Association of Interleukin-6 With the Progression of Carotid Atherosclerosis
A 9-Year Follow-Up Study

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Background and Purpose—Limited information is available on the long-term effects of interleukin-6 (IL-6) on systemic atherosclerosis. The purpose of the present study was to clarify the relationship between chronic elevation of IL-6 and the long-term progression of carotid atherosclerosis.

Methods—We prospectively evaluated 210 patients with ≥1 vascular risk factors for 9.0±1.0 years. Carotid mean-maximal intima-media thickness (mmIMT), the serum high-sensitivity C-reactive protein (hs-CRP) level, and the serum IL-6 level were measured at baseline and every 3 years. The associations between the progression of mmIMT and the long-term average levels of hs-CRP and IL-6 were analyzed.

Results—Carotid mmIMT increased throughout the study period (0.031±0.026 mm/y). Baseline mmIMT was significantly associated with baseline hs-CRP (P=0.002) and baseline IL-6 (P<0.001) levels. Progression of mmIMT was positively correlated with average hs-CRP (P=0.001) and average IL-6 (P<0.001) levels. When adjusted for age, sex, traditional risk factors, and baseline mmIMT, mmIMT progression remained significantly associated only with the average IL-6 level (standardized β=0.17; P=0.02), but not with the average hs-CRP level (standardized β=0.10; P=0.18).

Conclusions—Chronic elevation of serum IL-6 was associated with the progression of atherosclerosis in patients with vascular risk factors. IL-6 could be used as a quantitative marker and a potential therapeutic target for accelerated atherosclerosis. (Stroke. 2014;45:00-00.)

Key Words: atherosclerosis ■ follow-up studies ■ inflammation ■ interleukins ■ risk factors

Atherosclerosis is the main cause of cardiovascular disease, and preventing the progression of atherosclerosis is therefore important. However, in atherosclerotic high-risk patients, the conventional management of traditional risk factors is insufficient to prevent the development of atherosclerosis. Recent studies have shown that proinflammatory cytokines, such as interleukin-6 (IL-6), play a central role in the pathogenesis of atherosclerosis.1 IL-6 and its downstream signaling factors contribute to both atherosclerotic plaque development and plaque destabilization.2-3 Although regulation of IL-6 has been suggested as a therapeutic target in atherosclerosis,4,5 there is a lack of prospective data on the long-term effects of IL-6 on the progression of atherosclerosis. Here, we report the results of a 9-year follow-up study of carotid atherosclerosis in patients with vascular risk factors aimed at elucidating the association between the long-term progression of atherosclerosis and chronic elevation of IL-6 levels.

Materials and Methods

Subjects
The study subjects were recruited from participants in the Osaka Follow-up Study for Carotid Atherosclerosis part 2 (OSACA2), a multi-institutional prospective cohort study of atherosclerotic high-risk patients aged ≥40 years. The methods used for the recruitment of participants and the definitions of atherosclerotic risk factors have been described elsewhere.4 In brief, subjects were enrolled if they had ≥1 vascular risk factors such as hypertension, diabetes mellitus, dyslipidemia, or a current smoking status or a history of cardiovascular disease. Patients with collagen disease, malignant disease, and chronic inflammatory disease were excluded. If patients were scheduled for carotid endarterectomy or carotid artery stenting at enrollment, they were also excluded from the study. Of the 513 patients who were enrolled in the OSACA2 study at Osaka University Hospital between January 2001 and December 2003, 298 agreed to participate in this long-term single-center observational study. No significant differences in age, sex, body mass index, systolic and diastolic blood pressure values, serum cholesterol levels, and smoking rates were observed between participants and nonparticipants at enrollment. However, baseline glycosylated hemoglobin (HbA1c) level...
of participants was significantly lower than that of nonparticipants (5.4±0.9 versus 5.7±1.1%; *P*<0.002). Participants were followed up every 3 to 6 months at outpatient clinics in Osaka University Hospital until December 2011. Traditional risk factors were strictly controlled according to the updated guidelines during the follow-up period, and smoking cessation was strongly recommended for all smokers. Professional smoking-cessation programs and pharmacological treatments were also recommended to chronic smokers. Carotid ultrasonography and measurement of inflammatory markers were performed every 3 years. During the follow-up period, 18 patients died (including 4 cardiovascular deaths), 54 had relocated or did not wish to participate in the study, and 5 underwent carotid artery stenting or carotid endarterectomy. Of the remaining 221 participants, 11 were excluded because follow-up carotid ultrasonography examinations at 9 years were not performed. Consequently, all analyses were based on 210 participants. The study was approved by the Ethics Committee of Osaka University Graduate School of Medicine. All participants provided written informed consent.

**Measurement of Carotid Atherosclerosis**

Carotid ultrasonography investigations were performed at baseline and at 3, 6, and 9 years. We used mean-maximal intima-media thickness (mmIMT) as a marker of systemic atherosclerosis. The mmIMT was measured by high-resolution B-mode sonography using a SONOS 5500 (Philips) equipped with a 7.5-MHz linear-array transducer. A maximal IMT was measured as the distance between the luminal-intimal interface and the medial-adventitial interface including atheromatous plaque. The maximal IMT was manually measured in 12 segments (the near and far walls of the right and left common carotid arteries, carotid bifurcations, and internal carotid arteries) on a longitudinal image, and the mmIMT was calculated by averaging the 12 measurements (Figure 1). All measurements were performed by expert stroke neurologists who were blinded to patient data. Intraobserver correlation between repeated mmIMT measurements in 36 patients was 0.98, with similar averages for the 2 sets of readings (1.45±0.66 versus 1.45±0.62 mm, difference not significant). Interobserver correlation for 47 patients was 0.94, with similar mmIMT averages (1.27±0.43 versus 1.25±0.43 mm, difference not significant). The progression of carotid atherosclerosis was defined as the difference between the last and the first mmIMT measurement and was normalized as the annual change of mmIMT. The degree of carotid stenosis was assessed according to the European Carotid Surgery Trial (ECST) criteria.

**Physical and Laboratory Examinations**

Blood pressure and body mass index were measured in outpatient clinics at baseline and at every follow-up visit. Fasting venous blood samples were collected at baseline and every 3 years for acquisition of biochemical data and measurement of the levels of inflammatory markers such as high-sensitivity C-reactive protein (hs-CRP) and IL-6. Blood was centrifuged at 3000 rpm at 4°C for 15 minutes, and serum was stored at ~80°C. Circulating hs-CRP was measured by the latex turbidimetric immunoassay with a sensitivity of 0.01 mg/dl (SRL Inc, Tokyo, Japan). Serum IL-6 was measured by ELISA (High-sensitivity Quantikine Kit, R&D Systems Inc, Minneapolis, MN). The detection limit for IL-6 was 0.10 pg/mL, the intra-assay variation was 7.8%, and the corresponding interassay coefficient was 7.2%.

**Statistical Analysis**

All values are expressed as mean±SD, median and interquartile range, or counts and percentages. Because distributions of hs-CRP and IL-6 levels seemed to be left skewed, they were normalized by log10 transformation. Relationships between mmIMT progression and continuous variables were examined by Pearson correlation analysis. For categorical variables, differences in mmIMT progression were examined using an unpaired *t* test. To assess trends in the longitudinal changes of carotid mmIMT and inflammatory markers, *P* for trend was calculated by a linear regression method in which follow-up period (0, 3, 6, and 9 years) was a continuous variable. Multiple linear regression analysis was performed to examine the association between mmIMT progression and inflammatory markers. We sequentially introduced groups of variables into the model, (1) age and sex, (2) conventional atherosclerotic risk factors such as body mass index, diastolic blood pressure, estimated glomerular filtration rate, low-density lipoprotein cholesterol level, HbA1c level, and use of statins, and then (3) baseline IMT, because these are known to be associated with inflammation and progression of atherosclerosis.11–15 Multicollinearity was assessed using the variance inflation factor. A variance inflation factor >10 is regarded as indicating serious multicollinearity, and values >4.0 may be a cause for concern. If a patient used a medicine for more than half of the follow-up period, the patient was categorized as a user of the medication. All analyses were performed using JMP Pro 10.0 software (SAS Institute Inc, Cary, NC). Probability values were 2-tailed, and *P*<0.05 was considered significant.

**Results**

The baseline and follow-up characteristics of the 210 study participants are listed in Table 1. All patients had ≥1 atherosclerotic risk factors, 29% had 1, 41% had 2, and 30% had ≥3 risk factors. Each conventional risk factor was well controlled throughout the follow-up period. Smoking rates declined from 19% to 10%. The mean follow-up period was 9.0±1.0 years. At the baseline examination, 128 patients (61%) had ≥1 plaques >1.5 mm, 21 (10%) had 50% to 70% stenosis, and 4 (2%) had >70% stenosis. Figure 2 shows the longitudinal changes of carotid mmIMT and inflammatory markers. The average mmIMT increased throughout the study period (*P* for trend <0.001). IL-6 also tended to increase gradually during follow-up (*P* for trend <0.001), whereas no change was observed in the levels of hs-CRP (*P* for trend=0.18). During the follow-up period, the absolute change in mmIMT was 0.28±0.236 mm and the average yearly progression was 0.03±0.026 mm/y.

At the baseline examination, baseline mmIMT was significantly associated with baseline hs-CRP (*r*=0.21; *P*=0.002) and baseline IL-6 (*r*=0.25; *P*<0.001) levels. After controlling for age, sex, baseline body mass index, baseline systolic blood pressure, baseline estimated glomerular filtration rate, baseline low-density lipoprotein cholesterol, baseline HbA1c, and baseline statin treatment, these associations remained significant for hs-CRP (standardized β=0.15; *P*=0.04) and IL-6 (standardized β=0.17; *P*=0.03). The associations among annual mmIMT progression, the prevalence of conventional risk factors, and the average

![Figure 1. Evaluation of carotid mean-maximal intima-media thickness. The carotid artery was divided into 3 parts, each 15 mm in length, beginning at the flow divider. Severity of carotid atherosclerosis was evaluated by the mean-maximal intima-media thickness (mmIMT), which is the mean of maximal wall thickness including atheromatous plaque at 12 carotid segments (near and far wall of the left and right common carotid artery [CCA], carotid bifurcation [BIF], and internal carotid artery [ICA]). Carotid mmIMT=(a+b+c+d+e+f×contralateral sum)/12 (mm).](http://stroke.ahajournals.org/).
values of variables during the follow-up period are shown in Table 2. Male sex and the presence of diabetes mellitus were significant predictors of mmIMT progression. Age, baseline mmIMT, average glucose, average HbA1c, average hs-CRP level, and average IL-6 level were positively associated with mmIMT progression. A negative correlation was observed between average diastolic blood pressure and mmIMT progression. Table 3 shows the associations of mmIMT progression with baseline and long-term average concentrations of hs-CRP and IL-6. In univariate analyses, baseline IL-6, average hs-CRP, and average IL-6 levels were positively correlated with mmIMT progression (Figure 3). When adjusted for age, sex, and conventional risk factors, average hs-CRP and average IL-6 levels remained significantly associated with mmIMT progression. When additionally adjusted for baseline mmIMT, only average IL-6 level showed a significant association with mmIMT progression. In this multivariate analysis model (Table 3; model 3), male sex (standardized β=0.20; P=0.01), baseline mmIMT (standardized β=0.18; P=0.02), and average IL-6 level (standardized β=0.17; P=0.02) were independently associated with mmIMT progression. Evidence for multicollinearity was absent because the variance inflation factors for independent variables in all models in Table 3 were <3.0.

Discussion
The main finding of the present study was that long-term average IL-6 level is a predictor of the progression of mmIMT independently of conventional risk factors. However, neither baseline IL-6, baseline hs-CRP, nor average hs-CRP level was independently associated with mmIMT progression.

Several studies have reported on the risk factors for the progression of carotid IMT. Age, male sex, smoking, and total- or low-density lipoprotein cholesterol were strong predictors of IMT progression in most studies.12–14,16 Some studies also reported that hypertension and systolic blood pressure,14 high-density-lipoprotein cholesterol,13 and diabetes mellitus and HbA1c13,16 were associated with IMT progression; however, these results are inconsistent across studies. In the present study, mmIMT progression was not significantly associated with smoking, systolic blood pressure, or low-density lipoprotein cholesterol. Our population included relatively few smokers (19%), and more than half of the participants received antihypertensive drugs and statins during follow-up. This may have affected the association between risk factors and IMT progression. Interestingly, diastolic blood pressure was negatively associated with IMT progression. Studies on the elderly population reported a similar negative correlation between diastolic blood pressure and carotid atherosclerosis.17,18

Figure 2. Longitudinal changes of mean-maximal intima-media thickness (IMT) and inflammatory marker levels during follow-up. Box plots represent median and interquartile ranges and whiskers indicate the range of values that fall within 1.5 box lengths. The number of patients is shown in parentheses. hs-CRP indicates high-sensitivity C-reactive protein.
Table 2. Correlation Among Annual Intima-Media Thickness Progression, Conventional Risk Factors, and Mean Values of Variables During Follow-Up

<table>
<thead>
<tr>
<th>Factors</th>
<th>Annual IMT Progression Mean±SD, μm or R</th>
<th>P Value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mean=SD, μm or R</td>
<td></td>
</tr>
<tr>
<td>Men/Women</td>
<td>37±29/25±21</td>
<td>0.001*</td>
</tr>
<tr>
<td>Risk factors</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Hypertension, yes/no</td>
<td>32±27/29±36</td>
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<tr>
<td>Dyslipidemia, yes/no</td>
<td>31±26/33±27</td>
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<tr>
<td>Diabetes mellitus, yes/no</td>
<td>46±29/29±25</td>
<td>0.003*</td>
</tr>
<tr>
<td>Previous cardiovascular diseases, yes/no</td>
<td>37±30/29±24</td>
<td>0.06</td>
</tr>
<tr>
<td>Current smoking, yes/no</td>
<td>35±27/31±26</td>
<td>0.30</td>
</tr>
<tr>
<td>Medication</td>
<td></td>
<td></td>
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<tr>
<td>Statin use, yes/no</td>
<td>33±29/25±24</td>
<td>0.21</td>
</tr>
<tr>
<td>Age, y</td>
<td>0.20</td>
<td>0.003*</td>
</tr>
<tr>
<td>Baseline body mass index, kg/m²</td>
<td>−0.03</td>
<td>0.66</td>
</tr>
<tr>
<td>Average systolic blood pressure, mm Hg</td>
<td>0.07</td>
<td>0.31</td>
</tr>
<tr>
<td>Average diastolic blood pressure, mm Hg</td>
<td>−0.21</td>
<td>0.002*</td>
</tr>
<tr>
<td>Average eGFR, mL/min per 1.73 m²</td>
<td>−0.13</td>
<td>0.06</td>
</tr>
<tr>
<td>Average LDL-cholesterol, mg/dL</td>
<td>0.00</td>
<td>0.96</td>
</tr>
<tr>
<td>Average HDL-cholesterol, mg/dL</td>
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<td>0.13</td>
</tr>
<tr>
<td>Average triglyceride, mg/dL</td>
<td>0.06</td>
<td>0.37</td>
</tr>
<tr>
<td>Average glucose, mg/dL</td>
<td>0.18</td>
<td>0.008*</td>
</tr>
<tr>
<td>Average HbA1c, %</td>
<td>0.15</td>
<td>0.03*</td>
</tr>
<tr>
<td>Average hs-CRP, mg/dL (log)</td>
<td>0.22</td>
<td>0.001*</td>
</tr>
<tr>
<td>Average interleukin-6, pg/mL (log)</td>
<td>0.27</td>
<td>&lt;0.001*</td>
</tr>
</tbody>
</table>

P<0.05.

eGFR indicates estimated glomerular filtration rate; HbA1c, glycosylated hemoglobin; HDL, high-density lipoprotein; hs-CRP, high-sensitivity C-reactive protein; IMT, intima-media thickness; and LDL, low-density lipoprotein.

Previous studies relating hs-CRP levels to the progression of atherosclerosis have showed conflicting results. Two large population-based studies reported a significant association between hs-CRP and IMT progression, but this association disappeared after adjustment for cardiovascular risk factors. Meanwhile, our group previously reported that hs-CRP level is an independent predictor of the short-term progression of carotid plaque as determined by plaque number and plaque score in patients with vascular risk factors. These inconsistent results suggested that the association between hs-CRP levels and the progression of atherosclerosis can be strongly affected by the population, coexisting risk factors, and measuring methods of atherosclerosis.

Meanwhile, investigation of the association between IL-6 and the progression of atherosclerosis has been limited. Lee et al reported an association between baseline IL-6 levels and carotid IMT after 12 years even after adjusting for atherosclerotic risk factors; however, these authors did not report the baseline IMT and the progression of IMT was unclear. Some cross-sectional studies showed a strong association between IL-6 and IMT, but this was not confirmed in other studies. To our knowledge, this is the first study to demonstrate the association between IL-6 and the progression of carotid atherosclerosis. Because proinflammatory cytokines such as IL-6 are short acting and prone to fluctuations, comparisons using only baseline values generally tend to underestimate the actual association of IMT progression with chronic inflammatory conditions because of the regression dilution effect. To avoid this potential bias, we used long-term average IL-6 values as a quantitative marker of chronic inflammatory conditions. Danesh et al noted that the long-term average IL-6 value can be a better predictor of future cardiovascular events than a single measurement of the baseline IL-6 level. Our results suggested that the long-term average IL-6 values are also a better predictor of atherosclerotic progression than baseline IL-6 values. This superiority of IL-6 as a marker may be explained by the central role of IL-6 in mediating inflammatory cascades. IL-6 not only stimulates the secretion of acute phase proteins such as hs-CRP but also promotes a variety of atherosclerotic mechanisms such as endothelial dysfunction, recruitment and activation of other inflammatory cells, and oxidation of lipoproteins. Therefore, the circulating IL-6 level could be more closely associated with the progression of atherosclerosis than the level of downstream factors such as hs-CRP.

Our study had several limitations. First, the study only included patients who could continue to attend the hospital and excluded those who died or had severe disabilities.
preventing hospital visits. These excluded patients were expected to have lower adherence to therapies and more severe atherosclerosis than the participants did, hence this sampling bias might have attenuated the expected association between inflammatory markers and carotid IMT progression. Second, we used mmIMT for the assessment of carotid atherosclerosis instead of conventional common carotid artery-IMT, because this index could predict the severity of systemic atherosclerosis more sensitive than conventional common carotid artery-IMT in patients with vascular risk factors.7,9 Because mmIMT is affected by plaque thickness and the number of plaques, mmIMT might be correlated more closely with inflammatory markers than conventional common carotid artery-IMT.22 Thus, our findings should be interpreted with caution. Finally, an event analysis could not be performed because of the small sample size and few cardiovascular events. Lorenz et al31 conducted a meta-analysis be performed because of the small sample size and few car-

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