Original Article

CD40 Ligand and MCP-1 as Predictors of Cardiovascular Events in Diabetic Patients with Stroke

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***Background and Aims*: Up-regulation of soluble CD40 ligand (sCD40L) and of monocyte chemoattractant protein-1 (MCP-1) has been found in diabetes and in patients with acute cerebral ischemia. We asked whether (i) the two molecules are similarly upregulated among non-lacunar and lacunar diabetic strokes and (ii) sCD40L and/or MCP-1 predict the risk of cardiovascular events in this setting.**
***Methods and Results*: Ninety patients with type 2 diabetes mellitus presenting with an acute ischemic stroke (compared with 45 control subjects) were evaluated on admission and up to 36 months (median 24 months) after the event. Diabetic patients with acute stroke had higher plasma CD40L and MCP-1 than controls ($p < 0.0001$), with no significant differences among lacunar and non-lacunar strokes. On multiple regression analysis, only higher sCD40L quartiles and older age were associated with higher MCP-1 quartiles. Forty-eight percent of patients experienced vascular events. Cox regression analysis showed that only the presence of higher sCD40L values independently predicted the recurrence of vascular events.**
***Conclusion*: Up-regulation of inflammatory molecules, such as CD40L and MCP-1, is involved in the advanced stage of atherosclerotic cerebro-vascular disease and is associated with increased risk of recurrence of cardiovascular events.**

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Key words; Cytokines, Chemokines, Cerebral ischemia, Diabetes mellitus

Introduction

Diabetes is a major risk factor for stroke[1, 2] and is associated with an increase in overall stroke mortality attributable to higher-than-optimum blood glucose concentration[3]. Admitted patients with diabetes mellitus show a higher prevalence of lacunar stroke subtype than non-diabetics[4]. Whether lacunar strokes are associated with a different outcome vs non-lacunar strokes in diabetics is controversial[5, 6].

Cytokines and cytokine-inducible inflammatory molecules are regarded as predictive markers of cardiovascular events[7]. It is known that CD40 signaling mediates many inflammatory responses in atherosclerosis[8]. Enhanced CD40 ligand (CD40L) release is associated with thromboxane-dependent platelet activation in type 2 diabetes[9]. Activated platelets induce monocyte chemoattractant protein-1 (MCP-1) release from endothelial cells in a CD40L-dependent manner[10]. MCP-1 is a chemokine responsible for the recruitment of monocytes to inflammation sites and its expression level is directly related to the extent of atherosclerosis and macrophage infiltration into the atherosclerotic lesion[11]. MCP-1 levels are increased in type 2 diabetes and are associated with increased risk of cardiovascular mortality[12]. Upregulation of soluble CD40L (sCD40L) and MCP-1 has been found in patients with acute cerebral ischemia, even though only 22% of patients with stroke or transient ischemic attack were diabetics[13].

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Table 1. Clinical characteristics of patients and controls

Variable	Patients, $n=90$	p value	Controls, $n=45$
Age,* y	72 ± 10	0.89	72 ± 9
Sex,* F/M	39/51	0.90	19/26
TOAST			
0	36	–	–
1	41	–	–
2	13	–	–
SSS Score	37 ± 10.2	–	–
Plasma glucose on admission mmol/L (mg/dL)	12.5 ± 3.4 (225 ± 62)	< 0.0001	5.2 ± 0.5 (94.5 ± 7.9)
HbA_{1c}, %	7.7 ± 1.6	–	–
Diabetes duration, yrs	9.5 ± 3.9	–	–
BMI, kg/m	30.7 ± 1.7	< 0.0001	27.4 ± 3.4
Hypertension,* n (%)	55 (61)	< 0.01	38 (84)
SBP, mmHg	153 ± 16	< 0.0001	131 ± 1.5
DBP, mmHg	92 ± 20	< 0.0001	81 ± 11
Total cholesterol, mmol/L (mg/dL)	5.9 ± 1.0 (229 ± 37)	> 0.05	5.8 ± 1.0 (226 ± 38)
Triglycerides, mmol/L (mg/dL)	2.1 ± 1.3 (191 ± 117)	> 0.05	2.1 ± 1.3 (186 ± 106)
Previous TIA,* n (%)	46 (51)	< 0.001	0 (0)
Previous stroke,* n (%)	38 (42)	< 0.001	0 (0)
Antiplatelet agents,* n, (%)	42 (47)	< 0.001	0 (0)
Lipid-lowering drugs,* n (%)	24 (27)	0.16	18 (40)
ACE-I/ARBs,* n (%)	61 (68)	< 0.001	14 (31)
Body temperature, ℃	37 ± 0.5	–	–
Leukocytes TSD/μL	8.5 ± 2.1	< 0.0001	5.4 ± 0.8
sCD40L, Ü pg/mL	1,252 (813–2,468)	< 0.0001	456 (326–650)
MCP-1, Ü pg/mL	210 (157–348)	< 0.0001	135 (85–198)

Data are the mean ± SD, t test for independent samples. *Chi-square test. Ü Median (range), Mann-Whitney U test.
TOAST = Trial of Org 10172 in Acute Stroke Treatment, SSS = Scandinavian Stroke Scale, BMI = Body Mass Index, SBP = systolic blood pressure, DBP = diastolic blood pressure, ACE-I = Angiotensin converting enzyme inhibitors, ARBs = Angiotensin receptor blockers, TSD = thousand, CD40L = CD40 ligand, MCP-1 = Monocyte chemoattractant protein-1.

We asked whether, in a cohort of diabetic subjects with acute ischemic stroke (i) the two molecules are similarly up-regulated among non-lacunar and lacunar strokes and (ii) CD40L and/or MCP-1 may predict the risk of cardiovascular events in ischemic stroke.

Subjects

Between November 2002 and February 2006, we evaluated a cohort of 90 patients with type 2 diabetes mellitus, defined according to the World Health Organization diagnostic criteria for diabetes[14] presenting with an acute ischemic stroke, as diagnosed on the basis of neurological signs and symptoms and compatible CT findings, at the Department of Internal Medicine of Palermo University Hospital within 72 hours after the onset of symptoms. Patients with symptoms lasting for >72 hours on admission were excluded from the study. Further exclusion criteria were acute and chronic inflammatory diseases, autoimmune diseases or malignancies, acute coronary syndromes, intracerebral haemorrhage, and treatment with hormonal replacement therapy, steroids, and non-steroidal anti-inflammatory drugs. A detailed history was obtained (**Table 1**).

Standard diagnostic tests included cranial computed tomography to exclude intracerebral haemorrhage, duplex sonography to detect or exclude significant stenosis of carotid arteries, and echocardiography to exclude the presence of an intracardiac thrombus.

Acute cerebral ischemias were classified according to the Trial of Org 10172 in Acute Stroke Treatment (TOAST) criteria[15, 16]. Severity of stroke was evaluated by the Scandinavian Stroke Scale (SSS) score on acute admission[17]. The Scandinavian Stroke Scale is an acute stroke assessment scale based on scored assessments of: level of consciousness, eye movement, arm motor power, hand motor power, leg motor power,

orientation, speech, facial palsy, and gait. Forty-five subjects (26 males and 19 females, **Table 1**) were also recruited for the study as a control group. They were recruited from our vascular event prevention clinic and they were free of any previous vascular event. Controls were matched for sex and age with stroke patients. All patients gave written informed consent. The study was approved by the local ethics committee.

Patients were re-evaluated in our clinic every three months up to 36 months after the event (median follow-up 32 months; range 4–36) or when they experienced cardiovascular events (myocardial infarction, angina, stroke, transient ischemic attack, critical limb ischemia, need for percutaneous revascularization procedures or coronary artery by-pass).

Methods

Biochemical Measurements

Blood samples, collected within 72 hours of the ischemic event, were processed according to the recent recommendations regarding the appropriate specimen and preparation for laboratory evaluation of sCD40L[18, 19]. Thus, blood samples were anticoagulated in Na citrate 3.8% (1:9 v:v), centrifuged at 2,000 g for 10 minutes at 4℃. Supernatants were collected and platelets and other cell types were counted (Beckman Coulter). Plasma was stored at −80℃ until analysis. Plasma CD40L and MCP-1 levels were measured by enzymelinked immunosorbent assay (R&D Systems, Minneapolis, Minnesota).

Statistical Analysis

Statistical analysis was performed by chi-square statistics, Pearson's correlation coefficient *t* test for independent samples and by one-way analysis of variance with Bonferroni adjustment to assess differences among groups. When necessary, appropriate nonparametric tests were used (Spearman correlation coefficient and Mann-Whitney *U* test). Because the distribution of sCD40L and MCP-1 was skewed (Shapiro-Wilk test) the concentration of these variables was also analyzed statistically by quartiles. Multiple linear regression analysis was performed to further quantify the relationship between MCP-1 (divided into quartiles) and the possible explanatory factor. Specifically, the dependent variable, MCP-1, was regressed for sCD40L (divided into quartiles), age, SSS score, sex, TOAST group, HbA1c, arterial blood pressure, total cholesterol, BMI, diabetes duration, and previous vascular events. The clinical relevance of parameters used to predict all endpoints (vascular events, such as myocardial infarction, episodes of unstable angina, stroke, transient ischaemic attack, and critical limb ischemia) was estimated by univariate (log rank test) and multivariate (Cox proportional hazard model including variables that achieved statistical significance in univariate analysis) analyses. Only p values lower than 0.05 were regarded as significant. Data are presented as the mean (SD), or median (interquartile range) (SPSS by SPSS Inc. and EGRET by SERC city, state/country).

Results

According to the TOAST criteria, the etiology of stroke was large-artery atherosclerosis (LAA) in 36 (40%) patients, cardioembolism (CE) in 13 (14%) patients, and lacunar stroke [small artery occlusion (SAO)] in 41 (46%) patients.

Patients and controls were comparable for age (72 ± 10 vs 72 ± 9, $p=0.8989$) and sex [males: 51 (57%) vs 26 (58%), $p=0.90$).

Diabetic patients with acute stroke had higher plasma CD40L [median (interquartile range): 1,252 (813–2468) vs 456 (326–650) pg/mL $p<0.0001$] and MCP-1 [210 (157–348) vs 135 (85–198) pg/mL, $p<0.0001$] than controls (**Fig. 1**).

No significant differences in plasma CD40L or MCP-1 levels among different TOAST groups ($p=0.486$ and $p=0.378$, respectively, **Fig. 1** inside panels) were detected.

The SSS on admission showed the lowest values in cardioembolic stroke [median (interquartile range): 22 (19–29)] as compared with LAA [37.5 (31–40)] or lacunar stroke [42 (37–42)] ($p<0.0001$).

Among all patients, plasma MCP-1 directly correlated with sCD40L (Rho=0.69, $p<0.0001$). Because the distribution of sCD40L (Shapiro-Wilk W=0.75815, $p<0.00001$) and MCP-1 (Shapiro-Wilk W=0.87251, $p<0.0001$) was skewed, the concentration of these variables was also analyzed statistically by quartiles.

Thus, multiple-linear regression analysis was performed to further quantify the relationship between plasma sCD40L and MCP-1 and the other possible explanatory factors. Multiple regression analysis indicated that only higher sCD40L quartiles (b=0.70, SE=0.08; $p<0.0001$) and higher age (b=0.21, SE=0.08; $p<0.02$) were associated with higher MCP-1 quartiles, independently of SSS score, sex, TOAST group, HbA_{1c}, diabetes duration, body mass index, systolic and diastolic blood pressure, total cholesterol, and previous vascular events. In fact, 17 out of 24 patients (71%) with MCP-1 in the upper quartile showed sCD40L in the upper quartile.

At discharge from the stroke unit, the SSS score

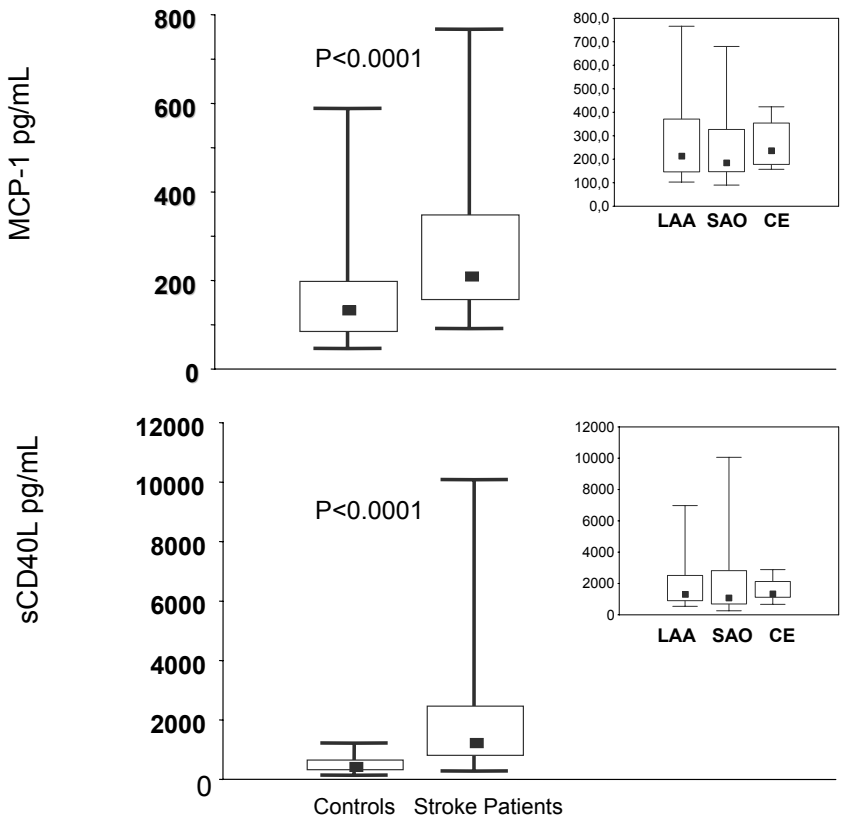

Fig. 1. Monocyte chemoattractant protein-1 (MCP-1) and soluble CD40 ligand (sCD40L) in control subjects and diabetic patients who experienced stroke of non-lacunar and lacunar type (inside panel).

LAA = large-artery atherosclerosis (non-lacunar stroke), CE = cardioembolic (non-lacunar stroke), SAO = small artery occlusion (lacunar stroke). Error bars represent minimum and maximum values, the heavy horizontal rules indicate median values, and the ends of the boxes indicate interquartile range. *Mann-Whitney *U* test

showed a significantly improvement in all included patients (36.7 ± 10.2 to 44.8 ± 10.6, t = − 12.73, median change = +29%, $p < 0.0001$). Nevertheless, MCP-1, sCD40L plasma levels, and the other risk factors did not show any significant correlation with SSS improvement.

Median follow-up was 24 months (12 to 36 months). No patient was lost to follow-up. Three patients died of causes unrelated to vascular events (censored data). Forty-three (48%) patients experienced vascular events (1 fatal and 4 nonfatal myocardial infarctions, 9 episodes of unstable angina, 7 strokes, 15 transient ischaemic attacks, 7 critical limb ischemia). No significant difference was observed in the rate of vascular events among patients with lacunar vs non-lacunar stroke (**Table 2**). No difference in the ongoing treatment (aspirin and/or statins) was observed between patients who experienced a vascular event and those who did not, or among CD40L and MCP-1 quartiles.

Median values of plasma CD40L [2,324 (1,355–3,310) vs 826 (670–1,147) pg/mL, $p < 0.0001$] and MCP-1 [327 (236–408) vs 161 (133–193) pg/mL, $p < 0.0001$] at the time of the inciting acute stroke were higher in those who experienced an event during follow-up than in those who remained event-free.

Univariate and multivariate analyses were performed by the Cox proportional hazard model: the first step was performed by the log rank test, and the covariates found to be associated were included in the Cox regression model (**Fig. 2**). Of the parameters listed in **Table 2**, only high sCD40L levels were entered in the Cox proportional hazard model. In fact, the presence of higher sCD40L values had an independent prognostic value in predicting the recurrence of vascular events (hazard ratio = 2.6, 95% confidence limits: 1.9–3.6, $p < 0.001$). In particular, 31 (72%) out of 43 patients who experienced an event during fol-

Table 2. Variables showing prognostic significance by the log-rank test and evaluated as prognostic indicators by cox model in the 90 patients

Variables	No. of Patients	% EndPoint	Log-Rank Test	*p* value
Sex,			0.03	>0.05
Females	39	48.72		
Males	51	47.06		
Age*			0.95	>0.05
<72 yrs	44	43.18		
>72 yrs	46	52.17		
TOAST			3.33	>0.05
LAA	36	58.33		
SAO	41	36.59		
CE	13	53.85		
Diabetes duration*			0.46	>0.05
≤8 yrs	46	39.13		
>8 yrs	44	56.81		
HbA_{1c}*			0.93	>0.05
<7%	34	41.18		
>7%	56	51.79		
BMI*			1.81	>0.05
≤31	58	44.82		
>31	32	49.09		
Hypertension			1.04	>0.05
No	35	54.29		
Yes	55	43.64		
Dyslipidemia			2.58	>0.05
No	48	56.25		
Yes	42	38.10		
Previous ischemic event			2.68	>0.05
No	20	30.00		
Yes	70	52.86		
Aspirin treatment			0.06	>0.05
No	48	45.83		
Yes	42	50.00		
sCD40L			41.60	<0.001
1st quartile	22	00.00		
2nd quartile	23	30.43		
3rd quartile	22	77.27		
4th quartile	23	82.61		
MCP-1			36.77	<0.001
1st quartile	22	13.64		
2nd quartile	23	21.74		
3rd quartile	21	76.19		
4th quartile	24	79.17		

*>50th of the stroke population
TOAST=Trial of Org 10172 in Acute Stroke Treatment; LAA=large-artery atherosclerosis; SAO=small artery occlusion; CE=cardioembolism; HbA_{1c}=Hemoglobin A_{1c}; BMI=Body Mass Index; sCD40L=soluble CD40 ligand; MCP-1=Monocyte chemoattractant protein-1.

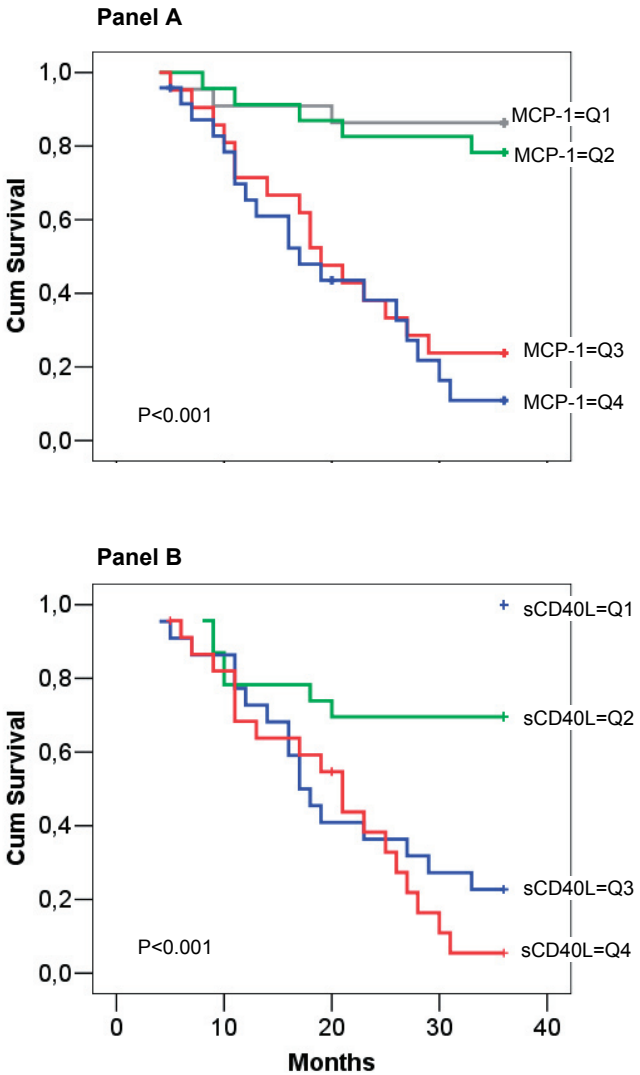

Fig. 2. Kaplan-Meier curves for patients grouped by their plasma levels of monocyte chemoattractant protein-1 (MCP-1) (Panel A) and soluble CD40 ligand (sCD40L) (Panel B).

Q=quartile. [MCP-1: Q1<157, Q2>157 and <210.5, Q3>210.5 and <348, Q4>348 pg/mL] [sCD40L: Q1<813, Q2>813 and <1,252, Q3>1,252 and <2,468, Q4>2,468 pg/mL]. *Cox proportional hazard model.

low-up had CD40L and MCP-1 in the third and fourth quartiles.

Discussion

Type 2 diabetes is characterized by persistent platelet activation[20], responsible for increased plasma levels of CD40L in this setting[9]. CD40L released from platelets induces inflammatory responses in the endothelium[21]. Activated platelets stimulate MCP-1 production from endothelial cells through enhanced

CD40L shedding[10] and this seems particularly relevant in type 2 diabetes[12, 22]. MCP-1 plays an important role in the early phase of atherosclerosis by initiating monocyte recruitment to the vessel wall[23]; thus, platelet-derived CD40L can trigger the recruitment of monocytes or promote their differentiation into macrophages[21], inducing MCP-1 release from the endothelium. Upregulation of MCP-1 and sCD40L has been found in patients with acute cerebral ischemia[13]. Platelets contribute to enhanced MCP-1 levels in patients with chronic heart failure[10]. In patients with acute coronary syndromes[24], as well as in type 2 diabetes[12], an elevated baseline level of MCP-1 is associated with an increased risk for cardiovascular death.

The evidence for a role of sCD40L in cardiovascular disease is even greater, suggesting that CD40L levels are a prominent candidate for early detection in cardiac disease[25, 26]. Activation of the CD40-CD40L dyad has been observed in atherosclerosis-related diseases[19] and CD40L levels may predict cardiovascular risk in women[27]. Acute cerebral ischemia is associated with persistent thromboxane-dependent platelet activation *in vivo*[5]. Up-regulation of CD40-CD40L has been also shown in patients with acute cerebral ischemia[13] and high levels of sCD40L may predict the risk of cardiovascular events both in high-risk plaques[28] and in asymptomatic low-grade carotid stenosis[29].

This study demonstrates for the first time that early elevation of plasma CD40L and MCP-1 after an acute stroke is associated with long-term risk of nonfatal vascular events in a large group of patients with diabetes and acute stroke. On multiple-linear regression analysis, all patients with MCP-1 levels in the third/fourth quartiles showed sCD40L values in the two highest quartiles. Moreover, we demonstrated the potential prognostic value of an elevated CD40L level in stroke patients during 2 years of follow-up, thus suggesting that CD40L shedding acting at the interface between endothelial cells and platelets, and thereby triggering both inflammation and platelet activation, is an early event upstream of vascular events. These findings strongly suggest that enhanced platelet-derived mediators of the inflammatory response may predict the risk of cardiovascular events after stroke.

The age-standardized incidence rates for the European population (per 100 000) regarding ischemic stroke subtypes were as follows: cardioembolism, 30.2; small-artery occlusion, 25.8; and large-artery atherosclerosis, 15.3. Two years after onset, patients in the small-artery occlusion subgroup were 3 times more likely to be alive than those with cardioembolism[15]. We found that sCD40L and MCP-1 are similarly up-regulated among nonlacunar and lacunar strokes, in agreement with a recent meta-analysis showing a similar risk of cardiac outcome in longer term surveillance between the two subtypes[6].

In conclusion, up-regulation of inflammatory molecules, such as CD40L and MCP-1, is involved in the advanced stage of diabetic cerebro-vascular disease. Elevated CD40L levels are associated with an increased risk of recurrence of cardiovascular events. Statins are able to downregulate both soluble MCP-1 and CD40L[30, 31] and a number of additional drugs, including glitazones, antioxidant and antiplatelets, have been shown to favorably modulate sCD40L[32]. Thus, both CD40L and MCP-1 are attractive as surrogate biomarkers and merit further study as potential therapeutic targets in diabetic acute cerebral ischemia.

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Conflict of Interest Statement

There is no conflict of interest that the authors should disclose.

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