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Effects of Pegylated Recombinant Human Megakaryocyte Growth and Development Factor on 5-Fluorouracil-induced Thrombocytopenia in Balloon-injured Rats

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Abstract

We examined whether pegylated recombinant human megakaryocyte growth and development factor (PEG-rHuMGDF) affected 5-fluorouracil-induced thrombocytopenia without inducing more severe intimal thickening after injury to rat carotid arteries. Rat carotid arteries were injured using a balloon catheter on day 0. 5-Fluorouracil ($100\,mg\,kg^{-1}$) or vehicle was intravenously administered on day 1 in balloon-injured rats. PEG-rHuMGDF ($100\,\mu g\,kg^{-1}$) or vehicle was intravenously administered once a day on days 1–5 to balloon-injured rats given 5-fluorouracil or vehicle.

5-Fluorouracil ($100\,mg\,kg^{-1}$, i.v.) caused a significant decrease in the platelet count from day 3 and peaked on days 7–9 in balloon-injured rats. PEG-rHuMGDF ($100\,\mu g\,kg^{-1}$, i.v.) reduced this decrease on days 9 and 11. The administration of PEG-rHuMGDF did not accelerate the intimal thickening of balloon-injured arteries in rats treated with 5-fluorouracil compared with control balloon-injured rats. PEG-rHuMGDF did not increase plasma tumour growth factor-β1 (TGF-β1) from days 0–9 in balloon-injured rats compared with control balloon-injured rats.

These results suggest that PEG-rHuMGDF ameliorated 5-fluorouracil-induced thrombocytopenia without accelerating the intimal thickening of balloon-injured arteries.

Pegylated recombinant human megakaryocyte growth and development factor (PEG-rHuMGDF) is a recombinant pegylated truncated polypeptide related to human thrombopoietin, the cloned ligand for the c-Mpl receptor (Bartley et al 1994; de Sauvage et al 1994; Lok et al 1994; Kato et al 1995). Thrombopoietin is thought to act on megakaryocytopoiesis leading to platelet production through c-Mpl. Thrombopoietin is a primary regulator of megakaryocytopoiesis and platelet production (Wendling et al 1994). Recently, it has been observed that PEG-rHuMGDF reduces the thrombocytopenia induced by cancer chemotherapy in animal models (Akahori et al 1995, 1996; Ulich et al 1995, 1996). Therefore, PEG-rHuMGDF is expected to be a clinically effective agent for cancer chemotherapy-induced thrombocytopenia. However, PEG-rHuMGDF, at suprapharmacological doses, is also reported to induce an increase in tumour growth factor-β1 (TGF-β1) in platelet-poor plasma of rats (Yanagida et al 1997).

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Balloon-injured rats are used as animal models for arteries damaged by percutaneous transluminal coronary angiography (PTCA) therapy for coronary disease due to atherosclerosis. It is known that balloon injury to carotid arteries induces intimal thickening due to the proliferation and migration of vascular smooth muscle cells. The increase in proliferation and migration of vascular smooth muscle cells is reported to be regulated by the cytokines platelet-derived growth factor (PDGF) and TGF-β1 (Creighton et al 1997; Panek et al 1997). Therefore, it is feared that PEG-rHuMGDF may not only ameliorate cancer chemotherapy-induced thrombocytopenia, but may also induce more severe intimal thickening of injured arteries by increasing TGF-β1 in platelet-poor plasma.

In this study, we examined whether PEG-rHuMGDF reduced the decrease in platelet count caused by cancer chemotherapy, and also whether

it accelerated intimal thickening of arteries in balloon-injured rats.

Materials and Methods

Materials and animals

Recombinant human megakaryocyte growth and development factor (rHuMGDF) was expressed in *Escherichia coli*, purified to homogeneity and derivitized 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 $< 0{\cdot}48\,\mathrm{EU\,mL^{-1}}$ by a limulus lysate assay. Male Crj: CD (SD) rats, weighing approximately 390 g, 10 weeks-of-age, 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.

Induction of balloon injury

The balloon injury operation was performed on rats on day 0. The rats were anaesthetized with sodium pentobarbital ($40\,\mathrm{mg\,kg^{-1}}$ i.p.). A 2F Forgarty catheter (Arterial emblectomy catheter, Baxter) was inserted through the internal carotid artery to the common carotid artery. The left common carotid artery was exposed for visual confirmation of catheter insertion. The inflated balloon (diameter of the inflated balloon: 2·5 mm) was pulled through the common carotid artery three times, and the left internal carotid artery was permanently ligated.

Effects of PEG-rHuMGDF on 5-fluorouracil-induced thrombocytopenia in balloon-injured rats

Three groups of rats were set up in the present study. The first group (control group) was treated with saline as vehicle for 5-fluorouracil and vehicle for PEG-rHuMGDF. The second group was treated with 5-fluorouracil and vehicle for PEG-rHuMGDF. The third group was treated with 5-fluorouracil and PEG-rHuMGDF. 5-Fluorouracil, at a dose of $100\,\mathrm{mg\,kg^{-1}}$, or saline was administered intravenously once on day 1. PEG-rHuMGDF, at a dose of $100\,\mu\mathrm{g\,kg^{-1}}$, or vehicle was administered intravenously once a day on days 1–5 in 5-fluorouracil-treated rats.

Platelet count. Peripheral blood samples for the measurement of platelet count were collected from the tails of rats. Rats were tail-bled by drawing 300 μL of freely flowing whole blood directly into a Gilson pipette and immediately transferring the blood into a blood collection tube containing EDTA-2K for platelet measurement and into a heparinized tube for TGF-β1 measurement. The blood for the measurement of platelet count was diluted with Cellpack diluent (Toa Medical Electronics, Kobe, Japan) according to the increase in platelet count. Platelet count was determined with the Sysmex cell counter (E-2000, Toa Medical Electronics, Kobe, Japan).

TGF-β1 levels. TGF-β1 levels were measured in platelet-poor plasma prepared from the blood samples by a modification of reported procedures (Castro-Malaspina et al 1981; Baglin et al 1988). Briefly, plasma was obtained from whole blood in a heparinized glass tube by centrifugation at 1500 rpm for 15 min. Then, platelet-poor plasma was obtained by further centrifugation at 1500 rpm for 15 min. TGF-β1 levels in the platelet-poor plasma were measured by immunoassay using a human TGF-β1 immunoassay kit (R & D Systems, Minneapolis, MN) as recommended by the supplier.

Intimal thickening. The intimal thickening of balloon-injured rats was measured on day 11. The rats were anaesthetized with ether and administered 0·5% Evans blue saline to confirm the point of injury in the balloon-injured artery, followed by 5% buffered formalin. Next, the left carotid artery was removed, postfixed and embedded in paraffin. Artery sections (3 mm thick) were then cut and stained with hematoxylin and eosin. The cross-sectional areas of the intima and the media on photographs were measured by use of an IBAS20 image analyzer (Carl Zeiss Vision Co., Ltd). The average of the ratio of the intimal area to the medial area (I/M ratio) in each artery was determined.

This study was conducted in accordance with the current guidelines for the care and use of experimental animals of our institution.

Data analysis

Differences were considered significant at $P < 0{\cdot}05$, using analysis of variance for multiple comparisons. When multiple comparisons were made with a single control, Dunnett's test was used to determine the levels of significance. Data are presented as the mean ± s.e.m.

Results

The effects of PEG-rHuMGDF on circulating platelets

We evaluated the effects of PEG-rHuMGDF on the decrease in platelet count induced by 5-fluorouracil

(100 mg kg^{-1} i.v.) in balloon-injured rats. In the control balloon-injured rats, no alternation in platelet count was observed. The decrease in the circulating platelet count produced by 5-fluorouracil began to be observed by day 3, and was maximal on day 9 and then gradually recovered (Figure 1). Treatment with PEG-rHuMGDF (100 μg kg^{-1} i.v.) reduced the severity of thrombocytopenia on days 9 and 11.

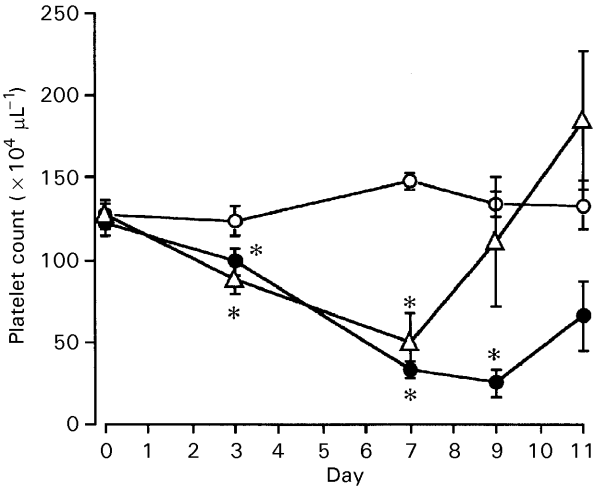

Figure 1. Effects of PEG-rHuMGDF on peripheral platelet counts. ○, Response in saline and vehicle-treated animals; ●, response in 5-fluorouracil (100 mg kg^{-1}, i.v.)- and vehicle-treated animals; △, response in 5-fluorouracil (100 mg kg^{-1}, i.v.)- and PEG-rHuMGDF (100 μg kg^{-1})-treated animals. Each point represents the mean ± s.e. of 6 animals per group. * $P < 0.05$ compared with saline and vehicle-treated group.

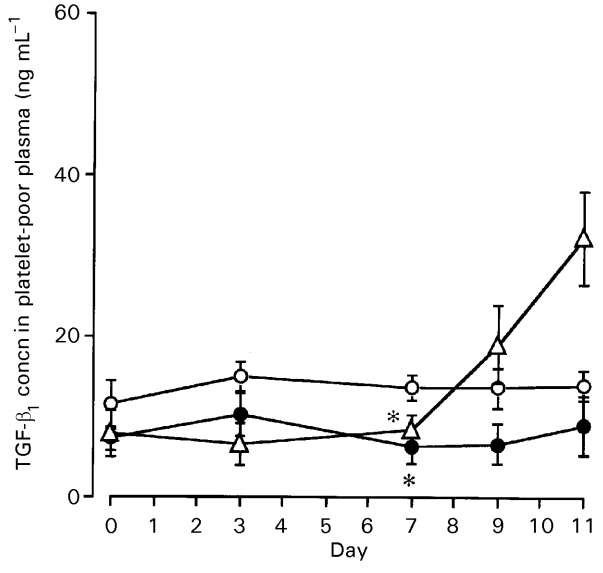

Figure 2. Effects of PEG-rHuMGDF on TGF-β1 in platelet-poor plasma. ○, Response in saline and vehicle-treated animals; ●, response in 5-fluorouracil (100 mg kg^{-1}, i.v.)- and vehicle-treated animals; △, response in 5-fluorouracil (100 mg kg^{-1}, i.v.)- and PEG-rHuMGDF (100 μg kg^{-1})-treated animals. Each point represents the mean ± s.e.m. of 6 animals per group. * $P < 0.05$ compared with the saline and vehicle-treated group.

The effects of PEG-rHuMGDF on TGF-β1

TGF-β1 was slightly decreased on day 7 in platelet-poor plasma of balloon-injured rats treated with 5-fluorouracil (Figure 2). TGF-β1 was also slightly decreased on day 7 and increased on day 11 in platelet-poor plasma of balloon-injured rats treated with 5-fluorouracil and PEG-rHuMGDF.

The effects of PEG-rHuMGDF on the I/M ratio

The intimal thickening of balloon-injured rats was measured on day 11 and the I/M ratio was calculated. The I/M ratio of balloon-injured rats treated with 5-fluorouracil was less than that of control balloon-injured rats (0·396 ± 0·202 vs 0·829 ± 0·261, respectively; n = 6; $P < 0.05$). The I/M ratio of balloon-injured rats treated with 5-FU and PEG-rHuMGDF was also less than that of control balloon-injured rats (0·341 ± 0·061 vs 0·829 ± 0·261, respectively; n = 6; $P < 0.05$).

Discussion

In the present study, 5-fluorouracil at a dose of 100 mg kg^{-1} significantly decreased the platelet count in balloon-injured rats, with the maximal decrease on day 9. PEG-rHuMGDF reduced the extent of this decrease on days 9 and 11. The decrease in platelet count produced by 5-fluorouracil is thought to be due to inhibition of megakaryocytopoiesis due to myelosuppression; similar effects are caused by other cancer chemotherapy agents. The effects of PEG-rHuMGDF on platelets noted in this study were also observed in earlier studies (Akahori et al 1995, 1996; Hokom et al 1995; Ulich et al 1995, 1996). PEG-rHuMGDF is known to increase the production of platelets due to megakaryocytopoiesis mediated through the c-Mpl receptor (Wendling et al 1994).

Suprapharmacological doses of PEG-rHuMGDF have also been reported to produce an increase in TGF-β1 in platelet-poor plasma in rats. Some cytokines (e.g., TGF-β1 and PDGF) are known to induce proliferation and migration of smooth muscle cells in balloon-injured rats (Creighton et al 1997; Panek et al 1997). It was feared, therefore, that PEG-rHuMGDF, in addition to the amelioration of thrombocytopenia produced by 5-FU, might also cause more severe internal thickening of injured arteries. However, PEG-rHuMGDF did not

accelerate the intimal thickening of injured arteries in balloon-injured rats treated with 5-fluorouracil compared with control balloon-injured rats. It is thus expected that clinical use of PEG-rHuMGDF will ameliorate chemotherapy-induced thrombocytopenia without inducing more severe damage to injured arteries. 5-Fluorouracil decreased the I/M ratio in balloon-injured rats compared with control balloon-injured rats. 5-Fluorouracil is known to decrease the proliferation of smooth muscle cells (Cragg et al 1992). Therefore, there is a possibility that 5-fluorouracil directly inhibits the proliferation of smooth muscle cells in injured arteries and thereby decreases the I/M ratio in balloon-injured rats. However, the present results also suggest that the change in the intimal thickening of balloon-injured arteries is modulated by TGF-β1 in platelet-poor plasma. TGF-β1 is known to be regulated by peripheral platelets and megakaryocytes in bone marrow (Yanagida et al 1997). The effect of PEG-rHuMGDF on the intimal thickening of balloon-injured arteries seems to be due to the change in TGF-β1 regulated by peripheral platelets. In the present study, PEG-rHuMGDF increased TGF-β1 on day 11 in platelet-poor plasma of balloon-injured rats treated with 5-fluorouracil. Therefore, PEG-rHuMGDF might accelerate the intimal thickening of arteries in 5-fluorouracil-treated balloon-injured rats after day 11. But this is unlikely, however. We observed that the I/M ratio in balloon-injured rats was not increased on day 13 by the subcutaneous administration, once a day for 5 days, of PEG-rHuMGDF at a dose of $100\,\mu g\,kg^{-1}$ in preliminary experiments (data not shown). Also, levels of TGF-β1 in platelet-poor plasma of normal rats was reported to be increased on days 6–10 (returning to baseline values on days 10–20) by the subcutaneous administration, once a day for 5 days, of PEG-rHuMGDF at a dose of $100\,\mu g\,kg^{-1}$ (Yanagida et al 1997).

In summary, we have demonstrated that PEG-rHuMGDF reduced thrombocytopenia produced by 5-fluorouracil in balloon-injured rats without accelerating the intimal thickening of injured arteries. These results suggest that PEG-rHuMGDF should be clinically useful even for patients with injured arteries.

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