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The influence of thermal trauma on pro- and anticoagulant activity of erythrocyte-derived microvesicles

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ABSTRACT

The goal of this research was to study the influence of erythrocyte-derived microvesicles on hemostasis parameters during burn. It was found that the number of microvesicles derived from washed erythrocytes of burn patients after 1 day of storage at 37 °C was 4.2 times bigger than the number of microvesicles derived from erythrocytes of healthy donors. Hemocoagulation properties of erythrocyte-derived microvesicles of burn patients also change: according to the results of thromboelastography their procoagulant activity increases significantly, at the same time their antithrombin and fibrinilytic activity decrease. Thus, we can conclude that hepercoagulation during burn is to a certain extent caused by the disruption of the balance between procoagulant activity of erythrocyte-derived microvesicles and their antithrombin and fibrinolytic activity. Hypercoagulation effect of erythrocyte-derived microvesicles increases during burn not just because of their changed properties but also due to their increased number after thermal trauma.

1. Introduction

A number of studies have examined hemostasis disorder after burn [1–3]. They mainly report pronounced hypercoagulation that may result in disseminated intravascular coagulation after thermal trauma. Multiple factors are believed to be responsible for the hypercoagulation: tissue factor release, hyperadrenalism, erythrocyte hemolysis, activation of lipid peroxidation and proteolysis and some other factors [3–6]. However, the possible role of microvesicles (MVs), RBC-derived in particular, in thrombophilia (hypercoagulability) after thermal trauma remains uninvestigated. At the same time, articles describing the impact of RBC-derived MVs on pathogenesis of a variety of clinical conditions (cancer [7], cardio-vascular diseases [8], hemolytic anemia [9], and sepsis [10]) have recently appeared. The connection between these disorders and the increase in concentration of MVs in blood has been reported.

The increased microvesiculation of erythrocytes can be explained by morphological and functional red blood cell disorders which are described in a number of researches on burn [11]. Besides, the increase in concentration of MVs in burn patients could be caused by hemotransfusions, since microvesiculation significantly increases during erythrocyte storage [12].

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In few studies that are dedicated to the role of RBC-derived MVs in the hemostasis process it has been shown that these MVs demonstrate prominent procoagulant activity, mostly due to the fact that phosphatidylserine clusters on their membranes act as catalyst surface for endogenous and exogenous tenase and prothrombinase complex [13].

The role of RBC-derived MVs in hemocoagulation process in burn remains practically unstudied. The present research is focused on this issue.

We have reported earlier that RBC-derived MVs display antithrombin activity as well [14].

2. Materials and methods

The research was made on 35 blood samples of healthy donors (aged 20–50) and 24 blood samples of patients with burn (II–III degree burns, more than 20% of body surface, aged 18–65). Blood of both burn patients and healthy donors was drawn at the same time of the day in the morning on an empty stomach. Blood was collected not earlier than 2 days after the patient had been fully resuscitated, not earlier than 2 days after necrectomy, not earlier than 1 day after blood transfusion.

The work has been carried out in accordance with The Code of Ethics of the World Medical Association (Declaration of Helsinki). The study was also approved by the Local Human Subjects Research Ethics Committee. The blood was stabilized by 3.8% Sodium citratum solution in the ratio 9:1 on days 3–14 after the thermal trauma. The whole blood was centrifugated for 20 min at 3000 × *g*, plasma and platelet-leukocyte film were removed and erythrocytes were derived. The latter were washed three times with saline, resuspended in Tris-buffered saline (TBS; 150 mmol/L NaCl, 10 mmol/L Tris–HCl, pH7.4) in the ratio 2:1 and then incubated at 37 °C for 24 h. During the storage of washed erythrocytes a big amount of microvesicles (MVs) is released from them [12]. After incubation the erythrocytes were pelleted by centrifugation at 3000 × *g* for 20 min. The supernatant was purified from cell debris according to Dey-Hazra method [15]. The number of MPs was determined by Navios/Gallios flow cytometry (Beckman Coulter, USA) and was standardized in the samples (5000 ± 512 microparticles/μL PBS). MPs were preliminarily separated by ultracentrifugation using a Sorvall MX 150 Micro-Ultracentrifuge (Thermo Scientific, USA) at 100,000 × *g* for 60 min [16].

The effect of RBC-derived MVs on hemocoagulation parameters during plasma recalcification was studied using thromboelastograph analyzer TEG 5000 (USA). The following parameters of thromboelastography were assessed: *R* (min) – time from the start of the test to the thromboelastogram split point, *K* (min) – time from the beginning of the split until it reaches the amplitude of 20 mm, *MA* (mm) – the maximum amplitude on the thromboelastogram tracing. Platelet-free plasma was stored at 4 °C before usage. To perform thromboelastography suspension of MVs derived from erythrocytes of a healthy donor or a burn patient was mixed with the same plasma (of a healthy donor or of a burn patient) in the ratio of 1:1, 340 μl of the mixture were placed into the cuvette and 20 μl 0.2 M $CaCl_2$ were added. In the control group Tris-buffered saline (TBS) was used instead of the suspension of RBC-derived MVs. To perform thromboelastography the number of microvesicles was not standardized in the samples.

Antithrombin activity of RBC-derived MVs was assessed by coagulation method (U. Abildgaard) using a coagulometer [17]. A solution of heparin in an imidazole buffer (150 mmol/L imidazole, 100 mmol/L NaCl, pH 7.35) (Sigma, USA), thrombin (10 NIH/mL) (Sigma, USA), fibrinogen 0.2% (Sigma, USA), and suspension of MVs was prepared. The time from the addition of fibrinogen to fibrin clot formation was recorded at 37 °C using a coagulometer (Sticker Coagulometer BC1, Germany). In the control group TBS was used instead of MV suspension.

The effect of MVs on the time of fibrin clot formation was also studied in the absence of heparin. TBS was added instead of imidazole buffer, containing heparin. A mixture containing an MV suspension and thrombin (10 NIH/mL) (Sigma, USA) was incubated for 2 h at 25 °C. After that, a solution of fibrinogen 0.2% (Sigma, USA) was added and the time of fibrin clot formation was determined. In the control group TBS was used instead of the suspension of MVs. The time from the addition of fibrinogen to the formation of the clot was recorded at 37 °C using a coagulometer.

Antithrombin activity was also assessed by optical method. The target of action of thrombin was not fibrinogen, but chromogenic substrate selective to thrombin (3 mmol/L) (Sigma, USA). The optical density (OD) of the chromogenic substrate, which changes under the influence of thrombin, was measured using a spectrophotometer (Spekol UV VIS, Zeiss, Germany) at λ = 405 nm. In the control group TBS was used instead of MVs. Like in the coagulation method, antithrombin activity was determined in the presence of heparin 3 units/ml (Sigma, USA) and without it. At that, we were able to evaluate the presence of both heparin-dependent and heparin-independent antithrombins.

The effect of MVs on the fibrinolytic activity of donor plasma was determined using the following methods:

1. According to the euglobulin lysis time. Euglobulin was prepared from 0.05% of kaolin-treated platelet-free plasma and TBS (pH 7.4) in the ratio of 1:1, 180 μl of a 1% acetic acid and 7.5 ml of distilled water were added to the mixture. The mixture was then incubated for 30 min at 37 °C and was then centrifuged at 650 × *g* for 6 min. Euglobulin precipitate was dissolved in Tris–HCl buffer and the 0.277% solution of $CaCl_2$ was then added at a ratio of 1:1. The time till the complete dissolution of the formed clot was recorded at 37 °C using a coagulometer. In the experimental samples, instead of Tris–HCl buffer, MVs resuspended in Tris–HCl buffer were added to the plasma. The difference in euglobulin lysis time obtained with and without the presence of MVs characterized the fibrinolytic activity of MVs.
2. According to the fibrinolytic activity induced by streptokinase. Euglobulin was obtained from plasma using the same method, but without the addition of kaolin, then the euglobulin was incubated for 30 min at 4 °C and centrifuged at 650 × *g* for 6 min. Streptokinase 100 μl (330 IU/ml) (Sigma, USA) and thrombin 100 μl (50 units NIH/ml) (Sigma, USA) were added to the euglobulin solution. The time period from the addition of these components to complete clot lysis was recorded at 37 °C using a coagulometer. In the experimental samples, instead of Tris–HCl buffer, MVs resuspended in

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