Homocysteine is an essential amino acid with a sulfhydryl group and is produced through the metabolism of methionine. Since McCully reported the possible involvement of homocysteine in atherosclerosis and thrombosis on the basis of dissection of some patients afflicted with a rare congenital metabolic disorder, hyperhomocysteinuria, elevated plasma concentrations of homocysteine have been investigated as a trigger for atherosclerosis in adults. Research has also been conducted into the mechanism by which homocysteine induces atherosclerosis and thrombosis/embolism. In 1993, Nishinaga et al reported that homocysteine caused the release of heparan sulfate-bound antithrombin III (AT III) into the blood, resulting in a decrease of AT III (ie, an anticoagulant effect) on the vascular endothelial surface. There is also the possibility that homocysteine may also cause the release of heparan sulfate-bound extracellular superoxide dismutase (EC-SOD) into the blood. Wilcken et al reported that patients with inherited homocysteinuria had a strong correlation between the blood concentrations of homocysteine and EC-SOD, and that the blood concentrations of released EC-SOD decreased after the concentration of homocysteine was decreased by treatment.

EC-SOD is a glycoprotein, discovered by Marklund et al in 1982, that contains copper (Cu) and zinc (Zn) and has an affinity with glycosaminoglycans such as heparan sulfate. It is a type of superoxide dismutase that performs a disproportion reaction to remove superoxide, and is mainly produced by vascular smooth muscle cells. Immunohistochemical analysis has revealed that the substance is present in high concentrations, particularly between vascular endothelial and smooth muscle cells. After its extracellular release, EC-SOD exists between the vascular endothelium and interstitium in a form bound to heparan sulfate. Its role remains unknown, but may prevent inactivation of nitric oxide (NO) by ambient superoxide when NO produced in vascular endothelial cells acts on smooth muscle, suggesting that EC-SOD may have anti-atherosclerotic activity by ensuring NO bioavailability. Endothelium-bound EC-SOD was found to be decreased in patients with coronary artery disease (CAD), compared with a control group, and the decrease positively correlated with the decline in plasma homocysteine concentration.

Hyperhomocysteinemia is Associated With Human Coronary Atherosclerosis Through the Reduction of the Ratio of Endothelium-Bound to Basal Extracellular Superoxide Dismutase

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**Background** Homocysteine is involved in coronary atherosclerosis through oxidative stress, so the present study investigated the association between plasma concentrations of homocysteine and extracellular superoxide dismutase (EC-SOD) in coronary artery disease (CAD).

**Methods and Results** The study group comprised 154 consecutive male patients with suspected CAD who had undergone angiography. Plasma concentrations of homocysteine and EC-SOD, which was determined before (basal) and after heparin therapy, were measured and the difference was designated as endothelium-bound EC-SOD. The EC-SOD ratio (endothelium-bound/basal EC-SOD) was also evaluated as an index of binding capacity. The plasma homocysteine concentration in the stenosis (+) group (n=97, 12.0±4.6 μmol/L) was significantly higher than that of the stenosis (−) group (n=57, 10.2±3.0 μmol/L, p=0.004). Plasma homocysteine correlated positively with the basal EC-SOD (r=0.377, p=0.001) and negatively with the EC-SOD ratio (r=−0.199, p=0.014). When the group was subdivided according to either homocysteine or the EC-SOD ratio, there were 2 groups with high homocysteine concentration and of these atherosclerosis was reduced in the group with a high EC-SOD ratio.

**Conclusions** In CAD patients, homocysteine is involved in the significant release of EC-SOD from the endothelium. Furthermore, the higher EC-SOD binding capacity, even at high concentrations of homocysteine, suggested that homocysteine-induced atherosclerosis was suppressed.

**Key Words:** Atherosclerosis; Coronary artery disease; Endothelium; Extracellular superoxide dismutase; Homocysteine
forearm blood-flow-dependent vasodilation. In the present study, based on the previous findings, we evaluated whether the plasma concentration of homocysteine correlates with the plasma concentration of EC-SOD or endothelium-bound EC-SOD released by heparin in patients with CAD, and investigated the correlation of these concentrations with coronary atherosclerosis.

### Methods

**Patients**

The subjects were 154 consecutive male patients (aged 62.1±9.8 years) who were suspected of having CAD and underwent coronary angiography. Patients with acute coronary syndrome were excluded because of upregulation of EC-SOD expression. Patients who were under hemodilysis therapy with heparin were excluded, because the heparin might modify EC-SOD metabolism. Fourteen patients (8.3%) were also excluded because their basal values of EC-SOD and those after heparin injection were extremely high, indicating the presence of a mutation that lacked affinity with the endothelium. Coronary angiography was performed and the degree of stenosis judged by two angiographers, with a third angiographer making the final decision when the 2 could not agree. Stenosis of 75% or greater by the American Heart Association classification was designated significant and the patients were classified into a stenosis (+) group, and the remainder comprised the stenosis (–) group. Furthermore, the degree of coronary atherosclerosis was expressed by a score (coronary score) based on the Gensini method. We obtained approval for the protocol from the institutional committee and then also obtained written consent from the individual patients for the protocol from the institutional committee and then also obtained written consent from the individual patients for participation.

**Subgroups According to Homocysteine and EC-SOD Ratio**

In order to evaluate the effects of the plasma homocysteine concentration and the EC-SOD ratio (endothelial binding capacity) on the coronary score (degree of coronary atherosclerosis), the patients were subdivided according to their concentration of homocysteine or the EC-SOD ratio. More particularly, the plasma concentration of homocysteine and the regression line of the EC-SOD ratio (y=–0.029x+2.28) were used to determine the value of the EC-SOD ratio (1.98) at homocysteine 10 μmol/L. Comparisons of the coronary score were also made in 4 groups (group A, low homocysteine/high EC-SOD ratio, n=28; group B, low/low, n=43; group C, high/high, n=38; and group D, high/low, n=45).

**Statistical Analysis**

In the comparative evaluations of the individual parameters on the basis of the presence or absence of significant stenosis, continuous variables were assessed by the unpaired Student’s t-test, whereas categorical data were assessed by the chi-square test. The data are expressed as the mean value±standard deviation. EC-SOD and homocysteine were assessed by Pearson’s simple correlation coefficient. Because the coronary score does not show a normal distribution, nonparametric analysis was used to create a description in terms of the median, minimum or maximum. The Mann-Whitney test was conducted to make intra-group comparisons, the Kruskal-Wallis test compared 3 or more groups, and the Scheffe test was conducted as a

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### Table 1 Characteristics of the 2 Subgroups According to Significant Stenosis

<table>
<thead>
<tr>
<th>Significant stenosis (–)</th>
<th>Significant stenosis (+)</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (years)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>n=57</td>
<td>62.3±11.3</td>
<td>62.9±9.8</td>
</tr>
<tr>
<td>Body mass index</td>
<td></td>
<td></td>
</tr>
<tr>
<td>n=57</td>
<td>23.2±3.7</td>
<td>23.7±2.9</td>
</tr>
<tr>
<td>Total cholesterol (mmol/L)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>n=57</td>
<td>4.42±0.85</td>
<td>4.81±1.05</td>
</tr>
<tr>
<td>LDL-cholesterol (mmol/L)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>n=57</td>
<td>2.16±0.82</td>
<td>2.52±1.22</td>
</tr>
<tr>
<td>HDL-cholesterol (mmol/L)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>n=57</td>
<td>0.80±0.22</td>
<td>0.73±0.26</td>
</tr>
<tr>
<td>Total triglycerides (mmol/L)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>n=57</td>
<td>1.68±0.22</td>
<td>1.75±0.64</td>
</tr>
<tr>
<td>No. of coronary risk factors</td>
<td></td>
<td></td>
</tr>
<tr>
<td>n=57</td>
<td>1.73±1.0</td>
<td>2.3±1.1</td>
</tr>
<tr>
<td>Smoking (+/-)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>n=57</td>
<td>26/21</td>
<td>59/38</td>
</tr>
<tr>
<td>Diabetes mellitus (+/-)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>n=57</td>
<td>22/25</td>
<td>45/52</td>
</tr>
<tr>
<td>Hypertension (+/-)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>n=57</td>
<td>30/27</td>
<td>54/43</td>
</tr>
<tr>
<td>Hyperlipidemia (+/-)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>n=57</td>
<td>21/26</td>
<td>58/39</td>
</tr>
<tr>
<td>Homocysteine (μmol/L)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>n=57</td>
<td>10.2±3.0</td>
<td>12.0±4.6</td>
</tr>
</tbody>
</table>

Values are mean±SD or number of patients.

NS, not significant; LDL, low-density lipoprotein; HDL, high-density lipoprotein.

Number of coronary risk factors is the sum of smoking, diabetes mellitus, hypertension and hyperlipidemia.

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Individual EC-SOD values were determined as follows: (i) basal EC-SOD: before administration of heparin; (ii) post-heparin EC-SOD: 15 min after administration of heparin (100 units/kg body weight); and (iii) endothelium-bound EC-SOD = (post-heparin EC-SOD) – (basal EC-SOD). Because vascular endothelium-bound EC-SOD can be considered as equivalent to blood liberated EC-SOD, the basal EC-SOD increases with an increase in the endothelium-bound EC-SOD. However, if its binding capacity to endothelial heparan sulfate continues to be affected by a pathophysiological state, such as hyperhomocysteinemia, EC-SOD will be more easily released from the endothelium into the blood. Thus, the EC-SOD ratio = endothelium-bound EC-SOD/basal EC-SOD was calculated as the fourth parameter and evaluated as an index for the binding capacity of endothelial EC-SOD.

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**Assay for Lipids, Homocysteine and EC-SOD**

Blood samples were collected early in the morning before the patients had eaten. Blood lipids were determined by an enzymatic method. Total blood homocysteine was determined by high-performance liquid chromatography. EC-SOD was collected as described below and determined by enzyme-linked immunosorbent assay. The difference between the blood EC-SOD concentrations before and after intravascular administration of heparin was used to estimate the quantity of vascular endothelium-bound EC-SOD.
Fig 1. Correlation of the 3 indices of extracellular superoxide dismutase (EC-SOD) with the plasma concentration of homocysteine. Basal EC-SOD (A), endothelium-bound EC-SOD (B) and the EC-SOD ratio (C) from 154 male patients were analyzed. Each EC-SOD value was obtained by the methods described in the text. The distinguishing value (1.98) of the EC-SOD ratio was calculated by the equation \(y = -0.029x + 2.28\) and the value (10 μmol/L) of homocysteine (C).

Fig 2. Comparison of the coronary score between subgroups according to the concentration of plasma homocysteine (A) or the extracellular superoxide dismutase (EC-SOD) ratio (B). The distinguishing value for homocysteine was 10 μmol/L and 1.98 for EC-SOD (Fig 1C). Mann-Whitney analysis was performed and each column is expressed as the center value and 1st and 3rd quartiles.
post-hoc test. All analysis was performed with StatView J5.0 (SAS Institute Inc., Cary, NC, USA). A value of p<0.05 was considered statistically significant.

Results

Comparison of Characteristics and Measurements

On the basis of coronary angiography, the stenosis (–) group consisted of 57 patients (aged 60.5±9.3 years) and the stenosis (+) group consisted of 97 patients (aged 62.9±9.8 years). With regard to the conventional parameters, low-density lipoprotein cholesterol was significantly higher in the stenosis (+) group (2.52±1.22 mmol/L) than in the stenosis (–) group (2.16±0.82 mmol/L, p=0.026), but no statistical significance was found in the other measurements. However, when the number of conventional coronary risk factors was compared, there were significantly more in the stenosis (+) group (2.3±1.1) than that in the stenosis (–) group (1.7±1.0, p=0.002). Comparison of the plasma concentrations of homocysteine in the stenosis (+) group (12.0±4.6 μmol/L) and stenosis (–) group (10.2±3.0 μmol/L) revealed a significantly higher value (p=0.004) in the stenosis (+) group (Table 1).

Correlation Between the Plasma Homocysteine Concentration and EC-SOD Concentration

The plasma homocysteine value correlated positively and significantly with the basal EC-SOD value (n=154, r=0.377, p<0.001) (Fig 1A), but did not correlate with endothelium-bound EC-SOD (Fig 1B). The EC-SOD ratio negatively and significantly correlated with the plasma concentration of homocysteine (r=−0.199, p=0.014) (Fig 1C).

Comparison of the Coronary Scores of the Subgroups

The patients were divided into 2 groups according to the plasma concentration of homocysteine and the coronary score of the high homocysteine group (7.5, 0–169) tended to be higher, but not significantly different, than that of the low homocysteine group (2.0, 0–202.0) (Fig 2A). When divided into 2 groups according to the EC-SOD ratio, the coronary score was significantly lower in the high EC-SOD ratio group with a better EC-SOD binding capacity (1.5, 0–103.0) than in the low EC-SOD ratio group (7.8, 0–202.0), suggesting that coronary atherosclerosis did not progress in the high EC-SOD ratio group (Fig 2B).

The same 2 parameters were used to divide the patients into 4 groups for comparison (Fig 3). Group A (low homocysteine/high EC-SOD ratio, n=28) had the lowest coronary score (1.5, 0–93) and group D (high/low, n=45) had the highest coronary score (28.0, 0–169), with a clear and significant difference between the groups (p=0.010 by Kruskal-Wallis and Scheffe tests). The most interesting result was that comparison of group C (high/high, n=38, homocysteine value, 13.0±2.7 μmol/L) with group D (high/low, n=45, homocysteine value, 14.8±4.5 μmol/L), both of which had similar high levels of homocysteine, revealed that the coronary score of group C (1.5, 0–103.0) was significantly lower than that of group D (28.0, 0–169). This finding was not obtained in the comparison between group A (1.5, 0–93) and group B (2.0, 0–202), which had a lower level of homocysteine, and it suggests that even when the homocysteine concentration was high, retaining a
high EC-SOD ratio could result in a lower degree of coronary atherosclerosis. Risk factors were compared to identify what caused the difference in the EC-SOD ratio between groups C and D. Group C was significantly lower in age and had a significantly lower basal EC-SOD and higher endothelium-bound EC-SOD. Intra-group comparison revealed no significant difference in the EC-SOD ratio between groups C and D. Group C was significantly lower in age and had a significantly lower basal EC-SOD and higher endothelium-bound EC-SOD. Intra-group comparison revealed no significant difference in the EC-SOD ratio between these pro-oxidative and anti-oxidative substances.

**Discussion**

In the present study, we measured the plasma concentrations of homocysteine and EC-SOD in 154 male patients with various degrees of CAD to assess the correlation between these pro-oxidative and anti-oxidative substances. We also elucidated the possibility that the retention of EC-SOD could attenuate the adverse effects of homocysteine on coronary atherosclerosis. This study demonstrates for the first time that homocysteine may aggravate coronary atherosclerosis through its action on the anti-oxidative substance, EC-SOD.

Since McCully reported the possible involvement of hyperhomocysteinemia in atherosclerosis, attention has been given to the correlation of homocysteine with atherosclerosis and thrombosis. Meta-analysis by Boushey et al revealed that 10% of the coronary atherosclerosis risk in the general population was related to homocysteine. We also considered the increased production of EC-SOD by homocysteine, the basal EC-SOD positively correlated with the homocysteine value to a great extent, which indicates that homocysteine may cause the elevation in plasma EC-SOD.

Regarding the correlation between EC-SOD and homocysteine, the basal EC-SOD positively correlated with the homocysteine value to a great extent, which indicates that homocysteine may cause the elevation in plasma EC-SOD. We speculate that there are 2 mechanisms: increased production of EC-SOD or increased release of endothelium-bound EC-SOD into the blood by homocysteine. Although clinical data were not available about the effect on the production of EC-SOD by homocysteine, Yamamoto et al suggested that homocysteine decreased the binding of EC-SOD to vascular endothelial cell surface by degrading endothelial heparan sulfate proteoglycan and that the high concentration of homocysteine decreased the production of EC-SOD. Nonaka et al also reported that homocysteine reduced the expression of EC-SOD mRNA and protein levels in cultured rat smooth muscle cells. Therefore, we surmised the increased production of EC-SOD by homocysteine was highly unlikely as the mechanism. When the previous AT III data were also taken into account, the mechanism of increased release of endothelium-bound EC-SOD into the blood was the most likely explanation for the elevation of plasma EC-SOD by homocysteine and is further supported by the fact that the EC-SOD ratio in the present study inversely correlated with homocysteine. Namely, if a patient has higher concentration of homocysteine, the patient tends to release EC-SOD from endothelium into the blood, resulting in a decrease of the EC-SOD ratio. We consider this is one of mechanisms by which oxidative stress is increased by homocysteine because EC-SOD effectively removes superoxide near the endothelium by binding it to the endothelium. It has been established in animal and human studies that for effective function of EC-SOD needs to be attached to the endothelium through its heparin-binding domain. We used 2 parameters to represent the effect of homocysteine on the amount of EC-SOD: one was the basal level and the other was the...
ratio of endothelium-bound to basal EC-SOD. The strongest correlation was between the levels of basal EC-SOD and homocysteine (Fig 1A). However, there were no significant differences among the coronary scores of the subgroups when we used the basal values instead of the EC-SOD ratio (data not shown). Taking into account the other reports that emphasize the location of EC-SOD[17,31,32] we selected the EC-SOD ratio (the ratio of vascular endothelium-bound EC-SOD to basal EC-SOD), not the basal level of EC-SOD, for the comparisons the coronary score.

The coronary score in the high EC-SOD ratio group was significantly lower than that in the low EC-SOD ratio group (Fig 2B), which suggested that atherosclerosis was suppressed when the ratio of vascular endothelium-bound EC-SOD to basal EC-SOD was high and concurred with the report by Landmesser et al.[14] Furthermore, when the patients were divided into 4 groups according to the EC-SOD ratio and homocysteine level (Fig 3), it appeared that atherosclerosis did not differ greatly according to the ratio of vascular endothelium-bound EC-SOD to basal EC-SOD as long as the blood homocysteine concentration was low (groups A and B). In contrast, when group C (high EC-SOD ratio/high homocysteine) and group D (low EC-SOD ratio/high homocysteine) were compared, the coronary score was significantly lower in group C, which suggested that homocysteine could aggravate atherosclerosis only when the ratio of vascular endothelium-bound EC-SOD to basal EC-SOD was low. Another study found that homocysteine reduced NO production in the vascular endothelium, increasing oxidative stress and promoting the growth of vascular smooth muscle cells.[34] Homocysteine is considered to play an important role in preventing the destruction by superoxide of NO released from the vascular endothelium. Recently, we reported the inhibitory action of EC-SOD on the oxidation of low-density lipoproteins[35] and Laukkanen et al also reported that local administration of EC-SOD to rabbit aorta after balloon injury successfully reduced restenosis[36] both of these finding suggesting the anti-atherosclerotic effects of EC-SOD.

Study Limitations

First, we could not confirm what caused the difference in the EC-SOD ratio between the high value group and the low value group despite the similar plasma concentration of homocysteine, and factors other than age not being significant. The finding that basal EC-SOD was more easily released in elderly patients was consistent with the report from Marklund et al[19] but this mechanism still remains unclear. Second, we could not clarify why the coronary score was similar irrespective of EC-SOD ratio when the homocysteine level was low (groups A and B). We speculate that EC-SOD might not be necessary to act against the oxidative stress induced by homocysteine when the homocysteine level is low. We need further in vitro experiments on the regulation of EC-SOD by homocysteine. Third, a further question is why the endothelium-bound EC-SOD was not negatively correlated with the plasma concentration of homocysteine. At present, we do not have data to clarify this problem and we will have to conduct further experiments. Finally, we enrolled and studied only men because the distribution of values in EC-SOD differs by gender[16] Therefore, we also need further population studies in Japan.

Conclusions

EC-SOD is released from the endothelium by the action of homocysteine, but when the ratio of endothelium-bound to basal EC-SOD is high, homocysteine-induced atherosclerosis may be inhibited. This finding indicates the mechanism by which homocysteine influences atherosclerosis and leads to the hypothesis that maintaining the level of endothelium-bound EC-SOD could be a target for homocysteine treatment in clinical practice.

Acknowledgments

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References


