Scanning Electron Microscopic Observations on Human Blood Platelets and Their Alterations Induced by Thrombin

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Since the introduction of transmission electron microscopy in hematology, blood platelets have frequently been the subjects of investigation using shadow cast technique or ultrathin sections on account of their unique native (circulating) form and their behavior (aggregation) and transformation during hemostasis (for review see; Johnson et al. 1961, David-Ferreira 1964, Marcus and Zucker 1965, Schulz 1968). Recent employment of glutaraldehyde in prefixation has added many new findings on the internal structure of the platelets. As for the surface or three dimensional structures, on the other hand, recently advanced scanning electron microscopy can give us more informations far more easily and directly than transmission electron microscopy has.

As far as the authors know, the blood platelets have been studied with scanning electron microscopy by two groups of investigators: Clarke and Salsbury (1967) and Inoshita et al. (1969). However, the description by the former group was not only insufficient but also inadequate probably owing to the lack of care in the treatment of the platelets; they described the platelets as irregular in outline with no characteristic appearance. The latter authors, though their method was more suitable, presented only two platelets. This paper describes the surface structure of human platelets and their alterations caused by thrombin.

Materials and Methods

Exsanguination was carefully done from the cubital vein of a healthy voluntary male with a siliconized needle (1 mm inner diameter) connected to a plastic tube 30 cm long. First several drops were discarded. For the investigation of native (circulating) platelets, a few drops of blood were directly fixed in 20 ml of fixative (Tokunaga, Fujita and Hattori, 1969) consisting of 1% glutaraldehyde in 0.1 M phosphate buffer adjusted at pH 7.4 (Sample I).

For the examination with thrombin, 27 ml of blood was collected in a siliconized tube containing 3 ml of 3.13% citrate. The mixture was centrifuged at about 100 g for 10 min at room temperature. The supernatant platelet rich plasma was incubated at 37° C for 30 min and divided into five siliconized tubes warmed at 37° C so that each tube contained 1.5 ml. Then 0.1 ml of bovine thrombin solution (10 unit per ml) was added. In this series, fibrin clots appeared about 14 sec after the addition of thrombin. Four samples were fixed at 5 sec intervals (5, 10, 15 and 20 sec after the addition of
Fig. 1. Two native platelets and three erythrocytes from whole blood of a normal individual (Sample I). Direct fixation in 1% glutaraldehyde in 0.1 M phosphate buffer (pH 7.4). The platelets are discoid and show some small elevations and depressions on the surface. ×12,000
thrombin) by adding 10 ml of a warmed fixative described above (Samples II).

The last sample was fixed without thrombin as a control (Sample III).

After 30 min fixation in the above warm fixative the experimental and control specimens were further kept overnight in the same fixative at the temperature of 4°C. The specimens were then washed twice with 0.1 M phosphate buffer, dehydrated by graded concentrations of acetone and finally dried on glass slides (for details see Tokunaga, Fujita and Hattori 1969).

The preparations were coated with carbon and gold and examined using a JSM-2 type scanning electron microscope with accelerating voltage of 25 kV.

**Results**

**Native platelets in Sample I**

In this sample platelets were only occasionally observed among other blood cells, mainly erythrocytes (Fig. 1, 2).

Although the authors observed only fifteen platelets, they were almost uniformly discoid or lentiform with a diameter of 1.8-3.0 μ (average 2.14 μ). An exceptional platelet was a sphere of 1.4 μ. The surface of the platelets was relatively smooth, but some platelets showed slight, small elevations and conic depressions on the surface. Four platelets had one or two short spiny projections at the margin (Fig. 2). No long pseudopod was encountered.

**Collected platelets in Sample III (control)**

Indicating the successive collection of the cells in this sample, abundant platelets were observed either solitarily dispersed or gathered in groups of rather small numbers (Fig. 3, 4).

Over one hundred (153) platelets were observed and measured for a quantitative analysis. About 78% of the platelets were discoid in form and 2.4-4.2 μ (2.62 ± 0.375 μ) in diameter. About 12.5% were spheroidal with a diameter of 1.6-3.0 μ (Fig. 5), and 5.5% were spherical with a diameter of 1.55-2.33 μ. The residual 4% were of irregular forms, which mainly were represented by large elliptic or sole-like plates (Fig. 6). Approximately 15% of the discoids and half of the other forms had one or sometimes several long pseudopods of about 0.16 μ in diameter (Fig. 4, 5, 6). In surveying the platelets with pseudopods it was noted that the pseudopods originated almost exclusively from the margin or its adjacent portion of the platelets (Fig. 5, 6, 7, 8), a rule which was also the case in the following samples.

In the specimen fixed, for comparison, in a more hypotonic fixative, 0.75% glutaraldehyde in 0.1 M phosphate buffer, the platelets appeared slightly swollen and the conic depressions were deeper and more distinct. (Fig. 7, 8)

**Altered platelets in Samples II**

Conspicuous changes appeared in the shape and size of the platelets following the addition of thrombin.

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*Fig. 2.* A native platelet and erythrocytes from the same sample as in Figure 1. The platelet has a small projection at the margin. ×12,000
Fig. 3. and 4. Platelets from platelet-rich plasma (Sample III: control). Most platelets are discoid and are not yet thickened. In Figure 4 some platelets have fine, long pseudopods at the margin. Fig. 3: $\times 2,400$, Fig. 4: $\times 12,000$
1. Five sec after the addition of thrombin

Although a small number of platelets preserved their discoid form, most platelets were thickened to various degrees into spheroids or spheres (Fig. 9, 10, 11). These showed a clear tendency to cohere forming smaller and larger aggregates.

Small lumpy cytoplasmic protrusions covered the surface of these cells (Fig. 10, 11, 12). These protrusions were generally hemispherical and seldom originated from the marginal region of the platelets. Thus they appeared to be quite different from the fine long pseudopods recognized in the margin of the cells. The latter were observed in some of the thickened as well as the discoid platelets in this sample. There was a tendency for the lumpy protrusions to be large (0.18–0.38 μ thick at the base and up to 0.3 μ high) in the spherically transformed platelets, and small and sparse in the less thickened platelets. Elevations of this category, though more inconspicuous, further occurred in about half of the discoid platelets in this specimen. This type of platelet may represent the first stage of transformation by thrombin (arrows in Fig. 10 and 11). There appeared to be no marked increase in the number of pseudopods.
2. Ten sec after the addition of thrombin

Surface alterations and transformation of the platelets were more prominent than in the previous sample (Fig. 13, 14). Most platelets were spherical as a whole, and it was difficult to find discoid forms. The diameters ranged from 1.00–2.60 µ (1.60 ± 0.261 µ), apparently smaller than those of the discoid platelets in the control. The round protrusions became so large (0.30–0.45 µ thick in the base) that the surface of the platelets was almost completely occupied by the protrusions. The aggregates often consisted of fairly numerous platelets.

Fig. 9. Platelets 5 sec after the addition of thrombin (Sample II–1). Most platelets are thickened and have hemispherical protrusions on their surface. Note that some platelets have retained discoid form, and that the platelets have begun to form small and loose aggregates. ×8,500

Fig. 10. Platelet 5 sec after the addition of thrombin (Sample II–1). Note that many platelets with hemispherical protrusions have retained the discoid form (arrows). ×8,500
Fig. 11. Transformed platelets 5 sec after the addition of thrombin (Sample II-1). Platelets are thickened to spheroids or spheres and many lumpy protrusions have appeared on the surface. Arrows indicate discoid platelets with the protrusions. ×12,000

Fig. 12. A higher magnification of Figure 11. Note many lumpy (hemispherical or irregular) protrusions on the surface of the spherically transformed platelet. ×22,000
Fig. 13. Aggregated platelets 10 sec after the addition of thrombin (Sample II-2). \( \times 6,000 \)

Fig. 14. Several platelets 10 sec after the addition of thrombin. Platelets are spherical as a whole and their protrusions are large. \( \times 18,000 \)
3. *Fifteen sec after the addition of thrombin*

The most striking feature in this sample was the appearance of a network of fibrin strands with a thickness of about 0.1 μ (Fig. 15, 16). At some places many strands formed thick fibers by cohering each other side by side (Fig. 17). Although no strict relationship could be clarified between the fibrin strands and the platelet pseudopods or protrusions, the platelets were apparently arrested in the knobs of the fibrin network. The shape of the platelets seemed to correspond to those in the preceding case.

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**Fig. 15.** Fibrin net and platelets 15 sec after the addition of thrombin (Sample II-3). Fine fibrin strands form a net work with transformed platelets. An erythrocyte is seen in the center. ×3,600

**Fig. 16.** A higher magnification of Figure 15. The platelets appear to be the knobs of fibrin net, but no strict relationship is found between them. ×12,000

**Fig. 17.** A thick fibrin fiber in Sample II-3. Fine fibrin strands cohered side by side forming a thick fiber. ×6,000

**Fig. 18.** Irregularly transformed and aggregated platelets 20 sec after the addition of thrombin (Sample II-4). ×3,600
Fig. 19. Irregularly transformed platelets 20 sec after the addition of thrombin (Sample II-4). ×12,000

Fig. 20. A solitary platelet in Sample II-4 (20 sec after the addition of thrombin). Enlarged cytoplasmic protrusions covered entire surface of the platelet. Several pseudopods have issued from the equatorial region. ×30,000
4. Twenty sec after the addition of thrombin

The platelets were gathered more firmly than in the previous sample (Fig. 18). In the aggregates mingled with the fibrin net it was often difficult to determine the strict configuration of each platelet (Fig. 18, 19). Observation of a few solitary platelets showed, however, that the platelets at this stage were rather irregular in shape and had more pseudopods than in the previous samples (Fig. 20).

Discussion

Native form of the platelets

For many years (Bizzozero, 1882), reporters have described that native or circulating blood platelets are discoid or lentiform in human beings as well as in several other animals (see review by Schulz, 1968). On the basis of either transmission electron microscopic observations or light microscopic findings, they only suggested a generalized shape. Owing to methods inadequate for such a purpose, they could not determine the precise shape of each platelet nor the frequency of the discoid and other atypical forms. As far as the authors know, the only study treating this problem was done by Inoshita and his coworkers (personal communications from Dr. Inoshita 1969). They reconstructed ten human platelets using serial ultrathin sections and found, by their method of fixation, that seven platelets were discoid and three were spherical. They considered the spherical form as the truly existing native form (Inoshita et al., 1969), a result confirmed in the present study.

The method of fixation employed in Sample I may be considered one of the best ones for the purpose of observing human blood cells in circulating state, since in vivo fixation is not adoptable. Strictly considered, the occurrence of possible alterations in the shape while running through the plastic tube and dropping from it into the fixative, and thereafter in the fixative can not be excluded. The fixative used in the present study is nearly isotonic and fixes the red blood cells in a most natural form (Tokunaga, Fujita and Hattori, 1969). Speculating on the basis of the reports of White (1968 a) and Barnhart and Riddle (1967), this fixative probably fixed the surface layer of the platelets in a few seconds so that the platelets did not transform later.

The results in Sample I, though the platelets observed were few in number, was in good accordance with the previous studies in ascertaining the discoid form as a general type. This form was more frequently seen than in the study by Inoshita (personal communication 1969).

The spiny, short projections shown in this study have been also observed in shadow-casted resting platelets of dogs by Barnhart and Riddle (1967). They reported that 38 % of in vivo fixed dog platelets and 20–40 % of canine and human platelets carefully collected and fixed in vitro had short projections. They supposed that the dendritic form, as they called this type of platelet, really existed in the circulating blood.

Some platelets had one or a few conic depressions on the surface of one side. The finding that the platelets, when fixed in a more hypotonic fixative, were slightly swollen and these depressions became prominent suggests that these depressions are related to certain consistent structures. It may be implied that they correspond to the openings of the endoplasmic reticulum found in the observation on sections (Behnke

Collected platelets

In the collected platelets as a control of the subsequent samples, a few signs of viscous metamorphosis i.e. pseudopod formation and spherification were apparent. These changes were thought to be produced during the procedures of separation, handling and incubation of the platelet rich plasma as a sign of minimal activation of the platelets though the authors intended to avoid it.

As for the pseudopod formation, many investigators have considered this change as one of the predominant changes in an early stage of thrombus formation or clotting (SCHULZ, 1968). The fine and long pseudopods were presumed to be the elongated spiny projections in Sample I. The original site of the pseudopods seems to have never been definitely described. The present study revealed that it corresponded to the margin of the discoid platelets. This is the same in sole like platelets (Fig. 6) and the platelets activated by thrombin (Fig. 20).

Studies using thin sections have demonstrated that the pseudopods often had a bundle of several microtubules running longitudinally (BEHNKE, 1966; WHITE and KRIVIT, 1967 a; SCHULZ, 1968; WHITE, 1968 a). These microtubules are thought to have been seperated from the marginal bundle of microtubules in unaltered, discoid platelets (RODMAN and PAINTER, 1967; WHITE and KRIVIT, 1967 a). In considering the circular arrangement of the original bundle of microtubules near the margin, and that some of the microtubules are issued to form cores of the pseudopods, it may be readily acceptable that the pseudopods grow at the margin or marginal portion.

Alterations by thrombin

Thrombin with appropriate divalent cations is known to convert fibrinogen to fibrin, to aggregate the platelets as well as to cause morphological changes such as pseudopod formation, swelling and degranulation, and the release of ADP and other adenosine nucleotides (KAESER-GLANZMANN and LUESCHER, 1962), serotonin (GRETTE, 1962; ZUCKER, 1967), and other substances (RODMAN, MASON and BRINKHOU S 1963). A divalent cation is not essential for thrombin-fibrinogen reaction, but essential, as a cofactor, for the induction of aggregation (SHERMER et al. 1960 and 1961; ROBINSON, MASON and WAGNER 1963). The ADP released from platelets by thrombin also causes their aggregation (GAARDER et al., 1961; BORN, 1962; BORN and CROSS, 1963; MAC-MILLAN, 1966). In citrated platelet rich plasma, Ca++ is supposed to be still available to cause platelet aggregation as well as the other changes with thrombin (Hovig, 1962; BARNHART and RIDDLE, 1967).

The results of Samples II can be summarized as follows. After the addition of thrombin the cytoplasmic protrusions began to appear, as the earliest change, on any portion of the discoid platelets except the marginal region. As the second change, the platelets became spheroid or spherical as a whole. The protrusions grew and the platelets formed aggregates. These changes were followed by the appearance of a fibrin network and irregular transformation of the platelets with more pseudopods.

The small elevations, which became large hemispherical or irregular protrusions in later stage, are believed to be the first change caused by thrombin and seem to have not been definitely described as yet. Though the physiological implication of
these structures is not known, they might be related to the so-called release reactions of the platelets (Grette, 1962). In an ultrastructural study on the effects of thrombin and ADP on the platelets using thin sections (Hattori, Ito and Matsuoka; in preparation) the authors noted the contour change corresponding to the elevations in this paper in the thrombin-activated platelets, but infrequently in the ADP-activated platelets. Besides this difference, thrombin-activated platelets seemed to differ from the ADP-activated platelets in the arrangement of the microtubules, the frequency of cores in the microtubules and of intragranular tubules as well as the destruction of the granules.

The shape change from discoid to sphere is generally associated with the aggregation of platelets with a few exceptional cases (Mitchell and Sharp, 1964; Zucker and Borrelli, 1964; Bull and Zucker, 1965; Behnke, 1967; White, 1968b; White and Krivit, 1967b). The spherification is now explained by the disarrangement or disappearance of the marginal bundle of microtubules which is considered to support the discoid shape as a cytoskeleton (Haydon and Taylor, 1965; Behnke, 1965; Bessis and Breton-Gorius, 1965; Sixma and Molenaar, 1966; Behnke, 1967). Bull and Zucker (1965) studied the volume change in human platelets induced by various fibrinolytic substances including thrombin. They found that the shape change was accompanied by an increase in the volume of at least 15% under all circumstances tested. In the case of thrombin the increase was 19% (average). It is interesting to note that the spherical platelets in Sample II-2 had diameters less than those of the discoid platelets in the control, though these spherical platelets had probably increased in volume.

The relationship between fibrin strands and the platelets or pseudopods remains to be investigated with reference to clot retraction (White, Krivit and Vernier, 1965; White, Silver and Krivit, 1966).

Scanning electron microscopy has given and will give us new information on the surface fine structures and their changes in the platelets. Further examination and discussions on both the native forms and the effects of thrombin compared with those of ADP and other agents are being prepared on more materials.

Summary

The surface structure of human blood platelets and their alterations by thrombin were studied by scanning electron microscopy.

The majority of the native platelets were discoid or lentiform as already reported by previous authors using the transmission electron microscope. The surface was relatively smooth, but some platelets had a few slight elevations and conic depressions which may correspond to the openings of the endoplasmic reticulum. A few platelets possessed at the margin one or two spiny short projections.

The platelets collected by careful centrifugation partly showed a few long pseudopods which were issued from the margin of the platelet and supposed to be the elongation of the spiny projections described above. About 78% of the collected platelets were discoid in form, 12.5% spheroidal, 5.5% spherical and 4% irregular.

The addition of thrombin to the citrated, platelet-rich plasma caused the following changes. At first small protrusions appeared on the entire surface of the platelets
except for their margin. The discoid platelets then transformed into spheres covered by the grown and rounded protrusions and aggregated, while the formation of pseudopods proceeded. Fibrin strands about 0.1 μ in thickness appeared to form networks into which the aggregated platelets were arrested more and more firmly.

ヒト血小板とそのトロンピンによる変化の走査電子顕微鏡による観察（内容自抄）

ヒト血小板の表面構造と、トロンピンによって起こったその変化を走査電子顕微鏡を用いてしらべた。

すでに透過電子顕微鏡によって報告されているように、未操作（全血中）の血小板の大半は類円板形または凸レンズ形であった。その表面は比較的滑らかであるが、僅かな隆起や亀裂を持つものがあった。この亀裂は小柱体の開口部に相応するかもしれない。少数の血小板は辺縁部に一列の短い突起を持っていった。

注意ぶかい血板によって集められた血小板は、一部辺縁部に上述の短突起が延長したものをと思われる二三本の細長い偽足を持っていた。この集められた血小板の約78%は類円板形で、12.5%は類球形、5.5%は球形、4%は不整形であった。

クエン酸加 血小板多血漿にトロンピンを加えると、次の変化が起こった。まず辺縁部を除き 血小板の全表面に小さな膨隆が出現した。次いで類円板形の血小板が球形化するとともに膨隆は大きく丸くなり、血小板は凝集し、一方偽足形成が進んだ。約0.1μの太さのフィブリン糸が析出して網目状になり、血小板凝集塊はさらに強固になった。

References


