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Serial measurements of levels of the chemokines CCL2, CCL3 and CCL5 in serum of patients with acute ischaemic stroke

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Abstract

Inflammation, involving cytokine/chemokine expression, occurs after stroke and deteriorates its course with leukocyte--mediated brain infarct progression. Chemokines are cytokines attracting selective leukocyte subsets and subgrouping into the four major subfamilies, CC, CXC, C, and CX3C. The CC subfamily preferentially acts on mononuclears.

The study aimed to define serum CCL2, CCL3 and CCL5 levels in stroke patients and their relationship to the extent of disease severity and outcome.

27 ischaemic stroke patients and 20 controls were studied. Blood sampling for the determination of chemokines was performed at days 1, 2 and 3 of stroke, while neurological and functional deficits were estimated, respectively, with the Scandinavian Stroke Scale (SSS) and Barthel Index (BI) at the same time points and at days 14 and 28.

Serum CCL3 levels at days 1, 2 and 3 of stroke were significantly higher than in controls. Serum CCL2 and CCL5 levels in stroke patients did not differ from those in controls at any of the time points examined. Serum chemokine levels in stroke studied separately did not differ between each other at any time point studied and demonstrated considerable variability. No correlation between serum chemokine levels and SSS scores was observed. Serum CCL2 and CCL3 levels at days 1, 2 and 3 of stroke correlated with BI scores at day 28. Serum CCL2 levels at days 2 and 3 of stroke also correlated with BI scores at day 14. Serum CCL5 levels at day 2 of stroke correlated with BI scores at day 28.

The early and sustained increase in serum CCL3 levels in stroke patients along with correlation between them and short-term poststroke functional disability could indicate that the chemokine response may predispose to poor stroke outcome. The relationship between non-increased serum CCL2 and CCL5 levels and worse stroke outcome seems to be coincidental. Overall, as a result of large interindividual variability, CCL2, CCL3 and CCL5 are not reliable candidates for surrogate markers in stroke.

Key words: *stroke, chemokines, CCL2, CCL3, CCL5*

Introduction

Inflammation occurs after stroke and contributes to its severity and outcome with leukocyte-mediated brain infarct progression. The leukocyte infiltration is initiated within hours of stroke by neutrophils followed during 24 h by monocytes and lymphocytes [6]. The sequence and timing of the leukocyte response is

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driven by temporal profile-dependent expression of inflammatory mediators, including chemokines [37].

Chemokines are a family of structurally and functionally related cytokines performing chemotactic activity on selective leukocyte subpopulations [20]. They can be divided into four subfamilies according to the number and spacing of their conserved cysteine residues in their sequences, and are classified as the CC, CXC, C and CX3C chemokines [20]. The two major subfamilies are CXC and CC chemokines, and the former preferentially chemoattract polymorphonuclear cells, whereas the latter mainly act on mononuclears [20].

In the settings of experimental and clinical stroke, CXC and CC chemokines have been found to be produced by CNS resident cells and/or leukocytes [12,28,31,39]. Animal models of cerebral ischaemia have shown that cellular expression of CXC and CC chemokines mRNAs and proteins precedes and accompanies the relevant leukocyte infiltration, implicating the molecules importance for promotion of brain infarct growth [28,50,51]. In human stroke, elevated number of CXCL8 mRNA expressing peripheral blood mononuclear cells has been demonstrated to correlate with the degree of neurological impairment [31].

Chemokines, like other inflammatory mediators, are not only expressed on cell surfaces but are also released into CSF or systemic circulation, reflecting – at least in part – the molecules' expression on activated immune cells. We and other authors have detected increased levels of CXC and CC chemokine subfamilies members in CSF and/or peripheral blood of acute ischaemic stroke patients [3,31,33,34,47,52,53]. Moreover, we have recently shown that the CSF levels of several CXC chemokines, including CXCL1, CXCL5 and CXCL6, correlate with the brain infarct size or with stroke severity and outcome [34,52,53]. To date, however, studies on relationships between CC chemokine levels in stroke and the disease-related findings have not been published.

CCL2 (monocyte chemoattractant protein-1 – MCP-1), CCL3 (macrophage inflammatory protein-1 alpha – MIP-1 alpha), and CCL5 (regulated on activation normal T cells expressed and secreted – RANTES) are the three best known and most extensively studied CC chemokines in primary and secondary inflammatory responses in humans [4,17,29].

CCL2, CCL3 and CCL5 are potent monocyte chemoattractants but the chemokines also act on other mononuclear cells, lymphocytes in particular [11,43,44].

The cellular expression and an increment in body fluids of CCL2, CCL3 or CCL5 have been indicated to be involved in monocyte/macrophage and lymphocyte response in several immunological/inflammatory conditions [16,17], including CNS diseases such as HIV-induced dementia and multiple sclerosis [12].

Also in experimental stroke, overexpression of CCL2 [48,50] and CCL3 [24,27,38] mRNAs and proteins has been shown in the brain of middle cerebral artery occluded-animals.

Studies in genetically modified mice have documented that CCL2 plays a pathogenetic role in leukocyte-mediated brain infarct growth; compared with wild-type animals, mice deficient for the chemokine develop smaller infarct together with reduced accumulation of macrophages [25], whereas mice overexpressing CCL2 display larger infarcts along with increased number of inflammatory cells [15]. In a rat model of hypoxic-ischaemic brain injury, a marked increase in CCL3 has been found in regions with the most pronounced neuronal damage and monocyte/microglia accumulation [18], and mRNA induction for CCL2, CCL3 and CCL5 has been shown to precede expression of markers for microglia/macrophages and lymphocytes [9].

The prominent role played by CC chemokines attracting mononuclear cells in the development of ischaemic brain lesion prompted us to perform serial measurements of CCL2, CCL3 and CCL5 in sera of acute ischaemic stroke patients and investigate whether chemokine levels may be related to stroke severity and outcome.

The study focused on three aims. The first was to determine serum CCL2, CCL3 and CCL5 levels in patients at days 1, 2 and 3 of ischaemic stroke and to compare the results with those of a control group. The second was to study whether the individual chemokine levels in stroke patients differ between each other at the time points studied. The third was to evaluate whether serum CCL2, CCL3 and CCL5 levels in patients at days 1, 2 and 3 of stroke may be related to neurological stroke severity as well as poststroke functional disability estimated at the same time points and at days 14 and 28 after disease onset.

Material and methods

Patients

Twenty-seven patients (mean age ±SD: 65.5±6.9 years, 16 women) with first-ever ischaemic stroke, hospitalized at the Departments of Neurology of the

University School of Medicine or the Municipal Hospital in Poznań, were included in the study. The diagnosis was based on clinical history, standardized neurological examination, and computed tomography of the brain. The patients had completed ischaemic stroke defined as clinical symptoms persisting for >24 h [10], and confined to the blood supply territory of middle or anterior cerebral artery.

Regarding stroke risk factors, 16 patients had hypertension, 7 had diabetes mellitus, and 4 had atrial fibrillation. Twenty healthy blood donors, without known stroke risk factors, balanced for age and sex with the stroke patients, were selected as a control group. To avoid the enrolment of subjects with concurrent diseases or conditions interfering with inflammatory mediator expression, the following exclusion criteria for stroke patients and controls were applied: history or coincidence of central nervous system diseases, hyperthermia, presence of infections, other inflammatory, autoimmune, haematological and malignant diseases, major renal or hepatic failure, intoxications, malnutrition, addiction, deep vein thrombosis, tissue injury related conditions within the last year, immunosuppression or treatment with anti-inflammatory drugs within the last six months. In addition, as acute stroke patients are prone to infections, especially of the chest and urinary tract [19], we included only patients without signs of infections on chest x-ray and urine tests performed after admission and repeated during the observational period of four weeks after stroke onset.

The study was conducted according to the principles established in the Declaration of Helsinki and was approved by the Local Ethics Committee. Both stroke and control patients gave their informed consent prior to their inclusion in the study.

Laboratory procedure

Blood samples from each stroke patient were collected at days 1, 2 and 3 after the onset of disease symptoms. Blood samples from the control group were drawn at one time point. The samples were allowed to clot at room temperature for 30 min, and after being centrifuged for 10 min the serum was immediately frozen and stored at –80°C.

CCL2, CCL3 and CCL5 levels in serum samples were determined with the ELISA method using human immunoassays (R & D Systems, USA). The minimum detectable doses of CCL2, CCL3 and CCL5 were less than 10.0, 5.0 and 8.0 pg/ml, respectively.

Evaluation of neurological stroke severity and stroke-related functional disability

Neurological stroke severity was determined with the Scandinavian Stroke Scale (SSS) [42]. The SSS has a score ranging from 58 (normal neurological status) to 0 (maximal neurological deficit of the scale).

The functional disability of stroke patients was estimated with the Barthel Index (BI) [35]. The BI has a score ranging from 100 (absence of disability) to 0 (complete dependence for activities of daily life).

Both the SSS and the BI scoring were performed at days 1, 2, 3, 14 and 28 after onset of stroke symptoms.

Statistical analysis

As the data on serum levels of the studied chemokines in the patients at days 1, 2 and 3 of stroke were not normally distributed, analysis was performed with nonparametric tests. The Mann-Whitney U test was applied to compare CCL2, CCL3 and CCL5 levels in serum of the stroke patients with control values. The Friedman ANOVA test and the Kendall Coefficient of Concordance were used to compare individual chemokine serum levels at days 1, 2 and 3 of stroke. The Spearman rank-order correlation test was adapted to examine correlations between serum chemokine levels at days 1, 2 and 3 of stroke and the SSS and the BI scores calculated at studied separate time points. The results are presented as mean ±SD, except the calculations of the SSS and the BI scores, using median. $P<0.05$ was considered statistically significant.

Results

CCL2, CCL3 and CCL5 levels in sera of patients at days 1, 2 and 3 after stroke onset

CCL2 levels in sera of stroke patients did not differ significantly from those in controls at any of the time points examined.

CCL3 levels in sera of stroke patients at all the measured time points, i.e. at days 1, 2 and 3 after disease onset, were significantly higher than the chemokine levels in controls.

CCL5 levels in sera of stroke patients did not differ significantly from those in controls at any of the time points examined.

Moreover, individual chemokine levels in stroke patients did not differ between each other at any

Table I. Serum CCL2, CCL3 and CCL5 levels [pg/ml] in patients at days 1, 2 and 3 of stroke and controls (values as mean ±SD and range /in parentheses/)

	CCL2	CCL3	CCL5
Stroke – Day 1	194.81±90.30 (66.13–508.31)	21.35±11.83* (13.60–79.64)	658.80±306.70 (113.62–1420.07)
Stroke – Day 2	204.39±109.10 (49.14–610.57)	25.92±30.29* (13.60–185.38)	665.99±308.66 (132.82–1464.59)
Stroke – Day 3	198.69±98.78 (73.88–586.63)	24.37±29.12* (9.95–171.51)	740.20±386.18 (164.36–2022.80)
Controls	159.03±41.03 (93.88–195.62)	14.26±2.63 (10.69–19.91)	701.99±255.21 (455.30–988.75)

** p<0.0001 compared with controls.*

Table II. The degree of neurological stroke severity and stroke-related functional disability determined, respectively, with the SSS and the BI scores (values as median and range /in parentheses/)

	Stroke – Day 1	Stroke – Day 2	Stroke – Day 3	Stroke – Day 14	Stroke – Day 28
SSS scores	42 (26–50)	45 (26–50)	48 (36–54)	54 (39–56)	56 (48–58)
BI scores	60 (25–80)	70 (25–80)	70 (30–90)	90 (50–90)	100 (80–100)

time point studied (P=0.507 for CCL2 levels, P=0.233 for CCL3 levels, and P=0.564 for CCL5 levels).

In stroke patients – except the serum CCL3 levels at day 1 – a large interindividual variability of the studied chemokines levels was observed (Table I).

The degree of neurological stroke severity and stroke-related functional disability determined, respectively, with the SSS and the BI scores calculated at days 1, 2, 3, 14 and 28 after disease onset

Follow-up 1 to 28 days after stroke onset, the degree of neurological stroke severity and stroke-related functional disability decreased with, respectively, the increase in the SSS and the BI scores (Table II).

Correlation between CCL2, CCL3 and CCL5 levels in sera of patients at days 1, 2 and 3 after stroke onset and the degree of neurological stroke severity and stroke-related functional disability determined, respectively, with the SSS and the BI scores calculated at days 1, 2, 3, 14 and 28 after disease onset

No significant correlation between serum levels of CCL2, CCL3 and CCL5 at days 1, 2 and 3 of stroke and the SSS scores calculated at days 1, 2, 3, 14 and 28 was observed.

Serum levels of CCL2 at days 1, 2 and 3 after stroke were inversely correlated with the BI scores at day 28; moreover, serum levels of CCL2 at days 2 and 3 of stroke were also inversely correlated with the BI scores at day 14. No significant correlation between serum levels of CCL2 at days 1, 2 and 3 after stroke and the BI scores calculated at other studied time points was observed (Table III).

Serum levels of CCL3 at days 1, 2 and 3 after stroke were inversely correlated with the BI scores at day 28. No significant correlation between serum levels of CCL3 at days 1, 2 and 3 after stroke and the BI scores calculated at other studied time points was observed (Table IV).

Serum levels of CCL5 at day 2 after stroke were inversely correlated with the BI scores at day 28. No significant correlation between serum levels of CCL5 at days 1, 2 and 3 after stroke and the BI scores calculated at other studied time points was observed (Table V).

Discussion

Among the studied chemokines, only CCL3 but not CCL2 and CCL5 displayed significantly higher serum levels in acute ischaemic stroke patients compared with controls. Moreover, the increased

Table III. Correlation (r) between serum CCL2 levels [pg/ml] in patients at days 1, 2 and 3 of stroke and BI scores determined at days 1, 2, 3, 14 and 28 after disease onset

	BI – Day 1		BI – Day 2		BI – Day 3		BI – Day 14		BI – Day 28	
	r	P	r	P	r	P	r	P	r	P
CCL2 Stroke – Day 1	0.07	NS	0.01	NS	0.03	NS	0.35	NS	–0.50	0.007
CCL2 Stroke – Day 2	0.25	NS	0.17	NS	0.18	NS	–0.44	0.021	–0.53	0.003
CCL2 Stroke – Day 3	0.17	NS	0.11	NS	0.18	NS	–0.44	0.021	–0.44	0.021

Table IV. Correlation (r) between serum CCL3 levels [pg/ml] in patients at days 1, 2 and 3 of stroke and BI scores determined at days 1, 2, 3, 14 and 28 after disease onset

	BI – Day 1		BI – Day 2		BI – Day 3		BI – Day 14		BI – Day 28	
	r	P	r	P	r	P	r	P	r	P
CCL3 Stroke – Day 1	0.05	NS	0.06	NS	–0.07	NS	0.11	NS	–0.45	0.018
CCL3 Stroke – Day 2	0.29	NS	0.35	NS	0.24	NS	0.28	0.021	–0.46	0.014
CCL3 Stroke – Day 3	0.10	NS	0.08	NS	0.12	NS	0.22	0.021	–0.39	0.041

Table V. Correlation (r) between serum CCL5 levels [pg/ml] in patients at days 1, 2 and 3 of stroke and BI scores determined at days 1, 2, 3, 14 and 28 after disease onset

	BI – Day 1		BI – Day 2		BI – Day 3		BI – Day 14		BI – Day 28	
	r	P	r	P	r	P	r	P	r	P
CCL5 Stroke – Day 1	–0.30	NS	–0.31	NS	–0.21	NS	–0.02	NS	0.03	NS
CCL5 Stroke – Day 2	–0.05	NS	0.02	NS	0.17	NS	0.29	NS	–0.38	0.045
CCL5 Stroke – Day 3	0.08	NS	0.10	NS	0.09	NS	0.12	NS	0.12	NS

serum CCL3 levels at all the measured time points, i.e. at days 1, 2 and 3 of stroke, did not differ between each other. These findings suggest rapid and persistent CCL3 overproduction during the acute phase of ischaemic stroke. The suggestion is supported by the results of studies on focal cerebral ischaemia in experimental animals.

Numerous investigators have observed the expression of CCL3 after middle cerebral artery occlusion in rodents, being found in cells located within ischaemic lesion at 6 h or at 8–16 h of experimental stroke and lasting until 48 or 72 h [24,27,38]. The above authors have also demonstrated that microglia/macrophages or astrocytes are responsible for the chemokine synthesis [24,27,38].

With the evidenced early and sustained poststroke intracerebral CCL3 expression [24,27,38] and stroke-related blood-brain barrier hyperpermeability [7], it is conceivable that the observed increment in serum CCL3 levels of our patients may be a result of chemokine production by cells localized within the ischaemic brain. This is further augmented by the study by Kostulas et al. [31], who observed that numbers of CCL3 mRNA expressing peripheral blood mononuclear cells did not differ between patients with ischaemic stroke and healthy individuals.

The role of CCL3 in stroke is still unclear. CCL3 is a well-known chemoattractant that can modulate macrophage function and trigger hydrogen peroxidase production in leukocytes [1, 22, 49], but it is not known if the chemokine may act in this manner following cerebral ischaemia. However, the primary function attributed to CCL3 is the attraction and activation of monocytes, and both systemic-originated macrophages and activated microglia may not only secrete neurotoxic substances such as

glutamate and nitric oxide but can also play a role in ischaemic brain injury healing [13,23,24].

Interestingly, CCL3 has also been reported to regulate the response of polymorphonuclear cells in different inflammatory conditions [2,46]. For instance, in a murine lung endotoxemia model, CCL3 mediates recruitment of first polymorphonuclears followed by macrophages [46]. CCL3 may play a similar role in stroke, where the chemokine three days' intracerebral expression is consistent with the timetable of polymorphonuclear and mononuclear cell recruitment into the ischaemic brain.

Thus, we are inclined to suggest that the presented increase in serum CCL3 levels in patients within the first three days of stroke may indicate the chemokine involvement in recruitment of leukocytes (probably both polymorphonuclears and mononuclears) to the ischaemic brain. Consequently, CCL3 might be implicated in the switch of recruited inflammatory cells from polymorphonuclears to macrophages at the site of inflammatory brain infarct.

The absence of increase in serum CCL2 levels of acute ischaemic stroke patients could partly be expected with the results of our previous study [33] documenting that in stroke subjects within the first 24 h of disease the chemokine elevation was restricted to CSF and did not appear in serum. Our studies suggest that CCL2 after stroke may be produced within the CNS. A similar concept is presented by Che et al. [14], who indicate that intracerebral CCL2 expression after middle cerebral artery occlusion is not a result of CCL2 entry from blood. Moreover, Kostulas et al. [30] have demonstrated no elevation in number of peripheral blood mononuclear cells expressing mRNA for CCL2 in ischaemic stroke patients. Arakelyan et al. [3] have found an increase in serum CCL2 levels of ischaemic stroke patients; however, the authors stressed substantial variability of the chemokine serum levels.

The temporal profile of intracerebral CCL2 expression after middle cerebral artery occlusion in rodents has initially been found to be similar to that of CCL3, i.e. at 6 or at 12 h of the experimental stroke; however, otherwise than in the case of CCL3, the expression of CCL2 has been observed even at 7–8 days postinsult [14,27,38,50]. Moreover, in comparison with CCL3, CCL2 is produced by more types of cells, i.e. not only microglia/macrophages and astrocytes but also neurons and endothelia [14,24,27,38,50].

Similarly to CCL3, the role of CCL2 after cerebral ischaemia is not clearly elucidated. In general, both chemokines perform akin functions [13,32]; however, CCL2 is a chemoattractant specific to monocytes (but not neutrophils) and induces expression of adhesion molecules and cytokine production in monocytes [8,36]. With the studies indicating that intracerebral CCL2 participates in the inflammatory cell response to the infarct [14,25,50], the observed lack of increase in serum chemokine levels in stroke patients indirectly suggests that the chemokine may predominantly act within the ischaemic brain.

The behaviour of CCL5 in human cerebral ischaemia, studied for the first time, revealed that the chemokine serum levels in acute ischaemic stroke patients did not differ from those in healthy subjects. Also in experimental stroke, to date, CCL5 has only been the subject of one study [45], showing that the chemokine mRNA remains unaffected in middle cerebral artery occluded-animals.

We observed a large interindividual variability of chemokine levels in stroke subjects. This may have two causes, at least. The first may be secondary to individual conditions before the stroke, atherosclerosis in particular; indeed, CCL2, CCL3 and CCL5 were found to be engaged in atherosclerotic lesion formation [40]. The second one may be associated with different extent and timing of reperfusion [5,26], the process influencing poststroke inflammation [21,41], including chemokine expression [24].

The attempt to correlate serum levels of CCL2, CCL3 and CCL5 at days 1, 2 and 3 of stroke with the degree of neurological stroke severity as measured by the SSS was not successful at any studied time point.

However, the test to correlate serum levels of CCL2, CCL3 and CCL5 at days 1, 2 and 3 of stroke with the degree of functional disability as measured by the BI brought different results, indicating presence of inverse correlations between the chemokine levels during acute phase of the disease and the BI scores estimated at day 28 or at days 14 and 28 after onset.

Such results could be associated with the aforementioned interindividual variability of the chemokine levels in stroke patients.

The importance of these relationships for stroke outcome requires reference to the results indicating that only CCL3 but not CCL2 and CCL5 levels in sera of stroke patients were significantly higher in comparison with the chemokine levels in controls. Thus, we suggest that measurement of CCL3 levels in serum of acute ischaemic stroke patients could have a predictive value for the short-term prognosis of poststroke functional disability. This suggestion is not related to serum CCL2 and CCL5 levels in acute

stroke as they did not differ from those in healthy controls, and the observed correlations between serum levels of these chemokines and the BI scores may be coincidental.

The above data indicate that measurement of the studied chemokines in sera of acute brain infarct subjects is not sufficient for the estimation of neurological stroke severity and poststroke functional disability in individual patients. This is also partly related to CCL3, as its increased serum levels during the first three days of stroke correlated with the BI scoring at one and the last studied time--point (i.e. at day 28 after disease onset) only. Moreover, it should be realized that chemokines other than CCL3 could play a more important role in the mechanisms responsible for stroke outcome.

The study results allow us to make some concluding remarks. Acute ischaemic stroke is accompanied by systemic increase in levels of CCL3 but not CCL2 and CCL5. The early and sustained increase in serum CCL3 levels in acute ischaemic stroke patients along with correlation between the chemokine levels and short-term poststroke functional disability could indicate that CCL3 response may predispose to poor stroke outcome. As serum CCL2 and CCL5 levels in stroke patients were not increased, the relationship between the chemokine levels and worse stroke outcome seems to be coincidental and not valuable for disease outcome prognosis. Overall, the large interindividual variability of serum CCL2, CCL3 and CCL5 levels means that these chemokines are not reliable candidates for surrogate markers in stroke.

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