The Demarcation Membrane System of the Megakaryocyte: A Misnomer?

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The concept that the demarcation membrane system delineates platelets within the cytoplasm of megakaryocytes has been examined. In short-term culture of mouse bone marrow, mature megakaryocytes extended long, attenuated processes that were found by electron microscopy to have a limited amount of invaginated membrane. When such megakaryocytes were exposed to microtubule depolymerizing agents, the attenuated processes retracted, became thicker, and an extensive demarcation membrane appeared. It is suggested from the results that the demarcation membrane system functions to provide a membrane reserve that undergoes evagination during the formation of attenuated processes and thereby envelopes putative platelets, rather than to demarcate platelets in the maturing megakaryocyte. The term "invaginated membrane system" is considered more appropriate than "demarcation membrane system."

Although it has been known for many years that platelets are derived from megakaryocytes, their mode of formation within the parent cell remains uncertain. Early electron micrographs of megakaryocytes revealed the existence of a membrane system permeating the cytoplasm, and the dogma arose that this system demarcates putative platelets. Ultrastructural inspection shows the demarcation membrane system to be in the form of tubules and flattened cisternae that often undergo branching. Tracer techniques have demonstrated that the space within the membranes is patent to the extracellular environment. The demarcation membrane system appears to arise from the plasma membrane by invagination, either in the form of tubules or of concentric fenestrated sheets. More recent studies involving freeze fracture have supported the idea that the system is tubular close to the plasma membrane and adopts a fenestrated sheet-like form deeper within the cell. Shaklai and Tavassoli sought to explain the transformation into sheets by proposing that parallel aligned tubules fuse together, the resulting structure then undergoing fission into two separate sheets that become part of the surface of adjacent platelets. The involvement of tubules over a three-dimensional matrix is envisaged to result in platelets being enveloped by their plasma membrane.

Many authors (e.g., Fig. 6, ref. 5) have designated certain sites in micrographs of mature megakaryocytes as "platelet areas." These contain organelles associated with the circulating platelet, but the marginal bundle of microtubules is absent. It is not unreasonable to suggest that such apparent "platelet areas" could arise by chance if the demarcation membrane system was distributed randomly through the cytoplasm. There is need therefore for further evidence that platelets are in fact delineated in the megakaryocyte cytoplasm by the demarcation membrane system. Any hypothesis concerning platelet formation must be compatible with their method of release from megakaryocytes. Wright showed that extravascularly located megakaryocytes produce cytoplasmic processes that penetrate the intravascular compartment of the bone marrow where they undergo fragmentation into platelets. Electron microscopic studies led to the suggestion that large fragments break off from megakaryocyte processes in the intravascular compartment and that these subsequently undergo further fragmentation into platelets, possibly as a result of the demarcation membrane becoming a fracture line. Serial sectioning, however, suggests that such fragments are sections through processes that are still attached to the parent megakaryocyte. This is consistent with scanning electron microscopic studies of bone marrow in which megakaryocytes are seen to extend processes into sinusoids where they undergo attenuation and constriction at their distal end into platelet-sized segments. Rupture of cytoplasmic bridges linking these segments is thought to release platelets into the circulation. Attention has been drawn to the ultrastructural similarity between these bridges and those linking cells in the terminal stages of cytokinesis, suggesting there is a common mechanism for separation and that extension of demarcation membranes is not involved in platelet release.

The demonstration of attenuated cytoplasmic processes in sinusoids with the scanning electron microscope has led Becker and De Bruyn to propose that megakaryocyte cytoplasm is demarcated into cylindrical processes rather than individual platelets, and that such processes are intertwined and compacted in the...

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parent megakaryocyte. A similar model had been proposed earlier by Bessis, following observations of process formation by megakaryocytes in vitro. However, ultrastructural evidence for cylindrical arrays of demarcation membrane within megakaryocytes has not yet been forthcoming.

Thus, it is by no means certain that the demarcation membrane system delineates putative platelets in the developing megakaryocyte. This has led us to reexamine the role of the demarcation membrane system in platelet formation and to consider how else the cytoplasm might be subdivided into platelet volumes and enveloped by membranes. Simple calculation shows that the surface membrane of the mature megakaryocyte suffices to cover less than half the surface of all the platelets it produces. While it is likely that the additional membrane is derived from the invaginated plasma membrane, the latter may not function to demarcate platelets. Instead, it could provide a membrane reserve that undergoes evagination during the formation of attenuated processes and thereby envelops putative platelets. This implies that a developing platelet would not become associated with its plasma membrane until the stage of process formation is reached. We have sought to elucidate this matter utilizing megakaryocyte process formation in short-term culture. After inducing retraction of developed processes, the ultrastructure of megakaryocytes has been examined, paying particular attention to the demarcation membrane system.

**MATERIALS AND METHODS**

Bone marrow was taken from femurs of male (BALB/c x C57BL/6F1) mice, aged 11 wk. The bones were split open while submerged in balanced salt solution containing 15 mM HEPES buffer (BSS) and 2% fetal calf serum (FCS) and fragments of marrow dissected out. These were placed on glass coverslips that were then inverted over depression slides, using BSS plus 2% FCS to fill the depression. The coverslips were sealed to the slides with wax and put in a 37°C room. Alternatively, fragments were placed in a 2-ml culture chamber consisting of 2 glass coverslips separated by a silicone rubber gasket. The formation of processes by megakaryocytes was observed by phase-contrast microscopy, and sometimes filmed using time-lapse photography. The processes were more easily perceived in those cells that had migrated out from a fragment. The number of isolated megakaryocytes could be increased by dislodging them from fragments with a quick flick of the slide, after about an hour of culture.

The effect of inhibition of protein synthesis on process formation was examined on slides to which cycloheximide (Sigma Chemical Co., St. Louis, Mo., 10 µg/ml or 50 µg/ml) had been added to the medium at the start of the culture. Preparations were transferred to a 4°C room to determine the effect of exposure of megakaryocytes to cold. Response to vincristine was determined by perfusion of prewarmed drug dissolved in the culture medium (60 ml/hr, concentrations ranging from 0.2 to 0.02 µg/ml Oncovin, Lilly, Sydney, Australia) into a culture chamber. Cells were fixed for electron microscopy with 2% glutaraldehyde and 1.5% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4) for 1 hr. Postfixation with 1% osmium tetroxide in the same buffer was carried out for 1 hr, following which the cells were dehydrated and embedded in Spurr resin. Sections cut for electron microscopy were stained with 2% aqueous uranium acetate and 1% lead citrate.

In separate experiments, marrow fragments were placed in BSS plus 2% FCS in 35-mm diameter plastic dishes with loose fitting tops (Lux Scientific Corporation, Thousand Oaks, Calif.) and incubated at 37°C. These dishes were gently agitated by hand at hourly intervals.

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**Fig. 1.** Megakaryocyte in culture, photographed using phase-contrast. (a) Four hours after setting up culture and immediately before exposure to 4°C. Note extensive development of attenuated processes, which have a "beaded" appearance. Long arrow points to the nucleus of the cell. A detached process is visible above the megakaryocyte (short arrow) (× 200). (b) Same cell after 2-hr exposure to cold. Processes are retracted around the nucleus (× 300). (c) Same cell after having been reexposed at 37°C for 1 hr. Processes again extended but not yet "beaded" (× 200).
intervals. This noticeably increased the number of megakaryocytes free in solution. Those that formed processes showed no tendency to attach themselves to the surface of the plastic dish and could easily be removed by gentle suction through a Pasteur pipette. Other cells showed better adhesion to the plastic but some were inevitably removed with the megakaryocytes; a purer preparation of the latter was obtained by transferring the aspirate to a new culture dish and again selectively harvesting with a pipette. The megakaryocytes were transferred to centrifuge tubes for further experimentation. After exposure to cold, or to vincristine, preparations were pelleted by centrifugation, the supernatent removed, and the cells fixed and processed for electron microscopy. Cells exposed at 4°C were kept at this temperature during centrifugation and fixation. Ruthenium red was included in the fixative, which was carried out according to the method of Wight and Ross.19

RESULTS

Light Microscopy

Cytoplasmic processes began to appear on some megakaryocytes within 2 hr of initiating the cultures. The megakaryocytes changed from a rounded shape and then formed several thick processes that became attenuated as they lengthened further. Eventually, the megakaryocyte consisted of a nucleus surrounded by a thin rim of cytoplasm and several attenuated processes of varying lengths and occasionally branched (Fig. 1a). Both thick and attenuated processes underwent continual irregular movements, generally in a lateral direction, but occasionally, twisting was evident. Slight translocations of the cell nucleus were also sometimes seen. The attenuated processes showed constrictions at intervals along their length; from the first signs of elongation to the development of these “beaded”-looking processes took 1-2 hr. Process formation also occurred in BSS alone, but the “beads” appeared to be more uniform in size when BSS plus 2% FCS was used. Occasionally, short lengths of “beaded” processes were found detached in cultures. However, fragmentation of processes into platelets was not observed, although some cells were filmed by time-lapse photography for up to 24 hr.

Transfer of slides to a cold room (4°C) for 2 hr resulted in marked retraction of megakaryocyte processes (Fig. 1b). After reexposing cells to a temperature of 37°C, movement of processes could be detected within 2-3 min. They initially appeared to shorten even more, sometimes merging completely to form a rounded cell, but within about 30 min, they began to reextend, and by 1 hr, process formation was extensive (Fig. 1c) although the “beaded” appearance took longer to develop. Cells were seen to redevelop processes following exposure at 4°C for up to 18 hr, the longest interval tested, but after this time only a minority were capable of doing so.

Exposure of megakaryocytes to vincristine also resulted in reversal of process formation. With a dose of 0.2 μg/ml, processes were considerably retracted after 30 min, while a dose level of 0.05 μg/ml required 70 min to achieve the same effect.

Ultrastructure of Megakaryocytes

Considerable difficulty was experienced in preparing megakaryocytes on coverslips for electron microscopy if they had developed processes and particularly if they had retracted them as a result of exposure to cold, because of the lability of these cells. Poor adhesion of megakaryocytes under culture conditions has been described before.20 Megakaryocytes were usually retained during preparation for electron microscopy if they were located within a narrow fragment. Although the protrusion of processes from a fragment was easily seen under the light microscope, it was often difficult to locate the nucleus within the mass of other cells. To circumvent these difficulties, most ultrastructural observations were made on megakaryocytes in enriched preparations that had been centrifuged down into pellets prior to fixation.

Sections through pellets prepared from cells that had been incubated at 37°C contained numerous megakaryocyte processes of about 1-1.5 μm diameter (Fig. 2). The processes contained microtubules, which

![Fig. 2. Attenuated processes in pellet harvested from cells kept at 37°C (7 hr after start of culture) (x 3360).](image-url)
were orientated longitudinally, as well as other organelles associated with platelets (Fig. 3). The surface of the processes generally appeared to be smooth, and there was relatively little invaginated membrane.

In contrast, processes of megakaryocytes fixed after exposure to cold for 2 hr were several microns in diameter (Fig. 4). They had a very irregular surface due to the formation of blebs and villi, the interior of which were amorphous in appearance. An extensive membrane system, generally in the form of dilated cisternae that were shown to be patent to the extracellular space by ruthenium red staining, permeated the processes. Microtubules were not present in these cells. Formation of the irregular surface does not appear to be part of the mechanism of retraction. The surfaces of megakaryocyte processes that had undergone retraction as a result of exposure to vincristine were smooth (Fig. 5). Such megakaryocytes also displayed an extensive demarcation membrane system. After cold-treated megakaryocytes were reincubated at 37°C for 30 min, the surface lost its roughness (Fig. 6).

In all preparations, large rounded megakaryocytes of varying stages of maturation were seen. In these cells, the demarcation membrane system was not

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Fig. 3. Attenuated process of megakaryocyte fixed on coverslip. Microtubules (arrows) are orientated longitudinally (x 23,000).

Fig. 4. Section from cells pelleted after 2 hr at 4°C (7 hr after start of culture). Processes are thicker than in Fig. 2, and the surface is highly irregular. The demarcation membrane system is extensive (x 3360).

Fig. 5. Cross-section of retracted process of a megakaryocyte from a pellet of cells prepared after 2 hr exposure to 0.2 μg/ml vincristine. Cells were kept at 37°C throughout the culture period of 7 hr. Ruthenium red staining demonstrates the extensive demarcation membrane system. Cisternae of the latter are generally dilated. Narrow channels to the surface are also evident (arrows). Microtubules were absent in these cells (x 4800).
dilated. It is thought that these were cells that had not participated in process formation because of their relative immaturity.

**Exposure to Cycloheximide**

When fragments of bone marrow were incubated on depression slides in the presence of cycloheximide (10 μg/ml or 50 μg/ml), processes were formed by megakaryocytes within 2–3 hr. They became attenuated and beaded in the same way as processes extended by megakaryocytes incubated without cycloheximide. After such cells had been exposed to cold (4°C) for 2 hr, the processes were retracted, but if the cells were returned to a temperature of 37°C, the processes reextended again.

**DISCUSSION**

The development of megakaryocyte processes in vitro, described many years ago by Thiéry and Bessis, and until now given little attention, appears to offer considerable potential for studying the mechanisms involved in the megakaryocyte–platelet transition. A useful attribute of the system is that with appropriate treatment, process formation can be reversed. The ultrastructure of attenuated processes that develop in vitro is similar to that previously found in vivo, and it would appear that microtubules have an integral role in their formation and maintenance. Process retraction when exposed to cold, or vincristine, is presumably related to the depolymerizing effects of these agents on the microtubules, although additional factors may be involved.

The reversibility of process formation has been utilized to gain insight into the way in which platelets become enveloped by their plasma membrane. It is clear from the results that redevelopment of the demarcation membrane system occurs concomitantly with the retraction of attenuated processes. It is difficult to explain this finding on the basis that the demarcation system delineates platelets, for this implies that a permanent association is formed between the membrane and cytoplasm of the developing platelet. Shaklai and Tavassoli, for example, proposed that demarcation resulted from the fission of opposite membranes of the demarcation system, progress towards platelet release being marked by the extent to which fission had occurred. With such a model it would be necessary to invoke extensive fusion to account for the reappearance of the demarcation membrane system during the retraction of attenuated processes. Becker and De Bruyn, in seeking to account for the formation of processes (proplatelets), have modified the idea that the demarcation system delineates individual platelets, suggesting instead that it progressively divides the cytoplasm of the developing cell into cylindrical processes that are intertwined and compacted about the nucleus. However, in the present study, when megakaryocytes were allowed to develop long processes that were then induced to retract, the resulting ultrastructural appearance did not suggest that the cytoplasm was composed of ravelled up process.

We believe the present results are explicable if the demarcation membrane system is considered not to delineate platelets in the developing megakaryocyte, but instead, to represent stored plasma membrane, the extent of which can be altered by invagination or evagination. Thus, the reappearance of a demarcation membrane system on retraction is interpreted to be due to invagination of the plasma membrane. It has already been concluded by several investigators, from studies of immature megakaryocytes, that the demarcation membrane system originates from the surface membrane in this way. Much other evidence attests to the similarity of the plasma and demarcation membranes, including surface reactivity to various stains, freeze-fracture, and virus budding studies. While invagination is believed to account for the origin of the demarcation membrane in young megakaryocytes, there is less certainty about its subsequent development as cells mature. Contact between dense coated vesicles, thought to be derived from the Golgi complex, and the demarcation membrane has been
seen, though their function has not been fully defined.3,9 "Dense compartments," short stacks of paired membranes in continuity with the demarcation membrane system, have been observed in some megakaryocytes and may represent sites of membrane assembly.3,5,8,9 However, although they are particularly abundant at the time extension of the demarcation membrane system is most intense, they are not found during early stages of its development. No evidence of dense compartments was found in the retracted processes in the present study, suggesting invagination may account for the entire redevelopment of the demarcation system. Certainly, experiments in which cycloheximide was present in the cultures indicate that de novo synthesis of proteins was not necessary for reformation of a demarcation membrane system.

If the membrane on the surface of attenuated processes undergoes invagination as the processes retract, it is reasonable to postulate that the reverse occurs as they extend. Thus, rather than envisaging the platelet to be almost completely enveloped by its plasma membrane in the maturing megakaryocyte, we suggest it acquires its membrane only as processes extend and become attenuated. Adjustment of the plasma membrane will also be necessary as progression towards platelet release occurs, since it involves the development of increasingly long and narrow cytoplasmic bridges between putative platelets.16 This, and the acquisition of the discoid shape of the platelet upon release, could conceivably involve either outflow or inflow of the demarcation membrane. It has been suggested that the open canalicular system of platelets represents the residue of the demarcation membrane system after platelet liberation has taken place.3 However, equating the two membrane systems may not be valid, since there is evidence from studies on human platelets that the membranes of the open canalicular system differ in enzyme composition to the plasma membrane.26 Whether this difference originates in the platelet or the megakaryocyte is unknown. Possibly the dense compartments may be associated with the production of these specialized membranes.

The idea that platelets do not become enveloped by their plasma membrane until the development of attenuated processes takes place does not preclude the possibility that there may be an underlying platelet substructure in the cytoplasm of the megakaryocyte. Such a substructure might be based, for example, on a specialized region of the demarcation membrane system destined to become the open canalicular system of the platelet, or on the dense tubule system. Close association of the two systems in platelets has been recognized.27

The mechanisms involved in invagination are not known. It may be relevant that microfilaments have been found in association with membranes of the demarcation and open canalicular systems as well as the plasma membranes of megakaryocytes and platelets.4,28 Cohen29 has discussed how actin filaments could participate in movement of membranes during platelet contraction. In many types of cell, microvilli and other surface protruberances are formed as a means of creating a reserve of membrane. This is seen, for example, during mitosis and after harvesting and reseeding of cells that are normally flattened during culture.30 The evolutionary history of the megakaryocyte is unknown,31 but it is probably more than mere coincidence that both the megakaryocyte and its progeny have an invaginated membrane system. This provides an alternative means for the storage of surplus membrane. In the platelet there is evidence that evagination of part at least of the open canalicular system can occur.32,33 Other functions for this system in the platelet, such as facilitating the uptake of exogenous substances and providing a conduit for the discharge of endogenous products have also been postulated.34 Progress in unravelling the mechanisms involved in controlling the flow between plasma and invaginated membranes should facilitate a better understanding of the role of the demarcation membrane in various diseases.35

Finally, it will be noted that the conventional term "demarcation membrane system" has been used in this text. However, it is suggested that this is a misnomer; "invaginated membrane system" would appear to be more appropriate.

REFERENCES

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