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Involvement of the TGF- β 1 Derived from Megakaryocyte in the PEG-rHuMGDF-Induced Myelofibrosis and Bone Formation

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Abstract: Bone marrow fibrosis and new bone formation were induced by Pegylated recombinant human megakaryocyte growth and development factor (PEG-rHuMGDF) injection in the rat. We investigated time course changes of megakaryocyte counts, circulating platelet counts, transforming growth factor- β 1 (TGF- β 1) levels in the bone marrow and those in platelet-poor plasma (PPP) when rats were injected with PEG-rHuMGDF at a dose of 0.1 mg/kg. Additionally, ultrastructural analysis of the circulating platelet and the bone marrow was performed by electron microscope. PEG-rHuMGDF injection daily for 5 days caused a megakaryocyte hyperplasia on days 5–7 [after the commencement of the treatment], myelofibrosis on days 7–10, and new bone formation on days 8–15. TGF- β 1 levels in the extracellular fluid of the marrow, megakaryocyte numbers, TGF- β 1 levels in the PPP, and circulating platelet counts increased by PEG-rHuMGDF injection, and reached to the maximum level on days 7, 7, 8, and 10, respectively. Ultrastructural analysis showed that circulating platelets had no prominent morphological changes in the PEG-rHuMGDF-treated rats on day 8, compared with vehicle-treated rats. Additionally, there were many platelets or fragments of megakaryocyte around mesenchymal cells, and those fragments deposited in the newly formed bone on day 10. These data suggested that myelofibrosis and new bone formation were induced by the increase of TGF- β 1 levels derived from bone marrow megakaryocytes. (*J Toxicol Pathol* 2002; **15**: 31–38)

Key words: megakaryocyte, TGF- β 1, myelofibrosis, platelet, new bone formation

Introduction

Pegylated recombinant human megakaryocyte growth and development factor (PEG-rHuMGDF) is a recombinant pegylated truncated c-Mpl ligand^{1–4}. PEG-rHuMGDF has been reported to cause an increase of the circulating platelet counts in normal mice, normal rats, and normal rhesus monkey^{1,5–7}. PEG-rHuMGDF prevented thrombocytopenia in many animal models^{8–13}. Therefore, PEG-rHuMGDF is expected to be effective in stimulating thrombopoiesis in clinical setting after myelosuppression.

However, PEG-rHuMGDF-overexpressing mice cause reversible myelofibrosis following new bone formation^{14–16}. Normal rats injected with the suprapharmacological doses of PEG-rHuMGDF is also reported to cause reversible myelofibrosis⁵. Several investigators have hypothesized that

growth factors derived from megakaryocytes, such as transforming growth factor- β 1 (TGF- β 1) and platelet-derived growth factor (PDGF), are responsible for the pathogenesis of idiopathic myelofibrosis^{17–19}. It is known that megakaryocyte have TGF- β 1 and PDGF in their cytoplasm, and TGF- β 1 is a strong inducer of bone formation^{20,21}. In our previous study, PEG-rHuMGDF caused a marked increase of bone marrow TGF- β 1 level in association with the induction of myelofibrosis in normal rats⁵. Although excretion of TGF- β 1 in the marrow would favor bone formation, the mechanism of excretion remains unexplained. In a patient with acute megakaryoblastic leukemia, severe thrombocytopenia, and myelofibrosis, electron microscopic study showed the presence of abnormal megakaryocyte with an α -granule defect²². These observation suggest that the local excretion of α -granule proteins triggers the myelofibrosis. A similar hypothesis has been also proposed to explain the myelofibrosis in the gray-platelet syndrome in which α -granules were absent from the platelets²³.

From our previous study, we presumed that TGF- β 1 leaked from megakaryocyte or circulating platelet played an important role in the development of the PEG-rHuMGDF-induced myelofibrosis⁵. To clarify the mechanism of TGF-

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β lincrease, we examined time course changes in megakaryocyte numbers, platelet counts, TGF- β 1 levels in the bone marrow and the plasma [quantity as TGF- β 1 levels in the platelet-poor plasma (PPP)]. Additionally, electron microscopic analysis was performed to examine whether platelet obtained from PEG-rHuMGDF treated rats cause morphologic changes, for example α -granule defect. We also tried to obtain the ultrastructural findings related to the cause of bone marrow fibrosis and new bone formation by using electron microscope.

Materials and Methods

MGDF reagents

PEG-rHuMGDF is a recombinant pegylated truncated polypeptide related to human thrombopoietin, the cloned ligand for the c-Mpl receptor. rHuMGDF was expressed in *E. coli*, purified to homogeneity and derivitized with polyethylene glycol. PEG-rHuMGDF was produced in our laboratory. Derivitizing rHuMGDF with poly-(ethylene glycol) is thought to delays its clearance from plasma.

Animals

Nine-week-old male Crj:CD(SD)IGS rats (n=72) weighing approximately 200 g (Charles River Japan, Tsukuba, Japan) were used. They had access to food and water *ad libitum*, and were housed in a barriered room at the Kirin Vivarium under pathogen-free conditions (room temperature, 20–25°C; relative humidity, 40–70%; air change, 10–15 times/hour; 12 h-light/12-dark cycle). The rats were divided into two groups (n=36 each), and PEG-rHuMGDF at a dose of 0.1 mg/kg or vehicle was administered by subcutaneous injection daily for 5 days (Day of the first injection: Day 1). The rats (n=4) of each group were then killed on days 1, 5, 6, 7, 8, 9, 10, 12, and 15 after the first injection. This study was conducted in accordance with the Animal Welfare Guidelines in the Index of experiments with animals in the Kirin brewery Co., Ltd.

The dose and duration of PEG-rHuMGDF treatment were determined according to the results of our study¹³. In the preliminary experiment using rats, PEG-rHuMGDF caused significant increase of platelet counts at more than 1 μ g/kg daily for 5 days in rats, therefore the dose of 0.1 mg/kg was thought to be the suprapharmacological dose.

Platelet counts

Each rat was anesthetized with ether, and then the peripheral blood was collected from the posterior vena cava. The blood obtained was transferred into a blood collection tube containing EDTA-2K. The peripheral platelet count was determined with a Sysmex cell counter (E-2000, Toaiyo, Tokyo, Japan).

Histology and quantitative analysis of megakaryocyte counts

After collection of blood samples, the femur was fixed by neutral buffered formalin. After fixation, the femurs were

decalcified, embedded in paraffin and cut to prepare 2–3 μ m longitudinal section. These sections were then stained with hematoxylin and eosin. Histological findings were expressed as grade. For the quantitative evaluation, megakaryocyte numbers in the bone marrow were measured in a 1.33 mm² area of the epiphysis as described before¹³.

TGF- β 1 levels

Bone marrow fluid and PPP were prepared as described before²⁴. Latent TGF- β 1 in the samples was activated to an immunoreactive form by acetic acid/urea treatment and TGF- β 1 levels were then measured. The TGF- β 1 levels were measured in duplicate by an immunoassay using a human TGF- β 1 immunoassay kit (Genzyme, Basel, Switzerland) as recommended by the supplier.

Electron microscopy

Bone marrow tissue and peripheral platelets were collected, fixed in 2.5% phosphate-buffered glutaraldehyde for 2 hr, postfixed in 1% osmium tetroxide for 1.5 h, and embedded in EPON812 (TAAB Laboratories, Berks., U.K.). Ultrathin sections were double-stained with uranyl acetate and lead citrate, and then observed with an electron microscopy (H-7000, Hitachi, Tokyo, Japan).

Statistical analysis

The results are presented as mean $\pm$ standard deviation (SD). The data were analyzed with the two-tailed Student's *t*-test.

Fig. 1. Effect of PEG-rHuMGDF on peripheral platelet counts. Rats received PEG-rHuMGDF once daily at 0.1 mg/kg (■) or vehicle (□) for up to 5 days. Each point represents the mean $\pm$ SD (n=4). Data were analyzed by the two-tailed student's *t*-test. ** $P < 0.01$ and *** $P < 0.001$ compared with the control.

Result

Platelet counts

PEG-rHuMGDF caused a gradual increase of platelet counts from day 6 and reached to the maximum level at day 10. Platelet counts of PEG-rHuMGDF-treated rat were about 3-fold higher than vehicle-treated rats on day 10 (Fig. 1).

Histology and quantitative analysis of megakaryocyte counts

In the rats injected PEG-rHuMGDF, the following result was obtained. Myelofibrosis was seen in the inner side of bone on days 7–10 with a peak frequency on day 9. Newly induced fibers replaced the bone on days 8–15 with a peak frequency on day 12 (Table 1, Fig. 2). Quantitative analysis showed that megakaryocyte counts were about 3-fold higher than vehicle-treated rats on day 7 (Fig. 3).

Table 1. Histological Findings of the Bone Marrow

Findings	+PEG-rHuMGDF					
	Day 7	Day 8	Day 9	Day 10	Day 12	Day 15
Myelofibrosis	1/4 (+:1)	3/4 ($\pm$:1, +:2)	4/4 (++:2)	4/4 (+:2, ++:2)	0/4	0/4
New bone formation	0/4	1/4 ($\pm$:1)	3/4 ($\pm$:2, +:1)	4/4 ($\pm$:1, +:3)	4/4 (+:1, +++:3)	3/4 (+:3)

Grades: ($\pm$), very slight; (+), slight; (++) moderate; (+++), severe.

Fig. 2. Histological changes of the bone marrow of vehicle-treated rats (A) or PEG-rHuMGDF-treated rat (B-D). Megakaryocytic hyperplasia is observed on day 6 (B). An increase of reticulin fibers is seen on day 8 (C). New and excessive bone formation is seen on day 13 (D). (Original magnification A-C: $\times 400$, D: $\times 200$ H & E stain).

Fig. 3. Time course changes of megakaryocyte counts in the bone marrow. Rats received PEG-rHuMGDF once daily at 0.1 mg/kg (■) or vehicle (□) for up to 5 days. Each point represents the mean $\pm$ SD (n=4). Data were analyzed by the two-tailed student's *t*-test. * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$ compared with the control.

Electron microscopic analysis

Circulating platelet obtained from PEG-rHuMGDF treated rats had many cytoplasmic organelle, mitochondria, α -granule, glycogen granules, and demarcation membrane system, similar to normal platelet on day 8. No significant difference was seen between normal platelet and platelet obtained from PEG-rHuMGDF treated rats (Fig. 4).

There were many platelets or megakaryocyte fragments between blood vessel and osteoblast, and mesenchymal cells were seen near the fragments on day 10. Additionally, small fragments of megakaryocyte including α -granule were seen in the newly-formed bone where hydroxyapatite began to attach to the collagen fibers (Fig. 5).

TGF- β 1 levels

Treatment with PEG-rHuMGDF caused an increase of TGF- β 1 in the bone marrow fluid and PPP from day 6 and reached to the maximum level on days 7 and 8, respectively (Fig. 6).

Discussion

The suprapharmacological dose of PEG-rHuMGDF caused myelofibrosis and new bone formation in rat bone marrow. We have obtained the following results: 1) TGF- β 1 levels in the bone marrow, megakaryocyte numbers, TGF- β 1 levels in the PPP, and circulating platelet counts were

significantly elevated by PEG-rHuMGDF injection from days 5, 5, 6, 6, and reached to the maximum level on days 7, 7, 8, and 10, respectively, 2) ultrastructural analysis showed that circulating platelets had no prominent morphological changes in the PEG-rHuMGDF-treated rats, 3) there were many platelets or fragments of megakaryocyte around mesenchymal cells, and those fragments deposited in the newly-formed bone.

Our previous study showed PEG-rHuMGDF caused an increase of TGF- β 1 in the bone marrow fluid⁵. Therefore, we initially suggested that the pathogenesis of myelofibrosis and osteogenesis in the PEG-rHuMGDF-injected rats was not a primary effect of PEG-rHuMGDF and that elevated cytokines such as PDGF and TGF- β 1 released from megakaryocyte could directly stimulate the fibrogenic and osteogenic response. In this study, we saw elevation of megakaryocyte and TGF- β 1 levels in the bone marrow simultaneously, following the increase of TGF- β 1 levels in the PPP and platelet counts. There is probably a relationship between megakaryocyte increase and TGF- β 1 increase in the bone marrow. Additionally, the maximum level of TGF- β 1 in the bone marrow was more than 20-times higher than that of the PPP. Our observation supports an earlier hypothesis that the release of TGF- β 1 from megakaryocyte, not the circulating platelet, played a critical role in the development of myelofibrosis and new bone formation. Additionally, we observed no abnormal platelets in the PEG-rHuMGDF-treated rat, such as α -granule defected platelet seen in the gray syndrome.

There was the time lag in the peak time between TGF- β 1 levels in the PPP and that in the bone marrow. These results suggest that there is another mechanism of the TGF- β 1 increase in the PPP. It is known that platelets are released from bone marrow megakaryocyte, trapped in the lung capillary, and then divided to the small size of platelet²⁵. Therefore, one possible cause of TGF- β 1 increase in the PPP is that TGF- β 1 was released when platelets divided in the capillary of lung.

Since our microscopic study showed that large fragments of megakaryocyte were deposited in the marrow parenchyma, it is probable that the disruption of megakaryocyte was the most critical cause of TGF- β 1 increase. But, its mechanism is still unclear. Apart from cytokines, the development of megakaryocyte is modulated by cell adhesive interactions²⁶. It is known that megakaryocytes attach capillary epithelial cell in the bone marrow, and then release many platelets²⁷. Therefore, it is probable that bone marrow microenvironment could not support excess megakaryocyte induced by PEG-rHuMGDF, and platelet or fragment of megakaryocyte were released in the marrow space, and then growth factors such as TGF- β 1 were erupted from these free fragments of megakaryocyte.

In this study, we focused TGF- β 1 on PEG-rHuMGDF-inducing bone formation. Not only TGF- β 1 but also several factors regulate the development of myelofibrosis and osteogenesis. Especially, PDGF and platelet factor 4 (PF4) which is located in alpha-granules of megakaryocytes, are

Fig. 4. Circulating platelet induced by PEG-rHuMGDF injection on day 8. Platelets have mitochondria, α -granule, glycogen granules, and demarcation membrane system. No abnormal changes were seen in the PEG-rHuMGDF-induced platelet (A), compared with vehicle-induced platelet (B) (Original magnification $\times 7,000$). Bar= $2\ \mu\text{m}$.

hypothesized to be responsible for the pathogenesis of idiopathic myelofibrosis^{17–19}.

In conclusion, we found that treatment of PEG-rHuMGDF caused an increase of TGF- β 1 levels in the bone marrow and megakaryocyte numbers followed by

myelofibrosis and bone formation. Additionally, ultrastructural analysis showed many platelets or fragments of megakaryocyte around mesenchymal cells, and those fragments deposited in the newly formed bone. These findings suggest that PEG-rHuMGDF at a

Fig. 5. There are many platelets or megakaryocyte between blood vessel (BV) and osteoblast (OB) (arrows). Undifferentiated mesenchymal cell (UM) is seen near the fragments on day 10 (A) (Original magnification $\times 2,500$ Bar= $4\ \mu\text{m}$). Many platelets or fragments of megakaryocyte are seen in the newly-formed bone on day 10 (B) (Original magnification D: $\times 3,900$ Bar= $3\ \mu\text{m}$).

suprapharmacological dose caused myelofibrosis by increasing growth factors (e.g. TGF- $\beta 1$, PDGF) derived from megakaryocyte.

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Fig. 6. Time course changes of TGF- β 1 levels in the bone marrow and PPP. PEG-rHuMGDF injected at a dose of 0.1 mg/kg () for up to 5 days caused increase of TGF- β 1 levels in the extracellular fluid of the marrow (A) and PPP (B) compared with vehicle-treated rats (). Each point represents the mean $\pm$ SD ($n=4$). Data were analyzed by the two-tailed student's t -test. * $P<0.05$, ** $P<0.01$, and *** $P<0.001$ compared with the control.

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