Pegylated Megakaryocyte Growth and Development Factor Abrogates the Lethal Thrombocytopenia Associated With Carboplatin and Irradiation in Mice

By Martha M. Hokom, David Lacey, Olaf B. Kinstler, Esther Choi, Steve Kaufman, Judy Faust, Chris Rowan, Erik Dwyer, Janet L. Nichol, Timothy Grasel, John Wilson, Randy Steinbrink, Randy Hecht, Dwight Winters, Thomas Boone, and Pamela Hunt

Megakaryocyte growth and development factor (MGDF) is a potent inducer of megakaryopoiesis in vitro and thrombopoiesis in vivo. The effects of MGDF appear to be lineage-selective, making this cytokine an ideal candidate for use in alleviating clinically relevant thrombocytopenias. This report describes a murine model of life-threatening thrombocytopenia that results from the combination treatment of carboplatin and sublethal irradiation. Mortality of this regimen is 94% and is associated with widespread internal bleeding. The daily administration of pegylated recombinant human MGDF (PEG-MGDF) significantly reduced mortality (to <15%) and ameliorated the depth and duration of thrombocytopenia. The severity of leukopenia and anemia was also reduced, although it was not clear whether these effects were direct. Platelets generated in response to PEG-MGDF were morphologically indistinguishable from normal platelets. PEG-MGDF administered in combination with murine granulocyte colony-stimulating factor completely prevented mortality and further reduced leukopenia and thrombocytopenia. These data support the concept that PEG-MGDF may be useful to treat iatrogenic thrombocytopenias.

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MATERIALS AND METHODS

rMGDF administration to normal or myelosuppressed mice. Balb/c mice (female, 8 to 11 weeks of age, and weighing 19 to 21 g) were purchased from Charles River Laboratories (Hollister, CA) and quarantined for at least 7 days before use. For normal mice, rMGDF or PEG-rMGDF was administered subcutaneously in a range of doses for 5 consecutive days. Platelet levels were determined on day 6. Myelosuppression was induced with a combination of carboplatin (Bristol Labs, Princeton, NJ; 1.25 mg/animal, single intraperitoneal dose) followed 4 hours later with sublethal gamma irradiation (500 rad, single dose) on day 0. Cytokines diluted into phosphate-buffered saline (PBS) and 0.1% homologous mouse serum were subcutaneously administered once daily, starting 24 hours later (day 1) after the chemotherapeutic/radiation treatment and throughout the duration of the experiments. The excipient control (placebo) was PBS and 0.1% homologous mouse serum.

Cytokines. A truncated version of rMGDF<sup>9,12</sup> was expressed in Escherichia coli cells and modified by the covalent attachment of polyethylene glycol (PEG-rMGDF). Both rMGDF species were purified to homogeneity before use (Amgen Inc, Thousand Oaks, CA). In vitro, the specific activities of rMGDF and PEG-rMGDF did not differ by more than 25%. Specific activities, based on the peptide molecular weight, were determined with a factor-dependent murine cell line transfected with the human c-mpl gene<sup>6</sup> in which 1 U results in half-maximal growth stimulation. Murine recombinant G-CSF was produced at Amgen Inc.

Hematology. Blood samples were taken on the indicated days from a 2-mm incision in the lateral tail vein. Blood cell counts were determined with the Sysmex Cell analyzer (TOA Medical Electronics, Kobe, Japan). Red blood cell (RBC) and white blood cell (WBC) counts were always within the linear range of the instrument. Samples were diluted for platelet determinations, if necessary.

Histopathology. Femurs were removed, fixed overnight in zinc-buffered formalin, and then decalcified in a formic acid solution. The bones were embedded in paraffin and sections were histochemically stained with hematoxylin and eosin. Addition-
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Fig 1. The effects of rMGDF on platelet counts in normal mice. The indicated dose of rMGDF was administered subcutaneously to normal mice for 5 consecutive days. Platelet counts (10^9/L) were determined 24 hours later. (•) PEG-rMGDF; (○) coil-rMGDF. Data from the linear portions of the curves are plotted according to the formula \( y = m \log x + b \). Each point is the mean ± SEM of counts from 4 to 19 animals.

ally, immunohistochemistry was performed using antibodies against factor VIII-related protein (Dako, Carpinteria, CA).

Electron microscopy. Whole blood was collected via cardiac puncture into a syringe containing 200 μL of 100% ACD and 8 μmol/L of prostaglandin E1 (PGE1) and was fixed immediately in 1.5% glutaraldehyde in 0.1 mol/L sodium cacodylate buffer, pH 7.4, for 2 hours at room temperature. The platelets were isolated by differential centrifugation, post-fixed in 1% osmium tetroxide for 1 hour, sequentially dehydrated in ethanol, infiltrated with L.R. White, and polymerized at 60°C. The sections were stained with uranyl acetate and lead citrate and then examined and photographed under a Philips CM120 transmission electron microscope (Philips, Eindhoven, The Netherlands).

Data analysis. Data are presented as the mean ± SEM. Statistical comparisons were performed using a two-tailed Student’s t-test.

RESULTS

The effects of rMGDF and PEG-rMGDF on normal murine blood cell counts. rMGDF and PEG-rMGDF showed different dose-dependent activities in increasing platelet counts in normal mice (Fig 1). The more biologically active species was PEG-rMGDF, with a response/dose ratio (slope) of 1,997. rMGDF was less biologically active, with a slope of 633. Even at doses of PEG-rMGDF that increased the platelet count fivefold, there were no statistically significant effects on WBC or RBC counts. Table 1 shows the mean values for WBC, RBC, and platelet counts in animals receiving PEG-rMGDF (n = 8) or excipient (n = 33) 24 hours after 5 consecutive days of treatment.

The effects of PEG-rMGDF on blood cell counts in myelosuppressed mice. PEG-rMGDF was selected for further evaluation and was administered to mice pretreated with carboplatin and irradiation. Effects on the degree and/or duration of thrombocytopenia are shown in Fig 2. Whereas mice receiving excipient after carboplatin and irradiation showed a 98% reduction in platelet counts by day 15 or 16 (17 ± 2 x 10^9/L), animals receiving PEG-rMGDF had improved platelet nadirs that occurred by day 9 or 10 (50 ± 8 x 10^9/L). Additionally, whereas platelet counts in mice receiving excipient never recovered to normal levels within the 23-day study, those in mice receiving PEG-rMGDF returned to normal by day 19 to 21. Studies comparing the efficacy of human rMGDF and PEG-rMGDF in this murine model were performed. It was necessary to administer 6 times the dose of rMGDF compared with PEG-rMGDF to achieve a similar therapeutic benefit (data not shown).

Platelets produced in response to PEG-rMGDF after carboplatin/irradiation were compared with platelets collected from normal animals. In Fig 3 are shown electron micrographs of platelets collected from platelet-rich plasma from normal animals (Fig 3A) or from carboplatin/irradiated ani-

Table 1. Daily Administration of PEG-rMGDF for 5 Days Has No Significant Effect on WBC or RBC Counts in Normal Animals

<table>
<thead>
<tr>
<th></th>
<th>WBC (10^9/L)</th>
<th>RBC (10^9/L)</th>
<th>Platelets (10^9/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PEG-rMGDF</td>
<td>8</td>
<td>8.03 ± 0.02</td>
<td>8.83 ± 0.05</td>
</tr>
<tr>
<td>Excipient</td>
<td>33</td>
<td>11.0 ± 0.9</td>
<td>10.3 ± 0.2</td>
</tr>
</tbody>
</table>

PEG-rMGDF preparation or excipient was administered at 50 μg/kg/d to normal mice for 5 consecutive days. Data are the mean ± SEM of multiple determinations. Platelet counts from the PEG-rMGDF-treated group were significantly different from those of the excipient-treated group (\( P \leq .01 \)). WBC and RBC counts from the PEG-rMGDF-treated group were not significantly different from those of the excipient-treated group (\( P \leq .01 \)).

Fig 2. The effects of PEG-rMGDF on platelet counts in myelosuppressed mice. Mice were treated on day 0 with carboplatin and irradiation and received PEG-rMGDF at 50 μg/kg/d (•; n = 17) or excipient (○; n = 25) from days 1 to 23. Platelet counts (10^9/L) were determined on the indicated days. Data are combined from five separate experiments.
mals treated with PEG-rMGDF for 21 days (Fig 3B). The platelets were virtually identical with respect to shape, size, and granule distribution.

PEG-rMGDF was also evaluated in this model for effects on the degree and/or duration of leukopenia and anemia (Fig 4). The leukopenic nadir in excipient-treated mice occurred on day 13 at approximately 6% of normal levels (0.6 ± 0.04 × 10^9/L). Administration of PEG-rMGDF did not affect this parameter. However, leukocyte recovery was significantly faster in PEG-rMGDF-treated animals (Fig 4A). RBC counts in excipient-treated animals decreased to 20% of normal levels by day 23 (1.8 ± 0.3 × 10^12/L). Mice receiving PEG-rMGDF exhibited a substantially milder reduction in RBC, to 60% of normal, which recovered to pretreatment levels by the end of study (Fig 4B).

The effects of PEG-rMGDF on survival after treatment with carboplatin and irradiation. Treatment with the combination of carboplatin and irradiation was lethal for most animals. Only 6% of excipient-treated animals survived the 23-day study period. In contrast, 86% of animals receiving PEG-rMGDF survived (Fig 5).

To investigate probable causes of death, histopathology studies were performed on mice treated with carboplatin and irradiation and treated with excipient or PEG-rMGDF for 15 days. Hemorrhagic foci were seen in the gastrointestinal tract, lung, urinary system, and brain in excipient-treated animals, but not in animals receiving PEG-rMGDF. No obvious signs of infection were present in either group at this time point (data not shown).

Sections of bone marrow from both groups are shown in Fig 6. Mice treated with excipient exhibited a 90% loss in marrow cellularity 15 days after carboplatin/irradiation (Fig 6A) and showed an almost total lack of marrow megakaryocytes (Fig 6B). In contrast, mice treated with PEG-rMGDF possessed 60% to 95% of normal marrow cellularity at this time (Fig 6C) and had an abundance of marrow megakaryocytes (Fig 6D). In addition to enhancing megakaryopoiesis, PEG-rMGDF exposure also led to an increase in myelopoietic and erythropoietic activity evident in foci of both lineages in bone marrows of treated mice (Fig 6E).

The effects of PEG-rMGDF and recombinant murine G-CSF on survival and blood cell counts after carboplatin and irradiation. The combination of PEG-rMGDF and G-CSF (recombinant murine G-CSF) was evaluated in mice pretreated with carboplatin and irradiation (Fig 7). In this experiment, none of the animals in the excipient-treated or the G-CSF-treated groups survived past day 15. However, 90% of the PEG-rMGDF-treated animals and 100% of the combination-treated animals survived (Fig 7A).

Animals treated with PEG-rMGDF plus G-CSF and animals treated with PEG-rMGDF alone exhibited similar platelet count kinetics, with nadirs of approximately 30 × 10^9/L
at day 9 and with recoveries to normal levels by days 19 to 23. However, by the end of the study, the cytokine combination-treated group had platelet counts that were significantly greater than those in the PEG-rMGDF-treated group (1,669 ± 234 × 10⁹/L and 497 ± 111 × 10⁹/L, respectively, P ≤ .01; Fig 7B).

Because of the mortality in the excipient-treated and G-CSF-treated groups, WBC counts were not available for those animals past day 13. We therefore compared WBC counts in the groups administered the cytokine combination or PEG-rMGDF alone. The leukocyte nadir in the former group was 0.70 ± 0.06 × 10⁹/L on day 5 and in the latter group was 0.38 ± 0.05 × 10⁹/L on day 8. At the end of study, the combination-treated group had a WBC count of 7.0 ± 1.6 × 10⁹/L compared with 2.2 ± 0.4 × 10⁹/L in the PEG-rMGDF-treated group (Fig 7C).

The coadministration of PEG-rMGDF plus G-CSF or treatment with PEG-rMGDF alone resulted in virtually identical RBC recovery kinetics (data not shown).

DISCUSSION

Damage to the hematopoietic system is frequently a serious complication of cancer therapy. In particular, damage to neutrophil and platelet progenitors can result in severe clinical consequences such as sepsis and hemorrhage, respectively. The severity and duration of neutropenia in patients can be reduced by administration of myeloid growth factors. However, corresponding cytokines for specific abrogation of thrombocytopenia have not been available. Preclinical and clinical work with cytokines such as interleukin-6 (IL-6), IL-11, IL-3, and IL-12 indicate some activity on thrombopoiesis, but amelioration of clinically important thrombocytopenia has not yet been achieved and the potential for adverse events perhaps due to the lack of specificity of these cytokines exists.

The lineage-selectivity of rMGDF and its potent in vitro effects on megakaryopoiesis suggest that this molecule may play a significant role in the treatment of thrombocytopenia. This study sought to identify effective rMGDF species that approximated the mass of circulating mammalian proteins. Molecules encoded by an N-terminal portion of the human cDNA sequence were produced as nonglycosylated or as pegylated versions. Both preparations had similar in vitro activities, indicating that the C-terminal portion of the molecule was not required for receptor/ligand interactions, as previously reported. However, the in vivo biologic activities of the two preparations were distinct. In particular, pegylation of rMGDF increased the in vivo potency of the molecule in normal animals roughly 20-fold. In preliminary studies, the circulating half-life of PEG-rMGDF was approximately 10 times that of r-MGDF (Cheung et al, unpublished data). Although other biophysical properties may also contribute to the improved efficacy of the pegylated molecule, the increase in the half-life must be considered as a primary reason.

PEG-rMGDF was evaluated further in a clinically relevant model of life-threatening thrombocytopenia. The model, which was more severe than a similar model developed by Leonard et al, used a combination of carboplatin and irradiation that induced severe myelosuppression, almost complete depletion of the red marrow, and widespread bleeding. In these experiments, mortality was almost 100% and correlated in time to the pancytopenic nadir. Although the definitive cause(s) of death is not known, the presence of microscopic hemorrhagic foci in multiple organs at a time when the survival was decreasing sharply from 50% to 0% strongly imply hemorrhagic complications.

PEG-rMGDF was effective at reducing mortality from 100% to 14% and in alleviating thrombocytopenia in this setting. PEG-rMGDF in combination with G-CSF eliminated the mortality and significantly shortened the duration of leukopenia as well. Interestingly, administration of PEG-rMGDF alone lessened the leukopenic duration. This observation was reproducibly observed in PEG-rMGDF-treated animals in comparison to the relatively few excipient-treated animals that survived to the point at which leukocyte recovery could be measured (day 19). The anemia caused by carboplatin and irradiation was also improved by administration of PEG-rMGDF. The available data do not allow a distinction between PEG-rMGDF acting either to stimulate erythropoiesis or to reduce bleeding. Enhanced erythropoiesis was observed in the marrows of myelosuppressed animals receiving the cytokine, supporting a direct or indirect action of PEG-rMGDF on the RBC lineage. Alternatively, the 24-day RBC survival time in myelosuppressed mice was significantly shorter than normal (50 to 60 days), implying RBC losses through bleeding. RBC recovery in PEG-rMGDF-treated mice occurred at a time when platelet counts were returning to normal (after day 15). The apparent benefits of PEG-rMGDF treatment to myeloid and erythroid recovery postmyelosuppression must be interpreted with caution at this point because it is not clear whether the effects...
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Fig 6. The effects of PEG-rMGDF on bone marrow histology in myelosuppressed animals. Mice were treated with carboplatin and irradiation as outlined in the Materials and Methods. Excipient (A and B) or PEG-rMGDF (C and D) were administered subcutaneously daily for 15 days before killing. Bones were embedded in paraffin and sections were stained with hematoxylin and eosin (A and C). Megakaryocytes were identified immunologically with antibodies against factor VIII/von Willebrand's factor (B and D). In (E), arrows and arrowheads denote erythroid and myeloid islands, respectively. Original magnifications: (A) through (D), ×100; inset and (E), ×400.

are direct or due to the fact that the (many) surviving animals in the PEG-rMGDF–treated group had significantly fewer complications caused by thrombocytopenia compared with the (few) survivors in the excipient-treated group. The hematologic microenvironments of the two groups of survivors would be expected to be distinct.

PEG-rMGDF is a molecule that has tremendous potential for the treatment of thrombocytopenia associated with cancer therapies. Studies evaluating the relationship of serum MGDF levels to platelet counts in human7 and rodent1,12,25,29 models of platelet recovery and work with mpl-deficient mice30 suggest that native thrombopoietin is an important physiologic regulator of thrombopoiesis. In a murine model of moderate thrombocytopenia induced by carboplatin alone,
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Fig 7. The effects of PEG-rMGDF and recombinant murine G-CSF on survival, platelet counts, and WBC counts in myelosuppressed mice. Mice (n = 10 each group) were treated on day 0 with carboplatin and irradiation as outlined in the Materials and Methods and received PEG-rMGDF at 50 μg/kg/d (●), G-CSF at 10 μg/kg/d (○), PEG-rMGDF and G-CSF in combination (●), or exipient (○) from days 1 to 23. Survival (A), platelet counts (10^10/L; B), and WBC counts (10^9/L; C) were determined on the indicated days.

PEG-MGDF completely prevented thrombocytopenia. However, because spontaneous bleeding in patients does not usually occur until platelet counts are severely suppressed, a more clinically relevant model of thrombocytopenia and its complications was required. The present study shows the unambiguous efficacy of PEG-rMGDF in a clinically relevant setting of severe thrombocytopenia. It also shows that G-CSF offers additional beneficial hematopoietic effects and that the combination is best for reducing myelosuppression and preventing mortality. This study provides a rationale for clinical studies of PEG-rMGDF alone or in combination with G-CSF.

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REFERENCES


29. Kuter D, Rosenberg RD: The reciprocal relationship of thrombopoietin (c-Mpl ligand) to changes in the platelet mass during busulfan-induced thrombocytopenia in the rabbit. Blood 85:2720, 1995

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