Expression of Heat Shock Protein 27 in Human Atherosclerotic Plaques and Increased Plasma Level of Heat Shock Protein 27 in Patients With Acute Coronary Syndrome

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Background—We intended to identify proteins that are differentially expressed in human atherosclerotic plaques.

Methods and Results—Comparative 2-dimensional electrophoretic analysis on carotid atherosclerotic endarterectomy specimens (n=10) revealed that heat shock protein 27 (Hsp27) expression was significantly increased in the nearby normal-appearing area compared with the plaque core area from the same vessel specimen, which was further confirmed by Western blot analysis. The Hsp27 expression in the adjacent normal-appearing vessel areas was much higher than that in nonatherosclerotic reference arteries. The phosphorylation of Hsp27 showed a gradation in the degree of phosphorylation: greatest in the reference arteries, intermediate in the adjacent normal-appearing area, and lowest in plaque core area. Immunohistochemical analysis showed that the phosphorylation of Hsp27 of smooth muscle cells in the carotid endarterectomy specimens was decreased compared with that in the reference artery specimen. The mean plasma level of Hsp27 was significantly higher in patients with acute coronary syndrome (ACS) (n=27; 106.1±74.1 ng/mL) than in the normal reference subjects (n=29; 45.8±29.5 ng/mL; P<0.005). The plasma levels of Hsp27 were significantly correlated with those of heat shock protein 70 (Hsp70) (r=0.422, P<0.0005), with adjustment for ACS/reference status.

Conclusions—In the atherosclerotic lesion, Hsp27 expression is increased in the normal-appearing vessel adjacent to atherosclerotic plaque, whereas levels in the plaque itself are significantly decreased. Both plaque and adjacent artery show decreased Hsp27 phosphorylation compared with reference vessel. In ACS, plasma Hsp27 and Hsp70 are increased, and levels of Hsp27 correlate with Hsp70, C-reactive protein, and CD40L levels. (Circulation. 2006;114:886-893.)

Key Words biological marker ▪ coronary disease ▪ heat shock proteins ▪ plaque ▪ proteomics

Heat shock proteins work as “chaperones” to affect protein folding of newly synthesized or denatured proteins.1 Heat shock proteins can be expressed during ischemic heart disease as the result of such stress as hypoxia, reperfusion, and oxidative stress, and they can confer antiapoptotic effects on myocytes after ischemia/reperfusion injury.2–4 Several heat shock proteins, including Hsp60/65 and Hsp70/72, have been implicated in atherosclerosis.5 These heat shock proteins were identified by performing immunohistochemistry on atherosclerotic plaques,6–8 and their serum levels have been significantly associated with the risk of coronary artery disease.9,10
carotid endarterectomy, we found a differential expression of heat shock protein 27 (Hsp27). Because there has not been a thorough examination of Hsp27 in atherosclerotic lesions or in the plasma of patients with coronary artery disease, we identified its localization in the atherosclerotic plaques and assessed the level of its soluble form in the plasma of patients with acute coronary syndrome (ACS).

Methods

Carotid Endarterectomy Specimen and Normal Vessel Specimen

Carotid endarterectomy specimens were obtained from 10 patients with carotid artery stenosis (age, 63 to 81 years) who underwent surgery at the Samsung Medical Center. The samples were taken from 2 different areas of each plaque: the core area and the adjacent normal-appearing area in the same vessel specimen. The tissue samples were washed with saline and were then frozen until analysis. The other 5 carotid endarterectomy specimens taken from other patients with carotid artery stenosis were embedded in optimal cutting temperature compound (OCT; Miles Laboratories) to make the frozen sections. Two renal artery specimens and 2 internal mammary artery specimens were also obtained from kidney transplantation donors and coronary artery bypass graft patients, respectively, and were used as normal reference vessels for Western blot analysis. This study was approved by the institutional review committee at our hospital, and informed consent was obtained from all the subjects.

Two-Dimensional Electrophoresis and Matrix-Assisted Laser Desorption-Ionization Time-of-Flight Mass Spectrometry

The specimens were suspended in sample buffer containing 7 mol/L urea, 2 mol/L thiourea, 4% (wt/vol) CHAPS, 40 mmol/L Tris, 0.1 mol/L diethiothreitol, and protease inhibitor cocktail (Complete; Roche, Mannheim, Germany). The suspensions were sonicated for 30 seconds and then centrifuged at 100 000 g for 45 minutes. Two-dimensional electrophoresis was performed as previously described.19 One milligram of the total protein was used for each electrophoresis. Aliquots of the specimen proteins in sample buffer were applied to immobilized nonlinear gradient strips (pH 3 to 10) (Amersham Pharmacia Biotech, Uppsala, Sweden), and the isoelectric focusing was performed for 90 000 volt-hours. The second dimension was resolved on 9% to 16% linear gradient SDS polyacrylamide gels (200×250×1.0 mm) at 15 mA per gel constant current for ~12 hours until the dye front reached the bottom of the gel. After this, the proteins were fixed in 40% methanol and 5% phosphoric acid for 12 hours, and the gels were then stained with Coomassie blue G250 for 24 hours. Gels were destained with H2O and scanned with a BioRad GS-800 densitometer; the data were next converted into electronic files, which were then analyzed with Melanie III computer software (GenBio, Geneva, Switzerland).

For mass spectrometry fingerprinting, the protein spots were cut from the gels, destained, and treated with trypsin as previously described.20 Aliquots of the peptide mixtures from the trypsin treatments were applied to a target disk and allowed to air dry. The spectra were obtained with the use of a Voyager-DE STR matrix-assisted laser desorption-ionization time-of-flight mass spectrometry (MALDI-TOF-MS) (PerSeptive Biosystems, Framingham, Mass.). The protein database searches were performed with ProFound (http://129.85.19.192/profound_bin/webprofound.exe) with the use of monoisotopic peaks. Mass tolerance was first allowed within 50 ppm, and then recalibration was performed with the protein lists obtained at 20 ppm.

Western Blot and Immunohistochemical Analysis

Specimen proteins were transferred onto polyvinylidene difluoride membranes, and the blots were then probed with specific antibodies and finally developed with the use of enhanced chemiluminescence reagents (ECL, Amersham, Del). For the immunohistochemical analysis, the specimens embedded in OCT were sectioned at 5-μm thickness and then stained with the use of the LSAB kit (DAKO) according to the manufacturer’s specifications. Monoclonal anti-Hsp27 and anti–phospho-Hsp27 (Ser82) antibodies were purchased from Cell Signaling Technology, and monoclonal antibodies to CD68 and smooth muscle cell (SMC) α-actin were obtained from DAKO. Monoclonal antibodies against the inducible forms of Hsp70 and Hsp60 were purchased from StressGen Biotechnologies. Other antibodies used in this study were purchased from Santa Cruz Biotechnology, Inc.

Blood Sampling

For the ACS group, 27 patients (age, 30 to 80 years) with acute myocardial infarction or unstable angina, who came to the emergency department and were then admitted to the coronary care unit of Samsung Medical Center, were entered into the study (male:female ratio, 18:9; age, 58.9±9.3 years). The blood samplings were done within 24 hours from presentation to the emergency department and before their coronary angiography. All patients in this study had their coronary stenosis proven by angiography. For the normal reference group, 29 sex- and age-matched healthy volunteers were recruited (male:female ratio, 19:10; age, 56.1±6.3 years). For the chronic stable angina (CSA) group, the patients with coronary artery disease who had been treated with antiangiinal medications, including statins, for >1 year, who had their risk factors adequately modified, and who had also been free of chest pain for >1 year were included in the study (17 male patients: age, 61.4±7.3 years). None of the reference subjects had any of the major risk factors for cardiovascular disease, and none were taking any medication. The risk group subjects who had ≥1 of the risk factors for coronary artery disease but who had no overt coronary artery disease (n=31; male:female ratio, 20:11; age, 59.2±6.5 years) were also enrolled in the study. We obtained informed consent from all the subjects before any blood work was sampled.

Enzyme-Linked Immunosorbent Assay

Plasma samples obtained from all study subjects were aliquoted and stored at −80°C until analysis. The plasma levels of Hsp27 were measured with the use of Oncogene Hsp27 enzyme-linked immunosorbent assay (ELISA) kits (catalog No. QIA119) according to the manufacturer’s directions. Briefly, the plasma was diluted 1:10 in assay buffer and then incubated for 1 hour at room temperature in ELISA wells that were precoated with anti-Hsp27 monoclonal antibody. The plates were washed and then were incubated with rabbit anti-human Hsp27 polyclonal IgG for an additional hour at room temperature. After washing was performed, peroxidase-conjugated goat anti-rabbit IgG was added to each well and then incubated overnight at 4°C. The enzymatic activity was assessed by the addition of 100 μL tetramethylbenzidine substrate solution to each well. After the reaction had been stopped by the addition of sulfuric acid, the absorbance was measured at 405 nm with a spectrophotometer. Each assay included positive controls with a known amount of human recombinant Hsp27 and a negative control (no human recombinant Hsp27), which were run in parallel. The plasma levels of adiponectin and CD40L were also determined by ELISA kits from R&D Systems, and ELISA kits from StressGen (StressXpress Hsp70 ELISA Kit) were used to measure the Hsp70 plasma levels.

Plasma α-Tocopherol Measurement

The plasma α-tocopherol concentration was measured by high-performance liquid chromatography (HPLC) with the use of a reverse-phase column (Novapak C18, 8×100-mm Radial-Pak Cartridge, Waters), as described previously.21 We used the Shimadzu HPLC system equipped with an autoinjector and a fluorescence detector at an excitation wavelength of 292 nm and an emission wavelength of 324 nm. α-Tocopherol acetate (Sigma-Aldrich Co, St Louis, Mo) was used as the internal standard.

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Louis, Mo) was used as an internal standard, and α-tocopherol was used as an external standard.

**Statistical Analysis**

Comparisons of continuous variables between ≥3 groups were performed by Kruskal-Wallis test or ANOVA test according to the normality of data distribution. The difference of continuous variables between 2 groups was identified with the Mann-Whitney test because of their non-normal distribution. Discrete variables were expressed as percentages and compared by Fisher exact test. Spearman correlation analysis and Pearson correlation analysis were used to analyze the interrelationship between variables. Probability values <0.05 were considered significant for all tests. All statistical analyses were performed with SPSS software (version 10.0).

The authors had full access to the data and take full responsibility for its integrity. All authors have read and agree to the manuscript as written.

**Results**

**Expression of Hsp27 in Human Carotid Atherosclerotic Plaques**

Proteome analysis was performed with the use of high-resolution 2D electrophoresis with human carotid endarterectomy specimens that were sampled from 2 regions: the atherosclerotic plaque core area and paired adjacent normal-appearing vessel area. More than 2000 protein spots were detected with SYPRO Ruby staining of proteins from the endarterectomy specimens; the representative diagrams for 2D electrophoresis maps of the 2 regions are shown in Figure 1. By computer-assisted comparative analysis of the respective spot patterns of the paired samples, we were able to detect ≥200 protein spots that showed a different expression. Each of the 21 spots that were upregulated or downregulated in the plaque area was selected, and they were subjected to protein identification by MALDI-TOF-MS analysis after trypsin digestion. We identified 17 plaque-associated proteins (Table 1), of which 4 proteins, including proapolipoprotein, ferritin light chain protein, and fibrinogen β-chain protein, were upregulated and 13 proteins, including vimentin, Hsp27, actin-binding protein (SM22-α), and β-tropomyosin, were downregulated at the atherosclerotic plaque core areas.

We were interested in the pattern of the decreased Hsp27 expression (spots indicated by arrows on representative 2D electrophoresis maps shown in Figure 1A and 1B). After separation of protein extracts by 2D electrophoresis, Western blot analysis revealed some additional anti-Hsp27 antibody–reactive spots other than the spot that had been identified previously as Hsp27 during MALDI-TOF-MS analysis (Figure 1C and 1D). Another MALDI-TOF-MS analysis on the spots responsive to anti-Hsp27 antibody in Figure 1C and 1D revealed that those spots were well matched with the peptide mapping for human Hsp27 (Figure 2) and that the spots numbered 1, 2, 5, and 8 in Figure 2 involved phosphorylations at the 15th and/or 82nd serine residue (data not shown).

In Figure 3, Western blot analysis showed that Hsp27 expression was significantly increased in the nearby normal-appearing area compared with the plaque core area from the same vessel specimen. Hsp27 expression in the adjacent normal-appearing vessel areas was much higher than those in nonatherosclerotic reference arteries from donor renal artery and internal mammary arteries. In the nearby normal-appearing areas, contrary to the higher Hsp27 expression, the phosphorylation of Hsp27 was decreased, which makes the ratios of phospho-Hsp27 to total Hsp27 expression much lower than those in reference artery specimens. The phospho-Hsp27 was hardly detectable at the atherosclerotic plaque core areas. On the other hand, the expression of Hsp70 was higher in both the plaque core areas and adjacent normal-appearing areas of carotid endarterectomy specimens compared with those in the reference artery specimens. There

![Figure 1. Differential Hsp27 expression in carotid endarterectomy specimens.](http://circ.ahajournals.org/)

Representative 2D electrophoresis maps of the proteome from a nearby normal-appearing area (A) and an atherosclerotic core region (B) and corresponding Western blot analysis (C and D) show the additional anti-Hsp27 antibody–reactive spots. The arrowhead indicates the spot identified as Hsp27 by comparative proteome analysis.
were no differences in the levels of \( \beta \)-actin among the specimen samples in this study.

Although immunohistochemical analysis revealed that the expression of Hsp27 overlapped with the SMC marker (\( \alpha \)-SM actin; data not shown) in the reference renal artery and carotid endarterectomy specimen (Figure 4), the phosphorylation of Hsp27 (phospho-Ser\(^{82} \) Hsp27) in those cells was detected to a much lower degree in the atherosclerotic plaque specimen than in the reference artery specimen (Figure 4F versus 4C).

### TABLE 1. List of Proteins the Expression of Which Was Lower (Negative Fold Change) or Higher (Positive Fold Change) in the Atherosclerotic Core Area Than in the Nearby Normal-Appearing Area in the Same Carotid Endarterectomy Specimen

<table>
<thead>
<tr>
<th>Spot</th>
<th>Protein Identity</th>
<th>Accession No.</th>
<th>Fold Change in Intensity</th>
<th>( P )</th>
<th>Sequence Coverage, %</th>
<th>PT</th>
<th>Mass, kDa</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Heat shock 27-kDa protein 1</td>
<td>4504517</td>
<td>((-2.8 \pm 0.34))</td>
<td>0.016</td>
<td>38</td>
<td>6.0</td>
<td>22.8</td>
</tr>
<tr>
<td>2</td>
<td>EGF-containing fibulin-like extracellular matrix protein 1 precursor</td>
<td>9665262</td>
<td>((-1.7 \pm 0.22))</td>
<td>0.025</td>
<td>20</td>
<td>5.3</td>
<td>45.5</td>
</tr>
<tr>
<td>3</td>
<td>Human thioredoxin peroxidase 1</td>
<td>2507169</td>
<td>((-2.4 \pm 0.53))</td>
<td>0.025</td>
<td>26</td>
<td>5.7</td>
<td>22.1</td>
</tr>
<tr>
<td>4</td>
<td>( \beta )-Tropomyosin</td>
<td>6573280</td>
<td>((-1.8 \pm 0.33))</td>
<td>0.006</td>
<td>33</td>
<td>4.7</td>
<td>29.9</td>
</tr>
<tr>
<td>5</td>
<td>Heat-shock protein 20-kDa-like protein P20</td>
<td>6166215</td>
<td>((-1.6 \pm 0.12))</td>
<td>0.016</td>
<td>31</td>
<td>6.0</td>
<td>16.9</td>
</tr>
<tr>
<td>6</td>
<td>Actin-binding protein; SM22-( \alpha )†</td>
<td>4507359</td>
<td>((-3.4 \pm 0.58))</td>
<td>0.016</td>
<td>40</td>
<td>8.6</td>
<td>22.5</td>
</tr>
<tr>
<td>7</td>
<td>Transgelin; SM22-( \alpha )†</td>
<td>4507359</td>
<td>((-3.6 \pm 0.31))</td>
<td>0.025</td>
<td>40</td>
<td>8.6</td>
<td>22.5</td>
</tr>
<tr>
<td>8</td>
<td>Transgelin 2</td>
<td>12803567</td>
<td>((-2.4 \pm 0.43))</td>
<td>0.025</td>
<td>35</td>
<td>8.6</td>
<td>22.6</td>
</tr>
<tr>
<td>9</td>
<td>Vimentin</td>
<td>4507895</td>
<td>((-3.5 \pm 0.17))</td>
<td>0.006</td>
<td>24</td>
<td>5.1</td>
<td>53.7</td>
</tr>
<tr>
<td>10</td>
<td>CTCL tumor antigen se20-7</td>
<td>11385652</td>
<td>((-4.2 \pm 1.12))</td>
<td>0.016</td>
<td>63</td>
<td>5.6</td>
<td>73.9</td>
</tr>
<tr>
<td>11</td>
<td>S100 calcium-binding protein A4</td>
<td>4506765</td>
<td>((-2.9 \pm 0.43))</td>
<td>0.025</td>
<td>41</td>
<td>5.9</td>
<td>11.9</td>
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<tr>
<td>12</td>
<td>Hypothetical protein XP_005833</td>
<td>14742615</td>
<td>((-1.7 \pm 0.44))</td>
<td>0.004</td>
<td>55</td>
<td>4.7</td>
<td>33.2</td>
</tr>
<tr>
<td>13</td>
<td>Hypothetical protein XP_032790†</td>
<td>14785937</td>
<td>((-3.3 \pm 0.18))</td>
<td>0.025</td>
<td>22</td>
<td>6.0</td>
<td>75.7</td>
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<td>Hypothetical protein XP_032790†</td>
<td>14785937</td>
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<td>0.025</td>
<td>22</td>
<td>6.0</td>
<td>75.7</td>
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<tr>
<td>15</td>
<td>Hypothetical protein XP_006432</td>
<td>11440003</td>
<td>((-2.4 \pm 0.47))</td>
<td>0.016</td>
<td>55</td>
<td>8.9</td>
<td>22.6</td>
</tr>
<tr>
<td>16</td>
<td>Human fibrinogen ( \beta )-chain precursor</td>
<td>399492</td>
<td>((+3.1 \pm 0.44))</td>
<td>0.006</td>
<td>27</td>
<td>9.0</td>
<td>56.7</td>
</tr>
<tr>
<td>17</td>
<td>Ferritin, light chain; ferritin light polypeptide-like 3†</td>
<td>4503797</td>
<td>((+2.8 \pm 0.27))</td>
<td>0.016</td>
<td>31</td>
<td>5.5</td>
<td>20.1</td>
</tr>
<tr>
<td>18</td>
<td>Ferritin, light chain; ferritin light polypeptide-like 3†</td>
<td>4503797</td>
<td>((+2.9 \pm 0.46))</td>
<td>0.006</td>
<td>42</td>
<td>5.5</td>
<td>20.1</td>
</tr>
<tr>
<td>19</td>
<td>Proapolipoprotein†</td>
<td>178775</td>
<td>((+1.5 \pm 0.16))</td>
<td>0.025</td>
<td>58</td>
<td>5.4</td>
<td>28.9</td>
</tr>
<tr>
<td>20</td>
<td>Proapolipoprotein†</td>
<td>178775</td>
<td>((+1.8 \pm 0.21))</td>
<td>0.016</td>
<td>43</td>
<td>5.4</td>
<td>28.9</td>
</tr>
<tr>
<td>21</td>
<td>Hypothetical protein XP_036825</td>
<td>16165168</td>
<td>((+2.6 \pm 0.52))</td>
<td>0.004</td>
<td>43</td>
<td>4.9</td>
<td>21.4</td>
</tr>
</tbody>
</table>

EGF indicates epidermal growth factor.

*Statistical significance was evaluated by the Mann-Whitney test.

†Both members in the pairs of same protein identity with different spot number were identified as the same protein after MALDI-TOF-MS analysis.

**Figure 2.** Identification of proteins reactive with antibody against Hsp27. High-magnification view of the 2D electrophoresis images shows the squared areas of the nearby normal-appearing region in Figure 1A (A) and atherosclerotic core region in Figure 1B (B). The spot numbers and arrows indicate the proteins shown in Figure 1C and 1D that were reactive with anti-Hsp27 antibody. Circles indicate the spots that were first identified. Spots 1 to 9 were all identified as Hsp27 by peptide mapping with the use of MALDI-TOF-MS followed by database searching. The human Hsp27 sequence is presented in C with the matched peptide sequences in bold. The observed phosphorylation sites are underlined.

**Figure 3.** Expression of Hsp27 and Hsp70 in human vessel specimens. Donors’ renal arteries from kidney transplantation and internal mammary arteries from coronary artery bypass grafting were used as normal reference vessels. P-Hsp27 indicates the phosphorylated forms of Hsp27 (phospho-Ser\(^{20} \) Hsp27) in those cells was detected to a much lower degree in the atherosclerotic plaque specimen than in the reference artery specimen (Figure 4F versus 4C).
On the contrary, the macrophage-infiltrated region (CD68 positive; data not shown) was colocalized with staining for Hsp27 phosphorylation (Figure 4F).

**Increased Plasma Levels of Hsp27 in ACS Patients**

The clinical characteristics and lipid profiles of each study group are summarized in Table 2. There were no significant differences between the ACS patients and the normal reference subjects for the serum levels of total cholesterol, triglyceride, and LDL, whereas the serum levels of HDL in patients with ACS were significantly lower than those in the reference subjects \( (P<0.001) \). There were significant differences for serum total cholesterol and LDL levels between the ACS and CSA groups \( (P<0.005 \text{ for both variables}) \) and between the reference and CSA groups \( (P<0.001 \text{ for both variables}) \).

The serum levels of C-reactive protein (CRP) in the ACS group \( (1.341±2.382 \text{ mg/dL}) \) were significantly higher than levels in both the reference group \( (0.101±0.096 \text{ mg/dL}; \ P<0.05) \) and the CSA group \( (0.088±0.056 \text{ mg/dL}; \ P<0.05) \) (Figure 5A). The plasma \( \alpha \)-tocopherol concentrations in the ACS group \( (29.5±7.8 \mu\text{mol/L}; \ P<0.05 \text{ versus control}) \) and the CSA group \( (33.7±10.1 \mu\text{mol/L}; \ P<0.05 \text{ versus control}) \) were significantly lower than those in the reference subjects \( (42.8±14.7 \mu\text{mol/L}) \) (Figure 5B). The levels of plasma Hsp70 were significantly higher in patients with ACS than in reference subjects \( (13.84±9.64 \text{ versus 8.21±7.00 ng/mL}; \ P<0.005) \) (Figure 5C), whereas the plasma levels of adiponectin in patients with ACS were significantly lower than the levels in the reference subjects \( (3.10±2.18 \text{ versus 5.25±3.99 g/mL}; \ P<0.05) \). The mean values for plasma CD40L were increased by almost 2-fold in patients with ACS compared with those in the reference group \( (2.07±1.92 \text{ versus 1.22±1.45 ng/mL}) \), but the differences did not reach statistical significance.

Between all groups, the plasma levels of Hsp27 \( (106.1±74.1 \text{ mg/mL}) \) were significantly higher in ACS patients than in the healthy reference subjects \( (45.8±29.5 \text{ mg/mL}; \ P<0.005) \) (Figure 6). As shown in Table 2, when the healthy reference group was compared with the group with risk factors for coronary artery disease, the plasma levels of Hsp27 were not significantly different between the 2 groups (reference group versus risk group, \( 45.8±29.5 \text{ versus 46.5±29.3 ng/mL} \)). Although the difference in the Hsp27 levels between the ACS and CSA groups was not statistically significant \( (P=0.066) \), marginal significance was found between them when the statistical analysis was limited to 3 groups involving the reference, ACS, and CSA groups \( (P<0.05) \).

**Correlations of Plasma Hsp27 Levels With Other Variables**

When statistical analysis was performed in the whole group of combined ACS and reference groups, the plasma levels of Hsp27 were significantly correlated with the plasma levels of Hsp70 \( (r=0.506, \ P<0.0001) \), serum levels of CRP \( (r=0.234, \ P<0.05) \), plasma levels of CD40L \( (r=0.417, \ P<0.005) \), and serum levels of total cholesterol \( (r=0.254, \ P<0.05) \). When we used Spearman partial correlation analysis to adjust for ACS/reference group status, these results of positive correlations remained significant except for between Hsp27 and CRP. The partial correlation coefficient was 0.422 for between Hsp27 and Hsp70 \( (P<0.005) \), 0.351 for between Hsp27 and CD40L \( (P<0.01) \), and 0.094 for between Hsp27 and CRP \( (P=0.494) \). The correlations between plasma levels of Hsp27 with the other serum or plasma factors such as triglyceride, HDL, LDL, \( \alpha \)-tocopherol, and adiponectin were not significant. The correlation between the plasma Hsp70 levels and the serum CRP levels was not significant \( (r=0.178, \ P=0.093) \). In our study groups, age, gender,
Hsp27 has not been much studied for its relationship with atherosclerosis. Hsp27, the reverse of our findings in the present study. In contrast to Martin-Ventura et al, who compared the patterns of protein secretion between carotid endarterectomy specimens and healthy reference arteries, we compared the proteome of the atheromatous plaque core areas and the adjacent normal-appearing areas in human carotid endarterectomy specimens. We found that Hsp27 expression was lower in the plaque core areas than in the nearby normal-appearing areas. These results were confirmed by Western blotting with identical results.

The reference artery specimens in our study showed a much higher ratio of phospho-Hsp27 to total Hsp27 expression compared with the nearby normal-appearing areas of the carotid plaque, and the plaque core areas showed markedly decreased expression of both Hsp27 and phospho-Hsp27 (Figure 3), suggesting that the nearby normal-appearing areas of the atheromatous plaque core areas and the adjacent normal-appearing areas of the atherosclerotic plaque is not a normal area free from atherosclerosis. Immunohistochemical analysis (Figure 4) showed that the phospho-Hsp27 stain in the SMCs was much lower in atherosclerotic plaque specimens than in normal artery specimens. Because many protective roles of Hsp27 are expressed by its phosphorylated form, those cells showing a lower ratio of phospho-Hsp27 to total Hsp27 might be more vulnerable to oxidative stress and inflammation. Hsp27 of the vulnerable to oxidative stress and inflammation. Hsp27 of the vascular smooth muscle cells in the atherosclerotic lesions was observed to be phosphorylated on the 82nd serine residue, which might be

smoking status, and the presence of diabetes mellitus or hypertension were not associated with the plasma Hsp27 levels.

Discussion
In contrast to Hsp60/65 and Hsp70/72, Hsp27 has not been much studied for its relationship with atherosclerosis. Hsp27 is highly expressed in the heart, and it has been previously studied in myocardial protection models. Hsp27 is induced by oxidative stress, and it is known to have an antiapoptotic effect.

Recently, Martin-Ventura et al. showed the decreased release of Hsp27 from buffer-soaked atherosclerotic plaques compared with those from normal reference arteries and concluded that these results might be associated with the observed low level of plasma Hsp27 in patients with carotid stenosis. However, the release of a certain protein from a specimen might differ according to the applied buffer system, and such a small area of diseased vessel of carotid arteries could not explain the systemic lower level of plasma Hsp27, the reverse of our findings in the present study. In contrast to Martin-Ventura et al, who compared the patterns of protein secretion between carotid endarterectomy speci-
involved in the oxidative defense mechanism\textsuperscript{24,26} and/or cell migration in atherosclerotic plaque.\textsuperscript{28,29}

We also found that the plasma levels of Hsp27 were significantly increased in patients with ACS compared with the healthy reference group (Figure 6). The risk factors for coronary artery disease may not be related to the increased plasma Hsp27 because the plasma Hsp27 levels of a group of subjects with risk factors for coronary artery disease but no symptomatic coronary stenosis were not different compared with those of the healthy reference group (Figure 6). The levels of plasma Hsp70 showed much stronger correlation with the levels of plasma Hsp27 ($r=0.422$, $P<0.005$) than any other variables in the ACS groups and reference group. It has been reported previously that the plasma Hsp70 levels were lower in patients at the time of diagnosis of coronary artery diseases by coronary angiography and that the patients with lower Hsp70 levels had a higher risk of coronary artery disease than the patients with higher Hsp70 levels.\textsuperscript{10} However, contrary to the association between plasma Hsp60 and coronary artery disease, which has been supported by multiple studies, the inverse relationship between the plasma Hsp70 level and the risk of coronary artery disease is not a settled issue. Moreover, some methodological aspects in the report by Zhu et al.\textsuperscript{10} are questionable because they evaluated the plasma Hsp70 level by the extrapolation method. In addition, it has been reported recently that the inflammatory status determines the serum level of Hsp70,\textsuperscript{30} which might support our results.

Although further research is needed to clarify the roles of Hsp27 and its phosphorylation in the pathogenesis and progression of atherosclerosis, these findings suggest that Hsp27 may increase in the earlier stage of atherosclerosis and the decreased potency of phosphorylating Hsp27 of the SMCs may be an important factor in the progression of atherosclerosis. We also observed increased levels of plasma Hsp27 in ACS patients, which may represent vulnerable or complicated plaque and an associated increase in systemic inflammatory or oxidative stress.

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**Disclosures**

None.

**References**

In our study we found differentially expressed proteins in atherosclerotic lesions by performing 2-dimensional gel electrophoresis on human carotid endarterectomy specimens. We divided the atherosclerotic plaque lesion into core area and nearby normal-appearing area, and we used the internal mammary artery and renal artery graft as reference arteries. Among the differentially expressed proteins, we were interested in heat shock protein 27 (Hsp27), which showed markedly decreased expression in the atherosclerotic core lesion compared with the nearby normal-appearing lesion. The mean plasma level of Hsp27 was significantly higher in patients with acute coronary syndrome than in the normal counterparts. The phosphorylation of Hsp27 showed a gradation in the degree of phosphorylation: greatest in the reference arteries, intermediate in the adjacent normal-appearing area, and lowest in the plaque core area. Immunohistochemical analysis showed that Hsp27 expression was observed mainly in the smooth muscle cells. Heat shock protein 70 (Hsp70) did not show any significant difference in the degree of expression between the plaque core area and nearby normal-appearing area. The mean plasma level of Hsp27 was significantly higher in patients with acute coronary syndrome than in the normal reference subjects. The plasma levels of Hsp27 were significantly correlated with those of Hsp70. Therefore, it can be suggested that Hsp27 may increase in the earlier stages of atherosclerosis and that the decreased potency of phosphorylating Hsp27 on the smooth muscle cells may be an important factor in the progression of atherosclerosis. Increased plasma levels of Hsp27 may also represent the vulnerable or complicated plaque and associated increase in systemic inflammatory or oxidative stress.
Expression of Heat Shock Protein 27 in Human Atherosclerotic Plaques and Increased Plasma Level of Heat Shock Protein 27 in Patients With Acute Coronary Syndrome

Haing Kee Park, Eui-Chul Park, Sung Won Bae, Mi Young Park, Seon Woon Kim, Hwan Soo Yoo, Munkhtsetseg Tudev, Young Hye Ko, Yoon-Ho Choi, Sungjoo Kim, Dong-Ik Kim, Young Wook Kim, Byung Boong Lee, Jong-Bok Yoon and Jeong Euy Park

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