Regulation of Platelet Production and Function by Megakaryocyte Growth and Development Factor in Nonhuman Primates

By Laurence A. Harker, Pamela Hunt, Ulla M. Marzec, Andrew B. Kelly, Aaron Tomer, Stephen R. Hanson, and Richard B. Stead

The primary physiologic regulator of platelet production, Mpl ligand, has recently been cloned and characterized. To define the regulatory role of Mpl ligand on platelet production and function we measured the effects of a recombinant truncated human Mpl ligand, megakaryocyte growth and development factor (rHu-MGDF) on megakaryocyteopoiesis, platelet function, and thrombogenesis in nonhuman primates. rHu-MGDF was administered to 10 baboons for 28 days while performing pharmacokinetics and repeated measurements of the following: (1) platelet count, volume, turnover, and function ex vivo and in vitro; (2) marrow megakaryocyte number, volume, and ploidy; and (3) platelet deposition and fibrin accumulation on segments of vascular graft and endarterectomized aorta in vivo. Daily subcutaneous injections of rHu-MGDF (5 μg/kg/d) attained plasma concentrations averaging 1.300 pg/mL 2 hours after injection with trough levels of 300 ± 65 pg/mL before the next dose. These levels of rHu-MGDF incrementally increased the peripheral platelet concentration threefold by day 7 and fivefold by day 28 (P < 10−4) associated with a reciprocal decrease of 25% in mean platelet volumes (P < 10−4). Platelet mass turnover, a steady-state measure of platelet production, increased fivefold (P < 10−4). Platelet morphology, life span, and recovery were normal. No significant change occurred in peripheral leukocyte, neutrophil, or erythrocyte counts (P > .1 in all cases). The platelet count gradually returned to baseline within 2 weeks after discontinuing rHu-MGDF injections. Marrow megakaryocyte volume doubled (P < 10−4) three days after initiating rHu-MGDF therapy and the modal ploidy shifted from 16N to 64N (P < 10−4). Marrow megakaryocyte number increased twofold by day 7, and nearly fourfold by day 28 (P < 10−4), resulting in a 6.5-fold increase in marrow megakaryocyte mass (P < 10−4). The effects of rHu-MGDF on thrombosis were determined by comparing baseline, day 5, and day 28 rHu-MGDF-treatment measurements of 111In-platelet deposition and 125I-fibrin accumulation on segments of homologous endarterectomized aorta (EA) and vascular graft (VG) interposed in arteriovenous femoral shunts. rHu-MGDF increased 111In-platelet deposition in direct proportion to the circulating concentration of platelets for both EA and VG (r = .98 in both cases), without significant changes in fibrin accumulation (P > .5 in both cases). During the first week of rHu-MGDF treatment ex vivo platelet aggregatory responsiveness was enhanced to physiologic agonists (adenosine diphosphate, collagen, and thrombin receptor agonist peptide, TRAP1,2) (P < .05 in all cases). Although in vitro platelet aggregation was not induced by any concentration of rHu-MGDF tested (P > .5), rHu-MGDF enhanced aggregatory responses to low doses of physiologic agonists, effects that were maximal at 10 ng/mL for baboon platelets and 100 ng/mL for human platelets, and were blocked by excess soluble c-Mpl receptor. Flow cytometric expression of platelet activation epitopes was not increased on resting platelets (ligand-induced binding sites, P-selectin, or Annexin V binding sites; P > .1 in all cases). Megakaryocyte growth and development factor regulates platelet production and function by stimulating endoreduplication and megakaryocyte formation from marrow progenitor cells, and transiently enhancing platelet functional responses ex vivo. rHu-MGDF has the potential for achieving platelet hemostatic protection with minimal thrombi-occlusive risk.

PERIPHERAL PLATELETS circulate with minimal day-to-day variation in concentration despite wide-ranging changes in platelet demand by modulating platelet production to compensate for changing peripheral requirements.1-6 Processes of platelet production involve terminal differentiation of megakaryocytes from multipotential hematopoietic stem cells by expanding the formation of megakaryocytes,7-9 endoproliferative cellular growth,10 subsequent cytoplasmic accumulation and differentiation, and final cytoplasmic fragmentation and release as functional platelets.11-13 The final cycles of cell division are concurrent with the initiation of cytoplasmic and endoreduplicative maturation of megakaryocytes.14 Recent research performed independently by several different groups of investigators shows that the ligand to the Mpl receptor15-18 is the long-sought factor regulating platelet production. This discovery stems from the finding by Souryi et al. of a cellular gene (c-Mpl) similar to genes encoding for a family of hematopoietic growth factor receptors, and the subsequent demonstration that c-Mpl antisense oligodeoxynucleotides selectively abolishes megakaryocyte colony formation in vitro.21 c-Mpl receptor was then used to isolate the ligand from thrombocytopenic plasma, followed by sequencing, cloning, and final biologic characterization of the ligand.19-22 Human Mpl ligand, alternatively known as thrombopoietin, is a heavily glycosylated protein composed of 332 amino acids exhibiting close similarity of its aminoterminal 152-residues with erythropoietin. mRNA analysis implicates the liver and the kidney as sites of thrombopoietin production.12,13 The regulatory role of thrombopoietin in the process of platelet production is evident from the capacity of excess c-
Mpl receptor to neutralize both the megakaryocyte colony-stimulating activity and platelet-elevating activity in thrombocytopenic plasma.\textsuperscript{14,17,19,23,24} Megakaryocyte growth and development factor (MGDF) stimulates megakaryocyte-poiesis in rodents.\textsuperscript{14,17,18} Conversely, mice engineered for deletion of the c-Mpl receptor exhibit platelet counts about one fifth of normal without affecting other blood cell counts.\textsuperscript{25} Endogenous thrombopoietin levels in patients with thrombocytopenia are elevated, and decrease after transfusional platelet replacement.\textsuperscript{26,28} Because thrombopoietin avidly binds to c-Mpl platelet receptors, the circulating levels of unbound thrombopoietin are postulated to induce concentration-dependent receptor-mediated proliferative and endo-proliferative maturation of megakaryocyte progenitors, implying that negative feedback regulation is produced via competitive binding of thrombopoietin to unbound Mpl receptors on circulating platelets and soluble Mpl receptor in plasma.

Recombinant human-MGDF (rHu-MGDF) is the truncated purified polypeptide produced by \textit{Escherichia coli} into which has been inserted a plasmid containing the human gene encoding the aminoterminal of the native molecule.\textsuperscript{14,15} This report describes studies investigating the role of rHu-MGDF in regulating marrow megakaryocytepoiesis and platelet function in baboons.

**MATERIALS AND METHODS**

\textit{Animals studied.} Juvenile male baboons (\textit{Papio anubis}) weighing 13 to 15 kg were used for this study.\textsuperscript{26} The animals were quarantined, tested for tuberculosis, and observed to be disease-free for 3 months before use. All procedures were approved by the Institutional Animal Care and Use Committee and conducted in accordance with Federal guidelines (Guide for the Care and Use of Laboratory Animals, National Institutes of Health, Bethesda, MD. NIH Publ. No. 85-23). For surgical procedures animals were given ketamine hydrochloride (20 mg/kg intramuscularly) for induction, isoflurane and oxygen by endotracheal tube for anesthetic maintenance, and buprenorphine (0.01 mg/kg every 8 hours as needed) postoperatively for pain management. Routine blood pressure and electrocardiographic monitoring was performed throughout the procedure and immediate postoperative period. Ketasine hydrochloride (5 to 20 mg/kg intramuscularly) was administered to achieve short-term immobilization for obtaining blood samples, bone marrow (BM) aspirates, and biopsy samples, and scintillation-camera imaging.

After performing baseline measurements of platelet production and function (see below), R Hu-MGDF, 5 \mu g/kg/d, was administered by subcutaneous injection to six baboons for 28 days, while repeating the measurements of platelet production and function during the 4 weeks of rHu-MGDF administration. Pharmacokinetic studies were performed in four additional animals.

\textit{Laboratory procedures.} Peripheral platelet counts, mean platelet volumes, red blood cell (RBC) counts, and total white blood cell (WBC) counts were determined in whole blood collected every other day in Na2EDTA (2 mg/mL) using Serono/Baker model 9000 whole blood analyzer (Serono/Baker, Allentown, PA).\textsuperscript{27-29} The absolute neutrophil counts (ANCs) were ascertained manually from WBC differential counts on Wright-Giemsa–stained peripheral blood (PB) films. The baseline platelet count was 353 $\pm$ 46 x 10$^3/\mu$L, RBC count 5.16 $\pm$ 0.60 x 10$^6/\mu$L, leukocyte count 14.4 $\pm$ 4.6 x 10$^9/\mu$L, and ANC was 3.5 $\pm$ 1.6 x 10$^9/\mu$L. Routine chemistry profiles were obtained before and at the end of rHu-MGDF treatment.

MGDF reagent. rHu-MGDF, a gift from AMGEN Inc (Thousand Oaks, CA), is a nonglycosylated polypeptide produced in \textit{E coli} transfected with a plasmid containing cDNA that encodes for the erythropoietin-like aminoterminal domain of human Mpl ligand.\textsuperscript{14,15} After extraction, refolding, and purification, this truncated protein was supplied as a sterile, clear, aqueous solution.

\textit{Plasma levels of rHu-MGDF.} Concentrations of plasma rHu-MGDF were determined using antibody-sandwich enzyme-linked immunosorbent assays (ELISAs). The assay system used a polyclonal antibody raised in rabbits against rHu-MGDF as the capture antibody. The same antibody conjugated to horseradish peroxidase (HRP) was used as the signal antibody. The sensitivity of the assay was 20 pg/mL. Immunospecificity was ensured for the assay system by documenting the loss of immunoreactivity in the presence of competing antibody.

\textit{Measurements of platelet function.} Template bleeding time measurements were performed on the shaved volar surface of the forearm; normal values averaged 4.0 $\pm$ 0.9 minutes.\textsuperscript{27-29}

Platelet aggregation was determined within 1 hour of drawing the blood using a Chrono-Log aggregometer (Havertown, PA) by recording the increase in light transmission through a stirred suspension of platelet-rich plasma (PRP) maintained at 37°C. PRP and platelet-poor plasmas were prepared by differential centrifugation, as previously described.\textsuperscript{27-29} The platelet count in the PRP was adjusted to 300 x 10$^9$ platelets/$\mu$L. Adenine diphosphate (ADP) (Sigma, St Louis, MO), Heparin (Heparin, Munich, Germany), and TRAP-1 (Peninsula Labs, Belmont, CA) were added at doses that spanned the range of responsiveness. The results were plotted and expressed as the concentration of agonist that induced half-maximal aggregation (AC50).\textsuperscript{27-29}

The appearance of activated platelets in the PB was evaluated by flow cytometry using fluoresceinated monoclonal antibodies (MoAbs) against neoantigens expressed on membrane surfaces of activated platelets, including conformationally altered glycoprotein (GP) IIb/IIIa, ligand-induced binding sites (LIBS) (a gift from Dr E. Plow, La Jolla, CA)\textsuperscript{30-31} and the secretory granular membrane, P-selectin (a gift from Biogen Inc, Cambridge, MA).\textsuperscript{32-35} In addition, enhanced binding to platelets by fluoresceinated Annexin V (a gift from Dr T. Yokoyama, Tokyo, Japan) was also examined using flow cytometry.\textsuperscript{33-35} Annexin V, a member of the multisegement family of calcium-dependent phospholipid binding proteins, exhibits high affinity for phosphatidylserine-rich negatively charged phospholipid platelet membrane surfaces promoting assembly of the macromolecular coagulation enzyme complexes.\textsuperscript{36-39} Flow cytometric platelet studies were performed on blood collected in 1/10 vol 3.8% sodium citrate. PRP was obtained by differential centrifugation; 5 \mu L PRP was diluted to 50 \mu L in phosphate-buffered saline (PBS), pH 7.4, containing saturating concentrations of fluoresceinated marker antibody and 1% bovine serum albumin, and incubated at 22°C. Annexin V was diluted in HEPES buffer (0.01 mol/L) in 0.15 mol/L sodium chloride and 2.5 mmol/L calcium chloride and 1% bovine serum albumin, pH 7.4. After 30 minutes of incubation with the appropriate antibody and buffer, the platelets were diluted 10-fold with the incubation buffer without albumin and placed on ice until analyzed by flow cytometry. The findings were standardized each day against calibrated fluorescent beads (Flow Cytometry Standards Corp, San Juan, Puerto Rico).

\textit{Measurements of platelet production.} Megakaryocyte number, size, and ploidy were measured by flow cytometry using a previously reported method for multiparameter correlative marrow analysis with a single-argon-ion-laser FACScan analyzer (Becton Dickinson, San Jose CA).\textsuperscript{40-44} Cell DNA in aspirated marrow was stained with
propidium iodide, and surface membrane receptors were analyzed with antibodies labeled with fluorescein and phycoerythrin. Megakaryocytes expressing GPIb/IIIa were enumerated in relation to the nucleated erythroid precursors expressing glycophorin A.\textsuperscript{4,5,44} Measurements of megakaryocyte diameters were based on the "time-of-flight" principle, ie, time required for a cell in suspension to pass through a focused light beam.\textsuperscript{10,40,42,44} Aspirated BM (3 mL) obtained from the pelvic cavity was collected into 10-mL plastic syringes containing 1/10 vol acid-citrate-dextrose (ACD formula A), 2.5 mMol/L EDTA and 2.2 \textmu mol/L prostaglandin E\textsubscript{2} (PGE\textsubscript{2}; Sigma) final concentrations. The marrow was gently pipetted, passed through a 120-\textmu m monofilament nylon filter, and diluted with cold Ca\textsuperscript{2+} and Mg\textsuperscript{2+}-free PBS containing 13.6 mMol/L sodium citrate, 2.2 \textmu mol/L PGE\textsubscript{2}, 1 \textmu mol/L theophylline (Sigma), 3% bovine serum albumin (BSA fraction V; Calbiochem, La Jolla, CA), 11 mMol/L glucose, and adjusted to a pH of 7.3 and an osmolarity of 290 mOsm/L. Megakaryocytes were analyzed in marrow aspirates fractionated with 1.06 density Percoll (Pharmacia Biotech Inc, Piscataway, NJ). The nucleated erythroid marrow cells were analyzed from marrow separated over 1.08 density Percoll (Pharmacia Biotech Inc). Megakaryocytes were selected on the basis of their distinct immunofluorescence at levels above that of control cells labeled with an unrelated MoAb. In each sample, 2,000 to 3,000 megakaryocytes were analyzed. Flow cytometric analysis was performed using FACSscan (Becton Dickinson). BM aspirates were obtained at baseline and after 3, 7, 14, and 28 days of rHu-MGDF treatment.

Estimates of marrow megakaryocyte mass were used to represent the marrow substrate giving rise to circulating platelets, and were calculated as the product of megakaryocyte numbers and mean megakaryocyte volumes.\textsuperscript{4,5,47} Megakaryocyte samples were obtained from the proximal femur before, and 4 weeks after, beginning rHu-MGDF therapy. The biopsy samples were processed for paraffin sectioning and stained for morphologic evaluation by light microscopy. Normal baboon marrow values (n = 10) averaged a megakaryocyte diameter of 39 \mu m (range: 21 \mu m for 2N to 56 \mu m for 64N cells), volume of 28.7 \pm 2.1 \times 10\textsuperscript{3} \mu m\textsuperscript{3}, and megakaryocyte number of 8.6 \pm 1.4 \times 10\textsuperscript{7}/kg, giving a total megakaryocyte mass of 24.6 \pm 4.3 \times 10\textsuperscript{8} fl/kg. The normal modal ploidy was 16N.

Steady-state platelet mass turnover (platelet concentration multiplied by mean platelet volume and divided by platelet life span and by platelet recovery) was used to estimate the rate at which viable platelet mass was delivered to the PB.\textsuperscript{46} To measure survival time, autologous platelets were labeled with \textsuperscript{111}In-oxine using the method described previously.\textsuperscript{48} Labeling efficiencies averaged 90%, and the labeled platelets functioned normally.\textsuperscript{46,48} After reinjection, daily blood samples were collected and analyzed for \textsuperscript{111}In-platelet activity to determine the rate at which \textsuperscript{111}In-platelets were cleared from the circulation. Platelet survival time, ie, the average time in circulation, was then calculated using computer least-squares fitting of the raw data to a gamma-function modeling program.\textsuperscript{48} The platelet survival time in normal baboons (n = 10) is 5.4 \pm 0.6 days. The recovery of labeled platelets in the circulation at equilibrium was estimated by extrapolating the survival curve to time zero and estimating the blood volume (70 mL/kg) using the formula: Recovery in Circulation = Total Circulating Platelet Radioactivity/Total Platelet Radioactivity Injected. Platelet recovery in normal baboons (n = 10) is 85% \pm 4%. Platelet mass turnover, a measure of steady-state platelet production and destruction, was calculated by the formula: Platelet Mass Turnover Rate (fL platelets/\mu L/d) = Platelet Count/\mu L multiplied by Mean Platelet Volume (fL) and divided by Platelet Survival Time (days) and Proportion of Platelets Recovered. Platelet mass turnover in normal baboons (n = 10) is 6.89 \pm 1.17 \times 10\textsuperscript{3} fl platelets/\mu L/d.

Measurements of thrombus formation. To determine the effects of rHu-MGDF–mediated increases in platelet production and function on the recruitment of platelets into vascular thrombi, we measured \textsuperscript{185}W–platelet and \textsuperscript{125}I–fibrin accumulation on thrombogenic segments of endarterectomized homologous aorta and Dacron vascular grafts. Surgically implanted exteriorized femoral arteriovenous (AV) "Scribbertype" access shunts were used for interposition of thrombogenic segments and blood sampling.\textsuperscript{26,27} AV shunts of this design do not detectably shorten platelet survival times or fibrinogen removal rates and do not produce measurable activation of platelets or fibrinogen.\textsuperscript{26,27} Thrombogenic segments were subsequently interposed between the arms of the permanent shunt system of awake animals for 1 hour, while measuring flow through the shunt using a C-clamp type flow probe interfaced with a Transonic T206 Blood Flow Analyzer (Transonic Corp, Ithaca, NY). Blood flows were maintained at 100 mL/min. Thrombus accumulation on these surfaces was measured with a gamma camera as radio labeled platelet and fibrinogen accumulation over time.\textsuperscript{51,52}

Fresh baboon aorta (5 to 6 mm internal diameter [id]) was obtained from other donor animals, flushed with saline, and divided into 4-cm lengths. Branches were ligated and specimens were stored in normal saline. Endarterectomies were performed by inverting the aortic segments and removing the intima and inner media for a distance of 1 cm in the central portion of the vessel using sharp dissection under 2.5X optical magnification. After completion of the endarterectomy, each segment was returned to its normal configuration and cannulated using 1-cm lengths of heat-shrinkable Teflon tubing (Small Parts, Inc, Miami, FL) attached to segments of 4-mm id silicone rubber medical tubing (Dow Corning, Inc, Corning, NY). The aortic segments were encased with heat-shrinkable Teflon tubing, sealing each end carefully by heating, but avoiding direct heat to the tissues. The resultant configuration maintained stable geometry with a smooth transition from vessel to tubing. For imaging, the entire apparatus was connected to additional lengths of 3 mm silicone rubber tubing and incorporated into the AV shunt system with 2-cm long tapered Teflon connectors (Small Parts, Inc).\textsuperscript{52,53}

Segments of knitted Dacron vascular graft, 4 mm id (Bioknit), were obtained from C.R. Bard, Inc (Billerica, MA). Segments 2 cm in length were rendered impervious to blood leakage by external wrapping in Parafilm (American Can Co, New York, NY) and 5.3-mm id "heat shrink" Teflon tubing. Butt joints were constructed that ensured smooth luminal flow surfaces.\textsuperscript{36,28,54}

For the platelet imaging procedures, autologous baboon platelets were labeled with 1 mCi \textsuperscript{111}In (\textsuperscript{111}In) oxine as previously described \textsuperscript{25,54} and reinjected at least 1 hour before imaging. Total deposited \textsuperscript{111}In-platelet activity was determined at endarterectomy sites or on the segments of vascular graft using 'region-of-interest' analysis. Images were acquired continuously for 60 minutes in 5-minute intervals using a GE 400T scintillation camera (General Electric, Milwaukee, WI). The data were stored and analyzed on a Medical Data System A\textsuperscript{3} Computer (Medasys Inc, Ann Arbor, MI). A medium-energy collimator was placed close to the animal. Total platelet deposition, including both labeled and unlabeled platelets, was calculated by dividing the deposited \textsuperscript{111}In-platelet activity by circulating \textsuperscript{111}In-platelet activity (blood standard) and multiplying by the circulating platelet count (platelets per milliliter of whole blood) as measured in the blood standard sample. The results were expressed as total deposited platelets. Radioactivity values in these calculations refer to platelet activity only, with blood and standard values corrected for the small fraction of non-platelet \textsuperscript{111}In-activity.
washed, and stored for 30 days until the $^{111}$In activity had decayed. Then the incorporated radioactivity was measured by gamma counter. Deposited fibrin (mg) was calculated by dividing the $^{125}$I-activity (cpm) in each segment by the coagulable plasma $^{111}$In-activity (cpm/mL) and multiplying by the plasma fibrinogen level (mg/mL). Results were expressed as total accumulated fibrin (mg/cm). The concentration of fibrinogen in plasma was estimated spectrophotometrically by a modification of Jacobsson's method.

Baseline control platelet and fibrin deposition onto segments of endarterectomized aorta and Dacron vascular graft were determined and repeat measurements were obtained after 4 week of rHu-MGDF treatment.

Morphology. For ultrastructural examination of platelets, 5 mL blood was drawn into 1/10 vol 3.8% sodium citrate anticoagulant. After removing erythrocytes and leukocytes by differential centrifugation, an equal volume of 0.1% glutaraldehyde in pH 7.4 PBS was added and platelets pelleted at room temperature by centrifugation at 800g for 5 minutes. The supernatant was decanted, and 3% glutaraldehyde in PBS pH 7.4 was layered over the pellet for 1 hour and then processed for transmission electron microscopy. For megakaryocyte morphology, the pelleting was performed at 180g for 5 minutes at 4°C. Marrow cores were obtained using clinical techniques, fixed in 10% buffered formalin solution, embedded in paraffin, sectioned, and stained with polychromatophilic dyes for examination at the light level.

Data analysis. Data were analyzed using SIGMA STAT (Jandel Scientific Software, San Rafael, CA). Comparisons between two groups were performed using the two-tailed Student's t-test, unless the data were not distributed randomly, in which case nonparametric analysis was performed. Analysis of variance was used to compare values for a particular group at various time points. Unless otherwise stated, variance about the mean is given as ±1 SD.

RESULTS

**rHu-MGDF blood levels.** The subcutaneous daily injection of 5 μg/kg rHu-MGDF to baboons produced peak plasma levels of 250 ± 150 pg/mL at 3 hours after the initial injection (basal levels were undetectable), and 900 ± 250 pg/mL 2 hours after the second injection. Thereafter, the peak levels 2 hours postinjection averaged 1,300 ± 300 pg/mL and trough levels before the next dose were 300 ± 65 pg/mL ($P < .01$, comparing peak and trough levels). One hour after injecting the initial dose of rHu-MGDF the peripheral platelet count decreased from 0.35 ± 0.05 to 0.29 ± 0.04 × $10^6$ platelets/μL ($P .04), and returned to baseline by the third hour. Subsequently, no change occurred in peripheral platelet counts following injections of rHu-MGDF.

**Effects of rHu-MGDF on platelet production.** The concentration of circulating platelets incrementally increased from the baseline value of 0.35 ± 0.05 to 1.21 ± 0.13 on day 7, 1.51 ± 0.21 on day 14, and 1.83 ± 0.40 × $10^9$ platelets/μL by day 28 of therapy ($P < 10^{-4}$ in all cases), after which the platelet counts decreased to baseline values within 2 weeks (Fig 1 and Table 1). The mean platelet volume reciprocally decreased by about 25% (8.2 ± 1.2 μL 6.0 ± 0.7 fL; $P < .001$), and normalized after discontinuation of rHu-MGDF administration (Fig 1). Despite the fivefold increase in platelet concentration, platelet ultrastructure was morphologically normal (Fig 2), and platelet lifespan was 114 ± 12 hours, compared with 130 ± 14 hours baseline ($P .12$). Platelet mass turnover, a steady-state measure of the rate at which platelet mass enters the peripheral circulation, progressively increased more than fivefold by day 28 (Table 1), ie, from 6.30 ± 1.91 to 31.8 ± 5.42 × $10^9$ fl platelets/μL/d ($P < .0001$). Peripheral leukocyte, neutrophil, or erythrocyte counts did not change significantly during rHu-MGDF administration (Fig 3; $P > .1$ in all cases).

**Effects of rHu-MGDF on megakaryocytes.** rHu-MGDF increased megakaryocyte number, size, and ploidy (Fig 4). Basal measurements were compared with findings obtained after 3, 7, 14, and 28 days of rHu-MGDF administration (Table 1). By day 3 the mean megakaryocyte volume doubled (28.7 ± 2.1 μm² 52.7 ± 10.0 × $10^9$ μL; $P < .001$; Table 1), and the modal megakaryocyte ploidy shifted from 16N to 64N (Fig 5). The striking increase in volume and ploidy on day 3 correlated with reciprocal reductions in the 16N, 8N, and 2N cohorts (Fig 5: $P < .01$ in all cases), reflecting the early direct stimulation of marrow megakaryocytes to undergo endoreduplication before inducing the formation of additional megakaryocytes. By day 7, the mean megakaryocyte volume remained increased (46.9 ± 2.84 × $10^9$ fl; $P < .0001$), corresponding to a modal ploidy value of 32N (Fig 5).

In determining the megakaryocyte number, the ratio of marrow megakaryocytes to marrow nucleated erythroid forms was measured by relating nucleated GPIb/IIIa-positive cells to nucleated glycophrin A-positive cells using MoAbs to erythroid-specific glycophrin and supravital nuclear staining. That ratio was then multiplied by the estimated total number of marrow nucleated erythroid precursors in normal baboons (assumed to be 5.3 × $10^9$ erythroid cells/kg, based on ferrokinetic studies in humans). The basal megakaryocyte:erythroid ratio was 1.62 ± 0.26 × $10^{-5}$, yielding basal megakaryocyte numbers of 8.59 ± 1.40 ×
Table 1. Effects of rHu-MGDF on Marrow Megakaryocytes and Platelet Production

<table>
<thead>
<tr>
<th>Duration</th>
<th>Platelets</th>
<th>Megakaryocytes</th>
<th>Platelets</th>
<th>Megakaryocytes</th>
</tr>
</thead>
<tbody>
<tr>
<td>rHu-MGDF Therapy 5 μg/kg/d (×10⁷)</td>
<td>Concentration (×10⁶/μL)</td>
<td>Volume (×10⁸ fL)</td>
<td>Mass (×10¹⁰ fL·kg⁻¹)</td>
<td>Increase (×N)</td>
</tr>
<tr>
<td>Baseline</td>
<td>0.35 ± 0.05</td>
<td>8.2 ± 1.2</td>
<td>6.30 ± 1.91</td>
<td>1.0</td>
</tr>
<tr>
<td>3 d</td>
<td>0.49 ± 0.08</td>
<td>6.2 ± 0.08</td>
<td>7.29 ± 2.01</td>
<td>1.2</td>
</tr>
<tr>
<td>7 d</td>
<td>1.21 ± 0.13</td>
<td>6.0 ± 0.7</td>
<td>17.8 ± 3.22</td>
<td>2.8</td>
</tr>
<tr>
<td>14 d</td>
<td>1.54 ± 0.20</td>
<td>6.0 ± 0.7</td>
<td>22.6 ± 4.34</td>
<td>3.6</td>
</tr>
<tr>
<td>28 d</td>
<td>1.83 ± 0.40</td>
<td>6.0 ± 0.7</td>
<td>31.8 ± 5.42</td>
<td>5.0</td>
</tr>
</tbody>
</table>

Abbreviations: NE, nucleated erythrocytes; xN, fold increase over baseline.

10⁶ megakaryocytes/kg. rHu-MGDF progressively expanded the number of marrow megakaryocytes throughout the period of administration, ie, twofold by day 7 (17.2 ± 10.4 × 10⁶ megakaryocytes/kg), and fourfold by day 28 (31.8 ± 22.0 × 10⁶ megakaryocytes/kg; P < .0001).

Total megakaryocytopoiesis was estimated as marrow megakaryocyte mass, which represented the product of the total number of megakaryocytes and their mean volume. The resultant megakaryocyte mass progressively increased sixfold to 161 ± 122 × 10⁸ fL/kg (Table 1; P < .01 compared with basal values), corresponding to a fivefold increase in platelet mass turnover (Table 1).

Effects of rHu-MGDF on platelet function. rHu-MGDF-dependent escalation of circulating platelet counts enhanced platelet hemostatic function, as indicated by the shortening of template bleeding time measurements during therapy, ie, baseline of 3.1 ± 0.5 minutes versus 1.9 ± 0.5 minutes at 1 week (P < .02). To assess intrinsic platelet function independent of changes in peripheral platelet counts, responsiveness to aggregatory agonists in PRP (with platelet counts normalized to 300,000 platelets/μL) and activation epitope expression were determined.

Ex vivo platelet aggregatory responsiveness to ADP and TRAP₆₆ was significantly enhanced at 2 hours (but not at 24 hours) after administering the initial dose of rHu-MGDF (Fig 6; P < .05 in both cases). By day 5 the enhancing effects of rHu-MGDF on platelet aggregation induced by ADP, collagen, and TRAP₆₆ was continuous (Fig 6; P < .05 in all cases), ie, the concentration of agonist inducing half-maximal aggregation decreased significantly for ADP (5.4 ± 1.2 v 3.0 ± 0.78 μmol/L; P = .002), collagen (2.5 ± 1.0 v 1.4 ± 0.7 μg/mL; P = .047), and TRAP₆₆ (48.2 ± 35 v 25 ± 14; P = .042). This enhancing effect on platelet aggregatory responsiveness was blocked by 100 μg/mL soluble c-Mpl (Fig 7). No significant augmentation of platelet aggregation was observed by day 8 despite continued rHu-

Fig 2. Effects of rHu-MGDF on platelet morphology. After attaining a fivefold increase in the concentration of circulating platelets, their morphology was compared with baseline morphology using transmission electron microscopy. (A) Low-power views of platelets before initiating rHu-MGDF therapy. (B) The ultrastructural appearance at the same magnification after 28 days of rHu-MGDF therapy. The platelets in (B) appear to be somewhat smaller.
MGDF administration (Fig 6). In vitro, no platelet aggregation was induced by concentrations of rHu-MGDF ranging from 1 ng/mL to 100 μg/mL (Fig 7; P > .5). However, in vitro aggregatory responses of baboon platelets to low-dose ADP (3.9 μmol/L) and collagen (1.9 μg/mL) were enhanced twofold by 10 ng/mL rHu-MGDF (Fig 7A; P < .05 in both cases), effects that were blocked by 100 μg/mL soluble c-Mpl receptor (Fig 7A). Similar enhancement of aggregatory responses and blockade by excess soluble c-Mpl receptor were observed for human platelets using ADP and collagen, albeit at concentrations of 0.3 μg/mL collagen, and 100 ng/mL rHu-MGDF to achieve maximal enhancing effects (Fig 7B).

The possibility that rHu-MGDF might inherently activate platelets was further examined using flow cytometric assessment of epitopes known to be expressed during platelet activation, ie, binding of MoAbs detecting the conformational change in GPIIb/IIIa (LIBS), and emergence of platelet secretory granular membrane protein (P-selectin) on platelet surfaces. The binding of fluorescein-labeled Annexin V to phosphatidylserine-rich activated platelet membranes was also evaluated by flow cytometry. There was no increased expression of any of these activation-related epitopes on resting platelets ex vivo during rHu-MGDF treatment (Table 2; P > .1 in all cases compared with baseline controls). Actually, the average number of LIBS epitopes on resting and stimulated platelets decreased (P < .0001 in both cases), whereas the number of inducible binding sites for Annexin V increased on platelets stimulated in vitro (P < .0001) during the course of treatment (Table 2). The mechanisms underlying these changes remain to be defined.

Effects of rHu-MGDF on thrombosis. The extent to which rHu-MGDF amplified thrombus formation was investigated by comparing basal versus day 5 and day 28 treatment measurements of 111In-platelet deposition and 125I-fibrin accumulation on segments of homologous endarterectomized aorta (EA) and vascular graft (VG) interposed in arteriovenous femoral shunts. Platelet accumulation into thrombus
Fig 5. Effects of rHu-MGDF on megakaryocyte ploidy distribution. Basal ploidy distribution for marrow megakaryocytes is depicted by (■) (± 1 SD); the modal ploidy value is 16N. The daily subcutaneous administration of rHu-MGDF (5 μg/kg) increases megakaryocyte ploidy, i.e., appearance of 128N ploidy class and reciprocal reduction in 2N–16N cells. The greatest increase in endoreduplication occurs by day 3 (△) and comprises a two-class shift of the modal ploidy value (P < .05) and a depletion of 2N–16N cells (P < .01 in all cases). The ploidy patterns are shown for day 3 by (■), day 7 by (●), day 14 by (▲), and day 28 by (○).

Fig 6. Effects of rHu-MGDF on platelet aggregation ex vivo. During rHu-MGDF dosing, aggregatory responses of platelets obtained from baboons receiving rHu-MGDF (5 μg/kg/d) are evaluated by comparing the agonist concentrations producing half-maximal aggregation (ACso). The concentrations of ADP (●), collagen (■), and TRAP1.6 (▲) inducing half-maximal platelet aggregation are decreased significantly during the 5 days of rHu-MGDF administration (P < .05 in all cases shown by asterisk). By day 8 there is no detectable enhancement of platelet aggregatory reactivity.

Fig 7. In vitro enhancement of baboon and human platelet aggregation by rHu-MGDF. The addition of rHu-MGDF in final concentrations of 1 to 1,000 ng/mL (○) to PRP fails to induce aggregation of either baboon (A) or human (B) resting platelets. By contrast, rHu-MGDF (final concentration 10 to 1,000 ng/mL) significantly enhances the aggregatory responses of minimally effective concentrations of ADP (3.9 μmol/L; ●; P < .05) and collagen (1.9 μg/mL for baboon platelets and 0.3 μg/mL for human platelets; ▲; P < .05). Excess soluble c-Mpl receptor (100 μg/mL) blocks the enhancing effects of rHu-MGDF on agonist-induced platelet aggregation (○). The depicted variation about the mean is ±1 SD.

forming on these thrombogenic segments was proportional to the elevation in the peripheral platelet count for both the early day 5 treatment when platelet aggregation ex vivo was enhanced, and late day 28 when no enhancement in platelet responsiveness was present (Figs 8 and 9). For example, on day 28 platelet deposition was 1.8 ± 0.1 versus 10 ± 2 × 10⁶ platelets on EA, and 4.4 ± 1.3 versus 12 ± 2 × 10⁶ platelets on VG (P < .0001 in both cases). However, no increase in fibrin accumulation was observed (1.8 ± 0.40 mg vs 1.9 ± 1.3 for EA and 1.9 ± 0.54 mg vs 2.5 ± 0.63 mg for VG; P > .5 in both cases). Thus, there were no excessive thrombotic responses attributable to enhanced platelet function.

DISCUSSION

This study shows that rHu-MGDF (5μg/kg/d) increases platelet production fivefold by inducing an early doubling of megakaryocyte cytoplasmic volume, coupled with a progressive fourfold expansion in the formation of megakaryocytes from marrow progenitor cells. The early wave of newly formed platelets rapidly elevates the peripheral platelet con-
centation threefold and is associated with transient enhancement of ex vivo platelet aggregatory responsiveness during the first week of therapy. Thereafter, platelet production increases to fivefold. Platelet morphology and function are normal, and platelet deposition onto thrombogenic segments is proportional to the concentration of platelets in the circulation.

After initiating rHu-MGDF therapy there is a 3-day delay before the peripheral platelet concentration increases, demonstrating that the processes of final megakaryocyte cytoplasmic maturation and platelet delivery are largely independent of Mpl ligand, a conclusion supported by studies investigating the effects of rHu-MGDF on platelet release mechanisms in vitro. The rapid increase in the circulating platelet concentration during the ensuing week is caused by the early increase in megakaryocyte cytoplasmic volume by day 3 together with a small increase in megakaryocyte numbers during that relatively short period of time (Table 1). Subsequently, platelet production increases exclusively by the progressive expansion in the number of marrow megakaryocytes (Table 1). Presumably, the intensity of early megakaryocyte responses is mediated by the very high plasma levels of rHu-MGDF, exceeding endogenous levels of baboon Mpl ligand by orders of magnitude. The circulating levels following the first dose are less than subsequent peak levels, due to pharmacokinetic accumulation of rHu-MGDF and initial binding with c-Mpl-receptor-bearing platelets (see Results). The early megakaryocyte changes (Table 1) emphasize the usefulness of ploidy as a sensitive morphologic indicator of MGDF stimulation. The steady decrease in the concentration of peripheral platelets after discontinuing rHu-MGDF therapy (Fig 1) reflects the 5- to 6-day survival time of circulating platelets together with several days of continuing platelet generation from already-formed marrow megakaryocytes, and underscores the complete dependence of amplified megakaryocytopoiesis produced in this study on exogenous rHu-MGDF.

As the concentration of circulating platelets increases in response to rHu-MGDF, the mean platelet volume decreases...
by approximately 25%, returning to normal only after rHu-MGDF injections are stopped (Fig 1). This reciprocal relationship between platelet volume and peripheral platelet count has been observed previously, interpreted to indicate that megakaryocytopoiesis is regulated by the circulating platelet mass (product of platelet volume and concentration). Because platelet mass indirectly reflects the concentration of platelet c-Mpl receptors competing for free Mpl ligand in plasma, platelet mass is a more meaningful determinant of the competition exhibited by circulating platelets for unbound Mpl ligand than the platelet count alone. Although the mechanism underlying the production of smaller platelets is not known, it may simply reflect incomplete accumulation of the full cytoplasmic complement during accelerated development. This association of stimulated megakaryocytopoiesis and smaller platelets also occurs during experimental thrombocytopenia induced by platelet consumption in non-human primates, but not in rodents when thrombocytopenia is produced by antiplatelet antibodies.

In this study the effects of rHu-MGDF on megakaryocyte number, volume, and ploidy are quantified in aspirated marrow using flow cytometric analyses. Because this technique is useful for measuring low-frequency cellular events in complex cell suspensions, it is particularly well suited for the evaluation of time-dependent changes in marrow megakaryocytes. Using a specific megakaryocyte probe combined with highly efficient DNA staining and a rapid method for analyzing marrow, it is feasible to obtain repeated comparisons of megakaryocyte size, ploidy, and number. The flow cytometric determination of marrow megakaryocyte number involves two basic assumptions. First, because the flow cytometric measurement of marrow megakaryocyte numbers is based on the ratio of megakaryocytes to nucleated erythroid cells, serial determinations depend on the assumption that the number of marrow nucleated erythroid cells remains constant. Since, in the present study, erythropoiesis is unchanged throughout the period of rHu-MGDF administration, this assumption appears to be justified. Second, it is assumed that the relative proportion of megakaryocytes capable of expressing the early differentiation marker GPIIb/IIa is quantitatively insignificant. Because GPIIb/IIa is present in very immature diploid megakaryocytes, it is likely that the proportion of total marrow megakaryocytes not expressing this membrane protein is less than 5%. At the very least, the analysis of GPIIb/IIa-positive marrow megakaryocytes is valid for comparing the changes produced by rHu-MGDF. Thus, the flow cytometric quantitative analysis of marrow megakaryocytes appears to be suitable for defining the regulatory role of rHu-MGDF on platelet production.

With the wave of newly formed platelets appearing in the circulation during the first week of rHu-MGDF therapy, ex vivo platelet aggregatory responses to physiologic agonists are transiently enhanced (Fig 6). Since this heightened responsiveness is blocked by excess c-Mpl receptor, the increased sensitivity is not attributable to artifacts of PRP preparation or contamination of the rHu-MGDF preparation or other reagents. Because the period of increased platelet responsiveness coincides with the entry of sufficient numbers of new platelets to amplify the peripheral platelet count threefold, the observed enhanced functional responses may be related, at least in part, to the as-yet undefined hyper-reactivity exhibited by newly formed platelets. Young platelets are reported to have enhanced hemostatic and aggregatory function, without spontaneously expressing activation epitopes, analogous to the features displayed by the cohort of platelets entering the circulation early after rHu-MGDF therapy is begun. In vitro, rHu-MGDF alone does not directly induce platelet aggregation at concentrations ranging from 1 ng/mL to 100 µg/mL (Fig 7). However, rHu-MGDF at concentrations of 10 to 100 ng/mL augment in vitro aggregatory responses to minimal doses of physiologic agonists (Fig 7), an effect that is abolished by excess soluble c-Mpl receptor (Fig 7), implying that the mechanism involves some inter-receptor reinforcement pathway that is most prominent in young platelets. However, the relevance of these findings for pathophysiologic processes in vivo remains uncertain because the evidence for transient platelet hyper-responsiveness is based on finding increased aggregatory responses ex vivo without corresponding expression of activation epitopes by circulating platelets (Table 2), and without direct physiologic evidence for enhanced platelet deposition onto thrombogenic surfaces independent of the elevated peripheral platelet count per se.

Evaluation of the potential thrombotic consequences associated with the administration of rHu-MGDF is relevant because of the observed enhancement in platelet aggregation ex vivo after rHu-MGDF treatment and the prospect of treating older cancer patients undergoing chemotherapy who may be at risk of developing thrombo-occlusive complications related to their underlying malignancy or associated vascular disease. In measuring the effects of rHu-MGDF on thrombus formation, the relative accumulation of 111In-platelets and 125I-fibrin are determined in thrombus forming on segments of two well-characterized thrombogenic surfaces, endarterectomized homologous aorta and knitted prosthetic vascular graft. These thrombogenic segments are interposed in arteriovenous shunts for fixed periods under standard flow conditions without systemic anticoagulation. Because these models are highly reproducible, they are well-suited for repeated comparisons. The number of deposited platelets is directly related to the peripheral platelet count over the range of 0.1 to 0.8 x 10^6 platelets/µL. In the present study this linear relationship is extended to nearly 2.0 x 10^6 platelets/µL for segments of endarterectomized aorta and vascular graft. These models show that thrombus formation does not exceed that predicted by the peripheral platelet concentration. Thus, the risk of developing thrombo-occlusive events in patients receiving rHu-MGDF should be small, because rHu-MGDF therapy is intended for thrombocytopenic patients, and any thrombotic risk is directly related to the concentration of circulating platelets, a response that is readily controlled by the dose and duration of rHu-MGDF therapy.

An important strength of this study is the use of quantitative nonhuman primate models. Baboons were selected for this study because (1) baboons regulate megakaryocytopoiesis, platelet kinetics, platelet activation, and mural...
thrombosis by mechanisms closely simulating humans; (2) there is considerable documentation regarding well-characterized, reproducible, and quantitative systems involving platelets in baboons; and (3) the thrombosis models discriminate between platelet and fibrin contributions during thrombus formation. Accordingly, we believe that the results may reasonably be expected to predict the effects of rHu-MGDF on human platelet production and function.

The Mpl ligand used in this study (rHu-MGDF) comprises a nonglycosylated truncated form of the native human molecule.

Thus, rHu-MGDF appears to be a suitable form of the molecule for development as a therapeutic agent. Assuming that rHu-MGDF is lineage-dominant with negligible toxicity, similar to that observed for the other two late-acting hematopoietic growth factors, erythropoietin and G-CSF, rHu-MGDF may have considerable clinical application in patients with platelet transfusion-dependent thrombocytopenia or other thrombocytopenic disorders responsive to exogenous stimulation of platelet production. For example, high-dose marrow-ablative chemotherapy with marrow or PB stem cell support are effective, potentially curative therapeutic modalities for patients with a variety of malignancies. The principal adverse effects resulting from such aggressive treatment are severe neutropenia and thrombocytopenia, leading to complications of infection and bleeding that generally require antibiotics and platelet transfusion support. Although recombinant human granulocyte colony-stimulating factor provides some protection from infections by shortening the period of neutropenic risk after chemotherapy, platelet transfusions are currently required to prevent hemorrhagic complications associated with severe thrombocytopenia in this setting. The present study in nonhuman primates indicates that rHu-MGDF may provide platelet-protective benefits without increasing the thrombo-occlusive complications in thrombosis-prone cancer patients undergoing marrow-ablative chemotherapy.

ACKNOWLEDGMENT

We gratefully acknowledge the technical expertise of Janis Wright, Deborah White, Evan Dessasau, and Birgitta Sandell-Ranby.

REFERENCES

18. deSauvage FJ, Hass PE, Spencer SD, Malloy BE, Gurney AL, Spencer SA, Darbonne WC, Henzel WJ, Worng SC, Kuang W-J.
PLATELET PRODUCTION & FUNCTION BY MGDF


53. Lumsden AB, Kelly AB, Schneider PA, Krupski WC, Dodson T, Hanson SR, Harker LA: Lasting safe interruption of endarterectomy thrombosis by transiently infused antithrombin peptide D-Phe-Pro-ArgCH2Cl in baboons. Blood 81:1762, 1993


Regulation of platelet production and function by megakaryocyte growth and development factor in nonhuman primates

LA Harker, P Hunt, UM Marzec, AB Kelly, A Tomer, SR Hanson and RB Stead

Updated information and services can be found at:
http://www.bloodjournal.org/content/87/5/1833.full.html

Information about reproducing this article in parts or in its entirety may be found online at:
http://www.bloodjournal.org/site/misc/rights.xhtml#repub_requests

Information about ordering reprints may be found online at:
http://www.bloodjournal.org/site/misc/rights.xhtml#reprints

Information about subscriptions and ASH membership may be found online at:
http://www.bloodjournal.org/site/subscriptions/index.xhtml