review
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EFFECTS OF RECOMBINANT HUMAN GRANULOCYTE COLONY-STIMULATING FACTOR ADMINISTRATION ON NEUTROPHIL PHENOTYPE AND FUNCTIONS

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ABSTRACT

Background and Objective. Recombinant human granulocyte colony-stimulating factor (rhG-CSF) is currently used for treatment of various types of neutropenia, treatment of aplastic anemia, mobilization of peripheral blood progenitor cells. However, rhG-CSF is not only a growth factor for the myeloid lineage, but it also acts as a modulator of neutrophil behavior. The aim of the present review article is to examine the following aspects of rhG-CSF therapy: 1) does rhG-CSF influence neutrophil functions, and in particular their microbicidal properties? 2) does rhG-CSF modify neutrophil phenotype? 3) If so, what are the mechanisms potentially involved?

Evidence and Information Sources. The author of the present article has been working in the field of rhG-CSF effects on neutrophil function, contributing original papers in peer-reviewed journals. In addition, the present review critically examines articles and abstracts published in journals covered by the Science Citation Index® and Medline®.

State of Art and Perspectives. Treatment with rhG-CSF causes enhancement of functions such as phagocytosis, superoxide anion generation, chemiluminescence, bacterial killing, and ADCC. Neutrophil phenotype changes after rhG-CSF administration: immediate effects cause direct activation of circulating neutrophils, but delayed effects are characterized by increased surface expression of important effector molecules directly involved in neutrophil functions, such as CD14, CD32, CD64. These effects may have useful clinical consequence in patients who show an increased risk of infections, such as cancer patients, subjects with hematologic diseases (myelodysplasia, aplastic anemia), congenital diseases characterized by neutropenia, and patients with AIDS. Other changes which characterize neutrophils after rhG-CSF administration are represented by significant impairment of CD16 expression, chemotaxis, and reduced *in vivo* migration of neutrophils to inflammatory sites. These effects may be explained by bone marrow modification due to rhG-CSF therapy. In fact, treatment with rhG-CSF causes a significant acceleration of transit time of cells belonging to the myeloid lineage, along with amplification of the mitotic pool and a relative decrease of elements of the post-mitotic pool. It is possible that, because of the accelerated bone marrow transit time of myeloid cells, rhG-CSF causes a relative immaturity of circulating neutrophils. It is known that both CD16 expression and chemotaxis properties are acquired by neutrophils in the late stages of maturation, but the time necessary to acquire full functional maturity seems to be shortened by rhG-CSF administration, and this kinetic aspect may play a non-negligible role in the modification of neutrophil behavior.

Key words: Recombinant human G-CSF, neutrophil function, therapy

Recombinant human granulocyte colony-stimulating factor (rhG-CSF) has been used on a wide scale in the clinical setting for almost 7 years now. It has been calculated that more than 1.2 million patients have been treated to date with this hemopoietic growth factor.[1]

The most common uses of rhG-CSF include treatment of drug-induced neutropenia, neutropenia associated with chemotherapy and radiotherapy, rescue from febrile neutropenia, treatment of various types of congenital neutropenia, acceleration of neutrophil recovery after bone marrow transplantation, mobilization of peripheral blood progenitor cells and supportive treatment for aplastic anemia. Autoimmune neutropenia can be treated successfully with rhG-CSF as well.[2]

Several randomized and non-randomized studies have shown that rhG-CSF administration results in significant improvement of neutrophil recovery after chemotherapy for various types of cancer. In addition, in the majority of these studies, therapy with rhG-CSF has been shown to have a significant effect in terms of reduction of complications from infections, the reduction of usage of antibiotics and minimized hospital stays.[3-15]

rhG-CSF administration has also shown signifi-

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Table 1. Relevant clinical studies concerning the effects of rhG-CSF on neutrophil count and infections and/or duration of fever.

Refs	*Disease*	*Patients (G-CSF/controls)*	*G-CSF dose (μg/kg or μg/m2)*	*Effects on ANC*	*Effects on infections or fever*
Randomized studies					
3	Breast cancer	25/26	5	yes	yes
4	NHL	34/34	5	yes	yes
5	urogenital cancer	32/38	2	yes	yes
6	SCLC	95/104	230/m^2	yes	yes
7	SCLC	34/31	5	yes	no
8	NHL	41/39	230/m^2	yes	no
9	various cancers	109/107	12	yes	yes
10	various cancers	186	5	yes	yes
16	SCN	63/60	3-11	yes	yes
17	newborn sepsis	36/9	1-10	yes	NA
Non-randomized studies					
11	SCLC	9/0	1-40	yes	yes
12	urogenital cancer	27/0	1-60	yes	yes
13	various cancers	15/0	1-60	yes	NA
14	various cancers	31/0	0.3-10	yes	NA
15	NHL	16/0	50-400/m^2	yes	yes

NHL: non-Hodgkin's lymphomas; SCLC: small cell lung cancer; SNC: severe chronic neutropenia; ANC: absolute neutrophil count; NA: not available

cant clinical utility in patients with various types of severe chronic neutropenia and in infants with severe sepsis: in these subjects as well, the growth factor has been shown to induce not only a rise in neutrophil count but also an evident reduction in infectious episodes.[16,17]

Table 1 summarizes the results of some of the most relevant studies published so far. The data obtained in such studies in terms of reduction of infections on duration of fever may depend, from a theoretical point of view, upon the effects of rhG-CSF as a growth factor. Thus, the increase in the number of neutrophils may be responsible for the clinical effect in such patients.

However, another hypothesis should be taken into consideration: part of the rhG-CSF-induced effects may depend upon the enhancement of some neutrophil functions, or more specifically, on the enhancement of the microbicidal properties of such cells.

Indeed, several papers dealing with the various effects of rhG-CSF on neutrophil behavior have already far been published, but these articles have dealt with only a partial aspect of the matter. Several excellent review articles have been published to date, all dealing with the most relevant biological characteristics and clinical applications of rhG-CSF,[1,18-25] and giving an economic analysis of the clinical uses of growth factors, including rhG-CSF.[26]

Table 2. Relevant effects of rhG-CSF administration in normal subjects.

Time	*Parameters*	
Immediate effects (up to 1 hr)	increased:	CD13, CD11B, CD16, CD45, CD16 plasmatic lactoferrin
	decreased:	ANC, LAP content
Early effects (up to 12 hrs)	increased:	ANC, LAP content CD14, CD64, SCD14, SCD16 plasmatic lactoferrin and elastase
	decreased:	cd16
Late effects (24-72 hrs)	increased:	ANC, LAP content, ADCC CD14, CD32, CD64, SCD16 plasmatic elastase
	decreased:	CD16,CD62L
Very late effects (> 96 hrs)	increased:	SCD16, plasmatic lactoferrin

Time: hours from rhG-CSF injection. ANC: absolute neutrophil count; LAP: leukocyte alkaline phosphatase. References: 27,28,30,31,34.

However, the various effects of rhG-CSF on neutrophil functions and on phenotype were not reviewed extensively in those articles.

Thus, several points deserve further analysis and will be taken into consideration in the present article: 1) does rhG-CSF influence neutrophil functions and, more specifically, their microbicidal properties? 2) does rhG-CSF modify the neutrophil phenotype? 3) If so, what are the mechanisms potentially involved?

In order to answer these questions, the present article provides a review of the numerous and relevant findings concerning the rhG-CSF-induced modifications of the biology of neutrophils. It should be pointed out that there are no randomized studies, only small series of subjects have been published, and rhG-CSF effects on neutrophils have not been correlated with the clinical outcome.

A single subcutaneous dose of rhG-CSF alters the phenotype of neutrophils from normal subjects

rhG-CSF is capable of modifying the phenotype of circulating neutrophils after a single subcutaneous dose (300 μg) in normal volunteers (Table 2). Some effects can be detected just a few minutes after injection, while other modifications occur later. On the basis of the data available, we can distinguish between immediate, early, delayed and very delayed effects.

Immediate effects (up to 1 hr)

Shortly after injection, a rapid decline in the absolute neutrophil count (ANC) can be detected with nadir at about 30 minutes; the decrease is

already apparent after 15 minutes.[27,28] This kinetic aspect has been found to be associated with an evident increase in the expression of surface antigens which are considered *activation molecules* because their expression is upregulated through neutrophil activation by a number of agonists. This increased expression included CD13, CD45, CD11b and CD66b, and was detected using whole blood methods, which excluded any aspecific effect due to isolation procedures. The level of activation showed 95% confidence intervals ranging from 101-189%, varying widely between the different molecules evaluated. However, it is possible that the level of activation created by flow cytometry is underestimated, because the peak of activation coincided with the most significant decrease in ANC, due in turn to the adhesion of activated neutrophils to endothelial cells. This hypothesis is supported by *in vitro* observations as well, as incubation of normal circulating neutrophils with rhG-CSF (10-100 ng/mL) induces CD11b upregulation, and this phenomenon is almost parallel to the increased adherence of neutrophils to nylon fiber.[29]

A mild upregulation of CD16 was detected at the same time the observation was made (30 min),[27,28] but this effect was not observed in all subjects. In addition, the levels of the circulating form of CD16 (sCD16) were found to be increased during this phase,[27] therefore the dishomogeneity of results may be explained by an increased shedding of the molecule in some subjects. However, incubation of normal neutrophils with 10-100 ng/mL rhG-CSF causes a rapid, transient CD16 upregulation.[27] Direct evidence of the mobilization of secretory vesicles was obtained in one study:[27] using immunoelectron microscopy to detect the presence of CD16, the number of these cytoplasmic structures was found to be decreased after rhG-CSF incubation; fusion of some vesicles with the surface membrane was observed as well.

These immediate effects of rhG-CSF on circulating neutrophils from normal subjects were followed by other signs of increased, neutrophil degranulation: the intracellular LAP content was decreased, and an initial increase in the plasmatic levels of lactoferrin and elastase-α1AT complexes was found 1 hour post-injection.[27] The increase in circulating lactoferrin levels was more evident than that of elastase.

When observed together, the modifications in neutrophil phenotype and the variations in LAP content and plasmatic lactoferrin concentrations show that neutrophil intracellular trafficking and degranulation involve mainly specific granules (CD11b, CD66b, lactoferrin) and secretory vesicles (CD11b, CD16).

These findings clearly suggest that the initial effect of rhG-CSF is due to a strong direct activation of circulating neutrophils by the high levels of rhG-CSF administered, while the subsequent modifications in elastase levels (see below) could be due to an additional effect provided by the endothelial cells on adherent neutrophils. This latter hypothesis could also explain why CD62L expression did not decrease shortly after rhG-CSF injection.[27]

Early effects (up to 12 hours)

The effects of a single 300 µg of rhG-CSF after 1 hour for up to 12 hours postinjection are characterized by the normalization of the most important activation markers. CD11b was found to return to the initial levels after 4-8 hours, and CD66b was normal after 8-12 hours.[26] A similar behavior was demonstrated by CD45 and CD13.[28]

In this phase, however, modifications in the expression of other important effector molecules have been detected. CD14 (LPS-binding protein) expression strongly increases[28,30] starting at 4 to 8 hours post-injection, and CD 64 (FcRI) was found to be actively synthesized and expressed by neutrophils in the late stage of this phase (about 8-12 hours postinjection).[28,30,31]

LAP behavior changes due to the increase in content.[27] Indeed, *in vitro* studies have shown that rhG-CSF is capable of stimulating active protein and specific LAP mRNA synthesis: this phenomenon is inhibited by actinomycin D and cycloheximide.[32] On the contrary, however, CD16 expression shows an opposite pattern with very low levels (significantly lower than before injection) about 8 hours postinjection[28] or even later.[30]

The levels of soluble CD14 (sCD14) and sCD16 were found to be increased during this phase,[27,28] with peak levels for sCD16 at 8 hours post-injection.[27] The results concerning sCD16 are very difficult to interpret because of the complex biology of this molecule: 1) CD16 is contained in secretory vesicles and, upon stimulation, it is translocated to the surface membrane; 2) a variable amount of the membrane CD16 is shed and released in the microenvironment; 3) sCD16 has a half life of 1.5 days;[33] this property may affect the measurement of the molecule during the study masking further release; 4) newly-formed neutrophils are responsible for a further increase in sCD16.

Whether sCD14 originates from monocytes, neutrophils or both is not clear. Monocytes express a functional G-CSF-R, and rhG-CSF is capable of inducing some modifications in monocyte phenotype,[34] but monocyte CD14 expression is not modified by rhG-CSF administration.[28] Therefore, it is likely that sCD14 derive from the neutrophil membrane. The circulating levels of lactoferrin and elastase-α1AT complexes are still very high during this phase, with peak values occurring 8 hours post-injection.[27] It has been hypothesized that elastase release by rhG-CSF-activated neutrophils is caused by the action of endothelial cells on adherent neutrophils. Indeed, endothelial cells express a func-

tional G-CSF-R, and rhG-CSF is capable of inducing stimulation of such cells.[35] Therefore, we can hypothesize that stimulation by rhG-CSF-activated endothelial cells on neutrophils may provide the stimulus sufficient for mobilizing primary granules.

Delayed effects (24-72 hours)

Delayed effects are characterized by a very high increase in CD64 expression and LAP content, a significant increase in CD14 expression, a significant, although variable, decrease in CD16 expression,[28,36] and a non-significant trend towards increased CD32 (FcRII) expression.[28]

sCD16 levels are still elevated during this phase,[28] as are the plasmatic levels of elastase-α1 AT complexes (two-fold levels 24 hours postinjection as compared with baseline values).[27]

Implementing a different study schedule (daily administration to normal volunteers of 50 μg/m^2 for seven days and blood sampling on day 8), Ohsaka *et al*.[34] observed a significant increase in the expression of CD11c, CD11b and CD35 (CR1) along with a significant decrease of CD62L expression. Interestingly enough, a significant enhancement of CD32 expression was detected in the same study.

Very delayed effects (96 hours or later)

No modifications in neutrophil phenotype were detected 96 hours postinjection or later. All neutrophil surface molecules showed baseline values during this phase, along with the complete normalization of ANC. However, high levels of sCD16 could be measured as late as 6 days post-injection,[28] and this phenomenon was considered as compatible with a release of CD16 from neutrophils which had migrated into the tissue compartment.

Plasma lactoferrin demonstrated similar behavior in that after the initial 4-hour peak post-injection, its concentrations increased again at 3 days post-injection and reached peak value on day 7. This second peak was also considered as reflecting the turn-over of neutrophils accumulated into tissues.[27]

rhG-CSF administration modifies the phenotype and the functions of neutrophils from patients

The studies carried out on normal volunteers involved very few subjects: nonetheless, they have provided very useful information because they were carried out with well-designed study schedules in terms of timing of blood sampling and the number of parameters taken into consideration. The various modifications found were obtained in subjects with normal myelopoiesis. On the contrary, the clinical investigations carried on in various patient populations appeared to be markedly dyshomogeneous in terms of type of underlying disease (hemopoietic cancers, other types of cancer, congenital neutropenias, etc.); initial ANC (patients either neutropenic or not); initial neutrophil behavior (patients with either baseline normal or defective neutrophil functions); dose of rhG-CSF and length of therapy; timing of blood sampling; number and type of parameters under investigation.

None of the clinical studies published to date have included patients with evidence of infection during rhG-CSF administration, but neutropenic patients have frequently been evaluated. This aspect can be very important and may play a non-negligible role as far as neutrophil behavior is concerned. The endogenous levels of G-CSF are frequently elevated in neutropenic patients and are particularly high in febrile patients.[37-39] Although the circulating levels of G-CSF in such patients are lower than the concentrations which can be obtained with rhG-CSF administration,[25] a cumulative effect of endogenous G-CSF and administered rhG-CSF on neutrophil behavior may be hypothesized in several patients. The matter is made more complex by the possible neutrophil-mediated clearance of G-CSF, which may involve both endogenous and exogenous G-CSF, and may amplify differences between neutropenic and non-neutropenic patients because the higher the number of circulating neutrophils the faster the G-CSF clearance.[40,41]

The majority of clinical studies included neutrophil analysis immediately before and 24 hours after subcutaneous rhG-CSF administration. In the study by Spiekermann *et al*.[30] a more detailed kinetic study was performed. It is necessary to underline the fact that we are still lacking randomized, placebo-controlled studies aimed at correlating modifications in neutrophil function with the clinical behavior (i.e. effects on incidence of infections or fever) of rhG-CSF-treated patients.

Several neutrophil functions are positively modified by rhG-CSF administration. The analysis of such modifications appears to be of particular interest because they are obtained in patients who show an increased risk of complications due to infection. Some types of diseases deserve particular attention, such as cancer patients (including lymphomas), patients with congenital diseases that are characterized by neutropenia and recurrent infections, patients with hematologic diseases, and AIDS patients.

Cancer patients

rhG-CSF-induced neutrophils from the majority of cancer patients evaluated in such studies did not show a significant CD11/CD18 complex upregulation after suspending the therapy,[30,42,43] although in some studies a neutrophil phenotype characterized by increased CD11b and decreased CD62L expression was reported.[44] This phenomenon was interpreted as a being consequence of partial neutrophil activation during rhG-CSF therapy, perhaps related

to the study schedule chosen by the authors.[45]

Few studies have dealt with eventual modifications in the chemotactic peptide-induced upregulation of CD11b/CD18 which is necessary for effective neutrophil adherence to endothelium and for transendothelial migration.[46] Patients with non-Hodgkin's lymphoma who where undergoing chemotherapy demonstrated the following behavior: fMLP-induced CD11b/CD18 upregulation was improved in patients with a previously defective function, but was down-regulated in patients with a previously normal function.[47]

Just as in normal subjects, cancer patients experienced a significant increase in CD64 expression as well,[30,48] which reached a maximum level at 3-5 days after therapy had been started.

Variable effects on CD16 expression were observed, considering that in some studies, this parameter did not change,[42] while in other studies,[30] it decreased continuously during treatment. However, clear evidence was reported for a dosage-dependent effect, as a significantly lower expression of CD16 was detected only in patients who received relatively high dosages of the growth factor (10-20 µg/kg).

Recently, CD32 expression was found to be enhanced by rhG-CSF therapy in patients treated with chemotherapy for non-Hodgkin's lymphoma as well.[42] In addition, another molecule that is involved in microbicidal activity, such as CD14, was reported to be upregulated by G-CSF administration in cancer patients,[30,49] thus demonstrating that neutrophil machinery for microorganism killing is strongly enhanced in such patients. In one study,[42] enhancement of phagocytosis was correlated with the increase in CD32 expression (Figure 1).

The phagocytic properties of neutrophils were significantly enhanced by rhG-CSF administration in patients with various types of cancer;[42,50,51] similar results were observed in normal subjects treated with the growth factor before leukapheresis.[52]

Similarly, the respiratory burst of rhG-CSF-induced neutrophils was found to be strongly enhanced in such patients.[42,43,51,53-55] Neutrophils appeared to have been primed by rhG-CSF therapy, and the most evident effects were observed when neutrophils were stimulated with receptor-dependent agonists, such as fMLP, opsonized zymosan, and C5a.

The killing activity against bacteria was also positively influenced in cancer patients treated with daily doses of lenograstim ranging 1-40 µg/kg,[50,51] but opposite results were observed in patients undergoing bone marrow transplantation.[56,57]

A positive modulation of neutrophil action against cancer cells also occurrs, as suggested by recent studies:[48] neutrophils from patients treated with rhG-CSF were found to kill neuroblastoma cells in the presence of anti-disialoganglioside/anti FcRI bispecific antibodies, thus suggesting new therapeutic strategies in cancer therapy. The increased ADCC function of neutrophils after rhG-CSF administration[31,58,59] is mediated by the induction of CD64 expression, as is demonstrated by blocking studies with specific anti-CD64 monoclonal antibodies.[28]

Despite the overall positive effects on several neutrophil functions, and despite the results of *in vitro*

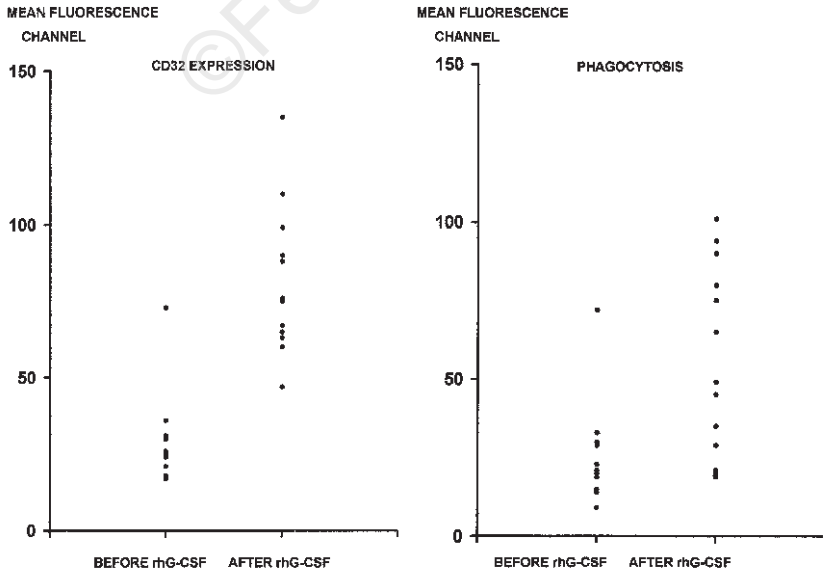

Figure 1. Effects of rhG-CSF administration on neutrophil CD32 expression and phagocytosis in 13 patients with non-Hodgkin's lymphoma. CD32 expression and phagocytosis were evaluated by means of flow cytometry (for technical details see ref. #42).

studies, neutrophil motility seems to be negatively influenced by rhG-CSF treatment in cancer patients as well as in normal subjects.[30,43,56,60] The majority of those studies were carried out by means of agarose assays, revealing some evidence of a dose-dependent effect after a negative impact on chemotaxis was detected by Spiekermann *et al*.[30] with the rhG-CSF dose higher than 10 μg/kg. However, in a recent study, Azzarà *et al*.[60] showed that the use of the Boyden chamber method, evaluated by a very sensitive image analysis device, detected a significant impairment of neutrophil motility with a rhG-CSF dosage of 5 μg/kg. In addition, both random and directed migration were impaired by rhG-CSF treatment, and it was interesting to observe that neutrophil kinetics during direct migration were strongly altered and displayed a gaussian curve (just like the random migration curve). This observation suggested that the specific orientation mechanisms of migrating neutrophils had been altered.

Interestingly, a recent study by Price *et al*.[61] showed that rhG-CSF administration to healthy volunteers at the dosage of 300 μg/day caused a significant impairment of the neutrophil inflammatory response when measured with the skin chamber technique, independently of the age of the subjects studied. Therefore, the altered chemotactic behavior of rhG-CSF-induced neutrophils may play an important role *in vivo*. The only study that presented a positive effect on chemotaxis concerned two healthy subjects undergoing leukapheresis for granulocyte transfusion.[52] However, neutrophils were probably activated during the apheresis procedure.

An interesting finding by Lindemann *et al*.[43] was represented by a weak but noticeable expression of IL-2R (receptor for IL-2) on the neutrophil surface during rhG-CSF treatment in some patients. The levels of soluble IL-2R were found to parallel total white blood cell counts, therefore suggesting that granulocyte lineage may be a source of soluble IL-2R in some pathological conditions and after some types of therapy.

G-CSF producing tumors may be responsible for an altered neutrophil phenotype. Ohsaka *et al*.[62] described the case of a patient with an undifferentiated mediastinal carcinoma; in this case, high endogenous G-CSF levels caused leukocytosis, high FcRI and CR1 expression, and low FcRIII and L-selectin expression on neutrophils. These parameters returned to normal levels after surgical resection of the tumor, suggesting the direct action of tumor-derived G-CSF on the activated neutrophil phenotype.

Congenital diseases characterized by neutropenia

Superoxide anion generation was positively influenced by rhG-CSF treatment of children with severe congenital neutropenia (SCN) or glycogen storage diseases type 1B.[63,64] In some SCN patients, quantization of cytochrome b559 showed a strong correlation with rhG-CSF-induced superoxide anion generation modifications.[63] However, the killing action against bacteria did not show significant enhancement in most rhG-CSF-treated SCN patients.[65]

Chemotaxis was found to be impaired in neutrophils from rhG-CSF-treated SCN children,[66] and this phenomenon appeared to be associated with a significant increase in neutrophil F-actin content.[65]

In addition, rhG-CSF-induced neutrophils showed an impaired fMLP-induced CD11b/CD18 upregulation in children suffering from SCN.[63] In a case of glycogen storage disease type 1B, *in vitro* stimulation of rhG-CSF-induced neutrophils with fMLP caused an up-modulation of CD11b that was comparable to that of control neutrophils, but with accelerated kinetics.[64]

Patients with hematologic diseases

Myelodysplastic syndromes (MDS) are frequently characterized by neutropenia and neutrophil dysfunction,[67,68] and recurrent infections are prominent characteristics of such diseases. Treatment with rhG-CSF not only increases neutrophil count in some MDS patients (thus reducing the incidence of neutropenia-related infections), but is also able to correct some neutrophil defects like impaired respiratory burst.[69] In addition, *in vitro* studies have demonstrated that LAP scores increase upon rhG-CSF incubation in MDS neutrophils,[70] the full induction of the enzyme requiring approximately 48 hrs.

Similarly, the respiratory burst activity of patients with aplastic anemia is positively influenced by rhG-CSF administration.[71]

In contrast with cancer patients, the expression of defective CD16 molecule (due to an anomaly involving the glycosyl-phosphatidylinositol anchor) was induced by rhG-CSF therapy in two patients with paroxysmal nocturnal hemoglobinuria.[72]

Recently, we have detected an impaired fMLP-induced F-actin polymerization in neutrophils from two rhG-CSF-treated patients suffering from autoimmune neutropenia, likewise, the same phenomenon has been detected in one patient affected by aplastic anemia (unpublished data).

AIDS patients

rhG-CSF can be useful in the treatment of AIDS patients. In fact, neutropenia is a common problem in such patients, due to the action of the virus itself and the effects of the various cytotoxic agents used to treat AIDS. It has been observed that neutrophils from AIDS patients show a modified phenotype and are characterized by various functional defects.[73] The fungicidal activity against *C. albicans* and *C. neoformans* of neutrophils from neutropenic patients with AIDS is reportedly enhanced after a single subcutaneous 5 μg/kg dose showing the

greatest activity against the two opportunistic fungi at 48-72 hours after rhG-CSF injection.[74] These effects, together with the correction of the neutrophil count, may explain some observations concerning the positive effects of low, intermittent doses of rhG-CSF aimed at the prevention of both neutropenia and fever in AIDS patients undergoing antiviral therapy.[75]

Some data from our laboratory (Minnucci S *et al*., manuscript in preparation) have shown that CD32 expression is significantly reduced in HIV-infected subjects, indicating that the positive effects of rhG-CSF on this important neutrophil receptor can be very useful.

Discussion of data from literature

During rhG-CSF administration, neutrophils demonstrate a distinct behavior. We can detect an immediate effect of activation which is due to the direct stimulation of circulating mature neutrophils. This phase coincides with the increase in rhG-CSF (Filgrastim) serum concentrations, which reach peak values at 120 to 480 minutes after a single subcutaneous injection, and at 4 to 12 hours after every single injection in patients undergoing daily subcutaneous therapy.[23,76] The immediate activation of the neutrophils explains the rapid decrease in ANC immediately after the start of therapy as a large amount of neutrophils is marginated because of increased adherence to endothelial cells. An additional effect of neutrophil activation may be provided by endothelial cells during transient adhesion of G-CSF-activated neutrophils.

At 24 hours, Filgrastim serum concentrations are < 10% of peak values,and the effects that are detected after this time are mainly due to the effects of rhG-CSF during bone marrow transit of myeloid cells.

The myeloid mitotic compartment is expanded by rhG-CSF action, with significant increases in promyelocytes and myelocytes and a relative depletion of mature neutrophils. This latter effect is due to a marked acceleration of bone marrow neutrophil transit time, which can reach values of 2.9±0.1 days with rhG-CSF doses of 300 µg/day, versus values of 6.4±0.3 days in untreated subjects, obtaining intermediate values with rhG-CSF doses of 30 µg/day. No modifications in the marginated pool were found and mean blood-neutrophil disappearance times were only slightly prolonged.[61]

Accelerated bone marrow transit is likely to induce several modifications in neutrophil biology. As far as neutrophil morphology is concerned, Price *et al*.[61] found that rhG-CSF-induced neutrophils are different from those produced in absence of G-CSF, in terms of size and ability of localizing at an inflammatory site. In addition, various authors observed that rhG-CSF-induced neutrophils displayed Dohle bodies, toxic granulations, vacuolization[13,43,77] and even various degrees of polarization.[78]

During and after rhG-CSF administration, important effector molecules such as CD64, CD32, and CD14 are actively expressed by newly-formed neutrophils. These phenotypic modifications play an important role in host defense because the molecules mentioned exert a key-role in functions such as phagocytosis, ADCC, and bacterial killing. These effects may explain the efficacy of the growth factor in decreasing morbidity and hospitalization of patients suffering from various diseases. However, other modifications, including defective chemotaxis and migration in skin chambers show that a relative immaturity of circulating neutrophil, does exist after rhG-CSF stimulation of bone marrow.

During differentiation, in fact, neutrophils undergo a particular development sequence in their functional properties.[79] Some effector molecules, such as Fc and complement receptors, are acquired early on differentiation,[79,80] with the exception of CD16, which is first expressed during the metamyelocyte stage.[81,82] Phagocytosis parallels the acquisition of the majority of Fc receptors, while oxygen-dependent bacterial killing is a later manifestation of functional differentiation[79,83] and chemotaxis represents a terminal stage of such a process.[79]

Thus, the phenotype of rhG-CSF-induced neutrophils can support these observations, and the following *scenario* could be proposed (Figure 2). During the accelerated bone marrow transit of rhG-CSF-stimulated myeloid cells, some cellular events, such as FcRI, FcRII, and CD14 expression are actively stimulated; these effects are concomitant with the stimulation of immature myeloid cells to

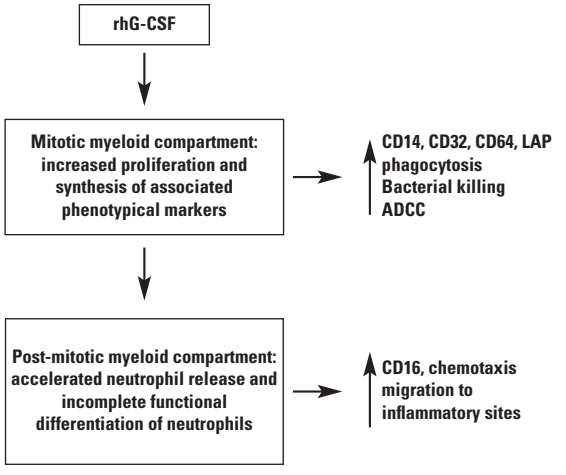

Figure 2. Effects of rhG-CSF on functional development of human neutrophils.

proliferate and differentiate. Stimulation of secondary granule formation by relatively mature granulocytic progenitor cells could occurr, as demonstrated by the increased LAP activity of rhG-CSF-induced neutrophils. However, a certain degree of incomplete maturation could follow, as the rapid release of mature neutrophils may shorten the time required by bone marrow neutrophils to fully express late functional markers, such as CD16 expression and chemotactic properties.

Role of the specific receptor for human G-CSF (G-CSF-R)

The biological activities of G-CSF are mediated by specific receptors expressed at the surface of responsive cells, such as myeloid progenitors and neutrophils.[84-86]

G-CSF-R, whose gene is located on chromosome 1p35-p34.3,[87] is a dimer of two gp130 chains that is homologous with gp130 IL-6 transducer.[88] This homology may have important consequences and may explain some overlapping effects of G-CSF and IL-6 as regards neutrophil stimulation. In fact, both are early acting cytokines in human myelopoiesis with overlapping effects on myeloid progenitor cells[89] and IL-6 is an activator of neutrophil function.[90]

Binding specificity for G-CSF resides in the hematopoietin domain of G-CSF-R, and the structural characteristics of this domain are important for the biological activities of the receptor. The 57 membrane-proximal cytoplasmic aminoacids are essential in the transduction of signals necessary for proliferation of myeloid cells, while the next segment of 30-35 aminoacids contains a domain that is responsible for differentiation.[91,92]

Recently, a somatic mutation in the G-CSF-R gene has been found in a patient with SCN;[93] moreover, two patients with acute myeloid leukemia and a history of SCN have been reported as having very similar mutations in the G-CSF-R gene.[94] In both reports, mutations produced a truncation in the C-terminal cytoplasmic region of the receptor, and were thought to cause abnormally high proliferation responses and an interruption in maturation of the myeloid lineage.

G-CSF-R lacks intrinsic tyrosine kinase activity, but after interaction with rhG-CSF, it undergoes homodimerization and activates cytoplasmic tyrosine kinases such as Jak1 and Jak2. In turn, Jak kinases induce tyrosine phosphorylation of STAT molecules such as STAT1 and STAT3 which translocate to the cell nucleus; they also induce tyrosine phosphorylation of the cytoplasmic domain of G-CSF-R. Furthermore, G-CSF-R stimulation activates other tyrosine kinases as well, such as Lyn and Syk, and the p21Ras/MAP kinase pathway.[84-86] The activation of these enzymatic pathways appears to be important in proliferating cells of the myeloid lineage, but not in mature neutrophils.

Studies carried out *in vitro* excluded the involvement of kinases of the Jak family in G-GSF-R signalling in normal mature neutrophils and, although G-CSF-R is tyrosine-phosphorylated after stimulation with rhG-CSF, this does not appear to be mediated by any of the known Jak kinases.[23] Similarly, tyrosine phosphorylation of STAT1 and STAT3 has not been detected in G-CSF-treated neutrophils.[84] However, it is possible that other members of the Jak family which are not yet identified, may be involved in G-CSF-R signalling in neutrophils. It should be pointed out that in a recent paper Rauprich *et al*.[95] reported that the Jak2 protein shows increased tyrosine phosphorylation in neutrophils from patients with severe congenital neutropenia as a result of treatment with rhG-CSF. In addition, a new STAT-like protein called StatG has recently been identified in rhG-CSF-stimulated neutrophils,[96] and rhG-CSF has been reported to activate the Src-family kinase Lyn as well as the non-Src related Syk tyrosine kinase in mature neutrophils.[97]

These effects may play an important role in rhG-CSF-induced modifications of neutrophil functions just as it occurrs in the case of neutrophil stimulation with various agonists.[98] However, at the present time, no data are available about the connection between these effects and specific modifications in neutrophil phenotype and function.

Future perspectives

In the future, some other points should be investigated. Different therapeutic schedules should be taken into consideration, expecially in terms of dosage of rhG-CSF. Adequate studies are needed in order to compare glycosylated rhG-CSF (lenograstim) with non-glycosylated rhG-CSF (filgrastim) regarding their effects on neutrophil phenotype and functions. The clinical effects of filgrastim and lenograstim are comparable, and both proved to be effective in neutropenia prevention and rescue. Differences in the *in vitro* effects on myelopoiesis, pharmacokinetics, and peripheral blood progenitor cell mobilization[99-101] are probably due to glysosylation, which characterizes lenograstim. However, data concerning possible differences between the two types of rhG-CSF in terms of effects on neutrophil phenotype and functions are not available. Preliminary results obtained in our laboratory (Azzarà A, unpublished data) suggest that treatment with lenograstim, on the contrary to treatment with filgrastim, may be associated with conserved neutrophil chemotaxis and normal random and stimulated migration patterns.

New forms of rhG-CSF could be available in the clinical setting, such as PEG-conjugated rhG-CSF,

which could cause different kinetic modifications of bone marrow myeloid transit and different neutrophil behavior. Indeed, PEG-conjugated G-CSF in rats shows a slower clearance and a higher mean residence time when compared to G-CSF. Accordingly, the relative potency of PEG-conjugated G-CSF/G-CSF is about 41%.[102]

rhG-CSF activity may also modify, at least from a theoretical point of view, the reciprocal effects of neutrophils and platelets. In fact, human platelets express the G-CSF-R because of their production by megakaryocytes, and some platelet functions are augmented by rhG-CSF.[103-105] Since neutrophils and platelets show reciprocal effects, activation and inhibition, depending on various physiological and pathological conditions,[106] the presence of a functional G-CSF-R on platelets may have *in vivo* consequences that ought to be investigated.

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