Effects of megakaryocyte growth and development factor on platelet production, platelet life span, and platelet function in healthy human volunteers


The effects of thrombopoietic stimulation on megakaryocytopoiesis, platelet production, and platelet viability and function were examined in normal volunteers randomized to receive single bolus subcutaneous injections of 3 µg/kg peglated recombinant megakaryocyte growth and development factor (PEG-rHuMGDF) or placebo in a 3:1 ratio. PEG-rHuMGDF transiently doubled circulating platelet counts, from 237 ± 41 × 10^3/µL to 522 ± 90 × 10^3/µL (P < .0001), peaking on day 12. Baseline and day-12 samples showed no differences in responsiveness of platelets to adenosine diphosphate or thrombin receptor agonist peptide (P > .4 in all cases); expression of platelet ligand-induced binding sites or annexin V binding sites (P > .5 in both cases); or density of platelet TPO-receptors (P > .5). Platelet counts normalized by day 28. The life span of autologous 111In-labeled platelets increased from 205 ± 18 hours (baseline) to 226 ± 22 hours (P < .01) on day 8. Platelet life span decreased from 226 ± 22 hours (day 8) to 178 ± 53 hours (P < .05) on day 18. The theoretical basis for senescence changes in mean platelet life span was illustrated by biomathematical modeling. Platelet turnover increased from 43.9 ± 11.9 × 10^3 platelets/µL/d (baseline) to 101 ± 27.6 × 10^3 platelets/µL/d (P = .0009), and marrow megakaryocyte mass expanded from 37.4 ± 18.5 fl/kg to 62 ± 17 × 10^10 fl/kg (P = .015). Although PEG-rHuMGDF initially increased megakaryocyte volume and ploidy, subsequently ploidy showed a transient reciprocal decrease when the platelet counts exceeded placebo values. In healthy human volunteers PEG-rHuMGDF transiently increases megakaryocytopoiesis 2-fold. Additionally, peripheral platelets expand correspondingly and exhibit normal function and viability during the ensuing 10 days. The induced perturbation in steady state thrombopoiesis resolves by 4 weeks. (Blood. 2000; 95:2514-2522)

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Introduction

Endogenous thrombopoietin (TPO) initiates receptor-dependent signaling in TPO-receptor–bearing hematopoietic precursors and megakaryocyte lineage cells that induces differentiation, proliferation and endoreduplication.1-9 TPO is produced constitutively by the liver and kidneys, and circulates in blood as unbound thrombopoietically active TPO and TPO bound to receptors on peripheral platelets and developing marrow megakaryocytes, in competitive equilibrium.7,10,11 Two recombinant thrombopoietic growth factors have been developed and evaluated in patients: a polyethylene glycol-conjugated, recombinant truncated polypeptide, PEG-rHuMGDF12-14; and the full-length, glycosylated recombinant protein, rHuTPO.5,15 In nonhuman primates, the subcutaneous administration of TPO or PEG-rHuMGDF produce molar-equivalent, log-linear increases in the concentrations of peripheral platelets. The purpose of this study is to examine the effects of single-dose thrombopoietic stimulation on megakaryocytopoiesis, platelet production, platelet life span, and platelet function in healthy human volunteers.

Patients and methods

Subjects and study design

This placebo-controlled, blinded study, approved by the Institutional Review Board, involved 16 healthy human volunteers recruited from the Emory campus community. Informed consent was obtained from each participant on admission to the General Clinical Research Center (GCRC) at Emory University, Atlanta, GA, and detailed clinical history, physical examination, complete profile of blood counts, routine serum chemistries, and urinalysis were obtained. The subjects were between the ages of 19 and 50 years, asymptomatic without known disease, and not taking any medications. The blood cell counts and routine blood chemistries were within the normal range for these laboratories.

Volunteers in each of the two 8-subject cohorts were randomized to receive either a single subcutaneous injection of 3 µg/kg PEG-rHuMGDF, or placebo in a 3:1 ratio on day 1. In the first group of 8 subjects (6 receiving active drug and 2 placebo), baseline measurements of platelet life span, platelet function, and megakaryocyte measurements were compared with the results obtained on day 12, the time when the concentration of
Mean TPO receptor numbers per platelet were estimated from platelet-Determination of thrombopoietin receptors on platelets human thrombopoietin. 12 After extraction, refolding, and purification, this transfected with cDNA encoding the 1 to 163 aminoterminal residues of Escherichia coli rHu-MGDF is a nonglycosylated polypeptide produced in Megakaryocyte growth factor

with active drug were compared with cohorts of 6 subjects on day 8, day 12, were calculated to be maximal, respectively. Thus, baseline (day 1) results receiving active drug and 2 placebo), platelet life span, platelet function, peripheral platelets was maximum. In the second cohort of 8 subjects (6

Scatchard analysis. 18,19 In brief, TPO receptors on platelets were to Scatchard analysis,18,19 In brief, TPO receptors on platelets were determined using purified rHu-TPO radiolabeled by Iodo-beads iodination reagent (Pierce, Rockford, IL), and incubating rHu-TPO with 50 mmol/L sodium phosphate buffer, pH 7.2, and 125I with Iodo-beads for 15 minutes. Platelets were obtained from blood drawn in acid-citrate-dextrose (ACD) anticoagulant (1:7 v/v), pelleting platelets from platelet-rich plasma by centrifuging at 500 × g for 15 minutes, and resuspending in Tyrode’s buffer containing ACD (1:7 v/v), pH 6.2, 1% bovine serum albumin (BSA), and 0.01% Tween. Binding isotherms were obtained by incubating platelets in plasma-free Tyrode’s buffer, ACD (1:7 v/v), 1% BSA, 0.01% Tween, pH 6.2, and various amounts of 125I-rHuTPO (40 to 640 ng/mL final concentrations) for 1 hour at room temperature. Nonspecific binding was assessed by comparing the effects of 100-fold excess unlabeled rHu-TPO 30 minutes before the addition of 125I-rHuTPO. Nonspecific binding ranged from 10% to 20%. Binding isotherms were analyzed using the Biosoft Ligand Program (Cambridge, UK) to determine the number of binding classes, the number of molecules bound/platelet, and the dissociation constant, Kd, TPO receptors on peripheral platelets were measured before, and 8, 12, and 18 days after initiating HuPEG-MGDF therapy. rHuTPO, a glycosylated protein produced in Chinese hamster ovary cells transfected with the cDNA encoding for human TPO, was provided as a gift from Kirin Pharmaceutical Laboratories, Takasaki, Japan. The purified molecular species was formulated in an aqueous buffer, and sterilized by filtration. Endotoxin levels in the final product were less than 2.5 EU/mL.

Determination of thrombopoietin receptors on platelets

Mean TPO receptor numbers per platelet were estimated from platelet-binding isotherms of 125I-labeled recombinant human (rHu) TPO according to Scatchard analysis.18,19 In brief, TPO receptors on platelets were determined using purified rHu-TPO radiolabeled by Iodo-beads iodination reagent (Pierce, Rockford, IL), and incubating rHu-TPO with 50 mmol/L sodium phosphate buffer, pH 7.2, and 125I with Iodo-beads for 15 minutes. Platelets were obtained from blood drawn in acid-citrate-dextrose (ACD) anticoagulant (1:7 v/v), pelleting platelets from platelet-rich plasma by centrifuging at 500 × g for 15 minutes, and resuspending in Tyrode’s buffer containing ACD (1:7 v/v), pH 6.2, 1% bovine serum albumin (BSA), and 0.01% Tween. Binding isotherms were obtained by incubating platelets in plasma-free Tyrode’s buffer, ACD (1:7 v/v), 1% BSA, 0.01% Tween, pH 6.2, and various amounts of 125I-rHuTPO (40 to 640 ng/mL final concentrations) for 1 hour at room temperature. Nonspecific binding was assessed by comparing the effects of 100-fold excess unlabeled rHu-TPO 30 minutes before the addition of 125I-rHuTPO. Nonspecific binding ranged from 10% to 20%. Binding isotherms were analyzed using the Biosoft Ligand Program (Cambridge, UK) to determine the number of binding classes, the number of molecules bound/platelet, and the dissociation constant, Kd, TPO receptors on peripheral platelets were measured before, and 8, 12, and 18 days after initiating HuPEG-MGDF therapy. rHuTPO, a glycosylated protein produced in Chinese hamster ovary cells transfected with the cDNA encoding for human TPO, was provided as a gift from Kirin Pharmaceutical Laboratories, Takasaki, Japan. The purified molecular species was formulated in an aqueous buffer, and sterilized by filtration. Endotoxin levels in the final product were less than 2.5 EU/mL.

Measurements of platelet function

Platelet aggregation was determined within 1 hour of drawing blood in 1/10 volume 3.2% citrate anticoagulant using a Chrono-Log aggregometer (Haverton, PA) by recording the increase in light transmission through a stirred suspension of platelet-rich plasma (PRP) maintained at 37°C. PRPs and platelet-poor plasmas (PPPs) were prepared by differential centrifugation, as previously described.20,21 The platelet count in the PRP was adjusted to 300 × 10^9/µL. ADP (Sigma, St Louis, MO), Heparin collagen (Nycomed Arzenmittel, Munich, Germany), and TRAP4a (Peninsula Labs, Belmont, CA) were added at doses spanning the range of responsiveness. The results were plotted and expressed as the agonist concentration inducing half-maximal aggregation (AC50).22,23

The appearance of activated platelets in the peripheral blood was evaluated by flow cytometry using fluoresceinated monoclonal antibodies (MoAbs) against neoantigens expressed on membrane surfaces of activated platelets, including conformationally altered integrin Ibβ ligand-bound binding sites (LIBS), a gift from Dr E. Flow (Cleveland, OH),24,25 and the secretory granule membrane, P-selectin, a gift from Biogen Inc, Cambridge, MA.26,27 In addition, enhanced binding to platelets by fluoresceinated annexin V, a gift from Dr T. Yokoyama, Tokyo, Japan, was also examined using flow cytometry.28,29 Annexin V, a member of the multigene family of calcium-dependent phospholipid binding proteins, exhibits high affinity for phosphatidylserine-rich, negatively charged, phospholipid platelet membrane binding sites promoting assembly of the macromolecular coagulation enzyme complexes.30,31 Flow cytometric platelet studies were performed on blood collected in 1/10 volume 3.8% sodium citrate. PRP was obtained by differential centrifugation; 5 µL PRP was diluted to 50 µL in phosphate buffered saline (PBS) pH 7.4 containing saturating concentrations of fluoresceinated marker protein and 1% bovine serum albumin (BSA), 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% BSA pH 7.4. After 30 minutes incubation with the appropriate antibody or binding protein 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, PR).

Measurements of platelet production

Megaekaryocyte number, size, and ploidy were measured by flow cytometry using a previously reported method for multiparameter correlrelative marrow analysis with a single-argon-ion-laser FACScan analyzer (Becton Dickinson, San Jose, CA).32-33 Cell DNA in aspired marrow was stained with propidium iodide, and surface membrane receptors were analyzed with antibodies labeled with fluorescein. Megaekaryocytes expressing Ibβ integrin (CD41/61) were enumerated in relation to the nucleated erythroid precursors expressing glycoporphrin A.34,35 Measurements of megaekaryocyte diameters were based on the “time-of-flight” principle, ie, time required for a cell in suspension to pass through a focused light beam.36 Aspired bone marrow (3 mL) obtained from the pelvic bones was collected into 10-mL plastic syringes containing equal volume acid-citrate-dextrose (ACD formula A), 2.5 mmol/L EDTA, and 2.2 µmol/L prostaglandin E1 (PGE1; Sigma, St Louis, MO) final concentrations. The marrow was gently pipetted, passed through a 120-µm monofilament nylon filter, and diluted with cold Ca++/Mg++-free PBS containing 13.6 mmol/L sodium citrate, 2.2 µmol/L PGE1, 1 mmol/L theophylline (Sigma), 3% BSA fraction V (Calbiochem, La Jolla, CA), 11 mmol/L glucose, and adjusted to a pH of 7.3 and an osmolality of 290 mosM/L. Megaekaryocytes 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). Megaekaryocytes were selected on the basis of their distinct immunofluorescence at levels above that of control cells labeled with an unrelated MoAb. In each sample, 2000 to 3000 megaekaryocytes were analyzed. Each subject had 2 bone marrow studies, ie, either baseline and at day 12, or day 8 and day 18. Estimates of marrow megaekaryocyte mass were used to represent the marrow substrate giving rise to circulating platelets, and were calculated as the product of megaekaryocyte numbers and mean megaekaryocyte volumes.37 Steady-state platelet mass turnover (platelet concentration multiplied by mean platelet volume and divided by platelet life span and by recovery of injected labeled platelets) was used to estimate the rate at which viable platelet mass was delivered to the peripheral blood.38,39 To measure platelet survival time, autologous platelets were labeled with 111In-oxine using the method described previously.38 Labeling efficiencies averaged 90%, and the labeled platelets functioned normally.40 After reinjection, daily blood samples were collected and analyzed for 111In-labeled platelet activity to
determine the rate at which $^{111}$In-labeled 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. The time course of $^{111}$In-labeled platelet activity in blood was modeled as:

$$L_a(t) = \frac{1}{n_a} \sum_{j=0}^{n_a-1} \left( n_a - j \right) \left( \frac{n_p}{\lambda} \right)^j e^{-\lambda t}$$

(1)

where $L_a(t)$ is the fraction of remaining $^{111}$In-platelet activity at time t, after injection of labeled platelets, and $\lambda$ is the mean platelet life span. The parameter, $n_a$, is the number of exponentials in the gamma function, which modulates the variance of $\lambda$. The recovery of labeled platelets in the circulation at equilibrium was estimated by extrapolating the survival curve to zero and estimating the blood volume (70 mL/kg) using the formula: recovery in circulation = total circulating platelet radioactivity/total platelet radioactivity injected.

**Nonstationary thrombokinetic model**

Peripheral platelet counts and $^{111}$In-labeled platelet activity curves were modeled simultaneously using a nonstationary gamma model. Modeling was conducted using SAAM II v. 1.1 (SAAM Institute, Seattle, WA). Megakaryocyte proliferation and maturation after stimulation by Mpl ligand were described by a system of $n_a$ differential equations as follows:

$$\frac{dM_1}{dt} = S_0 + k_{mpl}(C(t)\gamma) - \frac{n_m}{\tau_m} M_1$$

(2)

$$\frac{dM_2}{dt} = \frac{n_m}{\tau_m} M_1 - \frac{n_m}{\tau_m} M_2$$

(3)

$$\vdots$$

$$\frac{dM_{n_m}}{dt} = \frac{n_m}{\tau_m} M_{(n_m-1)} - \frac{n_m}{\tau_m} M_{n_m}$$

(4)

where $n_m$ is the number of caternary-linked megakaryocyte compartments ($n_m = 5$), $M_i$ is the mass in the $i$th megakaryocyte compartment, $S_0$ is the Mpl ligand-independent platelet production rate, $C(t)$ is the serum concentration of Mpl ligand at time t, $k_{mpl}$ is a proportionality constant relating mpl ligand serum concentration to Mpl ligand-dependent rate of thrombopoiesis, $\gamma$ modulates steepness of the concentration-response curve, and $\tau_m$ is the mean transit time of cells within the megakaryocyte maturation compartments. $C(t)$ was calculated by linear interpolation of the measured serum Mpl ligand concentrations. The initial condition for the $i$th megakaryocyte compartment, $M_i(0)$, was calculated as:

$$M_i(0) = (S_0 + k_{mpl}(C(t)\gamma)) \frac{\tau_m}{n_m}$$

(5)

where $C_0$ is the serum concentration of Mpl ligand at baseline. Megakaryocytopoiesis was described in the model by a black-box process. Without modeling of the time course of megakaryocyte mass, the model parameters estimate the Mpl ligand-dependent and Mpl ligand-independent platelet production rate:

$$S[S_0, C(t)] = S_0 + k_{mpl}(C(t)\gamma)$$

(6)

The model assumes that the rate of Mpl ligand-mediated platelet production is proportional to the serum concentration of Mpl ligand, $C(t)$, raised to the power $\lambda$. It is recognized that the relationship between serum Mpl ligand concentration and platelet production rate described in Equation 6 is applicable only over the range of Mpl ligand concentrations achieved in this study. At higher Mpl ligand concentrations, the relationship between platelet production rate and serum Mpl ligand may be appropriately described by a sigmoidal concentration-response relationship, such as a logistic function that could describe full receptor occupancy. Subsequent to stimulation of megakaryocytopoiesis, changes in platelet production rate are reflected by changes in platelet count after a transit time delay, $\tau_{trans}$.

Biologic parameters such as the rate of megakaryocytopoiesis, differential release rates of platelets from megakaryocytes of different ploidy, and megakaryocyt death rate, are not directly identifiable from observations of platelet kinetics in blood, per se. However, these biologic events affect the transit time, $\tau_{trans}$, and the variance of the transit time; hence, the thrombokinetic effects of these events are indirectly represented by the model.

 Destruction of peripheral platelets was modeled by random and senescent processes. The kinetics of peripheral platelets were described by the following system of $n_a$ differential equations:

$$\frac{dP_1}{dt} = \frac{n_m}{\tau_m} P_1 - \frac{k_p}{V_p} \frac{n_m}{\lambda} P_1$$

(7)

$$\frac{dP_2}{dt} = \frac{n_m}{\tau_m} P_1 - \frac{k_p}{V_p} \frac{n_m}{\lambda} P_2$$

(8)

$$\vdots$$

$$\frac{dP_{n_a}}{dt} = \frac{n_m}{\tau_m} P_{(n_a-1)} - \frac{k_p}{V_p} \frac{n_m}{\lambda} P_{n_a}$$

(9)

where $n_a$ is the number of caternary-linked platelet “age” compartments ($n_a = 20$), $P$ is the total number of peripheral platelets, $V_p$ is the apparent dilution volume of peripheral platelets (including splenic pooling), $P_i$ is the number of platelets in the $i$th platelet age compartment, $k_p$ is a constant rate of random platelet utilization, and $\lambda$ is intrinsic platelet longevity. The initial condition for the $i$th platelet age compartment, $P_i(0)$, was calculated as:

$$P_i(0) = \left( S_0 + k_{mpl}(C(t)\gamma) \right) \frac{\tau_m}{n_m}$$

(10)

where $P_0$ is the baseline peripheral platelet count. The peripheral platelet concentration at time $t$ was calculated as the sum of the platelet numbers within $n_a$ age compartments divided by the peripheral platelet dilution volume, $V_p$.

The $^{111}$In-labeled platelet activity curves were modeled simultaneously with peripheral platelet counts to account for the effects of platelet count and mean platelet age on the kinetics of $^{111}$In-labeled platelet decay:

$$\frac{dP_{1}^*}{dt} = - \frac{k_p}{V_p} \frac{n_m}{\lambda} P_1^*$$

(11)

$$\frac{dP_{2}^*}{dt} = \frac{n_m}{\tau_m} P_1^* - \frac{k_p}{V_p} \frac{n_m}{\lambda} P_2^*$$

(12)

$$\vdots$$

$$\frac{dP_{n_a}^*}{dt} = \frac{n_m}{\tau_m} P_{(n_a-1)}^* - \frac{k_p}{V_p} \frac{n_m}{\lambda} P_{n_a}^*$$

(13)

where $P_i^*$ is the platelet activity (cpm) associated with the $i$th age compartment. The initial amount of radiolabel, $P_1^*(t)$, within the $i$th age compartment after injection of the tracer bolus at time $t$ was assumed to be proportional to the fractional number of platelets within the $i$th age compartment, ie, incorporation of label was assumed to be independent of platelet age:

$$P_i^*(t) = \frac{P_i(t)}{P(t)} \cdot D^*$$

(14)

where $P_i(t)$ is the modeled number of platelets within the $i$th age compartment at time $t$, and $P(t)$ is the modeled number of peripheral platelets at time $t$, and $D^*$ is the dose (cpm) of $^{111}$In-labeled platelets.
The mean platelet age at time \( t \), \( A(t) \), was calculated from the weighted average of \( n_a \) platelet age compartments:

\[
A(t) = \frac{1}{P(t)} \sum_{j=1}^{n_a} P_j(t) \Lambda_j \tag{15}
\]

where \( \Lambda_j/n_a \) is the average platelet age in the \( j \)th age compartment.

Numerical integration was conducted in SAAM II using the Romberg method. Integration was conducted using an adjustable step size. A relative integration error of 0.001 (0.1%) was used.

**Blood cell counts**

Peripheral platelet counts, mean platelet volumes, red cell counts, and total white cell counts were determined in whole blood collected every day in K$_2$EDTA (2 mg/mL) using Serono/Baker model 9000 whole blood analyzer (Allentown, PA). The absolute neutrophil counts (ANCs) were ascertained manually from white cell differential counts on Wright-Giemsa–stained peripheral blood films.

**Statistical analysis**

The data were analyzed using SIGMA STAT (Jandel Scientific Software, San Rafael, CA). Comparisons between 2 groups were performed using the 2-tailed Student \( t \) test, unless the data were not distributed randomly, in which case nonparametric analysis was performed. Analysis of variance (ANOVA) was used to compare value for a particular group at various time points. Unless otherwise stated, variance about the mean is given as \( \pm 1 \) SD.

**Results**

**Serum levels of megakaryocyte growth factor**

Serum levels of megakaryocyte growth factor were measured immunometrically each day for 4 weeks (Figure 1, Table 1). In subjects receiving active agent, peak concentrations of Mpl ligand occurred 2 days after administering bolus subcutaneous injections of PEG-HuMGDF, and averaged 500 \( \pm \) 90 ng/mL (\( P < .001 \) compared with placebo). Compared with placebo subjects, significantly elevated concentrations were present in PEG-HuMGDF–treated subjects for more than 5 days. The average time after injecting PEG-HuMGDF to achieve half-maximal concentrations was 53 hours. The subjects receiving placebo exhibited serum endogenous TPO levels of 74 \( \pm \) 20 ng/mL, which remained unchanged throughout the study period (Figure 1; Table 1; \( P > .6 \) in all cases).

**Peripheral platelet concentrations and volumes**

The concentration of peripheral platelets after the single bolus subcutaneous injection of PEG-HuMGDF was detectably increased on day 6 (from baseline 230 \( \pm \) 43 \( \times 10^3/\mu L \) to 271 \( \pm \) 35 \( \times 10^3/\mu L \); \( P = .01 \); Figure 2), and doubled by day 12 (Figure 2; Table 1; from baseline 230 \( \pm \) 43 \( \times 10^3/\mu L \); \( P < .0001 \)). The increase in peripheral platelet counts was associated with a small reduction in mean platelet volume (MPV) from 8.1 \( \pm \) 0.6 fL on day 8 to 7.7 \( \pm \) 0.5 fL on day 12 (Figure 2; Table 1).
0.8 fL; \( P < .05 \)) on day 12. The peripheral platelet concentration and volume gradually normalized by day 28, ie, \( 243 \pm 43 \times 10^3 / \mu \text{L} \) compared with placebo of \( 230 \pm 43 \times 10^3 / \mu \text{L} \) (\( P > .6 \)) compared with baseline), and \( 8.0 \pm 0.6 \) fl to \( 8.0 \pm 1.0 \) fl (\( P > .8 \)) compared with baseline), respectively.

**Platelet responsiveness to physiologic agonists, and expression of platelet receptors**

The concentrations of ADP or TRAP inducing half-maximal platelet aggregation were not changed from baseline by administering PEG-rHuMGDF (Table 2; \( P > .4 \)) in all cases). Similarly, administering PEG-rHuMGDF did not alter the expression of TPO receptors, LIBS, or annexin V binding sites during the study period (data not shown).

**Platelet life span**

Life span measurements of autologous \(^{111}\text{In}}\text{-labeled platelets were computed using gamma function routines, modified for nonsteady-state analysis.}^{38,41,42} \) The disappearance pattern of \(^{111}\text{In}}\text{-labeled platelets obtained at baseline from 8 normal subjects is nearly linear, but exhibits slight curvilinearity (Figure 3). The computed average lifetime platelets remain in the circulation, ie, platelet life span (\( \lambda \)), was \( 205 \pm 18 \) hours (Table 1). These results agreed closely with the estimates of platelet life span obtained in placebo subjects at 8, 12, and 18 days (\( P > .5 \) for each of the 4 estimates). In PEG-rHuMGDF–treated subjects, platelet life span was prolonged to \( 226 \pm 22 \) hours on day 8 (Figure 3; \( P = .04 \)), the time when the cohort of platelets produced in response to PEG-rHuMGDF was youngest.

Peripheral platelet counts and \(^{111}\text{In}}\text{-labeled platelet kinetics were simultaneously modeled using a nonstationary thrombokinetic model (Figure 4). The model incorporated the effects of endogenous and exogenous Mpl ligand on megakaryopoiesis, elimination of platelets by senescence and endothelial use, and the effects of nonsteady-state alterations in mean platelet age on \(^{111}\text{In}}\text{-labeled platelet decay curves. Modeling of peripheral platelet counts and platelet tracer kinetics for a subject receiving autologous \(^{111}\text{In}}\text{-labeled platelets at baseline and on day 12 are illustrated in Figure 5 (upper and middle plots). Data fitting yielded an intrinsic platelet age (\( \Lambda \)) estimate (platelet life span in absence of external hazard) of 9.7 days and a constant rate of platelet utilization (\( k_u \)) of 8000 platelets per microliter per day. The apparent dilution volume (\( V_D \)) of platelets (including the splenic pool) was 7700 mL. The model predicted a linear platelet decay at baseline, due to a relatively uniform distribution of peripheral platelet ages. On day 12, the modeled tracer decay curve was slightly sigmoidal because of an increase in the proportion of younger platelets in circulation just before the platelet zenith. Modeling of peripheral platelet counts and platelet tracer kinetics for a subject receiving autologous \(^{111}\text{In}}\text{-labeled platelets on days 8 and 18 are illustrated in Figure 6 (upper and middle plots). In this subject, the intrinsic platelet longevity (\( \Lambda \)) was 10.3 days, platelet utilization (\( k_u \)) was 7700 platelets per microliter per day, and the platelet dilution volume (\( V_D \)) was 6200 mL. A pronounced sigmoidity in the platelet tracer decay curve on day 8 was noted, and was attributed to the high influx rate of new platelets into circulation. By contrast on day 18, the platelet tracer dose decayed rapidly and exponentially; the platelet tracer kinetics on day 18 were attributed to an increased proportion of old platelets in circulation with an associated increased destruction rate of the senescent cells.

The theoretical course of mean platelet age, \( A(t) \), during transient stimulation of megakaryocytopoiesis by a bolus dose of PEG-rHuMGDF was calculated (Equation 15) and is illustrated in Figures 5 and 6 (lower plot). The theoretical nadir in mean platelet age was predicted to be around day 9, and the theoretical zenith in mean platelet age was predicted to be around day 19. Modeling of the data calculated a difference in mean platelet age between the theoretical age zenith and nadir of approximately 2.0 to 2.5 days. The modeling projection corresponded closely to the observed platelet life span difference of 48 hours between days 8 and 18 (Table 1). After model correction for nonstationary alterations in the age distribution of peripheral platelets, the model estimates of intrinsic platelet longevity and endothelial use were similar to published steady state determinations (intrinsic platelet longevity,
10.5 days; endothelial utilization rate 7100 platelets per microliter per day) in absence of exogenous cytokine stimulation. 52,53

Platelet production

Platelet production was assessed in 2 ways: the rate at which viable platelets appeared in the circulation, calculated as platelet turnover (the peripheral concentration of platelets divided by the mean platelet life span, and corrected for splenic pooling); and megakaryocyte cytoplasmic substrate destined to give rise to platelets, calculated as megakaryocyte mass (the product of megakaryocyte number and megakaryocyte volume). The administration of PEG-rHuMGDF doubled platelet turnover, platelet mass turnover, and marrow megakaryocyte mass (Table 2), ie, platelet turnover increased from baseline of 44 ± 12 × 10^3 platelets/µL to 101 ± 28 × 10^3 platelets/µL/d (P = .0009), platelet mass turnover (Table 1) increased from 3.6 ± 1.1 fL/µL/d to 7.5 ± 2.1 fL/µL/d (P = .0009), and marrow megakaryocyte mass (Table 3) expanded from 37 ± 19 × 10^10 fL/kg to 62 ± 17 × 10^10 fL/kg (P = .015).

Megakaryocyte volume and ploidy

Megakaryocyte volume (Table 3) was significantly decreased on day 12 (from 30 ± 1.8 × 10^10 fL to 24 ± 3.7 × 10^10 fL; P = .0003). This decline was reciprocally related to the increase in peripheral platelet counts beyond the placebo values. Corresponding changes were noted in megakaryocyte ploidy (Figure 7). The proportion of megakaryocytes with ploidy of 16N and 32N was decreased on days 8 and 12 (P = .05 in both cases) when peripheral platelet counts in treated volunteers significantly exceeded placebo controls (Figure 2; P = .05 in both cases).

Table 2. Effects on Mpl ligand on platelet function

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<th>Baseline</th>
<th>Day 8</th>
<th>Day 12</th>
<th>Day 18</th>
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| Agonist concentration producing half-maximal aggregation.  
| ADP (µmol/L)     | 2.7 ± 1.5| 2.1 ± 1.4| 2.6 ± 1.9| 2.5 ± 2.0|
| TRAP (µmol/mL)   | 2.2 ± 1.9| 1.5 ± 1.2| 1.6 ± 1.4| 1.9 ± 1.8|
| Activation epitopes†  
| LIBS             | 11 ± 7   | 12 ± 8  | 18 ± 15 | 16 ± 11 |

*Binding sites × 10^5/platelet.
Discussion

This study demonstrates that administering single bolus subcutaneous PEG-rHuMGDF (3 µg/kg) to healthy human volunteers doubles the peripheral platelet concentration by stimulating megakaryocyte proliferation and endoreduplication, thereby doubling the mass of megakaryocyte cytoplasm destined for release as circulating platelets, without modifying platelet viability, platelet responsiveness to physiologic agonists, or platelet expression of activation epitopes.

Circulating concentrations of unbound endogenous TPO regulate physiologic platelet production to maintain constant peripheral platelet counts by compensating for changes in the peripheral demand for platelets.4,6,9,43 rHuTPO and PEG-rHuMGDF are 2 thrombopoietic growth factors that have been developed and evaluated in patients.3,9 These 2 agents produce molar-equivalent effects on megakaryocytopenesis. In healthy human volunteers, exogenous stimulation of thrombopoiesis by PEG-rHuMGDF produces measurable increases in marrow megakaryocyte endoreduplication and cytoplasmic volume within 24 hours, and in marrow megakaryocyte number several days later (Table 3).44 Corresponding increases in circulating platelets are detectable after 5 to 6 days and peak by 10 to 14 days.45,46 Under steady-state conditions PEG-rHuMGDF produces comparable log-linear dose responses in megakaryocyte mass and platelet mass turnover.47,48 This study in volunteers underscores the reliability of megakaryocyte ploidy as a morphologic indicator of thrombopoietic stimulus, as shown by the transient decreases in megakaryocyte ploidy during induced thrombocytosis.

In healthy human volunteers, minimal reduction in MPV develops as the peripheral platelet counts are elevated (Table 1), consistent with previous reports in nonhuman primates regarding the reciprocal relationship between MPV and the peripheral concentration of platelets.49 Presumably, the blunted decrease in MPV observed in normal subjects (Table 1), compared with the greater reduction in nonhuman primates, is due to the striking difference in the relative total dose and duration of PEG-rHuMGDF administration.

Modulation of peripheral platelet counts in healthy human volunteers provides an opportunity to evaluate the effects of platelet concentration and age on the measurements of platelet life span under physiologic conditions. Current understanding of platelet survival time has evolved from the Mills-Dornhorst equation,41,49 which postulates an intrinsic 10-day time-dependent pattern of mixed population 111In-labeled platelets is predominately from fixed random vascular utilization, so-called “external hazard function” (Figure 3).50,51 Gamma function analysis of these disappearance curves provides good estimates of the average time that platelets survive in the circulation.52,53 As peripheral platelet counts decrease below 50 000/μL the relative importance of vascular utilization progressively increases, resulting in shortened platelet survival times secondary to thrombocytopenia, per se, apart from any other mechanism(s) of platelet destruction.

In this study, platelet life span was measured for platelet populations that were (1) enriched with young, recently formed platelets (day 8); (2) enriched with platelets approaching senescence (day 18); and (3) platelets at twice the baseline concentration (day 12). Gamma function analysis revealed prolonged platelet life span for platelets enriched with newly formed platelets (day 8; Figure 3; Table 1), shortened platelet life span for platelets approaching senescence (day 18; Figure 3; Table 1), and minimally prolonged platelet life span at peak-induced thrombocytosis, compared with baseline (day 12; Figure 3; Table 1). These findings are concordant with the concepts that platelets are removed from the circulation predominantly by time-related senescent mechanism(s), with superimposed fixed vascular utilization that becomes proportionately less important when the peripheral concentration of platelets is increased. Clearly, the observed effects reported here would be accentuated by enhancing the degree of thrombocytosis induced by PEG-rHuMGDF or rHuTPO.

To provide a theoretical basis for the effects of changing platelet count and nonsteady-state alterations in mean peripheral platelet age, peripheral platelet counts, and 111In-labeled platelet decay curves were simultaneously modeled using a nonstationary model of platelet destruction and PEG-rHuMGDF-mediated
megakaryocytopenia (Figure 4). The gamma function used for calculating platelet life span from platelet tracer curves (Table 1) was derived using steady state assumptions. Although the gamma function can approximate mean platelet life span under nonsteady-state conditions, the gamma function, per se, does not correct for nonstationary alterations in platelet age distributions or the effects of changing platelet count on endothelial platelet clearance during the time course of tracer decay. Therefore, to estimate intrinsic platelet longevity and endothelial platelet utilization rate after transient stimulation of megakaryocytopenia, a nonstationary thrombokinetic model was implemented. Using this model, excellent fits to peripheral platelet counts and tracer decay curves were attained (Figures 5 and 6). Model estimates of intrinsic platelet longevity and endothelial utilization rate were consistent with published values determined under steady-state conditions, thereby supporting the notion that the observed shifts in mean platelet life span on days 8, 12, and 18 relative to baseline were attributable to anticipated effects of changing platelet count and mean peripheral platelet age.

Because the Mpl ligands, PEG-HuMGDF and rTPO, initiate receptor-dependent platelet signaling in vitro, and enhance platelet aggregative responsiveness to physiologic agonists in vitro and ex vivo, it has been postulated that their therapeutic use would increase the risk of developing vascular thrombosis. However, the negative results reported in Table 3 for normal subjects, and by others for cancer patients, support the conclusions from studies in nonhuman primates, that therapy with Mpl ligands does not increase the risk of developing vascular thrombotic events, at least for the dosing and indications evaluated to date. However, in patients with underlying vascular disease, the degree of thrombocytosis complicating Mpl ligand administration may be of importance in the genesis of thrombotic events.

The development of PEG-HuMGDF has been suspended because of the development of neutralizing antibodies that inactivate endogenous TPO in some individuals after repeated injections. This observation represents a significant impediment to development of Mpl ligands. Recent descriptions of short peptide Mpl agonists with amino acid sequences unrelated to TPO indicate a possible means of circumventing this problem. The findings in this study support the rationale for using modest doses of nonimmunogenic Mpl ligands in volunteer donors undergoing platelet apheresis, to improve yields and efficiency.

In conclusion, 1 week after bolus thrombopoietic stimulation with 3 mg/kg PEG-HuMGDF, megakaryocytopenia doubles, leading to corresponding doubling on day 12 of platelets that exhibit normal function and viability during the ensuing 10 days. All values return to baseline 4 weeks after bolus thrombopoietic stimulation.

References

Effects of megakaryocyte growth and development factor on platelet production, platelet life span, and platelet function in healthy human volunteers