Chemotherapy-Induced Thrombocytopenia Derives from the Selective Death of Megakaryocyte Progenitors and Can Be Rescued by Stem Cell Factor

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Abstract

Thrombocytopenia is a common side effect of chemotherapy, responsible for increased risk of bleeding and delay of treatment schedules in cancer patients. It is currently unknown how chemotherapeutic agents affect platelet production and whether the platelet precursors megakaryocytes represent a direct target of cytotoxic drugs. We investigated the effects of chemotherapeutic agents on primary megakaryocytes by using a culture system that recapitulates in vitro human megakaryopoiesis and found that cytotoxic drugs predominantly destroyed megakaryocytic progenitors at early stages of differentiation. Immature megakaryocytes could be protected from chemotherapeutic agents by the cytokine stem cell factor (SCF), which binds the c-kit receptor expressed on hematopoietic stem and progenitor cells. In chemotherapy-treated megakaryocytes, SCF activated Akt, neutralized the mitochondrial apoptotic machinery, and inhibited caspase activity. Interfering with Akt activation abrogated the anti-apoptotic effects of SCF, whereas exogenous expression of constitutively active Akt inhibited drug-induced apoptosis of primary megakaryocytes, indicating the Akt pathway as primarily responsible for SCF-mediated protection of megakaryocyte progenitors. These results indicate apoptosis of megakaryocyte progenitors as a major cause of chemotherapy-induced thrombocytopenia and suggest that SCF may be used to prevent platelet loss in cancer patients with c-kit-negative tumors. [Cancer Res 2007;67(10):4767–73]

Introduction

Thrombocytopenia is a detrimental side effect of cancer treatment, often resulting in chemotherapy dose reductions, schedule alterations, or the need for platelet transfusions. Although more frequent in leukemia patients, drug-induced thrombocytopenia and consequent bleeding episodes occur also in patients with solid tumors, where they are associated with increased morbidity and occasional mortality (1, 2). Despite the clinical importance of chemotherapy-induced thrombocytopenia, its causes are still unclear, thus considerably limiting the possibility to develop effective remedies. In particular, the effects of chemotherapeutic agents on the platelet precursors megakaryocytes have not been investigated in detail, leaving open the possibility that drug-induced toxicity on this cell population may represent a major cause of thrombocytopenia in cancer patients.

Megakaryopoiesis is a peculiar process that involves the gradual differentiation of immature megakaryocyte progenitors into diploid megakaryocytes, which undergo a progressive polyploidization and a subsequent process of cytoplasmic maturation leading to platelet release in the bone marrow sinusoids. Platelet formation and release involve a reorganization of the cytoskeleton that has recently been shown to rely on the compartmentalized activation of caspases (3). Caspase activation in megakaryocytes has been shown to occur also as part of the apoptotic program, which can be initiated either by cytotoxic agents or by activation of death receptors expressed on the surface of megakaryocytic precursors (3, 4). Megakaryocyte survival, proliferation, and differentiation are coordinated by combinations of cytokines and mediators present within specialized bone marrow niches (5). Besides thrombopoietin (TPO), which is the essential growth factor responsible for platelet production, several hematopoietic growth factors such as interleukin (IL)-3, IL-6, IL-11, stromal cell–derived factor 1, and stem cell factor (SCF) have been shown to influence megakaryopoiesis at different developmental stages (6–8). SCF, the ligand for the c-kit tyrosine kinase receptor, has recently been shown to play an important role in megakaryopoiesis, as mice with a double inactivation of TPO receptor and c-kit (c-Mpl−/−/KitWv/Wv) display lower levels of bone marrow megakaryocyte progenitors than c-Mpl−/− mice (9).

To investigate the basis of chemotherapy-induced thrombocytopenia, we analyzed the sensitivity to drug-induced cytotoxicity of primary human megakaryocytes at different maturation stages and explored the possibility to protect megakaryocytic progenitors from drug-induced apoptosis with the use of cytokines. We also investigated the molecular events activated during chemotherapy-induced apoptosis of megakaryocyte progenitors and during cytokine-mediated protection from cell death. These studies may help understand the causes of chemotherapy-induced thrombocytopenia and may support the development of therapeutic strategies to prevent platelet depletion in cancer patients.

Materials and Methods

Cytokines, antibodies, and chemicals. Human recombinant SCF and TPO were purchased from PeproTech, Inc. Anti-tubulin antibody and chemotherapeutic drugs were purchased from Sigma-Aldrich, Inc. Anti–Bcl-2 and anti–cytochrome c antibodies were from BD Pharmingen. Anti–Bel-XI (H5), anti–Akt (C20), anti–Bad (H-168), anti–extracellular signal–regulated kinase (ERK)-1 (K-23), anti–phospho-ERK1-2 (E-4), and anti–Mcl-1 (S-19) were from Santa Cruz Biotechnology. Anti–phospho-Bad
(Ser196) and anti–phospho-Akt (Ser273) were purchased from Cell Signaling. Anti-hemagglutinin (HA) antibody was from Babco. Anti–c-kit (AF32) was from R&D Systems. Annexin V-FITC, MitoTracker Red CMXRos, TOTO-3, antimitouse Alexa Fluor 488, antirabbit Alexa Fluor 488, and antimitouse Alexa Fluor 633 were purchased from Invitrogen-Molecular Probes. Antirabbit Texas red and antimitouse Texas red were from The Jackson Laboratory. L1299002 was from Sigma-Aldrich and U0126 from Promega.

Adult peripheral blood human progenitor cell purification and culture. Adult peripheral blood was obtained from male donors after their informed consent and approval by the institutional Committee for Human Studies. Human CD34+ precursor cells were purified from peripheral blood by using the mid-MACS immunomagnetic separation system (Miltenyi Biotec). CD34+ cells were cultured in serum-free medium prepared as previously described (10) and supplemented with 100 ng/mL TPO (from now on indicated as standard megakaryocyte medium) to induce unilineage megakaryocytic differentiation. In these conditions, a progeny of cells 97% to 99% CD61+/CD41+ is generated (11). The differentiation stage of megakaryocytes was routinely evaluated by May-Grünwald-Giemsa staining and cytologic analysis. Stained cells were observed through a Nikon Eclipse E1000 transmitted light microscope equipped with PlanFluor 40× oil objectives (numerical aperture, 1.4 for both). All objectives were from Nikon. Images were subsequently taken by using a Nikon DXM1200 RGB camera and the Nikon ACT-I software.

Western blotting. For detection of Bcl-2, Bcl-XL, Bad, and Akt, protein extracts were prepared by resuspending cell pellets in 1% NP40 lysis buffer [20 mM Tris-HCl (pH 7.2), 200 mM NaCl, 1% NP40] supplemented with Protease Inhibitor Cocktail and Phosphatase Inhibitor Cocktails I and II (all from Sigma-Aldrich). For phospho-Bad detection, protein extracts were passed through a 26-gauge (0.45 × 10 mm) syringe needle, loaded on a 15% polyacrylamide gel, and transferred to a nitrocellulose membrane with 0.05-μm pores. Samples were analyzed by standard immunoblot procedure and visualized by chemiluminescence (SuperSignal West Pico, Pierce). The intensity of bands representing relevant proteins was quantified using Scion Image (Scion Corp.).

Production of retroviral particles and transduction of hematopoietic progenitor cells. HA-myrr-Akt cDNA was cloned into a third-generation lentiviral vector pRRL-CMV-PGK-GFP-WPRE called TWEEN (12). Lentiviral supernatants were produced by calcium phosphate transient cotransfection of a three-plasmid expression system in the packaging human embryonic kidney cell line 293T. One cycle of infection with viral supernatant (with addition of 4 μg/mL polybrene and 100 ng/mL TPO) was done on megakaryocytes at day 3 of unilineage culture, and green fluorescent protein (GFP)-positive cells were separated by flow cytometry using a FACSVantage (Becton Dickinson).

Evaluation of caspase activity and apoptosis. Caspase-3/caspase-7 activity in megakaryocyte progenitors stimulated with chemotherapeutic drugs was determined with the Apo-ONE Homogeneous Caspase-3/7 Assay (Promega) based on cleavage of the z-DEVD-Rhodamine 110 substrate. Fluorescence values were read on a Victor 2 plate fluorometer (Wallac, Perkin-Elmer, Inc.) at a wavelength of 485/535 nm (excitation/emission). Apoptosis was assessed by ethidium bromide/acidine orange staining and fluorescence microscopy, by staining with Annexin V-FITC, by terminal deoxyribonucleotidyl transferase–mediated dUTP nick end labeling (TUNEL) staining (described below), or with the CellTiter 96 AQueous One Solution Assay (Promega).

Immunostaining and TUNEL staining. For immunofluorescence experiments with anti–cytokeratin e and anti-Bad, megakaryocytes were preloaded with 100 mM/L L MitoTracker CMXRos (30 min in culture medium at 37°C), fixed in 4% paraformaldehyde, permeabilized with blocking buffer (0.05% saponin, 3% bovine serum albumin, 0.1% sodium azide in PBS), and incubated overnight at 4°C with primary and secondary antibodies (Alexa Fluor 488 antimitouse immunoglobulin G for cytokeratin e and Alexa Fluor 633 antirabbit IgG for Bad). For immunofluorescence experiments with anti–Akt, anti–phospho-Akt, and anti-HA, cells were cytocentrifuged on glass slides, fixed with 4% paraformaldehyde, permeabilized with methanol at −20°C, and stained with primary and secondary antibodies (antigot Alex Flou 633 for anti-Akt, antirabbit Texas red for anti–phospho-Akt, and antimitouse Texas red for anti-HA). Slides were mounted using the LowFade Light Anti Fade Kit from Invitrogen-Molecular Probes. TUNEL staining was done with the In Situ Cell Death Detection Kit conjugated with FITC (Roche, Mannheim, Germany) on megakaryocytes fixed and cytopsin on glass slides. In immunofluorescence and TUNEL experiments done on lentivirally transduced cells, detergent-based permeabilization resulted in GFP extraction. Consequently, GFP fluorescence became undetectable and cells could be stained with Alexa Fluor 488–conjugated secondary antibodies or TUNEL FITC. Fluorescence images were taken by means of an Olympus FV-500 laser scanning confocal inverted microscope equipped with Argon ions, Green and Red Helium-Neon lasers, and with Planapo 40× dry, 60×, and 100× oil Olympus objectives (numerical apertures, 0.85, 1.4, and 1.35, respectively). Emission at different wavelengths was collected using the proper filters and overriding signal was subtracted. Digital zooming was applied where specified. Images were assembled with the Canvas 8 software (Deneba Systems, Inc.).

Statistical analysis. The statistical significance of the results shown in Figs. 2A, B, 3B-D, and 4B was evaluated by means of two-way ANOVA and Bonferroni posttests. Single asterisk represents P < 0.05, double asterisk indicates P < 0.01, and three asterisks indicates P < 0.001. All statistical analyses were done using GraphPad Prism 4.3.

Results

Megakaryocyte progenitors are the preferential target of chemotherapy-induced apoptosis. Purified CD34+ cells isolated from peripheral blood and cultivated in serum-free medium in the presence of TPO undergo unconditional megakaryocytic differentiation, reproducing in vitro all the stages of megakaryopoiesis (Fig. 1A; ref. 11). We used this unilineage culture system to investigate the sensitivity to chemotherapeutic drugs of the different stages of megakaryocytic maturation. To do so, we evaluated the extent of drug-induced apoptosis in CD34+ cells, megakaryocytic progenitors (megakaryoblasts and promegakaryocytes), and immature (bunucleated) and mature (multinucleated) megakaryocytes. Three chemotherapeutic drugs, cisplatin, cytosome arabinoside, and vincristine, were chosen because they can cause thrombocytopenia in treated cancer patients. The apoptotic rate of megakaryocytic progenitors treated with the three chemotherapeutic agents was significantly higher compared with that of CD34+ cells and polyploid megakaryocytes (Fig. 1B), pointing to a higher sensitivity of the early stages of megakaryopoiesis to drug-induced cytotoxicity.

SCF but not TPO protects immature megakaryocytes from drug-induced apoptosis. Because chemotherapeutic agents seemed to preferentially target the earlier forms of megakaryocytic maturation, we did subsequent studies on cells at day 6 of unilineage culture, which contain mostly megakaryoblasts and promegakaryocytes (as evaluated by morphologic features of cell size, cytoplasm abundance, and nucleus/cytoplasm ratio). To investigate whether drug-induced apoptosis of megakaryocytic progenitor population could be inhibited with the use of cytokines, we analyzed the antia apoptotic properties of TPO, the major megakaryocytic growth factor, and of SCF, which is a potent antia apoptotic factor for primary hematopoietic cells. Day 6 megakaryocytes were exposed to chemotherapeutic drugs in serum-free, cytokine-free medium in the presence or absence of TPO. However, no significant difference was observed in the amount of cell death induced by chemotherapeutic agents in either

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shown that SCF protects primary erythroid progenitor cells from chemotherapy-induced apoptosis through the up-regulation of antiapoptotic Bcl-2 family members (13). Differently, we found that SCF treatment did not alter the expression levels of either Bcl-2 or Bcl-X
 in megakaryocyte progenitors (Fig. 3A), indicating that the SCF receptor c-kit exploits different antiapoptotic signaling pathways in progenitors belonging to different hematopoietic lineages. To identify the pathways involved in SCF-mediated protection of megakaryocyte progenitors, we analyzed the activation of two major mediators of c-kit signaling involved in cell survival, Akt and ERK kinases. Treatment of day 6 megakaryocytic cultures with SCF resulted in a strong Akt activation and consequent phosphorylation of proapoptotic Bad on Ser
, which results in Bad inactivation and apoptosis inhibition. Conversely, SCF treatment resulted only in a modest activation of ERK kinases and did not modify the levels of Mcl-1, an important antiapoptotic mediator controlled by both the Akt and ERK pathways (ref. 14; Fig. 3A). To confirm the preferential involvement of the phosphatidylinositol 3-kinase-Akt pathway in megakaryoblast protection, we treated megakaryocytic cultures with vincristine and SCF in the presence of inhibitors of the phosphatidylinositol 3-kinase and ERK pathways.

Inhibition of the phosphatidylinositol 3-kinase pathway abrogated the protective effect of SCF, whereas inhibitors of the ERK pathway interfered marginally with SCF-mediated protection of megakaryoblasts (Fig. 3B). Mitochondria release cytochrome c in response to most anticancer drugs, resulting in apoptosome formation and subsequent activation of executioner caspases (15). We analyzed the localization of cytochrome c in immature megakaryocytes treated with anticancer drugs in the presence or absence of SCF and found that cytochrome c was sequestered in the mitochondria in untreated cells, released in the cytoplasm on drug treatment, and again retained in the mitochondria in the presence of SCF (Fig. 3C). As the presence of cytochrome c in the cytoplasm is essential for activation of executioner caspases, we evaluated the activation of caspase-3/caspase-7 induced by chemotherapeutic drugs in megakaryocyte progenitors and found that the levels of caspase activation were significantly lower in megakaryocyte progenitors treated with chemotherapeutic drugs in the presence of SCF (Fig. 3D).

These observations suggest that SCF-mediated megakaryocyte protection primarily relies on Akt activation and inhibition of the mitochondrial apoptotic pathway.

Akt activation protects megakaryocyte progenitors from drug-induced apoptosis. Akt promotes cell survival by phosphorylating several components of the intrinsic cell death machinery. To address whether the antiapoptotic effect of SCF in megakaryocyte progenitors could be ascribed to Akt activation, we retrovirally transduced a myristylated constitutively active form of Akt (myr-Akt) in immature megakaryocytes derived from CD34
 cells. Transduced cells were then sorted on the basis of GFP expression and the presence of phosphorylated HA-tagged Akt was confirmed by immunostaining (Fig. 4A). Megakaryocyte progenitors transduced with myr-Akt were then exposed to chemotherapeutic agents and the extent of cell death was assessed together with cytochrome c localization. The expression of myr-Akt rendered immature megakaryocytic cells significantly more resistant to apoptosis induced by chemotherapeutic agents (Fig. 4B and C). Moreover, the presence of myr-Akt in drug-treated megakaryocyte progenitors resulted in retention of cytochrome c in the mitochondria (Fig. 4D), thus explaining the increased survival condition (Fig. 2A). Differently, the presence of SCF in the megakaryocyte culture medium strongly increased the survival of megakaryocytic progenitors treated with chemotherapeutic agents (Fig. 2B). Flow cytometric analysis of cells at day 6 of unilineage culture showed that the presence of SCF inhibited drug-induced phosphatidylerine externalization (Fig. 2C), whereas TUNEL staining showed that SCF reduced the amount of damaged DNA in cells treated with chemotherapeutic agents (Fig. 2D). Some tumor types express cytokine receptors and may respond to hematopoietic growth factors with increased proliferation and survival. However, we did not observe c-kit expression or any antiapoptotic effect of SCF in primary breast cancer cells (Supplementary Fig. S1), suggesting that, in principle, SCF could be safely administered to patients with c-kit-negative tumors.

SCF activates Akt and inhibits the mitochondrial apoptotic pathway in immature megakaryocytes. We have previously
observed in this cell population. Altogether, these results indicate that Akt activation protects megakaryocyte progenitors from apoptosis induced by chemotherapeutic drugs and suggest that Akt phosphorylation mediated by SCF may be responsible for megakaryocyte resistance to the cytotoxic action of chemotherapeutic drugs.

Discussion

Chemotherapy-induced thrombocytopenia is a major clinical problem that, on estimate, affects 300,000 persons yearly worldwide (1). Management approaches to chemotherapy-induced thrombocytopenia are mainly based on platelet transfusions that, however, are associated with transfusion-transmitted disease, infection, refractoriness, and alloimmunization. Over the past decade, several pharmacologic agents with thrombopoietic activity have been evaluated for the supportive therapy of cancer patients including TPO, IL-3, IL-6, and IL-11 (16, 17). At present, IL-11 is the only cytokine licensed in the United States for the treatment of chemotherapy-induced thrombocytopenia, but its thrombopoietic activity is modest and its use is often associated with unfavorable side effects (17, 18). The administration of hematopoietic growth factors after chemotherapy offers a faster recovery from myelosuppression in patients with solid tumors. However, an important goal of anticancer therapies would be to avoid the occurrence of myelosuppression either through more selective therapeutic strategies that target only neoplastic cells (19) or through the use of antiapoptotic factors that specifically protect hematopoietic cells. The results presented in this study indicate that SCF is a potent antiapoptotic factor for megakaryoblasts, which are the preferential target of chemotherapeutic drugs among maturing megakaryocytic cells, and therefore may be useful in the prevention of drug-induced thrombocytopenia.

A potential use of SCF to prevent chemotherapy-induced thrombocytopenia is supported by the complete hematologic recovery and increased platelet counts observed in a cancer patient treated with recombinant SCF (Ancestim, Amgen) in combination with other hematopoietic growth factors (20). In this case, the use of SCF was associated with a particularly fast platelet recovery, possibly due to the protection of early megakaryocytic progenitors from drug-induced depletion. Our findings that SCF protects megakaryocyte progenitors from apoptosis by inhibiting the mitochondrial apoptotic machinery suggest that SCF would be mostly effective if administered simultaneously with the chemotherapeutic treatment and during all the duration of the chemotherapy cycle to prevent the occurrence of drug-induced apoptotic events.

The ability of SCF to protect megakaryocytic progenitors from chemotherapy-induced apoptosis reveals a link between c-kit signaling and thrombocytopenia that may influence the extent of...
megakaryocyte loss during chemotherapy. According to this hypothesis, pharmacologic agents that inhibit c-kit, such as imatinib mesylate, frequently produce megakaryocyte loss and thrombocytopenia in treated chronic myeloid leukemia patients (21, 22). Thrombocytopenia is particularly evident when imatinib is combined with standard chemotherapeutic drugs (23), suggesting that the interaction between c-kit and endogenously produced SCF is important for megakaryocyte survival in myelosuppressive conditions.

A prospective use of SCF to support hematopoietic protection and recovery in cancer patients should take into account the potential oncogenic effects of this cytokine on c-kit–expressing

Figure 3. SCF inhibits apoptosis through Akt activation and inhibition of the mitochondrial apoptotic pathway in megakaryocyte progenitors. A, megakaryocyte progenitors at day 6 of unilineage culture were treated for 8 h with (+SCF) or without (−SCF) 100 ng/mL SCF in standard megakaryocyte medium and then analyzed by Western blotting for protein expression. Top, Western blot results of three independent experiments are expressed as fold increase in protein expression as compared with untreated (−SCF) controls. B, day 6 megakaryocyte progenitors untreated or treated with SCF as above were incubated with 0.5 μmol/L vincristine in the presence or absence of 25 μmol/L LY294002 or 10 μmol/L U0126 (both administered 1 h before the chemotherapeutic agent). Cell death was assessed 16 h later by ethidium bromide/acridine orange staining. Columns, mean of three independent experiments; bars, SD. C, immunofluorescence analysis of cytochrome c localization in megakaryocyte progenitors treated with 0.5 μmol/L vincristine, preloaded with MitoTracker CMXRos, and stained with anti–cytochrome c antibody. Original magnification, ×1,000, 3× zoom. D, caspase-3/caspase-7 activation in megakaryocyte progenitors kept in the presence (+SCF) or absence (−SCF) of SCF as indicated above and incubated for 24 h with 80 μmol/L cytosine arabinoside, 0.5 μmol/L vincristine, or 5 μg/mL cisplatin. Columns, mean fold increase of caspase activity of three independent experiments done with cells from different donors, as compared with an untreated cell sample; bars, SD. \( P < 0.001 \), ANOVA.
tumor cells. In fact, some tumor cells, particularly from acute leukemias and colon and gastric cancers, have been shown to express c-kit, and some of these tumors respond to SCF with enhanced proliferation (24–26). The potential oncogenic effect of cytokines used in the supportive therapy of cancer patients is a general problem, as shown by the recent discovery that many tumors express the erythropoietin receptor and respond to recombinant erythropoietin with proliferation and chemotherapy resistance (27–31). Therefore, to avoid undesirable proliferative and antiapoptotic effects on tumor cells, SCF should be administered exclusively to patients with c-kit–negative tumors. A recent comprehensive study on c-kit expression in human tumors showed that 81 of 120 tumor types examined were negative for c-kit expression including hormone-refractory prostate carcinoma, Kaposi's sarcoma, non-Hodgkin's lymphomas, medulloblastoma, tubular breast carcinoma, and chronic myelogenous leukemia (32), whereas other investigators reported a complete absence of c-kit expression in malignant brain tumors and breast and ovarian cancers (33). Although an individual evaluation of receptor expression on cancer cells would be desirable before the administration of any hematopoietic cytokine, these observations suggest that the use of SCF should be safe in many common malignancies.

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**Figure 4.** Constitutively active Akt protects megakaryocyte progenitors from drug-induced apoptosis. Megakaryocyte progenitors at day 3 of unilineage culture were transduced with a lentiviral vector carrying a myristylated constitutively active form of Akt and GFP as a reporter, sorted on the basis of GFP expression, and analyzed after 3 additional days of culture. A, megakaryocyte progenitors transduced with the empty vector (Vector) or with myr-Akt (myrAkt) were immunostained with anti-Akt (αAKT; left), anti–phospho-Akt (αpAKT; center), or anti-HA (αHA; right). Original magnification, ×400. B, cells transduced with the empty vector or with myr-Akt were left untreated (Con) or treated with chemotherapeutic agents as in (C). Cell death percentage was evaluated by ethidium bromide-acridine orange staining. Columns, mean of three experiments done with cells from different donors; bars, SD. P < 0.001, ANOVA. C, transduced megakaryocyte progenitors were treated with 80 μmol/L cytosine arabinoside for 24 h, stained with TOTO-3 and TUNEL FITC (pseudocolored in blue and magenta, respectively), and analyzed on a confocal microscope (original magnification, ×400, 2× zoom). D, transduced megakaryocytes were treated with cytosine arabinoside, preloaded with MitoTracker CMXRos, and stained with anti–cytochrome c. Samples were analyzed on a confocal microscope (original magnification, ×1,000, 3× zoom).

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*4 Our unpublished data.*
References


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