Platelets Generated In Vitro From Proplatelet-Displaying Human Megakaryocytes Are Functional

By Esther S. Choi, Janet L. Nichol, Martha M. Hokom, Alex C. Hornkohl, and Pamela Hunt

An in vitro culture system demonstrating the transitions from megakaryocyte progenitors to functional platelets is described. CD34+-selected cells from normal human peripheral blood are cultured under conditions that promote megakaryocyte formation. After 8 to 11 days, enriched populations of mature megakaryocytes are replated under conditions that favor the development of proplatelets. Proplatelets express the platelet-specific proteins, glycoproteins Ib and IIb (GPIib and GPIIb), and fibrinogen and also contain microtubule cells equal in size to those found in plasma-derived platelets. In addition, proplatelets have ultrastructural features in common with plasma-derived platelets. Platelet-sized particles from the proplatelet culture supernatants are examined. Ultrastructurally, these particles are identical to plasma-derived platelets. Functionally, these culture-derived platelets aggregate in response to both thrombin and adenosine diphosphate (ADP) plus fibrinogen. This aggregation is specifically inhibited by the addition of a function-blocking anti-GPIIbIIIa antibody. Culture-derived platelets stimulated with agonists also express the activation-dependent antigens P-selectin and functional fibrinogen receptor. This is the first description of an in vitro culture system that sequentially demonstrates megakaryocyte growth, development, and platelet production.

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PLATELETS ORIGINATE from megakaryocytes during the terminal differentiation phase of thrombopoiesis. However, the precise mechanism by which platelets are shed from megakaryocytes is not fully understood. Studies of this process are hampered by the low frequency of megakaryocytes in bone marrow as well as by a lack of in vitro systems in which platelet shedding reproducibly occurs. Theoretic models of platelet shedding have been developed from observations of fixed cells. Megakaryocytes displaying membranous structures outlining platelet-fields form the basis of the demarcation membrane model of platelet shedding. Observations of long cytoplasmic processes emanating from megakaryocyte cell bodies have led to the proplatelet concept. Proplatelets, dependent on microtubules for stabilization, display constrictions along their lengths that define platelet-sized areas. The areas between the attenuation points contain platelet-specific organelles and proteins. Micrographs of bone marrow fixed in situ show proplatelets projecting through the endothelial cell layer of marrow venous sinuses into the circulation. It has been postulated that the circulatory shear force within the marrow, or possibly the lung, aids in the fragmentation of proplatelets into platelets.

The study of proplatelet development has been advanced through the use of tissue culture systems that permit this differentiation process to occur in vitro. Although these culture systems have been useful for the study of proplatelet development, they have been of limited value for the study of platelet fragmentation as observations of platelet-like particles in these cultures are reported as rare events and in no case has the identity and function of the particles been established. Furthermore, culture systems that rely on rodent or bovine megakaryocytes may be of limited value to the study of human thrombopoiesis.

This report describes a model system in which functional platelets are generated in vitro from human megakaryocytes. Platelet formation appears to occur via proplatelet intermediate structures. The system exploits earlier observations that human megakaryocytes can be generated from peripheral blood leukocytes stimulated with plasma from thrombocytopenic animals. In the present study, the frequency of culture-generated megakaryocytes was improved by preenriching the leucocyte population for CD34+ megakaryocyte progenitors. Mature megakaryocytes that appear within 8 to 11 days of culture are then available for studies of proplatelet development and, subsequently, for platelet fragmentation. This in vitro system is relevant to clinical conditions that affect the last phase of thrombopoiesis, platelet formation and release.

MATERIALS AND METHODS

Isolation of CD34+ Progenitor Cells From Peripheral Blood

Written informed consent was obtained from donors of leukapheresis units, normal AB plasma, and plasma-derived platelets. Leukapheresis units from a pool of normal healthy donors committed to the program were purchased from HemaCare (LeuKaPAK, Sherman Oaks, CA). Peripheral mononuclear cells were isolated by Ficoll-Paque (Pharmacia, Piscataway, NJ), rinsed, and adherence-depleted overnight. Nonadherent cells were collected and fractionated with countercflow centrifugal elutriation in a Beckman (Fullerton, CA) J2-M1 centrifuge with a JE-6B elutriation rotor to enrich for megakaryocyte progenitors. Rotor speed was 2,020 rpm, and the flow rate was increased by 0.5 mL/min from 8.5 mL/min to 13 mL/min over 63 minutes. Cells of interest were collected in 50-mL fractions at flow rates from 11 to 13 mL/min and frozen in 90% fetal bovine serum (FBS) and 10% dimethylsulfoxide (DMSO) in the cell freezer (Gordinier 9000) using Cryopack Software Version 3.2 (Gordinier Electronics Inc, Roseville, MI) until use. In some experiments, leukapheresis units were processed without elutriation. Mononuclear cells were isolated by Ficoll-Paque in Hank’s buffered saline solution (HBSS) containing phenol red (GIBCO, Grand Island, NY). After rinsing in HBSS, cells were either processed immediately or after overnight incubation in Iscove’s Modified Dulbecco’s medium (IMDM) and 10% FBS at 4°C.

From AMGEN, Inc Thousand Oaks, CA. Submitted March 17, 1994; accepted September 13, 1994. Address reprint requests to Pamela Hunt, PhD, AMGEN, Inc, Amgen Center, mall stop T-1A-206, Thousand Oaks, CA 91320. The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. section 1734 solely to indicate this fact.

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CD34+ progenitor cells were isolated using a magnetic cell sorting system, Mini-MACS (Miltenyi Biotec, Auburn, CA) by one of two methods. One method used anti-CD34–biotinylated antibody from AMAC (Westbrook, ME) followed by streptavidin microbeads (Miltenyi Biotec). The other method used the CD34 isolation kit (Miltenyi Biotec) in accordance with the manufacturer’s recommendations. When elutriated, frozen/thawed cells were processed, an average of 1.4 × 10^6 CD34+ cells were obtained per leukapheresis unit, with a range of 2.0 × 10^6 to 4.0 × 10^6 (n = 9). When nonelutriated, fresh cells were processed, an average of 4.8 × 10^6 CD34+ cells were obtained per leukapheresis unit, with a range of 2.9 × 10^6 to 6.2 × 10^6 (n = 4). Elutriated cell populations contained fewer CD34+ cells than nonelutriated cell populations as they had been preenriched for megakaryocyte progenitors. The wide range in the number of CD34+ cells obtained was attributed to donor variation.

The purity of CD34-selected cells was determined for each isolation by using a combination of anti-CD34 antibodies (HPCA-1 and HPCA-2, each at 1:10 in phosphate-buffered saline (PBS); Becton Dickinson, Lincoln Park, NJ) and the HistoMark Streptavidin–β-Gal system (Kirkegaard and Perry Labs, Gaithersburg, MD). Immunopositive blue cells were counted microscopically. On the average, cells selected with anti-CD34 biotinylated antibody followed by streptavidin microbeads were about 40% CD34+ (range, 15% to 93%; r = 19). Similarly, cells selected with the CD34 isolation kit averaged about 50% CD34+ (range, 22% to 90%; n = 8). When CD34-selected cells were reapplied to Mini-MACS columns, the percentage of CD34+ cells increased to an average of 90% (range, 83% to 93.5%; n = 4). The percentage of CD34+ cells obtained by this staining method and by flow cytometric analyses were virtually identical in every case studied.

### Culture of Human Megakaryocytes

CD34+ enriched cells were stimulated to form megakaryocytes in the culture system described by Mazur et al. All of the reagents were purchased from Gibco unless indicated otherwise. Briefly, the cells were plated at 5 × 10^5 cells per milliliter in growth medium (Meg GM) that consisted of IMDM with 1% penicillin/streptomycin/glutamine (P/S/G), 1% sodium pyruvate, 1 mm nonessential amino acids, 0.1 mmol/L 3-mercaptopropionin, 2 mg/mL L-asparagine, and 0.2% deionized bovine serum albumin (BSA; Calbiochem, San Diego, CA). Fresh Meg GM was prepared every 2 weeks. Meg GM was supplemented with 10% normal human platelet-poor heparinized (33 U/mL) AB plasma, and 10% aplastic canine serum. Some experiments were conducted in the absence of 10% AB plasma to determine the necessity of plasma for proplatelet induction. The cultures were examined daily for emergence of proplatelets. A megakaryocyte bearing one or more cytoplasmic processes was considered a proplatelet-displaying megakaryocyte. The processes were at least twice the length of the cell body diameter. The percentage of megakaryocytes displaying one or more cytoplasmic processes was determined by visual examination of video prints taken of the culture wells.

### Detection of Platelet-Specific GPIb/IIb/IIIb on Proplatelet Membranes

Megakaryocytes displaying proplatelets were fixed in situ by adding glutaraldehyde (1.6% final concentration) to the culture wells. The culture plates were then centrifuged at 800 rpm for 15 minutes. The cells were rinsed and blocked with 10% normal goat serum (NGS) for 20 minutes at room temperature. Anti-GPIb/IIb/IIIb cocktail and the HistoMark Streptavidin–β-Gal system was used as described above.

### Detection of Fibrinogen Within Proplatelets by Indirect Immunofluorescence

Megakaryocytes displaying proplatelets were stabilized with 0.38% formaldehyde and 100 mmol/L EDTA and sedimented onto silanized slides (Oncor, Gaithersburg, MD) by cytocentrifugation. These cells were fixed for 20 minutes in 4% paraformaldehyde and 1% Triton-X 100, rinsed three times in PBS, and then blocked for 20 minutes in 10% FBS-PBS at room temperature. Mouse anti-fibrinogen monoclonal antibody (MoAb; American Diagnostics, Greenwich, CT) was used at 1:50 in 10% FBS-PBS for 1 hour at room temperature. Goat anti-mouse–fluorescein isothiocyanate (FITC; Cappel, Durham, NC) was used at 1:1,000 in 10% FBS-PBS for 30 minutes at room temperature. Slides were mounted in Immunumount (Shandon) and visualized with an Olympus BH2 microscope (Olympus, Chatsworth, CA).

### Detection of Microtubules Within Proplatelets by Indirect Immunofluorescence

For indirect staining, megakaryocytes displaying proplatelets were treated as above except that 4% paraformaldehyde, 1% Triton-X 100, and 10% methanol was used as a fixative. Cells were blocked with 5% NGS in PBS for 20 minutes at room temperature. The anti-tubulin antibody cocktail (anti-α-tubulin MoAb, Amersham, Buckinghamshire, England; anti-β-tubulin MoAb, Amersham; and rabbit anti-tubulin antibody, Biomedical Technologies, Stoughton, MA, at manufacturers’ recommended dilutions) was incubated with cells at 37°C for 1 hour. Goat anti-mouse IgG-FITC and goat anti-rabbit IgG–FITC (Cappel; at 1:1,900 each) were used as secondary antibodies at 37°C for 30 minutes. Cells were rinsed and mounted in Immumount and visualized with a Nikon MICROPHOT-FXA microscope (Nikon, Irvine, CA).

### Electron Microscopy of Proplatelets and Platelets

Megakaryocytes displaying proplatelets were stabilized with 0.38% formaldehyde and 100 mmol/L EDTA, centrifuged at 800 rpm for 5 minutes and stained with a modification of Wright-Giemsa stain using Wescor’s automatic cell stainer (Wescor Inc, Logan, UT; model no. 7120). Megakaryocytes were staged according to the criteria of Levine.

### Induction of Proplatelets From Mature Megakaryocytes

The megakaryocyte-enriched population was replated in Meg GM at 5 × 10^6 cells per milliliter in 96-well plates (Falcon no. 3072) supplemented with 10% heparinized human AB plasma but without aplastic canine serum. Some experiments were conducted in the absence of 10% AB plasma to determine the necessity of plasma for proplatelet induction. The cultures were examined daily for emergence of proplatelets. A megakaryocyte bearing one or more cytoplasmic processes was considered a proplatelet-displaying megakaryocyte. The processes were at least twice the length of the cell body diameter. The percentage of megakaryocytes displaying one or more cytoplasmic processes was determined by visual examination of video prints taken of the culture wells.

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FIG 1. Mature megakaryocyte selection, proplatelet development, and presence of platelet-specific proteins detected on the surface and within proplatelets. (A) A representative Wright-Giemsa staining of megakaryocytes after 8 days of culture time enriched by velocity sedimentation (53% megakaryocytes; bar, 40 μm). (B) Phase micrograph of proplatelet development 3 days after replating (bar, 40 μm). (C) Anti-GPⅠb/Ⅰbα antibody staining on proplatelets detected by HistoMark, bright-field micrograph (bar, 15 μm). (D) Control panel for HistoMark, in which the primary antibody cocktail was omitted (bar, 13 μm). (E) Anti-fibrinogen antibody within proplatelets detected by indirect immunofluorescence (bar, 40 μm).

rpm for 15 minutes, resuspended in 1% glutaraldehyde, and allowed to settle on poly-L-lysine coated aclar film for 45 minutes. The cells were rinsed with PBS, treated with 1% OsO₄ for 1 hour, rinsed with PBS, and sequentially dehydrated with ethanol (30%, 50%, 70%, and 90%). Uranyl acetate (2%) was included during the 70% ethanol dehydration step. The cells were infiltrated with 50% L.R. White plastic (EM Sciences, Fort Washington, PA; medium grade) in ethanol at 52°C for 24 hours. Proplatelets were identified under the light microscope on polymerized plastic, sectioned, and viewed under the electron microscope (Phillips 300, Mahwah, NJ).

Platelet Aggregation Studies

**Thrombin aggregation.** Plasma-derived (citrated blood) or culture-derived platelets were rinsed three times with PBS and incubated with or without 0.8 U/mL thrombin (Sigma, St. Louis, MO) at 37°C for 10 minutes. Platelet preparations were then cytocentrifuged onto glass slides at 1,200 rpm for 5 minutes. The slides were air-dried and stained in a slide stainer.

**Adenosine diphosphate (ADP)/fibrinogen aggregation.** Plasma-derived or culture-derived platelets were prepared by gel filtration according to Shattil et al. on Sepharose-2B (Pharmacia) in gel filtration buffer (137 mmol/L NaCl, 2.7 mmol/L KCl, 1 mmol/L MgCl₂, 5.6 mmol/L glucose, 1 mg/mL BSA, and 20 mmol/L HEPES; pH 7.4). For aggregation studies, 1 × 10⁷ platelets were incubated for 5 minutes in 600 μg/mL of human fibrinogen (American Diagnostica, Greenwich, CT) and 2 mmol/L ADP (Calbiochem) in a final volume of 150 μL. Ten microliters of each platelet suspension were pipetted into wells of Terasaki-style microtiter plates (Vanguard International, Neptune, NJ) and fixed with glutaraldehyde (1.6% final concentration) for 15 minutes with low speed centrifugation (800 rpm; Sorvall 6000). Preliminary experiments were performed to determine the optimal concentrations of ADP and fibrinogen necessary to cause
aggregation of platelets at this concentration. Photographs of the platelet aggregates were taken through an inverted microscope.

Inhibition of Platelet Aggregation by Anti-GPIIb/IIIa Antibody

Culture- and plasma-derived platelets were washed by gel filtration. Platelets were diluted to 2,000 platelets per microliter in the gel filtration buffer. To $10^6$ platelets, 50 μg/mL (final concentration) of either isotype control antibody (anti-CD34; AMAC) or anti-GPIIb/IIIa antibody (AMAC; no. 0145) was added and allowed to incubate for 5 minutes at room temperature. 200 μg/ml of Human fibrinogen (200 μg/mL; American Diagnostica) and 900 μmol/L of ADP (Calbiochem) were then added with gentle pipetting and allowed to incubate for an additional 5 minutes. Platelet suspensions were pipetted into microtiter wells to observe for aggregation. Platelets were fixed and photographed as described above. Video prints were taken of each field containing aggregates, and the longest diameter of each aggregate was measured.

Detection of Activation-Dependent Antigens on Platelets

P-selectin. Culture- and plasma-derived platelets were washed by gel filtration. A set of platelets from each population was fixed immediately (resting platelets) with an equal volume of cold 1% paraformaldehyde, pH 7.4, for at least 2 hours. Another set of platelets was stimulated with 1 U/mL thrombin for 1 minute and then fixed as above (activated platelets). Preliminary experiments determined the minimal concentration of thrombin necessary for activation of platelets at this concentration. Fixed platelets were rinsed and incubated with goat anti-mouse-FITC (Cappel; 1:500) for 30 minutes, rinsed, and analyzed for fluorescence intensity by flow cytometry (FACScan Flow Cytometer, Becton Dickinson). The FACScan was calibrated using 2-μm beads (Becton Dickinson). Events were collected, without gating, on a log scale for forward side scatter (FSC) and side scatter (SSC; FSC, E90; SSC, 326 V; fluorescence 1 [FL1], 686 V; FSC threshold, 100 V). An analytical gate was drawn on the FSC/SSC dot plot of resting platelets, which excluded a few large contaminating cells and small debris. The same gate was transposed onto the FSC/SSC plot of the activated platelet populations. The histograms shown represent gated events. For plasma-derived platelets, 50,000 events were collected from resting and activated populations, of which 48,821 and 48,010 respective events were analyzed. For culture-derived platelets, 7,000 events were collected from resting and activated populations, of which 4,068 and 3,503 respective events were analyzed. Specific fluorescence-positive platelets were those with higher fluorescence intensity than seen with the isotype control. Percent specific fluorescence intensity was obtained by comparing specific fluorescence intensity to total fluorescence intensity.

Functional fibrinogen receptor (activated GPIIb/IIIa). Culture-derived and plasma-derived platelets were prepared by gel filtration. ADP stimulation and identification of activated GPIIb/IIIa by the PAC1 antibody was performed according to Shattil et al. Platelets were incubated with 50 μg/mL PAC1-FITC with or without 40 μmol/L ADP and without stirring at room temperature for 15 minutes. Preliminary experiments determined the concentration of ADP necessary for significant activation. The platelets were diluted with equal volume of the gel elution buffer and analyzed by flow cytometry without fixation. Platelets were collected through a live FCS/SSC gate preset on the platelet population within whole blood. This gate excluded white blood cells, red blood cells, and debris. For each histogram, 25,000 gated events were collected.

RESULTS

Proplatelet Induction From Mature Megakaryocytes

CD34-enriched human peripheral blood cells were stimulated to form megakaryocytes by the addition of 10% aplastic canine plasma to Meg GM. After 8 days in culture, 82%, 8%, 7%, and 3% of the megakaryocytes were classified as stage I, II, III, or IV, respectively. After 12 days in culture, the relative proportions of megakaryocytes within each stage had shifted to 48%, 19%, 13%, and 20%, respectively.

Megakaryocytes were enriched from the population of cultured cells by velocity sedimentation (Fig 1A). Cells selected by this procedure averaged 54% megakaryocytes (n = 13), with a range of 31% to 82%. An average of $2 \times 10^6$
mature megakaryocytes was obtained per leukapheresis unit, with a range of \(4 \times 10^5\) to \(3 \times 10^6\). The megakaryocyte percentage values obtained from immunostaining closely correlated with those obtained by morphologic analysis. Other cell types frequently present in the megakaryocyte-enriched population included myelomonocytic cells and blast cells of unknown origin. Very few lymphoid and no erythroid cells were detected (F. Naeim, UCLA Medical Center, University of California, Los Angeles, CA; July 1993).

Enriched megakaryocytes were replated in Meg GM supplemented with 10% heparinized human AB plasma but without aplastic canine serum. Within 2 to 3 days, megakaryocytes displaying proplatelets were observed (Fig 1B). Constrictions delineating platelet-sized areas were seen along the length of the proplatelets. The frequency of cells displaying proplatelets increased with time after replating. At the peak of proplatelet development, generally occurring after 3 to 4 days postreplating, an average of 40% ± 16% of the megakaryocytes exhibited proplatelets. In some experiments, enriched megakaryocytes were replated on Matrigel (Collaborative Biomedical Research, Bedford, MA) to determine if the presence of extracellular matrix proteins and proteoglycans enhanced proplatelet development. Megakaryocytes replated on plastic or on Matrigel-coated surfaces both developed proplatelets with a similar time course. The percentage of megakaryocytes undergoing differentiation was approximately 60% ± 5% in each case by day 3 postreplating (data not shown). To determine if plasma proteins affected proplatelet development, megakaryocyte-enriched populations were replated in Meg GM in the absence of heparinized human AB plasma. In these plasma-free experiments, proplatelet development still occurred, although at a decreased frequency, which was generally 30% to 50% of that observed when heparinized human AB plasma was included in the medium. To determine if serum proteins affected proplatelet development, megakaryocyte-enriched populations were replated in Meg GM in the presence of 10% serum. The presence of serum significantly inhibited proplatelet development (data not shown).

Detection of Platelet-Specific Proteins on the Surface and Within Proplatelets

GPIb and IIb are platelet-specific plasma membrane proteins found on the surface of megakaryocytes.\(^{39-43}\) Figure 1C shows that GPIb and IIb are present on the cell body of megakaryocytes and throughout proplatelets, all the way to the tip. Figure 1D shows the control experiment in which the primary antibody cocktail was omitted. The use of anti-GPIIIa was avoided due to its tendency for non-specific binding in the presence of serum or plasma.

Fibrinogen is a protein found in alpha-granules of mature megakaryocytes and resting platelets.\(^{44,45}\) Fibrinogen is localized within proplatelets as seen by indirect immunofluorescence of detergent-permeabilized megakaryocytes displaying proplatelets (Fig 1E). There was no detectable staining when detergent was omitted during the fixation step (data not shown), indicating that the fibrinogen molecules are not cell surface-associated.

Microtubule Coil Detection Within Proplatelets

The presence of microtubule coils circumferentially located within platelets has been well documented and is a
Fig 4. Observation of platelet-sized fragments from culture supernatants of megakaryocytes displaying proplatelets by phase microscopy. (A) The arrow indicates a row of platelet-sized fragments (putative culture-derived platelets), as if it had just been released from a proplatelet. (B) The arrow indicates platelet-sized doublets. Both panels contain images of the same magnification (bar, 40 μm).

Fig 5. Electron micrographs of culture-derived platelets (A,B) and plasma-derived platelets (C). (A) A culture-derived platelet with a smooth contour, resembling an inactivated platelet. (B) A culture-derived platelet with ruffled contour, resembling an activated platelet. (C) A plasma-derived (normal) platelet with activated morphology. All panels contain images of the same magnification (bar, 1 μm).
structural characteristic by which platelets are defined.\textsuperscript{46,47} Figure 2 demonstrates the presence of microtubule coils within proplatelets. The diameter of these microtubule coils is within the same range as those within plasma-derived platelets (approximately 3 to 5 \( \mu \text{m} \)). Some proplatelets displayed microtubule coils along the processes, whereas some displayed microtubule coils only at the tips (Fig 2A and B).

Some proplatelet tips were rounded with a microtubule coil enclosed and some were not, in which case the microtubule strands ended in a tapered fashion (arrow, Fig 2A).

**Ultrastructure Similarities Between Proplatelets and Platelets**

Electron micrographs demonstrate ultrastructural similarities between proplatelets and plasma-derived platelets. Pro-
FUNCTIONAL CULTURE-DERIVED HUMAN PLATELETS

Table 1. Platelet Aggregation is Blocked With Anti-GPllbllla Antibody

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Specific inhibition of platelet aggregation by anti-GPllbllla antibody. Gel-filtered culture- and plasma-derived platelets were incubated with 50 μg/mL of either isotype control antibody (anti-CD34) or anti-GPllbllla antibody in the presence of human fibrinogen and ADP as detailed in Materials and Methods. From each population, 20,000 platelets were aliquoted into microtiter wells, where they formed a monolayer suspension. Platelet aggregate diameters were measured from video prints.

Platelets contain electron-dense alpha granules (Fig 3A and B) similar to those found in normal platelets (Fig 3C). In the proplatelet sample shown in Fig 3B, platelet-sized areas defined by constrictions (arrows) can be seen that are similar in size (approximately 3 to 5 μm) and morphologic appearance to plasma-derived platelets.

Observation of Platelet-Size Fragments

Examination of proplatelet cultures showed the presence of platelet-sized fragments (putative culture-derived platelets; Fig 4). These fragments were observed in every proplatelet culture examined. Generally, these particles were first observed within 24 hours after proplatelet development. If the culture vessel was handled gently to minimize agitation, the putative culture-derived platelets could be seen lined-up, as if they had just undergone the transition from proplatelets to platelets (arrow, Fig 4A). Sometimes, two or three putative culture-derived platelets were seen linked together by thin cytoplasmic bridges (arrow, Fig 4B). The mean platelet volume (MPV) of the putative culture-derived platelets was similar to that of normal platelets (6 to 10 μL), according to analyses with an electronic cell counter (data not shown). The concentration of platelet-sized fragments in the culture supernatant was approximately 3.2 × 10^6/mL (range, 1.4 × 10^6/mL to 5.3 × 10^6/mL; n = 10).

Ultrastructural Similarities Between Culture-Derived and Plasma-Derived Platelets

Putative culture-derived platelets were examined by transmission electron microscopy. The size and ultrastructural composition of the culture-derived fragments (Fig 5A and B) were virtually identical to those of normal platelets derived from plasma (Fig 5C). The diameter of the culture-derived platelets was approximately 3 μm. A culture-derived platelet shown in Fig 5A displays a smooth contour and spherical shape. Another culture-derived platelet shown in Fig 5B displays a ruffled and irregular contour, similar in shape to the plasma-derived platelet shown in Fig 5C. The activated appearance of the platelets in Fig 5B and C is most likely due to centrifugation before fixation. In addition to the similarities in size and granular components of culture-derived and plasma-derived platelets, careful examinations of culture-derived platelet micrographs showed circumferential microtubule strands, although they were not as prominent as those found in plasma-derived platelets. Sometimes microfibrils were also observed in culture-derived platelets.

Culture-Derived Platelets Aggregate in Response to Thrombin and ADP/Fibrinogen

The function of culture-derived platelets was investigated. Culture-derived or plasma-derived platelets incubated in the absence of agonists do not aggregate, as shown in Fig 6A and B, respectively. After addition of the strong platelet agonist thrombin, both populations of platelets aggregated (Fig 6C and D). Because of the concern that the cytocentrifugation involved in the assay may have caused the aggregation, all these slides were examined with extreme caution, and the aggregates were found to be unique to platelet preparations treated with thrombin. Addition of the weak platelet agonist ADP together with fibrinogen also induced aggregation of gel-filtered, culture-derived and plasma-derived platelets (Fig 6E and F). ADP/fibrinogen-induced aggregation was dependent on an interaction between fibrinogen and the fibrinogen receptor as aggregation could be blocked with a neutralizing anti-GPllbllla antibody. Culture- and plasma-derived platelets incubated with either anti-GPllbllla or an isotype-control antibody (anti-CD34) were stimulated with

![Fig 7](image-url)
ADP and fibrinogen. Whereas both culture-derived and plasma-derived platelets aggregated in the presence of the control antibody, neither population aggregated in the presence of anti-GPIIbIIIa (Table 1).

Expression of Activation-Dependent Antigens on Culture-Derived Platelets

Normal functional platelets, when activated, express certain cell surface antigens not associated with resting platelets. Two antigens of this type are P-selectin (CD62, GMP-140, or PADGEM)\textsuperscript{37,59} and the functional fibrinogen receptor (activated GPIIbIIIa).\textsuperscript{36,50} P-selectin, present on the alpha granules of resting platelets, becomes mobilized to the plasma membrane after activation. As shown in Fig 7A, P-selectin (CD62) expression on culture-derived platelets increased significantly after activation with thrombin. The percentage of specific CD62\textsuperscript{+} platelets increased from 15.2% to 61.5% with activation. Figure 7B shows an identical experiment performed with plasma-derived platelets where the percentage of specific CD62\textsuperscript{+} platelets increased from 20.6% to 58.6% with activation. The presence of a CD62\textsuperscript{+} subpopulation of resting platelets is attributed to accidental activation during the platelet isolation procedures. The platelet surface protein complex GPIIbIIIa changes conformation after platelet activation in its conversion to a functional fibrinogen receptor. PAC1, a MoAb against the activated form of GPIIbIIIa, has been previously described to be specific for activated platelets.\textsuperscript{36,50} As shown in Fig 8A, PAC-1\textsuperscript{+} labeling of culture-derived platelets increased significantly after activation with ADP. The percentage of specific PAC-1\textsuperscript{+} platelets increased from 7.04% to 95.1% with activation. When this experiment was performed with plasma-derived platelets (Fig 8B), the percentage of specific PAC-1\textsuperscript{+} platelets increased from 2.13% to 97.9% with activation.

DISCUSSION

This report describes an in vitro culture system in which to study human megakaryocyte development and platelet formation. CD34\textsuperscript{+} megakaryocyte progenitors are selected from leukapheresis units that are now commonly prepared by many blood banks. The number of CD34\textsuperscript{+} cells recovered per leukapheresis unit is sufficient to generate as many as 3 \times 10\textsuperscript{6} megakaryocytes in culture. This fact, along with the observations that megakaryocytes generated in culture from peripheral blood progenitor cells are morphologically, antigenically, and endomitotically normal,\textsuperscript{51} make this an ideal system for the study of in vitro human platelet formation.

Human megakaryocytes will spontaneously form proplatelet structures in culture. While normal human plasma enhances the phenomenon, it is not required. Matrigel has no apparent effect on the frequency or the rate of in vitro proplatelet development. These data suggest that direct interactions between megakaryocytes and the extracellular matrix are perhaps not necessary for proplatelet development, in agreement with some\textsuperscript{23} but not all\textsuperscript{19,25,26} reports.

The developmental point at which megakaryocytes become capable of spontaneously producing proplatelets is not known but obviously occurs within the time frame of the culture system. Although at any given time 40% to 45% of the megakaryocytes display proplatelet morphologies, the possibility remains that all the megakaryocytes could eventually do so upon reaching the critical maturation point. One explanation for less than 100% of the megakaryocytes developing proplatelets at any given time may be due to the wide range in cytoplasmic and nuclear maturity displayed by the culture-generated megakaryocytes. Megakaryocyte differentiation is not synchronous in culture, despite the fact that the starting cell population is enriched for CD34\textsuperscript{+} cells. Another explanation may be related to the use of CATCH buffer. The metabolic inhibitors adenosine and theophylline in the buffer, necessary to maintain maximum megakaryocyte integrity,\textsuperscript{31} may differentially inhibit proplatelet development depending on cell-cycle or maturation stages of the megakaryocytes.

Microtubules are essential structural elements of proplatelets and of platelets. In proplatelets, longitudinal microtubules may guide or direct the membrane and organelle flow of the developing processes.\textsuperscript{12,15,36,42,55} in platelets, microtubule coils provide structural support and may also harbor microtubule-associated proteins important for platelet function, analogous to the situation found in neurites.\textsuperscript{34} In this report both longitudinal microtubules and circumferential microtubule coils.
FUNCTIONAL CULTURE-DERIVED HUMAN PLATELETS

Microtubule coils are observed in human proplatelets. Other investigators using rodent systems have not observed microtubule coils in proplatelets and have proposed that the structures may not form until after platelet fragmentation. The differences in these results may be due to species variation or to the possibility that the described culture system allows for additional maturation of proplatelets during the culture period. Human proplatelets also display the platelet antigens GPIbα and IIBβ37 and electron-dense alpha granules containing fibrinogen. Taken together, these antigenic and structural data emphasize the platelet-like nature of proplatelets and support the notion that proplatelets may be the structural precursors to platelets.

There have been scattered reports suggesting that platelet-like fragments can be found in megakaryocyte cultures. Radley and Hatshorn observed fragments consisting of 2 to 10 linked, putative platelets migrating out of mouse marrow explants. The presence of platelet strings suggests that platelet release may not occur sequentially from the distal proplatelet tip. More recently, the same group has identified proplatelet-fragments within murine long-term bone marrow cultures. The presence of fragments was reported as occurring rarely. Leven and Yee also reported fragmentation of guinea pig proplatelets, but only in response to thromboxytenic rabbit plasma and cytochalasin D in combination. Although elaborate proplatelet-like networks have been seen on cultured human megakaryocytes, evidence of detached platelet-sized particles in culture has not been presented.

This report presents the first formal identification and functional analysis of platelets most likely arising from proplatelet cultures of any species. These are de novo synthesized platelets and not residual platelets from leukapheresis units, as the average culture time before platelets are observed is 14 days. Furthermore, cultured cells are subjected to extensive processing, including Ficoll, counter-current flow elutriation, freeze-thaw, CD34* selection, and velocity sedimentation. The ultrastructural similarities between culture-derived and normal platelets strongly suggest that the populations are identical. The functional similarities between culture-derived and plasma-derived platelets with respect to thrombin- and ADP-induced aggregations and activation-dependent expression of P-selectin and activated GPIIbIIIa also fully support this notion. There are approximately 240 platelets recovered from these cultures for every proplatelet-bearing megakaryocyte. This ratio is certainly within the theoretical range proposed by others and is possibly an underestimate of the fragmentation capacity of megakaryocytes. Uncharacterized platelet-shedding factors or rheumatic shear forces may be necessary to increase the rate and/or number of platelets observed. It will be of interest to combine this megakaryocyte culture system with one of the emerging artificial capillary culture systems to test this idea.

ACKNOWLEDGMENT

We thank Dr F. Naeim, UCLA Medical Center, for expert morphologic differentiations of megakaryocyte cultures; Dr Sanford Shattil (University of Pennsylvania, Philadelphia, PA) for PAC1-FITC antibody; S. Richardson for excellent technical assistance in transmission electron microscopy, and UCSB Biological Sciences and UCSB Neuroscience Research Institute (University of California, Santa Barbara, CA) for the use of the electron microscope facilities. We additionally thank Dr Joan Egrie and Eric Mazur for critical reading of the manuscript.

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Platelets generated in vitro from proplatelet-displaying human megakaryocytes are functional

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