Contribition of an imbalance between oxidant–antioxidant systems to plaque vulnerability in patients with carotid artery stenosis

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Object. Reactive species of oxygen and nitrogen mediate the oxidative modification of low-density lipoprotein (LDL). Oxidation of LDL is inhibited by endogenous radical scavenging enzymes such as manganese superoxide dismutase (SOD) and Cu-ZnSOD that catalyze dismutation of oxygen to H2O2. Low-molecular antioxidants such as uric acid regulate the inactivation that appears to be linked to an increase in peroxynitrite resulting in oxidized LDL (OxLDL) elevation. The authors evaluated whether a focal imbalance between pro- and antioxidant systems induces plaque vulnerability in patients with carotid artery (CA) stenosis.

Methods. Carotid artery plaques obtained in 35 patients who had undergone carotid endarterectomy were classified as vulnerable or stable based on histopathological findings. In vulnerable plaques, OxLDL, measured using enzyme-linked immunosorbent assay, was significantly higher (p < 0.01) and SOD activity significantly lower than in stable plaques (p < 0.05). The plaque and plasma OxLDL levels were inversely correlated with plaque SOD activity (p < 0.01). The physiological uric acid level in all plaques was one fourth to one eighth of that in plasma and appeared to be unable to protect Cu-ZnSOD from degradation by H2O2. Immunohistochemical analysis showed increased peroxynitrite and OxLDL in vulnerable plaques. There was a significant correlation between plaque and plasma OxLDL levels (p < 0.01).

Conclusions. Analysis of the results suggests that a focal imbalance between pro- and antioxidant defense systems in patients with CA plaques induces an increase in plaque OxLDL levels and consequent plaque instability, contributing to high levels of plasma OxLDL.

KEY WORDS • vulnerable carotid artery plaque • oxidized low-density lipoprotein • superoxide dismutase

THE incidences of cerebral atherosclerotic infarctions has increased in developed countries including Japan.26 An understanding of the pathogenesis of atherosclerotic plaque is important for the development of effective therapies for cerebrovascular diseases. Several types of reactive species derived from oxygen (ROS; O2, H2O2, and OH-) or nitrogen (RNS; ONOO- and NO) are generated in the body as a result of metabolic reactions; these are called prooxidants.35 Another category of compounds, antioxidants, counteracts the effects of prooxidants. These compounds are produced endogenously or received from exogenous sources and include enzymes such as SOD, catalase, glutathione peroxidase and reductase, minerals, vitamins, bilirubin, and uric acid.5,7,28 In the healthy body, pro- and antioxidants maintain a ratio, and a shift in this ratio toward prooxidants gives rise to oxidative stress. Both ROS and RNS mediate the oxidative modification of LDL.29 The uptake of OxLDL via scavenger receptors and the interaction of macrophages with T cells and vascular cells through cytokines can lead to increased macrophage activation and the generation of ROS. Therefore, OxLDL may be a consequence of increased general or local oxidative stress and a cause of increased intracellular oxidative stress.32 In the vascular wall OxLDL plays an important role in the pathogenesis of atherosclerosis; however, the nature of OxLDL in vivo has remained unclear until recently.13,23,33,36 Itabe and coworkers13,19 developed a method to determine OxLDL in human plasma samples in which they used sandwich ELISA and anti-OxLDL mAb. Using their method, we first demonstrated that the OxLDL content in CA plaques is approximately 70-fold higher than in plasma and that the increase of OxLDL in plaques and plasma is associated with plaque vulnerability.20 Analysis of our previous results suggested that a focal imbalance between oxidant and antioxidant defense systems induces an increase in OxLDL, resulting in high levels of plasma OxLDL and plaque vulnerability.

Superoxide dismutases can regulate redox signals modulated by ROS and RNS. The local steady-state of oxygen

Abbreviations used in this paper: CA = carotid artery; CEA = carotid endarterectomy; EDTA = ethylenediaminetetraacetic acid; ELISA = enzyme-linked immunosorbent assay; IgG = immunoglobulin G; LDL = low-density lipoprotein; mAb = monoclonal antibody; NO = nitric oxide; OxLDL = oxidized LDL; PBS = phosphate-buffered saline; RNS = reactive nitrogen species; ROS = reactive oxygen species; SD = standard deviation; SOD = superoxide dismutase.
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depends on both the rate of superoxide production and the activity of endogenous SODs, cytosolic and extracellular Cu-ZnSOD,4 and mitochondrial MnSOD that catalyze dismutation of oxygen to \( \text{H}_2\text{O}_2 \). It has been shown that Cu-ZnSOD, but not MnSOD, is inactivated by \( \text{H}_2\text{O}_2 \). Hink, et al.,19 suggested that low-molecular antioxidants such as uric acid regulate this inactivation. Because the oxygen scavenged by SOD competes with NO for oxygen,21 we postulate that the reduction in SOD activity promotes the interaction with NO for oxygen, leading to an increase of peroxynitrite and OxLDL.

To date, few investigations have been conducted to evaluate the enzymatic antioxidant defenses involved in human CA plaques. In the present study we provide evidence of an imbalance between pro- and antioxidant systems in patients requiring CEA.

Materials and Methods

Prior informed consent was obtained from all study participants. Carotid artery plaque material and plasma samples obtained in 35 patients who underwent CEA at the University of Tokushima Hospital between February 2001 and March 2002 were examined. Samples were acquired in 32 men and three women whose mean age was 71.3 ± 6.1 years (mean ± SD). All CEAs were performed according to the criteria established by the North American Symptomatic Carotid Endarterectomy Trial1 and the Asymptomatic Carotid Atherosclerosis Study.2 In all symptomatic patients surgery was performed during the chronic stage more than 4 weeks after the last symptom onset. Table 1 provides a summary of the clinical data.

Control plasma was obtained in 19 age-matched healthy volunteers (nine men and 10 women who ranged in age from 34 to 74 years (mean 61.2 ± 9.6 years) with no history of ischemic vascular disease and no risk factors. In addition, nine CA samples were removed at autopsy in nonage-matched patients who ranged from 41 to 69 years at the time of death. In these samples there was no macroscopic evidence of atherosclerosis. The intima was dissected carefully for examination, immediately immersed in PBS buffer containing proteinase inhibitors and EDTA-2Na in the autopsy room, and examined using the same procedures for analysis as the CEA-treated plaques.

Carotid Artery Plaque and Plasma Samples

Carotid artery plaques obtained in patients undergoing CEA were immediately immersed in PBS buffer (pH 7.4) containing proteinase inhibitors and EDTA-2Na and kept until needed. For immunohistochemical studies the most severe portion of the plaque was excised and fixed. The remaining portion was weighed and homogenized; one part was processed for LDL isolation, and the other was stored at −80°C until use. Venous blood was drawn into tubes containing EDTA-2Na and separated by centrifugation at 4°C.

Isolation of LDL

Low-density lipoprotein isolation was performed by potassium bromide stepwise density-gradient ultracentrifugation; standard OxLDL was prepared by incubating LDL with 5 μM CuSO₄, at 37°C for 3 hours, and anti-OxLDL mAb was prepared as described previously.10,19

Determination of Plasma OxLDL Levels

The plasma OxLDL level was measured using the commonly accepted procedure described in our previous study.7,8,23,30,41 Briefly, sandwich ELISA was performed using mAb against oxidized phosphatidylcholine (POH1a/DLH3; DLH3) and apoB IgG antibody (Boehringer Mannheim, Pleasanton, CA) was performed.18 The complex was detected by phosphatase-conjugated donkey anti-sheep IgG antibody (Chemicon, Inc., Pittsburgh, PA) and visualized by incubating with substrate solution containing 1 mg/ml of disodium p-nitrophenyl-phosphate hexahydrate (Wako, Osaka, Japan). Absorbance was measured at 405 nm. Simultaneously, a parallel set of ELISA testing was conducted to determine the amount of apoB in the same lipoprotein fractions by using anti-apoB mAb (OEM, Egg Harbor, NJ). The OxLDL levels were expressed as the amount of OxLDL per microgram of apoB protein.

Determination of the Level and Activity of MnSOD, Cu-ZnSOD, and Uric Acid

Thawed, homogenized CA plaques were sonicated and centrifuged. The supernatant was used after filtration with Microcon YM-10 (Millipore, Billerica, MA), as necessary. The protein level was determined using a BCA kit (Pierce Biotechnology, Woburn, MA); the MnSOD and Cu-ZnSOD levels were determined by ELISA (Amersham Bioscience, Piscataway, NJ). Total SOD activity and uric acid levels in plaques and plasma were measured using the nitrite method described by Elsner and Heupel15 and a commercial kit (Wako).

Immunohistochemical Analysis

Serial paraffin-embedded sections were immunohistochemically stained using mouse mAb against macrophages (HAM-56; Dako, Kyoto, Japan), mouse mAb against OxLDL (DLH1), rabbit immunoaffinity purified IgG against peroxynitrite (antinitrotyrosine) and mouse mAb against leukocytes (CD66; Dako). For signal detection we used the Histofine simple stain MAX-PO (Nichirei, Tokyo, Japan) or fluorescence probes (Alexa Fluor 488 and 594; Moleculer Probes, Inc., Eugene, OR) as a secondary antibody. Slides were inspected under a fluorescence microscope (IX71–22 TFL; Olympus, Tokyo, Japan). Negative control slides were prepared by omitting the primary antibodies. Macrophage infiltration and the lipid core size were measured using a commercial image analysis program.

According to the criteria we have reported elsewhere19 and those described by Naghavi, et al.,22 we classified the CA plaques as vulnerable or stable based on the level of macrophage infiltration (> 5% of total area) and on histopathological findings that indicated that they were prone to rupture, manifested fibrous cap thinning, and had a large lipid core (> 10% of total area). Vulnerable plaques were significantly different from stable plaques with respect to their lipid-rich core, plaque rupture, fibrous cap thinning, and intraplaque hemorrhage (p < 0.001; Table 2). Although more patients with vulnerable plaques were symptomatic than those with stable plaques, the difference was not statistically significant (p = 0.08).

Statistical Analysis

Sequentially obtained data, expressed as the mean ± SD were analyzed using the Mann–Whitney U-test for two-group comparisons, analysis of variance, and Scheffe test. Correlations were examined by the Spearman rank correlation test. Statistical analyses were performed using a Macintosh computer running StatVie 5 (SAS Institute, Inc., Cary, NC) statistical software. Differences were considered statistically significant at a probability level less than 0.05.

<table>
<thead>
<tr>
<th>TABLE 1</th>
<th>Summary of characteristics in 35 patients who underwent CEA</th>
</tr>
</thead>
<tbody>
<tr>
<td>Factor</td>
<td>No. of Cases (%)</td>
</tr>
<tr>
<td>mean age ± SD (yrs)</td>
<td>71.3 ± 6.1</td>
</tr>
<tr>
<td>male/female</td>
<td>32:3</td>
</tr>
<tr>
<td>symptomatic/asymptomatic</td>
<td>23/12</td>
</tr>
<tr>
<td>risk factors</td>
<td></td>
</tr>
<tr>
<td>hypertension</td>
<td>25 (71.4)</td>
</tr>
<tr>
<td>smoking</td>
<td>24 (68.6)</td>
</tr>
<tr>
<td>hyperlipidemia</td>
<td>13 (37.1)</td>
</tr>
<tr>
<td>diabetes mellitus</td>
<td>11 (31.4)</td>
</tr>
<tr>
<td>ischemic heart disease</td>
<td>9 (25.7)</td>
</tr>
</tbody>
</table>
Results

We found no significant correlation between plasma lipid parameters and OxLDL levels in plasma. Moreover, the presence of risk factors appeared to have no effect on plasma and plaque OxLDL levels (data not shown).

Association Between OxLDL and SODs

Compared with patients with stable plaques and the controls, significantly higher plaque and plasma OxLDL levels were observed in patients with vulnerable plaques ($p < 0.01$; Fig. 1a and b). Plaque SOD activity was significantly lower in patients in whom plaques were vulnerable ($p < 0.05$; Fig. 1c). There was no difference among the three groups in terms of plasma SOD activity (Fig. 1d). Plaque SOD activity was inversely correlated with the plaque and plasma OxLDL level ($p < 0.01$; Fig. 2a and b), and there was a significant correlation between plaque and plasma OxLDL levels ($p < 0.01$; Fig. 2c). There was no significant correlation between the levels of OxLDL and SOD and clinical symptomatology; however, levels of plaque and 

TABLE 2
Summary of characteristics relating to CA plaques

<table>
<thead>
<tr>
<th>Factor</th>
<th>Vulnerable</th>
<th>Stable</th>
<th>p Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>no. of cases</td>
<td>16</td>
<td>19</td>
<td></td>
</tr>
<tr>
<td>histopathological findings</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>macrophage infiltration*</td>
<td>8.5 ± 4.4</td>
<td>2.8 ± 1.2</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>lipid core*</td>
<td>33.7 ± 13.4</td>
<td>9.5 ± 7.6</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>plaque rupture</td>
<td>10 (63)</td>
<td>1 (5)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>fibrous cap thinning</td>
<td>12 (75)</td>
<td>1 (5)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>intraplaque hemorrhage</td>
<td>14 (88)</td>
<td>8 (44)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>calcification</td>
<td>8 (50)</td>
<td>16 (84)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>ulcer</td>
<td>12 (75)</td>
<td>10 (56)</td>
<td>NS</td>
</tr>
<tr>
<td>clinical findings</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>symptomatic</td>
<td>13 (81.3)</td>
<td>10 (52.6)</td>
<td>NS</td>
</tr>
<tr>
<td>echolucent</td>
<td>4</td>
<td>4</td>
<td>NS</td>
</tr>
<tr>
<td>echorich</td>
<td>2</td>
<td>7</td>
<td>NS</td>
</tr>
<tr>
<td>angiographic stenosis*</td>
<td>80.9 ± 13.8</td>
<td>77.8 ± 14.0</td>
<td>NS</td>
</tr>
</tbody>
</table>

* Values (% of total area) are presented as the mean ± SD.

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Fig. 1. Bar graphs. The OxLDL (a and b) and SOD (c and d) in patients with CA plaques and in controls. a and b: In patients with vulnerable plaques (black bar), plaque and plasma OxLDL levels were significantly higher than in those with stable plaques (gray bar) and in the controls (white bar). c: In patients with vulnerable plaques, total SOD activity was significantly lower than in those with stable plaques and in the controls. d: The total plasma SOD activity was not significantly different among three groups. Data are presented as the mean ± SD. *$p < 0.05$, **$p < 0.01$ (Scheffé test).
plasma OxLDL in 13 symptomatic patients with vulnerable plaque were significantly higher than in nine asymptomatic patients in whom plaques were stable (31.9 ± 19.4 and 0.26 ± 0.063 ng/µg of apoB compared with 3.96 ± 2.14 and 0.142 ± 0.036 ng/µg of apoB, respectively; p < 0.01). On the other hand, plaque SOD activity in symptomatic patients with vulnerable plaque was significantly lower than that in asymptomatic patients with stable plaque (48.2 ± 8.1 and 73.3 ± 6.6%, respectively; p < 0.01).

**Levels of SOD and Uric Acid in Plaques and Plasma**

Compared with the controls, the plaque MnSOD level was significantly higher in patients with vulnerable plaques but not in those with stable plaques (p < 0.05; Fig. 3a). Plaque Cu-ZnSOD was significantly lower in patients with vulnerable and stable plaques than in the controls (p < 0.05; Fig. 3b). In cases involving vulnerable plaques, the physiological uric acid level was one fourth to one eighth that measured in plasma; however, the difference between vulnerable and stable plaques was not statistically significant (Fig. 3c).

**Immunohistochemical Findings**

As shown in Fig. 4, the number of cells positive for anti-nitrotyrosine and DLH3 (OxLDL) was markedly increased in vulnerable plaques. Nitrotyrosine was observed in the media, intima, and core of the CA plaques (Fig. 4a). The OxLDL antigen was colocalized with nitrotyrosine in the core of vulnerable plaques (Fig. 4c). The CD66-positive cells were also colocalized with OxLDL-positive cells (data not shown).

**Discussion**

Oxidation of LDL is inhibited by natural (vitamins C and E) and synthetic antioxidants as well as by certain free radical scavenging enzymes such as SODs but not by catalase and hydroxyl scavengers. Despite the potential ability of antioxidants to modulate atherogenesis, there is little information regarding the antioxidant constituents of human atherosclerotic tissue. In this study we demonstrated that plaque OxLDL levels were significantly higher in vulner-
able than stable plaques and that SOD activity was lower in patients with vulnerable than stable plaques. Additionally, we found that plaque and plasma OxLDL was inversely correlated with plaque SOD activity and that there was a significant correlation between the plaques and plasma OxLDL. These results confirm that in patients with high OxLDL levels the antioxidant system is not sufficiently effective against oxidative stress. Our findings also suggest that a focal imbalance between pro- and antioxidants induces an increase in OxLDL, leading to plaque instability, and that elevated plasma OxLDL may be predictive of the presence of vulnerable plaques. A schematic illustration of our findings is presented in Fig. 5.

In cerebrovascular biology, Cu-ZnSOD is a key isoform because it accounts for 50 to 80% of total SOD activity. We found that total SOD activity and Cu-ZnSOD levels were decreased in vulnerable plaques. Cytoplasmic Cu-ZnSOD not only catalyzes the dismutation of oxygen to H$_2$O$_2$ but also has peroxidase activity—with H$_2$O$_2$ as a substrate it forms a Cu-bound hydroxy radical. Several lines of evidence suggest that this bound hydroxy attacks at least one adjacent histidine residue that binds Cu, leading to the loss of Cu and inactivation of the enzyme. Our results support the hypothesis that in the atherosclerotic prooxidant state, these enzymes are partially inactivated. The physiological uric acid level in plasma can prevent the H$_2$O$_2$-induced inactivation of Cu-ZnSOD. We found that there was no significant difference in plasma and plaque uric acid levels between patients with vulnerable and stable plaques and that in patients with both types of plaques the uric acid level in the plaque was one fourth to one eighth that of plasma. Our observations are consistent with those of others.
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Fig. 5. Schematics showing the relationship between the imbalance of oxidants–antioxidants and plaque instability. a: Vulnerable plaques harbor macrophages containing OxLDL and have a thin fibrous cap. Prooxidants, represented by OxLDL, predominate over antioxidants represented by SODs. b: Stable plaques harbor few macrophages, with little OxLDL accumulation and have a thick fibrous cap. Antioxidants, represented by SODs, predominate or are equal to prooxidants represented by OxLDL.

analysis of our data also suggests that the plaque uric acid is unable to prevent SOD degradation and the consequent increase in oxygen radicals.

Ballinger, et al., have provided insight into the possible role of MnSOD in CA disease. In response to oxidative stress MnSOD is upregulated because its promoter region contains response elements for the redox-sensitive transcription factors of many inflammatory-related genes. The upregulation of MnSOD may be a key compensatory response to oxidative stress. Like a stress protein in macrophages, MnSOD is induced in distinct regions of the atherosclerotic plaque as a consequence of increased oxidative stress and/or specific activation due to exposure to modified LDL. We found that the MnSOD level was increased in vulnerable plaques, although the total activity of SOD was decreased. The MnSOD activity was significantly lower in advanced- than early-stage plaques, and MnSOD-positive subendothelial cells manifested nuclear DNA strand breaks, suggesting apoptotic changes. Even in the presence of overexpressed MnSOD, SOD activity appears to be reduced in vulnerable plaques and SOD supplementation failed to protect subendothelial cells. This finding is not in conflict with ours because overexpressed SOD may promote the production of H$_2$O$_2$, which, in turn, may cause inactivation of SOD. Although the increased toxicity of oxygen may be attributable to an intracellular imbalance between catalase or glutathione peroxidase and SOD, in the present study we did not address these antioxidant enzymes.

Nitric oxide or derived RNS is a candidate oxidant that can modify LDL based on the extent of their interaction with ROS. We hypothesized that as SOD activity continues to decrease, oxygen can react more easily with NO to produce peroxynitrite and that this then results in an increase in OxLDL levels. Immunohistochemically, there was an increase in the numbers of cells positive for antinitrotyrosine and OxLDL in vulnerable plaques; we also noted the colocalization of both antigens in the plaque core. This finding suggests that once the oxidative status overwhelms the antioxidative defense system the oxidation progresses rapidly.

We defined the vulnerable plaque based on the level of macrophage infiltration. Because vulnerable plaques exhibited a thin fibrous cap and a higher incidence of rupture, we expected patients in whom these vulnerable plaques were present to be symptomatic at a higher rate than those with stable plaques. Although this was the case (81.3 and 52.6%, respectively), the difference was not significant, and large patient populations must be studied to determine whether there is a correlation between the status of the plaques and symptomatology. Studies are being conducted in our laboratory to identify factors that distinguish symptomatic from asymptomatic patients.
There are several limitations to our study. First, because we used autopsy specimens as a control, we cannot rule out the possibility of postmortem artifacts, the most likely of which would be artifactual oxidation of lipids. The control autopsy specimens were obtained soon after the patients died and immediately immersed in PBS buffer containing proteinase inhibitors and EDTA-2Na. If significant artifactual oxidation had occurred, contrary to our data, the OxLDL level in the autopsy specimens would be higher than that observed in the fresh CEA material. This suggests that our autopsy samples had undergone little postmortem change. Second, nitrotyrosine is generated not only from peroxynitrite but also via myeloperoxidase-H₂O₂-Cl⁻. Therefore, we cannot rule out that our histochemical findings reflected increased nitrotyrosine and OxLDL positivity due to myeloperoxidase-catalyzed oxidation, although activated human monocytes oxidize LDL in vitro predominantly via a process mediated by two-electron oxidants such as peroxynitrite. 

Oxidative stress in the vascular system is more complex than LDL oxidation alone. The administration of supplemental antioxidant vitamins may be hazardous in patients with heart disease, and most endogenous vitamins remain intact. Human atherosclerotic lesions contain both oxidized lipids and relatively large amounts of antioxidants. In early-stage lesions, some oxidation of vitamin E occurs, and the oxidation product oxidizes lipids by a two-electron oxidation reaction rather than acting as a radical-scavenging antioxidant that protects LDL lipids from oxidation. At a later stage, lipid oxidation becomes more prevalent and is not inhibited by vitamin E; in fact, oxidized lipid and intact vitamin E have been found to coexist. These findings explain why antioxidant supplements are unlikely to prevent lipid oxidation in the vessel wall.

The goal of treatment in patients with CA stenosis is stabilization of vulnerable plaques. Efforts are underway in our laboratory to identify genetic polymorphisms in ROS-generating systems or cellular antioxidants.

Conclusions

To the best of our knowledge, ours is the first study to demonstrate an imbalance in the oxidant–antioxidant system due to decreased SOD activity in vulnerable compared with stable CA plaques. In cases involving vulnerable CA plaques, this imbalance may induce high levels of plaque OxLDL, which in turn may play an important role in plaque instability and contribute to high levels of plasma OxLDL.

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References


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