterial thrombi in patients with ACS.

Apoptosis is an active process of cell death, morphologically and biochemically different from necrosis, and it occurs under physiological and pathological conditions. In addition to vasculogenesis, apoptosis of endothelial cells is considered to contribute to vascular pathology. Endothelial cell apoptosis in culture is caused by a number of stimuli, including tumor necrosis factor-alpha (6), lipopolysaccharides combined with heat shock (7) or ionizing radiation (8), high glucose concentration (9) and low concentrations of hydrogen peroxide (10). However, it is unknown whether endothelial cell death in patients with ACS is attributable to apoptosis.
Substances produced by the heart are secreted into the pericardial space and also into the systemic circulation. These substances are accumulated in the pericardial fluid and present at high concentrations. For example, in patients with heart failure, levels of B-type natriuretic peptide are much higher in the pericardial fluid than in plasma (11). These findings prompted us to investigate whether factors inducing endothelial cell death may be produced by the heart and accumulated at high concentrations in pericardial fluid. Thus, we investigated whether pericardial fluid obtained during coronary artery bypass surgery would affect endothelial cell survival.

**METHODS**

**Patient profile.** All of the 31 patients in this study underwent coronary artery bypass surgery because of double-vessel (n = 8), triple-vessel (n = 12) or left main trunk disease (n = 11). The mean age of the patients (21 men and 10 women) was 68 ± 10 years. The patients were divided into two groups. Group A consisted of eight patients with UA. Group B consisted of 23 patients with stable angina. Of eight group A patients, six had class III UA according to Braunwald’s classification (12), and the remaining two had class II UA. All patients gave their written, informed consent. The study protocol was approved by the ethical committee on human research at Kyoto University.

**Sampling of pericardial fluid.** Immediately after incision of the pericardium, undiluted pericardial fluid was obtained and heparinized. The amount of non-hemorrhagic pericardial fluid obtained was 3 ± 2 ml in group A and 4 ± 2 ml in group B. The samples were collected in sterile tubes and immediately placed on ice. After clarification of cellular components by centrifugation at 3,000 g for 10 min at 4°C, these samples were rapidly frozen in liquid nitrogen and stored at −80°C until use.

**Measurement of vascular endothelial growth factor (VEGF).** Concentrations of VEGF in pericardial fluid were measured by an enzyme-linked immunosorbent assay as described previously (13). In brief, this assay uses a sandwich method of detection comprising a plate precoated with a monoclonal antibody specific for VEGF. After addition of the sample, an enzyme-linked polyclonal antibody was added. Color development was achieved by the addition of a substrate solution; the degree of color development correlated with the amount of VEGF. The limit of VEGF detection in this assay was 1 pg/ml. The difference in values between the two measurements was within 10%.

**Cell culture.** The mouse endothelial cell line (F2 cells) was obtained from a murine vascular endothelial cell line (14) and kept in media consisting of Dulbecco’s modified Eagle’s medium plus 19.6 mM NaHCO₃ (pH 7.1 to pH 7.2), penicillin, streptomycin, and 10% (vol/vol) fetal bovine serum (FBS) (all from GIBCO BRL, Gaithersburg, Maryland) at 37°C, 5% CO₂.

**Cell survival assay.** F2 cells were plated in triplicate in a 96-well microplate with 5 × 10³ cells/well with the plating medium and maintained at 5% CO₂ and 37°C for 24 h. The cells were then washed twice with the above medium containing 0.1% serum, followed by the addition of 10% pericardial fluid from each patient to the well. Forty-eight hours later, the cells were stained with 3-(4,5-dimethyl thiaziazol-2-yl)-2,5-diphenyl tetrazolium bromide (Sigma, St. Louis, Missouri), and viable cells were counted by measuring the optic density. The optic density of the well incubated with 10% fetal bovine serum was set to 100% in each experiment. In all experiments, we used identical lots of FBS. All of the experiments were performed in triplicate for each patient sample and repeated at least three times.

**DNA fragmentation on agarose gels.** F2 cells were plated on 10 cm plates with 5 × 10⁵ cells/plate and maintained at 5% CO₂ and 37°C for 24 h. The cells were then washed twice with the above medium containing 0.1% serum, followed by the addition of 10% pericardial fluid from each patient to the well. Forty-eight hours later, the cells were harvested by scraping them into the media. After centrifugation at 500 g for 5 min at 4°C, the cells were lysed in a lysis buffer and subjected to a nucleosomal ladder assay using a commercial kit (Takara Biomedicals, Kyoto, Japan) according to the manufacturer’s recommendations. The presence of characteristic 180- to 200-bp multiple oligonucleosomal fragmentation was examined on 2% agarose gels stained with SYBR™Green I (Takara Biomedicals).

**Protein fractionation of pericardial fluid with Centricon C-100.** To prepare the Centricon C-100 retentates and filtrates, 1 ml of pericardial fluid was centrifuged in a Centricon C-100 concentrator at 500 g for 60 min. The retentate was washed with N-2-hydroxyethylpiperazine-N’-2-ethanesulfonic acid buffer plus 100 mM KCl and centrifuged again. Then, the retentate was recovered by reverse spinning the concentrator for 3 min at 500 g. The first filtrate and the final retentate were used for the F2 cell survival assay.

**Statistical analysis.** All values were expressed as mean ± SD. The differences between the groups were analyzed with the Mann-Whitney U test. Proportional data concerning the ladder formation of DNA were analyzed using the chi-square test with Yates’ correction. A p value <0.05 was taken to indicate significance.
RESULTS

Patient profile. Table 1 shows the clinical characteristics of group A and B patients. There were no significant differences in age, gender distribution, extent of coronary artery stenosis and risk factors between group A and B patients. Six patients in group A and 16 in group B had old myocardial infarction, but none had acute or recent myocardial infarction. Echocardiographic data demonstrated that there were no differences in the left ventricular end-diastolic and end-systolic diameters and ejection fraction between these two groups. There were no differences between these two groups in pre-operative medications such as calcium antagonists, angiotensin-converting enzyme inhibitors, beta-blockers and intravenous heparin. However, group A patients received intravenous heparin more frequently than group B patients.

Pericardial fluid level of VEGF. We measured pericardial fluid levels of VEGF using an enzyme-linked immunosorbent assay. In a previous report, we demonstrated that pericardial fluid levels of VEGF increased only in patients with severe rest angina within two days before emergent coronary artery graft surgery, not in those with stable angina (13). These findings suggest that the increase in pericardial fluid levels of VEGF serves as a marker of recent ischemic insults. Pericardial fluid levels of VEGF were significantly higher in group A than in group B, consistent with the former having had more recent ischemic insults than the latter.

Effects of pericardial fluid on endothelial cell survival and apoptosis. To investigate whether pericardial fluid from patients with UA affects the survival of endothelial cells, we stimulated F2 cells with 10% pericardial fluid from each patient. Forty-eight hours later, we counted the viable cell number by measuring the optic density in the well. As shown in Figure 1, the cell viability in group A (UA) relative to 10% FBS was 33 ± 26%, which was much lower (p < 0.001) than that in group B (stable angina, 91 ± 22%). Incubation of endothelial cells in serum-free media resulted in mild loss of these cells (the cell viability was 87% relative to 10% FBS control). To investigate whether the decrease in viable F2 cells after stimulation with pericardial fluid from group A is attributable to apoptosis of these cells, we evaluated F2 cells for the presence of internucleosomal cleavage by monitoring for DNA laddering, a hallmark of apoptosis. Figure 2 illustrates representative DNA fragments equivalent in size to the mononucleosomes and oligonucleosomes in F2 cells 48 h after incubation with pericardial fluid from UA (left lane) but not in cells incubated with pericardial fluid from stable angina (right lane). The DNA fragmentation of F2 cells was detected in

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<tr>
<th>Table 1. Comparison of Patients’ Characteristics Between Group A (UA) and Group B (Stable Angina)</th>
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<tr>
<td><strong>Group A</strong></td>
</tr>
<tr>
<td><strong>(n = 8)</strong></td>
</tr>
<tr>
<td><strong>Age (yr)</strong></td>
</tr>
<tr>
<td>Male</td>
</tr>
<tr>
<td>History of myocardial infarction</td>
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<tr>
<td>Left main trunk disease</td>
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<td>3-vessel disease</td>
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<td>Hypertension</td>
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<td>Diabetes mellitus</td>
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<tr>
<td>Cigarette smoking</td>
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<tr>
<td>Serum total cholesterol (mg/dl)</td>
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<tr>
<td>LVDd (mm)</td>
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<tr>
<td>LVSD (mm)</td>
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<tr>
<td>LVEF (%)</td>
</tr>
<tr>
<td>VEGF in pericardial fluid (pg/ml)</td>
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<tr>
<td>TNF-alpha in pericardial fluid (pg/ml)</td>
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<tr>
<td>Intravenous heparin</td>
</tr>
<tr>
<td>Ca antagonists</td>
</tr>
<tr>
<td>ACE inhibitors</td>
</tr>
<tr>
<td>Beta-blockers</td>
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<td>Nitrates</td>
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<td>Anti-platelet agents</td>
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Data are presented as number (%) of patients or mean value ± SD. ACE = angiotensin converting enzyme; LVDd = left ventricular diastolic dimension; LVSD = left ventricular systolic dimension; TNF = tumor necrosis factor; VEGF = vascular endothelial growth factor.
7 of 8 group A patients but in only 1 of 23 group B patients (p < 0.01). The cells stimulated with pericardial fluid from patients with UA displayed the small condensed nuclei, cell shrinkage and nuclear fragmentation that are consistent with the morphologic features of apoptosis (Fig. 3B). Thus, F2 cell death caused by pericardial fluid was associated with the presence of DNA fragmentation and the morphologic features of apoptosis, indicating that pericardial fluid from group A patients induced endothelial cell death by apoptosis.

Characterization of apoptosis-inducible factors. We began to characterize apoptosis-inducible factor(s) in pericardial fluid from patients with UA. One of the most important factors involved in apoptosis in many cell types is tumor necrosis factor-alpha. Therefore, we measured pericardial fluid levels of tumor necrosis factor-alpha by an enzyme-linked immunosorbent assay. However, these levels were less than the level detectable by this assay and did not differ between group A and group B patients (Table 1). To examine the role of the Fas–Fas ligand system in endothelial cell apoptosis by pericardial fluid, we administered a neutralizing antibody against Fas ligand. However, as shown in Figure 4, it failed to inhibit the cell death–inducing activities of pericardial fluid from group A patients. Before the addition to the well, pericardial fluid from UA was heated to 55°C for 30 min or freeze and thawed twice. Each procedure eliminated the cell death–inducing activity of the pericardial fluid, suggesting the lability of the factor(s). To investigate whether apoptosis-inducible factor(s) in pericardial fluid exist in fractions of molecular weight greater or less than 100 kDa, pericardial fluid from group A and group B patients was fractioned with the Centricon C-100, and each retentate and filtrate was subjected to the F2 cell survival assay. As shown in Figure 5A, the Centricon C-100 retentates contain substances with molecular weights more than 100 kDa. However, such high molecular substances do

Figure 1. Pericardial fluid from patients with UA induces loss of F2 cells. F2 cells were plated in 96 well plates and maintained for 48 h in the presence of 10% pericardial fluid obtained during coronary artery bypass surgery. Cell viability was assayed using 3-(4,5-dimethyl thiaziazol-2-yl)-2,5-diphenyl tetrazolium bro-mide as described under “Methods.” In each experiment, the viability of the well incubated with 10% FBS was set to 100%. All of the experiments were performed in triplicate for each patient sample and repeated at least three times. Results are the mean ± SD of each patient group.

Figure 2. Fragmentation of genomic DNA in F2 cells incubated with pericardial fluid from a patient with UA. Genomic DNA was isolated from F2 cells maintained for 48 h in the presence of 10% pericardial fluid from a patient with UA (left lane) or stable angina (right lane) and loaded on a 2% agarose gel. The ladder assays were performed in all patients, and the data presented are representative.

Figure 3. Induction of apoptosis in F2 cells by pericardial fluid from a patient with UA. A representative photograph of F2 cells incubated with pericardial fluid from patients with stable angina (A) and UA (B). These cells were stained with hematoxylin. Arrow heads show cells with evidence of apoptosis, including chromatin condensation.
not exist in the filtrate. As shown in Figure 3, when the Centricon C-100 filtrates were added to the well, the percentage of optic density did not differ between the two groups (78 ± 12% in group A vs. 81 ± 16% in group B, NS). By contrast, with the Centricon C-100 retentates, the percentage of optic density in group A (19 ± 27%) was much lower (p < 0.001) than that in group B (86 ± 25%). These findings suggest that apoptosis-inducible factor(s) in pericardial fluid from patients with UA reside(s) in the Centricon C-100 retentates.

DISCUSSION

**Vascular endothelial cell death in UA.** Unstable angina is a disease status of high risk for subsequent coronary arterial thrombosis. Much evidence suggests that endothelial cell death is involved in the pathophysiology of ACS (1–5). Because vascular endothelial cells provide potent anti-coagulant properties, death of these cells promotes the coagulation process, leading to coronary arterial thrombosis. Despite the pathophysiological importance of endothelial cell death, little is known about its regulators in ACS. This study demonstrates that pericardial fluid from patients with UA, but not from those with stable angina, induced endothelial cell death. This was associated with internucleosomal DNA fragmentation, suggesting that cell death is mainly attributable to apoptosis. Considering the fact that the occurrence of apoptosis is determined by the balance between inducers and inhibitors of apoptosis, we cannot rule out the possibility that the pericardial fluid from UA patients lacks factors indispensable for cell survival. Even if this is the case, a marked decrease in F2 cell survival in this fluid suggests that it contains potent apoptosis-inducible factor(s).

**Myocardial ischemia and VEGF.** Ischemic myocardium produces protective factors against apoptosis (13,15). One of them is VEGF (16). We previously reported that severe myocardial ischemia causes release of this factor into the pericardial space (13). Compatible with that report, this study demonstrates that pericardial fluid levels of VEGF are elevated in patients with UA compared with those with stable angina. Despite the high levels of this protective factor in pericardial fluid from UA patients, the fluid induced apoptosis of endothelial cells. It should be further clarified whether endothelial cell apoptosis occurs via distinct pathways or the levels of VEGF are insufficient to
produce its protective effects. Nevertheless, our data clearly indicate that the hearts of group A patients had been subjected to more severe ischemia than those of group B.

**Apoptosis in vascular endothelial cells.** A number of factors can induce apoptosis in endothelial cells. One important factor involved in the apoptosis of many cell types is tumor necrosis factor-alpha. However, pericardial fluid levels of tumor necrosis factor-alpha were almost undetectable using an enzyme-linked immunosorbent assay. Other factors that have been reported to induce endothelial cell apoptosis include oxidized low density lipoproteins (17,18), sphingolipids (19,20) and proinflammatory cytokines other than tumor necrosis factor-alpha, such as interleukin-1beta. We demonstrated here that apoptosis-inducible activities exist in the Centricon C-100 retentates but not in the filtrates. These findings suggest that apoptosis-inducible factors are greater than 100 kDa in molecular weight, or that factors <100 kDa form complexes that are retained in the Centricon C-100. Because most factors reported to induce endothelial cell apoptosis are <100 kDa, it is probable that large complexes consist of single or multiple substances or that unknown new proteins >100 kDa are responsible for the apoptosis-inducible activities in pericardial fluid.

**Study limitations.** We should also consider the possibility that pre-existing molecules in pericardial fluid are modified by ischemic insults to gain apoptosis-inducible properties. Patients with UA received intravenous heparin more frequently than those with stable angina. However, the apoptosis-inducible factors contained in pericardial fluid from these patients are high molecular substances. Although we cannot totally rule out the possibility that heparin modulated the death-inducible activity of other substances, heparin can provide the growth of endothelial cells. Therefore, it is highly unlikely that heparin is responsible for the endothelial cell apoptosis observed in this study. Although the heart is a main source of pericardial fluid, cardiac tissue consists of a number of cell types. Therefore, for the identification of apoptosis-inducible factors produced by the heart, it should be clarified which cell type is responsible for their production. In any event, identifying factors that induce endothelial cell apoptosis in patients with UA will provide novel insights into the mechanisms that mediate plaque rupture of atherosclerotic coronary arteries.

**REFERENCES**


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