Proteomic studies of blood plasma of patients with the ischemic stroke in its most acute period

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Abstract

Pilot studies of the blood plasma proteome were performed for six patients with ischemic stroke in the most acute phase of its development. This investigation involved evaluation of the state of the platelet-vascular homeostasis and determination of individual factors of coagulation, anticoagulation and fibrinolysis. The analysis revealed proteins with molecular masses from 14 to 140 kDa. A protein of 118 kDa was found for the patient whose blood flow had not recovered after systemic thrombolysis. This protein was absent from the blood plasma of all other patients for whom the thrombolysis was effective. Mass spectrometric analysis identified this protein as haptoglobin.

Key words: Plasma proteome, thrombolysis, ischemic stroke.

1. Introduction

Acute disorders in the cerebral circulation remain one of the most important medical-social problems. In Russia, the mortality in the acute period of ischemic stroke is as high as 35% and increases almost by 15% to the end of the first year of the disease. In addition, the postapoplectic disability occupies a leading place among all possible reasons of disablement. Numerous experimental and clinic studies have demonstrated that restoration of a blood flow (removal or dissolution of a thrombus) is effective in the first hours of the development of the ischemic stroke, when most of the neurovisually detected changes are reversible, and a cascade of ischemic changes is at the initial stage of a decrease in the blood circulation \cite{12, 13}.

The systemic thrombolysis was performed by treatment with the rt-PA drug (0.9 mg/kg, with the maximum of 90 mg). 10% of the agent was administered as a bolus dosing with its subsequent infusion for 60 min. Time (the first 4.5 h after the beginning of the ischemic stroke) was a decisive factor of the thrombolysis efficacy. Such treatment achieves the highest efficiency (class I, level A) and is recommended for application in both European and North American guidelines for treatment of patients with ischemic stroke \cite{12, 13}. The frequency of blood flow recovery after the thrombolytic therapy is 43 – 71% \cite{13}.

Endothelium dysfunction plays an important role in the development of disorders of the cerebral circulation \cite{2, 10, 11}. Application of the proteomic technologies in a search for both diagnostic markers of diseases and potential targets for drug therapy is promising.

The goal of this study is identification of potential protein markers of efficacy and complications of systemic thrombolysis in the blood plasma proteome of patients with ischemic stroke in its most acute period.

2. Materials and Methods

The pilot investigation of the blood plasma proteome was performed for six patients suffering from acute ischemic stroke. Systemic thrombolysis was carried out using the alteplase drug (rt-PA, Actilyse) according to international and domestic recommendations. The examined patients were at the age of 61 (55, 67) in average. The average time-to-door time was 150 (118, 171) min. The average door-to-needle time was
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41 (30, 60) min. The average severity of the neurological symptoms proved to be 14 (10, 17) according to the NHSS scale. We determined a restoration of the blood circulation for all the patients by CT or MR angiography before and immediately after the thrombolysis. Reperfusion (blood supply to the brain area that was subjected to ischemia due to occlusion of the cerebral artery) was evaluated by the dynamic CT-perfusion (before the thrombolysis and on the next day after the thrombolysis). A complete restoration of the blood circulation was observed for five patients according to angineurovisualization.

The hemostasis investigation involved examination of the platelet-vascular hemostasis (the thrombocyte aggregation that was induced by ADP and adrenaline), general parameters of hemostasis, and determination of individual factors of coagulation, anticoagulation, and fibrinolysis [1, 2]. Aggregation of erythrocytes and thrombocytes was studied by the standard methods on Biola and LORCA aggregometers (the Netherlands).

The protein spectrum of the blood plasma was studied by 1D-electrophoresis in 8% and 14% PAAG according to Laemmli before the systemic thrombolysis, just after this procedure, and one day after the procedure. The electrophoresis was performed for 60 min at a current intensity of 200 mA and voltage of 200 V using a Mini-Protein Tetra System electrophoretic unit (Bio-Rad) with an El’f-8 power supply (DNK-Tekhnologiya, Russia). The blood plasma was centrifuged for 15 min at 18000 g and 4°C on an Eppendorf-5810R Centrifuge (Germany). For Sample delipidation: The supernatant was diluted in 2.5 times (0.25 ml of the plasma + 0.375 ml of a buffer) with the following buffer: 10 mM Tris-HCl (pH 7.4) and 0.15 M NaCl. The particles and aggregates were separated by centrifugation for 15 min at 18000 g and 4°C. The mixture (20 µl) of 50 mM Tris-HCl (pH 6.8), 1 mM EDTA, and 2% SDS (TES) and 40 µl of the dye for the samples (100 mM Tris-HCl, pH 6.8, 20% glycerol, 8% SDS, and 0.002% BrPh) were added to the supernatant (20µl). The reaction mixture was heated for 5 min at 95°C in a “Gnom” thermostat (DNK-Tekhnologiya, Russia) [14].

The protein preparations were subjected to trypsinolysis in a polyacrylamide gel and further analyzed by the method of fingerprint of peptide masses according to the procedure that was described earlier [3]. A mass spectrometric analysis of the tryptic hydrolysates was performed on an Ultraflex MALDI TOF mass spectrometer (Bruker Daltonics, Germany). The positively charged ions were registered in a Prism mode in the m/z range from 600 to 4000 Da. The proteins were identified using the Mascot search system (MatrixSciense) (http://www.matrixscience.com/home.html). The following parameters of the search system were used: Enzyme – Trypsin; Database – SwissProt; Taxonomy – Human; Fixed modification – none; Variable modification – none; Missed cleavages – 1; Peptide tolerance – 100 ppm; Mass values (monoisotopic) – MH+.

3. Results and Discussion

Analysis of the 1D-electrophoresis results demonstrated that the protein spectrum involved proteins (from 10 to 20 protein bands) with molecular masses (MW) from 14 to 140 kDa (Fig.1).

![Fig.1. 1-D-electrophoregram of the blood plasma proteins of the patients with CVD before the treatment, one hour after the Actilyse administration, and 24 hours after the thrombolysis.](image-url)
The results of the mass spectrometric investigation are presented in Fig. 2.

Fig. 2. MALDI-TOF MS spectrum of the peptides that were formed after the tryptic hydrolysis of the proteins.
Tryptic fragments of the peptides of the human haptoglobin that were determined by the method of peptide mass fingerprints. 30.3% of the protein sequence was covered. The sequences which were identified by the MALDI TOF mass spectrometry of the protein tryptic fragment are shown in Table 1.

<table>
<thead>
<tr>
<th>Measured m/z</th>
<th>Calculated MH+</th>
<th>Deviation (Da)</th>
<th>Deviation (ppm)</th>
<th>Range</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>1439.695</td>
<td>1439.701</td>
<td>-0.006</td>
<td>-4.388</td>
<td>60 - 72</td>
<td>TEGDVVYTLNDKK</td>
</tr>
<tr>
<td>856.529</td>
<td>856.468</td>
<td>0.062</td>
<td>72.301</td>
<td>113 - 118</td>
<td>NYKKLR</td>
</tr>
<tr>
<td>1708.871</td>
<td>1708.850</td>
<td>0.021</td>
<td>12.228</td>
<td>117 - 131</td>
<td>LRTEGDGVVYTLNNEK</td>
</tr>
<tr>
<td>688.371</td>
<td>688.378</td>
<td>-0.007</td>
<td>-9.545</td>
<td>132 - 136</td>
<td>QWINK</td>
</tr>
<tr>
<td>895.486</td>
<td>895.474</td>
<td>0.012</td>
<td>12.903</td>
<td>154 - 161</td>
<td>NPANPVQR</td>
</tr>
<tr>
<td>923.540</td>
<td>923.531</td>
<td>0.009</td>
<td>9.741</td>
<td>162 - 170</td>
<td>ILGGHLDAK</td>
</tr>
<tr>
<td>920.471</td>
<td>920.462</td>
<td>0.009</td>
<td>9.559</td>
<td>171 - 178</td>
<td>GSFPWQAK</td>
</tr>
<tr>
<td>1290.743</td>
<td>1290.730</td>
<td>0.013</td>
<td>9.951</td>
<td>216 - 227</td>
<td>DIAPTLTLVYGK</td>
</tr>
<tr>
<td>703.376</td>
<td>703.373</td>
<td>0.002</td>
<td>3.161</td>
<td>256 - 261</td>
<td>VSVNER</td>
</tr>
<tr>
<td>809.383</td>
<td>809.379</td>
<td>0.004</td>
<td>5.502</td>
<td>271 - 277</td>
<td>DYAEGVGR</td>
</tr>
<tr>
<td>980.512</td>
<td>980.495</td>
<td>0.017</td>
<td>17.544</td>
<td>278 - 286</td>
<td>VGYVSGWGGR</td>
</tr>
<tr>
<td>760.400</td>
<td>760.399</td>
<td>0.001</td>
<td>1.011</td>
<td>292 - 297</td>
<td>FTDHLK</td>
</tr>
<tr>
<td>1274.666</td>
<td>1274.638</td>
<td>0.028</td>
<td>22.203</td>
<td>312 - 322</td>
<td>HYEGSTVPPEKK</td>
</tr>
<tr>
<td>1146.557</td>
<td>1146.543</td>
<td>0.015</td>
<td>13.024</td>
<td>312 - 321</td>
<td>HYEGSTVPPEK</td>
</tr>
<tr>
<td>1203.650</td>
<td>1203.637</td>
<td>0.013</td>
<td>10.929</td>
<td>392 - 401</td>
<td>VTSIQDWVQK</td>
</tr>
</tbody>
</table>

A unique protein (118 kDa, see Fig. 1 tracks 1, 2, and 3) was shown to be present in the proteome of the patient whose blood circulation was not restored after the systemic thrombolysis. This protein was absent in all cases of effective thrombolysis. We identified this protein as haptoglobin by the mass spectrometric analysis. We repeated the mass spectrometric analysis taking into account that haptoglobin could have some homology with the tissue plasminogen activator, urokinase, and a number of other serine proteases of the blood plasma, because genes encoding these proteins were located closely [9]. The repeated analysis confirmed the identification of the examined protein as haptoglobin. According to the literature, haptoglobin is a protein of the acute phase of inflammation. It can bind the free hemoglobin that is released by erythrocytes. Thus, haptoglobin prevents hemoglobin-stimulated free-radical oxidation of membrane lipids of endothelial cells and formation of the hydroxyl radicals in the inflammation areas [4]. This is the way that haptoglobin realizes its anti-oxidative effect. It is also important that the HP2-2 phenotypic variation of haptoglobin is considered as a marker of ischemic stroke in experimental animal models [5] and is an unfavorable prognostic factor of development of the ischemic stroke for the patients suffering from diabetes [6, 8] and metabolic syndrome. The haptoglobin identification in all the three samples (tracks 1, 2, and 3) of the patient can point to the fact that the brain ischemia is accompanied by an inflammatory process and disorder in the function of vascular endothelium. It is known that haptoglobin affects re-vascularization processes and can modulate functions of endothelial cells [7].

Thrombolytic therapy is known to be effective during the first 4.5 hours (the so-called therapeutic window), but time is probably not the only reason of the treatment inefficacy. Possibly, the presence of haptoglobin in all the three samples of the patient whose cerebral circulation was not restored after the thrombolysis can be considered as a factor of negative prognosis of the thrombolytic treatment. In this case, a surgical correction for restoration of the blood flow will possibly be more effective.

Dynamics of the following parameters of the erythrocyte aggregation activity were normal: Amp (the aggregation amplitude) 4.4 – 13.3 RU, Tf (time of the formation of coin baculum) 5.5 – 4.1 sec, Ts (time of the formation of three-dimensional aggregates) 1.1 – 42.1 sec, γdis (the rate of the complete deaggregation, i.e. stability of the aggregates) 200 – 100 sec⁻¹, Dlmax (maximum deformability of erythrocytes) 0.58 – 0.33.
The only exception was aggregation index (AI) that reflected a quantitative involvement of erythrocytes in the aggregation process. It was enhanced to 72.1%, but decreased to 49.7% with time.

However, different picture was observed for the patient whose blood circulation was not restored after the systemic thrombolysis.

This patient's aggregation data was, like of the other patients, normal. The only difference detected was the haptoglobin in the proteome of this patient's plasma, which was absent in patients with effective treatment.

The positive dynamics of the parameters of the thrombocyte aggregation did not correlate with the neurological symptoms and expression of the 118-kDa protein that was found in all three samples of the blood plasma.

Proteomic studies of blood plasma are a necessary initial step in a clinic analysis of acute disorders of the cerebral circulation and give an opportunity to choose the key proteins the expression of which is changed in response to pharmacological agents or the brain ischemia.

Our results can be used for elaboration of monitoring test-systems (biochips) for prognosis of the efficacy of thrombolytic therapy and for creation of new methods of treatment. Therefore, the search for biomarkers of the disorders in hemostasis and functions of a vascular wall is an urgent problem of practical angioneurology.

References


