Defective Polymorphonuclear Leukocyte Functions in Children Receiving Chemotherapy for Cancer Are Partially Restored by Recombinant Human Granulocyte Colony-Stimulating Factor In Vitro

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Granulocyte colony-stimulating factor (G-CSF) has important direct and priming effects on different functions of normal mature polymorphonuclear leukocytes (PMNL). Previous study has shown an alteration in respiratory burst and bactericidal activities of PMNL harvested from children with cancer treated with chemotherapy. The present study evaluates the possibility that recombinant human (rh) G-CSF could correct these defective functions in vitro. Free radical formation in defective PMNL was enhanced by rhG-CSF to a level similar to that found in normal PMNL primed by rhG-CSF. The defective bactericidal activity against Escherichia coli and Staphylococcus aureus was also corrected. This bactericidal activity was not different from that observed in normal PMNL primed by rhG-CSF. In conclusion, correction of the altered free radical-formation pathway by rhG-CSF in these cells contributed to the restoration of normal bactericidal activity against both gram-positive and gram-negative microorganisms.

In vitro and in vivo studies have shown that G-CSF not only induces granulocyte lineage-specific colony growth but also increases a wide variety of functional activities, including microbialicidal activities in both normal and defective PMNL [11–22].

The aim of this study was to determine if recombinant human (rh) G-CSF could correct the defective respiratory burst activity and the decreased intracellular microorganism killing of PMNL from children with cancer who were treated with chemotherapy.

Materials and Methods

Patients. PMNL were obtained from 19 children (mean age, 9.5 ± 2.1 years; range, 3–15) who had been admitted to the Oncology Unit of the Hôpital Universitaire des Enfants for intensive chemotherapy treatment of acute leukemia or lymphoma or of a solid tumor. Leukemic patients were in complete clinical and biologic remission. Children with acute infections or severe anemia were excluded. None of the patients studied had been exposed in vivo to hematopoietic growth factors. Two blood samples were collected for each patient: 1 within 24 h after the latest chemotherapy exposure (early sample) and the other after recovery of drug-induced bone marrow aplasia (>500 PMNL/μL; late sample). Mean absolute PMNL counts were not significantly different for early (2164 ± 1136/μL) and late (mean, 2269 ± 1282/μL) samples. The children had been exposed to chemotherapy for a mean of 7 months. The clinical characteristics of patients are detailed in Table 1. Control samples from healthy adult volunteers were run in parallel with the patient samples. In our experience, PMNL functions do not vary between childhood and adulthood.

Isolation of PMNL. PMNL were isolated from calparinized blood of healthy volunteers and cancer patients as described [23]. After centrifugation on ficoll-hypaque, erythrocytes from the resulting pellet were lysed by NH₄Cl. The PMNL were washed and
resuspended in Hanks’ balanced salt solution (HBSS) containing calcium and magnesium (GIBCO, Paisley, Scotland). Contamination by cells other than PMNL was <2.5%, as judged by Giemsa staining. 96.5% ± 1.03% of PMNL were viable by trypan blue exclusion.

**Preincubation of PMNL with rhG-CSF.** rhG-CSF (Amgen, Thousand Oaks, CA) had a specific activity of 2.5 X 10^8 U/mg of protein. The preparation contained <0.1 ng/mg of endotoxin when tested by the limulus lysate assay. Preliminary experiments with various concentrations of rhG-CSF and various periods of incubation were done: Pretreatment for 15 min with 4000 U/mL rhG-CSF was sufficient to prime normal PMNL as measured by chemiluminescence assay and* Staphylococcus aureus* 42D killing.

**Luminol-dependent chemiluminescence.** These assays were done as described previously [24]. Light emission was measured in the presence of luminol (10^-5 M; Sigma, St Louis). The probe was delivered into tubes containing 125 μL of PMNL suspension (5000 cells/μL) pretreated or not with rhG-CSF. After a period of adaptation, the readings were started immediately with the addition of HBSS as blank and FMLP (3 X 10^-6 M) as stimulus. The effect of additives was evaluated over 20 min. The peak amplitudes were measured using an LKB 1251 luminometer (LKB Wallac, Turku, Finland) and recorded as millivolts (mV).

**Intracellular killing.** The assay was done using the method of Waterlot et al. [25]. In brief, *S. aureus* 42D and *Escherichia coli* O54 were grown overnight and adjusted to 3 X 10^7 bacteria/mL after preposonization with 10% serum. The microorganisms were mixed with PMNL that were primed or not primed with rhG-CSF, and the mixture (5 bacteria/phagocyte) was placed in a rotator for 7 min at 37°C to allow phagocytosis to occur. Bacteria that were not ingested were removed by washing the PMNL three times. The PMNL containing ingested bacteria were resuspended in 5% serum. Aliquots were removed, diluted with distilled water to lyse the cells, and then transferred to pour plates (killing [K]). The rest of the culture was incubated at 37°C, and after 60 and 120 min, intracellular killing was stopped by centrifugation at 0°C. The cells were lysed by adding distilled water and then transferred to pour plates. Viable bacterial colonies were counted. The bactericidal capacity was calculated as the ratio of the number of viable organisms at 30 (K30), 60 (K60), and 120 min (K120) to the number of organisms that were phagocytized (K0). Killing was optimal at 120 min for *S. aureus* 42D and at 60 min for *E. coli* O54.

**Statistical analyses.** Results were expressed as means ± SDs. Statistical analyses of PMNL functions with and without rhG-CSF were done for each child using Wilcoxon’s paired t test. Differences were considered statistically significant at P < .05. Comparisons with normal controls were done using the Wilcoxon Mann-Whitney test, taking into account a similar significance level.

**Results**

**Effects of rhG-CSF on the oxidative metabolism of PMNL from healthy controls and children with cancer.** The oxidative metabolism of PMNL from healthy controls and children with cancer is shown in figure 1. The results are expressed as mean ± SD of the peak of chemiluminescence (mV) from 10 experiments. Data on analyses of chemiluminescence in the presence of HBSS were subtracted from data obtained in the presence of FMLP. Chemiluminescence in the presence of HBSS was slightly higher in PMNL from some patients than from normal controls; however, there was not a statistically significant difference.
PMNL from healthy donors had a mean chemiluminescence of 42.47 ± 14.72 mV. After PMNL were preincubated with rhG-CSF (4000 U/mL), peak chemiluminescence increased to 73.71 ± 18.51 mV (P < .01). The production of free radicals by PMNL from patients recently exposed to chemotherapy (early samples) was significantly reduced (mean, 25.99 ± 8.16 mV; P < .01) compared with that for controls. By contrast, free radical production by PMNL obtained at bone marrow recovery (late samples) was not different from that by control PMNL (mean, 37.92 ± 12.60 mV; P > .5).

Chemiluminescence was significantly enhanced in early PMNL samples that were preincubated with rhG-CSF (mean, 60.04 ± 13.19 mV; P < .01). It was also significantly enhanced in late PMNL samples (mean, 58.32 ± 18.34 mV; P < .01). Of interest, there was not a significant difference between peak chemiluminescence in defective PMNL obtained from patients and preincubated with G-CSF and peak chemiluminescence in normal control PMNL exposed to G-CSF.

Bactericidal activity was significantly enhanced in early samples of PMNL that were preincubated with rhG-CSF: mean, 39.44% ± 17.56% (P < .01) after 60 min and 52.94% ± 12.39% (P < .05) after 120 min. Killing was also increased in late samples of PMNL preincubated with rhG-CSF: mean, 37.12% ± 14.31% (P < .01) after 60 min and 58.03% ± 12.40% (P < .05) after 120 min. Of interest, after 60 min of incubation, patient PMNL primed with rhG-CSF had significantly less bactericidal activity than control PMNL exposed to rhG-CSF. This difference was not detectable when PMNL were incubated for 120 min.

Effects of rhG-CSF on the bactericidal activity of patient and control PMNL against E. coli. Figure 3 shows the bactericidal activity of PMNL from normal controls and children with cancer. The mean percentage of killing for normal PMNL was 48.63% ± 18.71% after 30 min and 49.64% ± 15.96% after 60 min of incubation. Preincubation of control PMNL with rhG-CSF increased the bactericidal killing to 51.62% ± 20.09% (P < .01) after 30 min and to 58.7% ± 15.6% (P < .01) after 60 min.

The mean percentage of bacteria killed by early samples of PMNL was reduced after 30 min (29.80% ± 8.57%; P < .01) and 60 min (33.80% ± 14.54%; P < .05) of incubation. When PMNL were harvested at bone marrow recovery (late samples), the mean percentage of bacterial killing was 31.65% ± 10.63% (P < 0.05) after 30 min, but it was normal after 60 min (42.60% ± 13.45%; P > .5).

Bactericidal activity was enhanced in early patient samples of PMNL that were preincubated with rhG-CSF: mean, 43.01% ± 17.7% (P < .01) after 30 min and 48.06% ± 11.37% (P < .05) after 60 min. Similar results were found with late samples of PMNL that were primed with rhG-CSF: The mean percentage of killing was 44.67% ± 17.8% (P < .01) after 30 min and 55.84% ± 10.83% (P < .05) after 60 min. The positive effect of rhG-CSF was such that there was no difference in the bactericidal activity of patient and control PMNL primed with rhG-CSF.

Discussion

G-CSF increases the proliferation and differentiation of granulocyte progenitors and modulates several functions displayed by differentiated granulocytes [1]. Several clinical trials have evaluated the effects of G-CSF in treating a number of bone marrow failure syndromes, including those in which marrow damage is the result of intensive cytotoxic chemotherapy. In these conditions, G-CSF has been shown to accelerate the recovery of neutrophil counts [2–4].

In healthy volunteers, a variety of neutrophil functions have been shown to be influenced by in vitro or in vivo exposure of PMNL to G-CSF. Following in vivo administration of G-CSF, normal neutrophils show an increased expression of different membrane proteins, such as the β2 integrins CD11b–CD18 [11], and an increased oxidative burst in response to FMLP [12].

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**Figure 2.** % bactericidal killing (mean ± SD of 19 experiments) of *Staphylococcus aureus* 42D by PMNL from healthy donors and cancer patients receiving chemotherapy. PMNL were or were not primed with rhG-CSF. Early = results for PMNL obtained just after latest dose of chemotherapy; late = results for PMNL obtained at bone marrow recovery. *P < .05*, † .01, and ‡ .001 by Wilcoxon’s paired *t* test.

vitro, G-CSF serves as a chemoattractant [13], enhances superoxide production [12, 14–17], and increases antibody-dependant cellular cytotoxicity [18]; it also induces the expression of the high-affinity receptor FcRI (CD64) [19, 20] and increases the expression of the β2 integrins CD11b–CD18 [11]. Last, preincubation of normal adult PMNL and defective PMNL from human immunodeficiency virus type 1–infected children results in enhanced killing of *S. aureus* [21, 22].

We previously demonstrated that PMNL from children with cancer treated by chemotherapy presents multiple functional defects [10]. While phagocytosis and the expression of receptors for complement factors and immunoglobulins were not modified, H2O2 and O2− production were profoundly altered.

In the present study, we showed that rhG-CSF corrects in vitro the defective respiratory burst activity in children with cancer by increasing the production of free radicals. Thus, for these children, there were no significant differences in chemiluminescence in their healthy and their defective PMNL primed with rhG-CSF.

In addition, we previously demonstrated reduced intracellular killing of *S. aureus* and *E. coli* in children with cancer who had been exposed to chemotherapy or were recovering from drug-induced aplasia [10]. In the current study, rhG-CSF totally corrected the defective bactericidal activity against *E. coli* O54 and greatly improved the bactericidal activity against *S. aureus* 42D. These differences in the killing ability of gram-positive and gram-negative microorganisms might be secondary to biologic properties. In this regard, components from the cell wall specific for either gram-positive or gram-negative bacteria have been well documented [26].

The kinetics and pathophysiologic roles of G-CSF in the body have not been clarified. G-CSF is produced by monocytes,

**Figure 3.** % bactericidal killing (mean ± SD of 19 experiments) of *Escherichia coli* O54 by PMNL from healthy donors and cancer patients receiving chemotherapy. PMNL were or were not primed with rhG-CSF. Early = results for PMNL obtained just after latest dose of chemotherapy; late = results for PMNL obtained at bone marrow recovery. *P < .05* and † .01 by Wilcoxon’s paired *t* test.
macrophages, vascular endothelium, fibroblasts, mesothelium, and neutrophils in response to agents such as endotoxin, tumor necrosis factor, phorbol esters, GM-CSF, interleukin (IL)-1, IL-3, IL-4, and interferon-γ [27]. The production of the G-CSF protein is inducible but not constitutive in normal cells. Serum levels of G-CSF in healthy humans are usually <30 pg/mL, but in conditions of infection, cytotoxic drug therapy, or bone marrow transplantation, levels can rise to 2000 pg/mL [28, 29]. The concentration of G-CSF used in vitro (10^{-4} M) is achievable in patients treated with 1.5–3 μg/kg G-CSF intravenously or subcutaneously [30, 31]. However, at bone marrow recovery, G-CSF decreases to very low levels, which might be insufficient to overcome the long-term deleterious effect of chemotherapy on the bactericidal activity of PMNL. In addition, cytotoxic drugs that affect neutrophil functions might also interfere with molecular processes involved in the G-CSF priming mechanism [32].

G-CSF activates specific metabolic pathways depending on the proliferative or nonproliferative status of the target cell [33]. Thus, activation of the p21<sup>ras</sup>/MAP kinase pathway is limited to proliferative myeloid progenitor cells. In contrast, this pathway is not activated in terminally differentiated granulocytes, which do not show a proliferative response to G-CSF. Thus, PMNL proliferation and activation are controlled by G-CSF through different mechanisms, which might be differentially sensitive to the toxic effect of chemotherapy. This could partly explain why children in our study who had recovered from drug-induced granulocytopenia still presented defects in bactericidal activity even though proliferative activity had returned to normal levels.

Very few data exist on the effects of specific drugs on neutrophil functions. Most of the data were derived from in vitro studies done with nontherapeutic drug concentrations or non-physiologic times of drug exposure. In addition, drugs like cyclophosphamide that are metabolized in vivo have not been able to be evaluated, and results from the in vitro studies have been controversial. In some studies, phagocytosis, O<sub>2</sub>– production, chemotaxis, and cell killing were depressed [34–36], whereas in others there were no significant differences [34, 37–40]. Therefore, the clinical relevance of these studies has yet to be determined. Chemotherapy might interfere with PMNL function in a number of ways [41–43]. Alternatively, PMNL defects in patients exposed to chemotherapy might also be secondary to the deleterious effects of the drugs on accessory cells regulating PMNL functions.

In conclusion, our study demonstrates that, in vivo, chemotherapy induced–defective PMNL are still able to respond biologically to the hematopoietic growth factor G-CSF. Thus, rhG-CSF restores the altered oxidative pathway as well as the defective microbicidal activity present in PMNL from children with cancer. Granulocytes are also known to be functionally sensitive to other cytokines, such as IL-1, IL-6, and tumor necrosis factor [44, 45]. Dysregulation of these cytokines in patients treated with chemotherapy might also play a role in the observed defects in PMNL functions. These different hypotheses are under investigation in our laboratory.

References


