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A Mild Transient Decrease of Peripheral Red Blood Cell Counts Induced by a Suprapharmacological Dose of Pegylated Human Megakaryocyte Growth and Development Factor in Rats

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Abstract

Previous studies have shown that pegylated recombinant human megakaryocyte growth and development factor (PEG-rHuMGDF) at suprapharmacological dose induces a mild transient decrease of red blood cell counts according to thrombopoiesis in normal mice. To unravel the mechanism underlying this mild transient decrease of red blood cells, we have studied the effect of PEG-rHuMGDF on the circulating plasma and blood volume, and the serum biochemical parameters of anaemia and splenectomy. Also, we have performed histological studies of the bone marrow and the spleen of PEG-rHuMGDF-treated rats.

PEG-rHuMGDF (300 $\mu g\,kg^{-1}$) or vehicle was subcutaneously administered to rats once a day for up to five days. From day 6 after the start of PEG-rHuMGDF administration, the platelet counts and plateletcrit levels were significantly increased, reaching peak values on day 10, and recovering to normal by day 20. The red blood cell counts and the haematocrit levels were significantly decreased on day 6 to 13. The decreases in red blood cell levels and haematocrit produced by PEG-rHuMGDF treatment were mild and had recovered by day 15. The plasma and blood volumes were significantly increased on day 10 in PEG-rHuMGDF-treated rats. No alteration of the serum biochemical parameters for anaemia, iron or total bilirubin, were observed on day 10. The histological examination on day 10 revealed a marked increase in megakaryocytes and a slight decrease in erythropoiesis in the bone marrow of rats that received PEG-rHuMGDF (300 $\mu g\,kg^{-1}$). There was also a slight increase in splenic megakaryocytes and erythropoiesis. The decrease of red blood cells by PEG-rHuMGDF was not affected by splenectomy.

These results suggest that the mild transient decrease of red blood cells induced by PEG-rHuMGDF treatment for up to five days is based mainly on the increases in the plasma and blood volume. These events are secondary changes due to the regulation of the excess production of megakaryocytes in the marrow and the peripheral platelets.

The mechanism of megakaryocytopoiesis leading to platelet production has been thought to be regulated by a lineage-specific humoral factor. Several groups have recently reported the cloning of thrombopoietin as a novel haemopoietic cytokine (Bartley et al 1994; de Sauvage et al 1994; Lok et al 1994; Kato et al 1995). Since it has been thought that thrombopoietin acts on megakaryocytopoiesis leading to platelet production through c-Mpl, thrombopoietin is also called c-Mpl ligand. Thrombopoietin is a primary regulator of megakaryocytopoiesis and platelet production (Wendling et al 1994). The administration of throm-bopoietin to normal mice produces a several-fold increase in the number of circulating platelets and a marked expansion of the bone marrow megakaryocyte mass and the CFU-MK pool in both the femur and spleen (Lok et al 1994; Kaushansky et al 1994).

It has recently been reported in various pre-clinical studies that the administration of throm-bopoietin is effective in the amelioration of the decrease of peripheral platelet counts caused by chemotherapy-induced thrombocytopenia (Hokom

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et al 1995; Ulich et al 1995; Akahori et al 1996). Therefore, thrombopoietin is clinically expected to be an effective drug for improving chemotherapy-induced thrombocytopenia.

Pegylated human megakaryocyte growth and development factor (PEG-rHuMGDF) is a recombinant pegylated truncated polypeptide related to human thrombopoietin, the ligand for c-Mpl receptor. It has also been reported that a supra-pharmacological dose of PEG-rHuMGDF produces mild transient decreases in the peripheral red blood cell and haemoglobin, along with erythroid and lymphoid hypoplasia in the bone marrow of normal mice seven days after the start of PEG-rHuMGDF administration (Ulich et al 1996). Those authors hypothesized that pharmacologically extremely high doses of PEG-rHuMGDF might result in the discordant generation of megakaryocyte mass followed by the suppression of the production of erythropoiesis and a decrease of the red blood cell counts. However, the lifespan of a red blood cell (50–70 days) is relatively long compared with those of other types of blood cells (Berlin et al 1951; Burwell et al 1953; Davis et al 1955). Therefore, it is unlikely that the decrease of peripheral red blood cell counts caused by high doses of PEG-rHuMGDF is due only to the suppression of erythropoiesis.

To elucidate the pathophysiological mechanism responsible for this PEG-rHuMGDF-associated mild transient anaemia, we have studied a series of haematological, biochemical and histological changes in rats administered by PEG-rHuMGDF ($300\,\mu g\,kg^{-1}$) once a day for up to five days. In addition, the plasma and the blood volume were measured serially in rats, and the haematological changes in splenectomized rats were studied.

Materials and Methods

Materials

Recombinant human MGDF was expressed in *Escherichia coli*, purified to homogeneity and MGDF derivatized with polyethyleneglycol. This molecule, provided by Amgen Inc. (Thousand Oaks, CA) was formulated in an aqueous buffer and sterilized. The endotoxin levels of the product were shown to be $< 0{\cdot}48\,EU\,mL^{-1}$ by a limulus lysate assay. Male Crj:CD (SD) rats (Charles River Japan, Tsukuba, Japan), approximately 290 g, 8-weeks-old at the start of PEG-rHuMGDF or vehicle administration were used. They had free access to food and water, and were housed in a barriered room at the Kirin Vivarium under pathogen-free conditions. PEG-rHuMGDF at a dose of $300\,\mu g\,kg^{-1}$ or vehicle was subcutaneously administered once a day for up to five days. The first day of PEG-rHuMGDF or vehicle administration was day 1.

Peripheral blood samples for haematology

Blood samples were collected from the posterior vena cava under ether anaesthesia and transferred into a blood collection tube containing EDTA-2K. The blood was diluted with Cellpack diluent (Toa Medical Electronics, Kobe, Japan) according to the increase in platelet count. The following haematological parameters were determined with a Sysmex cell counter (E-2000, Toa Medical Electronics, Kobe, Japan): platelet counts, plateletcrit levels, red blood cell counts and haematocrit levels.

Peripheral blood samples for serum biochemistry

Blood samples were collected from the abdominal aorta under ether anaesthesia, and the serum was prepared. The serum biochemical parameters examined were iron (Fe) and total bilirubin. These biochemical parameters were determined using the respective general methods with an Automatic Analyzer (H-736-10, Hitachi, Tokyo, Japan) on day 10 after the start of the administration of PEG-rHuMGDF or vehicle.

Determination of plasma and blood volumes

Rats were placed under ether anaesthesia and 0·1% Evans blue dye (Sigma Chemical Co., St Louis, MO) was administered through the left carotid vein. Blood was collected from the right carotid vein 3 min after the administration of Evans blue dye. For the determination of plasma volume, blood was transferred to heparinized tubes. The absorbance of Evans blue dye in the plasma samples, or standard solution diluted 10-fold in distilled water, was measured at 620 nm using an Auto Sipper Photometer (U-1080, Hitachi, Tokyo, Japan). Preliminary measurements confirmed that the standard curve of Evans blue dye solution showed a linear regression during the range of 0·0001% to 0·001% ($r^2 = 0{\cdot}998$).

For the determination of blood volume, blood was transferred to heparinized haematocrit capillary tubes. After the separation of the plasma fraction in capillary tubes, the blood volume was calculated as follows: blood volume (mL) = plasma volume (mL) × 100/plasma fraction (%).

Histology

For histology, specimens were prepared according to the standard methods on day 10 after the start of

the administration of PEG-rHuMGDF or vehicle. Formalin-fixed paraffin-embedded tissues were stained with haematoxylin and eosin.

Splenectomized rats

After splenectomy or sham-operation, rats had a two-week recovery period before the start of PEG-rHuMGDF administration (once a day for up to 5 days). The platelet and red blood cell levels were measured on day 10 after the start of PEG-rHuMGDF administration.

Statistics

Data are presented as the mean ± s.e.m. Significance was accepted at a level of $P < 0{\cdot}05$, using Student's *t*-test.

Results

PEG-rHuMGDF ($300\,\mu g\,kg^{-1}$, s.c., for up to 5 days) increased the circulating platelet counts and plateletcrit levels (Figure 1). A significant increase in platelet counts and plateletcrit levels was observed on day 6 after the start of the administration of PEG-rHuMGDF. Values peaked on day 10 followed by a gradual normalization by day 20 (Figure 1). In parallel with these changes, PEG-rHuMGDF caused a decrease in the red blood cell and the haematocrit levels. Decreases in red blood cell counts and haematocrit reached their lowest levels at day 10 followed by a gradual normalization to day 15 (Figure 2).

To investigate the mechanisms of the decrease in red blood cell counts, the effects of PEG-rHuMGDF on the plasma volume and the blood volume were examined at day 10. PEG-rHuMGDF ($300\,\mu g\,kg^{-1}$) significantly increased the plasma volume and blood volume on day 10 (Table 1).

No alteration of anaemia, Fe and total bilirubin, by PEG-rHuMGDF ($300\,\mu g\,kg^{-1}$, s.c., for 5 days) was observed on day 10 (Table 1).

The histological examination on day 10 revealed that PEG-rHuMGDF ($300\,\mu g\,kg^{-1}$) produced an increase in the megakaryocyte counts in the bone marrow. PEG-rHuMGDF ($300\,\mu g\,kg^{-1}$) produced a slight decrease in the length of the erythroid (Figure 3). Also, PEG-rHuMGDF ($300\,\mu g\,kg^{-1}$) slightly increased the erythroid in lineage in the spleen (Figure 4).

The decrease in red blood cell count after PEG-rHuMGDF ($300\,\mu g\,kg^{-1}$) treatment was observed in the splenectomized rats and the sham-operated rats (Table 2).

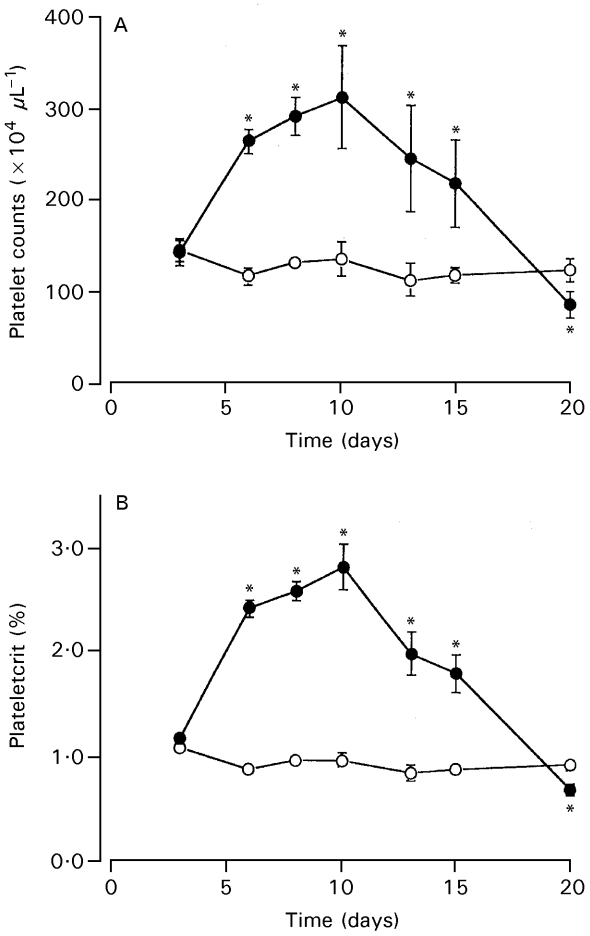

Figure 1. Effects of PEG-rHuMGDF on peripheral platelet counts (A) and plateletcrit levels. Response in vehicle-treated (○) and PEG-rHuMGDF ($300\,\mu g\,kg^{-1}$)-treated (●) animals. Each point represents the mean ± s.e.m. of five rats per group. $^*P < 0{\cdot}05$ compared with the corresponding value in the vehicle-treated group.

Discussion

PEG-rHuMGDF increased the peripheral platelet counts which peaked on day 10, and then gradually normalized by day 20. The PEG-rHuMGDF treatment also produced a mild transient decrease in red blood cell counts and haematocrit levels, reaching their lowest levels on day 15. Ulich et al (1996) also observed this decrease in red blood cell counts by subcutaneous PEG-rHuMGDF treatment in normal mice. The mechanism of this mild transient decrease in red blood cell counts is unknown. In our study, we observed that the suprapharmacological dose of PEG-rHuMGDF increased the plasma volume and the blood volume on day 10 and decreased the red blood cell count. Such an increase in the plasma volume has been produced by treatment with several cytokines, e.g. erythropoietin (Kawamura et al 1990) and interleukin-

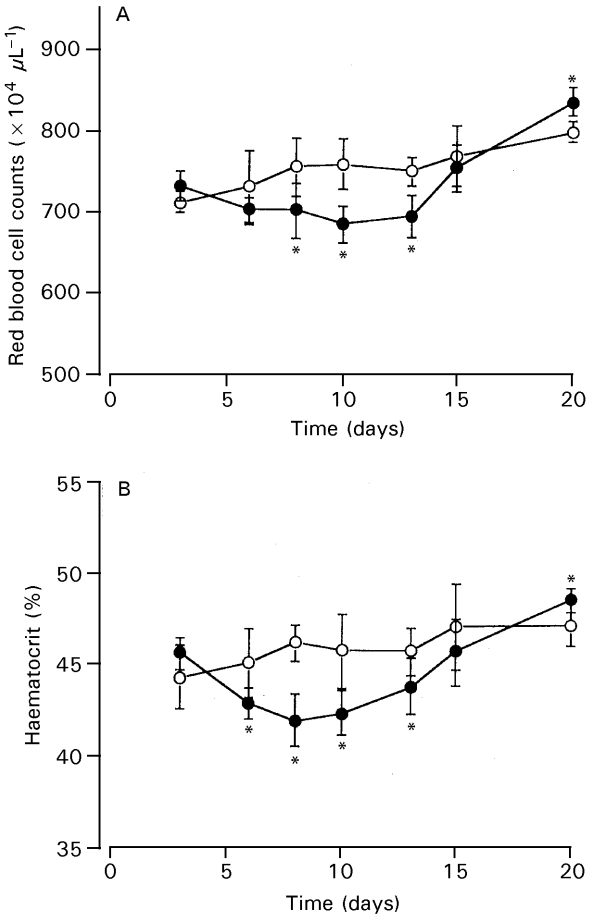

Figure 2. Effects of PEG-rHuMGDF on peripheral red blood cell counts (A) and peripheral haematocrit levels (B). Response in vehicle-treated (○) and PEG-rHuMGDF ($300\,\mu g\,kg^{-1}$)-treated (●) animals. Each point represents the mean ± s.e.m. of five rats per group. $^*P < 0{\cdot}05$ compared with the corresponding value in the vehicle-treated group.

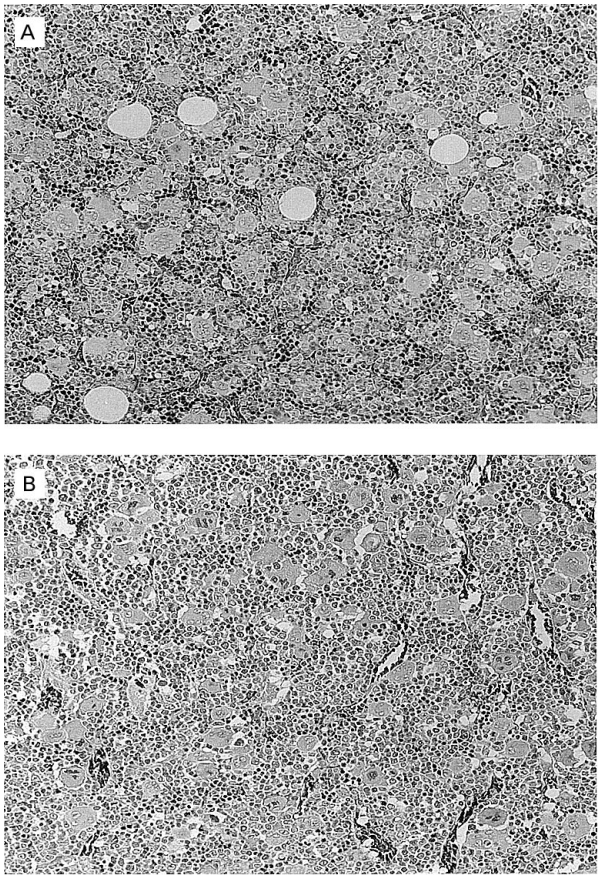

Figure 3. Histological changes of the bone marrow induced by vehicle (A) and PEG-rHuMGDF $300\,\mu g\,kg^{-1}$ (B) on day 10. PEG-rHuMGDF or vehicle was administered once a day for five days. Original magnification × 200.

6 (Nieken et al 1995). Erythropoietin stimulated the growth of erythroid progenitor cells (Nishi et al 1990), and increased the red blood cell count and the haematocrit (Nagano et al 1990). The increased haematocrit and blood viscosity induced by the high dose of erythropoietin are thought to be regulated by haemodilution due to the change in the plasma volume. In our study, PEG-rHuMGDF treatment produced an increase in the plateletcrit level in accordance with an increase in the platelet count. The increase in plateletcrit level may also induce the increase of blood viscosity regulated by the haemodilution due to the increase of the plasma volume.

Table 1. Effects of PEG-rHuMGDF on the circulating blood and the serum biochemical parameters of rats on day 10.

Parameters	Vehicle	PEG-rHuMGDF $300\,\mu g\,kg^{-1}$
Circulating blood parameter		
Plasma volume ($mL\,kg^{-1}$)	37·9 ± 1·4	42·2 ± 1·7*
Blood volume ($mL\,kg^{-1}$)	69·8 ± 2·2	80·0 ± 2·1*
Serum biochemical parameter		
Fe ($\mu g\,dL^{-1}$)	145·2 ± 19·9	157·4 ± 23·5
Total bilirubin ($mg\,dL^{-1}$)	0·064 ± 0·015	0·062 ± 0·015

Data are mean ± s.e.m. of five to eight animals. $^*P < 0{\cdot}05$ compared with the vehicle-treated animals.

In the histological examination, we observed that the high dose of PEG-rHuMGDF caused hyperplasia of megakaryocytes in the bone marrow with mild erythroid hypoplasia, but did not cause erythroid hypoplasia in the spleen. Therefore, the mild transient decrease in the red blood cell count caused by PEG-rHuMGDF may be partly based on erythroid hypoplasia in the marrow. However, since the lifespan of red blood cells is about 50–70 days, this is not likely (Berlin et al 1951; Burwell et al 1953; Davis et al 1955). Kaushansky et al (1995) reported that thrombopoietin greatly expanded the number of erythroid progenitors in the marrow and

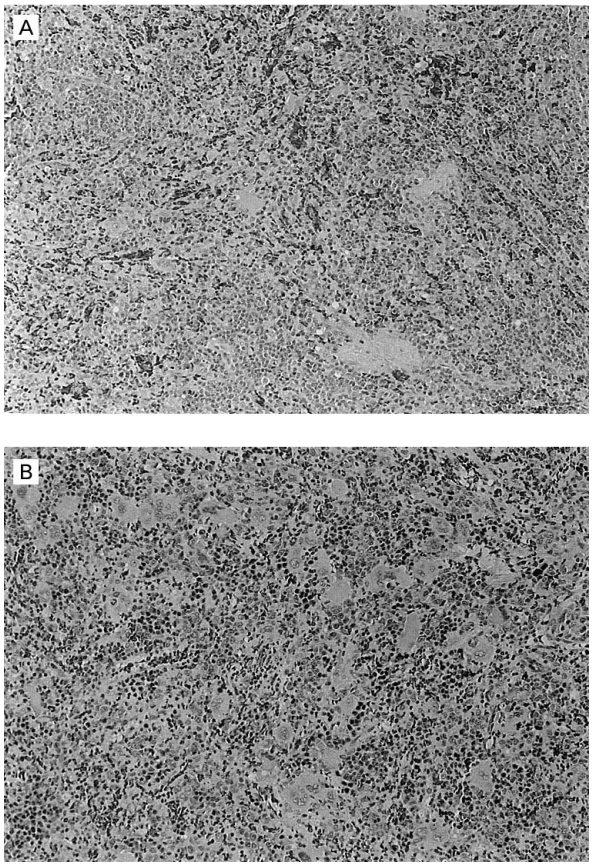

Figure 4. Histological changes of the spleen induced by vehicle (A) and PEG-rHuMGDF 300 $\mu g\,kg^{-1}$ (B) on day 10. PEG-rHuMGDF or vehicle was administered once a day for five days. Original magnification × 200.

blood reticulocytes and was associated with accelerated red blood cell recovery in myelosuppressed mice. There is a discrepancy in the action of thrombopoietin on the erythroid lineage in the marrow between our study of normal rats and Kaushansky's study of myelosuppressed mice. It is possible that the erythroid lineage in the marrow might be indirectly inhibited by a cytokine, such as transforming growth factor-β1 (TGF-β1), in normal rats treated with a high dose of PEG-MGDF. We observed that the high dose of PEG-rHuMGDF increased the levels of TGF-β1 in the extra fluid of marrow by more than 20-fold compared with controls (Yanagida et al 1997). In addition, Yan et al (1996) also observed that the level of TGF-β1 was increased 3- to 5-fold in thrombopoietin gene-transferred mice. TGF-β1 is an effective inhibitor of thrombopoiesis (Greenberg et al 1990; Cowley et al 1992; Kuter et al 1992). It was also reported that TGF-β1 inhibited the growth of erythroid progenitor cells, CFU-E and BFU-E, and stimulated the growth of the CFU-GM (Ottman & Pelus 1988). Therefore, it is postulated that TGF-β1 might play an important role in the regulation of megakaryocyte production in the marrow. The increased TGF-β1 in the marrow by the high dose of PEG-rHuMGDF may regulate the excess of megakaryocyte count and indirectly inhibit the erythroid lineage.

Table 2. Effects of PEG-rHuMGDF on platelet counts and red blood cell counts of splenectomized and sham-operated rats on day 10.

Parameters	Vehicle	PEG-rHuMGDF 300 $\mu g\,kg^{-1}$
Sham-operated rats		
Platelet counts ($\times 10^4\,\mu L^{-1}$)	156 ± 3	388 ± 26*
Red blood cell counts ($\times 10^4\,\mu L^{-1}$)	773 ± 12	720 ± 25*
Splenectomized rats		
Platelet counts ($\times 10^4\,\mu L^{-1}$)	141 ± 4	366 ± 26*
Red blood cell counts ($\times 10^4\,mL^{-1}$)	756 ± 12	688 ± 37*

Data are mean ± s.e.m. of five animals. *$P < 0{\cdot}05$ compared with the vehicle-treated animals.

Fe and total bilirubin are generally used as serum biochemical parameters for hypoferraemia and for haemolytic anaemia, respectively. No alteration of Fe and total bilirubin was observed in this study. Therefore, the mild transient decrease in the red blood cell count and haematocrit by PEG-rHuMGDF treatment is not based on hypoferraemia or haemolytic anaemia.

There is the possibility that red blood cells might be taken up in spleen with surplus platelets produced by the supraphamacological dose of PEG-rHuMGDF treatment, and the mild decrease in red blood cell count might be produced. But this seems unlikely because the anaemia induced by the supraphamacological dose of PEG-rHuMGDF was also observed in splenectomized rats.

In conclusion, these results suggest that the mild transient anaemia induced by a suprapharmacological dose of PEG-rHuMGDF for up to 5 days is based mainly on the increase of the plasma volume. These events are secondary changes due to the regulation of the excess production of megakaryocytes in the marrow and the peripheral platelets.

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