The levels of TNF-alpha in cerebrospinal fluid and serum do not correlate with the counts of the white blood cells in acute phase of ischaemic stroke

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[Introduced Article]

INTRODUCTION

Accumulated data strongly suggest that cerebral ischaemia induces an inflammatory reaction leading to secondary neuronal damage. This reaction is pivotally initiated by local intracerebral expression of proinflammatory cytokines, including tumour necrosis factor-alpha (TNF-alpha). Various events involving chemoattractant cytokines release, endothelial-leukocyte adhesion molecules upregulation, recruitment and migration of leukocytes from systemic compartment to the brain ischaemic zone, and transformation of endothelium to a prothrombotic state contribute to the development of local inflammation [1,6,13,20,30,32]. Thus, TNF-alpha may promote expansion of the brain infarct in a dual way, viz. (1) intracerebral accumulation of leukocytes plays a crucial role in the development of necrosis [26,35] whereas retention of leukocytes, which occurs in the brain microcirculation during reperfusion, decreases microvascular flow [36], (2) downregulation of two major antithrombotic mechanisms, i.e. tissue plasminogen activator and thrombomodulin [9,24,45]. Leukocytes, having invaded the brain, in turn, cause subsequent microvascular occlusion, increased vascular permeability, vasomotor reactivity and release of oxygen free radicals and cytoxic enzymes...

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Finally, this leads to enlargement of the ischaemic focus. Therefore, investigations of stroke-induced inflammatory phenomena seem to be very important, because neuronal cell death can appear in the core of the brain infarcted area if the blood flow is below 10 ml/100g tissue per minute, and may extend to the penumbral area even after reflow [27]. The evolution of TNF-alpha-induced inflammatory events was mainly described in experimental animal models of brain ischaemia [17,25,44,48,51]. In humans, elevated leukocyte counts have been reported to be a risk factor for cerebral infarction [34]. However, the literature on white blood cells (WBC) counts in human acute ischaemic stroke presents divergent data. Grau et al. [19] showed no significant increase of blood leukocytes during the first 3 days after the onset of ischaemic cerebrovascular event. Silvestrini et al. [37] observed significantly increased values of WBC counts within 6 days following ischaemic stroke. Then, Pozzilli et al. [33] demonstrated the significant increase in WBC counts measured on the 3rd day after the onset of ischaemic stroke in comparison with the values during the first 48 hours after stroke. Nevertheless, Pozzilli et al. [32] emphasised that the reinjected circulating WBCs of patients with brain infarct that were labelled in vitro with Indium-111 infiltrated into the infarcted hemisphere as early as two hours after stroke. We have recently demonstrated that ischaemic stroke patients displayed increased TNF-alpha levels in cerebrospinal fluid (CSF) and serum, which correlated with the volume of evolving brain infarct within the first 24 hours after the disease onset [50]. The aim of the present study was to investigate whether WBC counts can be increased in ischaemic stroke patients within the first 24 hours after the appearance of neurological symptoms in comparison with control group, and if so, to evaluate whether elevated WBC counts can be related to increased TNF-alpha levels in CSF and serum.

**MATERIAL AND METHODS**

**Patients**

Twenty-three patients with first-ever ischaemic stroke in a lifetime (mean age ± SD — 72.2 ± 10.8 years, 6 men and 17 women) entered the study consecutively. Patients with concurrent diseases or conditions interfering with the aim of the study, like haematological disorders, infections, autoimmune diseases, identified cardioembolic sources, myocardial infarctions, malignancies, undergoing surgical interventions within the previous 12 months, and those on immunosuppressive drugs, were excluded. All the patients presented completed ischaemic stroke, defined as clinical symptoms persisting for more than 24 hours [8]. All the patients with ischaemic stroke had symptoms confined to the carotid artery territory. The diagnosis of ischaemic stroke was confirmed by computerised tomography of the brain performed immediately after admission. Blood samples were obtained for the WBC counts within the first 24 hours after the onset of stroke. CSF, which was obtained for diagnosis, and serum samples were used for the analysis of TNF-alpha levels. Fifteen individuals (mean age ± SD — 70.1 ± 8.6 years, 4 men and 11 women) with the diagnosis of neurasthenia and tension headache served as a control group. The study was performed on the basis of written consent of each patient and approval of the Ethics Committee of the University School of Medicine in Poznan.

**Laboratory procedures**

Blood samples were taken from intravenous cannulae and WBC counts were performed by an automated haematology analyser. CSF samples for the estimation of TNF-alpha levels were centrifuged immediately after lumbar puncture and the supernatants were stored at −80°C until analysis. Blood samples destined for the analysis of TNF-alpha levels in serum were allowed to clot at room temperature for 30 minutes, and after blood centrifugation for 10 minutes, the obtained serum was stored at −80°C. TNF-alpha levels in CSF and serum samples were quantified by ELISA method (Quantikine R & D Systems, Minneapolis, MN, USA) according to the manufacturer’s instruction. The sensitivity of the method was 4.4 pg/ml.

**Statistical analysis**

Statistical analysis of WBC counts was performed using t-Student’s test for independent variables. Further statistical evaluations were based on the assumption that the data were not normally distributed, and the analysis was performed with nonparametric tests. U Mann-Whitney’s test was used to compare TNF-alpha levels in CSF and serum in stroke patients with control group. Spearman’s rank-order correlation test was used in the study of the group of ischaemic stroke patients to calculate the correlation between TNF-alpha levels in CSF and serum and the values of WBC counts. The results are presented as mean ± SD. P < 0.05 was considered statistically significant.
RESULTS

WBC counts in patients within 24 hours of the onset of stroke
The group of ischaemic stroke patients showed significantly higher values of WBC counts compared with control group (8.1 ± 2.4 x 10³ cells/µl versus 5.4 ± 1.1 x 10³ cells/µl; p < 0.001; Fig.1). The highest value of WBC counts in the group of ischaemic stroke patients was 11.8 x 10³ cells/µl, with the lowest being 4.3 x 10³ cells/µl. The highest value of WBC counts in control group was 7.1 x 10³ cells/µl, whereas the lowest was 3.1 x 10³ cells/µl.

CSF TNF-alpha levels in patients within 24 hours of the onset of stroke
The group of ischaemic stroke patients displayed statistically significant elevated levels of TNF-alpha in CSF compared with control group (9.1 ± 5.8 pg/ml versus 6.6 ± 0.5 pg/ml; p < 0.05; Fig. 2). The highest level of TNF-alpha in CSF in the group of ischaemic stroke patients was 95.0 pg/ml, whereas the lowest was 5.7 pg/ml. The highest level of CSF TNF-alpha in control group was 7.6 pg/ml, whereas the lowest was 5.3 pg/ml.

Serum TNF-alpha levels in patients within 24 hours of the onset of stroke
The group of ischaemic stroke patients demonstrated statistically significant increased levels of TNF-alpha in serum compared with control group (14.0 ± 10.2 pg/ml versus 9.1 ± 1.6 pg/ml; p < 0.05; Fig. 3). The highest level of TNF-alpha in serum in the group of ischaemic stroke patients was 55.0 pg/ml, the lowest being 6.8 pg/ml. The highest level of TNF-alpha in serum in control group was 13.0 pg/ml, whereas the lowest was 6.8 pg/ml.
CSF and serum TNF-alpha levels in relation to the values of WBC counts in patients within 24 hours of the onset of stroke

No statistically significant correlation was found between CSF and serum TNF-alpha levels and values of WBC counts in the group of ischaemic stroke patients (r = 0.09; p < 1.0 and r = 0.08; p < 1.0, respectively).

DISCUSSION

The present study demonstrated that ischaemic stroke patients within the first 24 hours of the disease onset displayed an increase in WBC counts and an increase in TNF-alpha levels in CSF and serum. However, neither increased CSF TNF-alpha levels nor increased serum TNF-alpha levels correlate with elevated WBC counts.

The elevated CSF TNF-alpha levels observed in this study may suggest intracerebral TNF-alpha synthesis during the early phase of ischaemic stroke. Many of the studies of acute cerebral ischaemia performed in animal models showed expression of TNF-alpha in ischaemic neurons, microglia, macrophages (especially in the cells present in ischaemic core and perifocal zone), astroglia and ependymal cells already within the first hours following the occlusion of cerebral artery [10,18,40,41,49]. Thus, the increased TNF-alpha levels in CSF shown in the present study may be the result of TNF-alpha production in various types of brain cells in the cerebral ischaemic environment and may reflect stroke-induced inflammatory reaction in the brain.

It is not clear if stroke can also induce systemic inflammatory response, because the cellular sources of systemic TNF-alpha production in acute ischaemic stroke are very poorly recognised. TNF-alpha is produced mainly by endothelial cells and activated mononuclear leukocytes, and both TNF-alpha and sTNFR-1 (soluble TNF receptor protein-1 p55, being the shedded extracellular portion of the TNF-alpha receptor) were found to be elevated in various infectious, inflammatory and malignant diseases [2,12]. Elneihoum et al. [14] showed that patients with acute ischaemic cerebrovascular diseases studied between 1 and 3 days after the event displayed higher serum sTNFR-1 (the marker for systemic leukocyte activation) levels than control subjects. Recently, Ferrarese et al. [16] reported high and significant increase of TNF-alpha release from stimulated blood leukocytes from day 1 or 2 until day 90 after stroke. These authors suggest that the ischaemic process in the central nervous system induces a long-lasting activation of TNF-alpha production in peripheral blood cells, which may be a major source of this cytokine in serum after stroke in humans. However, the inflammatory events described by Elneihoum et al. [14] and Ferrarese et al. [16] may exist already in chronic vascular injury before acute stroke. Further, TNF-alpha may play a significant role in the activation of leukocytes and in the initiation, formation and progression of arterial thrombosis [4,5,11,21,22,28,38,39,46]. This suggests a strong involvement of this cytokine in the development of arterial wall pathological changes which precede and can lead to stroke. Moreover, association of the main stroke risk factor, hypertension, with increased stimulated TNF-alpha release from blood vessels or cells, seems important [23]. Thus, in our opinion, the elevated TNF-alpha levels in serum of ischaemic stroke patients shown in this study may reflect the existence of systemic proinflammatory state in acute ischaemic stroke as well as the presence of stroke-induced systemic inflammatory reaction. We believe that further studies on the serum TNF-alpha levels in stroke would require the comparison of serum cytokine levels in the group of ischaemic stroke patients with the group of non-stroke patients that present stroke risk factors. This is planned to be involved in our future studies.

The significantly increased values of WBC counts in patients within the first 24 hours of the onset of ischaemic stroke, as observed in this study, are in accordance with the results obtained by Silvestrini et al. [37]. Pozzilli et al. [33] reported that WBC counts determined three days after the onset of ischaemic stroke correlate with the severity of neurological impairment and the size of brain infarct. However, similarly to the question raised regarding the increased levels of TNF-alpha in serum observed in our study, the problem appears if increased WBC counts found at early phase of stroke may be stroke-induced. Indeed, the stress that accompanies an acute ischaemic stroke seems to be responsible for adrenochrome, the process that has been shown to reflect the activation of leukocytes [42]. The stroke patients displayed significantly higher values of plasma oxidation of adrenaline to adrenochrome, the process that has been shown to reflect the activation of leukocytes [42]. However, elevated WBC counts observed at early phase of stroke are also characteristic of chronic vascular injury, because increased values of WBC counts are a significant predictor and risk factor for ischaemic stroke disease [15,34].
The lack of correlation between increased TNF-alpha levels in CSF and serum and elevated WBC counts within the first 24 hours of ischaemic stroke, as observed in this study, suggests that TNF-alpha overexpression is not dependent on increased total number of peripheral leukocytes.

Increase in CSF TNF-alpha levels may be considered an event which probably reflects the level of stroke-related acute brain inflammation. Numerous authors have stressed that the migration of leukocytes into evolving brain infarct occurs in very early phase of stroke. Garcia et al. [17] reported initiation of intravascular neutrophil recruitment following middle cerebral artery occlusion in a rat within 30 minutes of onset of cerebral ischaemia, with its intracerebral levels peaking at 24 hours. Hallenbeck et al. [20] showed migration of neutrophils into the brain ischaemic region within 60 minutes of ischaemia.

Increase in TNF-alpha levels in serum may reflect the level of systemic proinflammatory activity, because the absence of correlation between elevated serum TNF-alpha levels and increase of total WBC counts may suggest that not all WBCs synthesise TNF-alpha in peripheral circulation. This may also argue for involvement of not only WBCs but also endothelial cells in systemic TNF-alpha production.

CONCLUSIONS

Data obtained from our study on acute phase of ischaemic stroke allow the following conclusions:
— high CSF TNF-alpha levels suggest involvement of this cytokine in the initiation of stroke-induced intracerebral inflammation;
— high serum TNF-alpha levels suggest the presence of systemic proinflammatory state, which contributes to ischaemic inflammatory cerebrovascular damage;
— the elevated levels of TNF-alpha in CSF and serum are not directly linked to increase in WBC counts, suggesting that different cell types contribute to the TNF-alpha production during the initial phase of ischaemic stroke.

REFERENCES

50. Zaremba J, Skrobanski P, Losy J (In press) Tumour necrosis factor-alpha is increased in cerebrospinal fluid and serum of ischaemic stroke patients and correlates with the volume of evolving brain infarct.
