An Electron Microscopic Study of the Changes in Platelets during Viscous Metamorphosis

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The structure of the platelet has been studied by light and electron microscopy, and observations made on its morphologic changes in various in vitro coagulation systems. Published illustrations are of unsectioned material and do not clearly reveal changes in platelets during viscous metamorphosis. Phase contrast microscope studies of viscous metamorphosis have revealed distortion, fusion and disruption of platelets with release of granules.

Intact platelets and those involved in progressive stages of viscous metamorphosis have been studied with the phase contrast microscope, and ultrathin sections have been prepared for observation in the electron microscope. This paper reports the detail of the remarkable structural changes which occur in the platelet during viscous metamorphosis.

METHODS

All studies were carried out on platelets isolated from blood collected from normal humans using 1.5 per cent ethylene diamine tetra-acetic acid (EDTA) as anticoagulant. Siliconed glass or plastic surfaces were employed throughout.

1) Phase Contrast Microscopy

Platelets were isolated by differential centrifugation and then resuspended by gentle agitation in 2-3 ml. of plasma. A drop of plasma containing 800,000-1,000,000 platelets/cu. mm. was placed on a glass slide, recalcified with a drop of 0.2 molar calcium chloride, a coverslip applied and the changes observed continuously under the phase contrast microscope. The observations were recorded photographically, employing repeated exposures with electronic flash at 15-second intervals.

2) Electron Microscopy

Platelet rich plasma was prepared in a similar manner and maintained at 0 C. in a melting ice bath.

One sample of platelets was fixed immediately after isolation for their study in the intact state.

Two-three ml. aliquots of platelet plasma were recalcified with a similar volume of 0.2 molar calcium chloride after warming to 37 C. in a water bath. Coagulation was inhibited at intervals of 15, 45 and 90 seconds by returning the sample to a melting ice
bath and simultaneously adding an excess of osmic acid fixative. Another sample was allowed to proceed to the macroscopic appearance of fibrin at 160 seconds before returning to the bath and adding fixative.

In a similar manner, samples consisting of platelet free plasma and plasma containing 400,000 platelets/cu. mm. were recalcified and the process inhibited as soon as coagulation occurred. A final sample of platelet rich plasma was left for 2 hours at 37 C. after recalcification so that the changes of clot retraction could be observed.

After incubation, all samples were centrifuged at 1500 g for 15 minutes at 0 C. The supernatant plasma was discarded and the sediment immersed in veronal buffered osmic acid for 90 minutes. Fixation and the succeeding manipulation of the samples were all carried out at 0 C.

After fixation, the samples were washed twice with distilled water and passed through a series of graded acetones, 50 per cent, 75 per cent and 95 per cent for 15 minutes each. They were then immersed in 100 per cent acetone for 30 minutes, in an acetone-methacrylate mixture for 30 minutes and finally in 100 per cent N-butyl methyl methacrylate overnight prior to embedding. After embedding the tissue, the methacrylate was left to polymerize at 60 C. for 24 hours. Sections of approximately 0.02 μ thickness were cut with a Porter-Blum microtome. They were then mounted on Formvar coated grids, stained with half-saturated lead hydroxide and examined in a Phillips EM-100 electron microscope.

3) Granule Isolation

Approximately 250 ml. of platelet rich plasma was separated from whole blood which had been collected into plastic bags containing 1.5 per cent EDTA and spun at 320 g for 30 minutes in a refrigerated centrifuge. The platelets were then sedimented by centrifugation for 15 minutes at 1500 g and finally resuspended in one-third the original volume of plasma.

The platelet plasma was then warmed to 37 C. and recalcified with a half-volume of 0.02 molar calcium chloride. After 120 seconds the plasma was placed in a melting ice bath and maintained thereafter at 0 C.

The sample was then centrifuged at 1500 g for 15 minutes at 0 C. to sediment the intact platelets. The supernatant plasma, which was rich in platelet granules, was then decanted and centrifuged for one hour at 80,000 g. The small button of platelet granules was then prepared for electron microscopy as above.

RESULTS

Light Microscopy

The progressive changes occurring in platelets in recalcified plasma can be observed in some detail with the phase contrast light microscope. Within 30 seconds of recalcification, minute granule particles can be seen moving rapidly to and fro. Movement gradually ceases as the platelets clump, adhering to one another and to the glass surfaces. Processes may be seen on many platelets. Many platelets become swollen, and those in clumps lose their structural detail and become fused in an amorphous mass. Initially a few strands of fibrin appear, these increase in number to form a meshwork pattern and communicating strands bridge the intervals between the platelet masses. Frequently the fibrin strands are arranged in a stellate fashion around individual platelets. The appearance of the fibrin network does not differ from that observed in platelet poor plasma (fig. 1).

Electron Microscopy

When viewed with the electron microscope, intact platelets that have been fixed within a few minutes of collection of blood are spherical; however, as
Fig. 1.—A phase contrast micrograph illustrating the changes of viscous metamorphosis, including swollen platelets, platelet aggregates and large, fused masses of platelets with loss of definition. The fibrin network may also be seen frequently with stellate attachment to platelets.

the time between collection and fixation increases, hyaloplasmic processes appear. In our preparation, intact platelets were fixed 45 minutes after collection, and processes of varying length were almost universally present (fig. 2).

Many platelets sectioned at different levels allow an appreciation of their overall spherical shape to be gained. Low power electron microscope reproductions reveal that the majority of the platelets were intact, possessing a centrally placed granulomere, scattered vesicle-like spaces of varying shape, and a uniform hyaloplasm that varied slightly in electron density.

At higher magnification the granulomere is seen to consist of ovoid or spherical bodies which vary in content and electron density (fig. 3). The most common granule has a dense opaque appearance and contains an eccentric area of even greater density. Others are less dense and of uniform opacity.

Many spaces occur throughout the substance of the intact platelet. These vary considerably in size, have a recognizable limiting membrane and usually contain small quantities of lightly staining amorphous material. These spaces will be referred to as vesicles, although the nature of any possible contents is unknown. These vesicles are not evident in unsectioned platelets.

Mitochondria occur in small numbers, nearly always on the periphery of the granulomere (fig. 3). At most they are slightly larger than the other types of granules, have a definite double membrane but few cristae, and lack the complexity of those seen in other cells.
Fig. 2.—An electron micrograph of intact platelets. Hyaloplasmic processes may be seen on the platelets. Sections through the granulomere show the granules of varying density and scattered vesicle-like spaces. Scale in microns.

The platelet hyaloplasm forms a fairly uniform, greyish background. Distinct endoplasmic reticulum is not recognizable, although scattered throughout the hyaloplasm are small electron opaque structures 200-600 Å in diameter which may be free ribosomes. It is also possible that these small, granular opacities, staining darkly with lead hydroxide, are glycogen particles, as they resemble published illustrations of this material in both liver cells and platelets (fig. 3). Neither a centriole nor Golgi apparatus could be identified.

**Viscous Metamorphosis**

As a corollary to the changes observed with the light microscope, examination of ultra-thin sections during viscous metamorphosis reveals the details of a variety of structural changes occurring with great rapidity.

Within 15 seconds of the addition of calcium, many of the platelets have aggregated into close apposition. The membranes appear to be intact, but bridgelike processes can be seen crossing the interval between adjacent platelets (figs. 4 and 5). Occasional platelets show quite extensive areas of disintegration of their cytoplasm, even while the membrane appears intact (fig. 4.)

Sections taken during viscous metamorphosis 45 and 90 seconds after recalcification reveal a variety of changes. At the time when the appearance under the light microscope suggests fusion and loss of definition in the plate-
Fig. 3.—An electron micrograph of a section through the granulomere of an intact platelet. D.G: dense granule with eccentric opacity; P.G: paler, less common, granule; M: mitochondria; V: vacuole with limiting membrane. Bottom right there is a collection of small opacities which may be free ribosomes or aggregates of glycogen.

Let aggregates, the electron microscope demonstrates a remarkable degree of structural integrity in the majority of the platelets. It is possible to recognize the limiting membrane of most platelets although they are closely adherent to one another. In only occasional areas of any section is it possible to recognize fusion of adjacent platelets. The predominant change is that of intimate adherence between the platelets, although narrow spaces are still present between some cells. (fig. 6).

Various internal changes have occurred in the platelet structure. Some are swollen and pale. Many appear contracted, especially in the areas of closest apposition, and the hyaloplasm is of variable density. The granule content also varies from those that contain no recognizable granules to others in which granules are not depleted. The granulomere in other platelets is detectable but has lost definition (fig. 6). Those platelets that are empty or deficient of granules do not show any breach of their limiting membrane at this stage.

By the time of appearance of fibrin at 160 seconds, a variety of changes has developed (fig. 7). Some platelets remain intact but nearly all appear swollen in size. The hyaloplasm of the intact platelets is pallid and many show a
Fig. 4.—Early viscous metamorphosis (15 seconds). Illustrated are commencing aggregation of platelets and partial dissolution of the hyaloplasm of a centrally placed platelet.

crescent-like peripheral alignment of their granules. This peripheral grouping of granules is sometimes strikingly related to the site of close apposition to strands of fibrin (figs. 7 and 8).

Other platelets have developed gaps in their limiting membrane through which granules appear to be escaping (fig. 7). Still others are virtually empty of granules and contain only hyaloplasm. The impression of a progressive loss of granules from the platelets during viscous metamorphosis was confirmed by a count of 100 platelets before recalcification and at the stage of appearance of fibrin. This revealed that the average granule content had fallen from 10 to 4. Mitochondria may still be recognized at the stage of fibrin formation in some intact platelets, although they are not universally present. The dense granule, most common in the intact platelet, is only rarely seen as viscous metamorphosis proceeds and may be extruded or consumed in this process. In the same manner the vesicles become less evident in the intact platelets during viscous metamorphosis.

Examination of other areas of the newly formed clot shows a dense intermingling of platelet substance and scattered granules with particles and strands of fibrin (fig. 9).

Fibrin appears in the medium often quite unrelated to any platelet sub-
Fig. 5.—Early viscous metamorphosis (15 seconds) showing close adherence between adjacent platelets and some loss of detail in the granulomere.

stance (fig. 7). It is particulate but can often be seen in strands of considerable length. It is sometimes seen closely adherent to platelet membrane (fig. 8), which may appear thickened at the site of attachment to the fibrin. The characteristic cross striation of the fibrin particles is also illustrated (fig. 9).

Platelet Free Plasma

Sections of the fibrin clot formed in platelet free plasma have demonstrated the same particulate and linear arrangement of fibrin, as is seen in the newly-formed clot in the presence of platelets.

Platelet Plasma

The possibility of faulty polymerization of fibrinogen in the presence of an excess of platelets was examined by observing the changes in clotted plasma which contained 400,000 platelets/cu. mm., as against the highly-concentrated preparations used in the other studies. Sections of the newly formed clot illustrated the same platelet changes described above, and the same particulate arrangement of the fibrin. The only variation noted was the presence of a predominant proportion of empty, but intact, platelets. The loss of granules
Fig. 6.—Viscous metamorphosis at 90 seconds showing fusion of platelets into a dense mass in one area, and two swollen, pallid platelets, one being empty of granules.

thus appeared more complete when the platelet preparation was less concentrated.

Clot Retraction

Sections taken after clot retraction had developed in platelet rich plasma show a progression of the changes of viscous metamorphosis.

The majority of the platelets still appear to have intact membranes. Very few contain any granules. There is great variation in size and considerable distortion of the shape of many platelets. Many appear thin and elongated and this impression is supported by the small size of many of the platelets seen in cross-section. The close adherence of the platelets seen in viscous metamorphosis is even more marked when clot retraction has occurred. The spaces seen at 90 seconds (fig. 6) are now no longer evident, and any intervening areas are occupied by densely packed fibrin particles and strands (fig. 10).

High magnification demonstrates the persistence of mitochondria, but no other granules, within the platelets when clot retraction has occurred (fig. 11).
Fig. 7.—Coagulation in platelet plasma at 160 seconds. F.: fibrin apparently unrelated to platelets. Pallid platelets, one showing crescentic grouping of the granules and the other a defect in the membrane (arrowed). Loose platelet membrane is also illustrated (arrow).

The intimate apposition of platelet membranes is a striking feature in this illustration.

Isolated Granules

Granules isolated in the manner described, during viscous metamorphosis, were sectioned and examined in the electron microscope. Most of these appeared to be intact and to be of the more pallid variety. No mitochondria were recognized. Others appeared to have lost most of their contents and the membrane was often discontinuous. Some of these may have been vesicles, although the appearances observed may have represented the disintegration of granules either in the process of isolation or as part of their involvement in the coagulation process.

Discussion

The circulating platelet is a disc that rapidly becomes spherical after removal from the circulation. Intact, unsectioned platelets examined with the electron microscope often exhibit long hyaloplasmic processes. These become more evident as the time since collection increases, when the platelets are collected direct on to formvar grids.
Unspread, sectioned platelets are fairly regular in outline, having only short hyaloplasmic protrusions, and are roughly spherical in shape (fig. 2). Ultrathin sections reveal the structural detail of hyaloplasm and granulomere. The latter is composed of some mitochondria which are comparatively simple in structure and some less electron-dense and pallid granules that may be derived from mitochondria, as Rinehart has suggested.

The predominant granule is dense, round, and contains an area of greater density, usually eccentrically placed. This granule has little resemblance, except in size, to the mitochondria, and may contain the platelet factor active in thromboplastin generation. That platelet factor 3 activity may reside in the granules was originally suggested by Fonio, and subsequently supported by the experiments of Johnson et al., who isolated the granules by physical disruption of the platelets and observed the presence of factor 3 activity only as long as the granules remained in the suspension.

The initial disappearance of this dense granule from the platelets, during viscous metamorphosis, might also infer that it has been consumed in the process, and may be actively contributing to the generation of thromboplastin. In support of this we have observed a significant decrease in the recalcified coagulation time of platelet poor plasma in the presence of a saline suspension of platelet granules.

The paucity of free granules outside platelets in these preparations is due

Fig. 8.—Illustrating relationship of an intact platelet to a strand of fibrin. Granules are grouped towards the periphery.
Fig. 9.—An area of dense intermingling of fibrin strands, platelet material and free granules.

to the method of collection of the material. The final centrifugation in the preparation of each sample leaves the granules that have been liberated from platelets in the supernatant, and hence not included in the material for examination in the electron microscope. Nevertheless, their appearance in the plasma from disrupting platelets has been observed with the phase contrast microscope, and many platelets devoid of granules are evident in the sectioned samples.

The progressive changes seen with the electron microscope during viscous metamorphosis parallel in outline those observed with the light microscope. Platelets aggregate and fuse, become swollen and disintegrated and ultimately included in a fibrin network. However, ultra-thin sections under the electron microscope do reveal a variety of changes not evident in unsectioned material or in the light microscope. Some platelets are rapidly consumed, but the majority retain some structural integrity, and the persistence of intact platelet membranes throughout the whole process of coagulation is a remarkable finding. The dense platelet masses seen with the light microscope would appear from these studies to consist of densely aggregated platelets, many of which have lost their granules but retained their limiting membranes.

The rapid morphologic changes of viscous metamorphosis and coagulation in platelet plasma must be accompanied by complex biochemical changes. The dissolution of the platelet with release of granules and subsequent re-
traction of the clot would be expected to be accompanied by considerable chemical alterations. Born has demonstrated some changes in phosphorus distribution and a decline in adenosine triphosphate levels in platelets during clotting of platelet rich plasma.

There was no evident predilection for fibrin to appear in relation to more intact or disintegrated platelets. However, when related to more intact platelets, a crescentic alignment of granules within the platelet along the surface adjacent to the fibrin was observed. Most sections show fibrin as predominantly particulate. This may be the result of sectioning transversely most of the fibrin present. Certainly the occasional longitudinal section was obtained and revealed the characteristic cross-striations of the fibrin strand.

The orientation of fibrin and platelet material in the newly formed and retracted clot differs somewhat, in sectioned material, from the appearance of unsectioned preparations. The latter tend to show anchoring of fibrin strands to the granulomere, whereas the sections illustrated show rather a dense intermingling of disrupted and degranulated platelets with fibrin strands (figs. 9, 10 and 11). This is not a result of the highly concentrated preparation of platelets used, as a similar picture was observed when less concentrated preparations were examined. The only variation observed in the latter preparations was a more complete loss of granules from the platelets. If the granules are of importance in the generation of thromboplastin, this
observation may be accounted for on the basis of the concentration of platelet thromboplastic substance available according to the platelet numbers. Perhaps a similar finding of incomplete loss of granules may be expected in pathologic states of thrombocytosis where there are recognized abnormalities in thromboplastin generation and platelet factor 3 release.

The number of vesicles in the intact platelet also decreased during viscous metamorphosis. This phenomenon may be related to the release of some factor, such as a lipid, active in coagulation, or to the loss of some other platelet constituent, such as serotonin.13

**SUMMARY**

Viscous metamorphosis of platelets has been studied with the light microscope, and ultra-thin sections have been prepared at progressive stages for examination in the electron microscope.

The phase contrast light microscope reveals rapid aggregation and distortion of platelets and gives the impression of their fusion into structureless aggregates during viscous metamorphosis.

Sectioned material collected during viscous metamorphosis of platelets and examined in the electron microscope reveals a remarkable degree of retention of structure in a majority of the platelets. All become deficient in
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granules and devoid of vesicular spaces, but most retain intact cell membranes, and the structureless masses seen with the light microscope are found to consist of densely aggregated platelets. Fusion and complete loss of identity occurs in the minority.

The retracted clot was found to contain densely aggregated, distorted and elongated platelets, empty of granules and intimately related to fibrin particles.

SUMMARIO IN INTERLINGUA

Le viscose metamorphose de plachettas esseva studiate con le microscopio optic, e sectiones ultra-tenue esseva preparate a stadios progressive pro le examine electronomicroscopic.

Le microscopio optic a contrasto de phase revela un rapide aggregation e distortion del plachettas e lassa le impression que illos es fusionate in aggregatos sin structura durante le metamorphose viscose.

Sectionate materiales colligite durante le viscose metamorphose de plachettas e examinate per medio del microscopio electronic revela un remarcabile grado de retention de structura in un majoritate del plachettas. Omnes deveni deficiente in granulos e disproviste de spacios vesicular, sed le majoritate retine intacte membranas cellular, e il es trovate que le amorphe massas observate in le microscopio optic consiste de facto de densemente aggregate plachettas. Fusion e perdita complete de identitate occurre in solmente un minoritate del plachettas.

Esseva constatate que le retractionate coagulo contine densemente aggregate, distorquite, e elongate plachettas que es disproviste de granulos e que es intimemente relationate con particular de fibrina.

ADDENDUM

Since this paper was submitted for publication, a similar study has been reported. These authors obtained their sections of platelets during viscous metamorphosis, collecting specimens 10 and 13 minutes after recalcification, and of the partially retracted clot 26 minutes after recalcification. Their findings bear an overall similarity to those embodied in this report.

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