Circulating adhesion molecules are correlated with ultrasonic assessment of carotid plaques

Hiroyuki HASHIMOTO, Kazuo KITAGAWA, Keisuke KUWABARA,
Hidetaka HOUGAKU, Toshiho OHTSUKI, Masayasu MATSUMOTO
and Masatsugu HORI
Department of Internal Medicine and Therapeutics, Osaka University Graduate School of Medicine, 2-2 Yamadaoka, Suita,
Osaka 565-0871, Japan

ABSTRACT

The relationship between levels of circulating intercellular cell-adhesion molecule-1 (cICAM-1) or P-selectin (cP-selectin) and the severity of carotid atherosclerosis was examined in 301 outpatients undergoing duplex ultrasonographic examination. Carotid plaque was defined as an intima-media thickness greater than 1.0 mm, and a plaque score (PS) was calculated from the plaque thickness in both carotid arteries. Multivariate analysis demonstrated significant positive associations between cICAM-1 and the number of plaques $[\beta = 0.11; \text{confidence interval (CI), 0.007– 0.213}]$, maximum intima-media thickness ($[\beta = 0.11; \text{CI, 0.01– 0.219}]$, and PS ($[\beta = 0.10; \text{CI, 0.001– 0.205}]$). In contrast, no significant association was found for cP-selectin. cP-selectin did not increase until atherosclerosis was advanced (PS $> 10$), showing a marked increase in patients with $\geq 50\%$ stenosis. The circulating levels of both proteins are related to real measurements of plaque formation in the carotid arteries independently of classical risk factors. Marked elevation of cP-selectin occurs in advanced carotid atherosclerosis after gradual elevation of cICAM-1.

INTRODUCTION

In vitro and animal experiments have demonstrated that adherence of circulating leucocytes to endothelial cells and subsequent transendothelial migration are critical steps in the early stages of atherosclerosis [1]. Recent clinical investigations have focused on the relationship between the levels of circulating leucocyte–endothelial adhesion molecules and cardiovascular disease (CVD). Among the various circulating adhesion molecules, circulating intercellular cell-adhesion molecule-1 (cICAM-1) has received the most attention, and two large-scale studies have demonstrated that an elevated cICAM-1 level is a predictor of future cardiovascular events [2,3]. With respect to other leucocyte–endothelial adhesion molecules, several experimental and histological studies have shown a close relationship between P-selectin and ICAM-1, and some studies have revealed that circulating P-selectin (cP-selectin) levels are higher in patients who suffer a subsequent cardiovascular event than in patients who do not [4–6]. Of the other circulating adhesion molecules, cP-selectin is the strongest predictor of the risk of cardiovascular events after cICAM-1 [7].

Detection of carotid atherosclerosis is useful because it is an indicator of generalized atherosclerosis [8–11] and a reliable predictor of future CVD [12–15]. However, studies investigating the relationship between circulating levels of the above adhesion molecules and carotid atherosclerosis had different population sizes and different indicators to estimate the severity of

Key words: adhesion molecule, atherosclerosis, carotid artery, circulating intercellular cell-adhesion molecule-1 (cICAM-1), circulating P-selectin (cP-selectin), inflammation, ultrasound.

Abbreviations: (c)ICAM-1, (circulating) intercellular cell-adhesion molecule-1; CI, confidence interval; cP-selectin, circulating P-selectin; HbA1c, glycated haemoglobin; HDL, high-density lipoprotein; PS, plaque score; CVD, cardiovascular disease; IMT, intima-media thickness.

Correspondence: Dr Hiroyuki Hashimoto, Department of Internal Medicine, Osaka National Hospital 2-1-14, Hoenzaka, Chuoku, Osaka 540-0006, Japan (e-mail hashih@onh.go.jp).
carotid atherosclerosis, and therefore yielded inconsistent findings [2,16–21].

We have previously established the plaque score (PS) as an indicator of the severity of carotid atherosclerosis, and have investigated the relationships between this score and classical risk factors for CVD [22] and C-reactive protein [23], silent brain infarction [9], platelet accumulation in the carotid arteries [24] and future ischaemic cerebrovascular disease [14]. In the present study, we used the PS and several other indicators to estimate the severity of carotid atherosclerosis, and we investigated the relationship between cICAM-1 or cP-selectin levels and the severity of carotid atherosclerosis.

**MATERIALS AND METHODS**

Subjects

A total of 301 outpatients over 40 years old, who were being treated for classical risk factors and/or secondary prevention of CVD at the Department of Internal Medicine of Osaka University Hospital, were investigated for carotid atherosclerosis and provided blood samples. Patients were excluded from the study if they had experienced a clinical cardiovascular event in the previous year or if another disease that could increase the cICAM-1 or cP-selectin level was present, i.e. malignancy, collagen disease, chronic renal failure, infection or hepatic disease [25]. None of the subjects were receiving anti-oxidant vitamin supplements, oestrogen therapy or steroid therapy. Patients gave written informed consent to provide blood samples and participate in the study. The study was approved by the local ethics committees.

Risk factors for CVD

Hypertension was defined as a systolic blood pressure ≥140 mmHg and/or a diastolic blood pressure ≥90 mmHg, or the current use of antihypertensive medications. Hypercholesterolaemia was defined as total cholesterol level ≥220 mg/dl or the current use of cholesterol-lowering agents. Diabetes mellitus was defined as a glycated haemoglobin (HbA1c) level ≥5.8% or the current use of oral hypoglycaemic agents. Patients were considered to be smokers if they were current smokers or had stopped smoking less than 1 month before enrolment in the study. Cigarette pack-years were calculated for each patient as a measure of cumulative smoking exposure. Patients were considered to have CVD if they had a prior history of ischaemic heart disease, cerebrovascular disease, aortic aneurysm or peripheral vascular disease. Patients were defined as taking antiplatelet therapy if they were being treated with aspirin or ticlopidine.

Evaluation of carotid atherosclerosis and measurement of cICAM-1 and cP-selectin levels

High-resolution B-mode ultrasonography was performed with a 7.5-MHz duplex-type scanner (Hitachi EUB-450, 555) to evaluate carotid atherosclerosis. The upper limit of normal for the intima-media thickness (IMT) was defined as 1.0 mm [22], and lesions with an IMT ≥1.1 mm were defined as atheromatous plaques. We used the following indicators to assess the severity of carotid atherosclerosis: (1) maximum IMT measured in mm at the thickest point (including plaque) of the near and far walls of both carotid arteries, (2) the number of plaques in both carotid arteries, (3) the PS (calculated by summing all plaque thicknesses measured in both carotid arteries, as shown in Figure 1) [22], and (4) the maximum stenosis (calculated by measuring the residual luminal diameter and the original diameter at the site of maximum stenosis and dividing the difference by the original diameter, with the result being reported as a percentage).

Blood samples were collected into tubes containing citric acid and EDTA, and were stored at −80°C after centrifugation. The stored serum samples were assayed for cICAM-1, whereas the stored plasma samples were assayed for cP-selectin. Commercially available monoclonal antibody-based ELISA kits (R & D Systems, Minneapolis, MN, U.S.A.) were used for the determination of cICAM-1 and cP-selectin levels.

Statistical analysis

Data for the cICAM-1 and cP-selectin levels showed a normal distribution, whereas the maximum IMT, the number of plaques and the PS showed a skewed distribution. For univariate analysis, Spearman’s rank-order correlation coefficients were determined to assess the association between cICAM-1 or cP-selectin level and the parameters of carotid atherosclerosis, i.e. maximum IMT, number of plaques and PS. The associations between...
measured risk factors and PS were evaluated similarly. Differences of PS and the levels of circulating adhesion molecules in relation to categorical risk factors were evaluated by the Mann-Whitney U test and the unpaired t test respectively. Pearson’s test was used to evaluate the relationship between cICAM-1 and cP-selectin levels, as well as those between the levels of these molecules and age. Patients were classified into four groups on the basis of the severity of carotid atherosclerosis: no disease (PS = 0), mild disease (PS = 1.1–5.0), moderate disease (PS = 5.1–10) and severe disease (PS > 10) [9,14,24]. One-way ANOVA was performed with Scheffé’s multiple comparison test to assess differences in cICAM-1 and cP-selectin levels between these four groups. Patients were also classified into three groups on the basis of their maximum stenosis, i.e., no carotid atherosclerosis, less than 50% stenosis and 50% or more stenosis, after which the differences in cICAM-1 and cP-selectin levels between these three groups were similarly evaluated. To evaluate the differences, cICAM-1 and cP-selectin levels were adjusted for classical risk factors, statin use and a history of CVD. For multivariate analysis, multiple linear regression analysis was performed using each carotid atherosclerosis parameter as a dependent variable, while the age, sex, cigarette pack-years, presence/absence of hypertension, diabetes, hypercholesterolaemia, statin use, a history of CVD and the levels of circulating adhesion molecules were used as the predictive variables. The values of the maximum IMT, number of plaques and PS were log-transformed to normalize their distribution. 0.1 was assigned when number of plaques was 0, and 1.0 was assigned when PS was 0. All P values calculated were two-tailed and P < 0.05 was considered significant. All statistical analyses were conducted using SPSS/Windows software, version 9.0J (SPSS Japan Inc., Tokyo, Japan).

RESULTS

The clinical characteristics of the subjects are shown in Table 1. Of the 301 patients, 227 (75%) had carotid atherosclerosis, and 105 (35%) had a history of CVD and most of them were on antiplatelet therapy. In outpatients who were treated for hypertension, hypercholesterolaemia and diabetes mellitus, there were no significant differences in cICAM-1 and cP-selectin levels between the patients with or without these classical risk factors. As expected, the cICAM-1 level of current smokers was higher than that of non-current smokers (244.0 ± 86.1 ng/ml versus 189.0 ± 71.5 ng/ml, P = 0.007). The difference for cP-selectin was also significant (46.7 ± 26.5 ng/ml versus 38.5 ± 21.4 ng/ml, P = 0.03). The cICAM-1 level was higher in men than in women (212.8 ± 83.0 ng/ml versus 182.6 ± 66.3 ng/ml, P = 0.008), but no sex difference was found for cP-selectin. There were no statistical associations between the levels of the circulating adhesion molecules and age, nor were there any significant differences in cICAM-1 and cP-selectin levels between smokers and non-smokers. The associations between the levels of cICAM-1 or cP-selectin and age were not significant when they were adjusted for gender. There was a significant correlation between cICAM-1 and cP-selectin levels (r = 0.292, P < 0.001).

cICAM-1 and cP-selectin levels were shown in relation to the PS and maximum stenosis in Tables 2 and 3 respectively. Because the PS involves the factors of the number of plaques and maximum IMT, we showed Table 2 as a representative of the indicators. Unlike cICAM-1, cP-selectin did not increase until carotid atherosclerosis was severe (PS > 10). The cICAM-1 level of the severe group (PS > 10) was significantly higher than those of the group without atherosclerosis (PS = 0) and the group with mild atherosclerosis (PS = 1.1–5.0), whereas the cP-selectin level of the severe group was significantly higher than in all of the other three groups. The differences remained significant after adjustment for

Table 1 Clinical characteristics of the patients (n = 301)

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Classical risk factors for CVD</td>
<td></td>
</tr>
<tr>
<td>Age (years) (range)</td>
<td>64.0 ± 9.1</td>
</tr>
<tr>
<td>Male</td>
<td>53%</td>
</tr>
<tr>
<td>Hypertension</td>
<td>60%</td>
</tr>
<tr>
<td>Systolic/diastolic blood pressure (mmHg)</td>
<td>136 ± 17/80 ± 11</td>
</tr>
<tr>
<td>Hypercholesterolaemia</td>
<td>48%</td>
</tr>
<tr>
<td>Total cholesterol/HDL cholesterol (mg/dl)</td>
<td>207 ± 32/55 ± 17</td>
</tr>
<tr>
<td>Statin use</td>
<td>26%</td>
</tr>
<tr>
<td>Diabetes mellitus</td>
<td>18%</td>
</tr>
<tr>
<td>Fasting blood glucose (mg/dl)</td>
<td>104 ± 26</td>
</tr>
<tr>
<td>HbA1c (%)</td>
<td>5.3 ± 0.9</td>
</tr>
<tr>
<td>Current smoker</td>
<td>17%</td>
</tr>
<tr>
<td>Cigarette pack-years</td>
<td>11.1 ± 22.2</td>
</tr>
<tr>
<td>History of CVD</td>
<td>35%</td>
</tr>
<tr>
<td>Antiplatelet medication</td>
<td>33%</td>
</tr>
<tr>
<td>Severity of carotid atherosclerosis</td>
<td></td>
</tr>
<tr>
<td>Maximum IMT (mm)</td>
<td>1.9 ± 1.2</td>
</tr>
<tr>
<td>Number of plaques</td>
<td>2.7 ± 2.8</td>
</tr>
<tr>
<td>PS</td>
<td>4.8 ± 5.4</td>
</tr>
<tr>
<td>Maximum stenosis (0% &lt; 50%, ≥ 50%)</td>
<td>(74, 200, 27)</td>
</tr>
<tr>
<td>cICAM-1 (ng/ml)</td>
<td>198.3 ± 76.8</td>
</tr>
<tr>
<td>cP-selectin (ng/ml)</td>
<td>39.9 ± 22.5</td>
</tr>
</tbody>
</table>
cICAM-1 and cP-selectin levels with respect to atherosclerosis categories

Concentration of cICAM-1 and cP-selectin levels with respect to atherosclerosis categories and maximum stenosis

Relationships between PS and risk factors, including cICAM-1 and cP-selectin levels

Table 2: cICAM-1 and cP-selectin levels with respect to atherosclerosis categories

Table 3: cICAM-1 and cP-selectin levels with respect to maximum stenosis

Table 4: Relationships between PS and risk factors, including cICAM-1 and cP-selectin levels

Classical risk factors: hypertension, hypercholesterolaemia, cigarette smoking, diabetes mellitus, and antiplatelet medication.

Diabetes mellitus

Hypertension

Total cholesterol

HbA1c

Age

Men/women

Statin use

History of CVD

Current smoking

Cigarette pack-years

cICAM-1

cP-selectin

PS

P value
Table 5  Results of multivariate regression analysis for PS

<table>
<thead>
<tr>
<th>Variable</th>
<th>$\beta$ (95 % CI)</th>
<th>$\beta$ (95 % CI)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age</td>
<td>0.33 (0.232–0.431)</td>
<td>0.34 (0.214–0.437)</td>
</tr>
<tr>
<td>Sex</td>
<td>0.12 (0.002–0.231)</td>
<td>0.11 (–0.005–0.23)</td>
</tr>
<tr>
<td>Hypertension</td>
<td>0.15 (0.047–0.25)</td>
<td>0.14 (0.039–0.239)</td>
</tr>
<tr>
<td>Diabetes</td>
<td>0.12 (0.017–0.223)</td>
<td>0.12 (0.017–0.225)</td>
</tr>
<tr>
<td>Hypercholesterolaemia</td>
<td>0.14 (0.008–0.264)</td>
<td>0.14 (0.006–0.263)</td>
</tr>
<tr>
<td>Statin use</td>
<td>0.10 (–0.027–0.23)</td>
<td>0.097 (–0.033–0.228)</td>
</tr>
<tr>
<td>Cigarette pack-years</td>
<td>0.046 (–0.05–0.171)</td>
<td>0.078 (–0.032–0.19)</td>
</tr>
<tr>
<td>History of CVD</td>
<td>0.075 (–0.029–0.18)</td>
<td>0.074 (–0.032–0.179)</td>
</tr>
<tr>
<td>cICAM-1</td>
<td>0.10 (0.001–0.205)</td>
<td></td>
</tr>
<tr>
<td>cP-selectin</td>
<td>0.045 (–0.037–0.167)</td>
<td></td>
</tr>
</tbody>
</table>

the contribution of several classical risk factors (i.e. age, sex, hypertension, hypercholesterolaemia and diabetes mellitus), but the relationship for cP-selectin was no longer significant (Table 5).

With regard to the relationship between cICAM-1 or cP-selectin and other indicators, the cICAM-1 level was related to the maximum IMT ($r = 0.22, P = 0.001$) and to the number of plaques ($r = 0.23, P = 0.001$) by univariate analysis. When multivariate analysis was done, the relationship for the number of plaques [$\beta = 0.110$, confidence interval (CI), 0.007–0.213] and maximum IMT ($\beta = 0.114$; CI, 0.01–0.219) remained significant after accounting for the contribution of several classical risk factors. cP-selectin levels were related to maximum IMT ($r = 0.15, P = 0.008$), but not to the number of plaques, on univariate analysis. However, multivariate analysis did not confirm the relationship with maximum IMT.

DISCUSSION

A previous large-scale study showed that cICAM-1 levels were elevated in patients who had carotid atherosclerosis when compared with controls who had a normal IMT [2], while another study showed a significant relationship between cICAM-1 and the severity of carotid atherosclerosis (based on the mean IMT) [21]. However, studies that used the maximum IMT or maximum stenosis as an indicator have found no relationship with cICAM-1 [17–21]. The present study showed that cICAM-1 levels were related to the number of carotid plaques, maximum IMT and the PS. In contrast, there was no significant difference in cICAM-1 levels between groups that were stratified according to maximum stenosis. Because the present study was cross-sectional, we could not draw conclusions about the direction of association between cICAM-1 levels and carotid atherosclerosis. However, it is well known that adhesion molecules play important roles in the development of atherosclerosis and it has been reported that, unlike other adhesion molecules, ICAM-1 expression on endothelial cells is selectively upregulated by shear stress [26]. Strong focal expression of ICAM-1 is seen at the carotid bifurcation in patients with no or low-grade stenosis [27], whereas cICAM-1 levels are reported to increase in the early stage of carotid atherosclerosis [2]. Although the source of cICAM-1 in the serum is uncertain, circulating forms of adhesion molecules may be derived from vascular wall components, including endothelial and smooth muscle cells [28]. In the present study, atherosclerotic plaque was defined as an IMT $\geq 1.1$ mm based on our previous study [22], which is in accordance with those in several other studies [13,29]. Small atherosclerotic plaques in early stages of atherosclerosis comprise smooth muscle cells and macrophage-derived foam cells [30], and the macrophage is transendothelial migrated forms of a leucocyte [31]. The present study showed that cICAM-1 levels are significantly associated not only with maximum IMT but also with the number of plaques and PS. Taken together, these findings indicate that cICAM-1 levels may partly reflect the total extent of arterial involvement by atherosclerosis rather than the rate of carotid stenosis, and that elevation of cICAM-1 levels may precede the development of stenosis, which may help to explain previous inconsistent findings in studies based on different measures of carotid disease.

The strength of the relationship between cICAM-1 and cP-selectin levels in all of our subjects ($r = 0.292, P < 0.001$) was similar to that shown by a population-based study ($r = 0.23, P = 0.001$) [6], and that in patients with stable coronary syndrome ($r = 0.314, P = 0.007$) [32]. It was also similar to that found between cICAM-1 and circulating E-selectin ($r = 0.22, P = 0.0001$) and between cICAM-1 and circulating vascular cell-adhesion molecule-1 ($r = 0.17, P = 0.0001$) in a population-based study [2]. Focal expression of E-selectin in carotid arteries with no or low-grade stenosis is reported to be far weaker than expression of ICAM-1 [27]. Because we found that cP-selectin levels did not increase significantly until the advanced stage of carotid atherosclerosis, P-selectin may also show weak expression in carotid arteries with no or low-grade stenosis, and the increase of cP-selectin during the atherosclerotic process may follow the elevation of cICAM-1. Consistent with these findings, cP-selectin levels were previously reported to be higher in patients with carotid stenosis than in control subjects [20].

P-selectin is an adhesion molecule that supports the adherence of rolling leucocytes to the endothelium, and such adhesion is reinforced by ICAM-1-mediated bonding [31]. P-selectin and ICAM-1 are reported to be key adhesion molecules in the promotion of atherosclerosis [33–36], and expression of P-selectin in the endothelium overlying atherosclerotic plaques in humans is strongly correlated with ICAM-1 expression [37]. However, P-selectin is also expressed on the surface of activated platelets [38], and a previous study has
suggested that an elevated cP-selectin level may be a marker of platelet activation [39]. In fact, the origin of these circulating adhesion molecules has not yet been fully clarified. The increase of cP-selectin in patients with severe carotid atherosclerosis may be partly derived from activated platelets [39], since an experimental study has shown that P-selectin expression may play a role in platelet aggregation stimulated by pulsatile shear stress, which resembles blood flow in stenotic arteries [40]. This is also in accordance with our previous finding of Which resembles blood flow in stenotic arteries [40]. platelet aggregation stimulated by pulsatile shear stress, from activated platelets [39], since an experimental study with severe carotid atherosclerosis may be partly derived these circulating adhesion molecules has not yet been suggested that an elevated cP-selectin level may be a forms of the other adhesion molecules.

Because the present study focused on outpatients with classical risk factors for CVD, the confounding effects of antiplatelet and statin medication should be considered. Statins were used more frequently in patients with more severe carotid atherosclerosis in the present study. Because statins are reported to reduce cP-selectin levels [42], statin use may lead to an underestimation of true levels of cP-selectin in advanced carotid atherosclerosis and bias our findings toward a null result. The population of patients on antiplatelet medication was equal to that of those with a history of CVD. The relationship between cICAM-1 and the severity of carotid atherosclerosis remained significant, even after adjusting for the contribution of classical risk factors including statin use and history of CVD.

In conclusion, we found that cICAM-1 levels increased in parallel with the total extent of carotid arterial affected by atherosclerosis, but that cP-selectin levels did not increase until the stage of advanced carotid atherosclerosis, and both proteins predicted the severity of carotid atherosclerosis independently of classical risk factors. Ultrasonographic examination, which uses several scales to estimate the severity of carotid atherosclerosis, including measurement of carotid plaques, may be used to assess the origin of circulating forms of the other adhesion molecules.

REFERENCES


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