EDITORIAL

Does Stem Cell Exhaustion Result From Combining Hematopoietic Growth Factors With Chemotherapy? If So, How Do We Prevent It?

By Malcolm A.S. Moore

IN THE 6 years that data have been presented showing the efficacy of granulocyte-macrophage colony-stimulating factor (GM-CSF) and G-CSF in counteracting chemotherapy-induced myelosuppression, one of the most frequent questions has concerned the potential for exhausting stem cells by repeated growth factor stimulation, particularly in conjunction with repeated cycles of chemotherapy. This elicited a number of responses, which can be summarized as follows: (1) "G-CSF and GM-CSF do not act at the stem cell level, only on committed progenitors, and thus would not impart a continuous proliferative stimulus to the stem cell compartment." G-CSF or GM-CSF treatment in the absence of chemotherapy was considered unlikely to place undue strain on the pluripotent stem cell compartment because in vivo 3H Thd labeling studies in patients treated with G-CSF showed that the dramatic neutrophil expansion is produced by only 3.2 extra amplification divisions in the neutrophil developmental sequence. Similarly, after GM-CSF treatment, the rapid increase in granulocyte production is accounted for by the reserve capacity of the committed progenitor pool with shortening of the cell cycle time of colony-forming unit-GM (CFU-GM) and a doubling of the numbers of progenitors in S phase within 48 to 72 hours. However, CSF treatment does influence the stem cell compartment. After in vivo administration of CSFs in rodents, even lineage-restricted G-CSF produces a 5- to 10-fold absolute expansion of total body populations of erythroid, myeloid, and megakaryocytic progenitors and day 12 CFU-spleen (CFU-s), with most of this increase associated with mobilization of early cells into the circulation and expansion of the spleen as a major site of extramedullary hematopoiesis. Similar hematopoietic expansion occurs in primates and humans, but here the increase is associated with increased marrow cellularity. Thus, the expansion of the myeloid compartment seems to be matched by an expanded earlier pluripotential compartment, probably with an increased fraction of the stem cell pool entering the proliferative pool. This recruitment into cycle and expansion of more primitive precursors could be considered a response to the need to replenish the depleted progenitor compartment, ie, "pulling" early cells into an enhanced proliferative state. CSFs may also "push" early cells by acting in synergy with other cytokines, specifically interleukin-1 (IL-1), IL-6, or the c-kit ligand/stem cell factor to stimulate proliferation of primitive murine and human progenitors with functional (eg, CFU-blast, high proliferative potential-CFC,5,6 pre-CFU,6,7 long-term culture initiating cells9), or phenotypic (eg, murine Ly-6 A/E+, Thy1+, Lin-ve9 or human CD34+ Lin-ve)10,11 features of stem cells.

(2) "The stem cell reserve and the self-renewal potential of individual stem cell is so great that they cannot be exhausted except under extreme conditions such as serial transplantation at limiting dilutions in irradiated recipients." Much of the data on stem cell kinetics was obtained using the murine CFU-s assay. Serial passaging of individual spleen colonies in irradiated recipients showed that some colonies generated large numbers of secondary CFU-s whereas most generated few, and the probability of self-renewal versus differentiation was random at the single cell level. Calculations of self-renewal potential suggested that some CFU-s could undergo 50 to 75 population doublings. Based on total numbers of these cells in the marrow and the number of marrow cells required to reconstitute an irradiated recipient it was suggested that a single adult mouse could generate sufficient stem cells to fully engraft 106 recipient mice for their full life span. It now appears that most CFU-s are not the true stem cell and elutriation separation procedures can be used to isolate a marrow population that fails to form spleen colonies but can reconstitute irradiated recipients for a significant portion of their life span. On the other hand, a population of day 12 CFU-s defined as Ly 6 A/E+ve Lin-ve, Thy1+ve Rhodamine+ve can also repopulate in the long term with as few as 30 cells capable of resolving up to 50% of mice. Thus, we can conclude that long-term repopulating stem cell potential is a property of certain pre-CFU-s and a subpopulation of day 12 CFU-s.

Given the continuing debate as to the "quality" or "stemness" of cell populations defined by various properties, how best to measure, quantitatively and qualitatively, the stem cell compartment in mice and humans? Using both unfractionated and highly enriched populations of allelic, or retrovirally marked or sex mismatched marrow cells, various investigators demonstrated that large numbers of clones contribute to hematopoiesis early after transplantation with these more rapidly dividing stem cells having an early competitive repopulating advantage, but at the expense of longevity. Smaller numbers of long-term repopulating clones emerge later and have a life span at least as long as the life span of the mouse. Serial transplantation of marked cells in irradiated or genetically anemic (WW+) mice or competitive repopulation studies with mixtures of marked populations of donor cells provide the most reliable data on stem cell proliferative potential, but the assays are of necessity long term.

Shorter-term double-transplantation assays have been developed that measure stem cell decline. Hellman et al have measured the ratio of CFU-s produced in the bone marrow after 14 days to those originally injected into...
lethally irradiated mice. This ratio (Rₗ) correlates well with serial transplantability. Repeated proliferative demands on the stem cell pool provided by weekly irradiation of mice with shielding of one hind limb resulted in a permanent reduction in self-renewal capacity of shielded stem cells.²⁰ Similarly, the self-renewal capacity of stem cells in recipient mice transplanted with varying quantities of marrow demonstrated a narrow dose-dependent decrease in Rₗ. Repeated weekly cycles of alkylating agents such as busulfan and phenylalanine mustard permanently damaged stem cell proliferative capacity, but cytoxan at 80 to 100 mg/kg did not.²² Spangrude²³ has confirmed the validity of this double-transplant "pre-CFU-s" assay by showing that populations of murine Ly-6A/E positive, Rhodamine "dull" cells containing long-term repopulating stem cells generated the greatest number of secondary CFU-s relative to primary transplanted CFU-s whereas fractions depleted of long-term repopulating cells, ie, Ly-6A/E⁻neg or Rh-123hi generated very few secondary CFU-s.

The question of hematopoietic growth factor therapy combined with chemotherapy compromising stem cells introduces an additional complexity, namely the timing of initiation and cessation of CSF treatment relative to the administration of chemotherapy when repeated cycles of cycle-specific or proliferation-dependent chemotherapy are used. Sensitization of otherwise quiescent stem cells by activating proliferation places them at increased risk of massive depletion. Preclinical models in mice and monkeys demonstrated that continuous administration of CSFs, during or very shortly before or after chemotherapy, could be counterproductive. While this was probably caused by damage to progenitor pools that exhibited enhanced proliferation when exposed to CSFs, it is equally possible that stem cell damage could occur. Thus, it has proved necessary to build in certain "wash-out" periods before and after each chemotherapy cycle. Determining the duration of such "wash outs" has been less than an exact science in the design of cytokine clinical trials.

While kinetic studies addressed the rapidity with which progenitor cells increased their proliferation state after CSF treatment.¹² They also showed the rapidity with which progenitors become quiescent after cessation of cytokine treatment, briefly entering a proliferative state significantly less than in normal hematopoiesis. A precise understanding of the timing of this "rebound quiescence" and the duration of the window of opportunity presented for repeated chemotherapy should be a necessary component of any trial involving multi-cycle chemo-biotherapy. The mechanism of "rebound quiescence" has been the subject of long and acrimonious debate concerning the nature and specificity of negative regulatory influences; however, empirical observations clearly link an overshoot in production of mature cells with suppression of proliferation of marrow progenitors.

The entry of long-term repopulating cells into cycle also rapidly follows chemotherapy-induced depletion of more differentiated cells as shown in the study of Harrison and Lerner.¹⁹ They showed that in mice, after two doses of 5-fluorouracil (5-FU) separated by 3 to 5 days, the majority of these stem cells were destroyed whereas they were still resistant to a second dose after 1 day and resistance to 5-FU was restored by 8 days.

In the current issue of Blood, Hornung and Longo²⁴ report that treatment of mice with repeated cycles of high-dose cyclophosphamide (CTX) followed by restorative therapy with either G-CSF or GM-CSF led to a permanent impairment of stem cell function as measured by serial transplantation of allotype-marked marrow in lethally irradiated mice. The impairment did not compromise the hematopoietic restoration of the primary mice, but nevertheless the results raise troubling questions about the short-term advantages of CSF therapy in permitting chemotherapy dose and schedule intensification, if irreversible stem cell depletion were to occur in the clinical situation.

The animal model does not predict that the long-term consequence of the observed stem cell depletion is a catastrophic hematopoietic failure, but it does raise the possibility that patients treated intensively with repeated cycles of CTX and CSF may, in the long term, be less able to tolerate myelosuppressive insults. A number of strategies exist that may protect against stem cell damage. Hornung and Longo²⁴ demonstrated one approach that involved administration of a single dose of IL-1 24 hours before each cycle of CTX. This treatment on its own is not myeloprotective,²⁵ but it prevented the stem cell depletion seen with the subsequent CSF treatments.

The mechanism(s) by which IL-1 protects the stem cell compartment from both short-term CTX toxicity and long-term impairment in self-renewal capacity are potentially manifold.

(1) IL-1 pretreatment may activate stem cells into cycle from G₀ state and expand the stem cell pool, resulting in more stem cells surviving the chemotherapeutic insult. This "log-kill" argument requires that the timing of administration of IL-1 in the pretreatment phase is "fine tuned" because of the risk of sensitization of the proliferating stem cell to cytotoxic damage.

IL-1 administered alone or with G-CSF as single or multiple doses after CTX, 5-FU, or doxorubicin treatment enhances recovery of neutrophils and progenitor cells.⁵,²⁵,²⁸,³⁰ A single injection of IL-1 20 to 24 hours before CTX decreases mortality, but multiple daily administration for up to 5 to 7 days is more effective.²⁵,³⁰ With 5-FU treatment a single low dose of IL-1 24 hours before chemotherapy enhanced the accelerated neutrophil recovery obtained by subsequent post-chemotherapy treatments with IL-1 and G-CSF.⁷ One acute effect of IL-1 is to increase circulating CFU-GM and CFU-s by 30-fold and 10-fold, respectively, within 4 to 8 hours with an associated increase in circulating long-term repopulating cells.³¹ In Cynomolgus primates a single injection of IL-1 increased peripheral blood pre-CFU approximately 50-fold 1 day posttreatment, whereas CFU-GM did not begin to increase until 2 days and neither GM-CSF or IL-3 elicited increases in pre-CFU.³² These acute mobilizing actions of IL-1 will clearly alter the tissue distribution patterns of stem cells, possibly removing stem cells from environments where their proliferation is sup-
pressed to environments where rapid proliferation can be sustained (e.g., the spleen in rodents).

(2) IL-1 pretreatment initiates a cytokine cascade providing elevated levels of hematopoietic growth factors that promote accelerated regeneration of stem cells and progenitor cells surviving chemotherapy. IL-1 induces elevated serum levels of IL-6 and G-CSF, corticosterone, and tumor necrosis factor (TNF), and transcriptionally activates expression of GM-CSF in marrow stroma.

In vivo, in mice and humans IL-1 injection causes a rapid (2 to 6 hours) upregulation of type II IL-1 receptors on neutrophils and on bone marrow cells, including subpopulations enriched for marrow progenitors. The mechanism is indirect and appears to involve IL-1 interaction with type 1 IL-1 receptor (IL-1R) expressing accessory cells with rapid systemic elevation in corticosteroids and G-CSF, both individually and synergistically capable of directly upregulating IL-1 receptors on hematopoietic cells both in vivo and in vitro. The elevation in receptor expression, if it extends to stem cells, may enhance their sensitivity to endogenous or exogenous IL-1.

(3) Stem cell drug resistance could theoretically involve increased enzymatic inactivation, decreased drug influx, and/or increased drug efflux and increased DNA repair. The recent demonstration of a functional multidrug resistance P-glycoprotein expressed on early hematopoietic stem cells and progenitor cells raises the possibility of cytokine modulation of multi-drug resistant gene (MDR) expression on these cells with consequent changes in sensitivity to certain chemotherapeutic agents. Increased activity of CTX-detoxifying enzyme systems play an important role in resistance. Aldophosphamide, the penultimate cytotoxic metabolite of CTX, can be detoxified by an oxidative reaction catalyzed by certain cytosolic aldehyde dehydrogenase isozymes (ALDH) that are constitutively expressed by critical normal cells such as gut epithelium and CD34+ human stem and progenitor cells. Cytokine modulation of this detoxification system has been reported by Moreb et al., who showed that in vitro preincubation of human marrow with IL-1 or TNFα for 20 hours can protect early cells, detected by the CFU-blast assay, from 4-hydroperoxy-cyclophosphamide (4-HC) toxicity. Cycloheximide totally abolished this protection as did diethylaminobenzaldehyde, an inhibitor of ALDH. This enhanced transcription or stabilization of the ALDH mRNA in early hematopoietic cells may be directly induced by the cytokines or be secondary to alteration in the proliferative status of the cells. Other detoxifying systems may also protect from toxicity because early progenitors could be protected from inactivation by ALDH.

Glutathione and glutathione-dependent enzymes also confer protection particularly against the cardiac and skeletal muscle toxicities seen with very high doses of CTX; conversely, glutathione depletion significantly enhances such toxicities. An investigation of cytokine actions on cellular glutathione levels would be merited.

The ability of IL-1 or TNF to promote drug resistance would be of little practical value if the cytokines similarly protected neoplastic cells. The data so far suggest that they do not, certainly in the case of myeloid leukemic cell lines or in vivo in a murine primary breast tumor model and a renal tumor system. How can we determine if the human stem cell compartment has been damaged in patients that have received multiple chemotherapy cycles with G-CSF or GM-CSF rescue? Quantitation of the numbers of marrow cells with a stem cell phenotype, eg, CD34+ Thy1 Lin−, is unlikely to be informative because it does not define the proliferative potential of the cells. The long-term culture-initiating cell assay involving inoculating of test marrow onto irradiated allogeneic marrow stroma and measurement of progenitor output 5 to 8 weeks later is probably the best, most quantitative measure of human stem cell quality, but a more rapid and certainly easier pre-CFU, Delta, or secondary reconstituting assay may prove to provide comparable information. Information is critically needed on the proliferative status of human stem cells as a function of time after chemotherapy with or without hematopoietic growth factor stimulation. This could be obtained by 3H TdR suicide analysis of cell populations detected by the above in vitro assays. Furthermore, in vitro assays would allow measurement of the relative resistance of these early cells to in vitro exposure to 4-HC as a function of in vivo exposure to CSFs or IL-1. In the studies of Hornung and Longo, repeated CTX treatment was delayed for a full week after the last CSF treatment, during which time the peripheral neutrophil count would have recovered to normal, overshoot, and fell again. It is difficult to predict the proliferative kinetics of stem cells and progenitor cells in such a model apart from the likelihood that their proliferative status is reduced during the rebound phase. Some degree of synchrony of cell cycling and development of a temporary pattern of cyclic hematopoiesis would be anticipated. This further emphasizes the need to measure the cytokines of stem cells in both the experimental and clinical situations.

The use of IL-1 in conjunction with CSFs in clinical trials involving multiple cycles of CTX merits exploration. Initial clinical studies indicate that many of the potentially protective features of IL-1 action (acute hematopoietic cell mobilization, cytokine production, IL-R receptor upmodulation) and other less obvious effects (delayed elevation of platelet counts) were produced after a single injection of only 0.002 to 0.027 μg/kg of rhIL-1β. Dose-limiting toxicities involving hypotension were noted at higher doses (0.1 μg/kg) and the majority of patients experienced fever, rigors, and headache.

Hematopoietic protection strategies can also involve physiologic agents that mediate a reversible negative influence on hematopoietic cell proliferation. A single dose of TNF 24 hours before treatment with various chemotherapeutic agents partially protected mice from myelosuppression. Transforming growth factor (TGF-β) and macrophage inflammatory protein-1 (MIP-1) also had a selective
reversible inhibitory action on the cycling of early stem cells and precursors of later progenitors. The ability to "switch off" early hematopoiesis, particularly in protocols involving sequential administration of cytokines capable of inducing stem cell proliferation, would be of value in protecting the marrow from repeated cycles of high-dose chemotherapy. In addition, transforming growth factor may be protective to intestinal epithelium and oral mucosa.

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