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Adenosine Potentiates Stimulatory Effects on Granulocyte-Macrophage Hematopoietic Progenitor Cells in Vitro of IL-3 and SCF, but Not Those of G-CSF, GM-CSF and IL-11

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Summary

The aim of the studies was to ascertain if adenosine is able to co-operate with selected hematopoietic growth factors and cytokines, namely with granulocyte colony-stimulating factor (G-CSF), granulocyte-macrophage colony-stimulating factor (GM-CSF), stem cell factor (SCF), interleukin-3 (IL-3), and interleukin-11 (IL-11), in inducing the growth of colonies from hematopoietic progenitor cells for granulocytes and macrophages (GM-CFC) from normal bone marrow cells *in vitro*. Adenosine was found not to produce any colonies when present in the cultures as the only potential stimulator. All the tested cytokines and growth factors were observed to induce the growth of distinct numbers of GM-CFC colonies, with the exception of IL-11. When suboptimal concentrations of the evaluated cytokines and growth factors were tested in the cultures in which various concentrations of adenosine were concomitantly present, mutually potentiating effects were found in the case of IL-3 and SCF. These results confirm the role of adenosine in regulation of granulopoiesis and predict IL-3 and SCF as candidates for further *in vivo* studies of their combined administration with adenosine.

Key words

Hematopoiesis • Adenosine • Cytokines • Hematopoietic growth factors

Introduction

Hematopoiesis is a complex system of processes which is under the control of a network of numerous regulatory factors. Combining various cytokines and growth factors has been shown to increase therapeutic efficiency under myelosuppressive situations in experimental animals as well as in humans (e.g., Neta *et al*. 1988, Herodin *et al*. 2003, Wu *et al*. 2003). In our earlier studies we have shown that extracellular adenosine may also play a role as a positive regulator of hematopoiesis: it was found that combined administration of dipyridamole, a drug inhibiting the cellular uptake of adenosine, and adenosine monophosphate (AMP), an

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adenosine prodrug, enhances hematopoesis in normal and myelosuppressed mice and synergizes with the effects of granulocyte colony-stimulating factor (G-CSF) (Pospíšil *et al*. 1995, 1998, Hofer *et al*. 1999, 2001, 2002, Weiterová *et al*. 2000).

Interactions of various growth factors and cytokines have also been an object of numerous *in vitro* studies (e.g., McNiece *et al.* 1988, Lucas *et al.* 1999, Drouet *et al*. 2002, Gammaitoni *et al.* 2003, Zech *et al.* 2003). Such research makes it possible to obtain information about mechanisms of regulatory processes in hematopoiesis but may also point to drug combinations suitable for further *in vivo* testing or *ex vivo* procedures of clinical significance. In the present study, we have tested combinations of adenosine with selected hematopoietic growth factors (interleukin-3 (IL-3), interleukin-11 (IL-11), stem cell factor (SCF), granulocyte macrophage colony-stimulating factor (GM-CSF), and G-CSF) on the *in vitro* growth of colonies originating from granulocyte-macrophage colony-forming cells (GM-CFC). The principal aim of the study has been to find those factors which are able to synergize mutually with adenosine *in vitro* and which might be potentially effective when combined with adenosine or its analogs in the treatment of myelosuppressive states *in vivo*.

Materials and Methods

Recombinant murine granulocyte-macrophage colony-stimulating factor (GM-CSF), recombinant murine granulocyte colony-stimulating factor (G-CSF), recombinant murine stem cell factor (SCF), recombinant murine interleukin-3 (IL-3), recombinant murine interleukin-11 (IL-11), and adenosine were obtained from Sigma (St. Louis, MO, USA). For determination of granulocyte-macrophage colony-forming cells (GM-CFC), femoral bone marrow cells from the male $(CBAxC57BL)F_1$ mice were obtained 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, Pospíšil *et al.* 2004, Hofer *et al.* 2005) containing IMDM plus 20 % fetal calf serum, 10 % citrate bovine plasma, and $CaCl_2$ (1.5 mg/ml). Immediately after plating, the tested drugs were added to the cultures. The cultures were incubated in a thermostat (Forma Scientific, USA) for 7 days in a fully humidified atmosphere containing air with 5 % CO_2. Colonies of at least 50 cells were scored at 40x magnification. Values are presented as means ± S.E.M. Statistical significance of the differences was evaluated by Kruskal-Wallis ANOVA followed by Mann-Whitney U test or by t-test where appropriate. The significance level was set at $P<0.05$.

Results

Numbers of GM-CFC per 10^5 normal bone marrow cells were determined in a series of *in vitro* experiments in which the ability of various substances and their combinations to induce the growth of GM-CFC colonies was tested.

Adenosine alone, added to the bone marrow cultures in a wide range of concentrations (0.025 to 100 μM), did not induce any growth of GM-CFC (results not shown).

Experiments testing selected cytokines and hematopoietic growth factors alone served predominantly to find suitable concentrations of the mentioned substances which would be effective but suboptimal from the point of view of their abilities to induce the growth of GM-CFC. These concentrations could be suitable for subsequent experiments evaluating their combined action with adenosine. All of the mentioned cytokines and hematopoietic growth factors were able to induce the growth of GM-CFC in a wide range of concentrations with the exception of IL-11 which induced only the formation of low number of small cell clusters not complying with the definition of colonies (> 50 cells). GM-CSF was found to be the best GM-CFC stimulator among the cytokines and growth factors evaluated, producing about 130 GM-CFC per 10^5 bone marrow cells at a concentration of 0.06 nM (further results on the effects of the selected cytokines and growth factors alone are not presented).

A suboptimal concentration of 0.003 nM IL-3 was used for the evaluation of combined effects of IL-3 with adenosine. Adenosine was added to the cultures containing 0.003 nM IL-3 in concentrations ranging between 0.25 μM and 10 μM. The results are shown in Figure 1. Adenosine in concentrations of 0.10 μM, 0.25 μM and 0.50 μM was found to potentiate significantly the GM-CFC colony growth in comparison with cultures containing 0.003 nM IL-3 alone by 49 %, 38 % and 40 %, respectively.

Adenosine was not found to synergize with

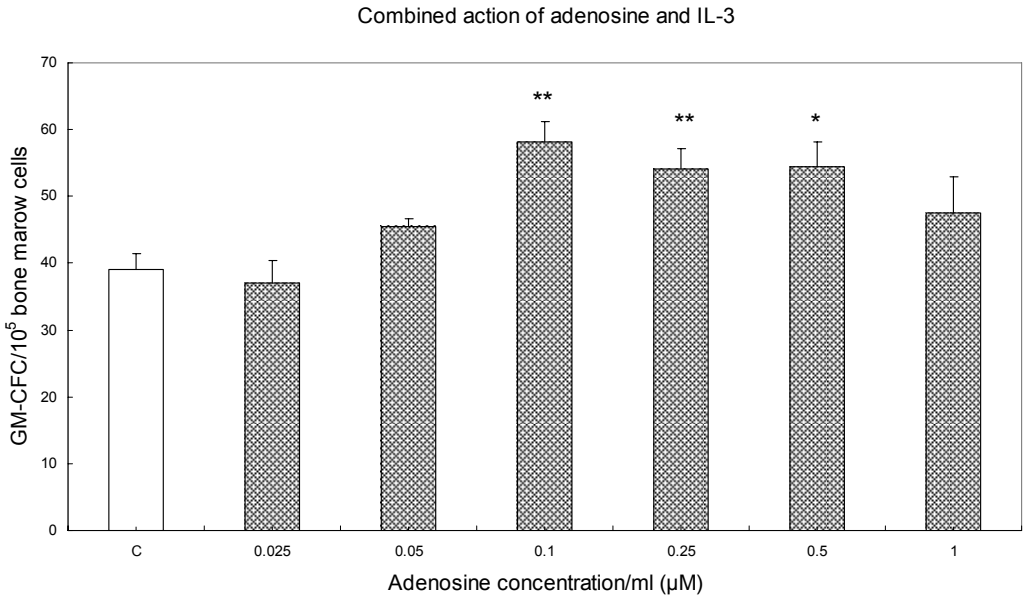

Fig. 1. GM-CFC per 10^5 normal bone marrow cells as a function of adenosine concentration. Open columns (C, controls) – cultures in which the growth of GM-CFC was stimulated with a suboptimal concentration of 0.003 nM IL-3. Shaded columns – cultures in which the growth of GM-CFC was stimulated by the combined action of adenosine in concentrations given and 0.003 nM IL-3. Values are means ± SEM. *,** P<0.05, P<0.01, respectively, in comparison with controls.

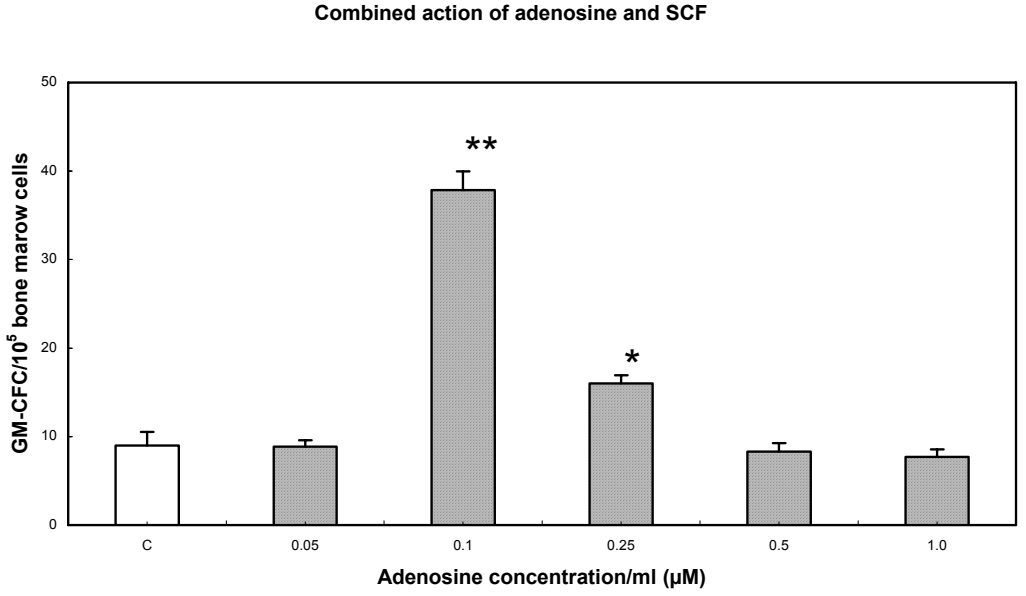

Fig. 2. GM-CFC per 10^5 normal bone marrow cells as a function of adenosine concentration. Open columns (C, controls) – cultures in which the growth of GM-CFC was stimulated with a suboptimal con-centration of 0.2 μM SCF. Shaded columns – cultures in which the growth of GM-CFC was stimulated by the combined action of adenosine in concentrations given and 0.2 μM SCF. Values are means ± SEM. *,** P<0.05, and P<0.01, respectively, in comparison with controls.

IL-11 to produce colonies of 50 or more cells.

A suboptimal concentration of 0.2 μM SCF was used for the evaluation of combined effects of SCF with adenosine (Fig. 2). Adenosine in concentrations of 0.10 μM and 0.25 μM was was found to potentiate significantly the GM-CFC colony growth in comparison with cultures containing 0.2 μM SCF alone by 321 % and 100 %, respectively.

Adenosine added in a wide range of concentrations to suboptimal concentrations of either G-CSF (0.05 nM) or GM-CSF (0.007 nM) did not potentiate the action of G-CSF or GM-CSF on the growth of GM-CFC (results not shown).

Discussion

Our results show that adenosine alone is not able to stimulate the growth of GM-CFC in cultures of normal bone marrow cells. However, adenosine can potentiate *in vitro* stimulatory effects of some hematopoietic growth factors and cytokines. These results confirm the previously postulated role of adenosine in regulation of myelopoiesis.

The bell-shaped synergistic effect of adenosine with IL-3 and SCF, especially pronounced in the case of SCF, bears evidence about the existence of a rather narrow optimum of effective adenosine *in vitro* concentrations.

If considered separately from our previously published papers, the present findings cannot determine, whether the observed effects of adenosine are extracellular, receptor-mediated, or intracellular, resulting from adenosine uptake. However, earlier *in vivo* studies performed in our laboratory have shown that the stimulatory action of AMP (an adenosine prodrug) given alone on the compartment of GM-CFC can be further potentiated by dipyridamole, which preserves adenosine in the extracellular space (Pospíšil *et al.*1992). Therefore, it may be suggested that the *in vitro* effects of adenosine presented here are also receptor-mediated. Final confirmation of this assumption requires additional experiments with synthetic adenosine receptor agonists having no intracellular mode of action.

Another question related to the mechanisms of the observed effects concerns direct vs. indirect action of adenosine on GM-CFC. Direct action would be the result of an interaction of adenosine with GM-CFC themselves. In the case of indirect action, the observed effects would be the result of an interaction of adenosine with other cells, which would subsequently stimulate the growth of GM-CFC by producting other stimulatory cytokines and/or hematopoietic growth factors. Whereas our previously performed *in vivo* studies (Pospíšil *et al.* 1992, 1995, 1998, Hofer *et al.* 1999, 2001, 2002, Weiterová *et al.* 2000) could not give an answer to this question, the results of these *in vitro* experiments might suggest a direct effect of adenosine on GM-CFC since the milieu of the cultures is permissive exclusively for the proliferation of GM-CFC. However, a final answer to this questions cannot be given, because at the moment of establishing the cultures in the presence of the drug tested all bone marrow cells are still present. Determination of adenosine receptors on various cells of the hematopoietic system may provide additional information in this direction. Currently available literary data refer only to the presence of adenosine receptors in mature blood cells (Cronstein *et al.* 1990, 1992, Walker *et al.* 1997, Gessi *et al.* 2000).

There is an interesting lack of the ability of adenosine to support the GM-CFC-stimulating action of G-CSF *in vitro* because previously obtained results from experiments in mice clearly documented the mutually potentiating effects on GM-CFC of drugs elevating extracellular adenosine and G-CSF *in vivo* (Hofer *et al.* 1999, 2001, 2002, Pospíšil *et al.* 1995, 1998, 2004). The striking difference between *in vitro* and *in vivo* findings on the ability of adenosine to co-operate with G-CSF in stimulating the GM-CFC colony-forming activity may be explained by a specific nature of their co-operation requiring the presence of another factor(s) not available in our *in vitro* cultures.

The differences in the percentage increase of GM-CFC after combined action of IL-3 and SCF with adenosine in comparison with the value obtained by the cytokine or growth factor alone cannot be taken as an expression of real differences in the *in vitro* and certainly not in the *in vivo* effectiveness of the substances tested. Even predications concerning comparisons of the *in vitro* efficacy itself would have to be based on the results of very extensive experiments comprising combinations of many concentrations of adenosine and of the cytokines and growth factors tested. Our experiments were not aimed at this goal.

In spite of the above mentioned open questions concerning mechanisms of the observed stimulatory effects of adenosine on the proliferation of hematopoietic progenitor cells, the data obtained in the *in vitro* experiments presented here support our previous conclusions (based upon results of extensive *in vivo* studies) about the potential of adenosine to find practical application in the treatment of myelosuppression. Besides adenosine itself, an open field for further studies is also represented by the investigation of the effects of synthetic adenosine receptor agonists. It has recently been reported by us (Pospíšil *et al.* 2004) as well as by others (Bar-Yehuda *et al.* 2002) that an adenosine receptor agonist selective for adenosine A_3 receptors, N^6-(3-iodobenzyl)adenosine-5'-N-methyluron-amide (IB-MECA), stimulates granulopoiesis. Therefore, our results obtained *in vitro* can serve as a starting point for *in vivo* experiments in which IL-3 and SCF, which have been shown to be effective in cooperating with adenosine in the stimulation of the growth of GM-CFC *in vitro*, will be

tested from the point of view of their effectiveness to act in concert with adenosine or IB-MECA in the treatment of myelosuppression *in vivo*. Without *in vivo* experiments combining pharmacological stimulation of adenosine receptors with the action of various cytokines and hematopoietic growth factors it will not be possible to give a more precise conclusion determining the most hopeful candidates among the cytokines and growth factors to be evaluated in future clinical studies.

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