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Efficacy of delayed administration of post-chemotherapy granulocyte colony-stimulating factor: evidence from murine studies of bone marrow cell kinetics

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Abstract

The optimal schedule of post-chemotherapy G-CSF administration has not been determined. G-CSF is customarily started 24 hours after chemotherapy; however, clinical data demonstrated that delaying the G-CSF until 5 days after completion of chemotherapy has not resulted in a longer duration of neutropenia. Here, we examined the optimal timing of post-chemotherapy G-CSF administration in a mouse model, to show that delayed administration does not postpone the appearance of mature granulocytes in the peripheral blood. We also investigated the mechanism of decreased efficacy of the early G-CSF application after chemotherapy by characterizing the changes in bone marrow cellular composition. To our knowledge, we demonstrate for the first time, that early after chemotherapy, the bone marrow is predominantly composed of mature residual granulocytes and very few progenitors and precursors, on which G-CSF would act to generate granulocytes. The point when immature progenitors reappear does not occur in murine bone marrow until 48 hours after a single dose of cyclophosphamide. Our results indicate that the bone marrow cellular composition early after discontinuation of chemotherapy is not optimal for G-CSF action on acceleration of myeloid recovery. Given the high cost of G-CSF prophylaxis, its delayed administration may potentially result in substantial economic benefits.

Introduction

Post-chemotherapy prophylactic granulocyte colony-stimulating factor (G-CSF) is customarily started 24 hours after chemotherapy in most of the current pediatric and adult solid tumors protocols to ameliorate the myelotoxicity of cytotoxic agents and reduce neutropenia-related complications. Previous studies in children with solid tumors and adult ALL patients^{1,2} have suggested that post-chemotherapy G-CSF administration can be delayed by several days without negative effect on acceleration of neutrophil recovery. Indirectly, these observations indicate that early post-chemotherapy doses do not contribute to the overall G-CSF effect on myeloid recovery enough to make the difference between early and delayed administration significant. Given the high cost of G-CSF prophylaxis, its delayed

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administration may potentially result in substantial economic savings. It could also improve the quality of patients' life by decreasing the numbers of injections.

The post-chemotherapy bone marrow represents a dynamic environment where the processes of chemotherapeutic damage and recovery lead to significant and rapid changes in cellular composition. The optimal schedule of post-chemotherapy G-CSF administration has not been established yet. Despite extensive use of G-CSF in the clinic, post-chemotherapy dynamics of its target bone marrow cell populations have never been examined to our knowledge. Thus, to understand why delayed start of G-CSF may still be equally effective compared to an early start, we studied post-chemotherapy kinetics of bone marrow cell populations in a mouse model. Assuming that accelerated hematopoietic recovery is mediated through direct G-CSF action on committed progenitors we hypothesized that 1) the majority of dividing myeloid progenitors are eliminated from the bone marrow early and will reappear later after chemotherapy; and 2) consequently, quiescent stem cells and residual mature granulocytes compose most of the bone marrow early after chemotherapy.

Thus, our specific goals were to determine the daily changes in mature granulocytes and progenitor cells in the post-chemotherapy bone marrow, and to determine the effect of different post-chemotherapy G-CSF schedules on blood count recovery, as well as progenitor and stem cell mobilization.

Materials and methods

Mice

Female or male C57Bl/6 or BALB/c mice, 6- to 8-weeks-old, were obtained from the Jackson Laboratories or bred in BCM animal barrier. Mice were maintained in a pathogen-free environment. Post-chemotherapy bone marrow cell kinetics experiments were initially performed on female BALB/c mice and later repeated on male C57Bl/6 animals. All other experiments were done with male C57Bl/6 mice.

G-CSF and chemotherapy administration

Cyclophosphamide (CPA, Bristol Myers Squibb, Pincerton, NJ) was given intraperitoneally (i.p.) at a single dose of 200 mg/kg, initially without subsequent G-CSF stimulation (to determine kinetics of bone marrow cells post-chemotherapy). Next, three groups of mice received CPA followed by daily i.p. injections of recombinant human G-CSF (rhG-CSF, Amgen, Thousand Oaks, CA) at a dose 250 mcg/kg on the following schedules: treatment with rhG-CSF starting 24 hours after CPA administration (early G-CSF group), 48 hours after CPA administration (intermediate G-CSF group), and 72 hours after CPA administration (delayed G-CSF group). Mice that received CPA only served as controls (control group). Both CPA and G-CSF dosing was done according to the methods of Morrison et al.³.

Cell harvesting and cell counts

Bone marrow cells were obtained by flushing the femurs of mice with a 3-ml syringe and 27-gauge needle, into Hanks' Balanced Salt Solution (HBSS). After RBC hemolysis, nucleated cell counts were performed using a hemocytometer and Coulter Z-1 automatic counter. Spleens were strained through a 70 μ m nylon cell strainer and re-suspended in HBSS.

After dissociation into cell suspension and RBC lysis, nucleated cell counts per spleen were determined using Coulter automatic particle counter Z-1 (Beckman Coulter Corporation).

Blood was obtained by retro-orbital venous plexus sampling in Microtainer® tubes containing EDTA. Complete blood counts were determined using a Cell Dyne 3500 automated CBC analyzer (Abbott, Abbott Park, IL).

Monoclonal antibodies and flow cytometry

Expression of Gr-1 and CD34 on bone marrow nucleated cells was examined at steady state, 24, 48, 72, 96, 120, 144, and 216 hours after a single dose (200 mg/kg) of cyclophosphamide. FITC- and PE- labeled anti-mouse anti-CD34 antibody (RAM34, PharMingen) and TC- (PE-Cy5 Tandem) or FITC-labeled anti-mouse anti-Gr-1 antibody (RM3006, Caltag; RB65, PharMingen) were used. Isotype-matched rat FITC, PE, and TC labeled IgG were used as controls. Flow cytometry was performed on either a Coulter Epics or FACScan cytometer.

Colony-forming unit in culture (CFU-C) assay

CFU-C assay was performed, according to the methods of Levesque et al.⁷, with minor modifications. In short, peripheral blood, bone marrow, and spleen cells were harvested from mice at 144 hours post-CPA injection using standard techniques, and the number of nucleated cells in these tissues was quantified using a Coulter Z1 automatic cell counter. Peripheral blood (20 µL), nucleated spleen cells (1×10^5), or nucleated bone marrow cells (2.5×10^4) were plated in 2.5 mL methylcellulose media supplemented with a cocktail of recombinant cytokines (MethoCult 3434; Stem Cell Technologies, Vancouver, BC, Canada). Cultures were plated in duplicate and placed in a cell incubator. Colonies containing at least 50 cells were scored on day 7 of culture.

Statistical analysis

Statistical analyses were performed using unpaired two-tailed Student's t-test, and P values < 0.05 were considered as significant.

Results

In order to test the hypothesis that the majority of dividing myeloid progenitors are eliminated from the bone marrow early and will reappear later after chemotherapy, we exposed five cohorts of mice to a single dose of CPA, sacrificed each group at the different time points after chemotherapy, and analyzed Gr-1 and CD34 expression on bone marrow cells with flow cytometry. These experiments were performed first with Balb/c mice and then repeated using C57Bl/6 to confirm results.

Post-chemotherapy bone marrow cellular composition Bone marrow cellularity

Bone marrow cellularity decreased 8-fold by 48 hour after chemotherapy. Mean absolute nucleated cell counts per femur (n= 3 - 5) were as follows: 2.2×10^7 - untreated control, 5.25×10^6 at 24 hours, 2.75×10^6 at 48 hours, 4.25×10^6 at 72 hours, and 7.8×10^6 at 96 hours after chemotherapy. This demonstrates that after reaching the nadir at 24 hours, bone marrow starts to recover at 48 hours after CPA administration.

Light scatter characteristics of bone marrow cells

Post-chemotherapy bone marrow cellular composition undergoes dramatic changes as evidenced by the light scatter properties dynamics. Untreated bone marrow can be roughly gated into granulocyte and lymphocyte gates and the area of blastoid morphology cells (Figure 1). We observed decline in the cells within the blastoid morphology area and relative predominance of neutrophils 24 hours post-CPA, followed by decline in neutrophils and expansion of the cells within the blast gate starting at 72 hours after chemotherapy. This

indicates elimination of progenitors early and their reappearance and expansion later after chemotherapy.

Post-chemotherapy kinetics of Gr-1^{hi}, Gr-1^{lo} and Gr-1^{neg} cells

We examined kinetics of Gr-1 expression to determine changes in mature granulocytes and myeloid progenitors/precursors. The Gr-1 antigen is selectively expressed on myeloid lineage cells in mouse bone marrow⁵. Hestdal et al.⁶ sorted bone marrow cells into Gr-1-negative, -low, and -high populations and demonstrated that mature neutrophils are highly Gr-1 positive, whereas myeloid precursors including immature blasts and myelocytes have low to intermediate level of Gr-1 expression. Based on these data and the intensity of fluorescence with antiGr-1^{TC} or FITC we gated bone marrow cells into two distinct subpopulations: 1) Gr-1 negative cells (Gr-1^{neg}), and 2) antiGr-1^{TC} highly fluorescent cells (Gr-1^{hi}). We also selected cells between Gr-1^{neg} and Gr-1^{hi} regions into a gate with low to intermediate fluorescence intensity (Gr-1^{lo}). Fluorescence intensity of Gr-1^{lo} cells was 50-fold higher than that of Gr-1^{neg} cells, whereas the Gr-1^{hi} cells were 500 times brighter than Gr-1^{neg} cells (Figure 2).

As shown in Figures 2 and 3, flow cytometric analysis with anti Gr-1 mAb revealed that bone marrow from untreated mice contained roughly 30% Gr-1^{high}, 20% Gr-1^{low}, and 50% of Gr-1^{neg} cells. By 24-48 hours post-chemotherapy (CPA) the proportion of Gr-1^{high} mature neutrophils increased to roughly 50%, and the proportion of Gr-1^{low} immature myeloid cells and Gr-1^{neg} cells decreased to roughly 15% and 35% respectively. Between 48 and 72 hrs post-CPA the relative proportions of mature and immature myeloid cell populations reversed. By 72 hours post-CPA the proportion of Gr-1^{high} neutrophils had markedly declined in both BALB/c and C57Bl/6 mice, and by 96 hours post-CPA, they had almost completely disappeared from the bone marrows of BALB/c mice, and were detectable at only low levels in the bone marrows of C57Bl/6 mice. Both Gr-1^{neg/lo} cells started to rise after day +2 and soon represented the majority of the post-chemotherapy bone marrow cells.

This shows that immature myeloid cells mostly disappear from the bone marrow after a single dose of CPA and start to expand 48 hours after injection. We did not observe complete disappearance of mature neutrophils from the bone marrow in C57Bl/6 strain, as opposed to the BALB/c strain, most likely due to an earlier onset of myeloid recovery in C57Bl/6.

Kinetics of CD34-positive cells

Krause et al.⁷ demonstrated that CD34-positive murine bone marrow cells comprise small lymphocyte-like cells, large blasts and myelomonocytic precursors. Evidence now suggests that most murine CD34-positive cells represent committed early and intermediate progenitors^{7,8}. Therefore, we conducted flow cytometry studies using CD34 as a progenitor cell marker. As shown in Figure 4, we found that approximately 5% of nucleated bone marrow cells from untreated mice were CD34-positive. The blastoid morphology gate contained the highest percentage of CD34-positive cells (10%), in absolute terms, the myeloid gate contained most of the CD34-positive cells. At 24 hours after chemotherapy the percentage of CD34-positive cells went down to 2-3%, representing a 10-fold decrease in absolute numbers (Figures 3, 4). This initial decline was followed by significant and rapid expansion. At 96 hours post-chemotherapy approximately 60% of bone marrow cells were CD34-positive in BALB/c mice. In C57Bl/6 mice CD34+ cells peaked even earlier, at 72 hours post-CPA (Figure 3). Additional analysis demonstrated that these changes in the total bone marrow CD34-positive population were preceded by an even earlier increase in CD34+ cells present in the blast gate on day +2 post-chemotherapy. Thus, though post-chemotherapy bone marrow is still hypocellular at days +3-+5 post-CPA, CD34+ cells predominate and their absolute numbers are elevated above steady state baseline. The observed kinetics of CD34 expression was similar to the changes in

Gr-1low/neg populations, indicating that both populations represent expansion of immature cells in the recovering bone marrow.

CD34/Gr-1 double positive myeloid progenitors post-CPA

We determined the proportion of CD34/Gr-1 double positive cells, representing immature myeloid progenitors, at several time points post-CPA in C57Bl/6 mice. These cells comprised roughly 27% of all bone marrow cells at 72 hours, 16% at 96 hours, and 11% at 120 hours post-CPA. Flow cytometric analysis with anti Gr-1 and anti CD34 mAb revealed that approximately 70% of all CD34-positive bone marrow cells expressed low to intermediate levels of Gr-1-FITC fluorescence intensity. Thus, the majority of CD34-positive cells were also Gr-1-low positive, representing committed myeloid precursors.

Effect of different timing of rhG-CSF administration on blood counts, and progenitor cell mobilization

In light of our detection of an apparent 2-3 day delay in the post-chemotherapy increase in immature myeloid precursors in the mouse bone marrow we proceeded to test the effects on the kinetics of marrow cell recovery post-chemotherapy of one and two-day delayed initiation of rhG-CSF administration. The different treatment protocols are illustrated in Figure 5.

Kinetics of white blood cell count (WBC) and absolute neutrophil count (ANC) recovery

Animals from the control group that did not receive G-CSF were still significantly leukopenic and neutropenic at 120 hours post-chemotherapy (Figure 6). All mice from 24 hours, 48 hours, and 72 hours G-CSF groups recovered their WBC and ANC counts well above baseline by 120 hours post-chemotherapy (Figure 6). By 168 hours post-CPA there was no significant difference in WBC and ANC counts between these 3 groups (Figure 6, A, C).

G-CSF causes decrease in platelet counts

A single dose of CPA did not result in thrombocytopenia in control animals. All mice treated with G-CSF post-CPA demonstrated some degree of thrombocytopenia. Animals started on G-CSF early (24 hrs) post-chemotherapy had significantly lower platelet counts compared to 48 hours, and 72 hours G-CSF groups (Figure 6D).

Methylcellulose CFU-c assay

To determine mobilization of colony-forming unit cells (CFU-C) out of bone marrow, peripheral blood, spleen, and bone marrow cells were harvested on day +7 (168 hours) after CPA and plated into MethoCult 3434 media. We chose the +168 hours time point because peripheral counts peaked at this time. By day +7 the 24 hr G-CSF group received 7 doses, 48 hr G-CSF group received 6 doses, and 72 hr G-CSF group received 5 doses of G-CSF (Figure 5). Control group did not receive G-CSF. The baseline level of CFU-C in the spleens of untreated mice was < 10,000/spleen. We observed significantly higher numbers of the circulating and splenic CFU-Cs in the 24 hr G-CSF group. As expected, mice that received CPA only did not have the same degree of CFU-C redistribution and the majority of their CFU-Cs remained in the bone marrow (Figure 7). Thus, by 168 hours post-CPA, delayed G-CSF administration resulted in a similar blood count recovery, but significantly lower mobilization of CFU-Cs. This shows that the processes of neutrophil recovery acceleration and stem cell mobilization are mediated via G-CSF action on different bone marrow cell populations, since the early G-CSF doses applied to progenitor-poor conditions effectively contributed to the overall CFU-C mobilization.

Discussion

The early G-CSF administration after chemotherapy is somewhat arbitrary, and is not based on formal demonstration of the post-chemotherapy kinetics of the G-CSF target cells. Here, we sought to fill gap by investigating the post-chemotherapy bone marrow cellular composition changes in a mouse model. We found that G-CSF initiated at different times post-chemotherapy act on different bone marrow cell populations. We further found that delayed G-CSF administration result in similar levels of WBC and ANC recovery compared to early G-CSF, but a lower degree of thrombocytopenia. This suggests that early post-chemotherapy doses of G-CSF do not significantly contribute to the acceleration of neutrophil recovery.

To our knowledge, we demonstrated for the first time, that early after chemotherapy (up to 48 hours), the bone marrow is predominantly composed of mature residual granulocytes and very few progenitors and precursors, and that the point when mature granulocytes significantly decline and immature progenitors reappear occurs in murine bone marrow on days 3 and 4 after a single dose of cyclophosphamide. Since human hematopoiesis is roughly two times slower than murine hematopoiesis, the post-chemotherapy reappearance of immature progenitors may occur even later in a human bone marrow.

Although primitive HSCs do not have G-CSF receptors, committed myeloid progenitors do, and are able to respond to direct stimulation by G-CSF⁹. Thus our findings suggest that early after chemotherapy, there may be few if any cells in the bone marrow capable of direct proliferative response to G-CSF.

We also investigated recovery of ANC and WBC after different timing of G-CSF administration and found that fewer G-CSF doses are needed to achieve similar counts if G-CSF is started later after chemotherapy. In accordance with these findings, at least one prospective randomized clinical study in children with cancer demonstrated that delaying G-CSF until 5 days after completion of chemotherapy has not resulted in a longer duration of neutropenia or increased incidence of neutropenia-related problems¹, which indirectly indicates that the doses of G-CSF applied within 24-48 hours after chemotherapy do not significantly contribute to acceleration of granulocytic recovery. We found that platelet counts were significantly lower after early administration of G-CSF. G-CSF has been shown to negatively affect thrombocytopenia in patients receiving chemotherapy and to cause a decline in platelet counts in healthy donors^{10,11}. The mechanism of this G-CSF action is not clear. The question remains whether delayed administration of G-CSF after repeat courses of chemotherapy will lessen thrombocytopenia in cancer patients.

We investigated mobilization of CFU-C to peripheral circulation and spleens after delayed versus early G-CSF administration and demonstrated that significantly higher numbers of CFU-C are redistributed from the bone marrow when G-CSF is started earlier post-chemotherapy. Thus, although G-CSF doses given 24 hours post-CPA did not significantly change recovery of the blood counts, they effectively contributed to the mobilization of CFU-C. Given the neutrophil rich and progenitor poor conditions in the bone marrow early after chemotherapy that we observe here, this was not an unexpected finding, since the mobilization of stem cells induced by G-CSF is mediated mainly via cytokine release from activated neutrophils and probably does not require substantial numbers of myeloid progenitors¹².

There are some publications suggesting that our murine studies could be translated into human research. Stewart et al.¹³ studied the effect of 5-FU pretreatment on stem cell characteristics and renewal properties of human bone marrow. They demonstrated that the generation of CFU-C and enhancement of bone marrow recovery in humans were consistent with those obtained in the murine system. They assumed that chemotherapy eliminates mature, nonproliferating cells from the bone marrow. This was experimentally confirmed in our murine study. Riccardi

et al.¹⁴ studied the kinetics of human bone marrow myeloid precursors in order to integrate cancer chemotherapy with granulocyte-macrophage colony-stimulating factor. They found that at 8-11 days after chemotherapy there was an increase in proliferating bone marrow myeloid precursors. Given the fact that hematopoiesis in humans is about twice as slow compared to mice, these data support our finding that murine bone marrow myeloid precursors reappear and quickly expand at 3-4 days after chemotherapy. Ostby I et al.¹⁵ assessed the effect of administering of G-CSF on neutrophil engraftment after high-dose chemotherapy and autologous stem cell transplant using mathematical modeling. Their model showed no major differences in neutrophils engraftment time if the initiation of G-CSF was postponed for up to 5 days after transplantation. Dolgoplov I et al.¹⁶ reported a study of G-CSF-stimulated mobilization and harvest CD34+ cells in pediatric patients undergoing chemotherapy. They demonstrated that delayed administration of G-CSF targeted to the time of neutrophil reappearance in peripheral blood after chemotherapy was at least as effective as its “prefixed” start 24 hours after chemotherapy.

Given the high cost of rhG-CSF, rational use of this growth factor is important. Most of the clinical studies evaluating delayed G-CSF demonstrated that not only parameters of hematological recovery, but the numbers of infectious episodes and days spent in hospital were not significantly different from the cycles followed by early G-CSF^{1, 2, 17, 18, 19}.

In conclusion, the observed bone marrow cell kinetics and the results of delayed G-CSF administration in mice suggest that delaying the use of G-CSF by several days post-chemotherapy in humans might permit retention of the benefits of G-CSF treatment at a considerably reduced cost.

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Figure 1. Post-chemotherapy changes of light scatter characteristics of bone marrow cells
 Untreated bone marrow cells were gated into granulocyte (A), lymphocyte (B), and blast (C) gates. Note neutrophil predominance/decrease in the blastoid morphology cells at 24 hours, and expansion of blastoid morphology cells/ decline of neutrophils at 72 and 96 hours time points after CPA

Figure 2. Expression of Gr-1 on bone marrow cells post-CPA

Gates represent selection of Gr-1^{high}, Gr-1^{lo}, Gr-1^{neg} cells. Note the decline of Gr-1^{lo} and Gr-1^{neg} cells 24 hours after chemotherapy and the expansion of the same populations at 72 hours.

Figure 3. Kinetics of bone marrow Gr-1 and CD34 expression post-chemotherapy

Graphs represent: kinetics of Gr-1^{hi}, Gr-1^{lo} and Gr-1^{neg} cells in BALB/c mice (A) and C57Bl/6 mice (B); kinetics of CD34-positive cells in BALB/c mice (C) and C57Bl/6 mice (D). n = 5 to 8 for each group of mice. Bars represent SD.

Figure 4. Kinetics of the bone marrow CD34+ cells after chemotherapy in C57Bl/6 mice
 Note distinct emerging population of large CD34-positive cells at 48 hours post-CPA time point

Figure 5. Different protocols of rhG-CSF administration post-CPA

Figure 6. WBC (A), % of neutrophils (B), ANC (C), and PLT count (D) post-CPA administration $n = 5$ to 7 for each point of analysis. Bars represent SD.

Figure 7. CFU-C per spleen, per 1 mL of blood, and per femur at 168 hours post-CPA administration
 n = 3 for each group of mice. Shown data include duplicates. Error bars represent SD. Arrows represent baseline levels.