Original Article
Association of plasma soluble CD40L and P-selectin with large-artery atherosclerosis stroke

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Abstract: Objective: To investigate the association of plasma soluble CD40L (sCD40L) and soluble P-selectin (sP-selectin) with large-artery atherosclerosis (LAA) stroke. Methods: We recruited 120 LAA stroke patients in acute phase and 60 controls. To evaluate the extent and severity of cerebral atherosclerosis, patients were divided into 3 subgroups by the number of cerebral-arteries with atherosclerotic stenosis (≥50%) or occlusion. The neurological function of patients was assessed with the NIHSS scores. Patients with responsible arteries involving the internal carotid artery system were detected microembolic signals (MES). The plasma levels of sCD40L and sP-selectin were measured by ELISA. The performance of sCD40L and sP-selectin in diagnosing LAA stroke were studied by an ROC curve. Results: The plasma sCD40L and sP-selectin levels were significantly increased in LAA stroke patients than controls. Significant variations were found that MES positive patients had higher sCD40L and sP-selectin levels than the MES negative patients. There was positive correlation of sCD40L and sP-selectin with the increase of the number of cerebral atherosclerotic arteries. Besides, the LAA stroke patients' sCD40L and sP-selectin levels were also correlated with the NIHSS scores separately. Both sCD40L and sP-selectin had good values in the diagnostic performance for the LAA stroke patients, whereas there was no difference between them. Conclusions: We conclude that LAA stroke patients have high levels of sCD40L and sP-selectin which could be used for assessing the worsening extent and severity of cerebral atherosclerosis, the destabilization of atherosclerotic plaque, and the severity of neurological dysfunction in LAA stroke patients.

Keywords: sCD40L, sP-selectin, LAA stroke, atherosclerosis, microembolic signals, the NIHSS score

Introduction

Large-artery atherosclerosis (LAA) stroke is a common type of cerebrovascular atherosclerosis according to the TOAST classification system, as the main pathogenesis of LAA stroke is atherosclerosis [1]. Platelet activation is a well-established critical process of atherosclerosis that releases inflammatory mediators and adhesive ligands, such as CD40L and P-selectin, which have been proven to play important roles in atherosclerosis [2]. CD40L is a 39-kDa trimeric transmembrane glycoprotein belonging to the tumor necrosis factor (TNF) family, rapidly appears on the surface of the membrane after platelet activation, and generates a soluble form that is sCD40L [2]. This soluble sCD-40L takes part in platelet activation, leading to an increased platelet P-selectin expression [3]. P-selectin is a 140-kDa granule membrane pro-

tein belonging to the selectin family and can release sP-selectin, which is a soluble fragment and is a specific biomarker that reflects platelet activation [4].

The levels of sCD40L and sP-selectin were separately reported to increase in patients with coronary artery disease (CAD) and cerebrovascular disease, and were considered to participate in the process of forming and extending atherosclerosis, including exacerbating the rupture of the atherosclerotic plaque [5-8]. Some studies have observed the relation of ischemic stroke patients' NHISS scores with sCD40L and sP-selectin respectively [7, 17]. While fewer researches referred to the microembolic signals [14]. At the same time, barely any of the studies focused on the relationship between the extent of cerebral atherosclerosis and the levels of sCD40L or sP-selectin in LAA stroke patients.

The aims of this study were to investigate the possibility of plasma sCD40L and sP-selectin as clinical markers of the extent and the severity of cerebral atherosclerosis, the destabilization of atherosclerotic plaque, and the severity of neurological dysfunction in LAA stroke patients.

Materials and methods

Subject selection and grouping

For this study, 120 patients who experienced an LAA stroke according to the Trial of Org10-172 in Acute Stroke Treatment (TOAST) classification system [1] were recruited and were admitted to the Department of Neurology at the Affiliated Hospital of Qingdao University within 7 days after stroke onset in 2013. All of the patients were examined by a CT scan and/or MRI of the brain. CT scans, or MR angiography of the brain arteries was performed for all of the patients to verify the cerebrovascular lesions. Digital subtraction angiography (DSA) was operated for the patients with unconfirmed images. All of the patients were examined by transcranial Doppler (TCD) to detected MES and were assessed with the National Institutes of Health Stroke Scale (NIHSS) within the first day of hospitalization [20]. All of the LAA stroke patients underwent an electrocardiography (ECG) and a cardiac ultrasound to exclude those patients with a cardiac embolism.

One hundred twenty patients were divided into 3 subgroups bases on the number of cerebral arteries with atherosclerotic stenosis (≥ 50%) or occlusion: single (n = 40), double (n = 34), and multiple (≥ 3, n = 46). This subgrouping was done to evaluate the extent and the severity of cerebral atherosclerosis. To reflect the instability of plaque, MES by TCD was detected in patients with a responsible artery belonging to the internal carotid artery system (ICA, n = 81), and the MES positive (MES+, n = 13) and MES negative (MES-, n = 68) groups were subdivided among them.

Sixty healthy control subjects were selected from the healthcare clinic at the hospital during the same time. These subjects were confirmed to have no previous history of a stroke and infarcts through a brain CT scan or MRI and to be without severe atherosclerosis or angiostenosis on TCD, a cerebrovascular CT scan, or an MR angiography.

In this research, the subjects with other subtypes of stroke, cerebrovascular malformation, severe heart disease, recent myocardial infarction, angina pectoris disorders, severe infections, severe nephrosis or liver disease, peripheral thrombotic diseases, autoimmune disease, arteritis, tumors, or severe trauma were excluded. The study was approved by the ethical committee of the Affiliated Hospital of Qingdao University. All of the participants gave their informed consent to participate in this study.

Biological samples and biochemical measurements

Peripheral blood was drawn from all of the subjects after an overnight fast. For the LAA patients, the blood samples were obtained within 24 hours of hospitalization. We detected the levels of triglycerides (TGs), total cholesterol (TC), low-density lipoprotein (LDL), high-density lipoprotein (HDL), glucose (GLU), leukocyte (WBC) count, platelet (PLT) count, and high sensitivity C-reactive protein (hs-CRP) with an automatic biochemical analyzer (Hitachi 7600-020, Hitachi, Tokyo, Japan) in the hospital.

sCD40L and sP-selectin measurements

All of the samples were isolated by centrifugation at 3000×g for 10 minutes; the serum and plasma samples were aliquoted and stored at -80°C. All of the samples were thawed only once.

The measurements of the plasma levels of sCD40L and sP-selectin were performed using commercial ELISA kits (R&D Systems, Abingdon, United Kingdom). The detection ranges of the assays separately were 20-1000 pg/ml and 50-1500 pg/mL, respectively.

Statistical analysis

SPSS statistical software 21.0 was used for statistical analysis. Data of normal distribution variables were presented as means ± standard deviation (SD). Data of non-normal distribution continuous variables were presented as median (lower-upper quartiles). Data of categorical variables were presented as frequency (percentage). The normal distribution continuous variables of the two independent groups were compared by a t-test; and the categorical variables were compared by a chi-square test. 3 subgroups bases on the number of cerebral

Table 1. Basic data and plasma levels of sCD40L and sP-selectin in the LAA stroke patients and the controls

Variables	LAA patients (n = 120)	Controls (n = 60)	P-value*
Age, year, mean ± SD	63.17±11.87	60.17±11.78	0.111
Gender, male (%)	78 (65)	35 (58.33)	0.383
Smoking, n (%)	67 (55.83)	15 (25)	0.000
Alcohol consumption, n (%)	64 (53.33)	18 (30)	0.003
Hypertension, n (%)	95 (79.17)	27 (45)	0.000
Diabetes, n (%)	38 (31.67)	13 (21.67)	0.160
Coronary disease, n (%)	22 (18.33)	13 (21.67)	0.594
TGs, mmol/L, mean ± SD	1.78±0.50	1.72±0.61	0.566
TC, mmol/L, mean ± SD	4.83±0.73	4.35±0.58	0.000
HDL, mmol/L, mean ± SD	1.06±0.22	1.23±0.26	0.000
LDL, mmol/L, mean ± SD	2.53±0.53	2.47±0.44	0.419
GLU, mmol/L, mean ± SD	6.29±2.66	5.35±1.32	0.002
hs-CRP, mg/L, mean ± SD	16.53±26.81	6.94±21.82	0.011
WBC, ×10⁹/L, mean ± SD	7.46±2.27	6.29±2.20	0.001
PLT, ×10⁹/L, mean ± SD	216.58±49.97	206.35±55.02	0.212
sCD40L, pg/ml, mean ± SD	586.52±335.84	200.44±78.28	0.000
sP-selectin, ng/ml, mean ± SD	42.32±19.52	17.42±5.34	0.000

*Differences between the LAA patients and the controls determined using a chi-square test or a *t*-test. n: case-number, TGs: triglycerides, TC: total cholesterol, HDL: high-density lipoprotein, LDL: low-density lipoprotein, GLU: fasting blood-glucose, hs-CRP: high sensitivity C-reactive protein, WBC: leukocyte count, PLT: platelet count.

years) and 60 healthy controls (35 men; average age 60.17±11.78 years). The patients and the controls had no difference in average age, gender, the presence of diabetes or coronary disease, or TGs, LDL and PLT levels (P > 0.05). The frequency of smoking, alcohol consumption, history of hypertension disease, the levels of TC, GLU, hs-CRP, and WBC count was significantly higher in patients than controls (P < 0.05). However, the HDL level was lower for the patients than controls (P < 0.001, **Table 1**).

Plasma levels of sCD40L and sP-selectin in LAA stroke patients and controls

The plasma sCD40L and sP-selectin levels were significantly higher in the LAA stroke patients than controls (P < 0.001, **Table 1**). With all of the listed risk factors being adjusted by the binary logistic regression analysis, significant differences in the plasma levels of sCD40L and sP-selectin were also noticed between the LAA patients and the controls (OR = 1.014; 95% CI 1.003-1.026, P = 0.015; OR = 1.276; 95% CI 1.086-1.50, P = 0.003, respectively).

Correlation analysis of sCD40L and sP-selectin levels

A Spearman correlation analysis showed that the levels of sCD40L and sP-selectin were positively correlated in the LAA stroke patients (r = 0.712, P < 0.001). However, there was no statistically signafecent relation between the levels of sCD40L and sP-selectin in the controls (P > 0.1).

Relationship of plasma levels of sCD40L and sP-selectin with the extent and severity of cerebral atherosclerosis

We found significant differences of the sCD40L and sP-selectin levels in the 3 subgroups based on the number of cerebral atherosclerotic arter-

arteries with atherosclerotic stenosis were compared by a rank test as the non-normal distribution continuous variables. A binary logistic regression analysis with adjustment was used for the analysis of the risk factors, such as smoking, alcohol consumption, history of hypertension disease, the levels of TC, GLU, hs-CRP, and WBC count. We analyzed the relativity with the Spearman test. ROC (receiver operating characteristic) curves analysis was used to examine the performance of sCD40L and sP-selectin for diagnosis. The optimal cut-off level of sCD40L and sP-selectin was ascertained by the Youden index. Furthermore, we compared the performances between sCD40L and sP-selectin for the diagnosis of the LAA stroke patients, assessing the AUCs by the method of Hanley&Mcneil with MedCalc statistical software. A two-tailed P < 0.05 was considered to indicate a statistically significant difference.

Results

Characteristics of LAA stroke patients and controls

We included 120 patients who experienced an LAA stroke (78 men; average age 63.17±11.87

Table 2. Plasma levels of sCD40L and sP-selectin in the 3 subgroups based on the number of cerebral atherosclerotic arteries

| Variables	Single-artery, n = 40	Double-artery, n = 34	Multiple-arterys (≥3), n = 46	P-value*
sCD40L, pg/ml, m (q1,q3)	344.08 (191.85, 728.72)	545.84 (394.37, 704.85)	654.45 (464.14, 821.62)	0.002
sP-selectin, ng/ml, m (q1,q3)	29.06 (20.00, 46.14)	39.13 (31.43, 50.30)	44.59 (37.91, 55.79)	0.000

*comparing the 3 subgroups by a Kruskal-Wallis test. n: case-number, m (q1, q3): median (lower-upper quartiles).

Table 3. Plasma levels of sCD40L and sP-selectin in the 2 subgroups of MES

Variables	MES+, n = 13	MES-, n = 68	P-value*
sCD40L, pg/ml, mean ± SD	1075.31±223.65	541.87±316.72	0.000
sP-selectin, ng/ml, mean ± SD	62.40±17.25	38.91±18.72	0.000

*comparing the 2 subgroups by t-test.
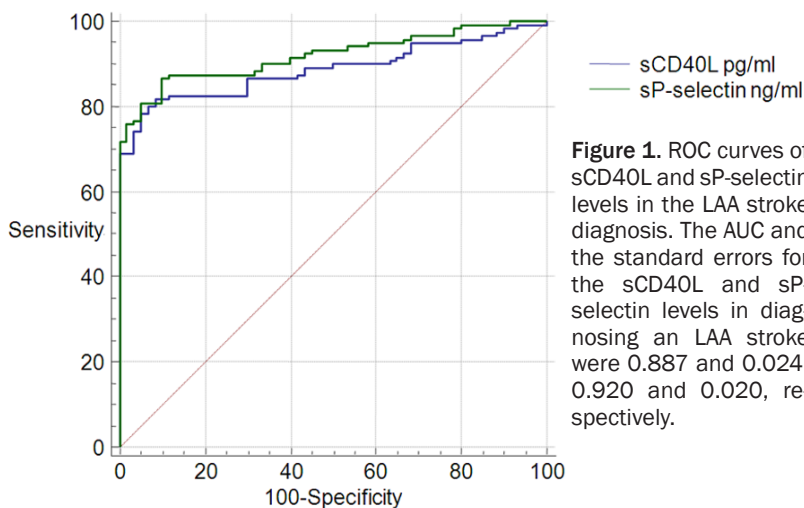
Figure 1. ROC curves of sCD40L and sP-selectin levels in the LAA stroke diagnosis. The AUC and the standard errors for the sCD40L and sP-selectin levels in diagnosing an LAA stroke were 0.887 and 0.024, 0.920 and 0.020, respectively.

(MES-, n = 68) groups. The plasma levels of sCD40L and sP-selectin were significantly higher in the MES+ patients than the MES- patients separately (P < 0.001, **Table 3**). Binary logistic regression analysis also showed both of sCD40L and sP-selectin were independent risk factor for microembolic signals in LAA patients (OR = 1.006; 95% CI 1.001-1.010, P = 0.016; OR = 1.100; 95% CI 1.008-1.200, P = 0.033, respectively).

Relationship of plasma levels of sCD40L and sP-selectin with NIHSS scores of LAA stroke patients

ies compared using the Kruskal-Wallis test (P < 0.05, **Table 2**). Further studies of post-hoc multiple comparisons with the Mann-Whitney test adjusted by Bonferroni method showed that the multiple group has higher levels of sCD40L and sP-selectin than the single group (P < 0.001 and P = 0.002, respectively). At the same time, the Spearman correlation analysis presented a positive correlation of the sCD40L and sP-selectin levels with the number of cerebral atherosclerotic arteries separately (r = 0.330, P < 0.001; r = 0.359, P < 0.001, respectively).

Relationship of plasma levels of sCD40L and sP-selectin with MES

There were 81 patients with a responsible artery belonging to the internal carotid artery system. These patients were divided into MES positive (MES+, n = 13) and MES negative

In the LAA stroke patients, the NIHSS scores were significantly correlated with the levels of sCD40L and sP-selectin by the Spearman test (r = 0.490, P < 0.001; r = 0.415, P < 0.001, respectively).

Association of plasma levels of sCD40L and sP-selectin and diognosis of LAA stroke

The AUC for the sCD40L and sP-selectin levels in diagnosing an LAA stroke was 0. 887 and 0. 920, respectively. The optimal cut-off values and the standard errors were 307.00 pg/mL and 0.024, 22.62 ng/mL and 0.020, respectively. The sensitivity and the specificity of the cut-off values mentioned above were 81.7% and 91.7%, 86.7% and 90%, respectively (**Figure 1**), but no statistically significant variation of the diagnostic performance for an LAA stroke was found within sCD40L and sP-selectin (Z = 1.26, P = 0.2077).

Discussion

In accordance with previous researches, we found that the plasma levels of both sCD40L and sP-selectin were significantly higher for the LAA stroke patients than the healthy controls. Furthermore, our data indicated that the higher levels of sCD40L and sP-selectin were significantly associated with the increased number of cerebral atherosclerotic arteries, MES+, and higher NIHSS scores in the LAA stroke patients. In addition, both sCD40L and sP-selectin had good value in the diagnostic performance for the LAA stroke patients, demonstrated by the ROC curve. Beyond that, a remarkable positive correlation of sCD40L and sP-selectin was observed in the LAA group; while no correlation was observed in the healthy controls. These results strongly support those previous reports presenting that sCD40L and sP-selectin are closely associated in the process of platelet activation [3].

Platelet activation plays a key role in forming and extending atherosclerotic plaques [2]. The activated platelets can release sCD40L, inducing platelet aggregation and platelet-leukocyte conjugation and making a direct contribution to endothelium injury that may lead to athero-thrombosis and inflammation [9]. In the meantime, sCD40L enhances platelet activation in an auto-amplification loop, resulting in the increased expression of P-selectin [9]. As an important adhesive molecule, P-selectin can stimulate atherothrombosis and inflammation by the formation of platelet-leukocyte aggregate and adhesion between leukocytes and endothelium [10]. Inflammation is well known to play significant roles in the occurrence and the development of atherosclerosis, which is an important mechanism of an LAA stroke [11]. Thus, high sCD40L and sP-selectin levels may have an accelerated role in atherosclerosis, consistent with the previous studies and our research. In these observations pointing to the carotid artery, sCD40L and sP-selectin were considered to relate with not only the formation of atherosclerosis plaque, but also the instability of the plaques [12-14]. Our data showed higher sCD40L and sP-selectin levels in the MES+ LAA stroke patients as well, indicating that both of them had an association with the instability of plaques. Otherwise, we first time noticed that the levels of sCD40L and sP-selec-

tin were elevated, accompanied with an increased number of cerebral atherosclerotic arteries in the LAA stroke patients. This reminded us that higher CD40L and sP-selectin levels might be useful markers for the worsening extent and severity of cerebral atherosclerosis.

The NIHSS scores have been widely applied in clinic to assess the neurological disability of ischemic stroke (IS) patients. There were relations between the NIHSS scores and the CT lesion volumes in IS patients, commonly accepted already [20]. In our study, a positive linear correlation was detected between the NIHSS scores and both of the sCD40L and sP-selectin levels in the LAA stroke patients during the acute phase. We considered that this was partly because both of them participated in the pathological process of the acute phase in an LAA stroke, which was proven in vivo studies by comparing the brain infarct volume in a middle cerebral artery occlusion (MCAO) model of mice [15, 16]. Wang Q et al. reported that the sP-selectin levels were higher in patients with a progressive IS than patients with a non-progressive IS [17]; studies in CAD proposed that sCD40L and sP-selectin levels might predict the severity of CAD [18, 19], confirming the accuracy of our research.

Conclusion

In conclusion, elevated plasma levels of sCD-40L and sP-selectin are supposedly involved in an LAA stroke by participating in multiple links of the disease and might be used for assessing the worsening extent and severity of cerebral atherosclerosis, the destabilization of athero-sclerotic plaque, and the severity of neurological dysfunction in LAA stroke patients. Further studies with larger sample sizes are required to estimate the utility of the sCD40L and sP-selectin levels in the clinic, and follow-up appointments are needed to judge the value of the prognosis.

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Disclosure of conflict of interest

None.

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