The Ultrastructural Localization and Release of Platelet Lipids

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Human platelets contain rich stores of a lipid substance which can markedly accelerate the clotting of blood. After initiation of intrinsic coagulation, the platelet lipid substance is made available for association with activated plasma protein clotting factors. The complex of clotting proteins and platelet phosphatides, in the presence of calcium and accelerating factors, effectuates the conversion of prothrombin to thrombin. Although other blood cells on extraction yield phospholipids which can replace platelets in in vitro clotting systems, only platelets appear capable of making their phospholipids available under the stimulus of physiologic blood coagulation.

Under normal conditions the platelets circulate with their active lipid substance unavailable for participation in clotting. This is due in part to the fact that the platelet phospholipids are bound into the cell as lipoprotein complexes. Disruption of platelets by ultrasound, freezing and thawing, repeated washing and centrifugation, liberate the lipid activity of the cells. Incubation with endotoxin will also release lipid activity, and exposure to kaolin will cause platelet adhesion to the particles and render active lipid substance available to clotting. This latter finding is of physiologic importance because it uncovers active platelet lipid without destroying the cells. The stimulus causing availability of platelet substance during intrinsic coagulation also appears to be associated with minimal cell damage. Electron micrographic studies have shown that the platelet membranes remain intact well beyond the time their phospholipids have been made available to the coagulation process.

Therefore, despite extensive knowledge of the chemistry of platelet clotting phospholipids, and the mechanism of their participation in coagulation, the localization of the active lipid substance in platelets and the mechanism of its availability to coagulation are not entirely clear. The present ultrastructural study will demonstrate that at an early stage of clot development...
in native platelet rich plasma, platelet granules are transformed into particles with the configuration of lipid micelles. The platelet micelles are ejected through the cell membranes into the surrounding plasma prior to cell aggregation. The structural similarity of the miceller platelet organelles to micelles of phospholipids capable of replacing platelets in in vitro coagulation systems, and their release from platelets in early clot development, suggest that the platelet micelles may be the active form of phospholipid contributed by platelets to coagulation.

**MATERIALS AND METHODS**

*Preparation of Platelet Rich Plasma*

Blood was obtained from donors used repeatedly in our laboratory for controls in platelet coagulation studies. Venisection was performed with cooled, silicone-coated syringes and needles. The blood obtained was immediately transferred to silicone-coated test tubes in an ice water bath. No anticoagulant was used. The samples were transferred to a Servall refrigerated centrifuge (2 C.), and platelet rich plasma was obtained by centrifugation at 800 r.p.m. for 15 minutes. After sedimentation, the plasma was immediately transferred to fresh, cooled, silicone-coated tubes, and returned to the centrifuge (2 C.) at 2400 r.p.m. for 30 minutes. Supernatant plasma was discarded, and the platelet buttons were transferred to a refrigerated water bath at 2 C. Fifteen separate samples of whole blood were processed in this manner.

*Fixation*

The platelet buttons were gently suspended in 5 ml. of cold 6.5 per cent gluteraldehyde, buffered to pH 7.3 with 0.05 M cacodylate buffer.10 Fixation was continued in the refrigerated water bath for 2 hours. At the end of this time the cells were sedimented in the refrigerated centrifuge, washed three times in .08 M cacodylate buffer containing .18 M sucrose, and returned to the refrigerated water bath. At this time 5 ml. of cold, veronal buffered 1 per cent osmic acid, pH 7.3, was added to the gluteraldehyde fixed platelets. The second fixation was continued for 1½ hours. The cells were then washed twice in veronal buffer, dehydrated in increasing concentrations of acetone and embedded in vestopal. Thin sections were obtained from the plastic embedded platelets with an LKB ultramicrotome. The sections were examined directly, or after poststaining with uranyl acetate, lead citrate, or both. The RCA EMU 3-D and the Phillips 200 electron microscopes were used to study the sectioned platelets.

*Control Preparations*

Additional samples of platelets were prepared in an identical manner to the samples of native platelet plasma, except for the addition of anticoagulant. A 2 per cent solution of the disodium salt of ethylenediaminetetraacetic acid (EDTA), and a 3.8 per cent solution of sodium citrate were mixed with whole blood in a ratio of 9 parts blood to 1 part anticoagulant. Twenty-two samples of whole blood were prepared with EDTA, and one with citrate.

*Extraction of Platelet Lipids*

The procedure described by Marcus et al.4 was followed closely in the preparation of a crude extract of platelet lipids. Platelets were harvested from 1000 ml. of whole blood collected in A.C.D. solution (120 ml. per 480 ml. of blood). After washing twice in buffered saline, the sedimented platelets were placed in a Waring blender bubbled with nitrogen. The blender was placed inside a clear plastic hood in which a constant atmosphere of nitrogen was maintained. All subsequent procedures were carried out in
this nitrogen atmosphere. Following homogenization, 100 ml. of chloroform methanol 2:1, previously bubbled with nitrogen, was added to the minced cells. The extraction mixture was thoroughly agitated, then transferred to a medium sintered glass filter joined to an Erlenmeyer flask. A constant suction was applied to the side arm of the flask, and the whole apparatus was maintained in the nitrogen atmosphere. After initial filtration the homogenized cells on the surface of the glass filter were resuspended in chloroform methanol. Reextraction was completed after 5 minutes incubation. The filtrate was passed through fine filter paper (S and S No. 589), and evaporated by constant suction. The faint yellow precipitate present after completion of evaporation was estimated to be less than 4 mg. It was vigorously dispersed in 40 ml. of 0.45 M phosphate buffered saline, pH 7.4, on the basis of 10 ml./mg. of lipid. Aliquots of this suspension were placed in siliconized test tubes (0-2 C.). The samples were spun at 2500 r.p.m. for 20 minutes at 2 C. Supernatant saline was discarded and 5 ml. of a cold fixative (1 per cent osmic acid or 6.5 per cent gluteraldehyde) was added to the sedimented lipid particles. Incubation in fixatives, postfixation, dehydration and embedding were carried out as described for platelet preparations. A sample of the residue of chloroform methanol extracted platelets was also prepared for electron microscopic examination.

Preparation of Derived Lipids

Cephalin extracted from human brain was dispersed in 0.85 M phosphate buffered saline (pH 7.4) as suggested for use in the one stage prothrombin test.44 0.5 Cm. portions of the lipid were suspended in 20, 50, and 100 mls. of buffered saline. After settling of large particles the supernates were transferred to fresh siliconized tubes, sedimented in the refrigerated centrifuge, and processed for electron microscopy as described above.

RESULTS

The ultrastructure of normal human platelets fixed in osmic acid and prepared for examination in the electron microscope has been established in previous investigations (Fig. 1). Osmophilic, oval-shaped granules (G.) are the outstanding internal structures of the cell. A few vacuoles (V.) and mitochondria (Mi) are also observed in the platelet substance, and the surface membrane of the cell is a typical unit membrane (C. M.).

Platelets prepared by dual fixation (gluteraldehyde first, then osmic acid) are similar in appearance to cells fixed in osmic acid alone (Fig. 2). The initial fixation in gluteraldehyde, however, reduces the avidity of the platelets for osmium. As a result, the electron density is decreased. The granules are distinguished by their compact, filamentous internal structure and unit membranes, rather than the electron opacity induced by osmic acid staining. Vacuoles and mitochondria are visible, but the unit membranes of the cell and its organelles are less apparent. Poststaining of thin sections of cells with uranyl acetate, lead citrate, or both compensates for the decreased electron density.

Preparation of platelet samples from native platelet rich plasma was originally undertaken for purposes of ultrastructural histochemistry. Preparation of derived lipids was selected in order to avoid trauma to the cells during separation, and to prevent possible toxic effects of anticoagulants on cellular enzyme systems. The rate of clot development was inhibited by the use of careful venisection, siliconized surfaces, and maintenance at cold temperatures (2 C.). The initial fixation with gluteraldehyde followed by postfixation in osmic acid gave excellent structural preservation.
Fig. 1.—Osmic acid fixed human platelets. Osmophilic granules (G.) are the predominate intracellular structures. A few mitochondria (Mi.), vacuoles (V.), tubules and vesicles and glycogen particles are also present in the platelet hyaloplasm (Hy.). A unit membrane surrounds the cell (C.M.), and each of the internal organelles. Fix.—O.A., Post Stain—U.A. and L.C. Mag. ×56,100.

In addition to the usual morphologic features of the osmic acid-fixed platelet, intracellular and extracellular structures were noted in platelets of native platelet rich plasma prepared by dual fixation, which had not been apparent in cells fixed in osmium alone (Fig. 3). Thick-walled vacuoles were present in the platelet hyaloplasm, and in the plasma outside the cells. The structure of these platelet organelles was unique (Fig. 4). Their walls were composed of multiple thin layers, closely applied to one another, and arranged in a concentric manner. The periodic interval of the dark layers was 39–41 Å.

Examination of multiple samples of native plasma indicated that the micelles developed by transformation of platelet granules (Figs. 5–7A). The granules in their unchanged state have a complex internal matrix surrounded by a unit membrane. In the native platelets one to several complex granules could be found in various stages of transformation to micelles (Figs. 5A, 5B). The
Fig. 2.—Platelet initially fixed in gluteraldehyde and postfixed in osmic acid. Intracellular structures are less electron dense than when cells are fixed in osmic acid alone. The granules appear to be composed of compacted filaments rather than discrete particles. Unit membranes of the cell and its organelles are evident, but have a diminished electron density. The hyaloplasm appears more compact. Aside from the staining differences the appearance of platelets prepared by dual fixation is practically identical to cells fixed in osmium alone. In. Fix.—Glut., Post Fix.—O.A., Post Stain—U.A. and L.C. Mag. ×90,700.

Initial indication of granule matrix alteration is the appearance of concentric electron dense lines just inside the granule unit membrane (Figs. 5A and 6A). More advanced stages find the entire periphery of the granule converted to concentric electron dense layers, while the central portion of the granule usually dissolves (Figs. 6B, 6C). Occasionally a granule appears to develop into a mass of lipid layers without dissolution of the central area (Fig. 6D). The mature micelles may remain individual, or coalesce with the micelles from other granules (Fig. 7A).

Release of the micelle particles through the cell membranes of platelets was observed in the same samples of native platelet rich plasma in which the
Fig. 3.—Native platelet rich plasma initially fixed in gluteraldehyde. This cell contains structures which were not recognized in platelets fixed in osmic acid alone. Thick-walled vacuoles (M.) are present inside the cell and in the surrounding plasma. The laminated vacuole (M.) at the top of the picture is in the process of transformation to a lipid micelle, the one below it is fully formed, and the micelle outside has been ejected from a platelet. Note the periodicity of the granule labelled with a star (*). In. Fix.—Glut., Post Fix.—O.A., Post Stain—L.C. Mag. ×62,500.
Fig. 4.—A thick-walled vacuole inside a platelet at high magnification. The wall of the vacuole is composed of multiple concentrically arranged layers with a periodic interval of 39 Å. This is the characteristic ultrastructural appearance of the molecular arrangement assumed by lipids in response to dispersion in an aqueous media, and is called a micelle. In. Fix.—Glut., Post Fix.—O.A., Post Stain—U.A. and L.C. Mag. ×504,600.
Fig. 5A.—An early stage in the transformation of a platelet granule into a lipid micelle. Lipid layers are beginning to form inside the unit membrane of the granule. In. Fix.—Glut., Post Fix.—O.A., Post Stain—U.A. Mag. ×78,500.

Fig. 5B.—A platelet in which seven different platelet granules can be observed transforming to micelles simultaneously. In. Fix.—Glut., Post Fix.—O.A., Post Stain—U.A. and L.C. Mag. ×58,500.
Fig. 6.—The ultrastructure of platelet granules during transformation to lipid micelles. In. Fix.—Glut., Post Fix.—O.A., Post Stain—U.A. and L.C.

Fig. 6A.—An early stage in the transformation of a platelet granule. Four short layers of the osmophilic lipid have been laid down just inside the granule membrane. The rest of the matrix of this granule, and that of adjacent granules, has not been transformed. Mag. x132,000.

Platelet granules were found in various stages of transformation (Figs. 7–8). The micelle in traversing the platelet membrane appears to push a portion of the cell wall ahead of it, incorporating this into its substance (Figs. 7A, 7B). The defect in the surface membrane appears to seal after the micelle has been extruded (Figs. 8A, 8B). Micelle formation and release were not observed in control samples.

The structure of platelet micelles formed de novo was compared with micelles formed by extracted platelet lipids and brain cephalin prepared as a platelet substitute. Extracted platelet lipids formed a colloidal suspension when dispersed in aqueous media. The particles of this colloid were similar in size to the platelet micelles (an average diameter of 1μ versus 0.3 μ for platelet micelles formed in cells). At high magnification the similarity of the lipid layering of the platelet micelle (Fig. 9A) and the micelles formed by extracted platelet lipid (Fig. 9B) was evident. The particles formed by aqueous dispersion of cephalin (Fig. 10B) were also similar to the platelet micelles (Fig. 10A). The periodic interval of the dense layers of extracted platelet lipid micelles and the micelles formed by brain cephalin was 35–45 Å.
This period is essentially the same as the 39–41 Å interval of platelet micelles formed de novo.

**DISCUSSION**

The active lipid substance of platelets is present in these cells in a hidden form. Injury to the platelet or initiation of the clotting mechanism is required to make the platelet lipid available for participation in coagulation. The clot promoting lipid of platelets is not present in pure form since particles of this nature would be visible in the electron microscope as fat droplets or micelles. Therefore, the platelet phospholipids important to clotting are probably present in the form of lipoprotein complexes. How this complexed lipid in the platelet is made available to coagulation has not been entirely clear.

The present study, using careful preparation of platelets to avoid damage, and an advanced fixation technique suggests a relationship between active platelet phospholipid and the platelet granules. In the platelets of native platelet rich maintained at 2 C. to inhibit the rate of clot development, platelet granules transform into lipid micelles. The micelles are extruded through the
cell membrane prior to platelet aggregation. All stages of granule transformation and micelle release were apparent simultaneously, suggesting that the conversion and release mechanism are rapid and concurrent processes. The number of granules undergoing alteration in thin sections of single platelets varied from one to seven. Only occasionally were platelets found in which most of the granules had converted to micelles to form a large lipid complex in the cell. Micelles in the plasma outside platelets whose granules were transforming and being released occurred with a frequency of one or two per cell in thin section. The origin of the micelles from platelet granules, their rupture into the plasma outside the cell in early coagulation, and the similarity of the platelet micelles to micelles of other active lipids which can effectively replace platelets in accelerating in vitro coagulation suggested to us that the process we had observed might be intimately related to platelet function in clotting.

Historically, Fonio\textsuperscript{22} was the first to associate the lipid clot promoting activity of platelets with the platelet granules. He observed the development of "vacuoles" within the platelet granulomere which ruptured through the cell membrane, and dissolved in the surrounding plasma. He considered this the source of, and mechanism for, release of platelet lipid activity.
Following Fonio's observations, many workers have suggested a relationship between platelet granules and the lipid substance contributed by these cells to clotting. Johnson and coworkers, Schulz and Heipler, Maupain, and Marcus and Zucker-Franklin have prepared isolated platelet granule fractions which were as effective as intact platelets in accelerating in vitro coagulation. Also, Ulutin related the extent of platelet degranulation to the rate of thromboplastin formation.

Recently, evidence has been presented suggesting that platelet membranes are the probable site of the platelet phospholipid activity important to coagulation. Marcus and Zucker-Franklin, using the technic of sucrose density gradient ultracentrifugation, prepared isolated platelet granules and membranes. The membrane fraction was as active as intact platelets in the thromboplastin generation test, while the isolated granules had only 20 per cent of the activity of the cell membranes. When the extracted phospholipids of their granule or membrane fractions were studied, however, the coagulation activities were similar. The authors concluded that membrane phospholipids were probably more available for reaction in the coagulation mechanism than granule phospholipids.

There are, therefore, conflicting views in the literature concerning the
Fig. 7.—The release of platelet micelles. Int. Fix.—Glut., Post Fix.—O.A., Post Stain—U.A. and L.C.

Fig. 7A.—A platelet from native platelet rich plasma. Several micelles are in various stages of development, and a fully formed micelle is beginning to break through the cell surface membrane. Small glycogen particles (Gly.) are evident in the platelet substance. Mag. ×99,000.

Fig. 7B.—The micelle of this platelet has penetrated about one-third of the way through the surface membrane. A portion of the cell wall is taken with the micelle during the extrusion process. Mag. ×110,500.

hidden site of platelet lipids active in clotting. Our study has not definitively resolved this argument, even though lipid micelles were shown to arise from the platelet granules. Whether the lipid particles which we observed were indeed active in the clotting mechanism could not be determined on a morphologic basis alone. Therefore, we turned to an examination of lipids prepared from platelets and brain and dispersed in aqueous media in a
Fig. 8.—The release of platelet micelles. Int. Fix.—Glut., Post Fix.—O.A., Post Stain—L.C.
Fig. 8A.—Only a portion of this micelle remains inside the platelet. Mag. ×105,600.

Fig. 8B.—A platelet micelle which has just been completely extruded. The membrane of the micelle is still attached to the platelet surface membrane (†). The defect produced by extrusion of the micelle does not appear incompatible with survival of the platelet. Mag. ×132,000.
Fig. 9A.—A solid micelle released from a platelet without completion of central vacuolization. This form is seen less frequently than the thick walled vacuole variety of platelet micelle. Compare with the particle in Figure 9B. In. Fix.—Glut., Post Fix.—O.A., Post Stain—U.A. and L.C. Mag. × 487,200.

Fig. 9B.—A solid type of micelle formed by aqueous dispersion of extracted platelet lipids. The multilayered appearance and the periodic interval of the dark lamellae are practically identical to platelet lipid micelles formed naturally within cells. Compare with platelet micelles in Figure 10A. In. Fix.—Glut., Post Fix.—O.A., Post Stain—U.A. and L.C. Mag. × 361,800.
Fig. 10A. — A miceller platelet particle with a satellite micelle. This type is commonly seen in platelets in which several altered granules appear to coalesce into a complex thick walled vacuole type of micelle. Compare with the particle in Figure 10B. In. Fix.—Glut., Post Fix.—O.A., Post Stain—U.A. and L.C. Mag. × 469,800.

Fig. 10B. — A micelle formed by colloidal dispersion of the extracted brain lipid, cephalin. Note the similarity in appearance to that of the platelet micelle in Figure 10A. The multilayered structure and periodicity are practically identical. In. Fix.—Glut., Post Fix.—O.A., Post Stain—U.A. and L.C. Mag. × 360,000.
manner known to supply lipid acceleratory activity to in vitro coagulation. The micelles formed by colloidal suspensions of the extracted lipids were essentially identical to the micelles which develop from the granules inside intact platelets.

The importance of physical structure to the clotting reactivity of lipids was demonstrated by Surgenor and Wallach.\textsuperscript{31,32} A purified phospholipid, phosphatidylethanolamine, derived from egg was shown by these workers to effectively replace the lipid substance of platelets in an in vitro coagulation system. The maximal efficiency of the phospholipid was dependent upon its assumption of a specific physical configuration in colloidal suspension. Electron micrographs of this preparation revealed tiny microspheres which in hemi-section were composed of multiple concentric lamellae. Other investigators\textsuperscript{33-38} have shown that this specific physical form, the micelle, is the characteristic arrangement of lipid molecules in response to aqueous dispersion and have confirmed the importance of structure to the activity of lipids in coagulation.\textsuperscript{39}

The platelet micelles shown in our present study are comparable in physical structure to the micelles described by Surgenor and Wallach.\textsuperscript{3} The specific form observed does not prove that platelet micelles are active in coagulation, but the origin of platelet micelles from platelet granules, and the importance of the micelle structure to lipid clotting activity, strongly indicate the likelihood that the platelet micelles may be the effective form of platelet lipid contributed by these cells to coagulation.

Important aspects of platelet micelle formation require further investigation. The transformation of platelet particles into micellar configurations is not limited to granules of blood platelets. Lysosomes of many different cell types undergo conversion into myelin bodies following release of their acid hydrolases.\textsuperscript{40,41} The hydrolytic enzymes appear to digest lysosomal protein permitting residual lipids to assume the micellar configuration necessary to protect their hydrophobic groups. Recently, Marcus and Zucker-Franklin\textsuperscript{80} have suggested that a portion of platelet granules contain acid hydrolase activity. They have thus far defined acid phosphatase, $\beta$ glucuronidase, and cathepsin in the subcellular platelet particles.\textsuperscript{42,43} Their observation that some platelet granules are probably lysosomes suggests a possible mechanism for the transformation of platelet granules into micelles, and the release of the lamellar particles from the platelets.

Following the initiation of intrinsic coagulation, or the action of an injurious agent, the platelet surface membrane is altered significantly. It is probable that the stimulus effecting changes in the cell wall could also affect platelet granule unit membranes. If the injury was sufficient, platelet granules with acid hydrolase activity could release their enzymes. The proteolysis of the

\textsuperscript{*The size of the particles studied by these authors was originally reported to be 0.5 millimicrons. Examination of their electron photomicrographs suggested that the micelles were considerably larger than this. Personal communication with Dr. Surgenor has confirmed the fact that the particle size is 0.5 microns rather than 0.5 millimicrons.
protein of the lipoprotein complexes of which platelet granules are composed would yield phospholipid residues. The free phospholipids would assume micellar forms spontaneously to protect their hydrophobic groups. Acid hydrolases diffusing through the platelet hyaloplasm might also cause alterations in the cell wall, preparing it for extrusion of micelles. The proposed sequence of events is purely speculative, but the lipoprotein structure of platelet granules, and the probable occurrence of acid hydrolase activity in some of them pose exciting possibilities.

In conclusion, the present study has demonstrated that in platelets of carefully prepared native platelet rich plasma fixed in gluteraldehyde, a process may be observed which has not been apparent by other technics. At an early stage of clot development platelet granules transform into lipid micelles and are released from the cells. The origin of lipid micelles from platelet granules, their release from platelets early in clot development, and the structural similarity to micelles of other lipids active in accelerating coagulation suggest that platelet micelles may be the active platelet lipid substance made available to coagulation. The lipoprotein complexes of platelet granules would thus appear to be the hidden form of active phospholipid in normal platelets.

**Summary**

Investigation of the ultrastructure of platelets of carefully prepared, native platelet rich plasma fixed in gluteraldehyde-osmium has revealed a process not apparent by other technics. Platelet granules undergo transformation into lamellar particles with the configuration of lipid micelles. The platelet micelles are released through the platelet cell membrane prior to aggregation. Comparison of the platelet micelles with micelles formed by aqueous dispersion of extracted platelet lipids and brain cephalin revealed similarities of particle size and micellar configuration. The origin of platelet micelles from platelet granules, their release from the cell in early clot development, and their similarity to other lipids active in coagulation are provocative. Platelet micelles may be the active lipid substance contributed by platelets to coagulation, and the lipoprotein complexes of platelet granules the “hidden form” of the platelet lipid in normal circulating cells.

**Summary in Interlingua**

Le investigation del ultrastructura de thrombocytos in cautemente preparate plasma a alte contento de thrombocytos native e fixate in gluteraldehyda a osmium ha revelate un processo non demonstrabile con altre technicas. Le granulos thrombocytic se transforma in particulas lamellar con le configuration de micellas lipidic. Le micellas thrombocytic es liberate a transverso le membrana cellular ante le aggregation. Un comparation del micellas thrombo-cytic con micellas formate per dispersion aquose de extrahite lipidos thrombo-cytic e cephalina cerebral revelava similaritates de magnitude e de configuration. Le origine del micellas thrombocytic in granulos thrombocytic, lor liberation ab le cellula in precoce phases del disveloppamento coagulatori, e lor similaritate con altre lipidos participante in le processo del coagulation pone interessantissime questiones. Il pare possibile que le micellas thrombo-
cytic es le active substantia lipidic contribuite per le thrombocytes al processo
del coagulation e que le complexos lipoproteinic de granulos thrombocytic es
le "forma celate" del lipido thrombocytic in le cellulas normalmente in circula-
tion.

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