IMPORTANCE OF NUMBER AND SHAPE OF PLATELETS FOR CONTACT ACTIVATION OF THE BLOOD KALLIKREIN–KININ SYSTEM *IN VITRO*

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Activation of Hageman factor (HF) on contact with artificial surfaces is a widely used model for the investigation of the blood kallikrein–kinin system (KKS). It has been shown that activation of HF systems in the micricirculation can be mediated by proteinases or functionally modified blood and endothelial cell membranes [6, 7, 9]. The role of platelets, structurally and functionally the most labile blood cells, carrying both HF itself [3, 4] on their surface and also a number of specific proteins which participate in activation of the blood clotting system, is particularly important in this respect. We know, in particular, that purified HF can be activated by platelets stimulated by ADP and collagen, and also that platelet factor 4 prevents contact activation of HF [7, 8]. The character of the activating principle is one of the key factors in the biochemical mechanism of regulation of KKS, linked functionally with clotting and fibrinolysis sytems [1].

The role of number and shape of platelets for contact activation of KKS was investigated. The main aim was an attempt to separate the plasma (biochemical) and cellular (membrane) components of activation of this system.

EXPERIMENTAL METHOD

Male Wistar rats weighing 180-300 g, kept on a standard diet, were used. To obtain the different platelet fractions, citrated blood (1:9), obtained from the right ventricle of animals anesthetized with pentabarbital, was used. The principal fraction containing platelets (1) was obtained by centrifugation of citrated blood at 1500 rpm for 10 min. This fraction contained from $5 \cdot 10^5$ to $3 \cdot 10^6$ cells in 1 µl. To obtain platelet-free plasma (2), fraction 1 was recentrifuged at 7000 rpm. This fraction contained from 0 to 25,000 cells/ml. Next, to obtain "washed" platelets the residue was washed with Tyrode solution not containing calcium or magnesium ions. Cells obtained from 2 ml of fraction 1 were resuspended in 0.5 ml of Tyrode solution. All the procedures indicated above were undertaken at room temperature, using plastic and siliconized vessels. The "washed" platelets were frozen and thawed 5 times, using dry ice. In this way up to 95% of the cells were disintegrated. The suspension of disintegrated platelets was ultracentrifuged at 100,000 g for 20 min. The supernatant fraction, into which the soluble contents of the platelets has passed, was used; the membrane fraction was resuspended in Tyrode solution. This fraction contained the disintegrated bodies of the platelets, their fragments, and fragments of membranes. The initial arginine esterase activity and the prekallikrein (PKK) level in the blood plasma were determined by the method in [2], using TAME (N-a-tosyl-L-arginine methyl ester, from Reanal, Hungary) as the substrate. The composition of the incubation mixture was changed depending on the series of the investigation, but the conditions of determination on the whole (ratio of the components, conditions of incubation, quantity of added reagents, etc.) always corresponded to the method indicated above. The shape of the nature and modified platelets, and also the character of membrane injuries were assessed on a scanning electron microscope (Hitachi, Japan) with accelerating voltage of 25 kV, using a "Polaroid" camera and materials for photographic recording. For this purpose samples were fixed for 45 min in 5% glutaraldehyde (pH 7.4) and centrifuged at 5000g for 10 min. The residue was washed with 0.1 M phosphate buffer (pH 7.4) and dehydrated in acetone of increasing concentrations. One drop of the material thus prepared was placed on a clean support and, after drying, was sprayed with gold. The degree of magnification

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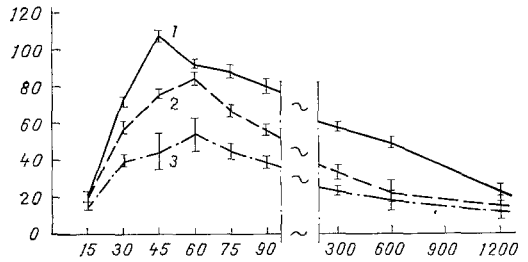

Fig. 1. Dynamics of contact activation of TAME-esterase activity in the presence of different numbers of platelets in the sample. Abscissa, incubation time (in sec); ordinate, TAME-esterase activity (in moles TAME/ml·h). 1) $4 \cdot 10^5$-$3 \cdot 10^6$ platelets, n = 9; 2) 0-25,000 platelets in 1 μl, n = 4; 3) fraction B obtained by diluting fraction 1 with platelet-free plasma ($12 \cdot 10^4$-$4 \cdot 10^5$ platelets in 1 μl, n = 9). At peak of activity for each fraction n = 15.

can be judged from the scale line drawn on each photograph. Platelets were counted in a Goryaev chamber (erythrocytes and other cells were not found in the preparations).

EXPERIMENTAL RESULTS

In the experiments of series I the kinetics of activation of kallikrein incubated with different numbers of platelets was investigated. The results (Fig. 1) show that addition of standard quantities of kaolin to the plasma containing different numbers of platelets leads to activation of PCC differing in rate and intensity. The greater the number of platelets in the incubation medium, the higher the peak of TAME-esterase activity, indicating conversion of PKK into kallikrein. The time of maximal activation for plasma with the highest platelet count was a little shorter (45 sec) than for the other fractions (60 sec). During the subsequent incubation period a gradual decline was observed in TAME-esterase activity, due to the influence of plasma kallikrein inhibitors. By the 20th minute no difference was observed between the platelet containing fractions, i.e., the number of platelets in the sample was immaterial for the effect of plasma kallikrein inhibitors.

Measurement of the initial arginine-esterase activity (before the addition of kaolin to the sample) showed virtually equal values for all three fractions: 25.9 ± 1.0 (1), 25.8 ± 1.8 (2), 25.8 ± 1.2 (3). This means that the number of platelets in the sample is a factor which facilitates but does not determine HF-dependent activation of KKS. A higher platelet concentration in the medium probably determines the larger number of HF molecules, but their activation, however, depends on the presence of a contracting surface, in the form of particles of finely dispersed kaolin.

Control experiments also showed that "washed" platelets, incubated in buffer solution, had a very low TAME-esterase activity, which was not increased by incubation with kaolin. In other words, "washed" platelets themselves did not contain enzymes determining TAME-esterase activity, or the combination of factors needed to activate PKK (HF, KK).

TABLE 1. Effect of Membrane and Supernatant Fractions of Disintegrated Platelets on TAME-Esterase Activity in Rat Blood Plasma

Parameter, μmoles TAME/ml·h	Washed intact platelets	Fraction of disintegrated		n
		supernatant	membrane	
Initial arginine esterase activity	27,4±1,7	9,9±1,2*	22,6±1,8	7
Arginine esterase activity by kaolin	80,6±3,2	47,9±2,1*	108,8±4,0*	7

Legend. *P < 0.05 compared with intact cells.

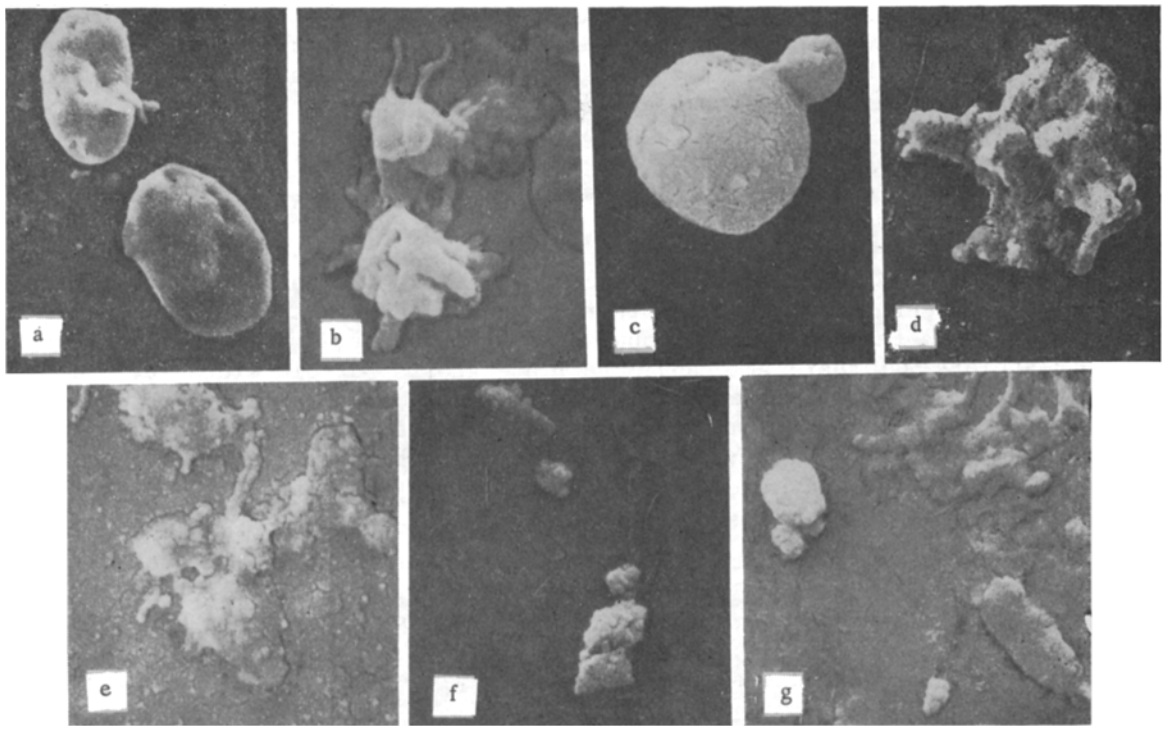

Fig. 2. Intact, activated, and disintegrated platelets in preparations studied with the scanning electron microscope: a) intact; b) washed platelets; c, d, e) platelets subjected to freezing and thawing; f, g) fragments of platelets and membrane fraction after ultracentrifugation.

In the experiments of series II the effect of the supernatant and membrane fractions obtained from disintegrated platelets on kallikrein activation was compared. The fraction of "washed" platelets obtained from fraction 1 was divided into two portions: the first was subjected to repeated freezing and thawing, the second served as the control. The number of platelets in the original fraction of "washed" platelets varied from $5 \cdot 10^5$ to $14 \cdot 10^5/\mu l$. The composition of the incubation medium was as follows: 0.05 ml of platelet-free plasma, 0.05 ml of a suspension of washed or disintegrated platelets (or supernatant fraction), 0.1 ml of kaolin suspension. The conditions of incubation were standard.

The results (Table 1) showed that after addition of washed platelets to the plasma, the level of TAME-esterase activity was identical with that for intact cells (Fig. 1, Table 1). However, after addition of the supernatant fraction of disintegrated platelets, activation of HF and PKK was 40.5% less than that found with the fraction of washed cells. Values of initial arginine esterase activity also were almost two-thirds lower in samples with the supernatant fraction, i.e., the absence of functionally intact platelets in the sample, despite the presence of soluble components of their contents, gave low values for both spontaneous and contact-induced TAME-esterase activity. The level of this activity was due only to substances added with the test plasma, and kaolin.

Addition of the membrane fraction of disintegrated platelets to the incubation medium, on the other hand, led to stronger activation of kallikrein than in samples with intact platelets (+34%). Probably the enlargement of the contact surface due to disintegrated platelet bodies, and also changes in its structure, led to increased activation of kallikrein. The level of spontaneous arginine esterase activity remained unchanged compared with the control.

The results of these experiments thus raise the question of correlation between the number, functional state, and integrity of platelets and the level of HF-dependent activation of kallikrein. Other arguments in support of this approach to the problem were obtained in morphological investigations using the scanning electron microscope. The results of morphological analysis indicate that half of the cells in original native plasma containing platelets are oval in shape, with a flat, smooth surface on which separate openings are visible. This picture is characteristic of intact, inactivated platelets. Besides these there were also partly activated platelets — during keeping the external features of the inactivated cell were characterized on the whole by the appearance of one or more pseudopodia (Fig. 2a).

An electron-microscopic study of preparations of washed platelets (Fig. 2b) showed that the procedure of frequent washing leads to marked activation of the cells, which is associated with typical morphological changes in their structure. Platelets throw out numerous pseudopodia, while the central part of the cell, its body, contracts. On the whole, the platelets change their shape from flat to spherical. Morphological changes in the cell of this kind are known to be accompanied by the release of physiologically active substances. A description of the changes undergone by platelets as a result of repeated freezing and thawing is particularly interesting (Fig. 2c, d, e). Characteristic features of preparations of this series were, first, the presence of platelets with traces of marked destruction and, second, the presence of many fragments of platelets of different shapes and sizes. One picture (Fig. 2c) shows a spherothrombocyte, another (Fig. 2c) shows a cell with a highly modified porous surface, and a third (Fig. 2e) shows a group of cells similar in shape to partially activated platelets, but there are many more flat "ghosts" of platelets (by analogy with "ghosts" of erythrocytes). In preparations obtained from fractions subjected to additional centrifugation after freezing and thawing, i.e., in preparations of the membrane fraction proper, many spherical fragments (Fig. 2f, g) and arbitrarily shaped splinters of platelets also could be found, the smallest of them not more than a fraction of a micron in size.

Platelets evidently play an important role in the regulation of activity of HF and the blood systems coupled with it. It was shown in [3-5] that HF, and also clotting factor XI are bound with the platelet membrane. Platelets stimulated by ADP and collagen stimulate clotting ability and fragmentation, as well as proteolysis of factor XI by a mechanism independent of HF [6, 7]. Intact platelets possess the same property, although weaker [7].

These findings show that the degree and rate of activation of kallikrein depend directly on the number of intact platelets. In this case it is a question of potentiation of HF-dependent contact activation of kallikrein due to addition of kaolin to the medium. Platelets themselves, whether intact or activated by repeated washing, have no direct ability to activate this system. Nevertheless, as comparison of the results of electron-microscopic and biochemical investigations shows, the shape of the platelets is important for this process. Disturbance of the integrity of the platelet membrane, separation of microspheres from its surface and, more especially, disturbance of the structure of the cell and the formation of membrane splinters lead to more marked contact activation of kallikrein. It must also be noted that the release of physiologically active substances accompanying platelet activation or the soluble fraction of disintegrated platelets does not yield factors which could increase the HF-dependent activation of kallikrein.

Model experiments described above show that the presence of disintegrated platelets and their fragments, with their ability to facilitate contact activation of HF-dependent blood systems, may play an important role in pathological processes accompanied by mass destruction of platelets. It can be tentatively suggested that direct activation of HF and, correspondingly, of the components of KKS in the microcirculation is due mainly to the effect of enzymatic factors (protease) and also to contact interaction with membranes of the vascular endothelium, platelets, and other blood cells.

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