Elevated plasma levels and monocyte-associated expression of CD137 ligand in patients with acute atherothrombotic stroke

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Abstract. – BACKGROUND: CD137 ligand (CD137L) is expressed by various immune cells and exists in membrane-bound and soluble forms. Recently, CD137L was found to be localized to macrophages in human atherosclerotic lesions and CD137L levels were much higher in atherosclerotic lesions than in normal arteries. However, the role of CD137L with different forms in atherothrombotic stroke remains unclear.

PATIENTS AND METHODS: The soluble CD137L (sCD137L) protein and CD137L expression on monocytes were analyzed by an enzyme-linked immunosorbent assay and flow cytometry in peripheral blood of patients with acute ischemic atherosclerotic stroke, atherosclerosis controls and normal controls.

RESULTS: During the initial 24h after onset, the stroke patients had elevated plasma sCD137L levels (133.2 pg/ml) and CD137L expression on monocytes [7.9±4.1%, 7.0±4.0 mean fluorescence intensity (MFI)] as compared with normal controls (75 pg/ml, $p < 0.05$; 4.6±2.4%, 4.1±2.7 MFI, $p < 0.05$).

CONCLUSIONS: The dysregulation of CD137L expression may reflect a persistent chronic inflammatory response that may have been induced during early stages of the disease. Our results strongly suggest that the abnormal expression of CD137L on monocytes may lead to dyregulated CD137L/CD137 signaling and consequently form part of a positive-feedback, inflammation-promoting circuit in stroke, while the elevated sCD137L protein levels may function as a self-regulatory mechanism of CD137/CD137L interaction and costimulation.

Key Words:
CD137L, Ischemic atherosclerotic stroke, Atherogenesis, Peripheral blood.

Introduction

Inflammation has received increasing attention in recent years as a cause of atherosclerosis[1,2]. So far, various studies have provided strong evidences that atherosclerosis involves immune cells, such as T cells, monocytes or macrophages in the vasculature, systemically elevated proinflammatory cytokines, chemokines, adhesion molecules, and tissue factor (TF)[3]. In the advanced stages, atherosclerosis lead to formation of an intravascular thrombus, which plays a critical role in the setting of acute arterial thrombosis, heart attack or stroke[4].

A variety of cytokines, including members of the tumor necrosis factor (TNF) superfamily (TNFSF), have been identified in atherosclerotic lesions and implicated in the pathogenesis of atherosclerosis[3,5]. TNF-α and CD40 ligand (CD40L) have been found to have pivotal roles in atherogenesis by eliciting immune responses such as secretion of proinflammatory cytokines, activation of matrix metalloproteinases (MMP) and induction of TF expression[6-8]. In addition, another TNFSF member of CD137 (4-1BB, TNFRSF9) has been recently reported to demonstrate an enhanced expression in human atherosclerotic plaques on T cells and endothelial cells as well as its ability to induce adhesion molecule expression on endothelial cells and reduce smooth muscle cell proliferation upon activation by CD137 ligand (CD137L, 4-1BBL, TNFSF9)[9]. Additionally, in one recent animal study of CD137-deficient apolipoprotein E-knockout mice (*ApoE$^{-/-}$CD137$^{-/-}$*) and LDL-receptor-knockout mice (*Ldlr$^{-/-}$CD137$^{-/-}$*), the deficiency of CD137 caused a reduction in atherosclerotic plaque lesions in both atherosclerosis mouse models, while stimulation of CD137L signaling by soluble CD137 protein activated monocytes/macrophages and augmented the production of proinflammatory cytokines in atherosclerotic vessels. These findings further support proatherogenic roles that CD137L/CD137 interaction plays in the process of

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Table I. Baseline characteristics of patients and normal controls.

	Ischemic stroke (n=20)	Asymptomatic carotid stenosis (n=19)	Normal controls (n=23)	p value
Sex (male/female)	12/8	13/6	14/9	-
Age (years)	62.2 ± 6.6	62.3 ± 5.3	59.9 ± 7.3	0.425
Hypertension (%)	50	47.4	-	-
Diabetes mellitus (%)	35	42.1	-	-
Hypercholerolemia (%)	25	21.1	-	-
Smoking (%)	55	52.6	52.2	-
Peripheral artery disease (%)	25	22.2	21.7	-
Leukocyte ($\times 10^3$/µl)	7.87 ± 1.37	7.03 ± 1.36	6.82 ± 1.59	0.0556
Monocyte ($\times 10^3$/µl)	0.46 ± 0.18	0.41 ± 0.12	0.39 ± 0.16	0.3947
Data are mean±SD				

atherosclerosis[10]. Moreover, two distinct studies showed a significantly increased CD137 expression on circulating monocytes and serum soluble CD137 levels in patients with acute coronary syndromes compared to patients with stable angina and healthy controls[11,12]. Nevertheless, there is still a great need for further studies of the CD137L expression as well as its possible role in the atherogenetic process of ischemic stroke. In this study, the surface expression of CD137L and soluble CD137L (sCD137L) proteins were analyzed in peripheral blood of patients with ischemic atherosclerotic stroke to further determine whether this molucule is aberrantly produced in this disease.

Patients and Methods

Patients and Controls

Twenty patients with acute ischemic atherosclerotic stroke (12 men and 8 women; mean age 62.2±6.6 years) were enrolled in this study. These patients had large-artery atherosclerosis (LAA) subtype, according to the Acute Stroke Treatment (TOAST) classification[13]. All patients were evaluated within 24h of ischemic stroke onset and diagnosed on the basis of medical history, clinical examination and results of brain MRI and MRA scans. National Institute of Health Stroke Scale (NIHSS) was adopted to reflect the disease severity at the time of blood sampling. Patients with risk factors for cardioembolic stroke such as atrial fibrillation, vascular heart disease, acute myocardial infarction and endocarditis were excluded. In addition, five of these patients with LAA stroke receiving treatment with simvastatin (40 mg qd) were chosen for serial study.

The atherosclerosis controls (ASC) consisted of nineteen patients with asymptomatic carotid stenosis (> 50%) (13 men and 6 women; mean age 62.3±5.3 years) on carotid duplex sonography. All the patients had no evidence of ischemic stroke events on clinical history and brain MRI. Twenty-three healthy subjects (14 men and 9 women; mean age 59.9±7.3 years) were included as normal controls (NC) without any evidence of large vessel atherosclerosis, clinical history of vascular events and vascular risk factors.

None of the participating individuals had (1) previously been taking statins and calcium channel blockers; (2) autoimmune, hepatic, renal or cancerous disorder; (3) an infection. The following diagnostic tests were performed in both patients and normal controls: complete blood counts, blood chemistry, erythrocyte sedimentation rate (ESR), C reactive protein (CRP), EKG, posterior-anterior chest radiography, transthoraric cardiac echocardiography, transcranial Doppler ultrasonography and carotid duplex sonography. All participating subjects gave their informed consent before the start of the study.

Sample Collection

All the heparinized blood specimens were collected between 09:00 am and 12:00 am. Peripheral blood mononuclear cells (PBMC) were isolated from the heparinized blood specimens by density gradient centrifugation using a standard protocol. After centrifugation, plasma was stored at −70°C in small aliquots and thawed just before further use.

Flow Cytometry

Peripheral blood mononuclear cells of patients and control subjects were characterized for the expression of CD137L by double-colour direct immunofluorescence and flow cytometry using a FACScan (Becton Dickinson, Carlsbad, CA,

USA). Cells were washed with PBS (pH 7.2) and resuspended in phoshate buffered saline (PBS) buffer. The following antibodies were added with $1 \leftrightarrow 10^5$ cells in 25 μl cell suspension: (1) IgG1 FITC, IgG1 PE (Becton Dickinson, Carlsbad, CA, USA); (2) CD14 FITC, CD137L PE (Pharmingen, San Diego, CA, USA). After 30 min incubation at 4°C, cells were washed twice with staining buffer and analysed on the FACScan using CellQuest software.

Enzyme-Linked Immunosorbent Assay (ELISA)

Plasma CD137L levels were measured by a commercial ELISA according to the supplier's directions (Immatics Biotechnologies, Tübingen, Germany) with a detection limit of 1 pg/ml. All assays were performed simultaneously in a blinded fashion.

Statistical Analysis

The data are presented as mean±standard deviation of the mean value (CD137L surface expression and blood monocyte) or median with range (sCD137L). Data with a normal distribution were analyzed with one-way analysis of variance (ANOVA) by Student-Newman-Keul's post-hoc test and Pearson's correlation test. Data with a non-normal distribution were analyzed with Kruskal-Wallis ANOVA and Spearman's correlation test. p value of less than 0.05 was considered to be statistically significant.

Results

Clinical and Laboratory Data

Patient's characteristics, including risk factors and laboratory data taken on the first day of sampling are shown in Table I. In addition, no signif- icant differences in blood pressure or levels of blood glucose, total cholesterol, high-density lipoprotein (HDL), low-density lipoprotein (LDL), ESR, CRP were found between the stroke patients and either of the control groups (data not shown).

### Cell Surface Expression of CD137L

The stroke patients showed increased expression of CD137L (Figure 1) on CD14+ monocytes [7.9±4.1%, 7.0±4.0 mean fluorescence intensity (MFI)] compared with NC (4.6±2.4%, 4.1±2.7 MFI, $p < 0.05$) (Table II), but no significant difference of CD137L expression was found on PBMC between stroke (8.7±4.9%, 8.2±5.0 MFI) and either of the control groups (7.8±5.4%, 7.4±5.6 MFI and 5.9±3.0%, 6.0±5.1 MFI) (Table II). Moreover, five stroke patients demonstrated a trend towards a continuous decrease in CD137L expression on CD14+ monocyte after 3 and 14 days of treatment, while the blood monocytes presented irregular changes (Figure 2).

Plasma sCD137L Levels

During the initial 24h after onset, the plasma sCD137L levels were significantly elevated in the patients with ischemic stroke (133.2 pg/ml) compared with normal controls (75 pg/ml, $p < 0.05$), but no significant difference was found between the stroke patients and the patients with asymptomatic carotid stenosis (84.1 pg/ml) (Figure 3). The plasma sCD137L levels within 24h were significantly higher in the stroke patients but showed a tendency of decrease after 3 and 14 days of treatment (Figure 4).

Correlation Analysis

CD137L surface expression levels on PBMC or CD14+ monocytes were not significantly correlat-
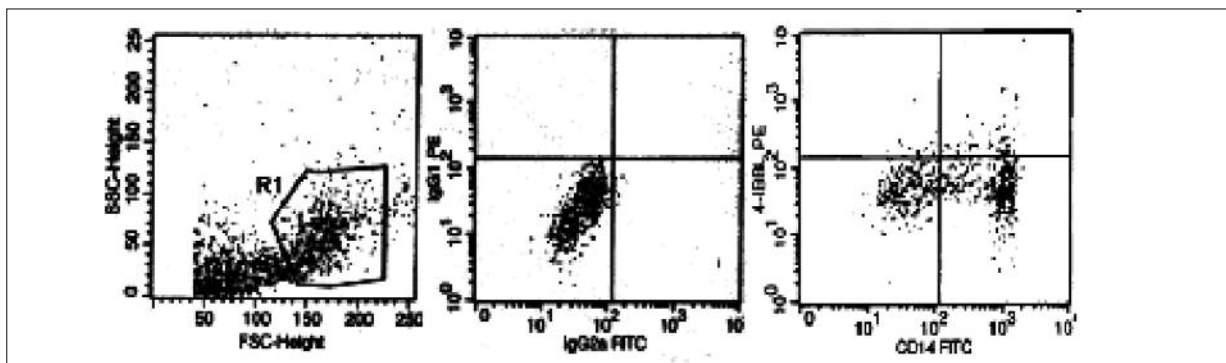
Figure 1. Region 1 (R1) is selected to set monocytes gate for CD137L analysis. Control staining with isotype control antibodies was used as control to define the gate.

Table II. Peripheral blood mononuclear cells (PBMC) and CD14+ monocytes spontaneously expressing CD137L in patients with acute ischemic atherosclerotic stroke, asymptomatic carotid stenosis (ACS) and normal controls (NC).

	CD137L (%)		CD137L (MFI)	
	PBMC	CD14+	PBMC	CD14+
Stroke (n=20)	8.7 ± 4.9	7.9 ± 4.1*	8.2 ± 5.0	7.0 ± 4.0*
ACS (n=19)	7.8 ± 5.4	6.3 ± 5.0	7.4 ± 5.6	5.2 ± 2.3
NC (n=23)	5.9 ± 3.0	4.6 ± 2.4	6.0 ± 5.1	4.1 ± 2.7
p value (ANOVA)	0.1380	0.0347	0.3978	0.0131

MFI = mean fluorescence intensity, *$p < 0.05$ for post hoc comparison with normal controls.
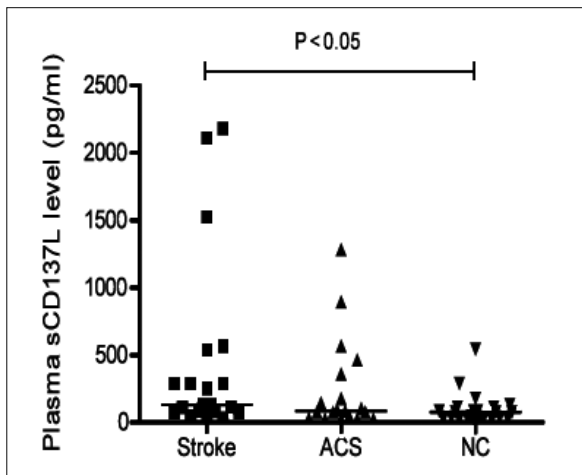
ed with plasma sCD137L levels in stroke patients. Additionally, there was no correlation between the sCD137 levels, CD137L surface expression on PBMC or CD14+ monocytes and NIHSS of stroke patients (data not shown).

Discussion

CD137L is expressed primarily on antigen presenting cells (dendritic cells, monocyte/macrophages and B cells), human primary T cells[14] and nonim-
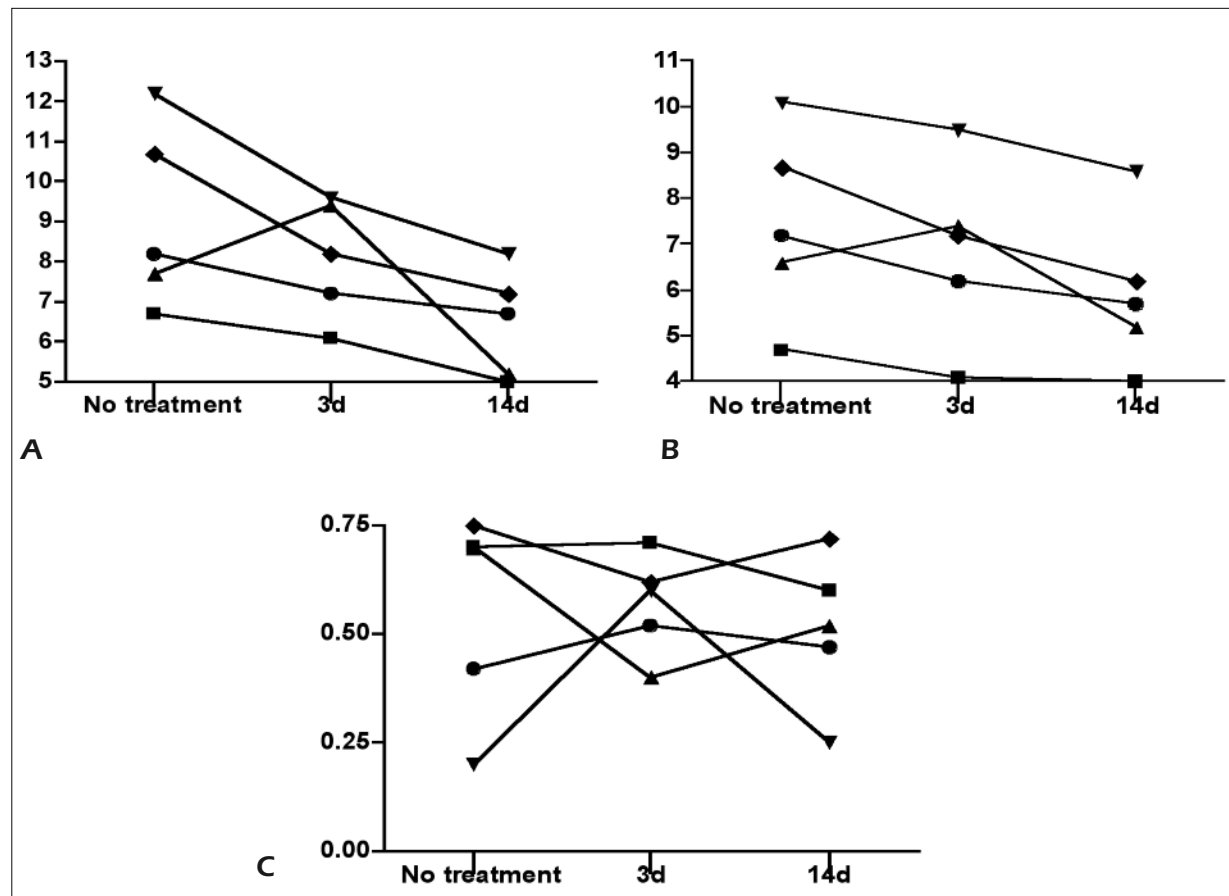
Figure 2. Changes of CD137L expression on CD14+ monocytes in patients with acute ischemic atherosclerotic stroke after treatment. Changes of CD137L expression on CD14+ monocyte (*A* and *B*) and blood monocyte count (*C*) were analysed in five patients with acute ischemic atherosclerotic stroke before treatment and after 3d and 14d of treatment with statin. 3d = 3 days; 14d = 14 days.
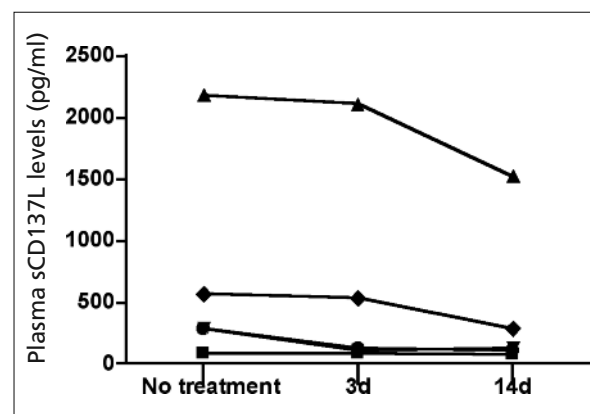
Figure 3. Comparison of plasma soluble CD137L (sCD137L) levels between acute ischemic atherosclerotic stroke and control groups. Plasma sCD137L levels are compared between patients with acute ischemic atherosclerotic stroke, asymptomatic carotid stenosis (ACS) and normal controls (NC) using an enzyme-linked immunosorbent assay (ELISA). Horizontal lines indicate median values, numerals on top are *p*-values

mune cells such as cardiac myocytes in myocarditis and aortic tissue in arteritis at sites of inflammation[15,16]. In addition to providing costimulatory signals for T cell activation and survival, CD137 ligation by CD137L can also trigger reverse signaling, which, in turn, promote the activation, migration and survival of monocytic cells that express CD137L[17,18]. Intriguingly, CD137L is produced in both membrane bound and soluble forms. The sCD137L protein, which is released at higher levels from leukocytes following cellular activation, has been found to stimulate interleukin-2 (IL-2) and interferon-γ (IFN-γ) secretion from peripheral T cells by binding to the CD137 receptor[13]. In a study from Olofsson et al[9] CD137L was mainly localized to macrophages in human atherosclerotic lesions and CD137L levels were much higher in atherosclerotic lesions than in normal arteries[19]. Additionally, the sCD137L protein has been shown to be increased in the tissue extracts from human atherosclerotic compared with normal arteries[9]. These findings suggest that the CD137L may be implicated in the inflammatory process of atherosclerosis.

In this study, patients with acute ischemic atherosclerotic stroke demonstrated an elevated plasma levels of sCD137L, indicating the involvement of CD137L in immune response during acute stage of the disease. Moreover, the increased CD137L expression on CD14+ monocytes further substantiates our findings and also suggests that the dys-

regulation of this molecule may occur predominantly in CD14+ monocytes. Similar to our results, a very recent study[19] reported that, besides the presence of CD137L on monocytes in circulation, patients with acute coronary syndrome (ACS) also exhibited increased CD137 levels in circulating CD4+CD28[null] T cells, which constituted an important proportion of CD4+ T cells in human atherosclerotic plaques. Taking into consideration all these findings, this suggests that there is an aberrant CD137L production existing in the early stage of stroke. Interestingly, no differences of CD137L levels could be found between the patients with asymptomatic carotid stenosis and normal controls, since this category of patients was previously reported to have higher levels of CD137L expression in atheromatous regions than in normal arteries[9].

So far, limited information is available on the CD137L/CD137 signaling in atherosclerosis, mainly due to the complexity of bidirectional signaling between CD137 and CD137L. As described in aforementioned animal study of ApoE^{-}/$^{-}$CD137$^{-/-}$ mice, activation of CD137L signaling by CD137 protein in *ex vivo* induces proinflammatory cytokine such as TNF-α and MCP-1 release in the CD137-deficient atherosclerotic aorta[10]. Another study of ACS patients also demonstrated that blockade of CD137 significantly downregulated the production of TNF-α and IFN-γ from peripheral CD4+CD28[null] T cells which were report to induce rupture of atherosclerotic lesions by directly lysing vascular

Figure 4. Changes of plasma soluble CD137L (sCD137L) levels in patients with acute ischemic atherosclerotic stroke after treatment. Changes of plasma sCD137L levels were analyzed in five patients with acute ischemic atherosclerotic stroke before treatment and after 3d and 14d of treatment with statin. 3d = 3 days; 14d = 14 days.

smooth muscle and endothelial cells[19,20]. In our serial study, although there was non-significant dynamic change of monocyte count in the stroke patients, CD137L expression on the CD14+ monocytes tended to decrease after 3 and 14 days of treatment with statin, which is known as an hydroxy-methyl-glutaryl-CoA (HMG-CoA) reductase inhibitor with multiple anti-inflammatory effects[21]. This is in parallel with a stroke study of a remarkably lower serum levels of TNF-α after a short term of statin therapy[22], indicating a positive regulatory role that CD137L could play in the disease pathogenesis.

To investigate the roles of CD137L activation by its receptor CD137 in stroke, we performed an *in vitro* study of TNF-α and monocyte chemoattractant protein-1 (MCP-1) release from isolated blood monocyte after treatment with a CD137 fusion protein in a few patients with ischemic atherosclerotic stroke (n=5), and consequently a remarkably increased supernatant levels of these two proinflammatory cytokines were found in the presence of the CD137 fusion protein as compared with control human IgG (data not shown). This further supports the notion that reverse CD137L signaling contributes to atherosclerosis-related disorders such as stroke. On the other hand, our findings also favor the costimulatory effect of CD137L on T cells, as our previous *in vitro* study showed that nonspecific T cells proliferation was significantly suppressed after treatment with an anti-CD137L monoclonal antibody (MoAb) compared with the control antibody in a few healthy subjects (n=5)[23]. Taken together, the abnormal expression of CD137L on the monocytes may lead to dyregulated CD137L/CD137 signaling and consequently form part of a positive-feedback, inflammation-promoting circuit in stroke.

In our study, increased sCD137L levels were found in peripheral blood of stroke patients compared with healthy subjects. This is noteworthy because previous studies have shown that the sCD137L protein might play a regulatory role by binding to the CD137 receptor and stimulate IL-2 and IFN-γ release from peripheral T cells as well as significant T cell proliferation[24,25]. We initially speculate that the increase in the sCD137L release may occur mainly because of the higher expression of CD137L in monocytes of stroke patients. However, we did not find a significant correlation between sCD137L protein levels and CD137L surface expression levels in the monocytes or PBMC. Together with similar findings reported in

other inflammatory diseases, such as rheumatoid arthritis and multiple sclerosis[23,26], this indicate that the soluble form of the molecule is not merely produced by shedding of the membrane-bound ligand, but perhaps due to production from distinct, differentially spliced mRNA, or proteolytic cleavage from the cell surface.

The role of the sCD137L in atherosclerosis remains elusive. Hence, we can only speculate about what induces the increase in the sCD137L release in ischemic atherothrombotic stroke and what function this molecule has in the development of the disease. An upregulation in peripheral blood of specific matrix metalloproteinase (MMP), such as gelatinase B and MMP-9, during episodes of acute stroke[27,28], could drive the cleavage of CD137L molecule from cell surface, is a possible theory[13]. Indeed, our study demonstrates that plasma sCD137L levels tend to decrease following treatment, in line with the decreased serum MMP-9 after the treatment with statin[29,30]. On the other hand, the other factors, such as potentially different mRNA splicing and cell type-dependent cleavage[13,26], may also be involved, as our results showed no significant correlation between the sCD137L levels and CD137L surface expression levels in the monocytes or PBMC. The shedding of CD137L from antigen presenting cells such as monocytes may function as a mechanism that limits inflammatory or costimulatory responses induced by the local cell-cell interaction[13]. The immunological derangement of CD137L could be a general phenomenon in chronic inflammatory disorders, as upregulation of sCD137L levels has been also described in other inflammatory conditions[13,23,26].

Conclusions

This study shows a remarkable dysregulation of CD137L expression in patients with acute ischemic atherosclerotic stroke. This may reflect a persistent chronic inflammatory response that may have been induced during early stages of the disease. Our results strongly suggest that the abnormal expression of CD137L on the monocytes may lead to dyregulated CD137L/CD137 signaling and consequently form part of a positive-feedback, inflammation-promoting circuit in stroke, while the elevated sCD137L protein levels in the stroke patients may function as a self-regulatory mechanism of CD137L/CD137 interaction and costimulation. Further studies on the influence of

different etiologies of ischemic stroke and other atherothrombotic diseases, on the effects of the CD137L are needed to gain more insight into the pathophysiological significance of this TNFSF member in human stroke.

Conflict of Interest

The Authors declare that there are no conflicts of interest.

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