Manganese superoxide dismutase polymorphism affects the oxidized low-density lipoprotein-induced apoptosis of macrophages and coronary artery disease

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Aims
Oxidative damage promotes atherosclerosis. Manganese superoxide dismutase (MnSOD) is an antioxidant enzyme localized in mitochondria. We investigated the associations of the MnSOD polymorphism (valine-to-alanine in the mitochondrial-targeting domain) with its activity in leukocytes, with macrophage apoptosis by oxidized low-density lipoprotein (oxLDL), and with coronary artery disease (CAD).

Methods and results
Blood samples were taken from 50 healthy subjects. The mitochondrial MnSOD activities in leukocytes were 542.4 ± 71.6 U/mg protein (alanine/alanine, n = 2), 302.0 ± 94.9 U/mg protein (alanine/valine, n = 12), and 134.0 ± 67.1 U/mg protein (valine/valine, n = 36; P < 0.0001 for non-valine/valine vs. valine/valine). Macrophages were treated with oxLDL. After incubation, the percentages of apoptotic macrophages were 48.6 ± 3.6% (alanine/alanine), 78.6 ± 9.8% (alanine/valine), and 87.5 ± 7.0% (valine/valine) (P < 0.0001, non-valine/valine vs. valine/valine). The association of the MnSOD polymorphism with CAD was investigated using blood samples collected from 498 CAD patients and 627 healthy subjects; the alanine allele was found to reduce the risk of CAD and acute myocardial infarction (AMI).

Conclusion
Our data indicate that the alanine variant of signal peptide increases the mitochondrial MnSOD activity, protects macrophages against the oxLDL-induced apoptosis, and reduces the risk of CAD and AMI.

Keywords
Apoptosis • Macrophage • Oxygen radicals • Coronary disease • Gene expression

Introduction
Oxidative damage is thought to play an important role in atherosclerosis.1–3 Reactive oxygen species (ROS) disturb the function of vascular wall cells. ROS also bring about lipid peroxidation, and oxidized low-density lipoprotein (oxLDL) promotes atherogenesis and destabilizes plaque via several pathways, including the induction of apoptosis of vascular wall cells.4–7

Manganese superoxide dismutase (MnSOD) is an endogenous antioxidant enzyme synthesized in the cytosol and is post-transcriptionally transported into mitochondria,8,9 where it catalyzes the dismutation of superoxide radicals, producing hydrogen peroxide and oxygen. There are several suggestions that MnSOD acts protectively against atherosclerosis and vulnerable plaque formation. MnSOD was reported to reduce the oxLDL-induced apoptosis of macrophages,10,11 protect against endothelial dysfunction,12 and inhibit the oxidation of LDL by endothelial cells.13

There are two genetic variants of MnSOD, arising from a C-to-T base transition which results in the substitution of an alanine (GCT) for a valine (GTT) at the 16th amino acid of the signal peptide in the
mitochondrial-targeting domain (Ala16Val). This substitution is thought to alter the conformational structure of the mitochondrial-targeting domain of MnSOD and change its efficacy in fighting oxidative damage.13,14

Several studies have suggested that MnSOD polymorphism is associated with certain kinds of diseases induced by oxidative damage including atherosclerosis.15,16 However, a difference in the MnSOD activity between the different genotypes has not been demonstrated, and the pathogenic function of each polymorphic variant is still unclear.

If Ala16Val modifies the mitochondrial MnSOD activity, MnSOD polymorphism may affect the susceptibility of vascular wall cells to oxLDL-induced apoptosis, thereby affecting atherosclerosis and the vulnerability of the plaque. In this study, to clarify the role of MnSOD polymorphism in atherogenesis, we first investigated the association of MnSOD polymorphism with the mitochondrial MnSOD activity in leukocytes and with the oxLDL-induced apoptosis of macrophages. Next, we investigated the association of the MnSOD polymorphism with coronary artery disease (CAD) and acute myocardial infarction (AMI).

Methods

Subjects

This study complied with the principles of the Declaration of Helsinki. The study protocol was approved by the Ethics Committee of each hospital participating in the study. The study was explained to every subject and written informed consent was obtained.

Fifty healthy Japanese subjects aged 20–27 years were enrolled to investigate the association of the MnSOD polymorphism with its activity in leukocytes (Group 1). None of the subjects had a history of hypertension (defined as having a systolic/diastolic blood pressure ≥ 140/90 mmHg or receiving antihypertensive therapy), hyperlipidaemia (defined as having a serum total cholesterol ≥ 220 mg/dL or a serum triglyceride ≥ 150 mg/dL or receiving lipid-lowering therapy), diabetes mellitus (defined in accordance with the criteria of the American Diabetes Association), hyperuricaemia (defined as having a serum uric acid ≥ 7.0 mg/dL or receiving uric acid-lowering therapy), obesity (defined as having a body mass index (BMI) ≥ 25.0), smoking, and other long-term medication.

To investigate the association between the MnSOD polymorphism and CAD, a total of 1125 Japanese subjects were enrolled, consisting of 627 healthy subjects aged 38–85 years, and 498 CAD patients aged 37 to 87 years (Group 2). The 627 healthy subjects were recruited at their annual health examination at Mitsui Memorial Hospital, Tokyo, Japan, and did not have any chest symptoms or electrocardiogram (ECG) abnormalities suggesting CAD, or a medical history for CAD. The 498 CAD patients were recruited at the University of Tokyo Hospital (Tokyo, Japan) and the Cardiovascular Institute Hospital (Tokyo, Japan). CAD patients were recruited by examination of 828 subjects who underwent coronary angiography (CAG) for the first time in their lives on suspicion of CAD because of chest symptoms or ECG abnormalities.

Quantitative coronary angiography

Coronary angiograms were assessed by two independent cardiologists unaware of the patients’ genotypes and other coronary risk profiles. All measurements were performed on cineangiograms recorded after the intracoronary administration of nitroglycerin.

We defined CAD patients as those who had at least one lesion with diastolic stenosis of 75% or more in their coronary arteries. Among the 828 patients, 498 patients were diagnosed with CAD.

Manganese superoxide dismutase genotyping

A 5 mL blood specimen was collected from every subject into a tube containing ethylenediaminetetraacetic acid (EDTA; final concentration 5 mM). The genomic DNA was extracted from leukocytes, using QiAamp blood kit (Qiagen, Germany). The polymerase chain reaction was performed using two amplification primers (forward primer: 5’-AGCCCGGAGTCTGGTGA-3’, reverse primer: 5’-GGCTG GTGCTTGGTGG-3’). An initial denaturation of DNA was accomplished at 95°C for 1 min, followed by 40 cycles of denaturation at 95°C for 10 s, annealing at 63°C for 10 s, and elongation at 72°C for 8 s. The melting curve analysis was performed using an upstream probe (5’-GCAGGTCGGGGAGGCC-TGTGCTTCTGCTGGACCC CA-3’-Fluorescein) and a downstream probe (LightCyclerRed640-5’- ATACCCCCAAAAACCGGAG-3’-phosphorylation). The DNA samples were selected randomly and assessed in the same batch by a laboratory technician blinded to the patient origin of the samples.

Measurement of the superoxide dismutase activity in leukocytes

A further 20 mL blood specimen was collected from each subject of Group 1 and centrifuged with an equal volume of polymorph prep (Daichigakagu, Tokyo, Japan) at 500 g for 35 min. Leukocytes were resuspended in 1.2 mL of cold solution (0.25 M sucrose, 10 mM Tris-HCl pH 7.4). Cells were homogenized with 10 strokes of glass and centrifuged at 900 g for 10 min. The supernatant was centrifuged

| Table 1 Characteristics of the 50 healthy subjects by genotype |
|-----------------|----------------|-----------------|-----------------|-----------------|
| Non-valine/valine (n = 14) | Valine/valine (n = 36) | P-value |
| Male, % (n) | 78.6 (11) | 77.8 (28) | 0.95 |
| Age, year | 22.2 ± 1.6 | 21.9 ± 1.4 | 0.53 |
| BMI, kg/m² | 22.2 ± 1.8 | 21.8 ± 2.2 | 0.56 |
| Total cholesterol, g/dL | 168.7 ± 22.6 | 162.6 ± 23.7 | 0.41 |
| HDL cholesterol, g/dL | 52.2 ± 8.0 | 54.2 ± 11.9 | 0.57 |
| LDL cholesterol, g/dL | 90.7 ± 19.2 | 90.7 ± 19.5 | 1.0 |
| Triglyceride | 104.4 ± 16.5 | 113.1 ± 16.3 | 0.10 |
| Uric acid, g/dL | 5.4 ± 1.0 | 5.3 ± 1.3 | 0.98 |
| Glucose, g/dL | 94.3 ± 8.1 | 94.9 ± 5.6 | 0.77 |
| HbA1c, % | 3.9 ± 0.4 | 4.2 ± 0.5 | 0.10 |
| Smoking, % (n) | 0 (0) | 0 (0) | — |
| Leukocytes, × 10³ per µL² | 4.9 ± 1.2 | 5.1 ± 0.8 | 0.37 |
| Monocytes, per µL² | 241.6 ± 51.0 | 257.7 ± 48.4 | 0.30 |

Non-valine/valine group included alanine/alanine genotype (n = 2) and alanine/valine genotype (n = 12).

Determined in peripheral blood.
at 5000 g for 10 min. The mitochondrial pellet was resuspended in 50 μL of cold solution and centrifuged at 100 000 g for 60 min. The resultant supernatant was used as cytosolic fraction. The quantity of protein in the cytosolic fractions and mitochondrial fractions was determined by the Bradford method (Bio-Rad Laboratories, Hercules, CA, USA).

The MnSOD activity and Cu/ZnSOD activity in each fraction were measured using an SOD Assay Kit-WST (Dojindo Molecular Technologies, Tokyo, Japan). In summary, the total SOD activity in each fraction was measured by the inhibition rate of 2-(4-iodophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium, monosodium salt (WST-1) reduction. The MnSOD activity was measured by adding 10 mM potassium cyanide to each fraction to inactivate the Cu/ZnSOD activity. The Cu/ZnSOD activity was calculated by subtracting the MnSOD activity from the total SOD activity. SOD activities were expressed as units per milligram of protein (1 U was defined as the amount of enzyme protein that inhibited the reduction of WST-1 by 50%).

Preparation of oxidized low-density lipoprotein

LDL was oxidized as previously described. Briefly, human-derived LDL (5 mg/mL; Biomedical Technologies, Stoughton, MA, USA) was oxidized by incubation with CuSO4 (5 μM) at 37°C for 16 h. The oxidation reaction was terminated by addition of EDTA (pH 7.4, final concentration 1 mM). Oxidation of LDL was confirmed by the difference in the mobility between the oxidized and normal LDL on agarose gels; this was possible because of a higher net negative charge of oxLDL. The quantity of protein in the oxLDL sample was confirmed by the Bradford method.

Analysis of oxidized low-density lipoprotein-induced apoptosis of macrophages

A further 20 mL blood specimen was taken from each subject of Group 1. A half volume of Nycodenz solution (NycoPrep 1.077; Axis- Shield, Oslo, Norway) was added and the sample was centrifuged at 400 g for 30 min to isolate the mononuclear cells. Mononuclear cells were collected and resuspended in RPMI-1640 containing 10% heat-inactivated fetal bovine serum (FBS) and then incubated on an MSP-P plate (JIMRO, Tokyo, Japan) for 1 h at 37°C. Non-adherent cells were removed by washing the plate three times with PBS (150 mM NaCl, 10 mM phosphate buffer, pH 7.2). To remove adherent monocytes-macrophages, 2 mM EDTA containing 10% FBS was added to the plate. After incubation at 4°C for 30 min, adherent monocytes-macrophages (>90% pure as judged by non-specific

Figure 1 Association of the manganese superoxide dismutase polymorphism and the activity of superoxide dismutase in leukocytes. The non-valine/valine group includes subjects with the alanine/alanine genotype (n = 2) and subjects with the alanine/valine genotype (n = 12), whereas the valine/valine group includes 36 subjects.
esterase staining) were collected and cultured in RPMI-1640 medium containing 10% heat-inactivated FBS, 2 mM l-glutamine, and penicillin (100 U/mL)/streptomycin (100 µg/mL) for 3 days. Morphological examination of Giemsa-stained preparations supported the conclusion that they were monocyte-derived macrophages. The macrophages were subsequently incubated with or without oxLDL (final concentration 200 µg protein/mL) at 37°C for 4 h. Apoptotic macrophages were identified by propidium iodide and annexin V staining using an annexin V-FITC kit (Trevigen, Gaithersburg, MD, USA) and counted with a fluorescence-activated cell sorter (Beckton Dickinson, Franklin Lakes, NJ, USA) using CellQuestPro software.

Statistical analysis
Quantitative data are presented as mean ± standard deviation, and the categorical data as frequencies (percentage). Continuous variables were compared using the unpaired t-test. Binary variables were compared by means of the Fisher exact test, and the variables comprising more than two categorical factors were compared by means of the χ² test. It is known that the frequency of the alanine allele of MnSOD is significantly lower than that of the valine allele in the Asian population.9,15 So, we first investigated the tendency of an allele to increase the mitochondrial MnSOD activity and the tolerance of macrophages against apoptosis. Next, we confirmed the statistical significance by comparing the valine/valine genotype group with the non-valine/valine (i.e. alanine/alanine and alanine/valine) genotype group. To identify the risk factors of CAD, univariable logistic regression analysis was performed using the valine/valine genotype and conventional coronary risk factors such as gender, age, hypertension, hyperlipidaemia, diabetes, hyperuricaemia, smoking, and obesity as independent variables. Then multivariable logistic regression model was used to test the

Figure 2 Association of manganese superoxide dismutase polymorphism with oxidized low-density lipoprotein-induced apoptosis of macrophages. (A) Representative cytograms of macrophages after 4 h incubation without oxidized low-density lipoprotein (upper panel) or with oxidized low-density lipoprotein (lower panel). In each cytogram, the left lower quadrant indicates the viable macrophages (those stained with neither propidium iodide nor annexin V); the right lower quadrant indicates the macrophages in early apoptotic state (those stained only with annexin V); the right upper quadrant indicates the macrophages in late apoptosis or necrosis (those stained with both propidium iodide and annexin V). (B) Percentages of macrophages in apoptosis stained with annexin V [those represented in the right upper square and the right lower square of the cytograms shown in (A)]. PI, propidium iodide. (b) Percentages of macrophages in late apoptosis or necrosis [those represented in the right upper square of the cytograms shown in (A)].
significance of the genotype after controlling for the other variables listed earlier. Odds ratios (ORs) were calculated as an estimate of relative risk of CAD associated with the valine/valine genotype. The same analysis was conducted to identify the effect of the genotype on AMI incidence. Statistical significance was defined as a $P$-value of $<0.05$. All statistical analyses were performed using JMP 5 software (SAS Institute, Cary, NC, USA).

Results

Superoxide dismutase activity in leukocytes

In the 50 subjects in Group 1, the distribution of the MnSOD genotypes was as follows: alanine/alanine 4.0% ($n = 2$), alanine/valine 24.0% ($n = 12$), and valine/valine 72.0% ($n = 36$).

There were no significant differences in the clinical characteristics of the subjects between the non-valine/valine genotype group (i.e. alanine/alanine and alanine/valine, $n = 14$) and the valine/valine genotype group in Group 1 (Table 1). The activities of Cu/ZnSOD in both the cytosol and the mitochondria and that of MnSOD in the cytosol were not significantly different between the non-valine/valine genotype group and the valine/valine genotype group. In contrast, the mitochondrial MnSOD activity in each genotype group was 542.4 $\pm$ 71.6 U/mg protein (alanine/alanine group), 302.0 $\pm$ 94.9 U/mg protein (alanine/valine group), and 134.0 $\pm$ 67.1 U/mg protein (valine/valine group). It was significantly higher in the non-valine/valine genotype group (336.3 $\pm$ 125.1 U/mg protein) than in the valine/valine genotype group ($P < 0.0001$, Figure 1).

Oxidized low-density lipoprotein-induced apoptosis of macrophages

After a 4 h incubation without oxLDL, the percentages of macrophages stained with annexin V (in apoptosis) and with both propidium iodide and annexin V (in late apoptosis or necrosis) were not statistically significantly different between the non-valine/valine group and the valine/valine group (Figure 2B). In contrast, after incubation with oxLDL for 4 h, the percentages of apoptotic macrophages were 48.6 $\pm$ 3.6% (alanine/alanine), 78.6 $\pm$ 9.8% (alanine/valine), and 87.5 $\pm$ 7.0% (valine/valine). It was significantly lower in the non-valine/valine genotype group (74.3 $\pm$ 14.2%) than in the valine/valine genotype group (87.5 $\pm$ 7.0%) ($P < 0.0001$, Figure 2B). After incubation, the percentage of macrophages in late apoptosis or necrosis was also significantly lower in the non-valine/valine genotype group than in the valine/valine genotype group (Figure 2B).

After incubation with oxLDL, the percentages of macrophages stained with annexin V and that of macrophages stained with both propidium iodide and annexin V were inversely correlated with the mitochondrial MnSOD activity (Figure 3). In other words, the tolerance of macrophages against oxLDL-induced apoptosis increased in proportion to the mitochondrial MnSOD activity.

Characteristics of the subjects enrolled to analyse the association of manganese superoxide dismutase polymorphism with coronary artery disease

The characteristics of the subjects enrolled to analyse the association of MnSOD polymorphism with CAD (Group 2) are shown in Table 2. The MnSOD polymorphism had no association with any of other coronary risk factors in either healthy subjects or CAD patients.

Association of manganese superoxide dismutase polymorphism and coronary artery disease

In the 627 healthy subjects, the allele frequency of the valine variant was 83.3%, which was similar to that previously reported.9,15 The distribution of the MnSOD genotypes was as follows: alanine/alanine 2.2%, alanine/valine 29.0%, and valine/valine 70.8% ($n = 451$) in the healthy subjects and alanine/alanine 1.4%, alanine/valine 31.2%, and valine/valine 67.4% ($n = 176$) in the CAD patients.
valine 68.8% (Table 2). This genotype distribution was compatible with the Hardy–Weinberg equilibrium.

In contrast, in the 498 CAD patients, the allele frequency of the valine allele was 88.5%, and the distribution of the MnSOD genotypes was as follows: alanine/alanine 1.2%, alanine/valine 20.7%, and valine/valine 78.1%. The frequencies of the valine allele and valine/valine genotype were significantly higher in the CAD group than in the healthy group [OR = 1.54, P = 0.0005 (valine allele), OR = 1.62, P = 0.0004 (valine/valine genotype)].

Among the 828 patients who underwent CAG in the two hospitals, 125 patients were diagnosed to have normal coronary arteries because they had no atherosclerotic coronary lesions with a diametric stenosis of >25%. Distribution of the MnSOD genotypes in those 125 patients was as follows: alanine/alanine 3.2%, alanine/valine 30.4%, and valine/valine 66.4%. This distribution was almost identical to that in the 627 healthy subjects mentioned earlier. So, there seemed to be no selection bias in this study.

Multivariable logistic regression analysis revealed that the valine/valine genotype was a statistically significant predictor for CAD independent of other coronary risk factors (OR = 1.49, 95% CI 1.06–2.11, P = 0.030, Table 3).

Among the 498 CAD patients, 87 were AMI patients (44 were non-ST-elevated AMI, and 43 were ST-elevated AMI). Genotype distribution of the 87 patients was as follows: alanine/alanine 0%, alanine/valine 13.8%, and valine/valine 86.2% (Table 2). The frequency of valine allele and valine/valine genotype was significantly higher in AMI patients than in healthy subjects [OR = 2.72, P = 0.0008 (valine allele), OR = 2.77, P = 0.0016 (valine/valine genotype)]. Multivariable logistic regression analysis revealed that valine/valine genotype was a genetic risk factor for AMI (OR = 2.89, 95% CI 1.47–5.68, P = 0.0021, Table 3).

**Discussion**

The major finding of our study is the significant association of MnSOD polymorphism with the mitochondrial MnSOD activity and with the oxLDL-induced apoptosis of macrophages. It was indicated that the alanine variant of MnSOD signal peptide increases the mitochondrial MnSOD activity and protects macrophages against oxLDL-induced apoptosis. Mitochondrial proteins are synthesized in the cytosol and imported into mitochondria via a mechanism that involves cleavage of the leader signal peptide. Without this process, mitochondrial enzymes cannot be efficiently transported into mitochondria. 19,20 The mitochondrial pre-sequences are thought to have an α-helical structure. 21 The alanine variant of MnSOD is thought to have an α-helical mitochondrial-targeting domain, whereas the valine variant of MnSOD appears to have a β-pleated sheet conformation. 22 This conformational difference is thought to result in a more efficient transport of the alanine variant of MnSOD into mitochondria than the valine variant. 14 But despite such speculations, no one has demonstrated a difference in the actual activity of mitochondrial MnSOD of the different genotypes, and the pathogenic function of each variant has remained unclear. In this study, it was indicated that the alanine allele increases the mitochondrial MnSOD activity and the tolerance of macrophages against...
oxLDL-induced apoptosis. Our results support the hypothesis that the alanine variant of MnSOD is more efficiently transported into mitochondria, thereby increasing its functional activity.

The role of macrophage apoptosis in atherogenesis is controversial. A study reported that a reduction in macrophage apoptosis stimulates atherosclerosis. On the other hand, macrophages are reported to have antioxidant and, potentially, antiatherogenic roles, indicating that a decrease in number of macrophages would promote atherosclerosis. Macrophage apoptosis may also promote vulnerable plaque formation. There is a hypothesis that the consequences of macrophage apoptosis are different in initial and advanced stages of atherosclerotic lesions. In the initial stage of atherosclerosis, accelerated apoptosis and rapid removal of the apoptotic macrophages by neighbouring macrophage may decrease inflammation. In contrast, in the late-stage atherosclerosis, defective phagocytosis of apoptotic macrophages may result in post-apoptotic necrosis, which promotes inflammation. Thus, the apoptosis of macrophage may have bimodal effects on atherogenesis and vulnerable plaque formation.

Another major finding of our study is the significant association of the MnSOD polymorphism with CAD. The valine/valine genotype was found to be an independent genetic coronary risk factor. These results provide a clinical meaning to the association of MnSOD polymorphism with the mitochondrial MnSOD activity and with oxLDL-induced apoptosis of macrophages. High activity of mitochondrial MnSOD and tolerance against oxLDL-induced apoptosis of macrophages due to the presence of the alanine allele may suppress atherosclerosis and stabilize the plaque, thereby reducing the risk of CAD and AMI in later life. Our result is compatible with the previous report that the valine variant of MnSOD is associated with carotid plaque thickness in those who have high plasma levels of LDL.

Oxidative stress may promote atherosclerosis via several pathways. Although we showed that higher activity of MnSOD of the alanine variant acts protectively against oxLDL-induced apoptosis and atherogenesis, cytoprotection against apoptosis may be one of the multiple antioxidant effects of MnSOD. Further study is necessary to fully clarify the role of MnSOD polymorphism in atherogenesis.

**Conclusions**

Our data indicate that the alanine variant of MnSOD signal peptide increases the mitochondrial MnSOD activity, protects macrophages against oxLDL-induced apoptosis, and reduces the risk of CAD and AMI. Our data provide an important clue to clarify the role of MnSOD polymorphism in CAD and AMI.

**Conflict of interest:** none declared.

**References**