Megakaryocyte Interaction With Subendothelial Extracellular Matrix Is Associated With Adhesion, Platelet-Like Shape Change, and Thromboxane A2 Production

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We have examined the morphological and secretory behavior of rat and guinea pig megakaryocytes exposed for up to 24 hours to extracellular matrix produced by cultured bovine endothelial cells. By phase-contrast microscopy of living cells and in more detail by scanning electron microscopy, the megakaryocytes showed a nonreversible adherence, an extensive formation of filopodia around the periphery like the rays of the sun, and a tendency toward flattening. These filopodia were generally linear with attenuated tips and were larger than, but resembled the filopodia of, rat or guinea pig platelets exposed to this extracellular matrix. In contrast, isolated megakaryocytes on glass or on uncoated plastic surfaces did not show these responses: adherence, in the face of gentle agitation before fixation, was minimal, with rare filopodia and no flattening. Megakaryocytes that interacted with the extracellular matrix produced significant amounts of thromboxane A2, but this did not occur on uncoated surfaces and could not be attributed to other contaminating cells in the megakaryocyte suspensions. The appearance in megakaryocytes of these typical platelet responses indicates that megakaryocytes acquire the functional capabilities of platelets by the synthesis and assembly of platelet substances and organelles. Thromboxane production by megakaryocytes stimulated by the extracellular matrix is a readily quantifiable measure of this capacity.

CULTURED BOVINE endothelial cells produce an extensive underlying extracellular matrix (ECM), which closely resembles the vascular subendothelial basal lamina in its origins and chemical composition. This ECM contains collagens (mostly type III, with smaller amounts of type I as well as IV and V), proteoglycans (mostly heparan and chondroitin sulfates), laminin, fibronectin, and elastin. This composition is characteristic of endothelial cells from different species and is somewhat unlike that of basement membranes from epithelial cells. The bovine ECM supports the attachment, proliferation, and differentiation of cell types of various species of origin.

We have previously reported the use of this ECM as a model to study the interaction of platelets with vascular subendothelium. The ECM was found to induce human platelet adhesion, aggregation, thromboxane A2 formation, and the release reaction in a manner similar to that observed with human platelets flowing over mechanically denuded rabbit aorta or over subendothelium from human arteries or veins.

It has become apparent that megakaryocytes synthesize and assemble platelet components and organelles, are capable of shape change and degranulation, and respond with a prompt but reversible flattening reaction on stimulation with known platelet agonists (adenosine diphosphate [ADP], thrombin, and arachidonic acid). However, unlike platelets, megakaryocytes do not adhere to glass or collagen-coated surfaces or undergo shape change when exposed to them. Thromboxane A2 formation has also been reported in megakaryocytes in response to the addition of arachidonic acid. In the present study we examined the morphological and secretory behavior of rat and guinea pig megakaryocytes and platelets stimulated by interaction with the isolated subendothelial ECM. Our results extend these earlier observations that platelet-like responses can be expressed in megakaryocytes prior to the actual shedding of the platelets.

METHODS AND MATERIALS

Preparation of ECM-coated plates. Cultures of bovine corneal endothelial cells were established as described previously. Stock cultures were maintained in Dulbecco's modified Eagle's medium (MEM, H-16) supplemented with 5% fetal calf serum and gentamicin (50 µg/mL) at 37°C in 10% CO2-humidified incubators. Cells were passaged weekly at a split ratio of 1:64 and fibroblast growth factor (FGF, 100 ng/mL) was added on alternate days during the phase of active cell growth. The FGF was purified from bovine brain as described previously. For the preparation of ECM-coated plates, cells were plated at an initial density of 4 x 10⁶ cells per 35-mm dish (Falcon Inc, Oxnard, Calif) or at 1 x 10⁶ cells per flat-bottomed microwell (96 well plate, Falcon, Inc) and 5% Dextran T-40 (Pharmacia Fine Chemicals, Uppsala, Sweden) was included in the growth medium. Six to eight days after reaching confluence, the cultures were washed once with phosphate-buffered saline (PBS) and exposed to 0.5% Triton X-100 (Fisher Scientific, Pittsburgh) in PBS for 30 minutes with gentle shaking. The cell layer then dissolved, leaving the underlying ECM intact and firmly attached to the tissue culture dish surface. The remaining nuclei and cytoskeletons were removed by a two- to three-minute exposure to 0.025 N NH₄OH, followed by four washes with PBS.

Megakaryocyte isolation and platelet preparation. Marrow tissue was harvested mechanically from the femora and humeri of rats or guinea pigs into calcium- and magnesium-free Hanks' solution containing 3.8% sodium citrate, 10⁻³ mmol/L of adenosine and 2 x 10⁻³ mmol/L of theophylline (CATCH medium). Single-cell suspensions were made by pipetting minced small pieces of the gelatinous marrow tissue in the same medium. These marrow cell suspensions were progressively enriched for megakaryocytes by...
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a discontinuous density gradient centrifugation, a velocity sedimentation, and a second velocity sedimentation.\(^{1,17}\) Resulting suspensions had 40% to 75% purity by number and yields of 3 \(\times 10^8\) to 1.2 \(\times 10^8\) megakaryocytes, depending on the species and number of animals used. Platelets were prepared from citrated blood (3.2% sodium citrate) obtained by cardiac puncture from the same animals from which the megakaryocytes were isolated. Platelet-rich plasma (PRP) was prepared as previously reported.\(^9\)

Megakaryocyte and platelet reactivity with ECM. Isolated megakaryocytes were incubated with Dulbecco’s MEM H-16 with 2.3% bovine serum albumin (Miles Laboratories, Kankakee, Ill) instead of serum, and with penicillin (50,000 U/mL) and streptomycin (\(\mu g/mL\)).\(^{12}\) Most cultures were done at 25,000 to 30,000 megakaryocytes per milliliter of medium, sometimes as high as 250,000 per milliliter. Immediately after isolation they were placed into 35-mm-diameter Petri dishes on in flat-bottomed 5-mm-diameter microtiter wells coated with ECM for up to 24 hours.

Control experiments were carried out in vessels of the same manufacture that were not coated with ECM. PRP from the same donor animals were incubated in similar vessels. Incubations were carried out in 5% CO\(_2\) in air in humidified incubators at 37°C.\(^{12}\) These conditions maintained a stable pH of 7.2 to 7.3 in the cell suspensions for up to 24 hours.

Thromboxane A\(_2\) (TXA\(_2\)) formation. Formation of TXA\(_2\), was measured by assay of residual TXB\(_2\), since TXA\(_2\), rapidly and spontaneously transforms into the stable product TXB\(_2\). Aliquots of 0.1 mL from each culture dish or well were quenched with 0.4 mL of indomethacin (10 \(\mu M\), Sigma Chemical Co., St Louis) in PBS. These suspensions were centrifuged at 10,000 g for one minute, frozen in liquid N\(_2\), and stored at –70°C until assayed by radioimmunoassay, as previously described.\(^9\) Highly specific anti-TXB\(_2\), antiserum was kindly supplied by Dr B.B. Weksler (Cornell University Medical College, Ithica, NY) and \(^3\)H-TXB\(_2\), was obtained from New England Nuclear, Boston. Results are expressed as amounts of TXB\(_2\) produced.

Morphological studies. Living cells in culture were examined in situ with an Olympus inverted phase microscope, at a magnification of 100 to 200. Petri dishes were prepared for scanning electron microscopy (SEM) by tilting the dishes and gently aspirating the medium three times in buffered (pH 7.3) 1% glutaraldehyde down the side of the dish until the bottom was completely immersed. The fixed cells were further prepared for examination as before.\(^9\) The samples were gold-palladium sputter-coated in situ and the entire 35-mm Petri dishes were 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 are adherent to the surface or are merely resting there without attachment, we used a simple test for adhesive-ness. At various times after placing megakaryocyte suspensions into 35-mm Petri dishes, the number of megakaryocytes per field were counted in four fields in the central portion of each dish, at a 200 times magnification. The dishes were tilted to 45° three times in three seconds; following the third time, the dish was kept at that angle and medium was gently aspirated. Four similar fields were immediately counted. The megakaryocytes remaining attached after this relatively reproducible force were judged to have become adherent and the percentage of megakaryocytes adhered was calculated as the ratio of the second count to the first for each dish. A potent inhibitor of platelet aggregation, the stable prostacyclin analogue, B-thio-imino-prostacyclin (Hoe 892), was tested for its effect on megakaryocyte adhesion. This drug was a gift of Hoechst AG, Frankfurt, West Germany.

RESULTS

Morphology of platelet and megakaryocyte interaction with plastic and ECM-coated surfaces. Guinea pig or rat platelets did not adhere to or aggregate on the surface of plastic dishes and, by phase microscopy and SEM, did not undergo any shape change. On ECM-coated plastic dishes, the platelets promptly adhered and underwent typical morphological transformation (Fig 1A through C). From the initial contact of platelets with the ECM, adherence was mediated by the extension of fine cytoplasmic processes, often called spikes or, more properly, pseudopods.\(^{27}\) but here termed filopodia after Leven and Nachmias.\(^{19,20}\) With time, the frequency of platelet interaction and surface contact with the ECM increased, from thin filopodia (Fig 1A and B; 0.10 to 0.15 \(\mu m\) wide and 1 to 3 \(\mu m\) long) to a stage at which these processes began to widen (Fig 1C, to 0.3 to 0.35 \(\mu m\)). Further spreading of the platelet was accomplished by extension of a cytoplasmic veil, filling the spaces between the filopodia (Fig 1D). As flattening progressed (Fig 1D and E), all platelets developed further extensions (Fig 1E, to 1 to 3 \(\mu m\) long and 0.10 to 0.13 \(\mu m\) wide), which formed extensive interdigitations with those of other nearby platelets (not shown).

These results are very similar to those of platelets interacting with glass surfaces, as reported by Allen et al.\(^{27}\)

When megakaryocytes were incubated in plastic vessels without ECM and the living cells were examined at various time periods up to 24 hours by phase-contrast microscopy, the megakaryocytes retained their characteristic spherical shape and bumpy appearance (Fig 2).\(^{17,28-30}\) No flattening or changes in contour were seen in the first hour of observation. After several hours, about 10% of the megakaryocytes initially developed wide filopodia and later attenuated filopodia, without adhering, as previously described.\(^{12}\) When such dishes were prepared for SEM, it was found that the most gentle aspiration of the culture medium and replacement with fixative resulted in the washing away of almost all of the megakaryocytes from the plastic surface. Examination by SEM showed that, of the few megakaryocytes remaining, only minimal filopodium formation could be seen (Fig 2).

In contrast, when megakaryocytes were incubated in dishes coated with ECM, all but a few megakaryocytes remained adherent, even after more vigorous washing of the dishes. In addition, the megakaryocytes frequently underwent a shape change, with formation of filopodia and concentric flattening (Fig 1F through J), very much like the changes that occurred in the platelets interacted with ECM (Fig 1A through E). Filopodia appeared, in apparent contact with the ECM, on approximately half of the megakaryocytes. These processes generally extended perpendicular to the perimeter of the main body of the cell, with a somewhat uniform width of 1.0 to 1.5 \(\mu m\) (Fig 1F through J). Filopodia originated either directly from the cell body (Fig 1F) or from the broad spreading veil of cytoplasm on the dish surface. The attenuated tips, occasionally branching (Fig 1H), gradually extended as far as 20 \(\mu m\) from the main cell body. Fig 1F, H, and I give some perspective on the broad veil of spreading adherent cytoplasm beneath the rounded cell body. The increasing area of cytoplasmic contact with the
Scanning electron micrographs of guinea pig platelets (A through E) and megalakaryocytes (F through J) at approximately parallel stages of response to ECM-coated surfaces. Because entire dishes were placed into the specimen chamber of the microscope, there was no removal of cells or ECM, and the actual sites of the interactions are seen. Platelets were exposed to ECM for 20 minutes without agitation in a 37 °C incubator. Megakaryocytes were incubated for 18 hours under the same conditions. White bars indicate 1 μm in A through E, 10 μm in F, G, I, and J, and 2 μm in H. (A) Initial contact of platelet; rounded cell body above but touching ECM surface and beginning formation of narrow filopodia (original magnification ×11,700; current magnification ×7,650). ECM material is seen as granular at high magnifications (A through E), or cratered at lower magnifications (F through J). Plastic substratum is seen as completely smooth and dark. (B) Increase in number and length of filopodia; cell body lowered or pulled closer, with greater cytoplasmic contact with surface (original magnification ×16,000; current magnification ×10,400). (C) Widening of existing filopodia (original magnification ×13,000; current magnification ×8,450). (D) Further widening of filopodia and filling in between them by centrifugal extension of the central cytoplasm, forming a thin veil (upper cell). Further flattening (lower cell) occurs by further filling in cytoplasm between filopodia, giving an almost rounded cell (original magnification ×11,700; current magnification ×7,605). (E) Further extension of narrow filopodia from the completely flattened platelet. These filopodia often interdigitate with those of adjacent platelets at a comparable stage (original magnification ×11,700; current magnification ×7,605). (F) Two rounded megakaryocytes adherent to the ECM surface show filopodia beneath the rounded cell body (original magnification ×500; current magnification ×325). (G) In the cell on the right, the cytoplasmic contact with the surface has increased and the entire circumference of the cell has fairly uniform filopodia extending from it. Megakaryocyte filopodia were generally 1.0 to 1.5 μm thick at the midpoint and 8 to 20 μm in length (original magnification ×1,000; current magnification ×650). (H) Details of the filopodia, often branching, may be seen (original magnification ×3,000; current magnification ×1,950). (I) The flat cytoplasmic base, in contact with the surface, has increased in area beyond the cell margin. This was the most common appearance of megakaryocytes incubated on ECM for 18 hours (approximately 50%) (original magnification ×1,260; current magnification ×819). (J) Complete flattening occurred in about 10% to 30%, apparently by further widening of existing filopodia, as can be seen at several places around the periphery, most notably at 2, 5, and 6 o’clock (original magnification ×1,260; current magnification ×819).
ECM seemed to redistribute the redundant cell surface membrane, giving it a smoother appearance (Fig 1F through I) than the typical bumpiness shown in Fig 2. In 10 to 30% of the megakaryocytes, spreading of the cytoplasmic veil progressed in a concentric fashion until only the nuclear lobes adhered, filopodium formation, and flattening started to appear within five minutes of incubation at 37 °C, progressively increased in frequency, and appeared to be irreversible. Adhesion was measured in a series of ECM-coated plastic dishes to which equal aliquots of freshly isolated megakaryocytes, prewarmed to 37 °C, were added. By this simple adhesion test, approximately 40% of the megakaryocytes were adherent at five minutes, 50% at ten minutes, 65% at 15 minutes, 80% at 20 minutes, and 90% at 30 minutes and after overnight incubation. Incubation with the prostacyclin analogue Hoe 892, at concentrations up to 1 μg/mL had no inhibitory effect on megakaryocyte adhesion over one hour of observation. Because the sedimentation rate of megakaryocytes was >1 mm/min and the height of the megakaryocyte-containing incubation medium in these experiments was approximately 1 mm, megakaryocytes can fall to the dish surface in no more than one minute. Therefore, contact with the surface was not rate limiting in the development of the adherence reaction.

By phase-contrast microscopy, extension of filopodia like that in Fig 1F could be seen as early as five minutes after the start of incubation. More extensive formation of filopodia, as in Figs 1G through H, occurred sometimes within one hour, but became more common over several hours. Flattening likewise was sometimes apparent after one hour, and by phase-contrast microscopy was usually but not always accompanied by the development of at least short filopodia. The use of Hoe 892 did not seem to prevent the initiation of filopodium formation or flattening over one hour of observation. Flattening on ECM also was more common over several hours, but it was not possible to determine by phase-contrast microscopy whether all megakaryocytes with filopodia progressed to actual flattening. We have avoided use of the term “spreading” to refer either to the development of extensive filopodia or to flattening, which are observationally specific and distinct, but have used it only in a general sense of increasing cytoplasmic contact with the substrate.

We also carried out similar studies using megakaryocytes isolated 24 hours prior to exposure to the ECM stimulus, as was the routine practice of Leven and Nachmias in their studies of agonist effects, by first maintaining the cells overnight in glass vials. The culture medium was replaced with fresh medium before placing the megakaryocytes on the ECM. The differences in megakaryocyte response were slight. A slight decrease in the percentage of megakaryocytes adhered, to a maximum of 80%, is consistent with the degree of cell death noted in this interval. Filopodium formation and flattening appeared to be more frequent in the first hour of observation, perhaps because of greater maturity of the pre-incubated megakaryocytes, but the degree of difference was not quantitated.

**Thromboxane B₂ production.** The quantities of TXB₂ produced by either guinea pig or rat PRP (1.2 to 1.6 × 10⁸ platelets per milliliter) on ECM for 60 minutes are in the range of 27 to 30 ng/mL. A smaller but significant amount of TXB₂ was produced by megakaryocytes on ECM, whereas negligible quantities were found in the medium of megakaryocytes incubated in plain plastic dishes (Fig 3). Control studies were performed on marrow cell populations from which the megakaryocytes were mostly removed in the second phase (velocity sedimentation) of the isolation process. These cells, referred to as waste cells in Fig 3, are mainly mononuclear with few granulocytes or red cells and no platelets. The TXB₂ levels from a much larger pool of these waste cells incubated for the same time period on ECM were very low and could be attributed to a small number of megakaryocytes present in this population. The coefficient of variation of TXB₂ production by megakaryocytes was 12.5% (n = 25, on ECM-coated microwells).

The time course of TXB₂ appearance in the medium of megakaryocytes incubated on ECM is shown in Fig 4. The TXB₂ generation was prompt, with half of the amount found after overnight culture having been produced within one hour. A similarly shaped curve was found for platelet TXB₂ levels measured in the medium after megakaryocytes were incubated for 20 hours in plastic uncoated or ECM-coated dishes. Each value is the mean of two assays. Megakaryocytes: a: 24 × 10⁶ megakaryocytes, 41 × 10⁶ total cells; waste cells: b: 3 × 10⁶ megakaryocytes, 190 × 10⁶ total cells.

**Fig 3.** TXB₂ levels measured in the medium after megakaryocytes were incubated for 20 hours in plastic uncoated or ECM-coated dishes. Each value is the mean of two assays. Megakaryocytes: a: 24 × 10⁶ megakaryocytes, 41 × 10⁶ total cells; waste cells: b: 3 × 10⁶ megakaryocytes, 190 × 10⁶ total cells.
secretion of thromboxane B₂, but the maximum level for platelets was reached in 90 minutes (data not shown).

The secretion of TXB₂ was generally proportional to the number of megakaryocytes present in Petri dishes coated with ECM (Fig 5). Different concentrations of cells gave the same net yield per 10⁴ megakaryocytes. However, the manufacture of an ECM coat in microwells with flat bottoms allowed a determination that the available ECM surface area was apparently rate-limiting for the stimulation of TX generation by megakaryocytes.

DISCUSSION

In the present study we have found two significant functional similarities between megakaryocytes and their progeny, the platelets. Megakaryocytes attached tightly to subendothelial ECM and concomitantly produced TXA₂. Adhesion and secretion occurred within the first hour, and in the next few hours a spreading typical of platelets occurred, with the formation of large numbers of long fibopodia extending from a veil of cytoplasm. The filopodia extended radially from the margin of the cell body in the plane of the surface and seemed to adhere tightly to the ECM. In the process of increasing membrane contact with the ECM surface, the typical megakaryocyte surface irregularity (bumpiness) disappeared, and with the marginal filopodia, the cells acquired

the graphic appearance of the rays of the sun. This common interaction with the ECM, which occurred in about 50% of the cells, has not been observed previously. A small number of cells (about 10%) continued to extend their cytoplasmic veil and flattened to the point at which only the nucleus extended above the plane of the ECM (Fig 1J). The adhesion of megakaryocytes to the ECM and their morphological transformations did not reverse under the conditions described.

Several investigators have described megakaryocyte secretion of H₂-serotonin, adenosine triphosphate (ATP), or TXB₂ in response to platelet agonists. These cellular transformations were observed when the stimulating agents were added to suspensions of isolated megakaryocytes. However, when megakaryocytes were exposed to glass surface or plastic tissue culture dishes coated with different types of collagen or fibronectin, they did not display the characteristic responses of platelets to these surfaces; neither adhesion nor shape was observed. 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 a combination of collagen and fibronectin could induce megakaryocyte flattening.

Although collagen and fibronectin are major components of the ECM, it is possible that other matrix components and/or the tertiary structure of this naturally produced basal lamina are involved in the multiple radial long filopodium formation that we observed in the present study.

Megakaryocyte flattening induced by soluble platelet agonists was reversible following the addition of agents that raise intracellular cyclic adenosine monophosphate (AMP). The megakaryocyte interaction with ECM was neither blocked nor reversed by a stable prostacyclin analogue (Hoe 892) at a concentration 100-fold higher than that needed to inhibit platelet aggregation. This is not surprising since the amounts of prostacyclin required to inhibit platelet adhesion to vascular subendothelium are much greater than those required for the inhibition of platelet aggregation. Similarly to platelets, the typical shape change of megakaryocytes is associated with the activation of contractile proteins. Studying the immunofluorescent localization of actin, myosin, filamin, and actinin. Leven and Nachmias demonstrated rearrangement of these proteins and filament formation in the flattened cytoplasm of stimulated megakaryocytes.

Moreover, megakaryocyte flattening was blocked by inhibiting actin polymerization with cytochalasin B, but not with colchicine, indicating that the actomyosin system rather than the microtubular apparatus is implicated in megakaryocyte shape change.

SEM of megakaryocytes in situ or in suspension characteristically shows most of the cell surface to be covered with rounded somewhat uniform projections often called “grapelike” or blebs (Fig 2). Several investigators have interpreted these blebs as young platelets about to be “budded off.” However, the fact that these projections disappear following megakaryocyte attachment to ECM or following stimulation with other platelet agonists implies that the grapelike appearance is due to excess surface membrane that is
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redistributed during megakaryocyte shape change. Furthermore, by transmission electron microscopy, these cellular projections do not contain the future platelet organelles and may thus be regarded as transient ectoplasmic blebs.\textsuperscript{18}

The present study extend and reinforces the concept of functional similarity between megakaryocytes and platelets. However, the time course of megakaryocyte adhesion and shape change induced by ECM was somewhat slower than that of platelets observed under the same conditions. TXA\textsubscript{2} synthesis by megakaryocytes was likewise slower than by platelets, and the absolute quantities were less. (The amount from 10\textsuperscript{6} megakaryocytes was only as much as that from 10\textsuperscript{5} platelets, and most other platelet substances, such as alpha granule contents, seem to be found in megakaryocytes and platelets in a ratio of approximately 1,000:1.\textsuperscript{32} Thus, megakaryocyte secretion was only 1% of that expected on a mass basis. This relative disparity in TXA\textsubscript{2} production between megakaryocytes and platelets may be explained by the differing ratio of activated surface area to volume, which is much higher in the platelets. It could also result from incomplete maturation of organization of surface membrane components in the megakaryocyte. This difference in TXA\textsubscript{2} production cannot be attributed to the fact that platelets aggregate on ECM and megakaryocytes do not, since we have recently found that platelets from patients with Glanzmann's thrombasthenia adhere to the ECM and secrete normal amounts of TXA\textsubscript{2} despite the complete absence of aggregation.\textsuperscript{33}

By the synthesis and assembly of platelet substances and organelles, megakaryocytes acquire the functional capabilities of platelets, including adherence, shape change, spreading, and secretion. The physiologic significance of megakaryocyte interaction with a subendothelial basal lamina is still unknown but might be related to egress of marrow megakaryocyte into vascular sinusoids.\textsuperscript{24} Thus, ECM-induced TXA\textsubscript{2} production is a readily quantifiable measure of megakaryocyte activation and is suitable for the study of the origins of abnormal platelet responses in disease states and for assays of drugs that might modify these conditions.

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