The role of oxidized albumin in blood cell aggregation disturbance in burn disease

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Original Article

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Abstract: The burn disease is found to be accompanied by increasing of the level of oxidized proteins of blood serum. We studied the influence of albumin oxidation rate on aggregation of platelets and erythrocytes, disaggregation of erythrocytes. The changes of blood cells aggregation associated with oxidation rate of albumin were found. Possible mechanisms of these effects are discussed.

Keywords: Platelet aggregation, erythrocyte aggregation, oxidized proteins, burn disease

Introduction

Oxidative stress plays an important role in pathogenesis of burn disease [1, 2]. Reactive oxygen species (ROS) act not only upon lipids but also upon proteins [3, 4]. The heightened interest of researchers is observed nowadays in studying the role of oxidatively modified proteins (OMP) in pathogenesis of various pathological conditions including burn disease. It was demonstrated that after thermal trauma the levels of carbonyl derivates of amino acids increased in skeletal muscles, rat liver, blister exudate and, during first hours after the trauma, in blood plasma [5-8]. Only oxidized fibrinogen was studied in most of investigations of the influence of OMP on aggregation of blood cells [9, 10]. There are hardly any studies devoted to the investigation of influence of serum OMP on aggregation of blood cells. The objective of the study: to measure levels of serum OMP in patients after thermal trauma and research influence of oxidized albumin on platelet and erythrocyte aggregation.

Materials and methods

The research was conducted on 20 blood samples from healthy people and 10 blood samples from patients in the acute period of burn disease (II– or III–degree burns of more than 20% of total body surface). For getting serum the blood was taken into vacuum blood tubes (BD Vacutainer® SST™ II), 30 minutes later it was centrifuged for 10 minutes at 3000 rpm. For studying the aggregation of platelets and erythrocytes the blood was stabilized with 3.8% sodium citrate solution at a v/v ratio 9: 1. Plasma enriched with platelets was obtained by centrifugation of citrated blood for 7 min at 1000 rpm. Then the blood was centrifuged for 20 min at 3000 rpm for getting platelet-free plasma and erythrocyte mass. During studies of induced and spontaneous aggregation of platelets, their number in the plasma enriched with platelets was standardized by adding autologous platelet-free plasma until the concentration of (200-250) x 10^9/l was attained. During studies of aggregation of erythrocytes, platelet-free plasma and erythrocyte mass were mixed at a v/v ratio 2: 1, respectively.

Spontaneous (shear-induced) aggregation of platelets and erythrocytes was investigated using a rheoscope designed according to the method of H. Schmid-Schönbein et al. [11], in modification of Levin et al. [12]. In this device the blood cells are placed between plane-parallel plates rotating in opposite directions. In the centre of the bottom plate there is a cylindrical excavation. So, a chamber is formed between the top and bottom plates. A suspension of
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Spontaneous aggregation of platelets was investigated under the condition of shear stress (shear rate is 160s⁻¹) with video recording of the aggregation process. Then the obtained micro-pictures were processed with a specially developed computer program, based on the principle of threshold binarization of an image. The degree and rate of spontaneous aggregation of platelets were evaluated by means of the following parameters: 1. Aggregation degree – the total maximal integral optical density of platelet aggregates (standard units) – Ma. 2. Aggregation rate - the total maximal integral optical density of platelet aggregates at 180th sec after the beginning of the aggregation process (standard units) – A₁₈₀.

Adenosine diphosphate (ADP)-induced (2x10⁻⁵ M) aggregation of platelets was studied by aggregometer according to the technique developed by C. Born [13].

The process of erythrocytes aggregation was registered by an automatic recorder after hydrodynamic mixing of erythrocyte suspension was stopped. The process of disaggregation of erythrocytes was recorded at shear rates 10 sec⁻¹ (D₁₀), 15 sec⁻¹ (D₁₅), 20 sec⁻¹ (D₂₀). We evaluated: degree of aggregation (Ma, mm), rate of aggregation – according to the amplitude of aggregatogram at 40th sec after the start of the aggregation process (A₄₀, mm), degree of disaggregation of erythrocytes – in percentage of Ma.

The influence of oxidized albumin on aggregation of blood cells was studied using donor blood. For oxidative modification, albumin solution (5%) was exposed to ultraviolet (UV) irradiation. When the albumin oxidation degree varied, its total concentration remained constant. The measurement of the level of OMP in blood serum and control of the degree of oxidative modification of albumin solution was made according to the technique of R.L. Levine et al. [14]; the technique is based on spectrophotometric detection of 2,4-dinitrophenylhydrazones, which are the products of reaction of oxidized amino acid residues and 2,4-dinitrophenyl-hydrazine (DNPH). It is known that neutral aliphatic aldehyde-dinitrophenylhydrazone have absorption range 260-558 nm, whereas the basic ones - 258-264 and 428-520 nm. The neutral aliphatic ketone-dinitrophenylhydrazone has absorption range 363-367 nm, the basic ones-430-434 and 524-535 nm [15]. Therefore, in order to test for all groups of hydrazones, the levels of carbonyl derivates in burned patients and healthy donors were detected at wavelengths 357 nm, 363 nm, 370 nm, 430 nm, 530 nm. The degree of albumin solution oxidation was derived from levels of carbonyl derivates having absorption peak at 370 nm [14]. Each series of experiments with albumin of various degrees of oxidation was performed on the blood samples from the same donor.

The results of studies were processed with the methods of non-parametric statistics using the criteria of Mann-Whitney, Wilcoxon matched pairs test.

Performing of this study was approved by the local ethics committee of Nizhny Novgorod Research Institute of Traumatology and Orthopaedics of Ministry of Health and Social Development of Russian Federation.

Results

This study revealed sharp rise of the level of OMP in blood serum during acute periods of burn disease (Table 1). Specifically, the concentration of dinitrophenylhydrazone registered at the wavelengths of 357 and 363 nm in burned

<table>
<thead>
<tr>
<th>groups</th>
<th>OMP (unit/mg)</th>
<th>total protein (mg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>λ=357 (nm)</td>
<td>λ=363 (nm)</td>
</tr>
<tr>
<td>norm (n=10)</td>
<td>19.29±0.73</td>
<td>18.48±0.75</td>
</tr>
<tr>
<td>burns (n=10)</td>
<td>26.21±1.87**</td>
<td>25.40±1.09**</td>
</tr>
</tbody>
</table>

Note: *p<0.05, **p<0.01, Mann-Whitney criteria.

Table 1. Changes of level of OMP in blood serum in burn disease
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Patients exceeded the norm by 36-37%. The levels of dinitrophenylhydrazine registered at the wavelengths of 370 and 430 nm at the acute periods of burn disease were 39% and 65% higher than the norm, respectively. The concentration of ketonodinitrophenyl–derivatives of base nature (530 nm) in severely burned patients exceeded the norm 2.2 times.

On the basis of data obtained we considered rational to estimate the influence of albumin, as the main and most promptly oxidizable blood serum protein, on blood cells aggregation. The degree of albumin oxidation in that part of the study corresponded to the degree of oxidation of blood serum proteins found in researches on burned patients.

It was found that 43%-increase of albumin oxidation (at the constant albumin concentration) resulted in the raise of ADP-induced platelet aggregation (Table 2). The further increase of albumin oxidation (68% and 95% increment, compared to the control group) lead to the progressive growth of ADP-induced platelet aggregation.

The different pattern was obtained in the course of studying influence of oxidized albumin on spontaneous platelet aggregation. The 43%-increase of albumin oxidation leads likewise to the raise of platelet aggregation. However, the further increase of albumin oxidation (68% increment, compared to the control group) resulted in statistically significant depletion of platelet aggregation down to the initial level of the control group (Table 2). The same changes were observed in studying platelet aggregation rate, yet they were not statistically significant.

Oxidized albumin caused the increase of aggregation of erythrocytes and the growth of strength of their aggregates. At that, both the rate and the degree of aggregation of erythrocytes elevated with increase of the degree of oxidation of albumin (Table 3). Similar to the experiments on spontaneous platelet aggregation, at the maximal albumin oxidation the rate of erythrocyte aggregation decreased. Disaggregation of erythrocytes also decreased progressively, especially at low shear rates, along with the increase of albumin oxidation (Table 3).

**Discussion**

For the investigation of the level of OMP the method based on the interaction of 2-4-dinitrophenylhydrazine with aldehyde and ketone

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**Table 2. Influence of oxidized albumin to platelet aggregation**

<table>
<thead>
<tr>
<th>Degree of albumin oxidation (unit/mg)</th>
<th>ADP-induced aggregation</th>
<th>Spontaneous (shear-induced) aggregation</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>λ=370 (nm)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>A (mm)</td>
<td>A20 (mm)</td>
</tr>
<tr>
<td>36.45±2.61 (control group) (n=10)</td>
<td>64.36±14.02</td>
<td>34.93±4.70</td>
</tr>
<tr>
<td>52.18±4.82 (n=10)</td>
<td>79.36±12.22*</td>
<td>38.43±6.17</td>
</tr>
<tr>
<td>61.20±6.03 (n=10)</td>
<td>102.71±13.56*</td>
<td>43.07±6.17</td>
</tr>
<tr>
<td>71.18±6.68 (n=10)</td>
<td>128.00±13.73*</td>
<td>57.79±8.87*</td>
</tr>
</tbody>
</table>

Note: *p<0.05, comparison with control group, Wilcoxon matched pairs test. - p<0.05, comparison with aggregation indices at albumin oxidation degree 52.18, Wilcoxon matched pairs test.

**Table 3. Influence of oxidized albumin on aggregation and disaggregation of erythrocytes**

<table>
<thead>
<tr>
<th>Degree of albumin oxidation (unit/mg)</th>
<th>aggregation</th>
<th>disaggregation</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>λ=370 (nm)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>A (mm)</td>
<td>A20 (mm)</td>
</tr>
<tr>
<td>36.45±2.61 (control group) (n=10)</td>
<td>72.14±3.89</td>
<td>9.29±0.97</td>
</tr>
<tr>
<td>52.18±4.82 (n=10)</td>
<td>81.71±5.55*</td>
<td>11.88±1.64</td>
</tr>
<tr>
<td>61.20±6.03 (n=10)</td>
<td>92.14±6.82*</td>
<td>18.29±3.60*</td>
</tr>
<tr>
<td>71.18±6.68 (n=10)</td>
<td>94.43±5.65*</td>
<td>13.86±0.88*</td>
</tr>
</tbody>
</table>

Note: *p<0.05, comparison with control group, Wilcoxon matched pairs test. - p<0.05, comparison with aggregation indices at albumin oxidation degree 52.18, Wilcoxon matched pairs test.
groups of amino-acid residues, formed as a result of protein oxidation, was used. It was found that in the acute period of burn disease nearly all kinds of carbonyl derivatives in the blood serum significantly exceeded the norm, and it indicated a sharp increase of OMP level.

In the previous work we demonstrated that in patients in the acute period of burn disease the level of oxidized fibrinogen exceeded the norm 2 times [16]. On the basis of the presented data, we may conclude that the activation of free radical oxidation that is observed after thermal trauma causes oxidative modification of not just lipids and fibrinogen of blood plasma, but also of proteins of blood serum. Among them albumin, which constitutes approximately 60% of the whole quantity of proteins of blood plasma, is of major consequence. The opinions concerning the influence of albumin on the aggregation of blood cells are ambiguous [17-20]; however, the fact that albumin does influence the aggregation is unobjectionable. Among the blood serum proteins albumin is known to be the most susceptible to oxidative modification [21]. Considering the aforesaid we investigated the influence of oxidized albumin on blood cells aggregation.

Despite the fact that a great number of receptors to different ligands were found on the membranes of both platelets and erythrocytes [22-25], aggregation mechanisms of these blood cells differ greatly. The platelet aggregation is primarily determined by the condition of “fibrinogen” receptors – GP IIb/IIIa. The action of many agonists (ADP, thrombin, catecholamines and others) is closely connected with the fact that interaction of these substances with appropriate receptors stimulates the transmission of the signal inside the cell. That finally stimulates transition of glycoprotein receptors to the active - high affinity - state when they can connect with ligands [26, 27].

The aggregation mechanism of erythrocytes is rather different. Though there are also receptors on the membranes of erythrocytes interacting specifically with fibrinogen, they define the aggregation of red blood cells for 18% only [28]. At present time the most recognized theory explaining the mechanism of erythrocyte aggregation is still the “bridge” theory, or the theory of macromolecular connection, according to which aggregation process is specified by adsorption of large proteins on the surface of erythrocytes [29].

We found that the growth of albumin oxidation degree increases erythrocytes aggregation and their aggregates strength. It is known that OMP may form aggregates of various sizes. That process is specified by disturbance of native confirmation of a number of OMP domains; as a result of it the number of hydrophobic residues on the surface of globules increases, which determines formation of large protein conglomerates [30]. Oligomers and polymers of albumin were shown to be proaggregants of erythrocytes [31].

Thereat, we can offer the following model for explanation of our results. During oxidation albumin molecules form quite large conglomerates having the mass which is enough for enhancing erythrocytes aggregation. Besides, these molecules have dipole moment, which means that in one part of the molecule more groups with negative charge are “accumulated”, and in the other – with positive one, and that leads to the appearance of electric potential difference between different parts of the molecule [32]. It is known, that as a result of oxidation, local conformational trans-formations take place in proteins, new polar chemical groups (e.g. carbonyl) appear, that can also promote redistribution of the molecule charge [33, 34]. It seems to give the molecules a chance to bring into action, by means of van der Waals forces, the dipole-dipole attraction between each other as well as between cells, which is consistent with the “bridge” theory.

There are some data that oxidized proteins themselves are able to act as stimulators of lipid peroxidation [35]. We demonstrated earlier (unpublished data) that the incubation of oxidatively modified albumin with erythrocytes of healthy persons lead to activation of lipid peroxidation in their membranes, as was justified by decrease of erythrocytes resistance towards peroxides (chemiluminescence technique) and considerable raise of malondialdehyde level in their membranes. During that process, like in the case of oxidative stress, the shift of phospholipids, that are localized on the internal side of the membrane, toward the outside takes place [36]. Thus, the mechanism of non-specific aggregation of erythrocytes can be realized as well with the participation of phosphatidyl-
serine which is located on the outer side of the erythrocyte membrane and is able to connect with high-molecular proteins such as the conglomerates of oxidized albumin. It is consistent to the data of increasing of non-specific erythrocytes aggregation with the increase of concentration of phosphatidylserine on the external side of the erythrocyte membrane [37, 38].

We showed that spontaneous (shear-induced) platelets aggregation grew under the influence of oxidized albumin. Earlier we found that oxidized fibrinogen increased spontaneous platelets aggregation [16]. There are data that oxidized proteins can be the source of free radicals [39, 40]. R.T. Dean et al. [41] consider that proteins of plasmatic membranes, including proteins-receptors, are “attacked” by ROS. In course of such attack the conformation of proteins-receptors changes and they can be activated. It leads to the increase in bonding fibrinogen with platelet receptors GPIIb/IIIa and GPIb. This is likely to explain the increase of spontaneous platelet aggregation at a low degree of albumin oxidation. However, further increase of the level of albumin oxidation leveled its pro-aggregative effect on spontaneous aggregation of platelets. It is known that shedding of the membrane cell fragments including the membrane parts with receptors may occur under the influence of ROS [42, 43]. The high concentration of oxidized protein, as it was noted above, leads to formation of a considerable quantity of ROS, which may damage platelets receptors, and this process is accompanied by the decrease of platelet aggregation. At the certain degree of albumin oxidation the decrease of number of the receptors and their activation counter balances the influence of oxidized albumin on aggregation, and the latter may approach the control values, as it was witnessed in our experiments. The same effect is observed under the influence of maximally oxidized albumin on the rate of erythrocytes aggregation. The less pronounced (in comparison with platelets) effect can be explained, as it was mentioned above, by the fact that the specific way of erythrocyte aggregation, associated with receptor interaction, determines only 18% of their aggregation. That is why the shedding effect of ROS is less important for aggregation of erythrocytes than of platelets.

Another situation was observed while studying the influence of oxidized albumin on ADP-induced platelet aggregation which was increasing alongside with the increase of degree of albumin oxidation. It could be associated with the different mechanism of ADP-induced aggregation. We demonstrated earlier the difference in intensity between shear-induced and ADP-induced platelet aggregation in burn disease [16]. Thus, we may conclude that not just oxidized fibrinogen but oxidized albumin as well has a certain impact on the development of hyperaggregation of blood cells observed in burn disease.

Conclusions

1. In the acute period of burn disease accompanied by the increase of free-radical oxidation the level of serum OMP significantly increases.
2. Oxidation of albumin causes the increase of platelet and erythrocyte aggregation and also the increase of erythrocytes aggregates strength. 3. The differences in influence of oxidized albumin on aggregation of erythrocytes and aggregation of platelets were revealed.

Competing interest statement

The authors declare that they have no competing financial interests.

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