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ADHESION, SPREADING AND FRAGMENTATION OF HUMAN MEGAKARYOCYTES EXPOSED TO SUBENDOTHELIAL EXTRACELLULAR MATRIX: A SCANNING ELECTRON MICROSCOPY STUDY

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Abstract

Platelet agonists and subendothelial extracellular matrix (ECM) induce morphological and biochemical changes in animal megakaryocytes, reminiscent of the response of platelets to the same substances. We have examined the behavior of human megakaryocytes exposed for up to 36 hours to the ECM produced by cultured bovine corneal endothelial cells. By phase contrast and scanning electron microscopy these megakaryocytes demonstrated non-reversible adherence and flattening with formation of long filopodia, thus confirming that human megakaryocytes acquire platelet functional capacities. In addition, megakaryocyte fragmentation into prospective platelets was apparently induced by the ECM. Up to 50% of the adherent megakaryocytes underwent spontaneous fragmentation into small particles which individually reacted like platelets on the ECM. The interaction of the megakaryocytes with the ECM was specific since no adherence, flattening or fragmentation occured upon incubation of the megakaryocytes on regular tissue culture plastic or glutaraldehyde fixed ECM. Thus we have demonstrated platelet like behaviour of human megakaryocytes in response to this physiological basement membrane and a possible role of the subendothelium in platelet production which may occur in vivo as megakaryocytes cross the sinusoid walls and enter the blood stream.

Key Words: Megakaryocytes, extracellular matrix, platelets, fragmentation, maturation, ploidy, adhesion, spreading, percoll, elutriation.

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Introduction

Megakaryocytes synthesize and assemble platelet components and organelles. They acquire many platelet properties and respond to known platelet agonists such as ADP, thrombin and arachidonic acid with shape change, degranulation and reversible flattening reaction (3,6,15-17,20-22,25,28). Unlike platelets, however, they do not adhere to nor demonstrate these morphological changes upon exposure to collagen coated surfaces (15,17). We have recently shown that guinea pig and rat megakaryocytes can be activated by interaction with an extracellular matrix (ECM) produced by cultured endothelial cells (18). This activation was associated with irreversible adhesion and spreading, with the formation of long filopodia and with the release of thromboxane A2 (18).

The ECM produced by cultured endothelial cells (bovine or human) closely resembles the vascular subendothelial basement membrane in it's supramolecular structure and chemical composition (9,27). It contains characteristic components of basement membranes such as types IV and V collagen, laminin, fibronectin and heparan sulfate (14,27,32). In addition the ECM has been shown to contain collagen types I and III, dermatan sulfate and chondroitin sulfate proteoglycans and elastin. We have previously reported on the use of this matrix as a model to study the interaction between platelets and the vascular subendothelium (5,32). Previous work by others has made clear the functional equivalence of bovine or human ECM and vessel wall subendothelium from rabbits and humans, and the generic nature of the interaction with it by platelets of various species (27). The uniformity, transparency and obvious biological relevance of this in vitro generated ECM make it a good model with which to study cell surface interactions important in hemostasis.

In the present study we extend our observations to human megakaryocytes, isolated from bone marrow specimens by centrifugal counterflow elutriation and a continuous Percoll density gradient. Our results demonstrate that human megakaryocytes respond to the stimulus of ECM by adhesion and shape change. We have also shown the spontaneous fragmentation of megakaryocytes on the ECM to form platelet like structures.

Methods and Materials

Preparation of ECM coated Plates:

Cultures of bovine corneal endothelial cells were established as described previously (7,9,32). Stock cultures were maintained in Dulbecco's modified Eagle's medium (DMEM, H-16) supplemented with 5% calf serum, 10% fetal calf serum and Gentamycin (50 ug/ml) (all from Grand Island Biological Co., Grand Island, NY) at 37°C in a 10% CO_2 humidified incubator. Cells were passaged weekly at a split ratio of 1:64 and fibroblast growth factor (100 ng/ml) was added on alternate days during the phase of active cell growth. The fibroblast growth factor was purified from bovine brain as described previously (8). For preparation of ECM coated surfaces, cells were plated at an initial density of $4x10^4$ cells per 35mm dish (Falcon Inc., Oxnard, CA) and 4% Dextran T-40 (Pharmacia, Sweden) was included in the growth medium. Six to eight days after reaching confluency the cell layer was dissolved by a 3 minute exposure to 0.5% (v/v) Triton X-100 (Sigma Chemicals Co., St Louis, Mo) and 0.025N NH_4OH in phosphate buffered saline (PBS) pH 7.3 at 22°C, followed by 4 washes in PBS. This treatment left the underlying ECM intact, firmly attached to the entire area of the tissue culture dish and free of any cellular debris as determined by SEM (9,10). Some of the ECM coated dishes were the kind gift of International Biotechnologies, Hadassah, Jerusalem.

Megakaryocyte Isolation:

Human bone marrow was obtained from the waste material of bone removed during orthopedic operations. Bone marrow tissue was collected into calcium and magnesium free Hank's Basic Salt Solution (HBSS) (Grand Island Biological Co., Grand Island, NY) containing 3.8% sodium citrate, 10^{-3}M adenosine and $2x10^{-3}$M theophylline (all Sigma Chemicals Co., St Louis, Mo) (CATCH medium) (16,-17). Single cell suspensions were made by repeatedly pipetting the material through a sterile, siliconized pasteur pipette, and then filtering the suspended material through a 150 mesh sieve. The resultant suspension was sedimented by centrifugation at 400g for 8-10 minutes, and the pellet was resuspended in the same "CATCH" medium. This proceedure was repeated twice. The final pellet was resuspended in "CATCH" medium and was then enriched by centrifugal, counterflow elutriation in a Beckman J2-21 centrifuge (Beckman Instruments, Palo Alto, Ca) using the JE-6 elutriation rotor at 2500rpm (2,23). The separation chamber was then removed and the pellet was resuspended in calcium and magnesium free HBSS, and passed once again through a 150 mesh sieve. This suspension was centrifuged at 400g for 10 minutes, and the cells resuspended in the same solution, at a cell density of $2-3x10^6$ cells/ml. For a further separation we developed a continuous Percoll density gradient, self generated around a median density of 1.057 gm/ml. The Percoll solution (Pharmacia, Sweden) was made up with HBSS (Ca++ and Mg++ free) to the correct density. The pH was corrected (when necessary) with 1N NaOH or HCl to 7.35-7.45, and the osmolarity was corrected to 290-295mosm/l with distilled water or HBSS x10, as necessary. The final solution was sterilized by filtration through a 0.22um filter (FP 030/3, Schleicher and Schuell, W. Germany). The solution was placed in polycarbonate bottles (Beckman, USA) and density marker beads (0.05-0.075ml each of numbers 1-7, Pharmacia, Sweden) were added to the standard. The gradient was generated at 20000g, for 30 minutes at 5°C in a Sorvall RC-5 centrifuge with the SM-24 rotor (Sorvall, Norwalk, Conn.). The cell suspension was layered upon the density gradient and centrifuged at 800g for 20 minutes (20°C). The layers of a density 1.015-1.045 gm/ml were gently removed, utilizing the standard as a guide. The layers 1.045-1.055gm/ml were also removed, giving a lower purity but a higher yield of young, immature forms. The fractions thus obtained were washed in HBSS 3 times until free of Percoll. The final fractions contained a total of $3-7x10^5$ megakaryocytes, with a purity of 60-80% in the lighter fractions, which were mainly large, mature forms. The heavier fractions (1.045-1.055 gm/ml) contained $1-2x10^5$ megakaryocytes with a purity of 30-40% mainly of the smaller immature forms.

Megakaryocyte reactivity with ECM:

Isolated megakaryocytes were incubated with Dulbecco's MEM H-16 with 2.3% bovine serum albumin (Miles Laboratories, Kankakee, Il) and with penicillin (50 units/ml) and streptomycin (50ug/ml) (Grand Island Biological Co., Grand Island, NY) (3). Most cultures were performed at $0.5-1x10^5$ megakaryocytes/ml of medium, and sometimes as high as $2.5x10^5$/ml. They were kept at 37°C in suspension for one hour to enable the megakaryocytes to "recover" from the repeated centrifugations and washings. The cell suspensions were then added to ECM coated dishes previously placed in the incubator to minimize agitation during the seeding of the megakaryocytes. Control experiments were carried out in tissue culture dishes of the same manufacture which were not coated with ECM, or in ECM coated dishes fixed with 1% glutaraldehyde for 1 hour. Incubations were carried out for up to 36 hours in a humidified incubator at 37°C, in 10% CO^2 in air. These conditions maintained a stable pH of 7.2-7.3 for the duration of the experiment.

Morphological studies:

Cells in culture were examined in situ with an Olympus inverted phase microscope, at 100-400x. Culture dishes (35mm) were prepared for scanning electron microscopy (SEM) by tilting the dishes, gently aspirating the culture medium and then slowly dripping 1ml of warm (37°C) phosphate buffered (pH7.3) 2.5% glutaraldehyde solution down the side of the dish. After fixation they were prepared for scanning by the GTGO method and air drying as previously described (32). The samples were gold-palladium sputter coated in situ and the entire 35mm dish was examined, using a specially constructed holder that was inserted into the specimen chamber of the JEOL JSM 35 scanning electron microscope.

Megakaryocyte adhesion:

In order to determine whether megakaryocytes on the ECM were firmly adherent to the surface or were merely resting there without attachment we used a simple test for adhesiveness, as described by Levine et al (18). At various intervals after seeding, the number of megakaryocytes per field was counted by phase contrast microscopy in five

fields in the central portion of each dish at 200x. The dishes were tilted to 45° three times in three seconds; following the third time the dish was kept at that angle and the medium was gently aspirated. A similar five fields were immediately counted. The megakaryocytes remaining attached to the ECM after this relatively reproducible force were judged to have become adherent and the percentage of adherence was calculated as the ratio of the second count to the first for each dish.

Results

Morphological appearance of megakaryocytes seeded upon plastic (non-coated) surfaces:

Human megakaryocytes, upon incubation in tissue culture plastic vessels without ECM, retained the spherical shape and irregular surface characteristic of these cells (Fig.1) (4,12,16,-24). Similar to our observations with guinea pig and rat megakaryocytes (18) this shape was maintained usually throughout the 36 hour period of incubation, without evidence of adhesion, flattening or fragmentation. To prepare the dishes for SEM without losing the cells, it was necessary to use care in removing the medium, as the slightest agitation of the fluid caused the cells to float off and be removed during the aspirations. In addition megakaryocytes did not adhere to, nor flatten, when seeded upon glutaraldehyde treated ECM.

Morphological appearance of megakaryocytes plated upon ECM coated surfaces:

Human megakaryocytes demonstrated rapid adhesion to ECM coated dishes. As the sedimentation rate of megakaryocytes is greater than 1mm/min (16) and the depth of the medium was approximately 2-3mm, the cells came into contact with the ECM within 2-3 minutes of placement upon the matrix. The adherence test described above was performed on identical samples (from the same batch) at various times ranging from 10 minutes to 36 hours after seeding the cells. Within 10 minutes 50% or more of the megakaryocytes adhered. Many of them also promptly demonstrated fine tenuous filopodia (Fig.2) or, in some cells, a concentric flattening to a broad spreading veil of cytoplasm, from the margins of which fine filopodia arose. By 30 minutes post seeding, over 75% of megakaryocytes had become adherent and the filopodia became more pronounced and thicker. Some megakaryocytes flattened so completely by this time that only a few bumps were observed protruding from the surface due to the large nuclear lobes beneath them (Fig.3). These flattened cells often had a total diameter of over 30μm, with mature filopodia extending from the cell periphery. After 12 hours of incubation, individual cells developed no further flattening or formation of filopodia. Beyond this time spontaneous fragmentation began to occur. Megakaryocyte cytoplasm dispersed into a network of globules interlocked by filopodia (Fig.4). The fragmented cytoplasm of each cell was spread out in an oval shape with a diameter of 70-150μm along the long axis, and when enlarged clearly showed the fine connections between the platelet like fragments (Fig. 5). Each of these fragments had a diameter of 2-12μm. They demonstrated platelet like morphological changes,

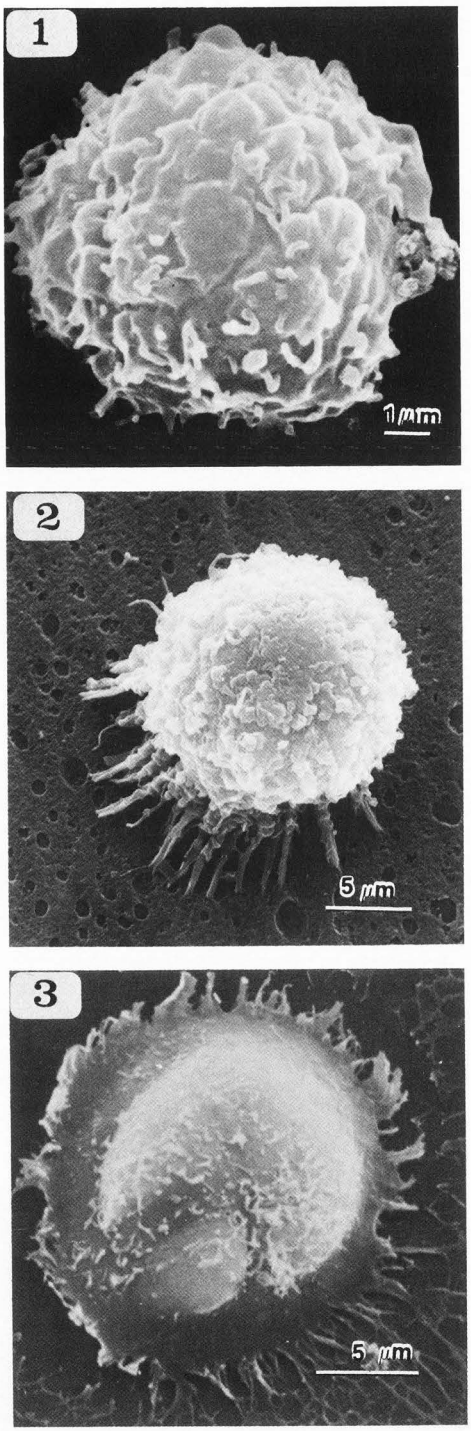

Figure 1: SEM of human megakarocytes incubated on tissue culture dish. Figure 2: Human megakaryocyte, 10 minutes on ECM, demonstrating initial formation of fine filopodia. Figure 3: Human megakaryocyte, 30 minutes on ECM. Note that the cell has already flattened noticeably, forming a spreading veil of cytoplasm in contact with the ECM.

adhering to the ECM and forming filopodia of their own (Fig.6), similar to those observed with human and animal platelets incubated on ECM (Fig.7). An occasional cell seemed to be spewing out young platelets from within (Fig.8). By the end of 36 hours of incubation, approximately 50% of the cells were found to have undergone fragmentation, 25% had flattened with peripheral filopodia and a further 25% remained round and non-adherent. The latter consisted primarily of dying and dead cells as shown by the trypan blue exclusion test. This proportion is consistent with our previous observations on cell mortality during incubation (16,18).

Discussion

Possession of platelet functional capacities by megakaryocytes was first demonstrated by Levine and Fedorko (16). Numerous studies since then have shown platelet-like responses to various soluble agonists (ADP etc') (15,22). In a recent study (18) we showed a marked similarity between the morphological and secretory response of guinea-pig and rat platelets and megakaryocytes to subendothelium. Here, we extend these observations for the first time to human megakaryocytes.In common with platelets, human megakaryocytes demonstrated classical platelet adhesion, filopodia formation and spreading upon contact with the ECM. The megakaryocytes became attached to the ECM within 10-30 minutes, immediately lost their irregular surface appearance and started to form filopodia and fine cytomembranous extensions towards the ECM. Some megakaryocytes progressively flattened, sometimes to the point of becoming thin and "pancake"-like in form, with just a bump where the nucleus was to be found. These morphological changes were dependent upon the presence of the ECM. In other experiments, guinea pig megakaryocytes cultured upon plastic tissue culture dishes, or plastic surfaces coated with fibronectin, types I,II,III and IV collagen (15,22) laminin or glutaraldehyde fixed ECM (unpublished observations), displayed neither adhesion nor shape changes. The typical activation of megakaryocytes by the ECM indicates that at least several components of the basal lamina are needed to elicit the responses of adhesion, shape change and release. Indeed, Leven and Nachmias showed that when a surface coated with collagen (type I,II,III or IV) had fibronectin added to it, megakaryocyte flattening was induced, but not the other morphological changes (15). While collagen type IV, laminin and fibronectin are major components of the ECM, it is possible that other matrix components, the relative proportions of the various constituents and/or the tertiary structure of this naturally produced basement membrane are involved in adhesion, flattening and in the development of multiple, radial long filopodia. The latter possibility is also suggested by the lack of response of megakaryocytes to contact with glutaraldehyde fixed ECM. Similarly to platelets, the typical shape change of megakaryocytes has been shown to be associated with activation of contractile proteins (6,15,22). As this study would suggest, the platelet forming process may differ and not be related, that is not one of "budding off". The observation of fragmentation of half of the megakaryocytes attached to the ECM suggests that a possible route to platelet production by megakaryocytes may be one of fragmentation, whereby the megakaryocyte "disintegrates" forming a large number of platelets. In these experiments, platelet-like fragments have apparently formed but -thanks to the ECM- have not detached from each other. The myriad individual small islands of megakaryocyte cytoplasm have attached to the ECM in a manner similar to that of platelets from peripheral blood. This would support the observations of previous authors (1,11,13,26,29,30,31), who have also noticed "fragmentation" or "disintegration" of megakaryocytes. This fragmentation of megakaryocytes was originally postulated by Wright in 1906 who observed the formation of pseudopods and protoplasmic elongations (33), and later observed by Thiery and Bessis (31) who utilized time lapse cinematography to show the cells forming long strands from which they concluded that the platelets are formed. Izak et al placed bone marrow aspirates in a culture medium for up to six days and observed the formation of possible platelets by the megakaryocytes (13). Tavassoli and Shaklai, along with others believe that the platelet surface membranes are formed from an extensively invaginated megakaryocyte surface membrane called the demarcation membrane system (DMS) (1,17,29, 30). They have suggested that individual platelets are freed by fusion of the membranes with subsequent fission from adjoining platelets (29,30). Recently Haller and Radley utilized time lapse cinematography and scanning electron microscopy to study the behaviour of mouse megakaryocytes in a bone marrow specimen placed between two glass surfaces (11). They observed the formation of globules, linked by strands of cytoplasm extending pseudopodia towards the glass surface. They hypothesized that the tubular invaginations observed by themselves and other authors served as a membrane reserve for the attenuated processes and ultimately became the exterior surface of the newly formed platelets (11). They preferred the term "invagination membrane system" instead of "demarcation membrane system" (11). Our finding (Figs. 4,8) that fragmentation seems to have begun inside the megakaryocyte would tend to support the views of Tavassoli and Shaklai (29), Behnke (1) and Levine (17). Due to technical limitations, (caused by the dispersion of the fragmented megakaryocytes upon the ECM) it has not yet been possible to perform transmission electron microscopy on these fragments to prove conclusively that they are -ultrastructurally- "true" platelets. Despite this, their behaviour upon the matrix is, as far as can be judged upon SEM, similar to that of washed platelets from peripheral blood.

Thus the interactions of human megakaryocytes and ECM illustrate two facets of megakaryocyte behaviour. One is the aquisition, even before platelet shedding, of the functional behavioral capacity of circulating platelets, namely the adhesion and shape change of megakaryocytes on ECM. These morphological responses are demonstrated here for the first time in human megakaryocytes. The other is a possible role of ECM as an anchor or inducer of platelet shedding in vivo,

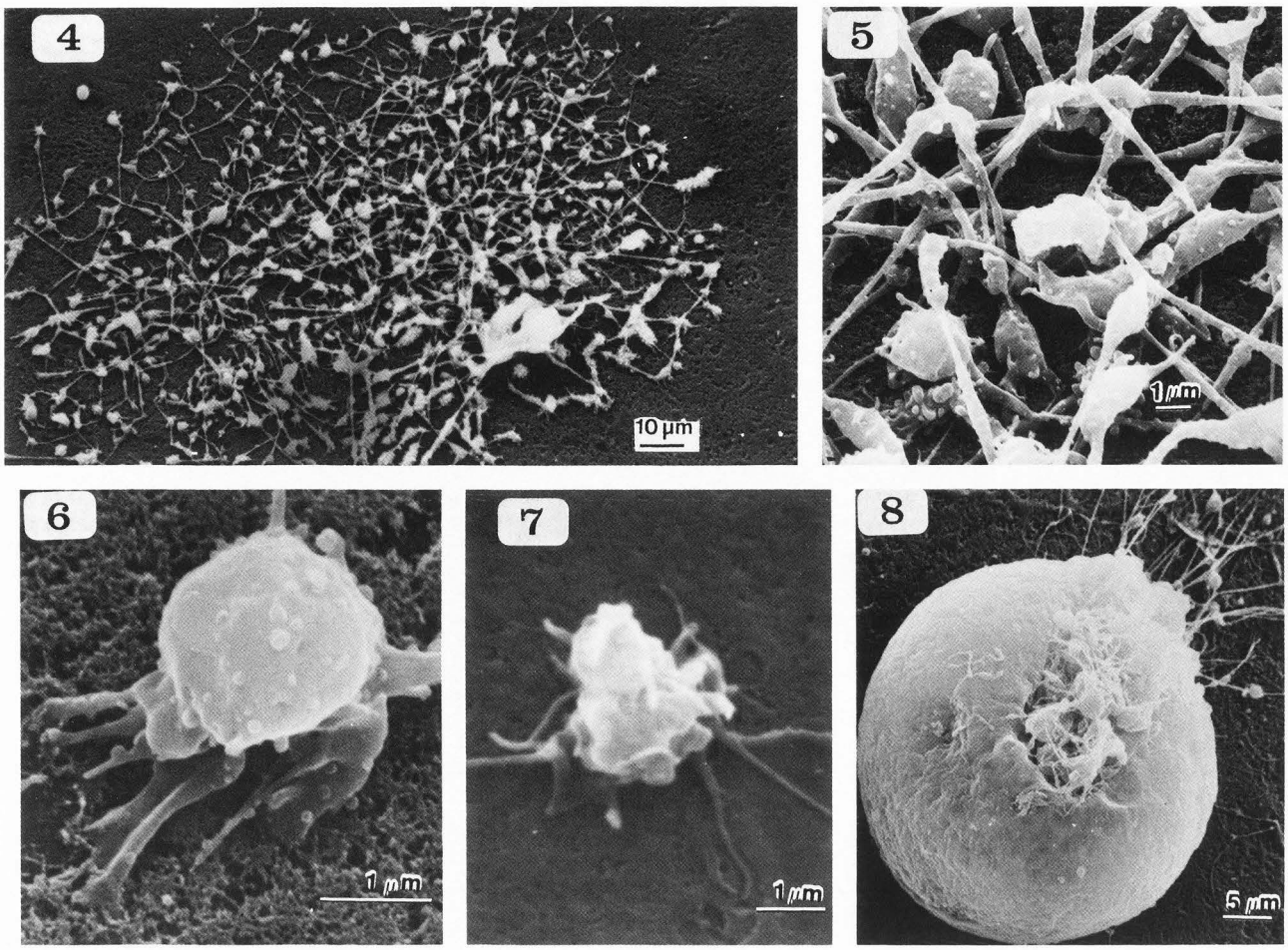

Figure 4: Fragmentation of human megakarocyte plated on ECM. Small globules are observed interlocked by filopodia-like strands. Figure 5: Fragments of megakaryocytes of different sizes linked to each other and the ECM by filamentous interconnections. Figure 6: Adherence of a megakaryocyte fragment to the ECM by filopodia extending from the platelet-like fragment. Figure 7: Platelets from peripheral blood on ECM. Adherence to the matrix is associated with filopodia formation. Note the resemblance to the fragmented megakaryocytes in terms of the type of adherence and formation of filopodia. Figure 8: Human megakaryocyte, in the process of fragmenting. Note the formation of fragments seemingly within the body of the cell, and the filopodia linking the fragments on the outside.

as the megakaryocytes are exposed to the subendothelium as they extend across the sinusoidal wall into the circulation (19). The molecular mechanism by which mature megakaryocytes would be induced to fragment into individual platelets is unknown. The possibilities include mutual repulsion by build-up of a sufficient surface membrane charge density, the removal of an adhesive substance between the membranes of prospective platelets, or the appearance of necessary inducers from within or from outside the cell. Although the megakaryocyte fragmentation that we have observed on the ECM is technically incomplete inasmuch as fine strands interconnect the cytoplasmic fragments, we feel that the model we have described for megakaryocyte activation and fragment formation will help in the further evaluation of the mechanism of platelet production.

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Discussion with Reviewers

M. Tavassoli: Could you give further details about the elutriation process. Why was it performed prior to the density gradients?
Authors: The elutriation was performed in the JE-6 rotor using the original Beckman chamber. Flow rates at 2500rpm for human cells were varied between 18ml/min and 39ml/min in 2-4ml/min increments with the steps at each 100ml eluted volume. Elutriation preceeded the density gradients because it removed the mass of erythrocytes, platelets and many of the other cells, leaving a relatively small pellet of cells for final seperation on the gradient. This gradient, whilst capable of high purity yields, is very sensitive to cell load/ gradient volume ratio. A large number of loaded cells would necessitate a very large volume of gradient.

M. Tavassoli: What was the viability after the proceedures?
Authors: >92% viability by the trypan blue exclusion method.

M. Tavassoli: Which other cells were in the final preperation?
Authors: The major contaminating cells were eosinophils. Erythrocytes and undefined mononuclears' were also commonly found.

M. Tavassoli: The authors obtain two fractions with young and old megakaryocytes. Was there any difference qualitatively or quantitatively in the interaction of these two preperations with ECM?
Authors: We made no attempt to quantitate a difference. This was because the identification of the cells on the plate (when dealing with immature cells) was exceedingly difficult, especially those of 2-4N and immature cytoplasm. We know that they were there from cytospin and staining preparations, but not many seem to have spread, and as far as we could tell practically none -if any- had fragmented.

M. Tavassoli: Fig. 4 is very reminiscent of the concept of proplatelet formation that inter alia has been discussed in ref. 30. The concept maintains that platelet formation occurs via formation of a dozen or so elongated strings of megakaryocyte cytoplasm, each can then form several hundred platelets. Would the authors comment on this view in the context of their own findings.
Authors: The term "proplatelets" was discussed by Dr. Tavassoli in his paper (ref. 30). The concept of largish chunks of cytoplasm, each equivalent to the volume of a few 10's - 100's of platelets flowing through the blood stream to eventual fragmentation would not be in conflict with the observed interaction described here in a static system. In another series of experiments (Manuscript in preperation), guinea pig megakaryocytes were placed on ECM in a flow chamber. After a few hours of exposure to the flowing medium, elongated strips of cytoplasm were formed by the flow, later detaching themselves and were carried downstream. These could be termed proplatelets. One must not forget that these experiments (in the present paper) were static in nature, and therefore there was no incentive for the megakaryocytes to form large chunks of cytoplasm, but were able to proceed to fragmentation in-situ.

M. Tavassoli: This association may explain why in the marrow megakaryocytes are preferentially located in the subendothelial region of marrow sinus endothelium as observed by Lichtman et al. (ref. 19).
Authors: Yes, we would certainly concur with this. If we attribute "migratory" properties to the megakaryocytes then they are going to adhere preferentially to the subendothelium. Carrying this one step further we could hypothesize that this adhesion activates them and then they can proceed to fragmentation after having been swept along by the blood stream to the lungs.

J. Breton-Gorius: Are immature megakaryocytes able to spread and to give long filopodia? If this occurs in megakaryoblasts with minimal maturation this formation of filopodia on ECM does not correspond to the long cytoplasmic processes observed before the platelet shedding by in vivo mature megakaryocytes.
Authors: As we commented above, in answer to Dr. Tavassoli's question, the identification of young megakaryocytes on SEM is difficult, but despite that fairly small megakaryocytes were seen to adhere and form filopodia. We would agree that these filopodia are not the long cytoplasmic processes from which platelet shedding occurs, according to some authors because, as one can see (fig. 8) there were no marked filopodia nor spreading as a prerequisite for fragmentation, a fact we refer to in our discussion about platelet formation.

J. Breton-Gorius: Leven & Nachmias (text ref. 15) showed that the flattening of megakaryocytes was

blocked by cytochalasin and not by colchicine. One can ask whether colchicine has an effect on the production of filopodia since the depolymerization of microtubules in megakaryocytes in short term cultures which were exhibiting long cytoplasmic processes triggered their retraction as shown by Radley and Haller (Blood 1982, (60)213).
Authors: One can conclude that two seperate mechanisms bring about flattening on the one hand, and filopodia retraction on the other. In this series we did not look at the mechanisms as we did not wish to add an additional variable which may have influenced the fragmentation.

D.B. Warheit: Can the authors confirm that the fragmentation products indeed are platelets? Have they looked for platelet granules using TEM?
Authors: We have been unable, as yet, to perform TEM. We base our conclusions on the functional and morphological behavior of the fragments when compared to platelets from peripheral blood.

D.B. Warheit: How do you reconcile internal fragmentation with the figure 8?
Authors: We believe that the photomicrograph shows a megakaryocyte in the process of fragmenting, and that this process is taking place whilst the general shape of the megakaryocyte is still preserved. The time frame from this stage until complete fragmentation is unknown, but is probably a matter of minutes, as this event is relatively a rare finding whilst fragmentation is common under these circumstances.

D.B. Warheit: What was the basis for using glutaraldehyde-fixed ECM and what is the source of the inhibitory effect on megakaryocyte adherence?
Authors: We chose glutaraldehyde as one method of denaturating the surface proteins of the ECM thereby changing their secondary and tertiary structure. This denaturation renders the adhesive glycoproteins of the matrix non-sticky to cells.

D.B. Warheit: Do the 25% of flattened cells represent a different population from the fragmented cells?
Authors: If by "different" you mean non-megakaryocytes, the answer is no, because only megakaryocytes were counted. It is possible that these represented cells at a different stage of development or maturation (nuclear and/or cytoplasmic). It is believed that only the most mature megakaryocytes produce platelets. There was no reason to assume that 100% of the cells would be equally mature and react in an identical manner.

D.B. Warheit: Is this a relevant model to platelet formation in-vivo?
Authors: As we have explained previously, we feel that this is a relevant model. As the complete mechanism and location of platelet formation has not yet been proven, confirming that these fragments are indeed platelets should help in clarifying this point.

K.M. Young: Are specific markers for endothelial cells available so that bone marrow endothelium could be isolated and evaluated in studies such as these? It would seem particularly relevant to study endothelium from that tissue if it is possible.
Authors: It is not possible at present, to the best of our knowledge, to isolate bone marrow endothelial cells for physical reasons such as the smallness of the sinusoids and disruption upon sampling, as well as the problems of overgrowth by other cells in culture.

K. M. Young: The entry of megakaryocytes into the vascular system as a routine phenomenon is controversial. Certainly, megakaryocyte cytoplasm contacts the sinus wall and extends into the sinus, but statements implying that the whole cell enters the sinus should be avoided.
Authors: We would beg to differ on this point. Many authors have described and discussed in detail the presence of whole MK's in the venous blood, in numbers approaching the theoretical figure for total platelet production. We do not feel that this is a controversial point, only the fate of those cells.
Following papers, as well as many other authors, have addressed this point.

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