Freeze-Etching Studies of Human Platelets

By John C. Hoak

The freeze-etching technique was used to study morphologic features of normal human platelets. A well-developed canalicular system was demonstrated within the platelet, with communication to the plasma surface of the platelets and with direct contact of the canalculi with organelles. Cross-section fractures of granules revealed compartmentalization in some while others appeared homogeneous. Several types of filamentous structures were observed within the matrix of the platelet. Microtubular subfilaments were approximately 80 Å in diameter. Microfilaments were observed in other areas of platelets and had a diameter of 50–70 Å, a size consistent with that described for actinoid filaments. The interior of the platelet membrane contained 85-Å particles, while the membrane surface had a more granular appearance. Earlier morphologic descriptions of platelets prepared by fixation methods have been confirmed and expanded with this technique.

FREEZE-ETCHING demonstrates promise as a valuable technique for the ultrastructural study of cells and subcellular components. When frozen specimens are cleaved, new surfaces originating from the interior of the specimen are exposed. The newly exposed surfaces are replicated by platinum-carbon shadowing under high vacuum. This technique avoids the artifacts of thin-section preparation induced by fixation, dehydration, embedding, and the use of heavy metal stains. In addition, it permits demonstration of areas of cellular structure in three-dimensional relief at the resolution of the transmission electron microscope (5–10 Å), which is considerably greater than that of the scanning electron microscopes (100 Å) that are available commercially.

Interpretation of membrane ultrastructure with the freeze-etching technique has been hampered because the actual plane of cleavage in membranes by the procedure had not been resolved. In studies with lipid bilayers, Deamer and Branton concluded that the freeze-fracture process actually splits membranes along a plane within the membrane itself, rather than cleaving along either its outer or cytoplasmic surface. In studies using ferritin or F-actin as membrane-surface markers prior to the freeze-etching of red blood cell ghosts, the markers were never observed on the fracture face. These results suggest that fracture alone does not show membrane surface detail. In contrast, sub-
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limation of the surrounding ice as a result of the etching procedure exposed the membrane-surface markers. These results suggest that etching is necessary to reveal the membrane surface of the cell.

In the present study, the freeze-etching technique was used to study the morphologic features of normal human platelets. With its application, we have confirmed many of the observations made earlier using thin-section and negative staining techniques. More important, new and more definitive information has been obtained concerning the morphologic features of the canalicular system, filamentous structures, and the membrane surface of the platelet.

MATERIALS AND METHODS

Blood from normal, human donors was drawn into Formula A-ACD solution in siliconized tubes (1 ml ACD solution and 10 ml of blood). Platelet-rich plasma was prepared by centrifugation of the blood at 300 g for 15 min at 20°C. Platelet pellets were prepared by centrifugation of the platelet-rich plasma at 750 g for 8 min at 20°C. In two studies with adenosine diphosphate, 5 ml of platelet-rich plasma were incubated with 0.05 ml of 0.1 mM for 5 min, and the platelet pellet was prepared as before.

Samples of platelet pellets were added to apposed specimen holders containing 15% glycerine and were frozen rapidly in Freon 22. The samples were then transferred to the Denton DFE-3 freeze-etch apparatus. Under vacuum, the specimen was cooled to −195°C with liquid nitrogen and then was fractured. The fractured specimens were then etched. Etching consisted of warming the specimens to −100°C for 1 min to permit vacuum sublimation of the surface ice. The etched specimen was cooled to −195°C, and the replica was prepared by shadowing with evaporated carbon and platinum. The frozen specimen was then thawed and separated from the replica. The replicas were cleaned in 4% sodium hypochlorite solution, rinsed in distilled water, and then were picked up on Formvar-coated grids. All specimens were examined in a Philips EM-300 electron microscope. All micrographs are mounted with shadow direction from bottom to top. Shadows are white.

Samples of platelets for thin sectioning were fixed in freshly prepared formalin for 24 hr at room temperature, washed in phosphate buffer, postfixed with osmium tetroxide, dehydrated in graded alcohol solutions and propylene oxide, and embedded in Epon-Araldite. Sections were cut on a Reichert Ultramicrotome, stained with uranyl acetate and lead acetate, and then examined in a Philips EM-300 electron microscope.

RESULTS

An example of a freeze-etch preparation of a normal platelet is shown in Fig. 1. An example of a thin-section preparation of a normal human platelet is shown in Fig. 2. In Fig. 1, a portion of the platelet membrane is shown that is irregular in contour and appears to be covered with particles. Vesicles and canalicular structures had a surface that appeared similar to that of the platelet membrane. Mitochondria appeared irregular, and typical cristae mitochondriales were not seen as clearly as in thin sections. Granules, in many instances, had the appearance of two sections or compartments. Difference in the density of parts of a single granule was a common finding when thin sections of platelets were examined.

The freeze-etch technique has been particularly useful in demonstrating the canalicular system of the platelet. Suggestive evidence for this system has been provided by examination of thin sections (Fig. 3), but more conclusive support can be seen in the freeze-etch preparation (Fig. 4A). The presence of 85-Å
particles on the membrane was also a feature of the topography of the canaliculus. These findings suggest that the plane of cleavage was within the platelet membrane. This etched preparation also demonstrated an area of surface membrane (Fig. 4B) that illustrates the particulate nature of the inner platelet membrane while the membrane surface was more smooth.

In the present study, we also confirmed the presence of filamentous structures within the platelet. Microfilaments were found within the more central areas of the platelet. An example is shown in Fig. 5A, B. The microfilaments had a mean diameter of 60 Å with a range of 50–70 Å. The most prominent of the filamentous system of the platelet is the circumferential band of microtubules. The freeze-etch technique demonstrated microtubular elements best in platelets that had been treated with adenosine diphosphate. An example is shown in Fig. 6. Microtubular structures, however, were also seen in untreated platelets. The subfilaments of microtubules resembled twisted ropes, and a space existed between the subfilaments. Each half of the subfilament had a diameter of approximately 80 Å.
DISCUSSION

The freeze-etching technique has been useful with the study of platelets in confirming and extending our knowledge of their morphologic features. In particular, it has provided considerable support for the existence of the canalicular form of the surface-connected membrane system. This system may operate in the transportation of contents both intracellularly and to the external environment. Similar observations have been made by Behnke in a limited report using the freeze-etching method. White had provided evidence earlier that the open canalicular system serves as the secretory pathway of the platelet.

Granules are the most numerous of the formed organelles in the platelet. Two zones of differing electron opacity are evident in the coarse matrix of the granule when fixed sections are studied with electron microscopy. The
basis for compartments is unknown, but it suggests that chemical constituents of the granules might be physically segregated. As with thin sections, not all granules exhibited compartmentalization in our studies using freeze-etching. These results suggest the possibility of different populations of platelet granules. In our studies with freeze-etching to date, we have not delineated characteristic features of the serotonin-containing dense bodies.

Several types of filamentous structures are present in the matrix of the platelet. Microfilaments constitute a system of fibers in the platelet sol-gel zone.\(^8\)\(^9\) They are so concentrated in unaltered platelets that they cannot be resolved in thin sections. They can be identified readily in whole mounts of spread platelets and in thin sections of platelet pseudopods.\(^10\)\(^-\)\(^11\) The freeze-etch technique permitted us to visualize microfilaments in normal platelets. These filaments were 60 Å in diameter with a range of 50–70 Å. This size is consistent with that found for actinoid filaments by Behnke et al.\(^12\) Zucker-Franklin et al. found platelet filaments of 50 Å in diameter that resembled actinlike filaments found in other cells.\(^10\)\(^-\)\(^11\)

The most prominent of the filamentous systems of the platelet is the circumferential band of microtubules. Considerable information concerning the substructural characteristics of the microtubules has also been provided by the work of White, who used negative staining of whole-mount platelet preparations.\(^13\) Each tubule is composed of 12–15 subfilaments in parallel association. The subfilaments resemble twisted ropes composed of globular subunits. In our freeze-etch studies, microtubular subfilaments were visualized best in
Fig. 4. (A). Platinum-carbon replica of a freeze-cleaved and etched normal platelet that shows a platelet canaliculus with its opening (1) to surface of platelet. CAN, canaliculus. Note 85-Å particles found on surface of canaliculus and adjoining portion of inner surface membrane. × 95,760. Fig. (B). Portion of replica shown in 4A at higher magnification. Cleaved convex face of platelet is covered with 85-Å particles and represents a part of inner membrane (IM). Sublimation of ice from surface of cleaved specimen during etching exposed a smooth face that is demarcated from the face containing the particles and is thought to represent the surface membrane (SM). × 159,070.
Fig. 5. (A). Platinum-carbon replica of part of a freeze-etched normal human platelet that demonstrates microfilaments (MF), (MICROFIL) that were 60 Å in diameter, × 191,500. Mitochondria (M) are also seen, × 59,850, (B). Portion of replica shown in 5A at higher magnification. Note microfilaments.
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Fig. 6. Platinum-carbon replica of part of freeze-etched platelet that had been treated with ADP prior to freezing. Microtubule subfilaments are shown adjacent to portion of platelet membrane (MEM). Each half of subfilament had diameter of approximately 80 Å. × 209,475.

platelets that had been treated with ADP. Some subfilaments were also seen in untreated normal platelets. The ropelike subfilaments formed longitudinal structures that occurred in pairs and appeared to be separated partially by a narrow space. The subfilament had a diameter of approximately 80 Å, which is similar to that described for thrombosethin.
Information concerning the features of the platelet surface membrane as determined with the freeze-etch method is neither detailed nor complete. Ruska and Schulz described 80–120-Å particles on the surface of the platelet membrane that stood up as filaments at the internal surface.5 Our findings would suggest that etching revealed a surface that was less particulate than that revealed by fracture cleavage and would be in accord with findings obtained by others with red cell membranes.2,3 Mason and Reddick observed that the external surface of the plasma membrane of normal human platelets in citrated plasma had a finely granular appearance.14 Further clarification of the nature of the platelet surface membrane is indicated. The application of surface markers prior to freeze-fracture and freeze-etch studies may provide useful information in the study of normal and abnormal platelets. Such studies are currently in progress.

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REFERENCES

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