Research article

Expression of autophagy and apoptosis biomarkers in patients with acute ischemic stroke

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ABSTRACT

Introduction and Aim: Apoptosis, autophagy, and necrosis are the main mechanisms of neuron death in acute ischemic stroke (AIS). This study aimed to evaluate the expression of apoptosis and autophagy biomarkers in peripheral blood of patients with AIS.

Materials and Methods: Sixty-eight patients (32 men and 36 women) aged 30-60 years with AIS underwent a clinical and neurological examination on the 1st, 7th, and 14th days after the disease onset. The expression of apoptosis and autophagy biomarkers in peripheral blood was evaluated by flow cytometry and compared with the severity of neurological deficit and injury on the 1st, 7th, and 14th days, using correlation analysis.

Results: There is a statistical significance compared with the control group and an increase in the expression of key biomarkers of apoptosis and autophagy was revealed. Increased expression levels of annexin A5 and caspase-3 positively correlate with the severity of neurological deficit and injury on the 1st and 7th days from the onset of the disease.

Conclusion: A direct correlation was revealed between elevated levels of apoptosis and autophagy biomarkers in peripheral blood and severity of neurological deficit and injury on the 1st, 7th, and 14th days from the onset of AIS.

Keywords: Apoptosis; autophagy; acute ischemic stroke; annexin A5; caspase-3.

INTRODUCTION

Acute ischemic stroke (AIS) is one type of stroke that remains a concern in neurology due to its wide prevalence, and high disability and mortality rates (1). Apoptosis, autophagy, and necrosis are the main mechanisms of neuron death in AIS (2, 3). But the mechanism of these processes prevails at a certain stage of the ischemic cascade and affects the outcome of the disease to a greater extent.

Like apoptosis, autophagy is also activated in the penumbra (2, 4, 5). It is believed that neurons damaged by apoptosis and autophagy in the penumbra zone can be restored, in contrast to neurons that died by the mechanism of necrosis, localized in the ischemic zone, and not subjected to regeneration (6). Signal proteins initiating both apoptotic and autophagic death of brain cells in AIS act either synergistically by creating common modules, or alternatively, as switches from a single cell program to another (7, 8).

A comparative assessment of the concentration of biomarkers of autophagy and apoptosis in the peripheral blood of patients with AIS in comparison with the severity of neurological deficit and injury will help in better understanding of cross interactions between these processes at different stages of the AIS. Thus, knowing biomarkers of apoptosis and autophagy is helpful in developing new diagnostic methods and predicting the outcome of AIS. This study aimed to evaluate the expression of biomarkers of apoptosis and autophagy in peripheral blood of patients with AIS.

MATERIALS AND METHODS

For comparative analysis, the 68 patients (32 men and 36 women) aged 28-65 years with AIS at the time of diagnosis were divided into two groups: case (n = 34); control (n = 34). As a case group, patients were included in the study in the first 24 hours from the onset of the disease and had not more than 13 points on the National Institutes of Health Stroke Scale (NIHSS) score. As a control group, 34 healthy people

were recruited. Patients with age less than 28 or more than 65 years, repeated stroke, hemorrhagic stroke, or other hemorrhages, and a history of diabetes mellitus were excluded.

Patients underwent a clinical and neurological examination with an assessment of the severity of neurological deficit according to the NIHSS, a study of the volume of brain damage on MRI, and testing according to the modified Rankin scale on the day 1, day 7, and day 14 from the onset of AIS from the onset of the AIS.

Expression of biomarkers of apoptosis and autophagy in leukocytes was assessed in the first 24 hours from the onset of the disease, on the day 7, and day 14 from the onset of AIS by flow cytometry. Blood was used for the analysis of each sample. To assess autophagy, membrane permeabilization was performed followed by staining of the studied samples with monoclonal antibodies (mAb) to the main indicators of autophagy, LC3, and p62 (Biorbyt, UK).

For quantitative CYTO-ID® Autophagy Detection Kit 2.0 containing the indicator dye, Cyto-ID conjugated with fluorochrome fluorescein-5-isothiocyanate was used to determine autophagosomes in circulating plasma cells. Then, experimental samples and control samples were analyzed on a MoFlo Astrios Cell Sorter (Ramcon, Denmark).

The level of apoptosis of leukocytes was analyzed by the amount of annexin A5- and caspase 3-positive cells using commercial kits for flow cytometry, Annexin V-FITC Apoptosis Detection Kit (Sigma–Aldrich, USA) and Caspase-3, Active Form, Apoptosis Kit (BD Biosciences, USA).

Intracellular expression of biomarkers of autophagy was assessed by the number of p62- and LC3-positive cells in each independent study population (lymphocytes, monocytes, granulocytes) and in the types of leukocytes.

The biomarkers of apoptosis and autophagy are compared with the severity of the neurological status, a correlation analysis was performed between the average values of biomarkers of apoptosis, and autophagy and the average values of neurological deficit and the volume of brain damage on the day 1, day 7, and day 14, respectively.

The Mann–Whitney test was used to assess the significance of differences between the groups. Statistical analysis was performed using Statistica v11.0 (StatSoft Inc., USA). Association between two groups was assessed using Spearman's rank correlation coefficient. A two-sided $p < 0.05$ was considered statistically significant.

RESULTS

During the observation period of 14 days in 52 (76.5%) patients, positive general condition and regression of cerebral symptoms were observed. In 16 (23.5%) patients, the condition remained unchanged.

During analyzing the neurological status of patients according to the NIHSS scale, it was revealed that the neurological deficit on the day 1 of AIS varied from 6 to 13 points (on average (11.4±2.1) points). On the day 7 in 78.6% of patients with AIS, the severity of the neurological deficit was significantly lower than on the day 1 ($p<0.05$) and averaged (8.3±3.2) points. On the day 14, there was a decrease in the average neurological deficit to (6.6±2.9) points.

The volume of brain damage, revealed by MRI on the day 1 from the onset of the disease left, varied from 20 to 280 cm^3 (average (102.3±9.2) cm^3). During the 14 days of the study, there are no statistically significant changes in the size of the lesion.

A statistically significant increase in the content of annexin A5- and caspase 3-positive cells, compared with the control group, was noted throughout the study in all populations of leukocytes with a maximum increase in the first 24 hours from the onset of AIS (Table 1). The highest values of apoptotic biomarkers were found in the granulocytes. There is a clear trend towards a decrease in the intensity of leukocyte apoptosis by the day 14, which probably indicates the activation of compensatory anti-apoptotic mechanisms.

Table 1: Biomarkers of apoptosis in the peripheral blood of patients with AIS

Populations of leukocytes									
I group									
Biomarkers of apoptosis	**Granulocytes**			**Monocytes**			**Lymphocytes**		
LC3, % of positive cells	1st day	7th day	14th day	1st day	7th day	14th day	1st day	7th day	14th day
	2.5	1.9	1.1	1.6	1.9	2.3	2.2	1.8	1.5
p62, % of positive cells	0.06	0.05	0.04	0.08	0.08	0.06	0.07	0.03	0.08
II group									
Biomarkers of apoptosis	**Granulocytes**			**Monocytes**			**Lymphocytes**		
LC3, % of positive cells	1st day	7th day	14th day	1st day	7th day	14th day	1st day	7th day	14th day
	65.7***	27.3**	14.3**	36.8***	20.6**	11.4*	28.2**	11.2*	6.4*
p62, % of positive cells	11.2**	4.9*	3.2*	9.6**	4.3*	1.7*	6.3*	2.9*	2.2*

Group I – control group (n=34); Group II (case) - patients with AIS (n=34); $p \leq 0.05$ = *, $p \leq 0.01$ = **, $p \leq 0.001$ = ***.

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Table 2: Evaluation of the biomarkers of autophagy in the peripheral blood of patients with AIS based on the volume of damage to the brain parenchyma

Populations of leukocytes									
IIa group									
Biomarkers of autophagy	**Granulocytes**			**Monocytes**			**Lymphocytes**		
LC3, % of positive cells	1st day	7th day	14th day	1st day	7th day	14th day	1st day	7th day	14th day
	3.2	8.8	2.1	2.4	14.7	2.8	2.5	11.9	1.4
p62, % of positive cells	7.1	2.8	2.6	1.3	2.9	1.7	1.9	5.3	3.4
Cyto-ID (MFI)	3.7	13.1	2.8	3.5	14.6	2.4	4.6	15.4	3.8
IIb group									
Biomarkers of autophagy	**Granulocytes**			**Monocytes**			**Lymphocytes**		
LC3, % of positive cells	1st day	7th day	14th day	1st day	7th day	14th day	1st day	7th day	14th day
	7.2*	11.2	10.5**	5.4*	9.9	8.7*	8.1**	11.5	8.8**
p62, % of positive cells	7.9	1.9	3.1	9.5**	3.2	2.4*	4.7*	2.8*	2.8
Cyto-ID (MFI)	10.6*	13.2	12.8**	7.2*	10.9	10.2**	11.7	9.6	9.9**

MFI = mean fluorescence intensity (median values are given); Group IIa = patients with AIS with a volume of damage to the brain (lesion less than 50 cm^3) and (n=42); Group IIb = patients with AIS with a volume of damage to the brain (lesion greater than 50 cm^3) and (n=26); $p \leq 0.05$ = *, $p \leq 0.01$ = **, $p \leq 0.001$ = ***.

The direct correlation between an increased level of annexin A5 expression in leukocytes and the severity of neurological deficit according to the NIHSS scale, as well as the volume of brain damage, was detected on the day 1 and day 7 from the onset of the AIS ($r=0.84$; $p<0.05$ and $r=0.76$; $p<0.01$). An increased level of caspase-3 expression positively correlated with the severity of neurological deficit (NIHSS>10) and the volume of brain damage (lesion greater than 50 cm^3) already on the day 1 after the onset of AIS ($r=0.81$; $p<0.01$ and $r=0.71$; $p<0.05$, respectively) and for the next seven days. It should be emphasized that the maximum values of caspase-3 expression (>10%) were detected in 12 patients, 2 of whom died on the day 26 and day 31 from the onset of the AIS, and in 10 the clinical and neurological status remained unchanged with high scores of neurological deficits.

When analyzing the expression of autophagy biomarkers, a strong direct correlation was found between elevated LC3 values, Cyto-ID fluorescence intensity, and volume of brain damage on the day 7 of the study ($r=0.82$; $p<0.05$ and $r= 0.79$; $p<0.05$). The expression of autophagy biomarkers is not detected in the peripheral blood of patients in the control group, the patients in the case group were divided into two subgroups (IIa and IIb): those with a lesion volume of fewer than 50 cm^3 and more than 50 cm^3 (Table 2). In patients of group IIb, there was a statistically significant increase in the expression of the LC3 protein and the average intensity of fluorescence of the Cyto-ID dye, compared with group IIa, on the day 1 and day 14 in all studied populations of leukocytes throughout the study. At the same time, high values of the p62 protein were found in leukocytes on the day 1 after a decline.

DISCUSSION

The lowest rates of apoptosis found in the lymphocytes indicate their escape from apoptosis, leading to penetration through the blood-brain barrier and actively involved in the immune-mediated destruction of the affected area of brain tissue (9).

The LC3 protein is a reliable marker of autophagy, which positively correlates with the number of active autophagosomes (3, 4) and the fluorescence intensity of the Cyto-ID also reflects the intensity of autophagy (10). In contrast to these indicators, the p62 correlates with the intensity of autophagy (7, 10). In group IIb, a high level of autophagy was observed in this study and positively correlated with the volume of brain parenchyma damaged. In group IIa, the maximum values of autophagy biomarkers were observed on the day 7 and decreased by the day 14 in all populations of leukocytes.

In this study, patients with AIS showed increased activation of apoptosis and autophagy in peripheral blood cells, which is statistically significant compared with the control group, increase in the expression of key biomarkers of apoptosis and autophagy in the leukocytes. There is increased spontaneous apoptosis in peripheral blood mononuclear cells and Li *et al.*, (11) reported an increase in the key autophagy biomarkers LC3 and Beclin-1 in the blood serum and CSF of patients with AIS.

In response to AIS, an immune response, neuroinflammation, apoptosis, and autophagy develop both in the lesion and systemically (12). In the AIS there is an imbalance of the cytokines with a deficiency of anti-inflammatory cytokines and an increase in the triad of pro-inflammatory cytokines such as Tumor necrosis factor-alpha (TNF-α), interleukin-1β (IL-1β) and IL-6, which are produced by both microglial cells and immunocompetent peripheral blood cells, as evidenced by an increase in the concentration of these cytokines in the serum of patients with AIS (13). TNF-α is a pleiotropic cytokine that plays a key role in many physiological

and pathological cellular processes, apoptosis and autophagy are complex multi-level processes, in the regulation of contents in the peripheral blood (2, 10, 12, 14). Thus, the signal for the activation of apoptosis and autophagy in peripheral blood cells can be received from several mediators of the immune response and neuroinflammation, the increased production of which in response to ischemic injury occurs not only in the lesion but also in the peripheral blood. Mediators that induce the activation of apoptosis in peripheral blood are TNF-α, IL-1β, soluble Fas ligand, and heat shock proteins-70 (14). Autophagy is involved in the regulation of the production of pro-inflammatory cytokines (IL-1β, IL-17, IL-18, IL-23, and chemokine (C-X-C motif) ligand 1 (10).

In this study, a direct correlation was revealed between elevated levels of apoptosis and autophagy biomarkers in peripheral blood and neurological status indicators (severity of neurological deficit and injury) on the day 1, day 7, and day 14 from the onset of AIS.

CONCLUSION

In the AIS, there is a statistically significance compared with the control group and increase in the expression of key biomarkers of apoptosis and autophagy was revealed. Increased expression levels of annexin A5 and caspase-3 positively correlate with the severity of neurological deficit according to the NIHSS scale and the volume of brain damage on the day 1, and day 14 from the onset of the disease.

A strong direct correlation was found between the key biomarkers of autophagy (LC3 and Cyto-ID), the amount of brain damage and the severity of neurological deficit on the day 7 of the AIS ($r=0.82$; $p<0.05$ and $r= 0.79$; $p<0.05$).

In patients with AIS having a brain parenchymal lesion volume of more than 50 cm^3, there is a statistically significant increase in the biomarkers of apoptosis and autophagy, indicating an increase in activation of both apoptosis and autophagy in peripheral blood cells in an extensive focus of brain tissue damage.

CONFLICT OF INTEREST

Authors declare that there is no conflict of interest.

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