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Action of Granulopoiesis-Stimulating Cytokines rhG-CSF, rhGM-CSF, and rmGM-CSF on Murine Hematopoietic Progenitor Cells for Granulocytes and Macrophages (GM-CFC)

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Summary

The aim of this study was to provide new data to the knowledge of mechanisms by which recombinant human granulocyte colony-stimulating factor (rhG-CSF), recombinant human granulocyte-macrophage colony-stimulating factor (rhGM-CSF) and recombinant murine granulocyte-macrophage colony-stimulating factor (rmGM-CSF) enhance the numbers of colonies growing from hematopoietic progenitor cells for granulocytes and macrophages (GM-CFC) in the murine bone marrow. The *in vitro* technique for cultivating GM-CFC from normal bone marrow cells was used. For evaluation of stimulatory actions of the drugs studied, the factors themselves or sera of mice given these factors were added to the cultures. The factors or the sera were present in the cultures either as the only potentially stimulatory agents or acted jointly with a suboptimum concentration of recombinant murine interleukin-3 (rmIL-3). It was found that both rhG-CSF and rmGM-CSF stimulate the proliferation of GM-CFC by a combination of direct mechanisms (direct actions on the target cells) and indirect effects (effects mediated through the induction of other cytokines and/or growth factors in the murine organism). The rhGM-CSF exhibited somewhat weaker *in vitro* effects in comparison with the other two factors and only indirect effects were noted. Additional *in vivo* experiments documented that, in spite of differences in mechanisms of action of the individual drugs studied on murine bone marrow cells *in vitro*, equal *in vivo* doses of the factors induce quantitatively similar effects on the production of GM-CFC *in vivo.*

Key words
Murine hematopoiesis • GM-CFC • rhG-CSF • rhGM-CSF • rmGM-CSF

Introduction

Granulocyte colony-stimulating factor (G-CSF) (Nagata 1994) and granulocyte-macrophage colony-stimulating factor (GM-CSF) (Rasko and Gough 1994) are at present widely clinically used for stimulation of hematopoiesis suppressed by various damaging agents, most often by cytotoxic chemotherapy or ionizing radiation (Lichtman *et al.* 1993, Welte *et al.* 1996, Honkoop *et al.* 1996, Oh *et al.* 2003, Muller *et al.* 2003, Virchis *et al.* 2004).

Both G-CSF and GM-CSF act in hematopoietic

tissues in a regulatory network of numerous cytokines and hematopoietic growth factors. In this network, individual factors may act directly on target hematopoietic cells or may stimulate the release of other factors by hematopoietic cells or by other, supporting cells present in hematopoietic or other tissues. In other words, the effects of cytokines and growth factors on the proliferation and differentiation of hematopoietic cells can be either direct or indirect. These circumstances may be of importance when estimating the time course of effectiveness of the pertinent factors after their administration and when assessing the best therapeutic combinations of growth factors and cytokines, both in experiments *in vitro* (Drouet *et al.* 2002) and *in vivo* (Herodin *et al.* 2003) as well as in clinical studies (Wu *et al.* 2003).

Recently our laboratory has published several reports on *in vitro* effects of various immunomodulatory agents or of sera of mice administered these agents added to cultures of normal murine bone marrow cells either alone as the only potentially stimulatory factors, or in combination with a known, positively acting cytokine, most often interleukin-3 (IL-3) (Vacek *et al.* 1999, 2000, 2001, 2002). These findings have added new knowledge about the manner in which these immunomodulators execute their hematopoiesis-stimulating activity.

Using this methodological approach, we have provided new information in this report on the mechanisms of the granulopoiesis-stimulating action of G-CSF and GM-CSF and present further data corroborating the fact that these hematopoietic growth factors are involved in the hematopoietic regulatory network. The results of a supplementary *in vivo* experiment show that equal doses of these two factors induce granulopoietic effects of similar intensites in spite of the observation of somewhat different mechanisms and intensities of their *in vitro* actions.

Methods

Drugs

Recombinant human granulocyte colony-stimulating factor (rhG-CSF, Neupogen) was purchased from F. Hoffman-LaRoche Ltd (Basel, Switzerland). Recombinant human granulocyte-macrophage colony-stimulating factor (rhGM-CSF, Leucomax) was obtained from Sandoz Pharma Ltd (Basel, Switzerland). Recombinant murine granulocyte-macrophage colony-stimulating factor (rmGM-CSF) was from Sigma (St. Louis, MO, USA).

In vitro experiments

For determination of granulocyte-macrophage colony-forming cells (GM-CFC), bone marrow cells from the femoral bone marrow were taken by flushing femoral diaphyseal cavity with Iscove's modification of Dulbecco's medium (IMDM), counted with a Coulter Counter (Model ZF, Coulter Electronics, UK) and kept in a melting ice bath until used. The cells were then plated in triplicate onto a semi-solid environment created by a plasma clot (in our modification - see Vacek *et al.* 1990, Pospíšil et al. 2004) containing IMDM plus 20 % fetal calf serum, 10 % citrate bovine plasma and $CaCl_2$ (1.5 mg/ml). For determination of GM-CFC from mice subjected to *in vivo* treatment, conditioned medium from recombinant murine interleukin-3 (rmIL-3)-producing myeloma cell line (Karasuyama and Melchers 1988). This was obtained from the Institute of Hematology and Blood Transfusion (Prague) in the final concentration of rmIL-3 being 1U/ml (1U/ml was defined as a concentration of rmIL-3 yielding the maximum colony number on a concentration-dependence curve). For determination of *in vitro* effects of combinations of various factors on the growth of GM-CFC from normal bone marrow cells, conditioned medium with rmIL-3 was used at a suboptimum concentration of rmIL-3 of 0.3 U/ml yielding approximately 30 % of the maximum colony numbers on a concentration-dependence curve. *In vitro* effects of rhG-CSF and rhGM-CSF were tested at concentrations of 30 ng/ml, those of rmGM-CSF at 3 ng/ml. These concentrations were chosen on the basis of results of preliminary experiments: the concentrations of 30 ng/mL of rhG-CSF and 3 mg/ml of rmGM-CSF were found to have similar effects on the growth of GM-CFC if these actors were present in the cultures as the only stimulatory agents; rhGM-CSF as the only potential stimulator did not induce any GM-CFC over a wide range of concentrations. RhGM-CSF concentration of 30 ng/ml was chosen for further experiments aimed at testing combined action of rhGM-CSF with IL-3. The cultures were incubated in a thermostat (Forma Scientific, USA) for 7 days in a fully humidified atmosphere containing 5 % CO_2 in air. Colonies of at least 50 cells were scored at 40x magnification.

In vivo experiments

Female ICR mice aged 3 months and weighing 26 g on the average were obtained from the breeding

facility of the Medical Faculty, Masaryk University, Brno, Czech Republic. The mice were kept under controlled conditions; standardized pelleted diet and HCl-treated tap water (pH 2-3) were given *ad libitum*. The mice were irradiated with the total whole-body sublethal dose of 6.5 Gy of ^{60}Co γ-rays (Chisostat, Chirana, Praha, Czech Republic) at a dose rate of 0.32 Gy/min. One hour after irradiation the mice received subcutaneously either 5 g of rhG-CSF dissolved in 5 % glucose, or 5 μg of rhGM-CSF dissolved in *aqua pro injectione* or 5 μg of rmGM-CSF dissolved in *aqua pro injectione* in volumes of 0.1 ml. Control mice received 0.1 ml of 5 % glucose. Sampling of femoral bone marrow was performed on day 10 after the irradiation. Numbers of GM-CFC per femur were determined as described above.

Preparation of serum for in vitro experiments

The mice were administered subcutaneously 5 g of rhG-CSF, rhGM-CSF, or rmGM-CSF. Three, 12, or 24 hours after administration of the drugs, the animals were anesthetized intraperitoneally with Narcamon/Rometar solution (5 % Narcamon and 2 % Rometar (both Spofa, Czech Republic) in the ratio of 2.63:1). Blood was collected by cardiac puncture, serum from three mice was pooled in each experimental group and frozen at –20 ^{0}C until *in vitro* testing.

Statistic

Values are presented as means ± S.E.M. Statistical significance of the differences was evaluated by Student's t-test. The significance level was set at $P<0.05$.

Results

The first experiment was aimed at assessing the ability of rhG-CSF, rhGM-CSF and rmGM-CSF to stimulate production of GM-CFC from normal murine bone marrow cells *in vitro*. The results are shown in Figure 1. If the factors were present in the cultures as the only potential hematopoietic stimulators, distinct positive effects were found in the presence of rhG-CSF and rmGM-CSF. RrhGM-CSF exhibited no effects under these conditions. When any of these three factors was added to the culture together with a suboptimum concentration of rmIL-3, a marked potentiation of the yield of GM-CFC occurred in all cases. The highest numbers of GM-CFC (129.3±9.2 GM-CFC per 10^5 bone marrow cells) were obtained when combining rmIL-3 with rhG-CSF, the lowest increase was observed at the combination of rmIL-3 with rhGM-CSF (61.3±5.0 GM-CFC per 10^5 bone marow cells).

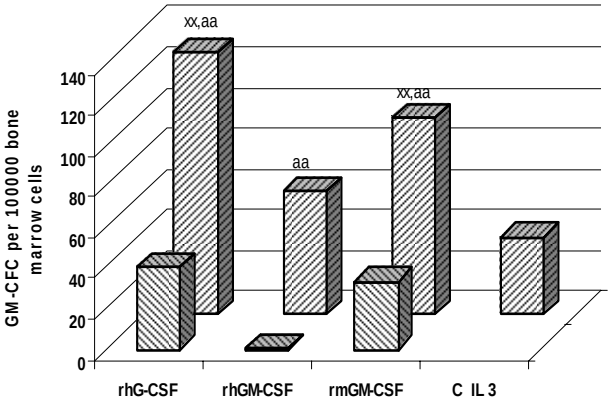

Fig. 1. Effects of rhG-CSF, rhGM-CSF or rmGM-CSF on GM-CFC *in vitro* RhG-CSF (30 ng/ml), rhGM-CSF (30 ng/ml) or rmGM-CSF (3 ng/mL) were tested for induction of GM-CFC proliferation as the only potentially stimulatory agents (fore row of columns) or in combination with a suboptimum concentration of rmIL-3 (rear row of columns). The data are means from three separate experiments. C IL-3 – effect of rmIL-3 alone. xx – $P<0.01$ in comparison with the effects of the drug (rhG-CSF or rmGM-CSF) alone. aa – $P<0.01$ in comparison with the effect of rmIL-3 alone. Statistical comparison between the effects of joint action of rhGM-CSF with rmIL-3 and effects of rhGM-CSF alone was not performed because of zero value of the effects of rhGM-CSF alone.

When rhG-CSF, rhGM-CSF or rmGM-CSF were administered to mice in equal doses of 5 μg and the sera of these mice sampled 3, 12 or 24 hours after the administration were tested in cultures of normal bone marrow cells as the only potential hematopoietic stimulators, different results were obtained when comparing the effects caused by rhG-CSF or rmGM-CSF with those caused by rhGM-CSF (Fig. 2). Rather a large growth of colonies was induced by sera of mice given rhG-CSF (66.8±8.8 GM-CFC per 10^5 bone marrow cells) and rmGM-CSF (54.3±6.2 GM-CFC per 10^5 bone marrow cells) if the sera were sampled in the early interval of 3 hours after administration of the drugs. In contrast no GM-CFC colonies were present in the cultures containing sera of mice administered rhGM-CSF. In the later intervals of 12 and 24 hours after administration of the drugs the numbers of GM-CFC colonies induced by the sera progressively dropped in mice given rhG-CSF or rmGM-CSF. At 24 hours, the sera of mice given any of the factors studied showed no significant effect on GM-CFC.

A somewhat different picture was observed

when the sera of mice given rhG-CSF, rhGM-CSF or rmGM-CSF were added to the cultures concomitantly with a suboptimum concentration of rmIL-3 (Fig. 3). Under these conditions, the serum from control mice induced the formation of only a relatively small number of GM-CFC colonies (39.5±6.2 per 10^5 bone marrow cells). However, a marked potentiating effect of the combined action of sera from mice given any of the three factors studied and rmIL-3 were observed when sera sampled at the early interval of 3 hours after administration of the drugs were tested (112.3±12.2, 98.3±10.3, and 112.4±8.4 GM-CFC per 10^5 bone marrow cells for combination of rmIL-3 with sera of mice given rhG-CSF, rhGM-CSF, and rmGM-CSF, respectively). Moreover, when the sera and rmIL-3 acted jointly, no marked drop of GM-CFC numbers was observed up to the sera sampling interval of 24 hours after administration of the drugs (105.0±8.3, 90.0±6.2, and 106.3±7.3 GM-CFC per 10^5 bone marrow cells for combination of rmIL-3 with sera of mice given rhG-CSF, rhGM-CSF, or rmGM-CSF, respectively). Thus, in combination with rmIL-3, stimulating activities of sera from mice administered any of the factors were retained after the long time interval of 24 hours.

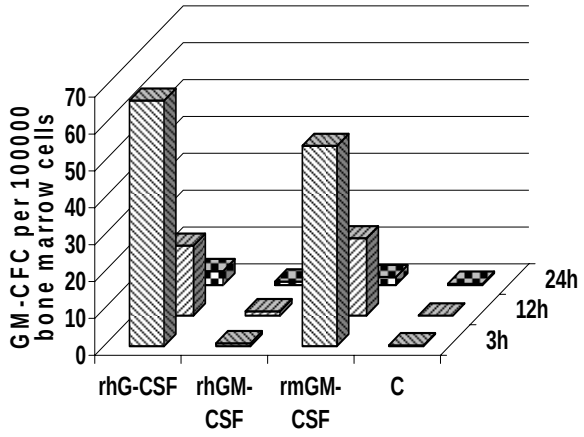

Fig. 2. Effects of sera of mice administered rhG-CSF, rhGM-CSF or rmGM-CSF on GM-CFC *in vitro.* RhG-CSF, rhGM-CSF or rmGM-CSF were administered to mice in doses of 5 μg per mouse. Sera were sampled after 3 hours (first row of columns), 12 hours (middle row of columns), or 24 hours (last row of columns) and tested for induction of GM-CFC proliferation as the only potentially stimulatory agents. The data are means from three separate experiments. C – effect of sera of control mice.

Since equal doses of 5 μg of rhG-CSF, rhGM-CSF, and rmGM-CSF were given to mice whose sera were used for *in vitro* experiments, we were interested in comparing the *in vivo* effects of these doses of the drugs on the number of GM-CFC in the bone marrow suppressed by a sublethal dose of 6.5 Gy γ-rays. As follows from Figure 4, the effects were positive and mutually comparable. Whereas in the controls 7.6±1.4 GM-CFC per femur were found on day 10 after irradiation, in mice treated with rhG-CSF, rhGM-CSF and rmGM-CSF the observed values of GM-CFC per femur were 26.3±2.2, 28.7±3.4, and 32.4±4.2, respectively.

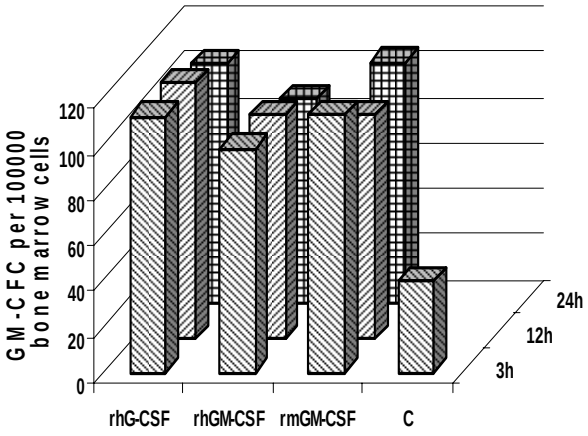

Fig. 3. Effects of combined action of sera of mice administered rhG-CSF, rhGM-CSF or rmGM-CSF and of rmIL-3 on GM-CFC *in vitro.* RhG-CSF, rhGM-CSF or rmGM-CSF were administered to mice in doses of 5 μg per mouse. Sera were sampled after 3 ours (first row of columns), 12 hours (middle row of columns) and 24 hours (last row of columns) and tested for induction of proliferation of GM-CFC in a joint action with a suboptimum concentration of rmIL-3. C – combined effect of sera of control mice and of rmIL-3. The data are means from three separate experiments. Values in all experimental groups and at all time time intervals are statistically significantly higher ($P<0.01$) in comparison with C.

Discussion

Several data obtained in the present study need to be discussed when evaluating the results obtained. RhG-CSF and rmGM-CSF, but not rhGM-CSF, were able to stimulate production of GM-CFC colonies from normal bone marrow cells when present in cultures as the only potential hematopoietic stimulators. This finding suggests that rhG-CSF and rmGM-CSF can directly stimulate the proliferation of hematopoietic progenitor cells for granulocytes and macrophages. Joint action of any of the factors with rmIL-3 evoked distinct potentiating effects on GM-CFC which were, however, weakest again in the case of rhGM-CSF. The serum of mice administered rhGM-CSF had no effect on GM-CFC if present in the cultures as the only potential stimulator.

It was reported that human and murine GM-CSF exhibit only 56 % amino acid sequence identity and do not manifest cross-species biological activity (Rasko and Gough 1994). Under our experimental conditions this is only partially true. As follows from our findings, some activity of rhGM-CSF on proliferation of murine GM-CFC was observed if rhGM-CSF could be geared to the hematopoietic growth factor and cytokine network by interaction with rmIL-3 in cultures and with the organism's milieu. On the other hand, there are no problems with the lack of cross-reactivity between human and murine G-CSF because of their higher amino acid sequence identity (74 %), (Nicola *et al.*1985). This fact is also reflected in our results.

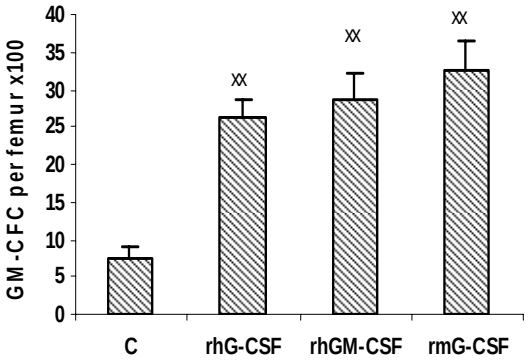

Fig. 4. Effects of rhG-CSF, rhGM-CSF or rmGM-CSF on GM-CFC *in vivo* in bone marrow of irradiated mice. Mice were irradiated with the dose of 6.5 Gy of γ-rays and given 5 μg of rhG-CSF, rhGM-CSF or rmGM-CSF 1 hour later. Numbers of GM-CFC per femur were determined on day 10 after irradiation and administration of the drugs. C – control irradiated mice. The data are means ± S.E.M. from two separate experiments; 10 mice per group were used. xx – $P<0.01$ in comparison with C.

The progressive drop in the effectiveness of sera from mice given rhG-CSF or rmGM-CSF sampled between 3 and 24 hours after administration of the drugs are illustrated in Figure 2. This opens a question whether the colony-stimulating activity of the serum in the early time intervals after injecting the drugs was mediated by factors induced by rhG-CSF or rmGM-CSF in the recipient's organism, or if simply the effects of these drugs alone persisting in the serum were observed. Both the drugs are relatively short-lived in the organism because the data on humans show that the half-life of rhG-CSF is about one hour (Akizawa *et al.* 1995, Watari *et al.* 1997) and that of rhGM-CSF 2 to 3 hours (Cetingul *et al.* 1997). Three hours after the administration of the drugs, the serum of the mice could still contain sufficient concentrations of the factors capable of stimulating the growth of colonies from GM-CFC purely by their direct effects. In that case, Figure 2 would present very similar data to those shown in a part of Figure 1. A new input into the decision-making process on the role of contingent indirect effects of the factors studied can be obtained from the assessment of Figure 3: In another experimental setting, i.e. in a situation when combined action of sera from mice administered the studied factors and of a suboptimum concentration of rmIL-3 was tested, the stimulatory effects of all the three factors were retained till 24 hours after their administration. Since during the interval between 3 and 24 hours the concentration of the injected factors into the serum had to fall progessively down and since the effect of rmIL-3 was the same in all cultures, the high numbers of GM-CFC found also in cultures containg serum sampled 24 hours after administration of the drugs can only be explained by indirect effects of the drugs studied, i.e. by the effects mediated by induced production of other substances stimulating the GM-CFC proliferation. Thus, under our experimental conditions, rhG-CSF and rmGM-CSF act on murine hematopoietic progenitor cells GM-CFC through a combination of direct and indirect effects, whereas rhGM-CSF is able to stimulate murine hematopoiesis only indirectly.

The ability to influence positively hematopoiesis suppressed by ionizing radiation is a known fact both for G-CSF and GM-CSF (Neta *et al.* 1988, Patchen *et al.* 1993). Our findings corroborate the usefulness of both these factors in the treatment of radiation-induced myelosuppression. They also show that even when operating through different mechanisms in terms of different participation of direct and indirect effects, all the three hematopoietic growth factors studied, i.e. rhG-CSF, rhGM-CSF, and rmGM-CSF, have very similar positive *in vivo* effects on the number of granulocyte-macrophage progenitor cells when given in equal doses to irradiated mice.

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Reprint requests

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