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CYTOKINE RECEPTORS, GROWTH FACTORS AND CELL CYCLE IN HUMAN BONE MARROW AND PERIPHERAL BLOOD HEMATOPOIETIC PROGENITORS◊

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ABSTRACT

Background and Objective. An increasing number of growth factors have been shown to be responsible for the proliferation, survival and enhanced function of many cell types within the hemopoietic system. The action of these hemopoietic growth factors in stimulating cell growth and survival applies both to cells within the progenitor compartment and mature cells. Whether a specific cytokine influences *in vivo* hematopoietic progenitor cell proliferation or survival depends on cytokine-mediated modulation or target cell cytokine receptors, cell proliferation, and cell death regulator genes and other pathways.

To address these issues, particularly in view of the current and future clinical use of hemopoietic growth factors, the Italian Society of Experimental Hematology organized a Meeting in Florence on July 4th, 1996.

Information sources. The material examined in the present review includes full papers and abstracts published in journals covered by the Science Citation Index® and Medline®. All the participants to the Meeting in Florence have been actively working in the field of biology and clinical application of hemopoietic growth factors. Summaries of their oral presentations at the Florence Meeting are reported in the *Appendix* to this article.

State of Art and Perspectives. Myelopoietic growth factors particularly granulocyte (G-) colony-stimulating factor (CSF) and granulocyte-macrophage (GM)-CSF, have been available for clinical use for only a few years but they have already markedly changed the management of chemotherapy-induced neutropenia, the use of dose-intensive chemotherapy regimens and the practice and safety of autologous stem cell transplantation. While these growth factors have been rapidly introduced as routine agents in the management of cancer patients, they have continued to generate a considerable amount of fundamental research into the biology of hematopoiesis as well as the growth regulation of normal and cancer cells. For instance, one goal of cancer treatment is to protect hematopoietic stem and progenitor cells from the damaging effects of chemotherapy, while maintaining their anticancer action. Any means of preferentially and reversibly suppressing the proliferation of normal hematopoietic stem and progenitor cells while leaving the proliferation of tumor cells and their susceptibility to chemotherapy unmodified, could potentially optimize treatment efficacy.

In this field, the possibility of using colony-stimulating factors as myeloprotective agents in dose-intensive chemotherapy to enhance anticancer activity could be an attractive goal of current anticancer treatment modalities.

Key words: hematopoietic growth factors, G-CSF, GM-CSF, cell cycle, apoptosis

Among the many hematopoietic growth factors which are currently known,[1] two glycoproteins involved in the control of the processes above in myeloid and monocyte cell lines have been isolated, synthesized with the recombinant DNA technique and finally made available for clinical use in humans. These two glycoproteins are the granulocyte colony-stimulating factor (G-) and the granulocyte-macrophage colony-stimulating factor (GM-CSF).

The G-CSF and the GM-CSF were developed experimentally and in pilot clinical studies; now they are both widely used in oncology to reduce chemotherapy-related neutropenia.[2] They are used in different clinical conditions ranging from the combination with conventional chemotherapy, which is the most frequent clinical use, to dose-intensive chemotherapy protocols, used mostly in clinical research, to the combination with myelosuppressive chemotherapy and support with stem

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cells from marrow or peripheral blood.[3] In relation to their combination with conventional/dose-intensive chemotherapy, we use primary and secondary administration of G-CSF and GM-CSF and treatment with the two growth factors. In addition, myeloid growth factors are currently used in the stem cell transplantation setting: in recent years, several reports on this use have appeared in this journal.[4-15]

A rational use of G-CSF or GM-CSF in combination with antibiotics seems to be a means of treating established febrile neutropenia or infection. Currently, however, few data support the clinical usefulness of this approach and the cost-effectiveness of G-CSF or GM-CSF therapy remains therefore debated. Their clinical use is usually reserved for patients requiring a prolonged hospital stay because of known infections or hypotension, sepsis, or fungal infections.

The so-called *secondary administration* consists of giving myelopoietic growth factors at the beginning of a treatment cycle (after the first one) to the patients who had experienced febrile neutropenia during a former cycle. In theory, this is a rationale choice because it limits the administration of the factors to the patients apparently needing them and prevents their use in those who probably do not need them, however, no randomized studies have yet proven the efficacy of secondary administration. G-CSF and GM-CSF are also used for primary administration, that is, before neutropenia is observed, in conventional or dose-intensive chemotherapy. As a rule, this protocol is reported to be advantageous in highly myelotoxic treatments and/or clinical situations causing increased medullary fragility. In both cases, the appropriate use of both growth factors can decrease the medullary toxicity related to anticancer therapies and thus permit different treatment regimens to be used to their fullest potential.

To optimize the use of these molecules, their effects on the biology and function of target cells must be considered. In particular, accurate information on the degree and time dynamics of marrow stimulation by G-CSF and GM-CSF is important in improving marrow support and simultaneously preventing possible depletion of the stem cell progenitor which is potentially sensitized by myelopoietic growth factors relative to subsequent chemotherapy in multicycle protocols.

Biological activity of myelopoietic growth factors

All the cells circulating in the blood of adults and children originate from a limited number of pluripotent stem cells, the only cells which can self-reproduce and have the potential for growing and differentiating into granulocyte, monocyte, erythroid, megakaryocyte and lymphoid cells.[1,11] Some stem cells divide and produce a progeny that loses its differentiation potentials and developes into a specific hematopoietic line. These *committed* progenitor cells differentiating into a single line continue to proliferate and differentiate into morphologically identifiable precursors which mature, further acquiring highly-specialized functions and losing their proliferative capabilities. The basic feature of stem cells is that they restore normal hemopoiesis in the recipient animal. Identifying and characterizing stem cells are the main goals of oncologic and hematologic research.

In 1961, stem cells were identified as the cell generating colonies in the spleen of irradiated mice (CFU-s). Later on, it was found that CFU-s include a heterogeneous population of hematopoietic precursors which include high proliferative potential colony-forming cells (HPP-CSF) and cells initiating long-term cultures (LTC-IC), as well as CFU-GEMM. Hemopoietic progenitors express the CD34 antigen.[4] More than 20 types of antibodies recognize different epitopes of the same CD34 antigen and most of them are used only in research. Clinically, the most commonly used antibodies are those derived from QBEND-10 and HPCA-2 clones.

Hematopoietic growth factors are cytokines that play a fundamental role in regulating hematopoietic cell differentiation and proliferation. Their action begins in the most immature progenitors where hematopoietic growth factors favor self-renewing and probably initiate differentiation, and continue in mature circulating and tissutal cells, regulating their functional activation. Moreover, apoptosis is inhibited in their presence.

Myelopoietic growth factors bind to cell receptors, and every cell has a specific receptor molecule or a subunit binding the growth factor. Recent studies have shown that there are two different receptor series, namely:

- tyrosine-kinase receptors (M-CSF, SCF),
- non-tyrosine-kinase receptors (G-CF, GM-CSF, IL-3, EPO).

The latter are heterodimers made of a β and α chains. The α chain binds to the low-affinity growth factor (the ligand) but, after this binding, it associates with the β chain into the high-affinity receptor. Every receptor has a specific chain, while it may share the chain with other receptors, as it happens for GM-CSF and IL3. Recent tecnological advances allowed quantitative expression of receptors using cytofluorometric analysis.[16-18]

We used these techniques to assess GM-CSF expression on different hemopoietic cells both *in vitro* and after *in vivo* treatment with HGF 13,6. The number of GM-CSFr binding sites on human neutrophils was assessed by using different bead standards. Results were compared with those from conventional receptor quantification, which was performed by using the radioligand binding assay. A

high degreee of correlation was found between the two methods, although a quantitative evaluation of GM-CSFr expression on neutrophils performed by flow cytometry (1653±333) revealed a somewhat higher number of receptor molecules per cell than that determined by Scatchard analysis (857±150). By this flow quantitative approach, we measured the number of GM-CSFr receptors on G-CSF mobilized CD34-positive cells, and the presence of a low (about 200) but significant number of receptors was demonstrated.[16, 19]

Cell line heterogeneity relative to the differentiation stage can explain the high number of identified growth factors. Another likely cause of the existence of several growth factors with similar functions is a possible cytokine hierarchy, where some cytokines regulate normal daily production and other weaker cytokines intervene only in emergencies. Alternatively, the various factors in a particular cell line may act sequentially, some in the early stages and others in the late stages of hemopoiesis.

Myelomonocytic growth factors act in different maturation stages of the hematopoietic system and play different roles. For instance, *in vivo* G-CSF administration shortens the S stage and markedly accelerates the cell cycle in myeloblasts and promyelocytes;[20] moreover, G-CSF and GM-CSF can mobilize many immature progenitors from bone marrow to peripheral blood.[2] These cells, which can restore the hematopoietic system in myeloablated subjects, are normally found in peripheral blood at very low concentrations and increase markedly after G-CSF or GM-CSF administration. Therefore, they can be used for treatment in autologous transplantation.

Clinical studies on the cytokinetic effect of hematopoietic growth factors

The *in vivo* effects of G-CSF, GM-CSF and IL-3 on the cell cycle of hematopoietic progenitors have been recently studied in some clinical trials in advanced cancer patients with preserved hemopoiesis. GM-CSF induces an increase in proliferation rate in committed progenitors, as shown by a significant increase in the number of granulocyte-macrophage colony-forming units (CFU-GM) in S phase.[20] GM-CSF also induces an increase in the percentage of morphologically recognizable marrow precursors in S phase (from 26% before GM-CSF to 41% after 3 days of treatment). The proliferative stimulus has been quantified more precisely with a double-labelling technique, by first infusing the patients with bromodeoxyuridine, then removing a sample of bone marrow cells and incubating them with 3H-thimidine. It was shown that GM-CSF treatment nearly tripled the marrow production of granulomonopoietic cells. Finally, GM-CSF significantly increases the half-life of circulating neutrophils, whereas it does not modify that of eosinophils and decreases that of monocytes. If this information is considered together with other studies[21-25] some conclusions can be drawn regarding why granulomonocytosis follows GM-CSF treatment. First of all, the monocytosis is sustained by a marked increase in marrow production of monocytes that is able to overcome a decreased half-life of circulating cells. Secondly, the eosinophilia is essentially caused by increased marrow production of eosinophils, whereas the neutrophilia depends on both an increased production and a prolonged half-life.

Although granulomonocytosis is the most evident effect of GM-CSF administration, other cells of the myeloid lineage also respond to GM-CSF treatment. GM-CSF treatment more than doubles the percentage of erythroid progenitors in S phase. Its action, however, does not extend beyond these early progenitor cells because the proliferative activity of erythroblasts does not change during GM-CSF treatment. This is probably caused by the loss of specific receptors during differentiation. Because this receptor loss is concomitant with the appearance of erythropoietin receptors, it is likely that effective stimulation of erythropoiesis could be accomplished with the sequential administration of GM-CSF and erythropoietin.[21,22]

Three observations suggest that GM-CSF plays a role in megakaryocytopoiesis. First of all, there is a highly significant increase in the proliferative activity of megakaryocyte progenitors during GM-CSF administration. Secondly, GM-CSF modifies the maturation profile of megakaryocytes, inducing a relative increase in the more immature forms. Thirdly, incubation with labelled GM-CSF and autoradiographic analysis shows the presence of specific GM-CSF receptors on megakaryocytes.[26]

Upon withdrawal of GM-CSF, the number of circulating leukocytes rapidly falls to the pretreatment level in patients with normal hemopoiesis. Examination of the marrow cell kinetics shows that the proliferative activity of the target cells quickly decreases to a level significantly lower than that observed before the administration of GM-CSF. The possible reasons underlying this phenomenon have been discussed.[21,22] Whatever the biological reason for this phenomenon, it is possible that the 48-hour to 96-hour phase after GM-CSF is discontinued represents a period of partial refractoriness of marrow cells to the action of cell-cycle-specific cytostatic drugs. This observation suggests that a short period of treatment with GM-CSF before chemotherapy might decrease the hemopoietic toxicity of chemotherapy.

To address this issue, two controlled trials in breast cancer and Hodgkin's disease patients have been performed.[25] Moreover, an additional study showed that, thanks to its quick action, a short

treatment with GM-CSF is effective after standard chemotherapy.[27]

Further insight into the action mechanism of GM-CSF and G-CSF comes from studies investigating the cytokinetic changes in $CD34^+$ medullary hematopoietic progenitors in 36 patients with breast carcinoma. The study investigated the increase and decrease in the proliferation of bone marrow CD34 hematopoietic progenitors in a protocol with 5-FU, epirubicin and cyclophosphamide, and mildly intensified FEC. This was effected by increasing the epirubicin dose to 100 mg/m^2 and the cyclophosphamide dose to 750 mg/m^2 scheduled every 22nd day for 6 cycles and supported by prophylactic (from the first cycle) and early (days 3-10) administration of GM-CSF or G-CSF.[28]

Cytokinetics were studied consecutively in this clinical trial, before treatment, after cycle 1 and at the end of cycle 4. Both the FEC + GM-CSF and the FEC + G-CSF combinations were confirmed as being able to significantly increase the myeloid burden and the proliferation of CD34 marrow cells. The latter parameter, however, returned to baseline values more quickly after GM-CSF than after G-CSF. Particularly, marrow response after completion of the treatment with the two growth factors was markedly different: while the rate of morphologically identifiable myeloid progenitors remained high in both cases, CD34 proliferation was strong at the 2nd, 4th, and part of the 8th day after G-CSF, but dropped significantly below baseline values after GM-CSF. At the beginning of cycle 4, both the myeloid burden and CD34 proliferation were the same as in cycle 1 in the patients receiving FEC + GM-CSF, while both parameters were lower than in cycle 1 in the patients receiving FEC + G-CSF.

We tried to correlate the biological data with some clinical results. Third degree alopecia was nearly always present; nausea and vomiting were frequent, but comparable in the two groups; mucositis was also frequent, but was severe only in two patients, with no significant intergroup differences. Two patients in both groups had reduced (by 10%) ventricular ejection fraction, but cardiac congestion was never significant. Asthenia, fever and first and second degree muscular pain were observed in 12 GM-CSF patients during the first three treatment cycles, while G-CSF patients exhibited only musculoskeletal pain. The mean neutrophil nadir in the FEC + G-CSF group was 2.48×10^9/L (range: .065-10.12) in 114 cycles vs 2.51×10^9/L (range: .061-11.9) in 120 FEC + GM-CSF cycles. Mean neutrophil nadir in the FEC + G-CSF group was lower after cycle 30 ($.87\times10^9$/L) than after cycle 1 (2.67×10^9/L) and no clinically significant anemia was found during the six treatment cycles. Thrombocytopenia occurred similarly in both groups (26% in FEC + G-CSF versus 19% in FEC + GM-CSF), but never exceeded the first degree; no bleeding related to thrombocytopenia itself was ever 44 observed.

Due to its action on a wider range of progenitors, IL-3 was considered a cytokine with interesting perspectives.[29] In a phase I/II trial, the complex effect of IL-3 on cell kinetics was investigated. IL-3 treatment affects BM proliferation by increasing the percentage of BM progenitors engaged in the S-phase of the cell cycle.[30] The effect is dose-dependent with the various progenitors showing different degrees of sensibility. The most sensitive progenitors are CFU-MK, BFU-E and finally CFU-GM, whose proliferative activity is stimulated at higher doses of IL-3 only. A slight increase in the proliferative activity of myeloblasts, promyelocytes and myelocytes is observed, whereas the activity of erythroblasts is unchanged. IL-3 has a priming effect: BM progenitors purified from patients treated with IL-3 produce more colonies *in vitro* in the presence of G-CSF, IL-5, GM-CSF. These data indicate that *in vivo* IL-3 acts essentially as a primer of the action of other cytokines.[31]

This hypothesis was pursued in another clinical trial including 18 patients with solid tumors that showed that proliferation suppression in $CD34^+$ hematopoietic progenitors after such growth factors as GM-CSF was increased in the patients pretreated with IL-3. This trial scheduled 6 cycles of a dose-intensive protocol with cyclophosphamide (1,100 mg/m^2), epirubicin (100 mg/m^2) and etoposide (200 mg/m^2) administered on day 1 and supported by IL3 on days +2 to +6 and with GM-CSF on days +7 to +12 and repeated on day 14.

In this trial, the effect of sequential IL3 and GM-CSF caused a sort of marked rebound on the proliferation of hematopoietic progenitors, which is suppressed since IL3 is discontinued but GM-CSF is still administered. This cytokinetic resistance in hyperplastic marrow lasts until day +14 and permits dose-intensive chemotherapy cycle as scheduled. From a clinical viewpoint, even though these trials were not randomized, the lack of cumulative toxicity on white blood cells and platelets shown in the following cycles of both trials can be hypothesized as being at least partially attributed to the effectiveness of cytokinetic protection relative to stem compartment exhaustion. This effect was particularly apparent after GM-CSF, and especially after sequential IL3 + GM-CSF administration, when the low proliferation window of CD34 cells was particularly apparent and lasted several days before the next chemotherapy cycle. Therefore, it does not seem advisable to begin chemotherapy immediately after G-CSF is discontinued, because this cytokine continues to stimulate the proliferation of CD34 cells for many days after it is discontinued.

Conclusion and future prospects

One of the main goals in oncology is to test new chemotherapy protocols very often intensifying the doses of the single drugs and attempting to increase their anticancer action. Another major goal is to protect stem cells and medullary hematopoietic progenitors from the action of chemotherapy drugs. In theory, this can be done by increasing the cytostatic toxicity that acts on the cell cycle of tumor cells, considering the cytokinetic action mechanisms of the drugs.

In fact, we should try to keep stem cells and hematopoietic progenitors kinetically quiescent, and thus protected from cycle-specific drugs during various chemotherapy schedules. The appropriate use of the growth factors currently available might achieve a lasting decrease in the medullary toxicity, due to their action on both the cell cycle and the induction/protection of apoptosis on $CD34^+$ cells[32,33] related to anticancer treatments.[34] Lastly, a correct use of cytokines might be helpful in *ex vivo* expansion of progenitors: this goal now seems obtainable.[35]

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Appendix

Papers presented at a meeting of the Italian Society of Experimental Hematology held in Florence, on July 4, 1996.

Control of the proliferation of primitive hematopoietic progenitors by positive regulators of growth

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Functionally mature circulating hematopoietic cells derive from a relatively small number of primitive and pluripotent hematopoietic cells, located mostly in the bone marrow (BM). The proliferation, the differentiation and the survival of primitive hematopoietic cells seem to be strictly dependent on cytokines having stimulatory or inhibitory activity.

In the human hematopoietic system the long-term culture-initiating cells (LTC-IC) represent the mùost primitive cell type that can be recognized and quantitated *in vitro* in virtue of their ability to give rise to clonogenic cells (CFC) for at least 5 wks when co-cultured on certain fibroblast-containing feeder layers. We validated a 16-hr tritiated thymidine (3H-Tdr) suicide assay to quantitate the proportion of LTC-IC and CFC in active DNA synthesis, and therefore to directly evaluate the cycling status of LTC-IC. By using this method, we have previously shown that circulating LTC-IC (like circulating CFC) are a population of quiescent cells. In contrast, LTC-IC in normal BM are a mixture of quiescent and cycling cells. Nevertheless, initially quiescent LTC-IC from both sources can be recruited into cycle within 72 hrs by a combination of Steel factor (SF), IL-3 and G-CSF.

To better understand the role of single growth factors on the proliferation of LTC-IC, we incubate $CD34^+/38^-$ BM cells for 8 d in serum-free containing either SF, Flt-3 ligand (FL), IL-3, the combination of SF+IL-3+FL+G-CSF+IL-6+NGF (as positive control) or no exogeneously added growth factors (as negative control). The number of LTC-IC within $CD34^+/38^-$ cells was maintained at input level only by the 6 cytokine combination and by FL, as single growth factor. In contrast, the recovery of LTC-IC within unpurified BM cells was complete even in the absence of any exogenously-added cytokine. and this was probably due to the presence of cytokines produced by accessory cells. These findings suggest that the survival of very primitive hematopoietic cells is strictly dependent on the presence of cytokines, and among single GFs, FL is the most potent survival factor for LTC-IC. A high proportion (> 80%) of LTC-IC was initially cycling both within the $CD34^+/38^-$ and the unseparated fraction. These initially quiescent LTC-IC were recruited into cycle only by the 6 GF combination, but not by any single cytokine, suggesting that primitive hematopoietic cells require a combination of stimuli to proliferate. Then, we evaluated the response of circulating hematopoietic progenitors to either SF, FL, IL-3 or the combination of SF+IL-3+G-CSF, within a 72-hr culture period. IL-3, as single GF, induced the proliferation of CFC and the proliferation and differentiation into CFC of LTC-IC; in contrast, SF and FL as single GFs, stimulated the survival of LTC-IC also.

All together these findings suggest that the survival and proliferaton of primitive hematopoietic cells is strictly dependent on growth factors, and that it is possible to differentiate LTC-IC and CFC on the basis of their response to cytokines. In fact, LTC-IC and CFC share only some pathways of activation, as circulating LTC-IC (but not circulating CFC) are responsive to SF and FL. Moreover, LTC-IC present in the circulation might be a subtype of BM LTC-IC, as they seem to respond differently to the cytokines tested.

Flow cytometric detection and quantification of the human GM-CSF receptor

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Granulocyte-macrophage colony-stimulating factor (GM-CSF) is a multilineage hemopoietic factor that stimulates the proliferation and differentiation of myeloid progenitor cells and regulates the functional activities of mature granulocyte and monocyte *in vitro* and *in vivo*. The human receptor for GM-CSF (GN-CSFr) consisting of an α and a β chain, is widely distributed on normal and neoplastic tissues. Until now, the most commonly used method for receptor quantification has been the radio-ligand binding assay. This method, however, requires relatively large quantities of cells not always easily obtainable and is not able to determine the receptor expression by different cell populations such as those recovered from peripheral blood (PB) or bone marrow specimens. Using a monoclonal antibody directed against the GM-CSFr α chain and a flow cytometric calibrations system (QSC sigma) that allows the measurement of antigen binding capacity, the number of GM-CSFr binding sites on human neutrophils was assessed and results were compared with those obtained using the conventional radiobinding assay. On 15 normal PB studied, we obtained a mean of 1653±333 SD receptors/cell (r/c) by flow cytometry and a mean of 857±150 SD r/c using the radiobinding assay. A high degree of correlation (r=0.91, p=0.004) was found between the two methods, although quantitative evaluation of GM-CSFr performed by flow cytometry revealed a somewhat higher number of r/c compared with that determined by Satchard analysis. By the flow quantitative approach we also measured the number of GM-CSF on mobilized $CD34^+$ cells (201±140 SD r/c) obtaining results in agreement with previously published data obtained using the radioassay.

Our data suggest that flow cytometric analysis is a simple and reproducible method in detecting and quantitating the presence of GM-CSFr per cell, thus allowing the study of receptor expression on different and also poorly-represented cell populations selected on the basis of their scatter parameters and surface markers.

Quantitative analysis by flow cytometry of cytokine receptors in normal progenitors and acute leukemia cells

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In the last few years, the molecular cloning and purification of several hemopoietic growth factors (GF) and cytokines such as the granulocyte colony stimulating factor (G-CSF), granulocyte-macrophage colony stimulating factor (GM-CSF), stem cell factor (SCF), erythropoietin (EPO), thrombopoietin (TPO), interleukin (IL) 2, IL3, IL4, IL5, IL6 and IL12 (among others) has made the use (at least for some of these GF) possible in the clinical setting. Apart from EPO, G-CSF and GM-CSF are the most widely used GF for the treatment of hematological malignancies. The main clinical use of G- and GM-CSF is to stimulate recovery of neutropenia following myeloablative chemotherapy, radiotherapy and/or bone marrow transplantation. Although the administration of G- and GM-CSF in patients affected by neoplastic proliferation of lymphoid origin (i.e. malignant lymphomas; multiple myeloma, Hodgkin's disease, acute lymphoblastic leukemias) is widely accepted, their clinical usefulness and safety in the management of acute myeloid leukemia (AML) and myelodysplastic syndrome (MDS) is still controversial. Based on experimental studies it has been shown that the biologic activities of GF on hemopoietic cells depends upon their binding to specific receptors, that are expressed on both normal and leukemic cells.

In this study, we analyzed by flow cytometry the expression of various cytokine receptors-CR (GM-CSF/R: Cw116; *c-kit*/R:CD117; IL3/R, G-CSF/R and FLT3) in patients with AML, ALL, MDS and healthy volunteers using a wide panel of monoclonal antibodies (MoAbs) directed against different forms of CR. Flow cytometry data were expressed either in the form of molecular equivalents of soluble fluorochrome (MESF) or of antibody binding capacities (ABC) per cell, by using quantitative microbead calibration standards of different sources and preparation (Quantum; QIFIKIT). In healthy subjects, GM-CSF/R was detectable on monocytes, neutrophils, myeloid precursors and a subset of $CD34^+$ progenitors which are committed to the myelo-monocytic pathway. Among AML samples, GM-CSF/R MoAb were found to be positive in 80% of the cases (range of ABC: 1,000-42,000). The highest ABC values were within the M4 and M5 FAB subvarieties, while ALL samples were negative except one case. The number of GM-CSF/R expressed by AML cells was significantly higher that their normal counterparts in 20% of the cases examined, allowing the possibility of using this marker for the monitoring of the minimal residual disease (MRD). Receptors for *c-kit* were found on 90% of AML, showing the highest ABC values in M1 and M2 FAB subtypes. All the patients with B-ALL resulted *c-kit*/R negative. 30% of AML cases showed a significantly higher number of *c-kit*/R than that of normal hemopoietic cells, suggesting the possibility of using this receptor expression for the detection of MRD. The highest expression for IL3/R was detectable in AML M5; however, most AML and ALL cases were IL3/R positive. FLT3 MoAbs were detected in 90% of AML cases, but the degree of positivity was rather low in most of the samples examined. Data derived from the cytofluorimetric analysis of G-CSF/R were doubtful due to the low expression levels detected

on both leukemic and normal progenitors.

In conclusion, the combined use of flow cytometry and calibration microbeads allows a precise and reliable quantitation of ABC values, permitting comparability of the data over time and between one laboratory and another. The determination of CR also offers the possibility of selecting patients suitable for growth factor therapy, reducing the risk of stimulating the proliferation of the leukemic clone. A dynamic evaluation of the density and affinity status of CR in normal and leukemic cells following the cell's exposure to GF could be of help for optimizing their use in acute leukemia.

Peripheral blood mobilized CD34⁺ cells express more amphotropic retrovirus receptor than bone marrow

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We have reported that hematopoietic progenitors mobilized into peripheral blood (PB) by anticancer chemotherapy and cytokine(s) are better targets for gene transfer by Moloney-based retroviral vectors than their bone marrow (BM) counterpars. This increased susceptibility may depend, among other factors, on their level of expression of the amphotropic receptor on the progenitor cell. We indirectly addressed this issue utilizing the 83A25 rat monoclonal antibody specific for the gp70 glycoprotein of the amphotropic viral envelope (*J Virol 64: 6176, 1990*).

We compared the binding of PA317-N2 vector to CD34⁺ cells derived from: 1) steady state BM; 2) steady state PB; 3) mobilized PB from cancer patients treated with high-dose cyclophosphamide + G- or GM-CSF. CD34⁺ cells were selected to a purity of ≥ 90% by a high-gradient magnetic cell separation system, cultured with SCF/IL3/IL6 for 48 hrs, incubated with N2 supernatant for 2 hrs and stained with 83A25 antibody. Antibody-virus-cell complexes were indirectly labelled and analyzed on a FACScan cytometer. We observed that the fluorescence intensity of mobilized CD34⁺ cells was one log higher than that of steady state BM or PB CD34⁺ cells, indicating that the expression of the amphotropic receptor is increased and may contribute to their high susceptibility to retroviral infection. Experiments aimed at evaluating the level of amphotropic receptor expression in steady state BM and mobilized PB CD34⁺ cells are in progress.

Cell cycle analysis of peripheral blood CD34⁺ cells mobilized by G-CSF in healthy donors

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Recent studies provide few data about the noncycling state of hemopoietic stem cells mobilized by growth factors in healthy adults. Our group studied the mobilization kinetics of hemopoietic progenitors in 20 normal donors stimulated with rhG-FSF (16 μg/kg/d × 5-7 days) and we attempted to establish the proliferative state of CD34⁺ stem cells in peripheral blood. Two to four leukaphereses were performed on each donor. The products were depleted of T-cells by E-rosetting with sheep red blood cells and density gradient separation, followed by positive immunological selection for CD34⁺ cells (CEPRATE system). CD34⁺ stem cells and S+G_2-M phase cells were assessed during G-CSF treatment by flow cytometry using anti HPCA-2 FITC and 50 μg propidium iodide respectively, on both daily peripheral blood samples, and on apheresis products in different manipulation phases. In peripheral blood samples the CD34⁺ cells behaved like the proliferating cells, reaching the maximum on the 5th day of G-CSF administration. Furthermore, we have got clear evidence that in peripheral blood the number of S+G_2-M phase cells/μL was greater than the number of CD34⁺ cells/μL during the whole G-CSF treatment (mean values on the 5th day: 874 S+G_2/M cells/<μL and 129 CD34⁺ cells/μL). The unfractionated apheresis products displayed the same kinetics trend as peripheral blood samples: proliferating cells and CD34⁺ stem cells reached the peak values on the 5th G-CSF stimulation day with mean values of 2.1×10^9 and 326.6×10^6, respectively. Cell cycle analysis on E-rosetting mononuclear negative cell fraction and CD34⁺ cell fraction showed that S+G_2-M phase cells were almost all in the CD34-negative fraction, whereas only CD34⁺ cells were in cycling status.

We conclude that G-CSF induces not only CD34 stem cell release but also mobilizes a large number of proliferating CD34-negative cells. Confirming other reports (*Roberts and Metcalf, Blood 1995; 86:1600; Letner et al., Br J Haematol 1996; 92:255*), we found growth factor mobilized CD34⁺ stem cells showing a low proliferative profile.

Proliferative response of human marrow myeloid progenitor cells to *in vivo* treatment with granulocyte colony-stimulating factor (G-CSF) and G-CSF in combination with interleukin-3 (IL-3): biological and clinical implications

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We have recently reported that the hematological recovery of non-Hodgkin's lymphoma (NHL) and Hodgkin's disease (HD) patients undergoing ABMT is significantly faster when recombinant human (rh) IL-3 is combined with rhG-CSF in comparison with patients receiving G-CSF alone.

We report here the kinetic response and concentration of bone marrow (BM) progenitor cells of 17 patients with lymphoid malignancies submitted to ABMT treated with G-CSF/IL-3 combination. The results were compared with those of 5 lymphoma patients receiving the same pretransplant conditioning regimen followed by G-CSF alone. RhG-CSF was administered as a single subcutaneous (sc) injection at a dose of 5 μg/kg/day from day +1 after reinfusion of autologous stem cells while rhIL-3 was added from day +6 at the dose of 10 μg/Kg/day sc (overlapping schedule). In both groups (i.e. G-CSF and G-CSF/IL-3-treated patients) cytokine administration was discontinued when the absolute neutrophil count (ANC) was 0.5×10^9/L of peripheral blood (PB) for 3 consecutive days. Following treatment with colony-stimulating factor (CSF) combination, the percentage of marrow granulocyte-macrophage colony forming unit (CFU-GM) and erythroid progenitors (BFU-E) in S-phase of cell cycle increased from 9.3±2% to 33.3±12% and from 14.6±3% to 35±6%, respectively ($p < .05$). Similarly, we observed an increased number of actively cycling megakaryocyte progenitors (CFU-MK and BFU-MK). Conversely, G-CSF augmented the proliferative rate of CFU-GM (22.6±6% compared to a baseline value of 11.5±3%; $p<.05$) but not of BFU-E, CFU-MK or BFU-MK, and the increase of S-phase CFU-GM was significantly lower than that observed in the postreatment samples of patients receiving IL-3 in addition to G-CSF. The frequency of hematopoietic precursors in the BM, expressed as the number of colonies formed per number of cells plated, was unchanged or slightly decreased in both groups of patients. However, due to the increase in marrow cellularity, a significant augmentation of the absolute number of both CFU-GM (3605±712/mL of BM vs 2213±580/mL; $p <.05$) and BFU-E (4373±608/mL vs 3027±516/mL; $p < .05$) was reported after treatment with G-CSF/IL-3 but not G-CSF alone. Similarly, administration of the cytokine combination resulted in a higher number of CD34⁺ cells/mL of BM and their concentration was significantly greater that that observed in the post-treatment samples of G-CSF patients. Finally, we investigated the responsiveness to CSFs, *in vitro*, of highly enriched CD34⁺ cells, collected after priming with G-CSF *in vivo* (i.e. after 5 days of G-CSF administration).

Our results demonstrated that pretreatment with G-CSF modified the response of BM cells to subsequent stimulation with additional CSF's. In conclusion, the results presented here indicate that *in vivo* administration of 2 cytokines increases the proliferative rate and concentration of BM progenitor cells to a greater degree than G-CSF alone and supports the role of growth factor combinations for accelerating hematopoietic recovery after high dose chemotherapy.

Cell cycle characteristics of bone marrow and blood-derived human hematopoietic progenitor cells

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The optimal use of different colony-stimulating factors (CSF) is still an area of intense clinical investigation. The best way to schedule and to combine the administration of these cytokines with chemotherapy (CT) is of critical importance for their most efficacious use both in the dose-intensive chemotherapy and in the bone marrow (BM) transplantation setting. For instance, it is important to consider that BM hematopoietic precursors are recruited into proliferative activity when CSF are sequenced with CT. This has underlined the problem that when CSF are used during multiple cycles of CT, progenitor cells may be sensitized to cycle-active agents if they are rapidly proliferating at the start of the next CT course. To go deeper into this problem, we have conducted different clinical studies of dose-intensification with CSF support in cancer patients.

In one of these studies, we have exploited the cell kinetic changes of the bone marrow $CD34^+$ cell subset after CT followed by the IL-3 + GM-CSF together with the clinical effects of this association. Twenty-four patients with advanced cancers and normal hematopoiesis were treated with an intensified CT course (mg/sqm: CTX 1300, Epirubicin 100, VP-16 200; iv day 1). Six cycles were planned at 14-day intervals with the support of IL-3 (5 µg/kg/day; from day 2 to 6) sequenced with GM-CSF (same dose; from day 7 to 11). DNA content and bromodeoxyuridine incorporation were evaluated using flow cytometry on immunomagnetically-sorted bone marrow $CD34^+$ cells, at baseline and at different times (day 5, 6,7, 8,11 and 14) after CT followed by IL-3+GM-CSF.

Treatment with IL-3 induced a marked increase in the percentage of myeloid precursors with respect to the baseline and in the percentage of $CD34^+$ cells in S-phase. However, while the first parameter remained elevated until day 14, the enhanced proliferative activity of the $CD34^+$ cell subset decreased after IL-3 was stopped and remained significantly low during GM-CSF administration. These data suggest a negative rebound on $CD34^+$ cell proliferation after IL-3 discontinuation which is maintained during GM-CSF, resulting in a kinetic refractoriness of the hyperplastic marrow. In the 99 courses completed, a rapid neutrophil and platelet recovery was obtained without cumulative multineage toxicity. The modifications of $CD34^+$ cell cycling after CT followed by IL-3+GM-CSF, could provide additional myeloprotection during multicyclic, dose-intensive programs.

On the other hand, hematopoietic progenitor cells mobilized into the blood (CPC) differ from their BM counterpart with respect to a number of biological characteristics such as cell cycle status and response to cytokines. Little information are available on the actual proportion of both BM and blood-derived $CD34^+$ cells that remain quiescent, or undergo apoptosis, after an *in vivo* treatment with CT + cytokines.

We have recently evaluated the impact of an epirubicin-based CT followed by G-CSF (utilized as a mobilizing sequence for CPC during a multicyclic, intensive CT program for breast cancer) on these two cell subsets. In 18 patients, using multiparameter FCM, nuclear DNA content and bromodeoxyuridine incorporation (BrdU-labelling index, LI) were measured on immunomagnetically-selected $CD34^+$ cells to define the proportion of cells undergoing G_0/G_1, S and G_2/M phases of the cell cycle. Apoptosis was also studied in $CD34^+$ cells, by labelling *in situ* the DNA strand breaks in genomic DNA with a tracer FITC-dUTP (green fluorescence) in a reaction catalized by exogenous terminal deoxynucleotidyl transferase. DNA strand breaks were then quantitated at a single cell level by FCM and simultaneously correlated with cell cycle phases.

Seven to nine days following CT + G-CSF the proportion of $CD34^+$ CPC in active S-phase was significantly lower than that of the BM counterpart at the same time (BrdU-LI=7.5±2% vs 26±6 %, $p < .005$) and in both cases an elevated percentage of $CD34^+$ cells was found in the G_0/G_1 phase. Apoptotic cells (deriving from G_1 or early S-phase) were detected in almost all the samples studied but in very little percentage both in the BM (3.5±3%) and in the PB (4.2±3%) $CD34^+$ cell population. The percentage of apoptotic $CD34^+$ cells increased significantly after a 48-hour incubation with culture medium without exogenous cytokines. These results confirm the feasibility of an *in vivo* simultaneous nuclear DNA content/BrdU incorporation analysis and detection of apoptosis in human BM and blood-derived hematopoietic progenitor cells. This approach is useful for generating data to be taken into account both for studies on myeloprotection during high-dose CT and for *ex vivo* cell manipulation (i.e. *ex vivo* expansion) or for retroviral gene tranfer experiments.

Peripheral blood and bone marrow human hemopoietic progenitors: proliferative differences

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In this study we assessed the functional and kinetic characteristics of highly purified hematopoietic $CD34^+$ cells from the apheresis products and the bone marrow (BM) of 16 normal donors undergoing glycosylated granulocyte colony-stimulating factor (G-CSF) treatment for peripheral blood stem cell (PBSC) mobilization and transplantation in allogeneic recipients. Mobilized and BM $CD34^+$ cells were evaluated for their colony-forming capacity and trilineage proliferative response to selected recombinant human (rh) CSF *in vitro*, and the content of very primitive long-term culture initiating cells (LTC-IC). In addition, the cycling status of circulating $CD34^+$ cells, including committed clonogenic progenitor cells and the more immature LTC-IC, was determined by the cytosine arabinoside (ARA-C) suicide test and the acridine orange (AO) flow cytometry technique. Clonogenic assays in methylcellulose showed the same frequency of colony-forming unit cells (CFU-C) when PB primed $CD34^+$ cells and BM cells were stimulated with PHA-conditioned medium. However, mobilized $CD34^+$ cells were significantly more responsive than their steady-state BM counterparts to interleukin-3 (IL-3) alone and stem cell factor (SCF) combined with G-CSF or IL-3 in presence of erythropoietin (Epo). In cultures added with SCF, IL-3 and Epo we found a mean of 1.5±1 SEM fold increase of PB CFU-GM and BFU-E as compared to BM $CD34^+$ cells ($p < .05$). Conversely, circulating and BM CFU-MK showed the same clonogenic efficiency in response to IL-3, GM-CSF and IL-3, IL-6 and Epo. After 5 weeks of liquid culture supported by the engineered murine stromal cell line M2-10B4 to produce G-CSF and IL-3, we reported 48.2±35 SEM and 62.5±54 SEM LTC-IC per 104 $CD34^+$ cells in PB and steady-state BM, respectively (p = n.s.). The Ara-C suicide assay demonstrated that 4±5% SD of committed precursors and 1±3% SEM of LTC-IC in PB are in S-phase as compared to 25.5±12% SD and 21±8% SEM of steady-state BM ($p < .001$), respectively. At day 5 of G-CSF treatment (i.e. the same day of PBPC collection) the percentage of S-phase CFU-C in BM increased to 34.6±11% SD. Notably, long-term incubation with Ara-C (16-18 hours) in presence of SCF, IL-3 and G-CSF or IL-6 shows that greater than 60% of PB LTC-IC are actively cycling with no differences with BM cells. Furthermore, studies of cell-cycle distribution on PB and BM $CD34^+$ cells confirmed the low number of circulating progenitor cells in S and G_2+M phase whereas simultaneous DNA/RNA analysis demonstrated that the majority of PB $CD34^+$ cells are not quiescent (i.e. G_0-phase) being in G_1-phase with a significant difference in baseline and G-CSF treated BM (80±5% SEM vs 61.9±6% SEM and 48±4% SEM, respectively, $p<.05$). Moreover, G-CSF administration prevented a small but significant proportion of mobilized $CD34^+$ cells from apoptosis. In summary, our results indicate that mobilized and $CD34^+$ cells can be considered equivalent for the frequency of both committed and more immature hematopoietic progenitors although they show different kinetic and functional profiles. In contrast with previous reports, we found that PB $CD34^+$ cells, including very primitive LTC-IC, are recruited in cell cycle.