Circulating Factors and Ultrasono-findings are Linked to Previous Atherosclerotic Burden and Recurrent Risk

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Abstract: Background: In the progression of atherosclerosis, platelet activation and the interaction of platelets with leukocytes play a crucial role in arterial thrombus formation and are associated with the pathophysiology of carotid and cerebrovascular disease (CVD), including ischemic stroke. With aged participants, we evaluated and followed up the change in circulating factor and platelet-leukocyte aggregate levels in participants with or without CVD history. This study investigated whether circulating factor changes and ultrasonographic characteristics link to CVD risk and other relating long-term outcomes.

Materials and Methods: Two hundred fifteen participants who enrolled in the study were divided into two groups with CVD and without CVD history. We evaluated and analyzed the correlation between ultrasonography-based morphological characteristics and circulating factor-based functional changes in both groups.

Results: There was no difference in p-selectin level between both groups. However, activated monocyte and platelet-monocyte aggregate levels were higher in patients with previous CVD than without previous CVD. Circulating factor and ultrasonographic characteristics were correlated in the group with CVD, whereas these factors were not correlated in the group without CVD.

Conclusion: We found that circulating blood factor levels showed a different tendency in participants with and without CVD history. The results depict that atherosclerotic severity might depend on the history of CVD and progression of atherosclerosis. We suggest that the circulating factor levels, atherosclerotic severity, and history of CVD are considered in the observation of pathologic progression to manage the development of CVD risks and CVD relating outcomes.

Keywords: Circulating factors, atherosclerosis, platelet monocyte-aggregation, p-selectin, Mac-1, ultrasonography, cerebrovascular disease.

1. INTRODUCTION

Carotid artery atherosclerosis is associated with subsequent risk of new or recurrent cerebrovascular diseases, such as transient ischemic attack, stroke, and post-stroke vascular dementia, particularly in the modern day society [1-5]. With this association, several noninvasive measures for subclinical atherosclerosis have been undertaken in the predictive diagnosis of carotid and cerebrovascular diseases (CVDs). The size and number of plaques; intima-media thickness (IMT) of carotid vessel on ultrasonographic measurement of the carotid artery; and circulating factor activation, such as platelet, leukocyte, and platelet-leukocyte aggregate (PLA) activation, according to flow cytometry of peripheral blood, have been shown to be closely correlated with atherosclerotic ischemic stroke [5-8]. However, previous studies investigating the relationship between circulating levels of the above mentioned hematologic factors and carotid atherosclerosis had different population sizes and indicators to estimate the severity of carotid atherosclerosis, and thus yielded inconsistent findings [9-11].

In this context, some prospective clinical studies have been performed to determine whether the ultrasonographic type of carotid artery plaques influences the rate of restenosis as well as the incidence of future cardiovascular events [12, 13]. The initial assumption of these studies was that different plaque types reflect different disease processes. Moreover, it could progress to a specific atheroma at carotid bifurcation, where atheroma is mostly detected after local resection [14]. However, our previous investigations showed that there were no significant differences among detailed categorized IMT or plaque size (diameter) and plaque numbers [2].

It has been reported that atherosclerotic plaques leading to acute coronary syndromes develop at sites wherein mild coronary-artery stenosis is angiographically detected [13, 15]. Several important questions remain unanswered, including the value of circulating factor activation markers and platelet-leukocyte interactions in asymptomatic subjects with high risk for stroke, correlation between circulating molecules and atherosclerotic vascular condition, and potentiality of this correlation analysis as a predictor of future CVD. Nevertheless, little is known about the potential participation of circulating molecules in the early stage of arterial disease before the development of complications.

The aim of this study was to compare the carotid vascular condition and circulating factor changes in asymptomatic patients with or without a CVD history. In this study, we measured circulating factor levels in participants with and without a known history of CVD, and explored their potential relationships with CVD risk factors and subclinical atherosclerosis assessed by ultrasonography for long-term follow-up care.

2. MATERIALS AND METHODS

2.1. Participant and clinical Assessment

The study enrolled 215 patients who had visited the neurology clinic of Severance Hospital between August 2007 and February 2008. The participants were divided into two groups: with previous CVD and without previous CVD. Participants with major trauma, surgery, severe liver disease, renal failure, cancer, and chronic in-
flammary diseases within three months were excluded to avoid external events or parallel infection that could also activate platelets or leukocytes. Two to three years later (from 2009 to 2010), 48 participants were followed up, PLAs level was re-assessed, and ultrasonography was also performed. Furthermore, in 12 participants, PLAs level was re-evaluated, and follow-up of over 10 years was performed. The two groups consisted of sex- and age-matched subjects with no inflammatory condition over the last three months before the study and with a comparable atherosclerotic risk profile such as hypertension, hypercholesterolemia, diabetes mellitus, and smoking. Hypertension was defined as a systolic blood pressure ≥140 mmHg and/or diastolic blood pressure ≥90 mmHg or the current use of antihypertensive medications. Hypercholesterolemia was defined as total cholesterol level ≥220 mg/dL or the current use of cholesterol-lowering agents. Diabetes mellitus was defined as a glycated hemoglobin (HbA1c) level ≥5.8% or the current use of oral hypoglycemic agents. Participants were considered smokers if they were current smokers or had stopped smoking less than one month before enrollment in the study. Participants were considered to have CVD if they had a history of ischemic heart disease, atherosclerosis, aortic aneurysm, peripheral vascular disease, or ischemic stroke. Detailed history is presented in Table 1. The study protocol was approved by the Committee for the Protection of Human Subjects in Research of Yonsei University, and written informed consent was obtained from all patients.

2.2. Carotid Ultrasonography

High-resolution B-mode ultrasonography was performed with a duplex-type scanner (Accuvix V10, Seoul, Korea) to evaluate carotid atherosclerosis. Carotid artery intima-media thickness (C-IMT) was measured offline in the far wall of the common carotid artery in a longitudinal view in a region free from plaques, using a computerized system. The upper normal limit for IMT was 1.0 mm, and lesions with IMT ≥1.1 mm were defined as atheromatous plaques. The measurements of plaques at the site of carotid artery were computed according to the distance from flow divider: above flow divider up 1.5 cm, internal carotid artery; below flow divider down to 1.5 cm, bifurcation; below flow divider down to 3 cm, common carotid artery [5]. According to Mannheim Consensus, carotid plaques were defined as focal protruding structures into the lumen with a size of at least 0.5 mm, 50% of IMT, or a distance of 1.5 mm from the media-adenitia interface to intima-lumen [16].

2.3. Blood Sampling and Flow Cytometry

Blood samples were taken from each participant and collected into Vacutainer tubes containing 0.5 mL of 3.2% buffered sodium citrate. Immediately thereafter, citrated blood sample was added to a fixation solution (4% formaldehyde) to minimize ex vivo platelet activation. In flow cytometry analysis, platelet activity was assessed by existing CVD history. Unlike platelet activation, monocye activation by Mac-1 expression had an apparently higher MFI value in the group with previous CVD than in the group without previous CVD (Fig. 1B, p < 0.00001). According to the pattern of Mac-1 expression, platelet-monocyte aggregation (PMA) had apparently higher percentage in the group with previous CVD than in the group without previous CVD (Fig. 1C, p < 0.00001). Similarly, the levels of platelet-lymphocyte aggregate (PLA) and platelet-granulocyte aggregate (PGA) were significantly higher in the group with previous CVD group than in the group without previous CVD (data not shown). In this study, we assumed that PMA had a more significant role in the difference between the two groups, and focused on monocye activation and interaction with platelets.

We assessed ultrasonography-based morphological characteristics as mean values of IMT and plaque numbers versus circulating factor-based functional changes in the two groups. Our previous investigations showed that there were no significant differences among detailed categorized IMT or plaque size (diameter) or plaque numbers [2]. Therefore, in this study, we used simple categories such as mean IMT and plaque numbers. The mean IMT and carotid atherosclerotic severity were significantly greater in the group with previous CVD than in the group without previous CVD (Fig. 2A, p = 0.0254). The number of carotid plaques also showed significant difference between the two groups (Fig. 2B, p = 0.0280).

Among participants enrolled in this study, there was a high prevalence of hypertension (without:with = 56.9%/69%), hyperlipidemia (without:with = 31.0%/22.0%), diabetes (without:with = 24.4%/24%), and smoking (without:with = 10.9%/22%). Although the participants were similar in age, the correlation between carotid atherosclerotic severity and vascular risk or circulating factors showed significant difference between the two groups (Table 2).
the group without CVD history, there was no correlation between circulating factors and atherosclerotic vessel factors, such as mean IMT or plaque number. In the group with CVD, the mean IMT was significantly correlated with PMA level ($p = 0.0321$). Furthermore, plaque numbers were deeply correlated with PMA level ($p = 0.0041$) and activated monocyte level (Mac-1, $p = 0.0098$).

Based on the results of the correlation analysis, we followed up with some patients to analyze circulating factor changes. Of all the variables listed in Table 1, the circulating factor changes that were statistically significant between the groups without and with previous CVD were:

- p-selectin positive platelets
- Mac-1-positive monocytes
- Monocytes aggregated with platelets

![Fig. (1). Circulating blood factor changes by FACS analysis between the groups without and with previous CVD.](image)

![Fig. (2). Vessel atherosclerotic determinant differences in the two groups.](image)
initial participants, 48 patients were followed up two to three years later (2009 to 2010), and 12 patients were followed up 10 years later (2016). They all belonged to the group with CVD history, and have been regularly checked as outpatients and were cared for with appropriate medication and daily life workout. In all followed-up participants, p-selectin expression in circulating platelets did not show differences among the groups (Fig. 3A). Meanwhile, Mac-1 expression in circulating monocytes showed a downward tendency according to the follow-up year. The difference between the two groups (with or without CVD history) showed a larger gap at the initial point \( p < 0.00001 \), but in the longer-period follow-up, Mac-1 level decreased compared to that in the initial point but was still higher compared to the group without CVD history \( p < 0.001 \). After the 10-year follow-up, Mac-1 level decreased more compared to that in the two-year follow-up \( (p < 0.05) \). In case of PMA, the downward trend was similar to that for Mac-1 level which was even more reduced, indicating no difference in the group without CVD history at 10-year follow-up (Fig. 3C).

4. DISCUSSION

Based on the previous studies as well as our previous reports and data, we hypothesized that there is a correlation between atherosclerotic severity and circulating factors according to CVD history, particularly in aged patients. Our results showed no differences in the levels of p-selectin expression in circulating platelets between the two groups. However, Mac-1 level in monocytes and circulating PMA was significantly increased in the group with previous CVD (Fig. 1). Likewise, the mean IMT and plaque numbers showed a more severe condition in the patient group with CVD history than in the group without CVD history (Fig. 2).

Several studies have reported that the ultrasonographic characteristics of carotid artery plaques are related to the development of future cardiovascular events [13, 17, 18]. Moreover, atherosclerotic severity is closely associated with new or recurrent stroke or other vascular diseases, and carotid IMT and plaque number present this association well [1, 9]. Thus, we investigated whether carotid mean IMT and plaque numbers, as indicators of atherosclerotic severity, have significant correlation in aged participants. Corresponding to our hypothesis, carotid mean IMT and plaque number were significantly correlated with aged patient group with CVD history (Table 2). In the present study, regression analysis did not show that CVD history was correlated with vascular risk factors such as mean IMT and plaque numbers, but revealed that circulating factors and PMA were independently correlated with CVD history. This might suggest a possibility for the prediction of CVD by analyzing the correlation between circulating factors and atherosclerotic severity in aged participants with previous CVD, as reflected in many previous studies [7, 8, 11, 14, 19].

We also confirmed that the levels of activated monocytes (Mac-1) and PMA increased as the plaque numbers or mean IMT increased in the group with previous CVD. However, in the group without previous CVD, circulating factors showed no significant changes, nor any tendency along with atherosclerotic severity, despite the participants’ old age. This result indicated that, in contrast to the group with previous CVD, other risk factors did not affect the levels of circulating factors with changes in atherosclerotic severity in the group without previous CVD. This result indicates that all participants with previous CVD have a higher risk for recurrent CVD than those without CVD history. For participants with previous CVD, we interpreted that circulating factors increased during the primary cerebrovascular events, including stroke, attack may contribute to atherosclerotic severity and eventually to the second cerebrovascular event. The results in patients who were followed up for 2 and 10 years showed that the circulating factor level and atherosclerotic severity were maintained as low or stable (Fig. 3). Among them, patients who developed recurrent CVD, including ischemic stroke, could not be observed, which might lead to sustained medical care.

Despite the long-term nature of our observation and follow-up, this study had several limitations. First, the sample sizes were small; 48 participants for 2-year and 12 participants for 10-year follow-up. During the follow-up period, many patients who failed to attend follow-up sessions died of non-vascular causes or developed another disease, such as severe inflammatory diseases. Considering the small size of our study subjects, further studies with a larger sample size during follow-up should be performed to confirm our findings and speculations on the incidence rate of CVD. Now, we have been recruiting patients for long-term follow-up for further study and concrete conclusion. Another limitation was that the circulating factor based on FACS analysis was also needed to determine a cutoff value for each blood cell population for standardization or generalization. Nonetheless, our findings may serve as a background for further evaluation of circulating factor changes, and might be applied to predict CVD in elderly people who need intensive care.
Table 2. Correlation analysis between without vs with previous CVD in each category.

| Without Previous CVD | With Previous CVD |
|----------------------|-------------------|
|                      | p-selectin | Macl | PMA | p-selectin | Macl | PMA |
| r                   | 0.097      | 0.0608 | 0.0965 | 0.1752 | 0.2148 | 0.3148 |
| R²                  | 0.0094     | 0.0037 | 0.0093 | 0.0307 | 0.0585 | 0.0991 |
| p                   | 0.3028     | 0.5649 | 0.3048 | 0.2141 | 0.0907 | 0.0312* |
|                      | 0.7350     | 0.9732 | 0.7641 | 0.2943 | 0.0098** | 0.0041** |

Ordinary oneway ANOVA test was performed. Pearson’s correlation coefficients, R squared, and associated two-tailed p-values are shown.

Collectively, we found that circulating factor levels showed a different tendency in aged people with and without previous CVD, and its correlation with atherosclerotic severity also depended on a history of CVD and progression of atherosclerosis. Ultrasonographic findings of carotid IMT and plaque number have not yet been used as reliable guidelines for the prediction of CVD or in the diagnosis of atherosclerotic ischemic stroke, since it remains unclear whether an increased carotid IMT or plaque number indicates the development of atherosclerotic ischemic stroke subsequently. Therefore, we suggest that pathologic progression in elderly patients with CVD history should be intensely observed while considering the circulating factor level and atherosclerotic severity. With further observational investigation, our results may be most effective in predicting the occurrence of atherosclerotic ischemic stroke.

CONCLUSION

Collectively, we found that the circulating factor levels showed a different tendency in aged people with and without previous CVD, and its correlation with atherosclerotic severity also depended on a history of CVD and progression of atherosclerosis. Ultrasonography findings of carotid IMT and plaque number have not yet been used as reliable guidelines for the prediction of CVD or in the diagnosis of atherosclerotic ischemic stroke, since it remains unclear whether an increased carotid IMT or plaque number indicates the development of atherosclerotic ischemic stroke subsequently. Therefore, we suggest that pathologic progression in elderly patients with CVD history should be intensely observed while considering the circulating factor level and atherosclerotic severity. With further observational investigation, our results may be most effective in predicting the occurrence of atherosclerotic ischemic stroke.

ETHICS APPROVAL AND CONSENT TO PARTICIPATE

The study protocol was approved by the Committee for the Protection of Human Subjects in Research of Yonsei University, Seoul, Republic of Korea.

HUMAN AND ANIMAL RIGHTS

No animals were used for studies that are the basis of this research. All the experiments on humans were in accordance with the Helsinki Declaration of 1975 as revised in 2013.

CONSENT FOR PUBLICATION

The written informed consent was obtained from all patients.

AVAILABILITY OF DATA AND MATERIALS

Not applicable.

FUNDING

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CONFLICT OF INTEREST

The authors declare no conflict of interest, financial or otherwise.

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Declared none.

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