Aging is a well-known independent risk factor for atherosclerosis. But the mechanisms of age-induced atherosclerosis are unclear. Several studies suggest that aging promotes atherosclerosis by increasing oxidative stress and reducing the effectiveness of defense systems against oxidative damage.1–3

Manganese superoxide dismutase (MnSOD) is an endogenous antioxidant enzyme that is localized in mitochondria and protects macrophages against apoptosis induced by oxidized low density lipoprotein (oxLDL). We previously reported that genetic polymorphism of MnSOD modifies mitochondrial MnSOD (mtMnSOD) activity and increases the risk of coronary artery disease. In this study, we investigated the association of mtMnSOD activity with aging.

Methods and Results: Blood samples were taken from 69 healthy participants aged 20–52. The MnSOD genotype was analyzed using real-time polymerase chain reaction. Leukocyte mtMnSOD activity was measured by inhibition of WST-1. Macrophages were treated with oxLDL and the apoptotic cells were counted. MtMnSOD activity was inversely correlated with the age of the participant regardless of the MnSOD genotype. The percentage of apoptotic macrophages after incubation with oxLDL correlated with age. Thus, the percentage of apoptotic macrophages after incubation with oxLDL was inversely related to mtMnSOD activity. Lecithinized SOD, which can easily transfer into cells, improved the tolerance of macrophages against oxLDL.

Conclusions: MtMnSOD activity decreases with age, thereby reducing the tolerance of macrophages against oxLDL-induced apoptosis. Our data may provide an important clue to clarify the mechanisms of age-induced atherosclerosis. (Circ J 2010; 74: 353–360)

Key Words: Apoptosis; Atherosclerosis; Gene expression; Macrophage; Oxygen radicals

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Sixty-nine healthy Japanese participants aged 20–52 were enrolled in the study. None of the participants had a history of hypertension (defined as having systolic/diastolic blood pressure $\geq 140/90$ mmHg, or receiving antihypertensive therapy), hyperlipidemia (defined as having serum total cholesterol $\geq 220$ mg/dl, or serum triglyceride $\geq 150$ mg/dl, or receiving lipid-lowering therapy), diabetes mellitus (defined in accordance with the criteria of the American Diabetes Association\(^1\)), hyperuricemia (defined as having serum uric acid $\geq 7.0$ mg/dl, or receiving uric acid-lowering therapy), obesity (defined as having a body mass index [BMI] $\geq 25.0$), smoking, or were taking long-term medication.

**MnSOD Genotyping**

As previously reported, the genetic polymorphism of MnSOD affects mitochondrial MnSOD activity.\(^2\) Thus, we first analyzed the MnSOD genotype of the participants. The MnSOD genotypes were determined as follows. A 5-ml blood specimen was collected from all participants into a tube containing 50% (0.25 mol/L sucrose, 10 mmol/L Tris-HCl; pH 7.4). Cells were homogenized with 10 strokes of glass homogenizer and centrifuged at 100,000 $\times$ g for 10 min. The supernatant was used as the cytosolic fraction. The quantity of protein in the cytosolic fraction was determined by the Bradford method (Bio-Rad Laboratories, Hercules, CA, USA). The polymerase chain reaction was performed using 2 amplification primers (forward primer: 5'-AGGCGTCCGCTGCTAGA-3', and reverse primer: 5'-GGCTGCGTCTTGCTGGC-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 30 s. Melting curve analysis was performed using an upstream probe (5'-GACGCGTCGGGAGGAGC-TGTCTCTGACTGG-3') and a downstream probe (5'-ATACC-CACAAACCGGAG-3'-phosphate). The DNA samples were removed randomly and assessed in the same batch by a laboratory technician blinded to the origin of the blood samples.

**Measurement of SOD Activity in the Leukocytes**

A further 20-ml blood specimen was collected from each participant and centrifuged with an equal volume of polymorph-prep (Daichikagaku, Tokyo, Japan) at 500 $\times$ g for 35 min. Leukocytes were resuspended in 1.2 ml of cold solution (0.25 mol/L sucrose, 10 mmol/L Tris-HCl; pH 7.4). Cells were homogenized with 10 strokes of glass homogenizer and centrifuged at 900 $\times$ g for 10 min. The supernatant was centrifuged at 5,000 $\times$ g for 10 min and the mitochondrial pellet was resuspended in 50 $\mu$l of cold solution and centrifuged at 100,000 $\times$ g for 60 min. The resultant supernatant was used as the cytosolic fraction. The quantity of protein in the cytosolic fraction and mitochondrial fraction was determined by the Bradford method (Bio-Rad Laboratories, Hercules, CA, USA).

MnSOD activity in the mitochondrial fraction was 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 of the rate of reduction of 2-(4-iodophenyl)-3-(4-nitrophenoxy)-5-(2,4-disulfophenyl)-2H-tetrazolium, monosodium salt (WST-1). The MnSOD activity was measured by adding 10 mmol/L potassium cyanide to each fraction to inactivate the Cu/ZnSOD. SOD activity was expressed as units per mg of protein (1 unit was defined as the amount of enzyme protein that inhibited the reduction of WST-1 by 50%).

**Measurement of Mitochondrial MnSOD Activity in Macrophages**

In order to directly measure the mitochondrial MnSOD activity in macrophages, a blood sample of approximately 100 ml was necessary. So we used leukocytes to measure the mitochondrial MnSOD, as described above. Next, we confirmed that there was a correlation between mtMnSOD activity in the leukocytes and macrophages using the 100-ml blood samples taken from 10 of the 69 participants from whom informed consent was obtained. Macrophages were collected as described above and mtMnSOD activity was measured. The relationship between mtMnSOD activity in the leukocytes and macrophages was analyzed.

**Preparation of oxLDL**

LDL was oxidized as previously described.\(^9\) Briefly, human-derived LDL (5 mg/ml; Biomedical Technologies, Stoughton, MA, USA) was oxidized by incubation with CuSO$_4$ (5 mmol/L) at 37°C for 16 h. The oxidation reaction was terminated by adding EDTA (pH 7.4, final concentration 1 mmol/L). 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 oxLDL-Induced Apoptosis of Macrophages**

A further 20-ml blood specimen was taken from each participant. A half volume of Nycodenz solution (NycosPrep 1.077; Aaxis-Shield, Oslo, Norway) was added and the sample was centrifuged at 400 $\times$ g for 30 min to isolate the mononuclear cells. Mononuclear cells were collected and resuspended in RPMI-1640 containing 10% fetal bovine serum (FBS) and then incubated on an MSP-P plate (JIMRO, Tokyo, Japan) for 1 h at 37°C. Nonadherent cells were removed by washing the plate 3 times with PBS (150 mmol/L NaCl, 10 mmol/L phosphate buffer, pH 7.2). To remove the adherent monocyte-macrophage layer, a solution containing 2 mmol/L EDTA and 10% FBS was added to the plate. After incubation at 4°C for 30 min, adherent monocyte-macrophages (>90% pure as judged by nonspecific esterase staining) were collected and cultured in RPMI-1640 medium 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. Nonadherent cells were removed by washing the plate 3 times with PBS (150 mmol/L NaCl, 10 mmol/L phosphate buffer, pH 7.2). To remove the adherent monocyte-macrophage layer, a solution containing 2 mmol/L EDTA and 10% FBS was added to the plate. After incubation at 4°C for 30 min, adherent monocyte-macrophages (>90% pure as judged by nonspecific esterase staining) were collected and cultured in RPMI-1640 medium containing 10% heat-inactivated FBS, 2 mmol/L L-glutamine and penicillin (100 units/ml) streptomycin (100 $\mu$g/ml) for 3 days. Morphologic 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 $\mu$g 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 (Trevenig, Gaithersburg, MD, USA) and counted with a fluorescence activated cell sorter (Becton Dickinson, Franklin Lakes, NJ, USA) using CellQuestPro software.

**Incubation of Macrophages With oxLDL and Lecithinized SOD**

Lecithinized SOD is known to transfer into the cytosol and mitochondria, and protects against apoptosis.\(^14\) Lecithinized SOD was prepared as previously reported.\(^15\) Purification was performed using ion-exchange column chromatography with Q-Sepharose FF (Pharmacia LKB Biotechnology Inc., Piscataway, NJ, USA) and isoelectrofocusing was used to identify the target protein. Macrophages taken from the 69 healthy participants were exposed to oxLDL and lecithinized SOD (final concentra-
MnSOD Activity and Macrophage Apoptosis

Table. Baseline Characteristics

<table>
<thead>
<tr>
<th></th>
<th>Alanine/alanine (n=3)</th>
<th>Alanine/valine (n=19)</th>
<th>Valine/valine (n=47)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Male, % (n)</td>
<td>66.7 (2)</td>
<td>68.4 (13)</td>
<td>72.3 (34)</td>
</tr>
<tr>
<td>Age (years)</td>
<td>30.3±10.4</td>
<td>29.1±7.5</td>
<td>30.8±8.6</td>
</tr>
<tr>
<td>Body mass index</td>
<td>22.1±1.9</td>
<td>21.3±3.1</td>
<td>21.9±2.1</td>
</tr>
<tr>
<td>Total cholesterol (g/dl)</td>
<td>166.7±24.4</td>
<td>172.7±20.2</td>
<td>164.5±24.6</td>
</tr>
<tr>
<td>HDL-cholesterol (g/dl)</td>
<td>59.3±12.9</td>
<td>65.5±8.4</td>
<td>60.5±9.8</td>
</tr>
<tr>
<td>LDL-cholesterol (g/dl)</td>
<td>90.3±11.7</td>
<td>93.4±17.7</td>
<td>91.5±18.6</td>
</tr>
<tr>
<td>Triglycerides (g/dl)</td>
<td>96.7±8.4</td>
<td>100.6±11.9</td>
<td>104.6±17.5</td>
</tr>
<tr>
<td>Uric acid (g/dl)</td>
<td>5.1±0.4</td>
<td>5.4±0.9</td>
<td>5.3±1.2</td>
</tr>
<tr>
<td>Glucose (g/dl)</td>
<td>95.3±5.6</td>
<td>95.0±7.6</td>
<td>94.5±5.8</td>
</tr>
<tr>
<td>HbA1c (%)</td>
<td>4.5±0.6</td>
<td>4.4±0.6</td>
<td>4.2±0.5</td>
</tr>
<tr>
<td>Leukocytes (×10^9/μl)</td>
<td>5.0±1.9</td>
<td>5.2±1.1</td>
<td>5.2±1.0</td>
</tr>
<tr>
<td>Monocytes (μl)</td>
<td>248±82</td>
<td>247±51</td>
<td>257±47</td>
</tr>
</tbody>
</table>

HDL, high-density lipoprotein; LDL, low-density lipoprotein. Values shown are mean±SD.

Figure 1. Association of mitochondrial manganese superoxide dismutase (MnSOD) activity and age in: (A) all participants (n=69); (B) the valine/valine genotype group (n=47); and (C) the non-valine/valine genotype groups (n=22), ie, the alanine/alanine genotype (n=3) and alanine/valine genotype (n=19).

Statistical Analysis

Quantitative data are presented as mean±SD and the categorical data as frequencies (%). Continuous variables were compared using Student’s unpaired t-test. Binary variables were compared using Fisher’s exact test and the variables...
comprising more than 2 categorical factors were compared using chi-squared test. 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**

**Baseline Characteristics**

Baseline characteristics of study population are shown in Table. The distribution of each genotype was comparable to that previously reported: alanine/alanine 4.3% (n=3), alanine/valine 27.5% (n=19) and valine/valine 68.2% (n=47). The mean age of participants in each genotype group was 30.3 ± 10.4 years for the alanine/alanine genotype group, 29.1 ± 7.5 years for the alanine/valine genotype group and 30.8 ± 8.6 years for the valine/valine genotype group. There were no significant differences in the characteristics between the 3 genotype groups.

**Association of Aging and Mitochondrial MnSOD Activity in the Leukocytes**

The mitochondrial MnSOD activity of each genotype group was as follows: 397.6 ± 97.1 units/mg protein (alanine/alanine), 305.1 ± 79.8 units/mg protein (alanine/valine) and 246.3 ± 68.2 units/mg protein (valine/valine). In all participants, mtMnSOD activity was inversely correlated with age (Spearman coefficient –0.713, P<0.0001, Figure 1A). Correlation of mtMnSOD activity was also found both in the valine/valine genotype group (Spearman coefficient –0.788, P<0.0001, Figure 1B) and in the non-valine/valine genotype groups, ie, alanine/alanine and alanine/valine genotypes (Spearman coefficient –0.627, P=0.0041, Figure 1C).

**Association of Aging and oxLDL-Induced Apoptosis of Macrophages**

After a 4-h incubation with oxLDL, the percentage of apoptotic macrophages correlated with age in all participants (Spearman coefficient 0.491, P<0.0001, Figure 2A). Correlation was also found between the percentage of apoptotic macrophages and age in both the valine/valine genotype group (Spearman coefficient 0.689, P<0.0001, Figure 2B) and in the non-valine/valine genotype groups, ie, alanine/alanine and alanine/valine genotypes (Spearman coefficient –0.627, P=0.0041, Figure 2C). Therefore, the percentage of apoptotic macrophages after incubation with oxLDL was inversely related to mtMnSOD activity in all participants (Spearman coefficient –0.677, P<0.0001, Figure 3A), in the valine/valine genotype group (Spearman coefficient –0.690, P<0.0001, Figure 3B), and in the non-valine/valine genotype groups (Spearman coefficient –0.627, P=0.0041, Figure 3C).
Figure 3. Association of patient age with the tolerance of macrophages against oxidized low density lipoprotein-induced apoptosis in: (A) all participants (n=69); (B) the valine/valine genotype group (n=47); and (C) the non-valine/valine genotype groups (n=22), ie, the alanine/alanine genotype (n=3) and alanine/valine genotype (n=19). *Stained with Annexine V. MnSOD, manganese superoxide dismutase.

Figure 4. Correlation between mitochondrial manganese superoxide dismutase (MnSOD) activity in leukocytes and macrophages. Measurements were made using blood samples obtained from 10 participants with alanine/alanine genotype (n=1), alanine/valine genotype (n=2) and valine/valine genotype (n=7).
Correlation Between mtMnSOD Activity in Leukocytes and Macrophages
The correlation of mtMnSOD activity in leukocytes and macrophages is shown in Figure 4. Thus, we confirmed that there was a good relationship between the activity of mtMnSOD in leukocytes and that in macrophages (Spearman coefficient 0.964, P=0.0038).

Cytoprotective Effect of Lecithinized SOD Against oxLDL-Induced Apoptosis
After incubation of the macrophages obtained from the 69 healthy participants with oxLDL alone for 4h, the percentage of apoptotic macrophages (those stained with propidium iodide and annexin V) was 77.6±12.4%. In contrast, after incubation of the macrophages with oxLDL with or without lecithinized SOD, the percentage of apoptotic cells was significantly lower in the cells not treated with lecithinized SOD (44.7±7.3%, P<0.0001, Figure 5A). Incubation with lecithinized SOD reduced the percentage of apoptotic macrophages both in the valine/valine genotype group compared with those not treated with lecithinized SOD (84.3±6.5% vs 48.2±4.8%, P<0.0001, Figure 5B) and in the non-valine/valine genotype group (63.3±9.5% vs 37.1±6.0%, P<0.0001, Figure 5C).

As with the 10 participants from whom macrophages were obtained, incubation with lecithinized SOD significantly increased the mitochondrial MnSOD activity in leukocytes compared with those not incubated with lecithinized SOD regardless the treatment with oxLDL (Figure 5D). Thus, the tolerance of macrophages against oxLDL-induced apoptosis depended on the SOD levels within the cells.

Discussion
The major findings of our study are that both the activity of mtMnSOD and the tolerance of macrophages against apoptosis induced by oxLDL decrease with age. Considering that lecithinized SOD reduced the oxLDL-induced apoptosis of macrophages, it could be construed that the reduction of the tolerance of macrophages against oxLDL with aging is mediated by the reduction of mtMnSOD activity.

We measured mtMnSOD activity using leukocytes, while
the percentage of apoptotic cells was counted using macrophages. We used the different cell types for the analysis because it is technically difficult to directly measure mtMnSOD activity in macrophages. To measure mtMnSOD activity in macrophages, a blood sample of approximately 200 ml must be taken from each participant. Thus, we measured mtMnSOD activity using leukocytes taken from all participants and confirmed the validity of the analysis by investigating the correlation between mtMnSOD activity in leukocytes and macrophages using blood samples taken from 10 participants who consented to providing a larger blood sample (Figure 4). Although the number of participants is small, we demonstrated that mtMnSOD activity in leukocytes has a good correlation with that in macrophages regardless of the genotype.

Aging is known as an independent risk factor for atherosclerosis. But the mechanisms whereby aging promotes atherosclerosis have never been clarified. Oxidative stress plays an important role in atherosclerosis. Several studies have suggested that oxidative stress increases with age and compromises the function of the vascular wall cells. Our data suggest that the activation of mtMnSOD decreases with age and reduces the tolerance of vascular wall cells against oxLDL, which, in turn, could promote atherosclerotic diseases such as coronary artery disease. In addition to the increase in the production of the reactive oxygen species that damage the vascular wall cells, the reduction of the endogenous antioxidant systems with aging may decrease the tolerance against such stress, thereby promoting atherosclerosis.

It has been previously reported that SOD activity decreases in older animals. In the present study, we showed that mtMnSOD activity decreases with age. But the mechanisms of age-induced reduction of mtMnSOD were not addressed in the present study. Several studies have shown that antioxidant enzyme gene expression and activity are lower in older animals compared with younger animals, and upregulation of antioxidant enzyme gene expression fails in an age-accelerated atherosclerosis animal model. Although we did not investigate potential changes in the regulation of the MnSOD gene, MnSOD gene expression may also be modified by aging.

Next, in our study, incubation of macrophages with lecithinized SOD increased mitochondrial SOD activity and the tolerance of macrophages against oxLDL-induced apoptosis. So, the tolerance of macrophages against oxLDL-induced apoptosis depends on the SOD levels within the cells. The effects of aging are usually considered to be irreversible, but lecithinized SOD may prove to be a possible intervention to retard acceleration of arterial aging.

Several studies have suggested that cardiovascular events including atherosclerosis may be induced by aging via multiple pathways. Here we showed that aging is associated with the reduction of activity of only one of the various antioxidant enzymes. In addition, we investigated the association of aging with the tolerance of macrophages against oxLDL. But the relationship of aging with the tolerance of endothelial cells or smooth muscle cells was not investigated in the present study because of the difficulty of collecting endothelial cells or smooth muscle cells from human participants. Further investigation is necessary to fully clarify the role of aging in atherosclerosis.

**Conclusion**

We showed that the activity of MnSOD in mitochondria and the tolerance of macrophages against apoptosis induced by oxLDL decreases with age. Our data may provide an important clue to clarify the mechanisms of age-induced atherosclerosis.

**References**


