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Activation of Adenosine A_3 Receptors Potentiates Stimulatory Effects of IL-3, SCF, and GM-CSF on Mouse Granulocyte-Macrophage Hematopoietic Progenitor Cells

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Summary

Adenosine A_3 receptor agonist N^6-(3-iodobenzyl)adenosine-5'-N-methyluronamide (IB-MECA) has been tested from the point of view of potentiating the effects of hematopoietic growth factors interleukin-3 (IL-3), stem cell factor (SCF), granulocyte-macrophage colony-stimulating factor (GM-CSF), and granulocyte colony-stimulating factor (G-CSF) on the growth of hematopoietic progenitor cells for granulocytes and macrophages (GM-CFC) in suspension of normal mouse bone marrow cells *in vitro*. IB-MECA alone induced no GM-CFC growth. Significant elevation of numbers of GM-CFC evoked by the combinations of IB-MECA with IL-3, SCF, or GM-CSF as compared with these growth factors alone has been noted. Combination of IB-MECA with G-CSF did not induce significantly higher numbers of GM-CFC in comparison with G-CSF alone. Joint action of three drugs, namely of IB-MECA + IL-3 + GM-CSF, produced significantly higher numbers of GM-CFC in comparison with the combinations of IB-MECA + IL-3, IB-MECA + GM-CSF, or IL-3 + GM-CSF. These results give evidence of a significant role of selective activation of adenosine A_3 receptors in stimulation of the growth of granulocyte/macrophage hematopoietic progenitor cells.

Key words

Hematopoiesis • Adenosine A_3 receptor • IB-MECA • Hematopoietic growth factors

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Introduction

Modulatory effects of activation of cell membrane adenosine receptors on hematopoiesis have been shown in several studies. Four types of adenosine receptors, i.e., A_1, A_{2a}, A_{2b}, and A_3 coupled to G proteins, have been classified (Fredholm *et al*. 2000). These receptors can be stimulated either non-selectively or selectively. A non-selective activation of adenosine receptors by their natural agonist adenosine has been achieved by a combination of drugs elevating extracellular adenosine, i.e., of adenosine monophosphate, an adenosine prodrug, with dipyridamole, which prevents the cellular uptake of adenosine. This drug combination has been found in our laboratory to stimulate hematopoiesis in normal and irradiated mice (Pospíšil *et al.* 1992, 1993a,b, 1995a, 2001, Hofer *et al.* 1995, Weiterová *et al.* 2007). Further studies carried out by our group as well as by others have revealed that an enhancement of hematopoietic processes can also been attained by selective activation of adenosine A_3 receptors using their selective agonist N^6-(3-iodobenzyl)adenosine-5'-N-methyluronamide (IB-MECA) (Fishman *et al.* 2000, Bar-Yehuda *et al.* 2002, Pospíšil *et al.* 2004, Hofer *et al.* 2006a, 2007).

Combined modality treatment of myelosuppression induced by various factors, most often by ionizing radiation or cytotoxic chemotherapy, is for a long time in the center of attention of hematologists seeking the best outcomes of hematopoiesis-stimulating approaches in combination with low incidence and intensity of undesirable side effects (Weiss *et al.* 1990, Pospíšil *et al.* 1995b, Herodin *et al.* 2003). Similar requirements may be

imposed on contingent combined therapies using adenosine receptor agonists. In several studies, positive results have been obtained when testing joint administration of non-selective adenosine receptor-activating drugs adenosine monophosphate and dipyridamole with granulocyte colony-stimulating factor (G-CSF) (Pospíšil *et al.* 1995b, 1998, Hofer *et al.* 1999, 2001, 2002, Weiterová *et al.* 2000). This above-mentioned topic is summarized in our review (Hofer and Pospíšil 2006).

The mentioned studies on hematopoietic effects of adenosine receptor agonists have been performed *in vivo*. However, testing combined modality treatments in all potentially promising variations may represent a long-termed task often going beyond the experimentator's possibilities. In these cases *in vitro* tests may be helpful in predicting factors with high probability of success in subsequent *in vivo* experiments. Recently we have published results of an *in vitro* study in which combined actions of the natural non-selective adenosine receptor agonist, adenosine, and several hematopoietic growth factors on the growth of hematopoietic progenitor cells for granulocytes and macrophages (GM-CFC) have been evaluated. Adenosine has been found to potentiate stimulatory effects on granulocyte-macrophage progenitor cells of stem cell factor (SCF) and interleukin-3 (IL-3) (Hofer *et al.* 2006b). However, the results of selective stimulation of adenosine A_3 receptors might lead to an even more detailed insight into mechanisms of interconnections between adenosine receptor activation and the action of hematopoietic growth factors. Therefore, a similar study has been performed testing the effects on GM-CFC growth induced by the joint action of the selective A_3 adenosine receptor agonist IB-MECA and certain growth factors, namely IL-3, SCF, G-CSF and granulocyte-macrophage colony stimulating factor (GM-CSF).

Methods

Drugs

N^6-(3-iodobenzyl)adenosine-5'-N methyluronamide (IB-MECA) was purchased from Tocris (Ellisville, MO, USA). Recombinant murine granulocyte-macrophage colony-stimulating factor (GM-CSF), recombinant murine granulocyte colony-stimulating factor (G-CSF), recombinant murine stem cell factor (SCF), and recombinant murine interleukin-3 (IL-3) were obtained from Sigma (St. Louis, MO, USA).

Animals

Normal mouse bone marrow for use in *in vitro* testing was obtained from male $B10CBAF_1$ mice aged 3 months and weighing about 30 g. The mice were kept under controlled conditions; standardized pelleted diet and HCl-treated tap water were available *ad libitum*. The use and treatment of the animals followed the European Community Guidelines as accepted principles for the use of experimental animals. The experiments were performed with the approval of the Institute's Ethics Committee.

Counts of granulocyte-macrophage progenitor cells (GM-CFC)

For determination of granulocyte-macrophage colony-forming cells (GM-CFC), femoral bone marrow cells from the male $(CBAxC57BL)F_1$ mice 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 (Pospíšil *et al.* 2004, Hofer *et al.* 2005, 2008) 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.

For experiments testing combined effects of IB-MECA and hematopoietic growth factors, a wide range of concentrations of IB-MECA together with one selected concentration of each hematopoietic growth factor were used. The concentrations of the hematopoietic factors chosen were effective but suboptimum from the point of view of their abilities to induce the growth of GM-CFC, so that they would be suitable for experiments evaluating their combined actions with IB-MECA. These suboptimum concentrations of the hematopoietic factors induced the growth of roughly 50 % of GM-CFC in comparison with the most effective concentrations and were equal to those used in the previous study assessing their combined effects with adenosine (Hofer *et al.* 2006b).

Statistics

The values obtained are presented as means ± S.E.M. Statistical significance of the differences was evaluated by Kruskal-Wallis ANOVA followed by Mann-Whitney U test. The significance level was set at $P<0.05$.

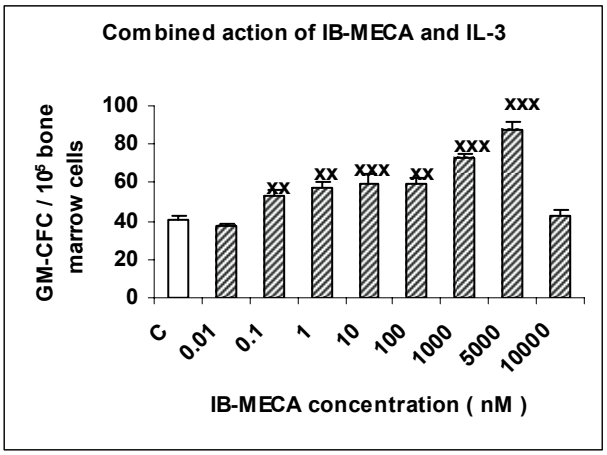

Fig. 1. GM-CFC per 10^5 normal bone marrow cells as a function of IB-MECA concentration acting in the presence of IL-3. Open column (C, controls) – cultures in which the growth of GM-CFC was stimulated with a suboptimum concentration of 0.003 nM IL-3. Shaded columns – cultures in which the growth of GM-CFC was stimulated by the combined action of IB-MECA in concentrations given and 0.003 nM IL-3. Values are means ± S.E.M. xx, xxx P<0.01, P<0.001, respectively, in comparison with controls.

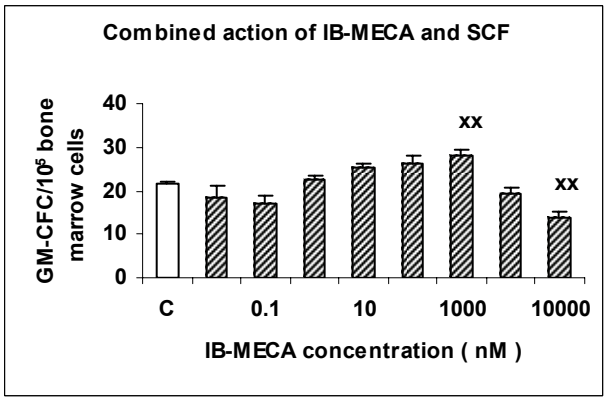

Fig. 2. GM-CFC per 10^5 normal bone marrow cells as a function of IB-MECA concentration acting in the presence of SCF. Open column (C, controls) – cultures in which the growth of GM-CFC was stimulated with a suboptimum concentration of 0.2 μM SCF. Shaded columns – cultures in which the growth of GM-CFC was stimulated by the combined action of IB-MECA in concentrations given and 0.2 μM SCF. Values are means ± S.E.M. xx P<0.01, in comparison with controls.

Results

Numbers of GM-CFC colonies per 10^5 bone marrow cells induced by IB-MECA alone

IB-MECA alone induced no GM-CFC colony growth in the range of concentrations between 0.1 and 10 000 nM (results not shown).

Combined effects of IB-MECA and IL-3

A suboptimum concentration of 0.003 nM IL-3 was used for the study. IB-MECA was found to potentiate significantly the stimulatory effects of the above concentration of IL-3 in a wide range of concentrations (0.1 to 5000 nM). The per cent elevation of the GM-CFC numbers amounted between 131 % at 0.1 nM IB-MECA and 214 % at 5000 nM IB-MECA tested in a combination with IL-3 as compared with IL-3 alone. When a high concentration of 10000 nM IB-MECA was tested, a drop in GM-CFC numbers was observed, probably because of IB-MECA toxicity. The results are shown in Figure 1.

Combined effects of IB-MECA and SCF

A suboptimum concentration of 0.2 μM SCF was used. Only a rather high concentration of 1000 nM IB-MECA was observed to potentiate significantly the effect of the above concentration of SCF. The per cent elevation of the GM-CFC numbers amounted to 132 % at this concentration of IB-MECA tested in a combination with SCF, in comparison with SCF alone. Toxicity was again found when testing the IB-MECA concentrations of 5000 and 10000 nM. The results are shown in Figure 2.

Combined effects of IB-MECA and GM-CSF

A suboptimum concentration of 0.007 nM GM-CSF was used. All the concentrations of IB-MECA between 0.01 and 1000 nM tested in combination with the above concentration of GM-CSF were found to induce higher numbers of GM-CFC in comparison with GM-CSF alone. The per cent elevation of the GM-CFC numbers in this concentration range was between 125 % to 136 % when testing IB-MECA in combination with GM-CSF, in comparison with GM-CSF alone. Nevertheless, none of the differences evaluated individually attained the statistical significance. However, the distribution of the values between 0.1 and 1000 nM IB-MECA in combined treatment with G-CSF made it possible to assess them for the purpose of an additional statistical evaluation as one entity. This entity was compared with the value for GM-CSF alone and significance of the difference (P<0.01) was obtained. Toxicity was again observed when high concentrations of IB-MECA were used. The results are shown in Figure 3.

Combined effects of IB-MECA and G-CSF

A suboptimum concentration of 0.05 nM G-CSF was used. No potentiation of the effects in the combined treatment with IB-MECA and G-CSF was found in

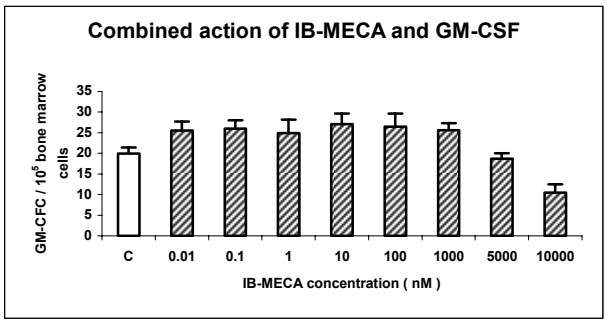

Fig. 3. GM-CFC per 10^5 normal bone marrow cells as a function of IB-MECA concentration acting in the presence of GM-CSF. Open column (C, controls) – cultures in which the growth of GM-CFC was stimulated with a suboptimum concentration of 0.007 nM GM-CSF. Shaded columns – cultures in which the growth of GM-CFC was stimulated by the combined action of IB-MECA in concentrations given and 0.007 nM GM-CSF. Values are means ± S.E.M. When the values corresponding to the concentrations of IB-MECA between 0.1 and 1000 nM were taken as one entity, significance (P<0.01) between this entity and the value for G-CSF alone (C) was observed.

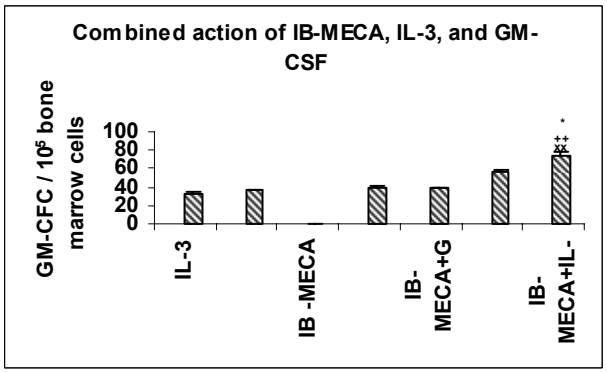

Fig. 4. GM-CFC per 10^5 normal bone marrow cells in cultures treated with various combinations of IB-MECA (0.1 nM), IL-3 (0.003 nM), and GM-CSF (0.007 nM). Values are means ± S.E.M. * P<0.05, in comparison with IL-3+GM-CSF. ++ P<0.01, in comparison with IB-MECA+IL-3. xx P<0.01, in comparison with IB-MECA+GM-CSF.

comparison with G-CSF in a wide range of IB-MECA concentrations studied (results not shown).

Combined effects of IB-MECA, IL-3, and GM-CSF

An additional, separate experiment was performed to test the effects of a three-drug combination of IB-MECA, IL-3, and GM-CSF. The combination of IB-MECA (0.1 nM) added to the cultures of the normal bone marrow cells concomitantly with IL-3 (0.003 nM) and GM-CSF (0.007 nM) induced significantly higher numbers of GM-CFC in comparison with the effects of combinations of two drugs, namely IB-MECA + IL-3, IB-MECA + GM-CSF, or IL-3 + GM-CSF. The statistical significances were at the levels of P<0.05, when the combination of the three drugs was compared with the combination of IL-3 + GM-CSF, and P<0.01, when the three-drug combination was compared to the combinations of IB-MECA + IL-3 or IB-MECA + GM-CSF. The results are shown in Figure 4.

Discussion

The results show that selective activation of adenosine A_3 receptors by IB-MECA added to cultures of normal mouse bone marrow cells as the only potential hematopoietic stimulator does not induce the growth of GM-CFC. However, IB-MECA can potentiate *in vitro* effects of some hematopoietic growth factors. These results are roughly similar to those reported earlier for non-selective natural adenosine receptor agonist, adenosine (Hofer *et al.* 2006b).

However, there exist several indispensable differences between the previously reported results on adenosine and those obtained with IB-MECA, as well as some new conclusions may be drawn taking into account the results of studies with IB-MECA.

The previous study (Hofer *et al.* 2006b) could not determine whether the observed effects of adenosine were extracellular, receptor-mediated, or intracellular, resulting from adenosine uptake. However, the similarity of the findings when utilizing the artificially prepared selective A_3 receptor agonist, which has been synthesized to interact with the cell only *via* the adenosine receptor system, strongly suggest that the previously observed effects of adenosine are extracellular, receptor-mediated. Thus, also the conclusions on receptor-mediated hematopoiesis-stimulating effects of adenosine made in a number of earlier *in vivo* studies utilizing dipyridamole, which prevents the cellular uptake of adenosine and supports thus its receptor action (for review see Hofer and Pospíšil 2006), are confirmed by results presented here.

Our results indicate higher *in vitro* efficacy of IB-MECA in comparison with the non-selective action of adenosine, if the ability of potentiation of their effects on the proliferation of GM-CFC in combination with some hematopoietic growth factors tested is assessed. The range of concentrations of IB-MECA which potentiate the effects of IL-3, an important hematopoietic growth factor, which was reported to have proliferation-stimulating effects on multiple hematopoietic progenitror cells (Eder *et al.* 1997), is wider in comparison with those of adenosine (Hofer *et al.* 2006b). Earlier we have observed no significant mutual potentiation of the GM-CFC growth by adenosine with GM-CSF (Hofer *et al.* 2006b), which is considered as a

major regulator governing granulocyte and macrophage lineage populations (Fleetwood *et al.* 2005). In the present study, an appropriate statistical treatment of the data has revealed a significant potentiation of the GM-CSF-induced growth of GM-CFC by IB-MECA in the range of concentrations of 0.01 - 1000 nM (see legend to Fig. 3).

SCF plays a fundamental role as both growth and survival factor for hematopoietic progenitor cells (Smith *et al.* 2001). Therefore, the finding of mutually potentiating effects of SCF and IB-MECA on the growth of GM-CFC is of interest. However, it should be taken into account that the range of effective concentrations of IB-MECA is very narrow (in fact, only one of the concentrations tested). Moreover, the effective IB-MECA concentration is rather high (1000 nM) in comparison with the effective concentrations of adenosine (100 and 250 nM) (Hofer *et al.* 2006b).

The previous *in vitro* study did not reveal any influence of various concentrations of adenosine on the growth-promoting effects of G-CSF on GM-CFC (Hofer *et al.* 2006b). This was a surprise because several *in vivo* studies documented mutually potentiating effects of extracellular adenosine and G-CSF (Hofer *et al.* 1999, 2001, 2002, Pospíšil *et al.* 1995b, 1998). However, as shown by this study, IB-MECA did not potentiate the effects of G-CSF in any of the concentrations tested. Since *in vivo* experiments evaluating the combined action of IB-MECA and G-CSF have not yet been carried out, it remains to be established whether the lack of mutual potentiation of the effects of these two drugs found *in vitro* reflects the situation *in vivo*.

The results presented here have shown that combination of three drugs, namely of IB-MECA with two hematopoietic growth factors, IL-3 and GM-CSF, significantly increases numbers of GM-CFC induced in normal bone marrow cells in comparison with both the combinations of IB-MECA + IL-3 and IB-MECA+GM-CSF. This observation is promising from the point of view of contingent future *in vivo* studies aimed at combined modality treatments, which are advisable because they may increase therapeutic effectiveness and keep undesirable side effects at a low level.

Conflict of Interest

There is no conflict of interest.

Acknowledgements

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