· Advances in Medical Sciences · Vol. 58(2) · 2013 · pp 298-303 · DOI: 10.2478/ams-2013-0014

The dynamics of soluble Fas/APO 1 apoptotic biochemical marker in acute ischemic stroke patients

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Received: 11.06.2012
Accepted: 09.05.2013
Advances in Medical Sciences
Vol. 58(2) 2013 · pp 298-303
DOI: 10.2478/ams-2013-0014

ABSTRACT

Purpose: Until recently, neuronal death in ischemic stroke infarction was ascribed exclusively to necrotic process. However, experimental animal models of cerebral ischemia suggest apoptosis to play a role in the pathogenesis of cerebral infarction. The aim of this study was to determine the level and monitor the dynamics of soluble Fas/APO 1 (sFas/APO 1) in serum and cerebrospinal fluid of acute ischemic stroke patients.

Material and Methods: This prospective study included 23 patients with first ever, computed tomography verified acute ischemic stroke and 20 control subjects with other functional neurologic disorders. Serum and cerebrospinal fluid sFas/APO 1 levels were determined on several occasions. Blood samples were obtained on day 1, 3 and 12, and lumbar puncture on day 3 and 12 of disease onset. Quantitative sandwich ELISA method was used on sFas/APO 1 determination.

Results: On day 1 of disease onset, serum and cerebrospinal fluid sFas/APO 1 levels were significantly higher in stroke patients as compared to control subjects, and then gradually declined during the period of monitoring.

Conclusion: Study results confirmed the dynamic pattern of sFas/APO 1 in serum and cerebrospinal fluid of patients with acute ischemic stroke, suggesting the possible role of apoptosis in the pathogenesis of cerebral infarction.

Key words: Ischemic stroke, apoptosis, sFas/APO 1, pathogenesis

INTRODUCTION

Stroke is the leading cause of disability and the third leading cause of death in industrialized countries [1]. Until recently, the pathophysiology of ischemic stroke was considered to be underlain exclusively by the pathologic processes in cerebral and extracerebral vasculature, i.e. blood coagulability. Thus, neuronal death in ischemic stroke used to be ascribed exclusively to necrotic process. However, recent studies do not deny those concepts, but point to the possibly active role of cerebral parenchyma and involvement of apoptosis in the pathophysiological mechanism of stroke [2-5].

The penumbra region, in earlier literature called edema zone, is currently considered to be of utmost importance for the outcome of stroke. Results of studies in experimental animal models point to the interaction of four pathophysiological processes in the area: excitotoxicity, peri-infarction depolarization, inflammation, and apoptosis [6]. It appears that apoptotic neuronal death is involved in the pathogenesis of some neurodegenerative diseases such as amyotrophic lateral sclerosis [7], Alzheimer's disease [8] and Parkinson's disease [9], and according to some animal models, also in cerebral infarction [10-12]. Experimental models of ischemia have shown the genes encoding for

proapoptotic and antiapoptotic proteins to be expressed in the early as well as in later stages of the disease [13]. The genes encoding for aspartate-specific cysteine proteases, i.e. caspases, have been most widely investigated [14]. Experimental animal models have suggested two possible pathways of caspase activation: activation *via* death receptors (extrinsic pathway) and mitochondrial pathway (intrinsic pathway). In the extrinsic pathway, caspase activation proceeds *via* Fas receptor (FasR), also known as apoptosis antigen 1 (APO-1). The Fas/Apo 1 receptor binding to its ligand (FasL) activates a number of intracellular proteins including procaspases, which trigger caspase activation by formation of the death-inducing signaling complex, resulting in neuronal death [15,16]. Soluble Fas/APO 1 (sFas/APO 1) is a soluble form of membrane protein of the TNF group, which prevents apoptosis by inhibiting the Fas/APO 1 to FasL binding reaction on the cell surface [17].

The aim of this study was to determine the level and monitor the dynamics of sFas/APO 1, apoptotic biochemical marker, in serum and cerebrospinal fluid (CSF) of patients with acute ischemic stroke.

MATERIAL AND METHODS

The study included 23 patients with acute ischemic stroke. On admission, patient medical history was obtained, and patients underwent standard neurologic examination including evaluation of the cranial nerve function, motor and sensory function, reflexes, muscle tone, coordination testing and cognitive function assessment. Clinical examination was followed by computed tomography (CT) of the brain to verify acute ischemic lesion in the middle cerebral artery supply territory. Stroke patients were evaluated on admission, then on day 3 and 12 of hospital stay with the use of respective scales (National Institutes of Health Stroke Scale (NIHSS) and Barthel index).

Inclusion criteria were: age <80 years, the onset of overt focal neurological deficit within 24 h before admission and CT verified ischemic stroke due to large artery atherosclerosis.

Exclusion criteria were: previous stroke, CT verified intracerebral hemorrhage, secondary hemorrhage after ischemic stroke, lacunar stroke, history of myocardial infarction, signs of cardiac insufficiency, febrile state or verified infection requiring antibiotic therapy, and malignant, autoimmune or neurodegenerative diseases.

Control group included 20 patients hospitalized for diagnostic work-up of neurologic symptoms, which included lumbar puncture. In this group, the presence of diseases or conditions used as exclusion criteria in patients with ischemic stroke was ruled out by thorough patient history. On clinical examination, control group patients were free from signs of neurologic deficit, whereas the existence of pathomorphological substrate was excluded by brain CT. Patient and control group characteristics are shown in *Tab. 1.* Dyslipidaemia was defined as condition diagnosed prior the stroke or level of LDL cholesterol >3 mmol/L at admission.

Ethics of experimentation

The study of serum and CSF level of sFas/APO 1 in acute ischemic stroke patients was performed as part of the project entitled Apoptosis in Central Nervous System Diseases, supported by the Ministry of Health and Social Welfare of the Republic of Croatia, that has received written approval by the Zagreb University Hospital Center Ethics Committee. An informed consent was obtained from all patients or their relatives.

Serum and CSF collection and storage

In stroke patients, blood samples were obtained on day 1, 3 and 12 of hospital stay. Lumbar puncture was performed twice, i.e. on day 3 and 12 of disease onset. Control group patients underwent single CSF and blood sampling for biochemistry parameters of apoptosis. Blood samples were allowed to clot at room temperature for 30 min and centrifuged at 3000 g for 10 min. Serum and CSF samples were aliquoted and stored at -20°C until analysis.

Determination of sFas/APO 1

sFas/APO 1 levels in serum and CSF samples were determined by enzyme-linked immunosorbent assay (ELISA) using a commercial kit (Quantikine Human sFas Immunoassay, R&D Systems, Minneapolis, USA) according to the manufacturer's instructions. Briefly, sFas/APO 1 standards, 10-fold diluted serum and undiluted CSF samples were pipetted into the wells coated with murine monoclonal antibody to human sFas/APO 1. After two hours of incubation, unbound substances were washed away and horseradish peroxidase conjugated polyclonal antibody against Fas/APO 1 was added to the wells. Following two hours of incubation and a wash, tetramethylbenzidine substrate solution was added to the wells. Color developed in proportion to the amount of sFas/APO 1 bound in the initial step. The color development was stopped after 30 minutes with sulfuric acid and the intensity

Table 1. **Basic characeristics of patients and controls.**

Characteristics	Stroke patients (n= 20)	Control group (n= 20)	P value
Age (years)	65 ± 10	62 ± 9	NS
Gender (female/male)	57 / 43%	65 / 35%	NS
Duration of hospitalization (days)	12 ± 2	10 ± 2	NS
Hypertension	65%	55%	NS
Diabetes mellitus	48%	40%	NS
Smoking	51%	45%	NS
Dyslipidemia	30%	20%	NS
Body mass index > 25	70%	70%	NS

of color was measured using a microplate reader set to 450 nm with wavelength correction at 540 nm. Serum and CSF sFas/APO 1 levels were calculated from a standard curve generated by plotting the mean optical density for each standard concentration on the y-axis against the sFas/APO 1 concentration on the x-axis.

Statistical analysis

Statistical analyses were performed using the commercial system SAS System for Windows, Version 9.3. Normality of distribution of the variables was tested with the Kolmogorov-Smirnov test. The results are expressed by mean value and standard deviation while non-normally distributed variables are expressed by median and interquartile range (IQR).

Differences in serum and CSF parameters between stroke patients and control group were assessed by Kruskal-Wallis test, followed by Mann-Whitney U-test with *P* value adjustment, i.e. reducing the level of significance depending on the number of comparisons (*P* 0.05/2=0.025 and *P* 0.05/3=0.017). Within-group differences were assessed by Wilcoxon rank-sum test on comparison of two dependent samples (analysis of difference in CSF parameters), whereas Friedman two-way analysis of variance by ranks was used on comparison of multiple dependent samples (analysis of difference in serum parameters). Correlations between particular variables were tested by Spearman rank correlation. Statistical significance was set at $P<0.05$, except for the Mann-Whitney U-test with multiple comparisons.

RESULTS

Patient clinical characteristics

Out of 23 patients initially enrolled, 3 (13%) patients with acute ischemic stroke were excluded from the study (febrile state – pneumonia, secondary hemorrhage after ischemic stroke and death of 5th day of admission). The rest of 20 patients had various grades of neurologic deficit quantified by the use of scoring systems. Impairment/loss of limb motor function was present in 11 (55%) and severe paresis in 8 (40%) patients, whereas 1 patient had lower extremity paralysis and severe upper extremity paresis. Twelve patients (60%) had hemisensory deficit, sensomotor aphasia was present in 9 (45 %), and 2 (20%) patients had motor dysphasia. Thirteen (65%) patients suffered neurologic deficit on the right side and 7 (35%) patients on the left side of the body.

On day 1 of disease onset, the grade of neurologic deficit, as assessed by the NIHSS, was 19 (IQR 14 – 27), with no improvement on day 3, and NIHSS score 15 (IQR 12 – 22) recorded on day 12 (day 1 *vs*. day 12, $P=0.005$).

The level of independence, as assessed by Barthel index, was 21 (IQR 15 – 25) on day 1, 23 (IQR 15 – 30) on day 3, and 27 (IQR 20 – 40) on day 12. There was no statistically significant difference in the Barthel index score between day 1 and 3, while a significant improvement of the patient functional abilities was recorded on day 12 as compared with the previous two measurements (day 1 *vs*. day 12, $P=0.004$; day 3 *vs*. day 12, $P=0.01$).

Serum concentration of sFas/APO 1 in stroke patients and control group

The mean serum concentration of sFas/APO 1 in ischemic stroke patients was 11656±3277 pg/mL on day 1, 9342±2804 pg/mL on day 3, and 7851±2167 pg/mL on day 12. In the control group, the mean serum concentration of sFas/APO 1 was 7076±1554 pg/mL. Analysis of differences in sFas/APO 1 level in stroke patients according to days of disease onset yielded the following results: day 1 *vs*. day 12, day 1 *vs*. day 3, and day 3 *vs*. day 12, $P=0.001$ all. Statistically significant differences were found in serum concentration of sFas/APO 1 between stroke patients on day 1 and day 3, and the control group ($P<0.001$ and $P=0.01$, respectively). There was no statistically significant difference in serum concentration of sFas/APO 1 between stroke patients on day 12 and the control group (*Fig. 1*).

CSF concentration of sFas/APO 1 in stroke patients and control group

In ischemic stroke patients, the mean CSF concentration of sFas/APO1 was significantly higher on day 3 as compared with day 12 (771±210 *vs*. 385±95 pg/mL; $P<0.001$). In the control group, the mean CSF concentration of sFas/APO 1 was 302±73 pg/mL. In comparison with the control group, CSF concentration of sFas/APO 1 in stroke patients was significantly higher on day 3 ($P<0.001$), whereas no statistically significant difference was recorded on day 12 (*Fig. 2*).

Correlation of clinical scales and biochemical parameters of apoptosis

In the group of stroke patients, there was no statistically significant correlation of serum concentration of sFas/APO 1 with clinical picture quantified by the use of NIHSS (day 1: r= 0.15, p=NS; day 3: r= 0.18, p=NS; day 12: r=0.2, p=NS) and Barthel index (day 1: r= -0.19, p=NS; day 3: r=−0.13, p=NS; day 12: r=−0.22, p=NS).

There was no statistically significant correlation of CSF concentration of sFas/APO 1 with clinical picture quantified by the use of NIHSS (day 3: r= 0.17, p=NS; day 12: r= 0.21, p=NS) and Barthel index (day 3: r= -0.18, p=NS; day 12: r=−0.16, p=NS).

Correlation of serum and CSF sFas/APO 1 concentration in stroke patients

There was no statistically significant correlation of serum and CSF sFas/APO 1 in stroke patients (day 3: r= 0.3, p=NS; day 12: r= 0.32, p=NS).

Figure 1. **Differences in serum concentration of sFas/APO 1 between stroke patients (pts.) and control group (mean ± standard deviation).**

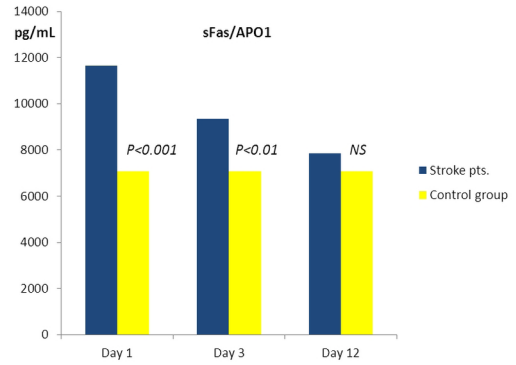
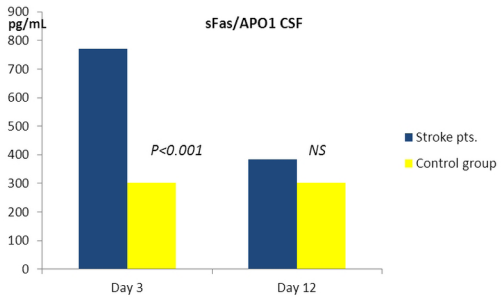


Figure 2. **Differences in cerebrospinal fluid concentration of sFas/APO 1 between stroke patients (pts.) and control group (mean ± standard deviation).**



DISCUSSION

Study results demonstrated the dynamics of biochemical parameters of apoptosis in serum and CSF of acute ischemic stroke patients. In comparison with the control group, serum and CSF concentrations of sFas/APO 1 were higher in stroke patients on day 1 of disease onset, and then gradually decreased during two-week monitoring. Similar results were also recorded in our pilot study using another commercially available kit [18].

In the experimental model of cerebral ischemia used by Rosenbaum et al. [19], evidence for apoptosis was determined by morphology and the presence of DNA fragmentation by the end labeling technique (TUNEL). Immunohistochemistry was performed to detect the expression of both Fas and FasL. Their results demonstrated the presence of apoptosis by morphological criteria and TUNEL technique in rat. In addition, the increased expression of Fas/APO 1 mRNA on glial cells and neurons of post-ischemic rat brain demonstrates the Fas-mediated apoptotic death of these cells. The soluble form of Fas/APO 1 blocks apoptosis by alternative binding to Fas mRNA, thus inhibiting the Fas/APO 1 to FasL binding reaction [20]. The inhibition of Fas/APO 1 has also been shown to reduce secondary brain damage in experimental models of brain ischemia [21]. Our results are in part consistent with those reported by Tarkowski et al. [22], who observed a reduction in sFas/APO 1 concentration in CSF of ischemic stroke patients over a period of 21 days. However, unlike our study, where sFas/APO 1 concentration normalized to the control group level in 12 days, in the study by Tarkowski et al. [22] the sFas/APO 1 concentrations were significantly higher in control group as compared with stroke patients at all time points. The authors tried to explain it by a reduced protein production or its increased breakdown. However, it is well known that any reaction occurring in the body leads to activation of antagonist mechanisms to establish due balance; therefore, one should expect that the enhanced expression of Fas and FasL that trigger apoptosis be accompanied by an increase in the concentration of proteins that inhibit these reactions, such as sFas/APO 1. The variation in the results could in part be ascribed to the use of different commercial kits or control group heterogeneity. As there are several different functional forms of sFas/APO 1, which are generated by alternative splicing and are expressed as soluble molecules [23], the same primary antibodies should be used in comparison studies.

Apoptotic neuronal death has also been investigated in some other neurologic diseases and conditions. Results reported by Lenzlinger et al. [24] suggest apoptosis to be one of the mechanisms of prolonged neuronal loss following traumatic brain injury. In these patients, serum and CSF sFas/APO 1 concentrations significantly exceeded the concentrations measured in control group. In addition, myocardial cells exposed to ischemia are known to be destroyed by apoptotic death. In the study by Ohtsuke et al. [25], serum concentrations of sFas/APO 1 were significantly higher in patients with acute infarction than in healthy volunteers.

sFAS/APO1 is extrinsic transmembrane protein and antagonistic pair of proapoptotic Fas / FasL with which it is in dynamic equilibrium. sFas/APO1 is therefore a reliable indicator of activation of programmed cell death (apoptosis). In our study, serum concentration of sFas/APO 1 significantly exceeded its CSF concentration. The absence of correlation between the two, suggested the synthesis of sFas/APO 1 by the central nervous system cells. Its concentration in serum is an expression of dynamic equilibrium with its antagonistic pair on endothelial cells of cardiovascular system. In the most studies with neural pathology examining its concentration in CSF is used as a benchmark to monitor its dynamics [24,26]. Low level of sFas in CSF (according to some authors in neurologically healthy individuals there is no sFas) is an expression of the fact that the cell surface of brain ventricles and subarachnoid space is far smaller than the vast cardiovascular area. In the case of organic brain pathology, such as cerebral infarction, proapoptotic mechanisms of cell vascular endothelial infarcted areas and elements of hematoencephalic barriers are activated in the penumbra

region and in the infarct zone equivalent mechanisms in astroglia and neurons. This leads to the activation of sFas/APO1 as antagonistic agents with the purpose to constraint propagation of infarct zone on the territory of the penumbra region. At the stage of clinical improvement of disease there is a reduction of infarcted areas and withdrawal of penumbra followed by decrease of sFas/APO1 in serum and CSF. The increased sFas/APO 1 concentration in CSF of stroke patients on day 1 of disease onset and its gradual decrease to the concentrations measured in the control group suggested the role of apoptosis in the pathogenesis of cerebral infarction. The main limitation of the study is a relatively small number of patients. It is necessary to conduct further studies on a larger sample to confirm the involvement of apoptosis in the pathogenesis of cerebral infarction. The confirmation of apoptosis and identification of antiapoptotical markers would provide potentially new therapeutic options in the form of reduced delayed brain damage in cerebral infarction.

CONCLUSIONS

Study results confirmed the dynamic pattern of sFas/APO 1 in serum and cerebrospinal fluid of patients with acute ischemic stroke, suggesting the possible role of apoptosis in the pathogenesis of cerebral infarction.

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