Comparison between early and late carotid endarterectomy for symptomatic carotid stenosis in relation to oxidized low-density lipoprotein and plaque vulnerability

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Objective: **Although carotid endarterectomy (CEA), the gold standard in stroke prevention, has been performed in the late stage after the insult, its optimal timing remains unclear. Using biomarkers in plaque and plasma, we evaluated oxidative stress and plaque vulnerability between early and late CEA in symptomatic patients.**

Methods: **We compared symptomatic stroke patients who underwent early CEA within 4 weeks of the last insult (group A; n = 15) with those who received CEA in the late stage beyond 4 weeks from the last symptom (group B; n = 57). They were divided into vulnerable (group Av, n = 13; group Bv, n = 33) and stable (group As, n = 2; group Bs, n = 24) subgroups according to the pathologic findings on their plaques. We studied the relationships among their primary symptoms, clinical findings, oxidized low-density lipoprotein levels, and gelatinase A (matrix metalloproteinase [MMP]-9) activity in their plaques and plasma.**

Results: **Group A had a variety of symptoms; there was no difference in the outcome of CEA between groups A and B. The plaque and plasma oxidized low-density lipoprotein levels were higher in group A than in group B ($P < .05$). The incidence of pathologically vulnerable plaque was higher in group A than in group B. Plaque oxidized low-density lipoprotein levels and MMP-9 activity were similar in group Av and group Bv and were higher in those groups than in group As and Bs.**

Conclusions: **We first demonstrated that vulnerable plaques in patients subjected to early CEA manifested a remarkable increase in oxidized low-density lipoprotein and MMP-9 activation. Our findings suggest that early CEA may be beneficial in the aspect of oxidative stress. (J Vasc Surg 2007;46:870-5.)**

Several studies have shown the efficacy of carotid endarterectomy (CEA) in patients with symptomatic carotid artery stenosis.[1,2] The optimal timing of CEA after stroke or transient ischemic attack (TIA) remains controversial. Although Blaisdell et al[3] recommended a delay of 6 to 8 weeks between the insult and operation to reduce the risk of conversion of a bland to a hemorrhagic infarct, more recent studies suggested that such a delay is not necessary. Rothwell et al[4] indicated that the timing of operation is important and that the procedure should be performed within 2 weeks of the patient's last symptoms. Particularly among women with symptomatic stenosis, the benefit derived from CEA was apparent only in those randomized within 2 weeks of their last symptom; it decreased rapidly during the first few weeks.[5] Other papers also suggested that CEA might be performed within 2 to 4 weeks of TIA or stroke onset.[6,7]

Oxidized low-density lipoprotein plays a crucial role in the development of atherosclerosis.[8,9] In our earlier studies[10,11] we classified plaques from patients who underwent CEA in the late phase (beyond 4 weeks from their last symptoms) into macrophage-rich (vulnerable) and macrophage-poor (stable) plaques and demonstrated that patients with the vulnerable plaques manifested high plaque and plasma oxidized LDL levels.[11] We also demonstrated[12] an oxidant/antioxidant imbalance in these patients. Together, our results suggested that patients with vulnerable plaque were under severe oxidative stress.

In our recent series of patients, we performed CEA within 4 weeks of the last insult. We compared the primary symptoms, clinical findings, and biomarker levels in the plaques and plasma with those in patients who underwent CEA beyond 4 weeks from their last symptoms. We also assessed whether early CEA benefits symptomatic patients in the aspect of oxidative stress and plaque vulnerability.

SUBJECTS AND METHODS

A total of 112 patients underwent CEA at the University of Tokushima Hospital between February 2001 and December 2005. Among those patients, 72 consecutive symptomatic patients were included in this study. Prior informed consent was obtained from all study participants. Fifteen patients

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(group A, 14 men and 1 woman; average age, 65.0 years; range, 54-74 years) underwent CEA in the early stage, and 57 (group B, 47 men and 10 women; average age, 69.2 years; range, 38-82 years) were treated in the late phase. CEA was considered as "early" if performed within 4 weeks of the last symptoms and "late" if performed after a minimum of 4 weeks following the most recent symptom.

All CEAs were performed according to the criteria established by the North American Symptomatic Carotid Endarterectomy Trial (NASCET).[2] Magnetic resonance imaging and cerebral angiography were performed in all patients. Although patients admitted within 4 weeks of the last attack underwent CEA as soon as possible, the choice of the timing of operation was made by the individual surgeons from the patient's state of ischemic heart disease and the results of magnetic resonance imaging findings with or without large cerebral infarction and hemorrhagic transformation. There is some pre-existing institutional bias toward performing CEA in a late fashion in patients who have experienced large preoperative cerebral infarction as a result of the early reports.[3] The interval from the last symptom to CEA in group A patients was as follows: 1 to 2 weeks, 5 (33.3%) of 15; and 3 to 4 weeks, 10 (66.7%) of 15. In group B patients it was as follows: 5 to 6 weeks, 8 (14.0%) of 57; 7 to 8 weeks, 13 (22.8%) of 57; 9 to 16 weeks, 25 (43.9%) of 57; and 17 to 52 weeks, 11 (19.3%) of 57 (Fig 1).

The Table provides a summary of the clinical data of group A and B patients. In group A, the severity of neurologic symptoms at admission was as follows: seven (46.7%) had no neurologic deficit (TIA), seven (46.7%) had a nondisabling stroke (National Institutes of Health Stroke Scale [NIHSS] 0-4), and one (6.6%) had a moderately disabling stroke (NIHSS). In group B, 27 (47.4%) had no neurologic deficit (TIA), 27 (47.4%) had a nondisabling stroke (NIHSS 0-4), and 3 (5.2%) had a moderately disabling stroke (NIHSS). There was no significant difference in the demographics between groups.

In group A, fewer patients had diabetes mellitus than in group B, and there was no significant difference in the ratio of other risk factors: hypertension, 7 (46.7%) in group A vs 41 (71.9%) in group B; hyperlipidemia, 5 (30%) vs 23 (40.4%); diabetes mellitus, 0 vs 22 (38.6%); ischemic heart disease, 1 (6.7%) vs 16 (28.1%); and smoking, 9 (60%) vs 39 (68.9%), respectively.

Carotid plaque and plasma samples. Carotid plaques obtained at CEA were immediately immersed in phosphate-buffered saline (pH 7.4) containing proteinase inhibitors and ethylenediaminetetra-acetic acid-2Na. For immunohistochemical studies, we cut out and fixed the most severe portion of the plaque, which was the most severe stenotic portion at the cross-sectional distance and had the greatest lipid core at the common carotid artery bifurcation or just distal to the bifurcation in almost all cases. The remaining portion was weighed and homogenized in a 5× volume of phosphate-buffered saline of each plaque weight; the other was stored at −80°C until use. Venous blood was drawn into tubes containing ethylenediaminetetra-acetic acid-2Na and separated by centrifugation at 4°C.

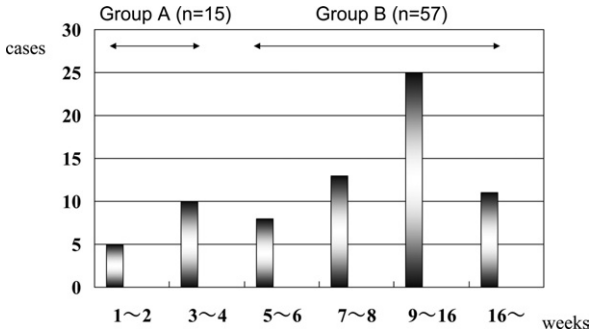

Fig 1. Distribution of the timing of carotid endarterectomy after the last neurologic symptoms in groups A and B.

Isolation of LDL. LDL isolation was by potassium bromide stepwise density-gradient ultracentrifugation; standard oxidized LDL was prepared by incubating LDL with 5 μmol/L $CuSO_4$ (37°C for 3 hours). Antioxidized LDL monoclonal antibodies (mAbs) were prepared as described previously.[13,14]

Determination of plasma oxidized LDL levels. The plasma oxidized LDL level was measured with a commonly accepted procedure[11,15-18] by using a sandwich enzyme-linked immunosorbent assay with mAbs against oxidized phosphatidylcholine (FOH1a/DLH3; DLH3) and apolipoprotein B immunoglobulin G antibody (Boehringer-Manheim, Ridgefield, Conn).[13,14] The complex was detected by phosphatase-conjugated donkey anti-sheep immunoglobulin G antibody (Chemicon, Temecula, Calif) and visualized by incubation 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 enzyme-linked immunosorbent assays was run to determine the amount of apolipoprotein B in the same lipoprotein fractions by using anti-apolipoprotein B mAb (OEM, Toms River, NJ). The oxidized LDL levels were expressed as the amount of oxidized LDL per microgram of apolipoprotein B protein.

Zymography. Gelatinase A (matrix metalloproteinase [MMP]-2; 72-kDa type IV collagenase) and B (MMP-9; 92-kDa type IV collagenase), members of the MMP family, are able to degrade components of the extracellular matrix. Gelatinase activity in the plaques was assessed zymographically. Thawed, homogenized carotid plaques were sonicated and centrifuged. The supernatant was used after the protein level was determined with the BCA kit (Pierce, Rockford, Ill). Each plaque sample was adjusted to a 10-μg protein level, and 0.5 mmol/L calcium chloride and 1 μmol/L zinc chloride were added to remove the effect of ethylenediaminetetra-acetic acid. The plaque sample was prepared for analysis by dilution in sample buffer consisting of 126 mmol/L Tris (pH 6.8), 4% sodium dodecyl sulfate, 20% glycerol, and 0.03% bromophenol blue and loaded onto gels. To standardize and normalize the gelatinase activity, we used gelatinase zymography standards for human MMP-2 and MMP-9 (Chemicon) with a molecular

marker (GE Healthcare, Tokyo, Japan) in each electrophoresis. After electrophoresis at 125 V, the gels were incubated in renaturing buffer for 60 minutes at room temperature and then for 24 hours at 37°C in a developing buffer containing 50 mmol/L Tris (pH 7.5), 200 mmol/L NaCl, 4 mmol/L $CaCl_2$, and 0.02% Brij 35. The gels were stained with 0.5% Coomassie blue G-250 in 30% methanol/10% acetic acid and then destained in 30% methanol/10% acetic acid. Gelatinase activity appears as unstained bands in the gel. Digestion was quantified on a gel scanner featuring an interface to the computer. The gels were analyzed with the aid of Image J software (National Institutes of Health), and the relative value was calculated against the standard. These experiments were individually repeated three times in each plaque sample.

Immunohistochemical analysis. Serial paraffin sections were immunohistochemically stained by using mouse mAb against macrophages (HAM-56; DAKO, Kyoto, Japan), DLH3, and rabbit polyclonal antibody against MMP-9 (Chemicon). For signal detection we used the Histofine simple stain MAX-PO (Nichirei, Tokyo, Japan) followed by diaminobentidine or a fluorescence probe (Alexa Fluor 488 and 594; Molecular Probes, Eugene, Ore) as a secondary antibody and observed by using a fluorescent microscope (IX71-22 TFL; Olympus, Tokyo, Japan). Negative control slides were prepared by omitting the primary antibodies. To determine macrophage infiltration and the size of the lipid core, we used an image-analysis program (Mac Scope, Mitani Co, Osaka, Japan). Carotid plaques were classified as vulnerable or stable according to the level of macrophage infiltration (≥5% of the total area)[10-12,19] by researchers (K.T.K., K.Y., and T.T.) who were not cognizant of either the plaque oxidized LDL levels or the patients' clinical data. Groups A (n = 15) and B (n = 57), operated on in the early or late phase, respectively, were subdivided according to whether the plaque was judged to be vulnerable (group v) or stable (group s).

Statistics. Sequentially obtained data, expressed as the mean ± SD, were analyzed with the Mann-Whitney *U* test for two-group comparisons and with analysis of variance followed by the Scheffé test for multigroup comparisons. Correlations were examined by the Spearman rank correlation test, the χ^2 test, and the Fisher test. Statistical analyses were performed with Stat View 5 (SAS Institute, Tokyo, Japan). Differences were considered statistically significant at $P < .05$.

RESULTS

Although two group A patients (13.3%) experienced transient post-CEA neurologic deterioration, recovery was immediate (within 24 hours after operation), and the mortality and morbidity rate was 0%. One of the 57 group B patients experienced hyperperfusion syndrome, with deterioration in his cognitive status; however, magnetic resonance imaging at discharge did not show a new lesion. Another patient experienced TIA. The mortality and morbidity rate in group B was therefore 3.5%.

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

Table. Summary of characteristics of patients in groups A and B

Variable	*Group A (n = 15)*	*Group B (n = 57)*
Age (y)	65.4 ± 7.0 (54-74)	69.2 ± 7.4 (38-82)
Men:women	14:1	47:10
Degree of ICA stenosis (%)	81.5 ± 14.6 (50-95)	83.8 ± 12.7 (60-99)
Clinical symptom		
TIA	7 (46.7%)	27 (47.4%)
Nondisabling stroke	7 (46.7%)	27 (47.4%)
Moderately disabling stroke	1 (6.6%)	3 (5.2%)

ICA, Internal carotid artery; *TIA,* transient ischemic attack.
Data are mean ± SD (range), ratios, or percentages.

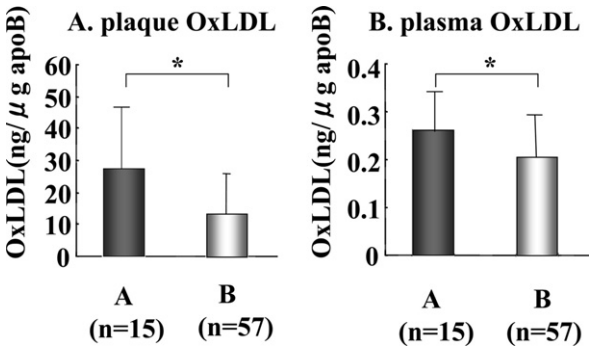

Fig 2. Plaques and plasma oxidized low-density lipoprotein *(OxLDL)* levels in group A and B patients. *$P < .05$.

The plaque and plasma oxidized LDL levels in group A (27.3 ± 18.5 ng/μg and 0.27 ± 0.08 ng/μg apolipoprotein B, respectively) were significantly higher than in group B (13.5 ± 12.8 ng/μg and 0.21 ± 0.08 ng/μg apolipoprotein B, respectively; $P < .05$; Fig 2). In group A, 13 plaques (86.7%) were vulnerable, and 2 (13.3%) were stable. In group B, 33 (57.8%) were vulnerable, and 24 (42.2%) were stable. This ratio of vulnerable to stable plaques was significantly different between groups ($P < .05$; Fig 3, *A*).

In our study population of 72 patients, the plaque oxidized LDL level in vulnerable plaques (n = 46) was significantly higher than that in stable plaques (n = 26). However, when we compared group Av and group Bv, we found that the plaque oxidized LDL level was not significantly different (Fig 3, *B*). Figure 3, *C* to *E*, presents our plaque zymography results. Although MMP-2 was present in all subgroups, only vulnerable plaques were positive for MMP-9 (Fig 3, *E*). There was no significant difference with respect to the activity of MMP-9 between groups Av and Bv (Fig 3, *D*), although it was greater in the vulnerable than the stable plaques (Fig 3, *D*).

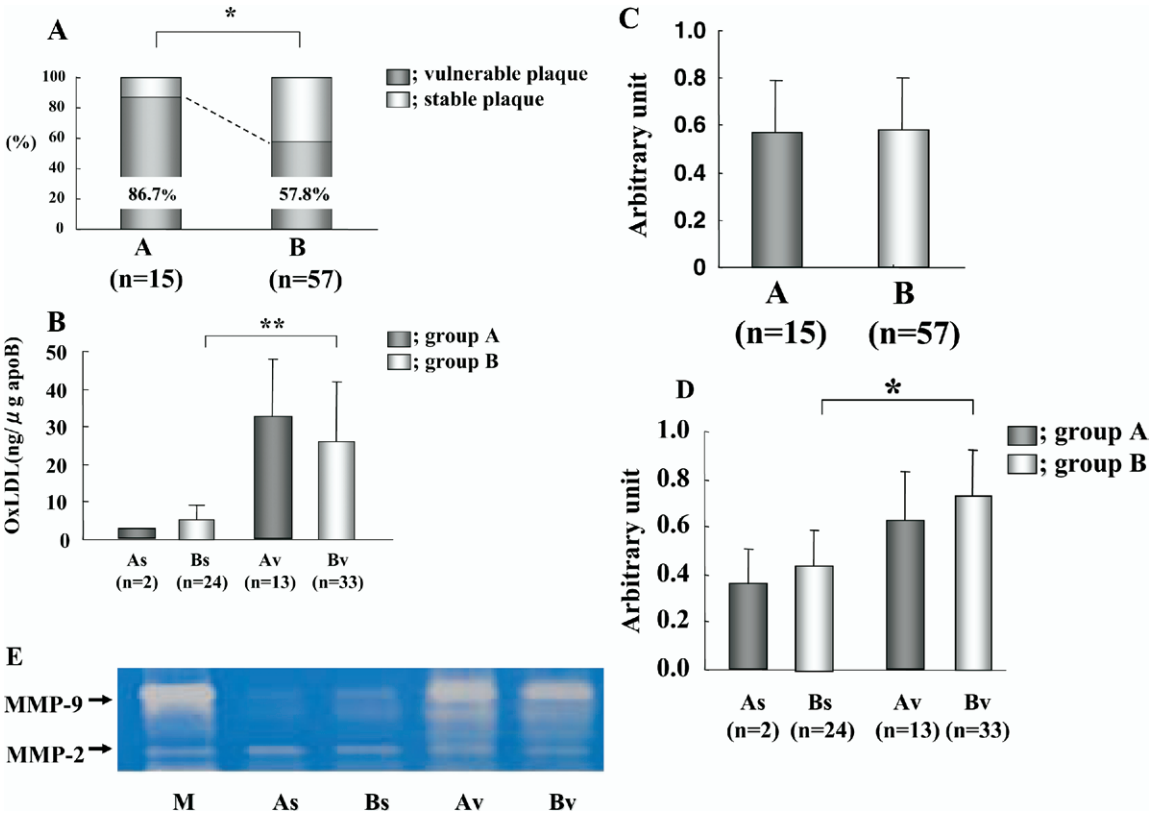

Fig 3. A, Ratio of vulnerable plaques in each group (*A,* group A; *B,* group B). *$P < .05$. **B,** Plaque oxidized low-density lipoprotein *(OxLDL)* level in each group. **$P < .01$. **C,** Plaque matrix metalloproteinase *(MMP)*-9 activity in groups A and B. **D,** Plaque MMP-9 activity in each group. *$P < .05$. **E,** Plaque zymography in each group. The individual three experiments were repeated in each plaque sample. MMP-9 was strongly positive in groups Av and Bv. *M,* maker (positive control).

Histopathologically, in both groups, vulnerable plaques were significantly different from stable plaques with respect to the lipid-rich core, rupture, fibrous cap thinning, and intraplaque hemorrhage. Immunohistochemically, oxidized LDL and MMP-9 antigen were colocalized (Fig 4). Moreover, MMP-9 and oxidized LDL were strongly positive in vulnerable compared with stable plaque (Figs 4 and 5). In the vulnerable plaque of group Av, the histopathologic changes were adjacent to the vascular lumen (Fig 4).

DISCUSSION

This is the first demonstration that the incidence of vulnerable plaques was significantly higher in patients who underwent CEA in the early stage (group A; 86.7%) than in those who were treated in the late stage (group B; 57.8%). Moreover, the plaque and plasma oxidized LDL levels were significantly higher in group A than group B.

Early CEA risks transforming a bland ischemic infarct into a hemorrhagic infarct,[3] whereas delaying CEA risks occlusion of the stenotic carotid artery, TIA, or stroke recurrence. Blaser et al[20] reported that 10.5% of patients with recent stroke were at risk of developing subsequent cerebral ischemia during a median follow-up period of 19 days. The reported benefits of early CEA[4,5] recommended it as a treatment for symptomatic patients with severe carotid artery stenosis. However, only few studies suggest that plaque vulnerability and oxidized stress are higher in the acute than in the late stage. According to Redgrave et al,[21] plaques removed earlier than 60 days after the most recent stroke were more unstable than those removed at more than 180 days after last recent stroke, thus suggesting that vulnerable plaques in the acute phase may become stable. Our finding that the incidence of vulnerable plaques was higher in group A than in group B may support this hypothesis.

Rothwell et al[5] reported that with respect to treatment outcome, there was a sex difference in the effect of time from symptom onset to CEA; the benefits derived by women decreased rapidly in the first few weeks, whereas men benefited even from delayed CEA.[5] They postulated that plaque in women was more frequently attributable to endothelial erosion than to plaque rupture. We cannot comment on this point because too few (n = 11; 15.3%) of our 72 patients were women, and 57.8% of all group B patients manifested vulnerable plaques.

The importance of oxidized LDL in the pathogenesis of atherosclerosis has been documented.[8,9] Oxidized LDL ex-

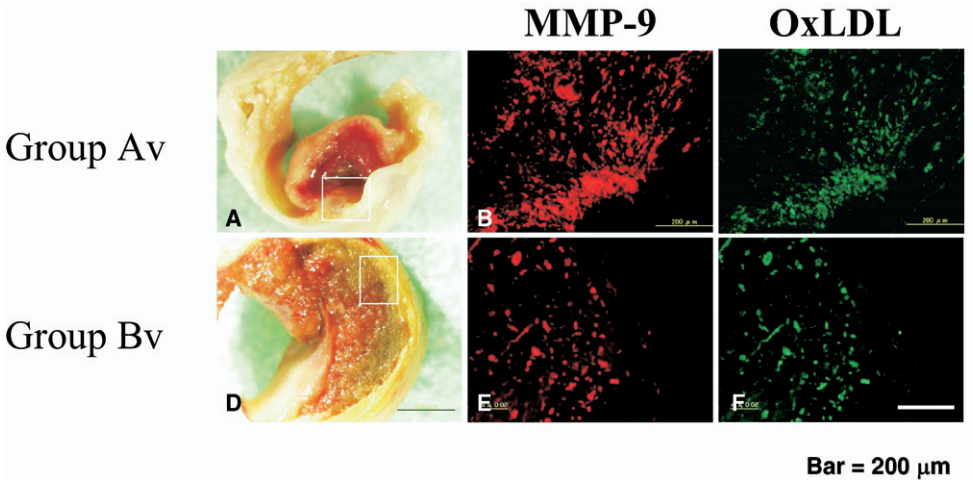

Fig 4. Matrix metalloproteinase *(MMP)*-9 and oxidized low-density lipoprotein *(OxLDL)* staining of vulnerable plaques in groups Av and Bv. In sequential specimens from each plaque sample, the immunostaining against each antigen was performed at least two times.

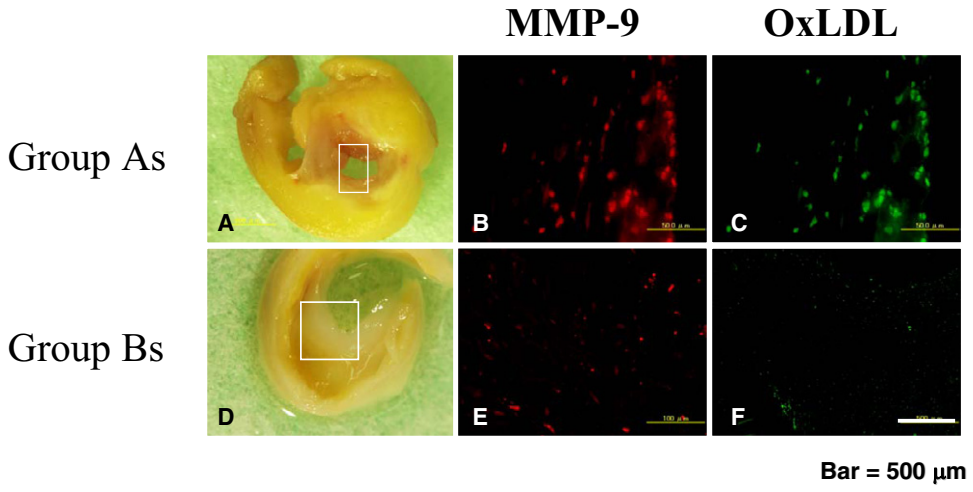

Fig 5. Matrix metalloproteinase *(MMP)*-9 and oxidized low-density lipoprotein *(OxLDL)* staining of stable plaques in groups As and Bs. In sequential specimens from each plaque sample, the immunostaining against each antigen was performed at least two times.

hibits a number of specific biological properties in vitro and in vivo, including inducing foam cell formation from macrophages. In our previous studies, we determined plasma and plaque oxidized LDL levels by sandwich enzyme-linked immunosorbent assay by using DLH3 and anti-apolipoprotein B antibodies and explored their correlation with carotid atherosclerosis.[11] We found that in patients with vulnerable plaque that was harvested more than 4 weeks after the last symptoms, the oxidized LDL levels were significantly higher than in the controls and patients with stable plaques, thus suggesting that plaque oxidized LDL is associated with plaque instability.[11] There was a strong correlation between plaque oxidized LDL levels and plaque instability. Our current findings confirm our earlier observations that higher oxidized LDL levels were correlated with plaque vulnerability in both the acute and late stages. Furthermore, in vulnerable plaques, high oxidized LDL expression was correlated with high MMP-9 activity.

Macrophages in human atherosclerotic plaques produce a family of MMP that may affect vascular remodeling and plaque disruption.[22] Because oxidized LDL upregulates MMP-9 expression,[23] it may contribute to a macrophage-mediated matrix breakdown in atherosclerotic plaques. In our series, the plaque MMP-9 activity did not differ significantly between group A and group B, but it was higher in vulnerable than in stable plaques. We previously demonstrated that an imbalance in the oxidant/antioxidant system correlated with plaque vulnerability.[12] The manganese superoxide dismutase gene is upregulated in response to oxidative stress, and, in fact,

increased plaque oxidized LDL was accompanied by an increase in MnSOD. Thus, the antioxidant system may contribute to the stabilization of vulnerable plaques, and in some cases, an ischemic episode may be followed by plaque healing and a relatively quiescent state in the late phase. Conversely, 57.8% of our symptomatic patients were exposed to high oxidant stress even in the late phase, thus suggesting that the persistent increase of oxidized LDL may lead to MMP-9 upregulation, rendering the plaque vulnerable during the late phase, and that this may lead to the induction of further ischemic attacks. Although we did not assess the balance of matrix degradation enzymes and their inhibitors in this study, a reduction in MMP-9 inhibitors such as tissue inhibitor of metalloproteinases 1 and 2 may be associated with plaque vulnerability.[23]

In conclusion, here we first demonstrated that vulnerable plaques in patients subjected to early CEA manifested a remarkable increase in oxidized LDL and MMP-9 activation. Our findings suggest that early CEA may be beneficial in the aspect of oxidative stress.

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AUTHOR CONTRIBUTIONS

Conception and design: AS, MU
Analysis and interpretation: HL, TT
Data collection: KN, KY
Writing the article: AS, MU
Critical revision of the article: MU, SN
Final approval of the article: MU, SN
Statistical analysis: MU, KTK
Obtained funding: MU, SN
Overall responsibility: MU, SN

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