Megakaryocyte Growth and Development Factor Accelerates Platelet Recovery in Peripheral Blood Progenitor Cell Transplant Recipients

By Graham Molineux, Cynthia Hartley, Patricia McElroy, Clay McCrea, and Ian K. McNiece

We have investigated the potential of PEGylated recombinant human megakaryocyte growth and development factor (PEG-rHuMGDF), a molecule related to thrombopoietin (mpl ligand or TPO) in minimizing the thrombocytopenia associated with hematopoietic ablation and peripheral blood progenitor cell (PBPC) transplant. Irradiated mice that received PBPC mobilized by PEG-rHuMGDF or granulocyte colony-stimulating factor (G-CSF) had a reduced number of thrombocytopenic days with platelets below 100 × 10⁹ per mL of blood. Recipients of unmobilized PBPC had a 9 day thrombocytopenic phase which was shortened to 7 days if they were given granulocyte-macrophage colony-stimulating factor (GM-CSF)—mobilized PBPC. This was further reduced to 2 or 3 days of thrombocytopenia in recipients of G-CSF- or PEG-MGDF—mobilized PBPC. Despite our observation that PEG-rHuMGDF is a relatively modest stimulator of the mobilization of myeloid progenitors to the blood, MGDF—mobilized PBPC do effect accelerated recovery of platelets after transplantation. However, the most effective use of PEG-rHuMGDF is when it is given during the recovery phase after PBPC transplantation to hematopoietically ablated mice. Posttransplant treatment with PEG-rHuMGDF reduces thrombocytopenia to a single day or less, in recipients of most types of PBPC. Mice that were treated during the first 2 weeks after PBPC transplant with PEG-rHuMGDF had 1 thrombocytopenic day compared to 9 days in carrier-treated recipients of unmobilized PBPC and 2 to 3 days in carrier-treated recipients of the optimally mobilized PBPC from G-CSF or G-CSF/PEG-rHuMGDF treated donors. In groups where PEG-rHuMGDF was included in the mobilization protocol and used to treat recipients as well thrombocytopenia was effectively eliminated. These data show that PEG-rHuMGDF is a highly effective agent in eliminating the thrombocytopenia associated with PBPC transplantation.

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PERIPHERAL BLOOD progenitor cell (PBPC) harvests are replacing bone marrow (BM) as the tissue of choice for hematopoietic support of ablated patients and for improving the rate of hematopoietic recovery in myelosuppressed patients. PBPC are normally present in very low numbers in the blood so large volumes of blood need to be processed to obtain sufficient cells for engraftment. Hematopoietic growth factors (HGF) can substantially increase the number of PBPC collected at each round of leukapheresis and harvests from HGF-treated patients have been shown to outperform conventional BM grafts in terms of the rate of recovery of PB populations. PBPC are also at least equivalent to BM in terms of the ability to sustain stable long-term engraftment. The ease of collection together with the advantages outlined above make HGF mobilized PBPC preferable to BM in many situations.

The usefulness of HGFs has also been shown in the posttransplant setting where blood cell recovery rates can be improved by treatment with granulocyte colony-stimulating factor (G-CSF) or granulocyte macrophage CSF (GM-CSF) in the period after transplantation. Traditionally the improvement of platelet recovery has proven more refractory to growth factor therapy, and though clinical studies have indicated that G-CSF mobilized PBPC can sustain accelerated platelet recovery in patients, a concern remained that the potential of PBPC may not be fully exploited in transplant recipients lacking the support of an effective thrombopoietin (TPO). The recent cloning of the mpl ligand (also called TPO) has raised the possibility of artificially stimulating accelerated recovery of platelets in various settings including myeloablative recipients of hematopoietic cell grafts. Megakaryocyte growth and development factor (MGDF), a molecule related to TPO, may also have potential as a hematopoietic cell mobilizer in PBPC donors.

The experiments described here were designed to address several potential uses of MGDF treatment. Firstly, by treating PBPC donors we determined whether PEG-rHuMGDF could increase the number of PBPC available for harvest. Secondly, by combining PEG-rHuMGDF treatment with either G-CSF or GM-CSF we attempted to modulate the quality of the product obtained in terms of its subsequent performance in transplant recipients. Thirdly, we undertook treatment of PBPC recipients with PEG-rHuMGDF to attempt to improve platelet recovery above even that obtained with CSF-mobilized PBPC. And finally, we combined PEG-rHuMGDF treatment of the PBPC donor with PEG-rHuMGDF treatment of the recipient. In this setting we attempted to both improve the quality of the graft and maximize the exploitation of any improvement by treating the recipient with an effective TPO.

Our data show the usefulness of PEG-rHuMGDF in the posttransplant setting and PEG-rHuMGDF presents us with the possibility of maximizing platelet recovery in recipients of PBPC mobilized by HGF as well as in idiopathic and chemotherapy-associated thrombocytopenic conditions.

MATERIALS AND METHODS

Mice
Splenectomized male C3H × DBA2 (BDF1) mice 12 to 20 weeks of age were used as donors and female BDF, mice 8 to 12 weeks of age were used as transplant recipients. Preparative irradiation was 1,200 cGy (¹³¹Cs, dose rate 106.7 cGy/min) given as a split dose of 2 × 600 cGy, 4 hours apart. All transplant experiments were repeated three times.
Donor Treatment
Mice were implanted with Alzet Micro-osmotic (1007D) pumps (Alza Corp, Palo Alto, CA). The pump, which was handled and filled under aseptic conditions, was inserted subcutaneously through a small incision made through the skin between the scapulae of anesthetized (Aerrane, Ohmeda Carbide Inc, Guayama PR) mice. Seven days later mice were killed by carbon dioxide inhalation and blood withdrawn via cardiac puncture. This blood was collected in evacuated glass tubes containing 50 μL 15% EDTA (Vacutainer 6536; Becton Dickinson, Rutherford, NJ). Between 800 and 1,200 μL was collected from each donor, typically groups of 10 donors were used. Complete blood counts were performed on individual blood samples on a Technicon H-1E (Technicon Instruments Corp, Tarrytown, NY) calibrated for the analysis of mouse blood. Blood from individual donors was assayed for GM-CFC content. Five hundred microliters of blood from each donor in a treatment group was then pooled and diluted to 6 mL with carrier and carefully layered over a 25 mL cushion of density separation medium (Accudyn, Accurate Chemical and Scientific Corp, Westbury, NY). After centrifuging at 800g for 20 minutes, buoyant density cells were collected, washed 2 times, counted on a Technicon H-1E, the count was confirmed manually with a hemocytometer and cells were resuspended at 4 × 10^6 white blood cell (WBC)/mL.

Recipient Treatment
Twenty-four hours before irradiation recipient mice were implanted with Alzet 2002 mini-osmotic pumps containing dilutions of PEG-rHuMGDF, which corresponded to a calculated delivered dose of 50 μg PEG-rHuMGDF/kg body weight/d or the equivalent volume of carrier solution (phosphate-buffered saline [PBS], Life Technologies, Grand Island, NJ) supplemented with 0.1% bovine serum albumin ([BSA]/PBS, Sigma, St Louis, MO), which was also used to dilute growth factors for infusion. After irradiation these mice were injected intravenously with 500 μL of the cell suspension previously described containing 2 × 10^6 PB cells from donors that had been variously treated. Groups of 10 recipients were divided into two groups of five and blood was withdrawn from the retroorbital sinus of anesthetized animals via heparinized glass capillaries into tubes containing 10 μL 3% EDTA. This blood was analyzed on a Technicon H-1E. Blood samples were collected from either group of 5 mice alternately, thus one cohort of 5 mice was sampled on days 5, 9, 12, 16, and 21, and the other cohort on days 7, 10, 14, and 19 after transplant. Transplant experiments were repeated three times. Mononuclear cell (MNC) preparations from groups of 5 to 20 donor mice were injected into groups of 10 recipient mice that were divided as outlined above. In total 270 donor and more than 350 recipient mice were used.

Growth Factors
Recombinant human MGDF is a molecule related to a truncated form of TPO, which is expressed in Escherichia coli and PEGylated. All growth factors were prepared at appropriate dilutions to give the dose levels indicated in the Results section. A dose of 200 μg/kg/d of rhG-CSF was used in comparison with a dose 200 μg/kg/d rmGM-CSF and PEG-rHuMGDF at 50 μg/kg/d. Treatment was administered by continuous subcutaneous infusion via Alzet mini-osmotic pumps. Delivery from these pumps could be expected to begin within 4 hours and continue for up to 18 days. Carrier solution was PBS/BSA and was used to dilute growth factors for infusion, or given alone to carrier-treated animals.

 Colony-Forming Assay
Aliquots of between 5 and 50 μL of whole blood were removed from individual blood samples obtained as previously described.

RESULTS
Analysis of PBPC Donors
Implantation sites in mice were inspected regularly. All donor mice were healthy and had pumps still in position and in good condition at the end of the mobilization protocol. Recipients of rHuPEG-MGDF showed increased numbers of platelets. At the 7 day time point shown in Fig 1, levels of over 4,700 × 10^6 platelets/mL, around 3.5 fold more than for carrier-treated mice, were noted in PEG-rHuMGDF treated mice. Platelet volume was increased from 5.4 ± 0.19 fL in normals to 5.9 ± 0.17 fL. WBC, neutrophils, and monocytes were increased by a relatively small amount in PEG-rHuMGDF treated donors and red blood cell (RBC) numbers were unchanged.
Predictably, G-CSF and GM-CSF increased the numbers of circulating WBC from 21.8 × 10^6/mL in carrier-treated mice to 93.1 ± 21 × 10^6 and 44.2 ± 15.3 × 10^6/mL, respectively. This was accounted for almost entirely by an 18-fold increase in neutrophils and an 11-fold increase in monocytes in G-CSF treated mice and fivefold increases in both populations in GM-CSF recipients. Platelet numbers were reduced by 34% in G-CSF and 54% in GM-CSF treated recipients. Mean platelet volume was increased to 5.78 by 34% in G-CSF and 54% in GM-CSF treated recipients. Platelets numbers were reduced interesting modifications to the CSF-only response. G-CSF/GM-CSF treated mice, but was slightly subnormal at 5.34 in G-CSF treated mice and fivefold increases in both populations of GM-CSF when it was combined with PEG-rHuMGDF (Table 1).

**Analysis of PBPC Recipients**

Results are presented from the recovery period between transplant (day 0) and up to 21 days later. Presented data

<table>
<thead>
<tr>
<th>Treatment Group*</th>
<th>GM-CFC per mL of Blood</th>
<th>GM-CFC/10^9 WBC</th>
</tr>
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<tbody>
<tr>
<td>Carrier</td>
<td>383 ± 380 (n = 45)</td>
<td>17</td>
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<tr>
<td>PEG-MGDF (50 μg/kg/d)</td>
<td>412 ± 173 (n = 45)</td>
<td>18</td>
</tr>
<tr>
<td>G-CSF (200 μg/kg/d)</td>
<td>42,091 ± 10,389 (n = 45)</td>
<td>452</td>
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<tr>
<td>G-CSF + PEG-MGDF</td>
<td>50,321 ± 9,701 (n = 45)</td>
<td>460</td>
</tr>
<tr>
<td>GM-CSF + PEG-MGDF</td>
<td>21,041 ± 873 (n = 45)</td>
<td>423</td>
</tr>
<tr>
<td>GM-CSF (200 μg/kg/d)</td>
<td>16,242 ± 4,231 (n = 45)</td>
<td>367</td>
</tr>
</tbody>
</table>

**Analysis of progenitor numbers in the blood of PBPC donors.** * Doses given to the mice were: PEG-MGDF 50 μg/kg/d, G-CSF 200 μg/kg/d, and GM-CSF 200 μg/kg/d, combination mice received the same doses. Carrier mice got 12 μL PBS/BSA per day.
Platelet recovery though the improvement was much less than was seen in recipients of G-CSF mobilized PBPC. Com-
resulted in approximately additive improvement in WBC (A.) Donor blood
ated slightly the recovery of WBC in subsequent transplant
are limited to recovery of WBC and platelets since these parameters reflect the major clinical concerns, which can be addressed by this type of experiment. Recipient mice were treated in two ways. For 1 day before transplant and for 16 days after transplant, the mice were exposed continuously to either carrier, or to PEG-rHuMGDF at 50 pg/kg/d deliv-
a detailed more pre-
are the same data as shown in Fig 2A and 2B, but regrouped to allow a direct comparison to be made. G-CSF was the more effective mobilizer whether WBC or platelet recovery in the recipients was considered as the evaluable endpoint.
PEG-rHuMGDF treated recipients. In general, the recover-
Table 3. Cellular Composition of PBPC Grafts
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<th>Parameter</th>
<th>Carrier</th>
<th>PEG-MGDF</th>
<th>G-CSF</th>
<th>G-CSF + PEG-MGDF</th>
<th>GM-CSF + PEG-MGDF</th>
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<td><strong>(A.) Donor blood</strong></td>
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<td></td>
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<tr>
<td>WBC (x10^6/mL)</td>
<td>21.78</td>
<td>23.59</td>
<td>93.13</td>
<td>106.46</td>
<td>49.76</td>
<td>44.24</td>
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<td>3.27</td>
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<td>58.46</td>
<td>70.12</td>
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<td>RBC (x10^6/mL)</td>
<td>9.32</td>
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<td>8.22</td>
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<td>Platelets (x10^6/mL)</td>
<td>1,315</td>
<td>4,784</td>
<td>866</td>
<td>1,739</td>
<td>1,056</td>
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<td>WBC (x10^6/mL)</td>
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<td>10</td>
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<td>MNCs %</td>
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<td>92.7</td>
<td>86.5</td>
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<tr>
<td>Neutrophils</td>
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<td>0.59</td>
<td>0.87</td>
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<td>MNCs</td>
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<td>1.96</td>
<td>1.87</td>
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<td>GM-CFC*</td>
<td>34</td>
<td>36</td>
<td>904</td>
<td>920</td>
<td>846</td>
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Analysis of the mobilized PBPC product as harvested from donors, after MNC enrichment, and as transferred to irradiated recipients. Cells were counted on a Technicon H-1E at each stage (see Materials and Methods). (A.) Cellular analysis of PBPC donor blood. Errors in all groups are omitted for clarity, but from between 35 and 45 individually measured donors in each case a SD of less than 10% was noted. It should be noted that the lymphocyte category probably also includes progenitor cells. These cells were measured by their functional capacity to form colonies of granulocyte/macrophages (GM-CFC). (B.) Cellular analysis of MNC preparation from PBPC donors. (C.) Analysis of graft (million/mL).

* Calculated from Table 1 and assuming 100% recovery in MNC fraction. Assumed number of GM-CFC transplanted, not in millions.
engrafted with PBPC from carrier-treated donors. PEG-rHuMGDF mobilized PBPC performed comparably to non-mobilized PBPC when the recipients also received PEG-rHuMGDF (Fig 3A). The accelerated platelet recovery obtained with G-CSF mobilized PBPC (Fig 2A) was duplicated or exceeded in recipients of nonmobilized PBPC when the recipients were treated with PEG-rHuMGDF. However, a slight advantage in WBC recovery (between days 9 and 14) was still obtained by treating the donor with G-CSF to mobilize PBPC. PBPC mobilized by combination G-CSF/PEG-rHuMGDF showed comparable recovery to other treatment groups in terms of WBC recovery (Fig 3C). Platelet recovery in all groups was faster and occurred with little or no delay in all recipients that received PEG-rHuMGDF in addition to the PBPC graft. The exception to this was PBPC derived from GM-CSF treated donors (Fig 3D). This dele-
PERSPECTIVE

The PB analyses of the PBPC donor mice indicate (Fig 1) that PEG-rHuMGDF had no effect on nucleated white cell counts, but increased platelet counts from around 1,300 \( \times 10^6 \) mL in controls to more than 4,500 \( \times 10^6 \) mL in PEG-rHuMGDF treated mice. G-CSF and GM-CSF, on the other hand, increased WBC from around 20 \( \times 10^6 \) /mL (which is normal in these older splenectomized mice) to about 80 \( \times 10^6 \) /mL in these mice was broadly equivalent. In donors that received the combination treatments this slight reduction in platelet numbers was fully reversed in G-CSF/PEG-MGDF recipients but remained slightly subnormal in GM-CSF/PEG-MGDF recipients. In further experiments where the dose of G-CSF was reduced to 40 to 70 \( \mu g \)/kg/d a similar degree of leukocytosis was induced in recipients of 200 \( \mu g \) GM-CSF/kg/d. In these lower dose G-CSF-treated mice platelet numbers remained normal. Therefore, it was unclear what doses of the two CSFs were directly comparable in terms of biological response and so the majority of our studies used doses of 200 \( \mu g \)/kg/d for each of the CSFs without further consideration of the leukocytosis-inducing activity of the material.

Both G-CSF and GM-CSF were effective mobilizers of potentially transplantable hematopoietic progenitors and one of our aims here was to evaluate PEG-rHuMGDF in this setting also. It was found that PEG-rHuMGDF was not an effective mobilizer in comparison with G- or GM-CSF, but was about as effective as some other reported materials such as MIP-1a. As a measure of mobilization of PBPC we used an assay of progenitor colony formation in the presence of rSF and rMIL-3. The progenitor population identified in this plating system may not be representative of the mobilization of so-called stem cells. It is possibly unrepresentative of megakaryocytic progenitor numbers also. However, it is apparent from several studies, including those of McNiee et al\(^{10}\) that progenitor numbers of any functional type (including CFU-Meg) are equally predictive of patient platelet recovery. The studies of McNiee et al\(^ {10}\) and clinical experience first illustrated by Sheridan et al\(^ {2}\) indicate that G-CSF is a potent mobilizer of multiple progenitor types including megakaryocyte progenitors. It is possible of course that the mobilization of committed platelet progenitors occurs as an inadvertent side effect of myeloid progenitor mobilization by G-CSF. It is also a possibility that among the PBPC population harvested from G-CSF treated patients is a precursor population common to both lineages. Whichever hap-
happens to be the case the precise mechanism is less interesting than the observation that the mobilization of progenitor cells capable of supporting platelet formation occurs under the influence of G-CSF. From the accumulated experience of clinical PBPC transplantation typified by Sheridan et al. and analyses such as that of McNiece et al. it is apparent that G-CSF mobilizes sufficient megakaryocyte progenitors to speed platelet recovery, but also that the potential of transferred CFU-Meg above ~200/kg is not fully exploited by the cytokine environment of the ablated host. These data indicate that there is a diminishing return with respect to acceleration of platelet recovery as higher progenitor numbers are transferred. Thus the transplantation of increasing numbers of progenitors to the recipient does not continue to translate fully to further reduction in the duration of thrombocytopenia. The possibility of assisting the host in fully utiliz-

Fig 3. Leukocyte and platelet recovery in mice transplanted with PBPC. The legend shows Donor/Recipient treatment. Recipient mice were treated with carrier only (---) or PEG-MGDF (----). Donor mice had been variably treated. (O) Represent carrier-treated donors; (■) PEG-MGDF treated donors. (∇ and △) Denotes G-CSF and GM-CSF donor treatment, respectively, and (∇ and △) the same growth factors in combination with PEG-MGDF. (A) Carrier or PEG-MGDF donor treatment combined with carrier or PEG-MGDF recipient treatment. (B) G-CSF mobilized PBPC transplanted into carrier or PEG-MGDF treated recipients. (C) G-CSF or G-CSF/PEG-MGDF mobilized PBPC transplanted into carrier or PEG-MGDF treated recipient mice.
The potential of the PBPC graft was the next area we investigated. From the effects of PEG-rHuMGDF in normal mice it was predictable that an effect on platelet recovery rate might be expected in irradiated, transplanted mice treated with PEG-rHuMGDF. This proved to be the case, but the degree of the effect was greater than we had predicted. The elimination of thrombocytopenia (taken somewhat arbitrarily as platelet numbers below $100 \times 10^9$ mL, around 10% of normal platelet numbers in mice) in some recipients and a single time point of thrombocytopenia in recipients of even a suboptimal PBPC graft (obtained from carrier-treated donors) indicate the unprecedented potency of PEG-rHuMGDF in this setting.

A second unexpected finding was the improved rate of recovery of WBC in PEG-rHuMGDF treated recipients, es-
especially because normal mice treated with PEG-rHuMGDF had shown little or no response in WBC numbers. MGDF also has little detectable colony-stimulating activity on myeloid progenitors and so it was reasoned that the observed effects may well be indirect. We also observed (though data are not presented) that recovery of erythrocyte numbers is improved in PEG-rHuMGDF treated PBPC recipients. It is unclear whether either of these effects are mediated via a direct effect of PEG-rHuMGDF on the populations in question. Several recent publications have indicated a role for some TPOs as cofactors in the growth of erythroid progenitor cells, and shown erythropoietic effects in some models of myelosuppression or BMT donor treatment. Thus we cannot eliminate the possibility that PEG-rHuMGDF may have a direct beneficial effect on erythropoiesis, despite the lack of evidence to this effect in normal rodents or primates. An intriguing possibility exists that platelets may influence myeloid and/or erythroid recovery via the effects of platelet derived materials on the functional recovery of other tissues including the hematopoietic microenvironment. It has been widely documented that cells grown in vitro, which have a functional role in the marrow stroma can be influenced by platelet derived factors such as PDGF. It is not clear which of these other candidate mechanisms may play a part in the PEG-rHuMGDF supported recovery of either leukocytes or erythrocytes, but it is clear that recovery of either population is certainly not suppressed in animals receiving highly effective doses of PEG-rHuMGDF confounding any concern over possible interlineage competition between the thrombopoietic and myeloid lineages which may have been inferred from data obtained in normal mice.

Previous experience with mouse models of hematopoietic transplantation have shown generally predictive of the response obtained in the clinic. Despite this it is always a concern that rodent data may not be readily extrapolated to the clinical setting. For instance, in our experience the advantages of PBPC grafting over marrow transplantation in terms of myeloid cell recovery rates are not easily reproduced in mice, though they have been widely reported in the clinic. This may be the result of the differing ablative regimes employed or because of intrinsic differences between species. Nevertheless, comparisons between various rodent mobilization regimens have proven generally applicable to humans and the advantages of HGF (especially G-CSF) mobilized PBPC over harvests from carrier-treated donor blood have been shown again in these studies. However, the effects of recipient treatment with PEG-rHuMGDF are of sufficient magnitude that even unmobilized PBPC perform remarkably well. So does mobilization of PBPC in the donor still offer any advantage? Undoubtedly the answer is yes because there is considerable benefit to be gained from transplanting HGF-mobilized PBPC whether the recipient receives PEG-rHuMGDF posttransplant or not. Firstly, preliminary experiments have shown that recipients of optimally mobilized PBPC enjoy a considerable survival advantage over recipients of nonmobilized PBPC. Secondly, irradiated mice that receive no graft but are treated with PEG-rHuMGDF show improved platelet recovery, but no parallel increase in survival rate. This infers that at least some graft is required and studies such as those previously discussed show that quantitatively, HGF mobilized PBPC are superior to other sources. The use of PEG-rHuMGDF treatment in the postransplant period either alone or with agents such as G-CSF, may allow the minimum number of PBPC required for engraftment to be reduced, raising the possibility of splitting apheresis product for multiple treatments over successive rounds of therapy, using nonexpanded cord blood as a viable tissue source in adults, and also of engrafting with the limited cell numbers attainable with current fluorescence-activated cell sorting and gene therapy protocols.

Overall we have shown the efficacy of PEG-rHuMGDF in hematopoietic transplantation and conclude that this may be a significant potential application for this novel therapeutic.

ACKNOWLEDGMENT

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REFERENCES


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