

Megakaryocyte Growth and Development Factor

Analyses of In Vitro Effects on Human Megakaryopoiesis and Endogenous Serum Levels during Chemotherapy-induced Thrombocytopenia

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Abstract

The present study shows that recombinant human megakaryocyte growth and development factor (r-HuMGDF) behaves both as a megakaryocyte colony stimulating factor and as a differentiation factor in human progenitor cell cultures. Megakaryocyte colony formation induced with r-HuMGDF is synergistically affected by stem cell factor but not by interleukin 3. Megakaryocytes stimulated with r-HuMGDF demonstrate progressive cytoplasmic and nuclear maturation. Measurable levels of megakaryocyte growth and development factor in serum from patients undergoing myeloablative therapy and transplantation are shown to be elaborated in response to thrombocytopenic stress. These data support the concept that megakaryocyte growth and development factor is a physiologically regulated cytokine that is capable of supporting several aspects of megakaryopoiesis. (*J. Clin. Invest.* 1995. 95:2973-2978.) Key words: colony forming unit-megakaryocyte • thrombopoietin • ploidy • cytokine • patient serum

Introduction

The hematopoietic factor(s) directing the processes of megakaryocyte development and platelet formation have been the object of intense study for nearly 40 yr (1, 2). Traditionally, regulation of this lineage has been attributed to separate factors controlling growth (megakaryocyte colony-stimulating factors, Meg-CSF¹) versus differentiation (thrombopoietin, TPO) (3-10). Recently, reports concerning c-mpl, a gene encoding a cytokine receptor expressed on CD34+ cells, megakaryocytes,

and platelets (11, 12) strongly suggested that the unknown ligand for this receptor might be an important megakaryocyte regulatory cytokine. This has now been confirmed by several groups that have cloned and expressed the gene for this new factor (13-16). This cytokine has been named: TPO invoking the historical name for a platelet-forming factor (15), Mpl ligand (ML) to reflect the ligand/receptor relationship with Mpl (14), or megakaryocyte growth and development factor (MGDF), to reflect the multiple biological properties of the molecule (13).

The in vitro characterization of the recombinant, human form of this cytokine (r-HuMGDF) on human megakaryopoiesis is presented here. r-HuMGDF behaves both as a meg-CSF and as a megakaryocyte maturation factor (TPO) on human CD34+ cells. Measurable levels of MGDF in serum collected from patients undergoing myeloablative therapy and transplantation demonstrate the physiological relevance of this molecule. These data support the concept that all of the megakaryopoietic and thrombopoietic activities present in plasma, sera, or urine from models of bone marrow aplasia or thrombocytopenia (2, 11, 17-20) can be substantially, if not wholly, accounted for by the cytokine MGDF.

Methods

Human subjects. Normal AB plasma was obtained from healthy adult volunteers. Leukapheresis units were purchased from Hemacare (Sherman Oaks, CA) (5). All donors provided informed consent.

Enrichment of CD34+ cells from leukapheresis packs. Low density mononuclear cells from leukapheresis units obtained by centrifugation over Ficoll-Paque from Pharmacia LKB Biotechnology, Inc., (Piscataway, NJ), (460 × g, 25 min) were stored overnight at 4°C until the CD34-selection procedure. Cells were prepared for selection using the CD34-Isolation Kit from Miltenyi Biotec Inc. (Auburn, CA) following the manufacturer's recommended protocol. Mini-MACS magnetic cell separation columns (Miltenyi Biotec Inc.) were used as recommended with the positive cells being passed over a new set of columns. A mean of 4.4 ± 1.9 million CD34+ cells per leukapheresis unit were obtained with this method (*n* = 25, range from 2.2 to 10.5 million). Purity of the CD34-selected population was determined by immunostaining as described elsewhere (5) and averaged 90 ± 5% (*n* = 12, range from 81 to 96%). Cells were aliquoted and frozen until use (5).

Megakaryocyte colony-forming assay. Colony forming units megakaryocyte (CFU-meg) were detected by a modification of the plasma clot assay (17). CD34+ cells were plated in triplicate wells of 24-well tissue culture plates from Falcon Labware (Lincoln Park, NJ) at the indicated cell concentration. Culture media consisted of 15% citrated, platelet-poor, human AB plasma in Iscove's modified Dulbecco's me-

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1. Abbreviations used in this paper: MGDF, megakaryocyte growth and development factor; r-HuMGDF, recombinant human MGDF; SCF, stem cell factor; TPO, thrombopoietin.

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dium from GIBCO BRL (Grand Island, NY) with designated growth factors and additives as described (21). Megakaryocyte colonies were stained with antibodies to GPIb and GPIIb/IIIa from BIODSIGN International (Kennebunkport, ME) and a secondary goat-anti-mouse conjugated with FITC from Southern Biotechnology Associates, Inc. (Birmingham, AL). Nuclei were counter-stained with propidium iodide (PI) at 2.5 $\mu\text{g}/\text{ml}$ from Sigma Chemical Co. (St. Louis, MO) in 0.1% citrate. Colonies were defined as three or more brightly green fluorescent cells by inverted fluorescent microscopy from Olympus Corp. (Lake Success, NY) at $\times 100$ magnification. Photomicrographs were obtained on a PM-20 microphotography system also from Olympus Corp.

Liquid culture of megakaryocyte progenitors. Megakaryocyte cultures were established as previously described (5) except for the substitution of r-HuMGDF for aplastic canine plasma. Cytospins of cultured megakaryocytes were stained by a modified Wright-Giemsa method in the Aerospray slide stainer by Wescor Inc. (Logan, UT). Slides were photographed with the Microphot-FXA microphotography system from Nikon Inc. (Melville, NY). Stages were assigned according to the method of Levine et al. (22).

Growth factors. The following human recombinant factors were added to cultures as indicated: stem cell factor (SCF; Amgen, Inc., Thousand Oaks, CA), and IL-3 (Genzyme Corp., Cambridge, MA). Chinese hamster ovary cell-derived r-HuMGDF (13) was produced and purified to homogeneity at Amgen, Inc.

FACS® Analysis. Flow cytometry was performed on a FACScan® flow cytometer (Becton Dickinson and Co., San Jose, CA) equipped with a 15-mW laser and a 100- μm diameter flow tip. An excitation wavelength of 488 nm was used, and fluorescence emission for FITC and propidium iodide were collected using a 530-nm band pass filter and a long wavelength (> 650 nm) band pass filter, respectively.

Megakaryocytes were stained with a cocktail of antibodies to GPIb, GPIIb, GPIIb/IIIa from BIODSIGN International and GPIIb from Dako Corp. (Carpinteria, CA) followed by a secondary goat-anti-mouse-FITC. Stained cells were washed and resuspended in propidium iodide and RNase both from Sigma Chemical Co. each at 50 $\mu\text{g}/\text{ml}$. The 2N ploidy peak, where 2N is the DNA content of a normal diploid cell, was established with data acquired from a CD34+ cell population. Data acquisition of cultured cells was restricted to megakaryocytes by gating on the FITC fluorescence. At least 10,000–50,000 megakaryocytes per sample were acquired.

Evaluation of MGDF in human patient sera. Serum samples were collected and platelet counts taken from patients with lymphoid malignancies: non-Hodgkin's lymphoma ($n = 6$), acute lymphoblastic leukemia ($n = 4$), and Hodgkin's disease ($n = 1$). Patients were treated (day -7 through -2) with high dose busulfan and cyclophosphamide before transplant with autologous peripheral blood progenitor cells with ($n = 9$) or without ($n = 2$) autologous bone marrow on day 0. All patients received Filgrastim (G-CSF) during the recovery period (23, 24). At the time of the treatment serum was separated, aliquoted, and stored frozen at -80°C . Serum MGDF levels were measured in the thawed samples with factor-dependent murine cells expressing human c-mpl (32D/huMpl+ cells) as described (13). For analysis, MGDF levels were normalized to the maximum MGDF level observed for each patient. MGDF activity in the samples could be completely inhibited by the truncated form of c-mpl.

Data analysis. Data are expressed as the mean \pm SEM.

Results

r-HuMGDF stimulates megakaryocyte colony formation. CD34+ cells incubated with r-HuMGDF for 12 d formed megakaryocyte colonies in a dose-dependent manner (Fig. 1 A). Maximum colony formation was observed with r-HuMGDF concentrations of 5–10 ng/ml or greater. A typical megakaryocyte colony as observed in these cultures is pictured in Fig. 1 B. No other types of colonies were observed even though the

use of propidium iodide allowed visualization of all cells in the culture dish.

Cultures were established with decreasing numbers of CD34+ cells to determine if colony formation was linearly related to the number of cells plated. Cell concentrations ranged from 150 to 3,000 cells per well. r-HuMGDF was used at 50 ng/ml. As shown in Fig. 2, a linear relationship was observed ($r^2 = 0.997$) such that the ratio of cell number to colony number was constant at 0.04 ± 0.01 , or one colony per 40–50 plated cells. Colonies were seen at all cell concentrations.

Cytokine combinations. r-HuMGDF was examined alone in combination with SCF or IL-3 for effects on megakaryocyte colony formation. Preliminary experiments were performed to determine the optimal concentration of each cytokine. Each cytokine was also tested alone for its megakaryocyte colony stimulating ability. SCF, which did not induce colony formation at any concentration tested, was used at 50 ng/ml. IL-3 was used at the maximally effective concentration of 10 ng/ml and r-HuMGDF was used in a dose range from 0.1 to 100 ng/ml.

The effects of these cytokines on colony number are shown in Fig. 3. In this experiment, IL-3 induced 12 ± 2 colonies. r-HuMGDF maximally induced from 120 to 140 colonies. However, the combination of r-HuMGDF and IL-3 resulted in fewer colonies than induced with r-HuMGDF alone. This type of "inhibitory" response was seen in two experiments. In two other experiments of equivalent design, the cytokine combination resulted in a number of colonies that was more than observed with either individual factor, but less than the additive number. In no case was a synergistic reaction observed. This was in contrast to the result of combining r-HuMGDF with SCF where, in four independent experiments, the combination consistently resulted in more colonies (120–200%) than generated with r-HuMGDF alone.

The effects of these cytokines on colony size (megakaryocytes per colony) are shown in Fig. 4. In panel A are data relating the number of cells per colony to increasing r-HuMGDF concentrations. To avoid a selection bias all colonies in the cultures were evaluated. A maximum number of cells per colony was observed with r-HuMGDF concentrations of 5 ng/ml or greater. In panel B, the effect of r-HuMGDF alone (10 ng/ml) or in combination with IL-3 or SCF is shown. r-HuMGDF or IL-3 induced 11 ± 1.3 or 11.5 ± 1.3 cells per colony, respectively. When used in combination, 17 ± 1.9 cells per colony were observed. In contrast, the combination of r-HuMGDF and SCF did not affect colony size which stayed constant at 9.7 ± 0.7 cells per colony.

r-HuMGDF stimulates megakaryocyte differentiation. r-HuMGDF was examined for the ability to induce differentiation and endomitosis in a megakaryocyte precursor cell population (Fig. 5). The cellular morphology and ploidy profile of a 2N CD34+ starting population are shown in Fig. 5, A and D, respectively. After 8 d of culture in r-HuMGDF, all four stages of mature megakaryocytes were observed in the population (B). The dominant maturity stages were stage I and II, each representing 37% of the megakaryocyte population. Stage IV cells comprised 7% of the population. At this time, the major ploidy peaks were 2N and 4N with smaller 8N and 16N populations visible (Fig. 5 E). Cultures were also established for 12 d. The dominant maturity stages at this time were stage II and III, each representing 29% of the megakaryocyte population while the percentage of stage IV cells was 21% (Fig. 5 C). The ploidy profile clearly shows evidence of continued endomitotic activity

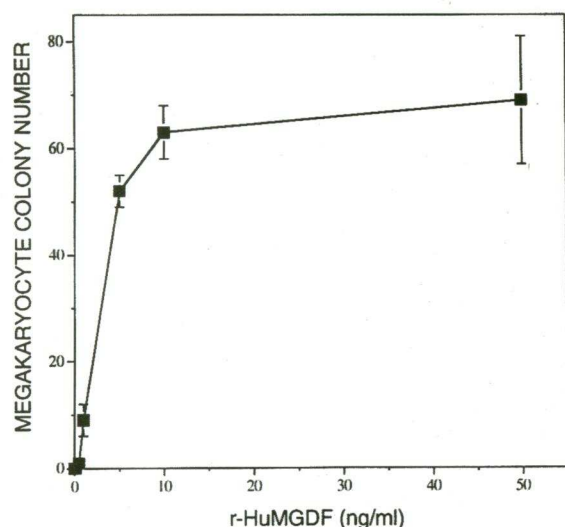
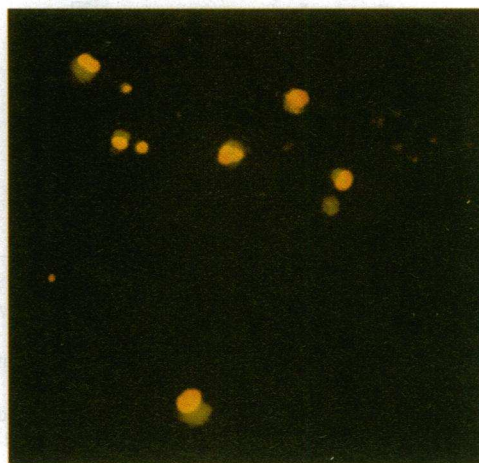
A**B**

Figure 1. r-HuMGDF is a megakaryocyte colony stimulating factor. (A) CD34⁺ cells were plated in the plasma clot assay at 1,500 cells per well (5,000/ml) with media alone or with r-HuMGDF at concentrations ranging from 0.1 to 50 ng/ml. (B) A typical megakaryocyte colony stimulated with 10 ng/ml r-HuMGDF. The pale halo of cytoplasm reflects FITC-labeled α -platelet glycoprotein Ib and IIb/IIIa. The bright nuclei are stained with propidium iodide. ($\times 25$) Data are expressed as the mean of triplicate data points \pm SEM. This experiment is representative of three identical experiments.

in the population as the main ploidy peaks have shifted to 4N and 8N and 16N and 32 N peaks have become more prominent (Fig. 5 F).

Serum MGDF levels in thrombocytopenic patients. Platelet levels and serum MGDF levels were determined in a population of patients treated with myeloablative therapy (day -7 through -2) and progenitor cell transplant (day 0) (Fig. 6). All of the patients exhibited a period of severe thrombocytopenia (days 5-10) followed by a rise in platelet counts as engraftment occurred. In all 11 of the patients evaluated, serum MGDF levels were low to undetectable through the treatment period, then rose and peaked during the thrombocytopenic nadir. MGDF levels dropped after this point and slowly returned toward baseline levels. Although all of the patients showed the same pattern over the time course, the absolute MGDF levels

varied considerably between patients. Levels ranged from 0 to 3,200 U/ml in the treatment phase and rose to from 1,600 to 102,400 U/ml during the peak phase. Because of this variation, MGDF levels within each patient series were calculated as the percentage of the maximum value seen in that patient series.

Discussion

The activity of r-HuMGDF on human CD34⁺ cells is consistent with that expected for a lineage-restricted, megakaryocyte colony-stimulating factor. Colonies of 3-30 megakaryocytes were observed with 12 d of culture, an accepted standard for this type of factor (25). r-HuMGDF appears to function without accessory cell contribution since the relationship between the number of CD34⁺ cells plated to the number of colonies formed

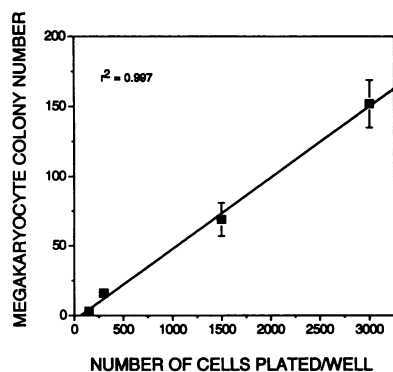


Figure 2. A linear relationship exists between CD34⁺ cells plated and r-HuMGDF-stimulated colonies. CD34⁺ cells were plated from 150 to 3,000 cells per well (500-10,000 cells/ml) in the plasma clot assay in 50 ng/ml r-HuMGDF. The cell dose response was linear over the ranges tested ($r^2 = 0.997$). Colony growth was seen in all

culture conditions. Data are expressed as the mean of triplicate data points \pm SEM. This experiment is representative of three identical experiments.

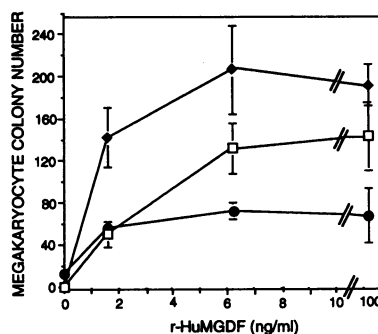


Figure 3. r-HuMGDF synergizes with SCF but not with IL3 in megakaryocyte colony formation. CD34⁺ cells were plated at 3,000 cells per well (10,000 cells/ml) in increasing concentrations of r-HuMGDF (open squares) or in MGDF in combination with SCF at 50 ng/ml (solid diamonds) or IL-3 at 10 ng/ml (solid circles).

The data points on the Y-axis are from cultures incubated without MGDF (media alone, open circle; SCF alone, solid diamond; IL3 alone, solid circle). Data are expressed as the mean of triplicate data points \pm SEM. This experiment is representative of three identical experiments.

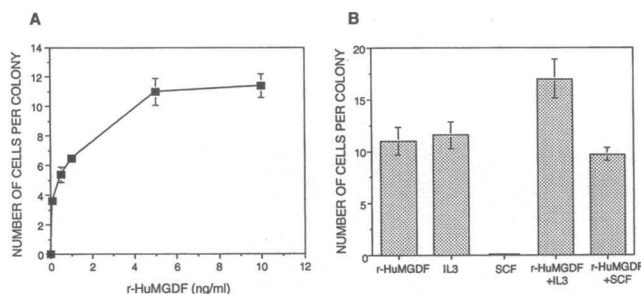


Figure 4. The effect of r-HuMGDF, alone or in combination with IL-3 or SCF, on colony size. (A) Colony size (number of cells per colony) was measured as a function of r-HuMGDF concentration (0.1–10 ng/ml). All colonies in the culture well were evaluated (0.1 ng/ml, $n = 5$; at 0.5 ng/ml, $n = 63$; at 1 ng/ml, $n = 70$; at 5 ng/ml, $n = 50$, at 10 ng/ml, $n = 70$). (B) Cytokine combinations were examined for effects on colony size. IL-3 (10 ng/ml) or SCF (50 ng/ml) were added to cultures containing r-HuMGDF (10 ng/ml). All colonies in the culture well were evaluated ($n = 37$, MGDF; $n = 21$, IL-3; $n = 0$, SCF; $n = 52$, MGDF + IL3; $n = 43$, MGDF + SCF). Data are expressed as the mean of multiple data points \pm SEM. This experiment is representative of three identical experiments.

was constant, even at low cell density. This function of r-HuMGDF was also not dependent on plasma-derived cofactors since megakaryocyte colony formation could also be observed in the serum-depleted fibrin clot assay described by Bruno et

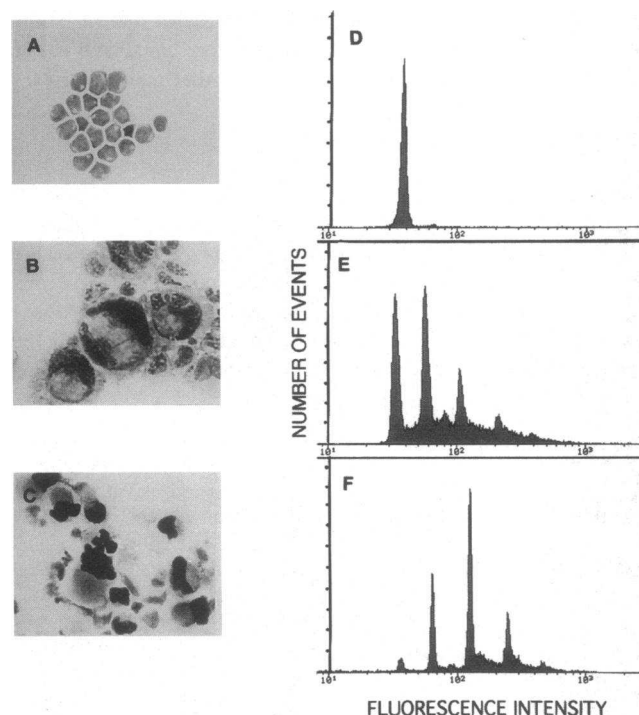


Figure 5. The effect of r-HuMGDF on megakaryocyte differentiation and endomitosis. CD34⁺ cells (shown in A and D) were set up in liquid culture in 50 ng/ml r-HuMGDF for 8 d (shown in B and E) or 12 d (shown in C and F). (A–C) Photomicrographs of cells stained with a modified Wright-Giemsa stain ($\times 90$). (D–F) Ploidy profiles from FACS[®] analysis of cells stained with α -platelet antibodies-FITC and propidium iodide.

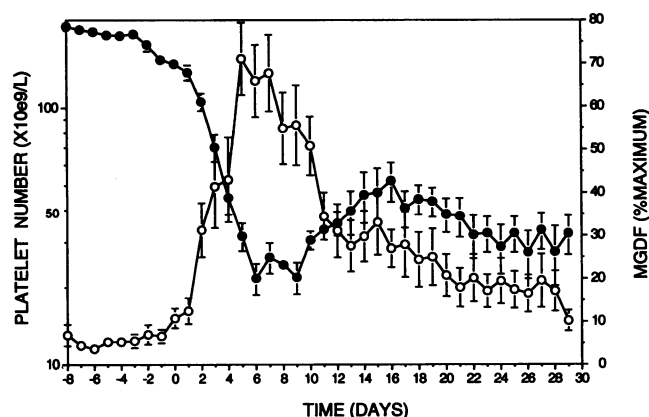


Figure 6. Serum MGDF levels in thrombocytopenic patients. Patients ($n = 11$) were treated with myeloablative therapy (day -7 through -2) and progenitor cell transplant (day 0). MGDF levels were determined from serum samples using the 32D/huMpl⁺ bioassay. MGDF levels are presented as the percentage of the maximum level observed within each patient series. Data are the mean \pm SEM.

al. (26) (Briddell, R. personal communication). r-HuMGDF, like other lineage-restricted colony stimulating factors (27, 28), is synergistic with the earlier acting factor, SCF. The MGDF-responsive progenitor cell has been partially characterized. It is a mononuclear cell of ~ 8 – $10 \mu m$ in diameter with a large nuclear/cytoplasmic ratio. In earlier studies, using a plasma source of MGDF, the phenotype was defined as CD34⁺/CD38⁺ and CD34⁺/HLADR⁺. Responsive cells were also included in both CD34⁺/CD41⁺ and CD34⁺/CD41[–] populations (5).

The activity of r-HuMGDF on human CD34⁺ cells is also consistent with that expected for a megakaryocyte differentiation factor (29, 30). r-HuMGDF was sufficient to induce the development of progenitor cell populations into stage IV megakaryocytes exhibiting classic features of eosinophilic granulation and nuclear condensation and eccentricity. Endomitotic activity, another *in vitro* parameter of maturity (29, 31, 32) was also evident in these cultures. In addition, r-HuMGDF-induced megakaryocytes are fully competent to complete the natural life cycle of this lineage, which is to fragment into functional platelets (Choi, E. S., M. Hokom, T. Bartley, Y.-S. Li, H. Oh-shi, T. Kato, J. L. Nichol, J. Skrine, A. Knudten, J. Chen, et al., manuscript submitted for publication). These data are in full agreement with published reports on the ability of this molecule to raise the platelet counts of recipient animals (14, 15).

The concept that factor(s) present in aplastic sera control both mitosis and endomitosis has previously been suggested by Arriaga et al. in 1987 (25), and more recently by Wendling et al. (33) and de Sauvage et al. (14). Other hematopoietic cytokines such as macrophage colony-stimulating factor, interleukin 5, erythropoietin, and granulocyte colony-stimulating factor (34–36) have also been shown to stimulate not only growth but also differentiation of their target cells. Although the exact mechanism for the dual function of MGDF has yet to be determined, this could involve stimulation of alternative tyrosine kinase pathways (35) or be accomplished through occupation of specific regions of the receptor (36).

Relationships between r-HuMGDF and the cytokines IL-3 and SCF can be inferred from the combination studies pre-

sented. SCF increases the number of colonies induced by r-HuMGDF without effecting the number of cells per colony. This implies that SCF may expand a megakaryocyte precursor pool responsive to the mitotic stimulation provided by r-HuMGDF. The r-HuMGDF/IL-3 combination had a different response. Colony numbers either remained below the levels seen with r-HuMGDF alone or they were slightly less than additive when each cytokine was present at a maximally effective concentration. These different results may be due to an inconsistency in the proportions of responsive subpopulations from donor to donor. However, the observation that this combination consistently resulted in an increase in the number of cells per colony also implies that at least some of the target cells are responsive to both factors. This mitotic effect of IL-3 on r-HuMGDF-stimulated cultures may provide an additional example of the relationship suspected between mitosis and endomitosis as described by Arriaga et al. (25), Hegyi et al. (1), and Debeli et al. (37).

MGDF is a cytokine present in several experimental models of bone marrow aplasia (13–16). Therefore, it was of interest to consider the relationship between serum MGDF and platelet levels in a patient population that experiences severe, but reversible, thrombocytopenia. In myeloablated patients undergoing progenitor cell transplant, the platelet nadir was always associated with the peak of serum MGDF which returned towards baseline as platelet counts recovered. This time course is consistent with earlier work that tracked megakaryopoietic activity in similarly compromised patients (38). The peak levels produced (1,600–102,400 U/ml) were sufficient to support in vitro colony formation. The mean unit value of aplastic canine plasma, a potent source of megakaryocyte colony stimulating activity (13, 17), is $4,222 \pm 353$ U/ml. Although it is tempting to calculate the MGDF protein concentrations in these samples, it would not be accurate to do so. Interfering substances present in serum make this a semiquantitative assay. The development of specific immunoassays will allow direct measurement of MGDF levels.

The data presented here support the concept that MGDF acts on at least two aspects of megakaryocyte development, growth, and maturation. MGDF is also elaborated into the plasma of patients experiencing thrombocytopenic stress in a manner that implies it is responding to this stress. These data suggest that MGDF is a physiologically regulated cytokine that is capable of supporting several aspects of megakaryopoiesis.

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