Platelet-derived growth factor enhances platelet recovery in a murine model of radiation-induced thrombocytopenia and reduces apoptosis in megakaryocytes via its receptors and the PI3-k/Akt pathway

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ABSTRACT

Background
Platelet-derived growth factor is involved in the regulation of hematopoiesis. Imatinib mesylate, a platelet-derived growth factor receptor inhibitor, induces thrombocytopenia in a significant proportion of patients with chronic myeloid leukemia. Although our previous studies showed that platelet-derived growth factor enhances megakaryocytopoiesis *in vitro*, the *in vivo* effect of platelet-derived growth factor in a model of radiation-induced thrombocytopenia has not been reported.

Design and Methods
In this study, we investigated the effect of platelet-derived growth factor on hematopoietic stem/progenitor cells and platelet production using an irradiated-mouse model. We also explored the potential molecular mechanisms of platelet-derived growth factor on thrombopoiesis in M-07e cells.

Results
Platelet-derived growth factor, like thrombopoietin, significantly promoted the recovery of platelets and the formation of bone marrow colony-forming unit-megakaryocyte in irradiated mice. Histology confirmed the protective effect of platelet-derived growth factor, as shown by an increased number of hematopoietic stem/progenitor cells and a reduction of apoptosis. In a megakaryocytic apoptotic model, platelet-derived growth factor had a similar anti-apoptotic effect as thrombopoietin on megakaryocytes. We also demonstrated that platelet-derived growth factor activated the PI3-k/Akt signaling pathway, while addition of imatinib mesylate reduced p-Akt expression.

Conclusions
Our findings show that platelet-derived growth factor enhances platelet recovery in mice with radiation-induced thrombocytopenia. This radioprotective effect is likely to be mediated via platelet-derived growth factor receptors with subsequent activation of the PI3-k/Akt pathway. We also provide a possible explanation that blockage of platelet-derived growth factor receptors may reduce thrombopoiesis and play a role in imatinib mesylate-induced thrombocytopenia.

Key words: platelet-derived growth factor, thrombopoiesis, imatinib mesylate, thrombocytopenia, apoptosis.

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Introduction

Platelet-derived growth factor (PDGF) is a 28-30 kDa protein originally found in and purified from the α-granules of platelets.[1] The PDGF family comprises four different genes that form at least five different dimers,[2] including PDGF-AA, AB, BB, CC and DD, which exert their biological functions through binding to PDGF receptors (PDGFR)-α and β.[2,3] Only PDGF-BB interacts with both PDGFR-α and -β with high affinity.[2-4] PDGF was originally recognized as a serum-derived growth factor in a variety of cell types.[1,4] Genetic studies have shown that both PDGF-B and PDGFR-β knockout embryos develop thrombocytopenia and other lethal defects during development.[5,6] PDGF also stimulates the proliferation of megakaryocytes, erythrocytes, leukocytes, and their progenitors,[7-11] presumably through the multiple endogenous growth factors released from mesenchymal stem/stromal cells.[8,10,12] More importantly, we have identified both PDGFR-α and β on human megakaryocytes and platelets.[13,14] We have postulated that PDGF may directly enhance megakaryocytopoiesis via its receptors.[9,10,13]

Clinical data also suggest a potential connection between PDGF/PDGFR and thrombopoiesis. Imatinib mesylate (Gleevec or STI-571) has been used to treat patients with chronic myeloid leukemia (CML).[15] However, a significant number of CML patients treated with imatinib mesylate have developed thrombocytopenia.[16] The rate of imatinib mesylate-induced thrombocytopenia was found to increase from 24% in those with low-grade CML to 60%-72% in those with blast crisis. This imatinib mesylate-induced thrombocytopenia is associated with decreased megakaryocytic progenitors.[16] In line with these clinical findings, recent *in vitro* studies have shed light on the possible mechanism of imatinib mesylate.[17] As a tyrosine kinase inhibitor, imatinib mesylate has been found to be a potent inhibitor of both PDGFR-α and -β and their respective signaling pathways.[18-20] These data point to a significant role of PDGF receptors in imatinib mesylate-induced thrombocytopenia. Moreover, imatinib mesylate also has anti-proliferation and anti-differentiation effects on human mesenchymal stem/stromal cells,[21] which play an important role in supporting thrombopoiesis.

We previously found that PDGF enhances the proliferation of megakaryocytic progenitor cells and up-regulates the expression of transcription factors NF-E2, GATA-1 and c-Fos in megakaryocytes.[10,13,22] However, the *in vivo* effect of PDGF in thrombocytopenic animals has not been reported. In this study, we investigated the *in vivo* effect of PDGF on hematopoietic stem/progenitor cells, and its effect on platelet recovery in radiation-treated mice. We particularly focused on the anti-apoptotic effect on megakaryocytes, and the PI3-k/Akt pathway, which has previously been shown to be involved in PDGF-dependent anti-apoptosis and proliferation in a wide variety of cell types.[23,24]

Design and Methods

Radiation-induced thrombocytopenia in mice and peripheral blood cell counts

Male Balb/c mice (7 or 8 weeks old) were obtained from Charles River (Yokohama, Japan) and given free access to food and water. Ethical permission for the studies was granted by the Animal Research Welfare Committee of the University of Hong Kong. The murine model of myelosuppression with thrombocytopenia was established by irradiating mice with 4-Gy irradiation.[25] Animals were divided into three groups: a PDGF treatment group, a thrombopoietin (TPO) treatment group, and a saline control group. Mice were injected intraperitoneally with PDGF-BB (1 μg/kg/day) (PeproTech, NJ, USA), or TPO (1 μg/kg/day) (PeproTech, NJ, USA) or saline. The injections were performed on a daily basis starting from the day of irradiation. Peripheral blood platelets, red blood cells (RBC) and white blood cells (WBC) were counted in blood samples collected on days 0, 7, 14 and 21. Mice were sacrificed on day 21 and their bone marrow samples were harvested for colony-forming unit (CFU) assays and histological analysis.

Murine colony-forming unit-megakaryocyte assay

Bone marrow cells (2×10^5) were collected from the three groups of mice, after the animals had been sacrificed on day 21, and cultured in Petri dishes (35 mm, Lux) using the plasma clot culture method.[10,26] The system contains 1% deionized bovine serum albumin (Sigma, MO, USA), 0.34 mg $CaCl_2$, 10% citrated bovine plasma (Sigma), and Iscove's modified Dulbecco's medium (IMDM) with TPO (50 ng/mL) in a total volume of 1 mL. Dishes were incubated at 37°C in a fully humidified atmosphere with 5% CO_2 for 7 days. After 7 days of incubation, the acetylcholine esterase staining method was used for identification of colony- forming unit-megakaryocyte (CFU-MK). A CFU-MK was defined as a cluster of three or more acetylcholine esterase-positive cells counted under an inverted microscope.

Murine bone marrow colony-forming unit-fibroblast assay

Mouse bone marrow cells (2×10^6 cells) from different groups were seeded in 2 mL of IMDM with 10% fetal calf serum using 35 mm Petri dishes in triplicate and maintained in a fully humidified incubator at 37°C with 5% CO_2 for 9 days.[8,26] Adherent cells were stained with Giemsa. The number of colony-forming unit-fibroblast (CFU-F) colonies was counted under an inverted microscope. An aggregate containing 20 or more fibroblasts was defined as a CFU-F.[26]

Other murine bone marrow colony-forming unit assays

To analyze the effect of PDGF and TPO on hematologic stem/progenitor cells, the CFU-mix assay was performed.[26] Mouse bone marrow cells (2×10^5 cells) from different groups were plated into 35 mm culture dishes in duplicate in 1% of methylcellulose (Sigma) supplemented with 30% of fetal calf serum, 1% of bovine serum albumin, 0.1 mM of β-mercaptoethanol (Sigma), 10 ng/mL of interleukin-3 (PeproTech, Rocky Hill, NJ, USA), 50 ng/mL of stem cell factor (PeproTech), 3 U/mL of erythropoietin (Calig, Zug, Switzerland), and 10 ng/mL granulocyte-macrophage colony-stimulating factor (GM-CSF; Sandoz, Basel, Switzerland). The dishes were incubated at 37°C with 5% CO_2 in a humidified atmosphere. Colony-forming unit - granulocyte-macrophage (CFU-GM), burst-forming unit/colony-forming unit - erythroid (BFU/CFU-E), and colony-forming unit - mixed (CFU-GEMM) were scored after 7 days.[26]

Bone marrow histology

Bone marrow samples from three groups of animals were collected on day 21, after the mice had been sacrificed. Tha samples were frozen in cryo-molds and cut into 5-μm sections. The slides were stained using the Wright-Giemsa staining method. Twenty-five high-power (400×) fields from each bone marrow sample were randomly selected and photographs were taken

for analysis. The number of erythroid, granulocytes and megakaryocytes and their morphological changes were examined.[25]

Annexin V, caspase-3, and mitochondrial membrane potential analysis of M-07e cells by flow cytometry

The megakaryoblastic cell line M-07e (American Type Culture Collection, Manassas, USA) was maintained in IMDM supplemented with GM-CSF (20 ng/mL) and 10% fetal calf serum.[26] To investigate the anti-apoptotic effect of PDGF, cells were resuspended in cytokine and serum-depleted IMDM medium. PDGF (50 ng/mL) or TPO (50 ng/mL) was added to the culture for 72 h. Cells were then collected for flow cytometry analysis. The population of apoptotic cells was measured by three complementary methods: annexinV-FITC/PI, active caspase-3-PE, and JC-1 (5,5',6,6'-tetrachloro-1,1',3,3'-tetraethylbenzimidazolcarbocyanine iodide) (BD Biosciences, San Diego).[26,27] Ten thousand events were acquired for each sample and analyzed by flow cytometry using the Lysis II software (FACScan; BD Pharmingen).

Phospho-Akt analysis of M-07e cells by flow cytometry

M-07e cells were maintained in IMDM supplemented with GM-CSF (20 ng/mL) and 10% fetal bovine serum as described previously.[26] Apoptosis was induced by serum and cytokine depletion. Cells were pretreated with wortmannin (100 nM) (Sigma) and imatinib mesylate (1 μM) (Novartis, MO, USA) for 30 min and 60 min, respectively, and then incubated with PDGF-BB (100-200 ng/mL) for 30 min. Cells from different treatment groups were collected and stained with anti-phospho-Akt (p-Akt)/PE antibody (BD Biosciences, San Jose, USA) (*http://www.bdbiosciences.com/external_files/pm/doc/tds/cell_bio/live/web_enabled/558275.pdf*) for flow cytometry analysis. Ten thousand events were acquired for each sample and the population of p-Akt positive cells was analyzed using flow cytometry.

Western blotting

M-07e cells were maintained in IMDM supplemented with GM-CSF (20 ng/mL) and 10% fetal bovine serum. Subconfluent cells were starved and pre-incubated with wortmannin for 30 min and imatinib mesylate for 60 min, and then stimulated with PDGF-BB for 30 mins. Cells were washed with ice-cold phosphate-buffered saline and lysed. Protein concentrations were determined by the Bradford protein assay. Proteins were then loaded on sodium dodecyl sulfate polyacrylamide gels and separated by electrophoresis. The gels were transferred to nitrocellulose membrane and blocked with 5% bovine serum albumin in Tris-buffered saline solution containing 0.1% Tween-20.[28] Primary antibodies, phospho-Akt or Akt (Cell Signaling, MA, USA) were incubated overnight at 4°C. After washing, the membrane was incubated with secondary antibody, goat anti-rabbit antibody (Santa Cruz, CA, USA). Total Akt and phospho-Akt were visualized by ECL detection systems.

Colony-forming unit-megakaryocyte assay for imatinib mesylate study

Bone marrow cells (2×10^5) from Balb/c mice were cultured in the system as described before, with PDGF-BB (50 ng/mL) and/or imatinib mesylate (1 μM) and stained with acetylcholine esterase after 7 days of incubation. A CFU-MK was defined as a cluster of three or more acetylcholine esterase-positive cells.[26]

Statistical analysis

Data from different treatment groups were compared using Student's paired t test and are expressed as mean ± SEM. A *P* value of less than 0.05 was considered statistically significant. Three different symbols are used to denote *P* values: * means *P* less than 0.05, # means *P* less than 0.01, and + means *P* less than 0.001.

Results

In vivo effects of platelet-derived growth factor on peripheral blood cell counts in thrombocytopenic models

On day 0, the basal numbers of peripheral blood platelets in experimental mice were around 580×10^9/L,

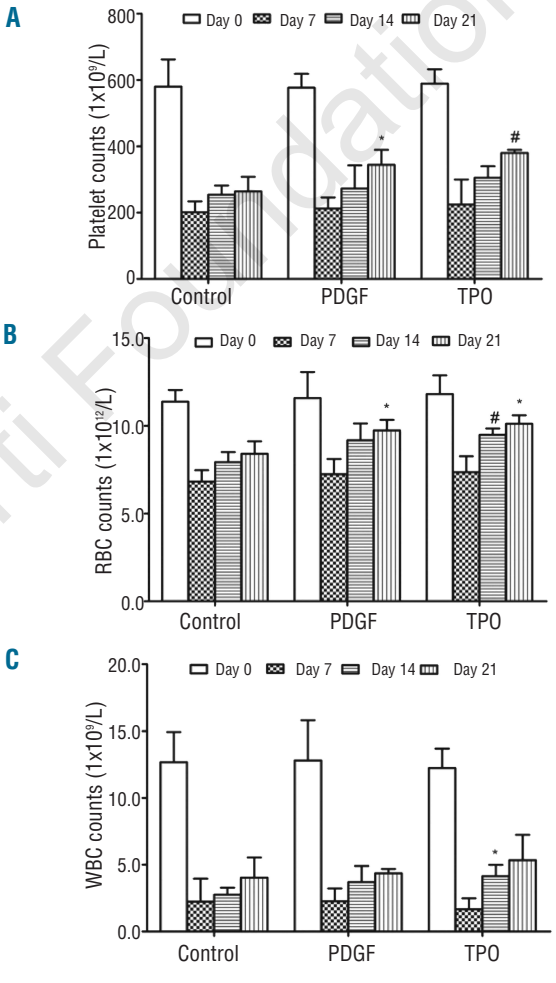

Figure 1. Effects of PDGF on blood cell counts in a radiated mouse model. (A) Hematopoietic suppression was induced by radiation. The platelet count reached a nadir (~200 x 10^9/L) on day 7, and recovered gradually to day 14 without significant difference among the differently treated groups. On day 21, animals treated with PDGF (1 μg/kg/day) and TPO (1 μg/kg/day) showed significantly higher platelet counts than the control group (n=5, control *versus* PDGF, * *P*=0.0284 and n=4, control *versus* TPO, # *P*=0.0078). (B) The irradiation-induced suppression in RBC was modest, and significant increases were observed in the PDGF-treated group on day 21 (n=5, control *versus* PDGF, * *P*=0.0249) and in the TPO-treated group on day 14 (n=4, control *versus* TPO, # *P*=0.0098) and day 21 (n=4, control *versus* TPO, * *P*=0.0103). (C) WBC count also decreased in the irradiated control group. Although there was no statistical significance between the PDGF-treated group and the saline-treated control group, PDGF tended to improve WBC recovery. The WBC count in the TPO-treated group increased by day 14 (n=4, control *versus* TPO, * *P*=0.0137).

and decreased after irradiation. The platelet counts of the irradiated mice reached a nadir (200×10⁹/L) on day 7, but recovered gradually. There was no difference in platelet counts among the three groups at day 0 or day 7. Recovery was better in the PDGF-treated group than in the saline control group, and the PDGF-treated animals had significantly higher platelet counts at day 21 (344±18.37×10⁹/L *versus* 264±19.65×10⁹/L, n=5, $P<0.05$). Both PDGF and TPO had similar potency in accelerating platelet recovery in this thrombocytopenic model (Figure 1A). These results showed that PDGF, like TPO, had radioprotective effect on platelets.

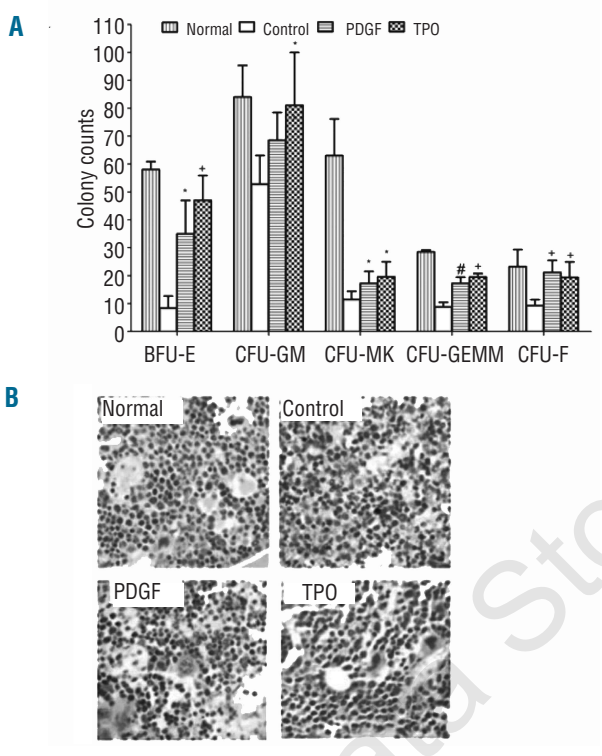

Figure 2. Effects of PDGF on bone marrow CFU formation and histology in irradiated mice. (A) Mouse bone marrow cells (2x10⁵ cells for CFU-MK, CFU-GM, CFU-GEMM and BFU-E assays; 2x10⁶ cells for CFU-F assay) were cultured from differently treated and normal untreated animals using various CFU formation systems. The numbers of CFU-MK in PDGF-treated group were significantly higher than those in the control group (n=8, control *versus* PDGF, * P=0.0255), and similar to those in the TPO-treated group (n=7, control *versus* TPO, * P=0.0222). In addition, PDGF promoted the formation of other CFU as effectively as TPO did, including BFU-E (n=4, control *versus* PDGF, * P=0.0109 and n=4, control *versus* TPO, + P=0.0008) and CFU-GEMM (n=4, control *versus* PDGF, # P=0.0084 and n=4, control *versus* TPO, + P=0.0001). The effect of PDGF on CFU-F formation was more significant than that of TPO (n=8, control *versus* PDGF, + P<0.0001 and n=8, control *versus* TPO, + P=0.0007). (B) The bone marrow cells from differently treated mice were collected on day 21 and stained with Wright-Giemsa for histological examination. Samples were analyzed under a microscope at high-power (400×). Compared to the normal untreated group, the numbers of hematopoietic cells and their progenitors in the irradiated control group were significantly decreased with notable apoptosis. Both PDGF- and TPO-treated samples showed enhanced recovery on megakaryocytes and hematopoietic stem and progenitor cells. A reduction of apoptotic and necrotic cells was also observed. PDGF: platelet-derived growth factor; TPO: thrombopoietin; CFU: colony-forming unit; CFU-MK: colony-forming unit-megakaryocyte; BFU: burst-forming unit-erythroid; CFU-GM: colony-forming unit – granulocyte-macrophage, CFU-GEMM: colony-forming unit-mixed; CFU-F: colony-forming unit-fibroblast.

The peripheral WBC and RBC counts also decreased following irradiation, with the nadir at day 7, and started increasing thereafter. No significant differences were found on day 0 or day 7 among different treatment groups. Compared to saline, administered to the control group, PDGF caused a significant increase in the number of RBC on day 21 (9.74±0.24×10¹²/L *versus* 8.40±0.32×10¹²/L, n=5, $P<0.05$), while TPO treatment produced better recovery after day 14 (Figure 1B). Although no statistically significant difference was observed in WBC recovery between the PDGF group and the saline control group, a trend suggestive of an increased recovery rate was evident for the PDGF group (Figure 1C). On the other hand, a significant difference was observed between the TPO group and the saline group. Our results demonstrated that PDGF has positive effects on RBC and WBC recovery, although the effect of PDGF is weaker than that of TPO.

Effects of platelet-derived growth factor on murine bone marrow colony-forming unit formation and bone marrow histology

Consistent with peripheral blood cell counts, there was a significant increase in CFU formation in the PDGF group compared to that in the saline group (Figure 2A). CFU-MK were cultured using the plasma clot method. PDGF, similar to TPO, significantly promoted CFU-MK formation (17.25±0.92 *versus* 11.50±1.50, n=8, $P<0.05$). CFU, including BFU-E, CFU-GM, and CFU-GEMM, formation assays were performed using the methylcellulose method. PDGF promoted the formation of BFU-E (35±5.97 *versus* 8.4±1.93, n=4, $P<0.05$) and CFU-GEMM (17.25±1.11 *versus* 8.8±0.73, n=4, $P<0.01$), but not that of CFU-GM, suggesting that PDGF enhanced hematopoietic recovery, except for the granulocytic lineage (Figure 2A). CFU-F were detected using a fibroblastic colony-forming assay. More CFU-F were found in PDGF-treated mice than in TPO-treated mice (Figure 2A), indicating that PDGF protected bone marrow stromal cells better than TPO did (21.13±1.53 *versus* 9.3±0.67, n=8, $P<0.0001$). The above data suggest that PDGF has a similar effect as TPO on CFU-MK, CFU-GEMM and BFU-E formation, further confirming the radioprotection of PDGF in hematopoiesis. PDGF had more significant and convincing effects on the megakaryocytic and erythrocytic lineages.

Bone marrow histology, assessed on day 21, revealed impaired hematopoiesis in irradiated control mice, as evidenced by decreased numbers of total cells. The numbers of necrotic and apoptotic cells were also higher than in normal mice that had not been irradiated. However, radioprotective effects were observed in both the PDGF- and TPO-treated groups as hematopoiesis was maintained, especially in the megakaryocytic lineage. Bone marrow was hyperplasic in the PDGF-treated mice. Significantly more megakaryocytes and their progenitors were found in PDGF-treated mice than in control mice. In addition, there was a significant reduction of apoptotic cells in the PDGF-treated samples (Figure 2B). In the TPO-treated mice, the recovery of megakaryocytes and their progenitor cells was better than the recovery of the granulocytic lineage and erythroid cells. Overall, there were more hematopoietic cells in the TPO-treated mice than in the PDGF-treated mice. These data suggest that PDGF significantly enhanced the recovery of the

megakaryocytic lineage as well as hematopoietic stem and progenitor cells, although the effect was slightly weaker than that of TPO.

Effects of platelet-derived growth factor on in vitro *megakaryocytopoiesis. Platelet-derived growth factor exerted anti-apoptotic effects on the Mo7e cell line*

Apoptosis assays were performed to elucidate whether the radioprotective potential of PDGF on megakaryocytopoiesis may be a result of reducing apoptosis of megakaryocytes and their progenitors. Nutrition-depleted M-07e cells were treated or not with recombinant PDGF-BB or TPO. As shown in Figure 3, there was a marked increase in the proportions of early apoptotic cells [annexin V positive, propidium iodide (PI)-negative, R2], late apoptotic and necrotic cells (annexin V-positive, PI-positive, R1) and total apoptotic cells (annexin V positive, R1+R2) in nutrient-depleted cells compared to in the normal samples that had not undergone nutritional depletion. PDGF significantly reduced the proportions of early apoptotic cells (n=8, $P<0.001$) and total apoptotic cells ($P<0.01$) (Figure 3A). These data suggest that PDGF has an anti-apoptotic effect on megakaryocytes, and that its effect is similar to that of TPO.

Caspase-3 is an effector protein of the caspase family, which plays an essential role in apoptosis. Levels of active caspase-3 in serum and cytokine-depleted controls were significantly higher than those in normal samples that had not undergone serum and cytokine depletion (n=5, $P<0.01$), suggesting that apoptosis was induced. Addition of PDGF suppressed active caspase-3 expression ($P<0.01$) (Figure 3B). Similar results were also obtained in the TPO group, suggesting a decrease of apoptosis in PDGF- and TPO-treated cells. The data again showed an anti-apoptotic effect of PDGF on megakaryocytes, possibly through down-regulation of caspase-3 activity.

JC-1 can be rapidly taken up into mitochondria by the polarized mitochondrial membrane potential ($\Delta\psi$) to form JC-1 aggregates in living cells. Depolarization of $\Delta\psi$

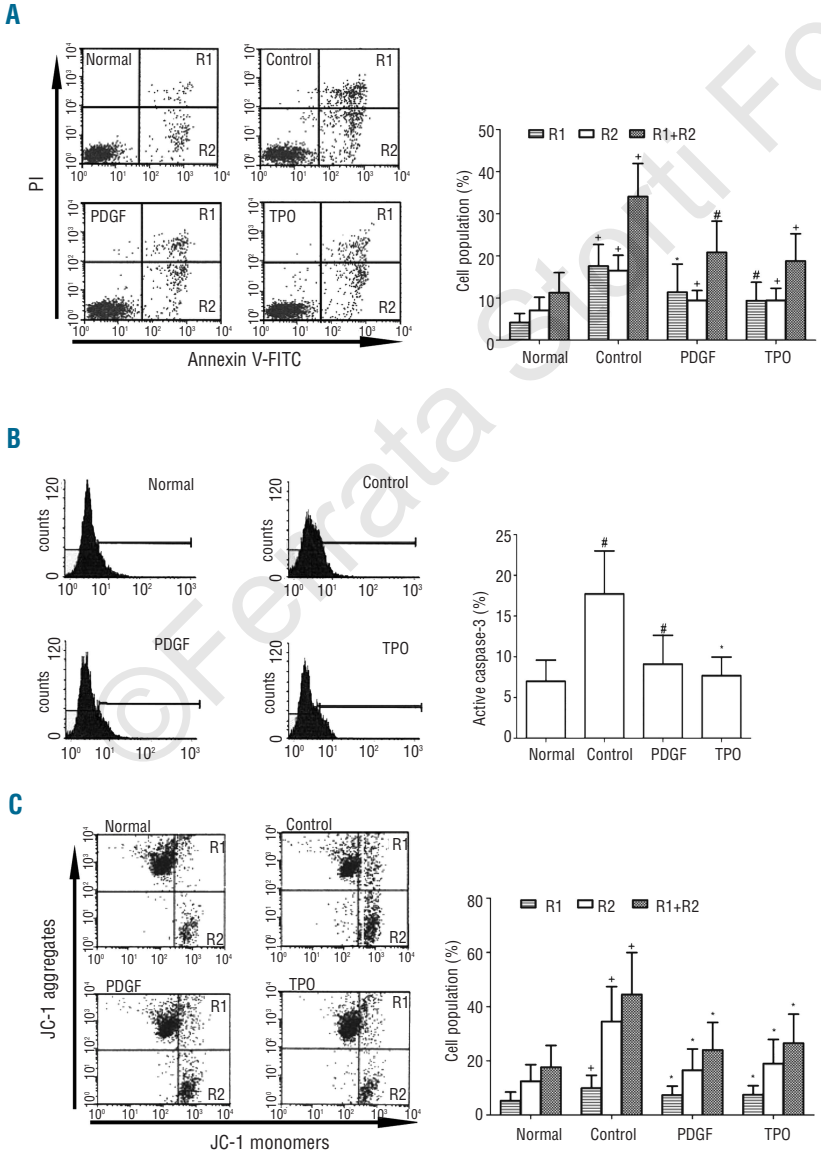

Figure 3. Anti-apoptotic effects of PDGF on M-07e cells. (A) To investigate the anti-apoptotic effect of PDGF, M-07e cells were resuspended in cytokine- and serum-depleted IMDM, and then incubated with PDGF (50 ng/mL) or TPO (50 ng/mL) for 72 h. The cells were then stained with annexinV/FITC and propidium iodide (PI) antibodies, and examined by flow cytometry. Early apoptotic cells (R2), late apoptotic and necrotic cells (R1) and total apoptotic cells (R1 + R2) were increased in nutrient-depleted M-07e culture (Control) (R1, R2 and R1+R2, n=8, Normal *versus* Control, + $P<0.0001$). PDGF significantly reduced the proportion of early apoptotic (R2) (n=8, control *versus* PDGF, + $P=0.0006$) as well as total apoptotic cells (R1+R2) (n=8, control *versus* PDGF, # $P=0.0017$). The anti-apoptotic effect of PDGF was similar to that of TPO (n=8, R2, control *versus* TPO, + $P=0.0005$ and R1+R2, + $P=0.0008$). (B) M-07e cells under different treatments were stained with anti-active caspase-3/PE antibody. The expression of active caspase-3 increased significantly in serum and cytokine-depleted M-07e cells (n=5, normal *versus* control, # $P=0.0084$). Cells treated with PDGF or TPO had impaired caspase-3 expression (n=5, control *versus* PDGF, # $P=0.0018$, control *versus* TPO, * $P=0.0167$). (C) M-07e cells under different treatments were stained with JC-1 reagent. A proportion of apoptotic cells contains JC-1 monomers (R2), and a subset of transitional cells contains both monomers and aggregates (R1). The total apoptotic cells (R1+R2) increased significantly in serum- and cytokine-depleted control samples (R1, R2 and R1+R2, n=7, normal *versus* control, + $P=0.0004$, 0.0005, and 0.0002). These populations of apoptotic cells reduced considerably in cultures treated with PDGF (R1, R2 and R1+R2, n=7, control *versus* PDGF, * P=0.0318, 0.0100, and 0.0103) or TPO (R1, R2 and R1+R2, n=7, control *versus* TPO, * $P=0.0428$, 0.0225, and 0.0230). PDGF, platelet-derived growth factor; TPO, thrombopoietin; JC-1, 5,5_,6,6_-tetrachloro-1,1_,3,3_- Tetraethylbenzimidazolcarbocyanine iodide.

indicates altered mitochondrial function which could be used as a marker of apoptosis. JC-1 fluorescence in these apoptotic cells does not accumulate in mitochondria and remains as monomers (R2). There is a subset of transitional cells containing both monomers and aggregates (R1). Our data suggest that the proportion of cells containing JC-1 monomers (R2) increased significantly in serum- and cytokine-depleted cells compared with in normal samples that had not undergone nutritional depletion (n=7, $P<0.001$), indicating a drop in $\Delta\psi$ and an increase in the proportion of apoptotic cells. Treatment with PDGF reduced the population of apoptotic cells ($P=0.01$) (Figure 3C) considerably. The total population of apoptotic cells (R1+R2) in each group exhibited a similar fluctuation (Figure 3C), suggesting the anti-apoptotic effect of PDGF may be mediated by the intrinsic mitochondrial pathway.

Effects of platelet-derived growth factor may be mediated via platelet-derived growth factor receptor and PI3-k/Akt signal pathway

PI3-k/Akt is a well-characterized pathway that plays an important role in both anti-apoptosis and proliferation processes. To determine whether PI3-k/Akt is involved in PDGF-induced megakaryocytopoiesis, nutrition-depleted M-07e cells were incubated with PDGF alone or in combination with wortmannin (an inhibitor of PI3-k). Cells were then stained with anti-p-Akt/PE antibody. PDGF increased the expression of p-Akt (n=9, $P<0.05$) (Figure 4A), indicating Akt activation. The addition of wortmannin to PDGF cultures reduced the expression level of p-Akt ($P<0.05$). This PDGF-induced Akt phosphorylation was also confirmed by the western blot. Reduced p-Akt expression was found in the presence of wortmannin (Figure 4B). Wortmannin alone did not change the pattern of p-Akt expression in the two experiments. This observation indicates that Akt activation was abrogated by the PI3-k inhibitor.

In a further investigation for an upstream executor that triggers the PI3-k/Akt signal cascade, nutrition-depleted M-07e cells were treated with PDGF alone or PDGF plus imatinib mesylate. As shown in Figure 5, PDGF alone increased the production of p-Akt, determined by either flow cytometry (Figure 5A) (n=4, $P<0.05$) or western blot, while the addition of imatinib mesylate compromised the effects of PDGF (Figure 5B). Imatinib mesylate alone did not affect the expression of p-Akt. This indicates that PDGF triggered the PI3-k/Akt pathway via PDGFR, and that imatinib mesylate was able to block this effect. These results suggest that PDGFR are essential for activating the PI3-k/Akt pathway involved in the PDGF-conducted anti-apoptotic process in megakaryocytes. In summary, the megakaryocytopoietic effect of PDGF is likely to be mediated via its receptor with sub-

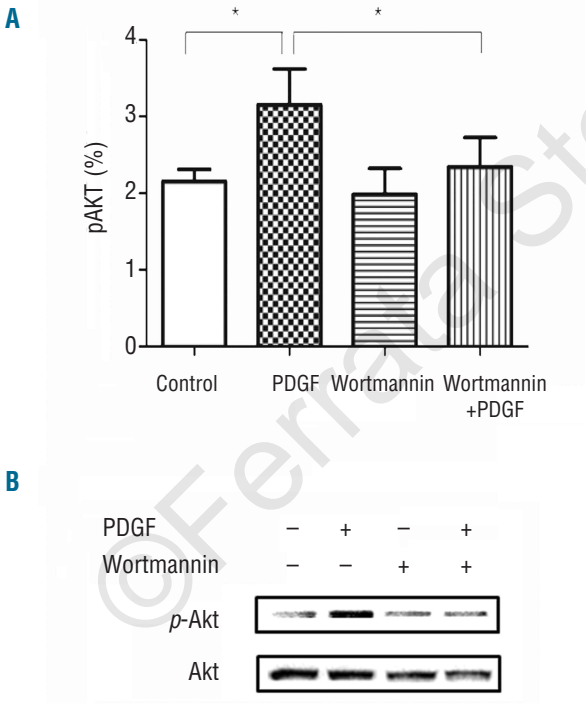

Figure 4. PDGF exerts an anti-apoptotic effect through the PI3-k/Akt signaling pathway. (A) M-07e cells were serum- and nutrient depleted (control). PDGF-BB (100-200 ng/mL) and wortmannin (100 nM) were added to the culture. Examined cells were stained with p-Akt /PE antibody. PDGF alone increased the expression of p-Akt significantly (n=9, control *versus* PDGF, * $P=0.0140$), whereas, the expression level of p-Akt decreased when the PDGF was combined with wortmannin (n=9, PDGF *versus* PDGF + wortmannin, *$P=0.0131$). (B) Phosphorylated Akt and total Akt of total cell lysates were examined by western blot. Expression of p-Akt was induced by PDGF, and reduced in the presence of wortmannin, an inhibitor of phosphoinositide 3-kinases.

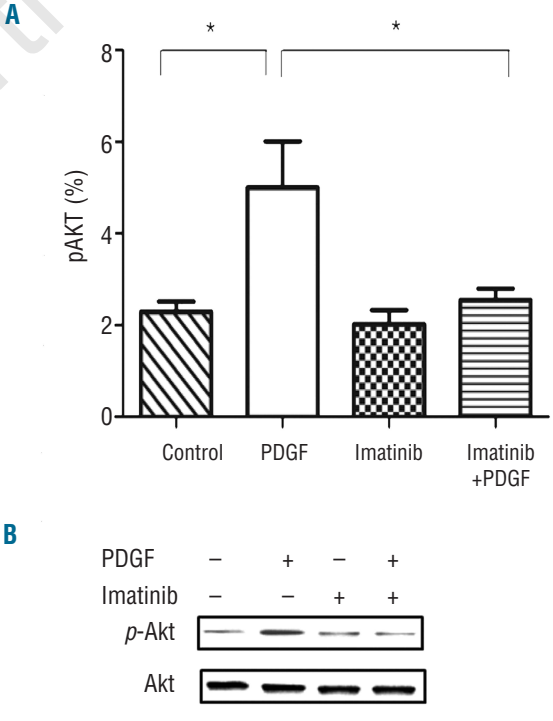

Figure 5. Effects of PDGFR on PDGF activating p-Akt. (A) M-07e cells were serum- and nutrient-depleted (control), then treated with PDGF-BB (100-200 ng/mL) and imatinib mesylate (1 μM). PDGF alone increased the expression of p-Akt as compared to expression in the control group (n=4, control *versus* PDGF, *$P=0.0409$), while PDGF plus imatinib reduced this population significantly (n=4, PDGF *versus* PDGF+ imatinib, *$P=0.0313$). (B) Phosphorylated Akt and total Akt of total cell lysates were examined by western blot. Production of p-Akt was increased by PDGF, and this effect was impaired by the administration of imatinib mesylate.

sequent activation of PI3-k/Akt pathway. Blockage of PDGFR by imatinib mesylate was capable of inhibiting the downstream phosphorylation of Akt.

Effects of imatinib mesylate on platelet-derived growth factor-induced colony-forming unit-megakaryocyte formation

A CFU-MK assay of mice that had not undergone irradiation treatment was performed to determine whether PDGF was able to directly promote megakaryopoiesis and whether it would be affected by imatinib mesylate. PDGF considerably stimulated the size of acetylcholine esterase-positive megakaryocytic colonies (Figure 6A, upper right) as well as the number of CFU-MK compared with untreated control (n=6, $P<0.005$) (Figure 6B). Imatinib mesylate treatment reduced both the size (Figure 6A, lower right) and number of the colonies. This finding indicates that PDGF is capable of inducing megakaryocyte proliferation, suggesting an additional mechanism for PDGF-mediated radioprotection.

Discussion

Here we report that PDGF effectively enhances *in vivo* platelet recovery in a murine model of radiation-induced thrombocytopenia. We further demonstrated that this protective effect is likely to be mediated via PDGF receptors with subsequent activation of the PI3-k/Akt pathway, which inhibits apoptosis in megakaryocytes.

In our previous studies, we found that megakaryocytes and platelets express both PDGFR α and β.[13] PDGFR-β is also expressed in early hematopoietic/endothelial precursors.[29] Activation of the various PDGFR triggers multiple cellular activities including cell growth, actin reorganization and migration.[30-32] In this study, we showed that PDGF exerts an anti-apoptotic effect via an interaction with its receptors. Stimulation of PDGFR induces dimerization and stabilization which leads to auto-phosphorylation and hence provides the docking sites for different signaling molecules, including PI3-k. PI3-k then activates a subset of down-stream signaling executors, including serine kinases, such as Akt,[33] by phosphorylation (Figure 7). It is well known that the PI3-k/Akt pathway transmits a cell survival signal in a large spectrum of cell types, and activation of this signaling confers resistance to apoptosis. However, the anti-apoptotic role of PI3-k in hematopoietic cells has been rarely reported. By using imatinib mesylate, a tyrosine kinase inhibitor, and wortmannin, a PI3-kinase inhibitor, we confirmed an involve-

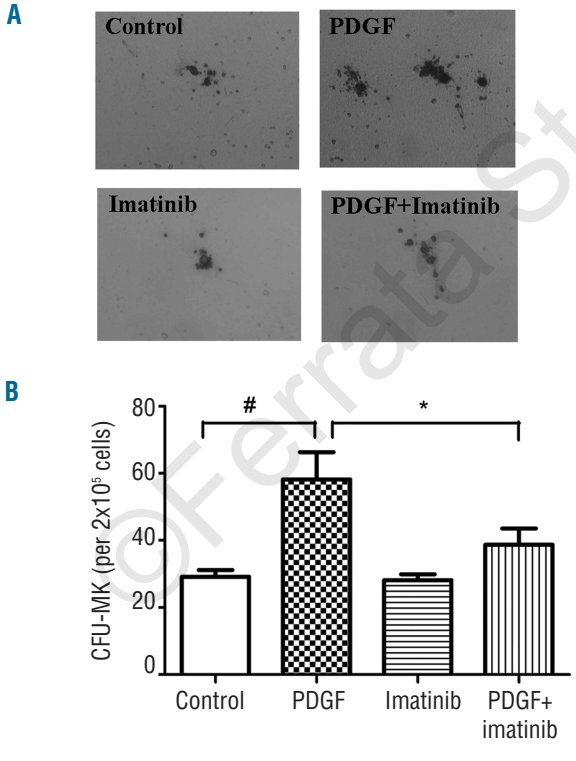

Figure 6. Effects of imatinib mesylate on PDGF-induced murine bone marrow CFU-MK formation. (A) Bone marrow cells from mice not subjected to irradiation were collected to perform the CFU-MK assay. PDGF increased the size of acetylcholine esterase-positive megakaryocytic colonies (upper right), which was reduced with addition of imatinib (lower right). (B) PDGF considerably stimulated the number of CFU-MK compared with the untreated control (n=6, #P=0.0030). Colony numbers were decreased by imatinib administration (n=6, *P=0.0331).

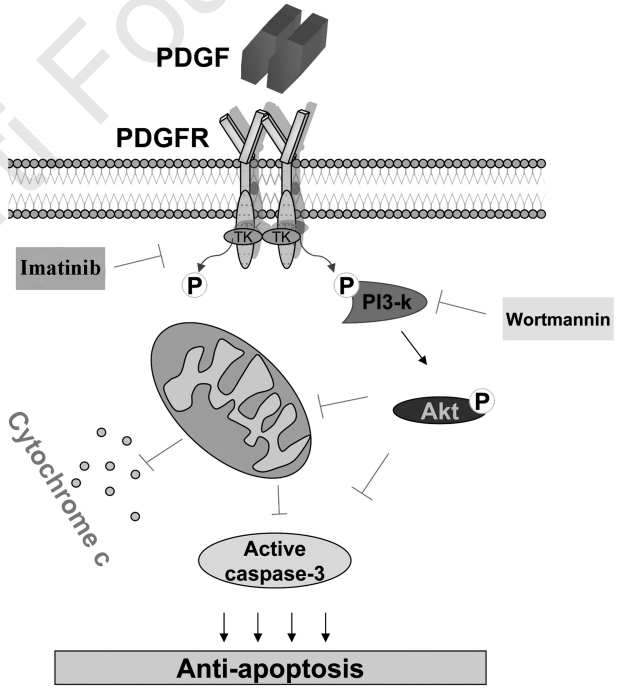

Figure 7. A schematic illustration of the PI3-k/Akt pathway initiated by PDGF/PDGFR which leads to proliferation and anti-apoptosis in megakaryocytes. An interaction between PDGF and PDGFR induces receptor dimerization and auto-phosphorylation on tyrosine kinase. The phospho-tyrosine functions as the docking sites for PI3-k molecules. Activated PI3-k leads to downstream Akt phosphorylation. Activation of Akt protects the mitochondrial membrane from depolarization and inhibits the release of cytochrome c, which negatively regulates the expression of activated caspase-3. Additionally, phosphorylation of Akt also inactivates caspase-3 directly. These signaling cascades lead to inhibition of megakaryocytes apoptosis. Imatinib mesylate used as a tyrosine kinase inhibitor, blocks the activation of the PDGF receptor. Wortmannin specifically inhibits PI3-kinase activity. Abbreviations: TK, tyrosine kinase; P, phosphorylation; PI3-k, phosphoinositide 3-kinases; Arrows (|) indicate experimentally verified stimulatory interactions, which may not be direct. Inhibitory interactions are denoted as ⊥.

ment of PDGFR and activation of the downstream PI3-k/Akt pathway in PDGF-initiated megakaryocytopoiesis.

Phosphorylation of Akt prevents cytochrome c, a major component of the mitochondrial respiratory chain, from leaking out of mitochondria.[34] Mitochondria play an essential role in the intrinsic apoptotic pathway through mobilization of cytochrome c and subsequent activation of caspase-9 and caspase-3, leading to apoptosis.[35] On the other hand, blocking the release of cytochrome c from mitochondria inactivates caspase-3 and prevents cell apoptosis. Akt phosphorylation also directly inhibits caspase-3 activation (Figure 7).[36] Our flow cytometry data showed that PDGF reduced the collapse of $\Delta\psi$ and attenuated the expression of active caspse-3, indicating that PDGF prevented megakaryocytic apoptosis through preventing the leakage of mitochondria membrane and reducing active caspase-3 expression (Figure 7).

The PDGF-initiated anti-apoptosis presented here at least partially elucidates the radioprotective effect of this growth factor. However, the mechanism may not be exclusive. Our previous studies and data shown in Figure 6 suggest a direct stimulatory role of PDGF on megakaryopoiesis and hematopoiesis. PDGFR are also found to be crucial in these biological processes.[9,10] The administration of a PDGFR blocker may, therefore, impair the factor's stimulatory and anti-apoptotic effects. Imatinib mesylate is a tyrosine kinase inhibitor, and recent studies suggested that it can potently block both PDGFR-α[18] and -β[20] by inhibiting PDGFR tyrosine kinase activity in fibroblasts and various tumor cells. There are reports on CML patients[15,16] and on patients with non-hematologic malignancies, such as gastrointestinal stromal tumors[37] and glioma,[38] who had had varying degrees of thrombocytopenia after treatment with imatinib mesylate. However, the underlying mechanism of the imatinib mesylate-associated thrombocytopenia has not been revealed. *In vivo* and *in vitro* evidence suggests that imatinib mesylate may directly inhibit normal hematopoiesis.[39,40] In our present study, we found that inhibition of PDGFR by imatinib mesylate inactivates PI3-k/Akt signaling and PDGF-induced anti-apoptosis in megakaryocytes. This suggests that blockade of PDGFR by imatinib mesylate may reduce megakaryocytopoiesis leading to thrombocytopenia, highlighting a crucial role for PDGF receptors in megakaryocytopoiesis. Since PDGFR appear to participate in the pathogenesis of many human malignancies, it is not surprising that newer anti-tyrosine kinase inhibitors other than imatinib mesylate, such as sunitinib[41] and nilotinib[42], which also inhibit PDGFR kinase activity and their downstream signal molecules, could induce thrombocytopenia.

Essential thrombocythemia is a chronic disease characterized by thrombocytosis. Increased plasma levels of PDGF were found in patients with this disease.[43] Increased PDGF expression was also observed in coronary artery smooth muscle cells from a patient with Kawasaki disease,[44] a vasculitis associated with thrombocytosis. Serum isolated from patients with Kawasaki disease may also induce PDGF-B expression in vascular endothelial cells.[45] Moreover, PDGF has been associated with idiopathic myelofibrosis,[46] a chronic myeloproliferative disorder. This growing clinical evidence suggests that platelet overproduction in these patients is accompanied by increased PDGF expression or release. Hence, these disorders may be a consequence of PDGF-driven pathological thrombopoiesis, again suggesting a positive regulatory effect of PDGF in hematopoiesis, particularly in platelet production

Authorship and Disclosures

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