Prevention of chemotherapy-induced anemia and thrombocytopenia by constant administration of Stem Cell Factor

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Chemotherapy-induced anemia and thrombocytopenia occur in a large portion of patients receiving myelosuppressive chemotherapy, leading to therapy-related complications and to treatment delay/reduction/discontinuation that negatively influence patient survival. The development of effective supportive strategies for the treatment of chemotherapy-induced myelosuppression is therefore essential to improve the outcome of both conventional and targeted antineoplastic therapies.

Stem Cell Factor (SCF) is mainly used in second-line treatment regimens for mobilization-refractory patients. Here we show that SCF protects the bone marrow from chemotherapy-induced damage and prevents the occurrence of anemia and thrombocytopenia in the peripheral blood of treated mice. We also show for the first time that the efficacy of SCF as a myeloprotective agent depends on the schedule of administration.

The observation that SCF is able to protect erythroid and megakaryocytic precursors in vivo provides a rationale for a future use of this cytokine to prevent drug-induced anemia and thrombocytopenia in cancer patients.
Abstract

**Purpose:** Chemotherapy-induced apoptosis of immature hematopoietic cells is a major cause of anemia and thrombocytopenia in cancer patients. While hematopoietic growth factors such as erythropoietin and colony-stimulating factors cannot prevent the occurrence of drug-induced myelosuppression, Stem Cell Factor (SCF) has been previously shown to protect immature erythroid and megakaryocytic cells *in vitro* from drug-induced apoptosis. However, the effect of SCF *in vivo* as a single myeloprotective agent has never been elucidated.

**Experimental Design:** The ability of SCF to prevent the occurrence of chemotherapy-induced anemia and thrombocytopenia was tested in a mouse model of cisplatin-induced myelosuppression. To highlight the importance of maintaining a continuous antiapoptotic signal in immature hematopoietic cells, we compared two treatment schedules: in the first schedule SCF administration was interrupted during chemotherapy treatment and resumed thereafter, while in the second schedule SCF was administered without interruption for seven days including the day of chemotherapy treatment.

**Results:** The administration of SCF to cisplatin-treated mice was able to preserve bone marrow integrity, to inhibit apoptosis of erythroid and megakaryocytic precursors, to prevent chemotherapy-induced anemia and to rapidly restore normal platelet production. Treatment with SCF increased the frequency of Bcl-2/Bcl-XL-positive bone marrow erythroid cells and sustained Akt activation in megakaryocytes. Myeloprotection was observed only when SCF was administered concomitantly with cisplatin and kept constantly present during the days following chemotherapy treatment.

**Conclusions:** SCF treatment is able to prevent the occurrence of chemotherapy-induced anemia and thrombocytopenia in mice, indicating a potential use of this cytokine in the supportive therapy of cancer patients.
Introduction

Chemotherapy-induced bone marrow damage results in anemia and thrombocytopenia that threaten the patients’ quality of life and the overall efficacy of anticancer treatments. Hematopoietic growth factors such as erythropoietin and colony-stimulating factors are commonly used to promote hematopoietic recovery following chemotherapy but they cannot prevent the occurrence of drug-induced myelosuppression. Stem Cell Factor (SCF) is produced by stromal cells of the bone marrow and binds the receptor c-kit expressed on hematopoietic stem and progenitor cells (1). SCF is essential for erythroid homeostasis as mice defective for SCF or its receptor c-kit display severe macrocytic anemia and inefficient response to stress erythropoiesis (2-7). SCF administration has been demonstrated to stimulate hematopoiesis in rodents, primates and humans by increasing the number of bone marrow stem and progenitor cells (8-10) and to promote recovery after cytotoxic damage (11). Besides stimulating hematopoietic cell expansion, SCF is a potent antiapoptotic factor for erythroid and megakaryocytic cells (12-15). In vitro, SCF is able to prevent chemotherapy-induced apoptosis of immature erythroblasts and megakaryocytes, which are exquisitely vulnerable to cytotoxic agents (14, 15). In this report, we show that SCF acts as a myeloprotective agent in vivo, being able to protect bone marrow cells from cisplatin-induced damage. In particular, SCF protected bone marrow erythroid (TER119⁺) and megakaryocytic (CD41⁺) precursors from chemotherapy-induced depletion and prevented the occurrence of anemia and thrombocytopenia in the peripheral blood of chemotherapy-treated mice. Importantly, only an administration schedule that maintained a constant presence of SCF during and after chemotherapy treatment was able to prevent bone marrow damage and consequent anemia/thrombocytopenia, thus indicating the need for an uninterrupted delivery of survival stimuli in order to achieve effective myeloprotection.
Materials and Methods

Mice treatment

Animal experiments were conducted according to the national animal experimentation guidelines (D.L.116/92) upon approval of the experimental protocol by the Institutional Animal Experimentation Committee. Six-weeks old C57/BL6 female mice weighing approximately 20g were purchased from Jackson Laboratories (Bar Harbor, ME) and maintained with food and water ad libitum for the duration of the studies. Mice were treated as follows: the “Control” group received matched PBS injections, the “Cisplatin” group received a single intraperitoneal dose of 7.5 mg/Kg cisplatin (Sigma Aldrich, St. Louis, MO) at day 0, the “SCF” group received 50 μg/Kg recombinant murine SCF (mSCF; Peprotech, Rocky Hill, NJ) dissolved in PBS twice a day from day -1 to day 7, “Interrupted” mice received 7.5 mg/Kg cisplatin at day 0 and 100 μg/Kg mSCF at day -1 and once a day from day 1 to day 7 (being SCF treatment interrupted during cisplatin administration), “Constant” mice received cisplatin at day 0 and 50μg/Kg mSCF twice a day from day 0 to day 7 (covering also the day of cisplatin administration). SCF was administered subcutaneously.

Microscopy and flow cytometry

Bone marrow sections were prepared as follows: at the end of the treatment mice were sacrificed, femurs were removed, fixed in buffered paraformaldehyde 10% for 24 h, washed and exposed to decalcifying solution (EDTA 0.05M, NaOH 5 N). Samples were deparaffinized and hydrated and staining reactions were then performed on 6 μm-thick sections. To evaluate bone marrow cellularity, histological sections were stained with hematoxylin/eosin. TUNEL staining was performed with the In Situ Cell Death Detection Kit (Roche Molecular Biochemicals, Indianapolis, IN) according to the manufacturer’s instructions. For immunofluorescence analysis and May-Grünwald-Giemsa staining, mice were sacrificed at the end of the treatment, femurs were harvested and marrow flushed with a 23G (0.45x10mm) syringe needle to collect single cell suspensions. For immunofluorescence analysis of mouse bone marrow cells double positive for CD41/phospho-Akt, TER119/Bcl-2 or TER119/Bcl-XL, bone marrow cells were stained with PE-conjugated anti-CD41 (BD Pharmingen, San Diego, CA) or anti-TER119 (eBioscience, San Diego, CA) and sorted with a FACSaria (Becton Dickinson). Sorted cells were then stained with primary antibodies against phospho-Akt (Cell Signaling, Danvers, MA), Bcl-2 (clone C-21) or Bcl-XL (clone H5) (Santa Cruz Biotechnology, Santa Cruz, CA). Alexa-Fluor 488 secondary antibody was from Invitrogen.
Molecular Probes (Carlsbad, CA). Bone marrow sections and May-Grünwald-Giemsa-stained cells were analyzed with a Nikon Eclipse E1000 microscope equipped with PlanFluor 40X dry objectives (numerical aperture 0.75) and PlanApo 60X oil objectives (numerical aperture 1.4) respectively (Nikon, Melville, NY). Images were taken by using a Nikon DXM1200 RGB camera and the Nikon ACT-1 software. Immunofluorescence images were taken with an FV1000 confocal microscope (Olympus) equipped with a 60X oil immersion objective and the Olympus Fluoview software. To evaluate CD41+ and TER119+ bone marrow populations by flow cytometry, cells were harvested as above and stained with PE-conjugated anti-CD41 or FITC-conjugated anti-TER119 (eBioscience, San Diego, CA). Samples were analyzed with a FACSCanto flow cytometer equipped with DIVA software.

Peripheral blood analyses
Peripheral blood was obtained from retroorbital bleeding of mice treated as described above. Bleeding was performed at day 10 for hemoglobin analysis and at days 2+10 for platelet analysis. Blood was dripped directly after removal into tubes containing 0.5M of EDTA. Analysis of peripheral blood parameters was conducted by a contract laboratory (AppiaLab, Rome, Italy) within two hours from bleeding. For hemoglobin and platelet evaluation, mice received two injections of cisplatin 5 mg/Kg at day 0 and day 4, which was necessary to produce a constant decrease in platelet number avoiding rebound effects that occur after a single chemotherapy treatment (16).

Statistical analysis. The statistical significance of results was calculated using GraphPad Prism 4 (GraphPad Software Inc., www.graphpad.com) and analyzed by means of one-way ANOVA and Bonferroni’s Multiple Comparison Tests. A p value <.05 is represented by a single asterisk, p<.01 is indicated by a double asterisk while three asterisks indicate p<.001.
Results and discussion

To investigate whether SCF was able to protect bone marrow cells from chemotherapy-induced destruction in vivo, we induced myelosuppression in mice with a single dose of cisplatin and administered SCF according to two treatment schedules. In the cohort of mice indicated as “Interrupted”, SCF was administered 24 hours before and 24 hours after cisplatin injection and once daily for 5 additional days. In the cohort of mice indicated as “Constant”, SCF was administered 4 hours before and 4 hours after cisplatin injection and then twice a day for 6 additional days. In the “Constant” group the schedule of administration ensured a constant availability of SCF throughout the treatment period, considering that SCF has an elimination half-life of approximately 8 hours (17), whereas in the “Interrupted” group this condition was intentionally not fulfilled (Fig. 1).

First we analyzed bone marrow sections from control mice (treated with PBS or with SCF alone) and mice treated with cisplatin (in the presence or in the absence of SCF delivered according to the schedules described above) stained with TUNEL to reveal the presence of apoptotic cells or with hematoxylin/eosin to display bone marrow structure. TUNEL-stained bone marrow sections showed a high number of apoptotic cells both in mice treated with cisplatin alone (36±6 per field) and in “Interrupted” mice (33±3), whereas in “Constant” mice the number of dying cells was significantly lower (9±2) (P<0.001) (Fig. 2A). Upon hematoxylin/eosin staining the bone marrow of cisplatin-treated mice showed a strong hypoplasia with myelofibrosis and clusters of dysplastic megakaryocytes (Fig. 2B, left panel, arrows). The bone marrow of “Interrupted” mice was similar to that of mice treated with cisplatin alone, indicating that an intermittent administration of SCF compromises the ability of this cytokine to prevent bone marrow destruction (Fig. 2B, left). These observations were confirmed by counting the absolute number of cells extracted from mouse femurs (Fig. 2B, right). By contrast, bone marrow structure and cell numbers of “Constant” mice resembled those of mice treated with PBS, indicating that constant administration of SCF protects bone marrow cells from chemotherapy-induced damage. The analysis of May-Grünwald-Giemsa-stained cells extracted from femurs of mice treated as described above showed that megakaryocytes and erythroblasts were significantly more abundant in “Constant” mice as compared to mice treated with cisplatin alone (Fig. 3A) (P<0.001 and P<0.05 respectively).

An analysis of bone marrow cells of the granulocytic lineage on May-Grünwald-Giemsa-stained slides revealed a significant decrease of granulocytic precursors in the “Cisplatin” and in the “Interrupted” samples whereas SCF treatment restored the levels of immature granulocytes in the “Constant” group (data not shown). However, a massive presence of mature granulocytes in the “Cisplatin” and in the “Interrupted” samples (likely represented by the long-lived bone marrow...
reservoir (18)) rendered difficult to prove a protective effect of SCF towards the granulocytic lineage. The relative abundance of megakaryocytic and erythroid cells was respectively confirmed by FACS analysis of CD41\(^+\) and TER119\(^+\) cells in the bone marrow of control and treated mice (Fig. 3B and Supplementary Fig. 1).

SCF was previously shown to prevent chemotherapy-induced apoptosis of purified human erythroblasts and megakaryocytes (which were shown to represent the preferential target of drug-induced damage within the erythroid and megakaryocytic lineage, respectively) by inducing an increase in antiapoptotic factors. Specifically, SCF upregulates Bcl-2/Bcl-XL in erythroid precursors while in megakaryocytes it increases Akt activation with subsequent Bad phosphorylation (14, 15). To investigate whether such antiapoptotic mechanisms were activated in murine hematopoietic precursors upon SCF treatment in vivo, we sorted cells positive for TER119 (erythroid precursors) or CD41 (megakaryocytic progenitors and megakaryocytes) from the bone marrow of mice treated either with vehicle, cisplatin, SCF only or cisplatin plus SCF in the “Interrupted” or “Constant” modality. Subsequent staining of sorted bone marrow cells with anti-phosphorylated Akt (for the CD41\(^+\) population) or with Bcl-2 or anti-Bcl-XL (for the TER119\(^+\) population) showed an increased frequency of Akt phosphorylation in CD41\(^+\) megakaryocytic cells of “Constant” mice as compared to mice treated with cisplatin alone (Fig. 4). Similarly, an increased number of TER119\(^+\) bone marrow erythroid cells expressed Bcl-2 and Bcl-XL in “Constant” mice as compared to mice treated with cisplatin alone (Fig. 4). In both cases, “Interrupted” mice did not differ significantly from mice treated with cisplatin alone. These results indicate that only a continuous stimulation with SCF is able to elicit antiapoptotic signals that act through the mitochondrial pathway, thus preventing the depletion of bone marrow erythroid and megakaryocytic cells.

Then, we wanted to investigate the consequences of SCF-induced myeloprotection on peripheral blood red cells and platelets. In order to obtain a sustained decrease of hemoglobin and platelets in the peripheral blood, mice were subjected to two injections of cisplatin respectively at day 0 and day 4 of treatment. Subsequent peripheral blood analyses revealed that at day 10 hemoglobin levels of “Constant” mice were significantly higher than those of cisplatin-treated animals (P<0.01) and similar to those of vehicle-treated controls, indicating that SCF efficiently prevented the occurrence of cisplatin-induced anemia (Fig. 5A). An assessment of platelet levels showed a progressive decrease of platelet levels in the “Cisplatin” group and, at a lesser extent, in the “Interrupted” group (Fig. 5B). Conversely, platelet levels of the “Constant” group, after an initial reduction started to increase and, ten days after the first chemotherapy insult, they reached the levels detected in control.
animals (Fig. 5B). Altogether, these results indicate that a constant administration of SCF can prevent the occurrence of chemotherapy-induced anemia and thrombocytopenia through a specific protection of immature erythroid and megakaryocytic cells. Investigations are ongoing in our laboratory to determine whether the protective effects of SCF on the bone marrow may involve other immature hematopoietic cells, such as CD34+ hematopoietic progenitors. A comprehensive analysis of pathways activated by SCF in immature hematopoietic cells performed by reverse phase proteomic arrays shows activation of multiple antiapoptotic pathways besides Bcl-2/Bcl-XL increase and Akt phosphorylation (Pedini F. and Zeuner A., manuscript in preparation), suggesting that a network of signals activated by c-kit contributes to SCF-mediated hematopoietic protection. The ability of SCF to activate an extensive network of survival signals may explain the multilineage antiapoptotic activity of this cytokine, which would provide an advantage over more restricted growth factors (erythropoiesis- and granulopoiesis-stimulating agents) currently used in the supportive therapy of cancer patients. Although further in vivo experiments with nonhuman primates would be useful to further confirm the myeloprotective activity of SCF, this cytokine may find a future use to prevent drug-induced anemia and thrombocytopenia in cancer patients with tumors that do not rely on c-kit signaling for growth and survival.
Legend to the figures

Figure 1. Representation of the treatment schedule with SCF and cisplatin. Five groups of mice were treated as follows: the “Control” group received matched PBS injections, the “Cisplatin” group received a single intraperitoneal dose of 7.5 mg/Kg cisplatin at day 0 (bolt), the “SCF” group subcutaneously received 50 μg/Kg recombinant murine SCF twice a day from day -1 to day 7, the “Interrupted” group received 7.5 mg/Kg cisplatin at day 0 (bolt) and 100 μg/Kg mSCF at day -1 and once a day from day 1 to day 7, the “Constant” group received cisplatin at day 0 (bolt) and 50 μg/Kg mSCF twice a day from day 0 to day 7.

Figure 2. Constant administration of SCF protects bone the marrow from chemotherapy-induced damage. Mice were treated as described in Figure 1. A, TUNEL staining of femur sections obtained at day 4 from the five groups of mice described above (left) and number of TUNEL-positive cells assessed in three independent experiments performed with four mice for each group (right). Images were taken with 40X magnification. One-way analysis of variance (ANOVA) and Bonferroni’s Multiple Comparison Tests showed a statistical significance of ***P<0.001 between Cisplatin and Constant mice. B, Hematoxylin/eosin staining of femur sections derived at day 7 from the five groups of mice described above (left) and absolute numbers of bone marrow cells extracted from contralateral femurs and counted on a Burker counting chamber (right). Images were taken with 40X magnification. Arrows on the “Cisplatin” panels indicate clusters of dysplastic megakaryocytes. The experiment was repeated four times with four mice for each group. The comparison between Cisplatin and Constant mice with one-way ANOVA and Bonferroni’s Multiple Comparison Tests showed a statistical significance of **P<0.01.

Figure 3. SCF specifically protects erythroid and megakaryocytic precursors from chemotherapy-induced depletion. A, May-Grünwald-Giemsa staining of bone marrow cells extracted from the femurs of mice treated as described above (upper panels) and numbers of megakaryocytes (lower left) and erythroblasts (lower right) counted on May-Grünwald-Giemsa-stained slides derived from four independent experiments. Images were taken with 60X magnification. The difference in megakaryocyte numbers according to one-way ANOVA and Bonferroni’s Multiple Comparison Tests was statistically significant with ***P<0.001 between Cisplatin and Constant mice. The difference in erythroblast numbers according to one-way ANOVA and Bonferroni’s Multiple Comparison Tests was statistically significant with *P<0.05 between Cisplatin and Constant mice. B, Flow cytometry analysis of CD41 (upper panels. MK, megakaryocytes) and TER119 expression...
(lower panels. Erythroid, erythroid precursors) in bone marrow cells extracted at day 7 from C57/BL6 mice treated as described above. The panels show a representative set of results obtained from two independent experiments performed with six mice for each group.

**Figure 4.** SCF activates antiapoptotic pathways in erythroid and megakaryocytic cells *in vivo.* Immunofluorescence analysis of Bcl-2, Bcl-XL and phospho-Akt in bone marrow cells extracted from mice treated as described in Figure 1. Cells were sorted for the expression of TER119 (Erythroid, erythroid precursors) or CD41 (MK, megakaryocytes) and then stained respectively with anti-Bcl-2, anti-Bcl-XL or anti-phospho-Akt. Images were taken with an Olympus FV1000 confocal microscope with 60X magnification and 6x zoom. The experiment was repeated twice with six mice for each group.

**Figure 5.** SCF prevents chemotherapy-induced decrease of hemoglobin and platelets in the peripheral blood. A, Hemoglobin levels in the peripheral blood of mice treated with SCF as described in Figure 1 and with Cisplatin 5 mg/Kg at day 0 and at day 4. The blood was withdrawn at day 10 by retroorbital bleeding and analyzed with an automated analyzer. The experiment was repeated six times with groups of four mice each. One-way ANOVA analysis with Bonferroni’s Multiple Comparison Tests showed a statistical significance of **P<0.01 between Cisplatin and Constant mice. B, Platelet numbers in the peripheral blood of mice treated as in A. Mice belonging to the five treatment groups were treated with cisplatin 5 mg/Kg at day 0 and day 4 (bolt). Blood was obtained by retroorbital bleeding at days 2 and 10 and analyzed with an automated analyzer. The experiment was repeated six times with groups of four mice each.

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References


Figure 2
Figure 4
Figure 5

(A) Graph showing Hb levels with different treatments. The graph indicates a significant difference between the groups marked with **.

(B) Graph showing PLT levels over the course of days for different treatments. The lines represent Control, Cisplatin, SCF, Interrupted, and Constant treatments. PLT levels are normalized to 10^3/μL.
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