The pharmacological activation of adenosine A₁ and A₃ receptors does not modulate the long- or short-term repopulating ability of hematopoietic stem and multipotent progenitor cells in mice

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Abstract This study continues our earlier findings on the hematopoiesis-modulating effects of adenosine A₁ and A₃ receptor agonists that were performed on committed hematopoietic progenitor and precursor cell populations. In the earlier experiments, N⁶-cyclopentyladenosine (CPA), an adenosine A₁ receptor agonist, was found to inhibit proliferation in the above-mentioned hematopoietic cell systems, whereas N⁶-(3-iodobenzyl)adenosine-5'-N-methyluronamide (IB-MECA), an adenosine A₃ receptor agonist, was found to stimulate it. The topic of this study was to evaluate the possibility that the above-mentioned adenosine receptor agonists modulate the behavior of early hematopoietic progenitor cells and hematopoietic stem cells. Flow cytometric analysis of hematopoietic stem cells in mice was employed, as well as a functional test of hematopoietic stem and progenitor cells (HSPCs). These techniques enabled us to study the effect of the agonists on both short-term repopulating ability and long-term repopulating ability, representing multipotent progenitors and hematopoietic stem cells, respectively. In a series of studies, we did not

find any significant effect of adenosine agonists on HSPCs in terms of their numbers, proliferation, or functional activity. Thus, it can be concluded that CPA and IB-MECA do not significantly influence the primitive hematopoietic stem and progenitor cell pool and that the hematopoiesis-modulating action of these adenosine receptor agonists is restricted to more mature compartments of hematopoietic progenitor and precursor cells.

Keywords Adenosine receptor agonists · CPA · IB-MECA · Hematopoietic stem cells · Long-term repopulating ability

Introduction

Hematopoiesis is under the control of numerous regulatory factors which act at various levels of the hematopoietic cell hierarchy, including stem, progenitor and precursor cells, as well as differentiated functional peripheral blood cells [1].

Adenosine, a ubiquitous purine nucleoside released into the extracellular environment from metabolically active or stressed cells, acts as paracrine regulator of many cellular functions, including those of cell proliferation and differentiation [2–5]. The regulatory role of extracellular adenosine is based on the activation of cell surface receptors, denoted as A₁, A_{2a}, A_{2b}, and A₃. Receptor activation can be achieved either nonselectively by adenosine or selectively using various adenosine analogs [6, 7].

The system of adenosine receptors and their agonists has been shown to play a distinct regulatory role in the processes of cell proliferation and differentiation in normal, as well as suppressed hematopoiesis [for a review, see 8, 9]. Numerous

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studies have revealed the significant role of the nonselective activation of adenosine receptors in stimulating hematopoiesis [e.g., 10–14]. Discrimination between the hematopoiesis-modulating effects of the activation of individual adenosine receptor types was made possible when selective adenosine receptor agonists became available. These agents, adenosine analogs, neither undergo facilitated uptake nor represent a substrate for cellular metabolism [7]. In our initial experiment investigating the effects of selective adenosine receptor agonists on murine hematopoiesis, agonists selective for A₁, A_{2a}, and A₃ adenosine receptors were compared in terms of their ability to modulate the cell cycling of bone marrow granulocyte/macrophage and erythroid progenitors as inferred from the action of the cell cycle-specific drug 5-fluorouracil (5-FU) administered to mice *in vivo*. The most interesting findings of these experiments were the opposite hematopoiesis-modulating effects of the selective agonists for the A₁ and A₃ receptors, i.e. of N⁶-cyclopentyladenosine (CPA) and N⁶-(3-iodobenzyl)adenosine-5'-N-methyluronamide (IB-MECA), respectively; these opposite effects manifested themselves as inhibitory (CPA) or stimulatory (IB-MECA) actions on cell cycling in the compartments of granulocyte/macrophage and erythroid bone marrow progenitor cells [15].

In most of the above studies, the hematopoiesis-modulating effects of the adenosine receptor agonists have been observed for both granulopoietic and erythroid lineages [10, 11, 13, 15]. These results have been obtained from the levels in the peripheral blood or from the compartments of bone marrow precursor and committed progenitor cells. It could be hypothesized on the basis of these findings that the target of the adenosine receptor agonists could be the hematopoietic precursor and committed progenitor cell populations. However, another hypothesis could also be postulated, namely that the interaction of adenosine receptor agonists with common ancestors of cells of the granulopoietic and erythroid lineages, i.e., hematopoietic stem and progenitor cells (HSPCs) could significantly contribute to the multi-lineage effectiveness of adenosine receptor agonists.

This study has been aimed at investigating the validity of the latter hypothesis. For this purpose, a new set of long-term experiments has been carried out. The techniques of flow cytometric analysis of hematopoietic stem cells as well as a functional test of HSPCs have been employed. The function of HSPCs has been tested by a competitive repopulation of the hematopoietic system after the transplantation of congenic bone marrow cells into sublethally irradiated recipients. These techniques have enabled us to study the effect of agonists on both short-term repopulating ability (STRA) and long-term repopulating ability (LTRA) in mice, representing multipotent progenitors and hematopoietic stem cells, respectively [16]. Since, as mentioned above, adenosine A₁ and A₃ receptor agonists, CPA, and IB-MECA, respectively, were found in a previous study to

significantly influence the cycling of hematopoietic progenitor cells, these two agents have been tested also in the study whose results are presented here. The effects of the administration of these adenosine receptor agonists on steady-state hematopoiesis and/or hematopoiesis perturbed with irradiation or 5-FU have been studied. Moreover, we also tested the effect of adenosine receptor agonists on the hematopoietic microenvironment recovering from combined effects of cytostatics and irradiation in terms of their ability to support the engraftment of transplanted bone marrow cells.

Materials and methods

Animals

C57BL/6NCrl mice (CD45.2) and congenic B6.SJL-Ptprca Pepcb/BoyJ mice (CD45.1) were bred in the specific pathogen-free barrier part of the institutional animal facility and maintained in a clean conventional part of the facility during the experiments (breeding pairs were obtained from Charles River Laboratory, Germany). Three- to six-month-old mice were used in the experiments. All experiments were approved by the Laboratory Animal Care and Use Committee of the First Faculty of Medicine, Charles University in Prague, and were performed in accordance with national and international guidelines for laboratory animal care.

Irradiation

Total body irradiation from a ⁶⁰Co source was used, with ~0.58 Gy/min from a distance of 123.5 cm.

Drug application

5-Fluorouracil (Sigma, St. Louis, MO) and cyclophosphamide (CY, Farnos, Oulunsalo, Finland) were injected intraperitoneally (i.p.) at a dose of 150 mg/kg and 135 mg/kg body weight (b.w.), respectively. N⁶-cyclopentyladenosine (Sigma), a selective agonist for the A₁ receptor, and N⁶-(3-iodobenzyl)adenosine-5'-N-methyluronamide (Sigma), a selective agonist for the A₃ receptor, were diluted initially in DMSO, then diluted further with sterile PBS and injected i.p. in doses of 200 or 1,000 nmol/kg, where the doses were always being isomolar in each experiment. The final concentration of DMSO was 2 %. The control mice were injected with PBS/DMSO alone. The agonists were administered with CY or 5-FU in a 2-day treatment regimen starting 24 h after irradiation. One or two injections per day (8:00 a.m. or 8:00 a.m. and 8:00 p.m., respectively) were given. The collections of the bone marrow (in case of donors) or irradiation prior to bone marrow transplantation (in case of recipients) were done 24 h after the last dose of

adenosine receptor agonists. The choice of a dose of 200 nmol/kg and the timing of the injections were based on when the maximum effects on the bone marrow progenitor and precursor cell populations were observed in our previous studies [17, 18]. The dose of 1,000 nmol/kg was used to test the contingent effect of a high dose of the drugs not used previously with the aim to assess the effectiveness of the agonists also in the case when the compartment of hematopoietic stem cells would be less sensitive to the action of the agonists in a comparison with the more differentiated compartments.

Bone marrow collection and transplantation

Bone marrow cells (BMCs) were collected from the femurs of mice sacrificed by cervical dislocation. Femurs were repeatedly flushed with 1 mL phosphate buffer saline (PBS, pH 7.5) +0.5 % bovine serum albumin (BSA) (hereafter referred to as PBS–BSA) using a 25 G needle. The cells were counted (Cellometer, Nexcelom Bioscience, Lawrence, MA). Aliquots containing BMCs from 0.2 of a femur (approx. 5×10^6 cells) from a nonmyeloablated mouse, or a 0.9 femur (approx. 23×10^6 cells) from a myeloablated mouse were injected intravenously in a volume of 0.5 mL into 5-Gy-irradiated recipients, respectively. In experiments focused on the transplantation of control bone marrow cells to myeloablated recipients treated with adenosine A₁ and A₃ receptor agonists, four million bone marrow cells were transplanted. Three to six recipient mice per group were used. All experiments were repeated at least three times.

Donor chimerism analysis

A sample of heparinized peripheral blood was taken from the retrobulbar venous plexus 1 and 6 months after transplantation to distinguish the STRA and LTRA as described previously [19]. Briefly, blood samples were lysed with ammonium chloride lysing buffer, washed twice with PBS–BSA, resuspended in 100 μ L PBS–BSA, and stained with phycoerythrin (PE)-conjugated anti-CD45.1 (BioLegend, San Diego, CA) and FITC-conjugated anti-CD4.2 antibodies (BioLegend) for 30 min on ice in the dark. After washing with PBS–BSA, samples were analyzed by flow cytometry (FACS Canto II, BD Biosciences, San Jose, CA). A gating for CD45.1+ and CD45.2+ cells was performed. When CD45.1+/CD45.2+ artificial doublets were present, they were omitted from the analysis.

Cytometric analysis of HSCs and blood cell lineages

Bone marrow cells were counted and stained for the presence of LSK SLAM (Lin[−]Sca-1⁺c-Kit⁺ CD150⁺CD48[−]) HSCs according to Kiel et al. [20]. Cells were washed with PBS–

BSA and stained with a mouse lineage biotin-antibody cocktail (Miltenyi Biotec GmbH, Bergisch Gladbach, Germany; 3 μ L per sample): APC-conjugated anti-mouse Ly-6A/E (Sca-1), APC/H7-conjugated anti-mouse CD117 (c-kit), PE-conjugated anti-mouse CD150 and FITC-conjugated anti-mouse CD48 (all antibodies from BioLegend, 1 μ L per sample) for 30 min on ice in the dark. After washing with PBS–BSA, 3 μ L streptavidin PE/Cy7 per sample (BioLegend) was added, and cells were incubated again for 30 min on ice in the dark. Cells were washed with PBS–BSA and resuspended in 300 μ L of PBS–BSA. Samples were subjected to flow cytometric analysis (FACS Aria II, BD Biosciences). Doublets and debris were discriminated, and the LSK SLAM population was gated as indicated in Supplementary Fig. S1A.

For detecting and quantifying blood cell lineages in the bone marrow, 300 μ L of a bone marrow cell suspension, containing approximately six million cells, was incubated with FITC-conjugated anti-mouse CD45R/B220, APC-conjugated anti-mouse Ly-6 G/Ly-6C (Gr-1), APC-conjugated anti-mouse CD11b (Mac-1), and PE-conjugated anti-mouse TER-119 (all from BioLegend, 1 μ L per sample) on ice for 30 min in the dark. After washing with PBS–BSA, the samples were resuspended in 300 μ L of PBS–BSA and analyzed for particular subpopulations. Blood samples were lysed as described above and stained with FITC-conjugated anti-mouse CD45R/B220, APC-conjugated anti-mouse Ly-6 G/Ly-6C (Gr-1), APC-conjugated anti-mouse CD11b (Mac-1), and PE-conjugated anti-mouse TER-119 (all from BioLegend, 1 μ L per sample) on ice for 30 min in the dark.

HSC proliferation

A Click-iT EdU Pacific blue flow cytometry assay kit (Life Technologies, Carlsbad, CA) was used for the labeling and detection of DNA synthesis according to the manufacturer's recommendations. This kit is not compatible with use of PE and PE conjugates, which restricts the number of fluorochromes that can be utilized for cell staining. Some antigens can be stained with PE-based dyes after completing the EDU reaction, but it must be carefully determined that permeabilization and fixation does not interfere with the availability of these antigens. Only the Sca-1 antigen fulfilled this requirement. In order to detect the proliferation of HSCs, we used a simplified SK SLAM (Sca-1⁺c-Kit⁺ CD150⁺CD48[−]) staining method which enables the detection of virtually the same HSCs as the LSK SLAM method (manuscript in preparation). In order to prove that the SK SLAM gating is identical to the LSK SLAM one also on fixed and permeabilized cells, we compared the both staining methods on 12 individual samples from control and regenerating bone marrow. A representative sample is shown in Supplementary Fig. S1B.

In order to determine the percentage of HSCs in S-phase of the cell cycle, bone marrow cells were incubated with 10- μ M EdU solution and stained with the following surface antibodies: APC-Cy7-conjugated anti-mouse c-Kit, FITC-conjugated anti-mouse CD48, and APC-conjugated anti-mouse CD150 (BioLegend), and then fixed in Click-iT fixative and permeabilized in Click-iT saponin-based permeabilization reagent. Permeabilized cells were incubated with Click-iT reaction cocktail and then stained with the surface antibody PE-conjugated anti-mouse Sca1 (BioLegend). For measuring DNA content, Ribonuclease A and 7AAD were added. Cells were analyzed in a BD FACS Canto II flow cytometer (BD Biosciences).

Statistical analysis

Statistical analysis was performed with GraphPad Prism software (GraphPad Software, San Diego, USA, www.graphpad.com). Data are presented as the mean $\pm$ standard error of the

mean. One-way ANOVA using Dunnett's post hoc test was used to compare each group to the control. *P* values <0.05 were considered statistically significant.

Results

Flow cytometric analysis of mouse bone marrow hematopoietic stem cells after treatment with adenosine A_1 and A_3 receptor agonists

LSK SLAM cells representing the HSC population were stained according to Kiel et al. [20]. In order to determine the effect of adenosine agonists on the proliferation of HSCs, a simplified SK SLAM staining was used. LSK and SK SLAM gating reveal identical cells (see "Materials and methods" and Supplementary Fig. S1). SK SLAM population was stained for DNA synthesis in order to determine the proportion of the cells in S-phase. Mice

Fig. 1 Flow cytometric analysis of HSCs (SK SLAM: Sca1⁺c-Kit⁺CD150⁺CD48⁺) and their proliferation activity in the bone marrow of mice treated for 2 days with daily doses of CPA and IB-

MECA (200 nmol/kg b.w.). **a** Control mice. **b** Mice irradiated with 4 Gy 1 day before the administration of the first dose of the drugs

were irradiated with a 4-Gy dose of γ -rays. Two daily doses of CPA, an adenosine A₁ receptor agonist, and IB-MECA, an adenosine A₃ receptor agonist, were administered to the mice 24 and 48 h after irradiation. Sampling of the bone marrow was performed 24 h after the administration of the second dose of the agonists. The numbers and percentage in the S-phase of SK SLAM cells did not differ in control mice (Fig. 1a) and in mice recovering from 4-Gy irradiation (Fig. 1b).

Fig. 2 STRA and LTRA of bone marrow of donor mice treated for 2 days with CPA and IB-MECA (200 or 1,000 nmol/kg per dose). The bone marrow of the donor mice was sampled and transplanted to the irradiated recipient mice 24 h after the administration of the second dose of the agonists in the amount of 0.2 of a femur or 0.9 of a femur taken from nonmyeloablated or myeloablated donors, respectively. The recipient mice were irradiated with a 4-Gy dose of γ -rays 24 h after the last dose of agonists, except for 5-FU-treated mice, where a dose of 5 Gy was used. STRC and LTRC were represented in the bone marrow recipients by the donor-derived chimerism in the peripheral blood 1 and 6 months after transplantation. **a** Percentage of peripheral blood nucleated cells derived from control donor mice. **b** Percentage of peripheral blood nucleated cells derived from donor mice irradiated with 4 Gy of γ -rays 1 day before the administration of the first dose of the drugs. **c** Percentage of peripheral blood nucleated cells derived from donor mice treated with 5-fluorouracil (150 mg/kg) 1 day before the administration of the first dose of the drugs

Functional assay of primitive bone marrow hematopoiesis treated with adenosine A₁ and A₃ receptor agonists

a) Effect of adenosine A₁ and A₃ receptor agonists on hematopoietic stem cells

The STRA and LTRA of bone marrow cells from donors pretreated with IB-MECA or CPA were measured after transplantation to 4-Gy-irradiated congenic recipients.

Donor mice were either nonmyeloablated (Fig. 2a), recovering from irradiation with the 4-Gy dose of γ -rays (Fig. 2b) or from 5-FU treatment (Fig. 2c). Two doses of agonists were used in untreated and irradiated mice: 200 and 1,000 nmol/kg b.w. 5-FU-treated mice were injected with a 200-nmol/kg dose twice a day for 2 days (four doses in total). No significant differences were observed between the control- and agonist-treated groups.

b) Effect of adenosine A_1 and A_3 receptor agonists on bone marrow microenvironment in HSC recipients

Mice were injected with 5-FU or CY, treated with adenosine agonists, irradiated with 4 Gy, and transplanted with four million normal congenic bone marrow cells. STRA and LTRA were determined in peripheral blood 1 and 6 months after transplantation, respectively (Fig. 3a). Bone marrow chimerism at the end of the experiment was also assessed (Fig. 3b). There were no significant changes in the hematopoiesis-supporting activity of the bone marrow microenvironment of the recipients treated with medium alone or adenosine A_1 or A_3 receptor agonists.

Discussion

The data obtained in this study have shown that the administration to mice of agonists for the adenosine A_1 and A_3 receptors, CPA and IB-MECA, respectively, neither influences their bone marrow LTRA nor their bone marrow STRA. These hematopoietic repopulating abilities are provided by HSCs and cells close to becoming multipotent progenitor cells,

respectively [21]. Flow cytometric analysis of the nucleated peripheral blood cells of recipients of the bone marrow from donors treated with CPA or IB-MECA revealed no significant influence of the drug treatment compared to the control mice. The lack of effect of CPA and IB-MECA on the compartments of HSCs and multipotent progenitor cells included both effects on the numbers of the cells and their proliferation status. Thus, it can be concluded that HSCs and multipotent progenitor cells are not functional targets of adenosine A_1 and A_3 receptor agonists. Further studies should clarify whether the inability of adenosine A_1 and A_3 receptor agonists to influence cell proliferation in the above-mentioned hematopoietic cell compartments is caused by the inability of the cells to express the corresponding membrane receptors or whether the ineffectiveness of the agonists is for other reasons.

We also focused on the question of whether the activation of adenosine A_1 or A_3 receptors influences bone marrow stromal cells in mice recovering from damage induced by the combined action of cytostatic drugs and ionizing radiation. In this experiment, recipients of normal bone marrow cells were pretreated with 5-FU or CY. Two daily doses of CPA or IB-MECA were then administered, followed by 4 Gy of γ -ray irradiation. 5-FU was selected because of our previous finding that these adenosine A_1 and A_3 receptor agonists influence the cycling of committed progenitor cells when applied to mice with 5-FU-depleted bone marrow [15] and because of its ability to induce a deep hematopoietic depression in mice soon (within days) after its administration [17]. As an opposite model, CY triggers a very rapid regeneration of hematopoietic tissue [22]. Thus, CPA and IB-MECA were administered to mice whose hematopoiesis was depressed (5-FU) or activated (CY). Nevertheless, we did not find adenosine A_1 or A_3 receptor

Fig. 3 Hematopoietic supportive function of bone marrow of mice treated for 2 days with CPA and IB-MECA (200 nmol/kg per dose). Mice were treated with 5-fluorouracil (150 mg/kg) or cyclophosphamide (135 mg/kg) on day 4, with CPA or IB-MECA on days 3 and 2, and irradiated with the 4-Gy dose of γ -rays on day 1. Four million bone marrow cells were transplanted on day 0. STRC and LTRC were

represented in the bone marrow recipients by the chimerism in peripheral blood 1 and 6 months after transplantation. **a** Chimerism in the peripheral blood 1 month (STRC) and 6 months (LTRC) after transplantation. **b** Chimerism in the bone marrow 6 months after transplantation

agonists to have any influence on the ability of the bone marrow stroma to support transplanted bone marrow cells.

It follows from these findings that it is probably the first hypothesis of the two mentioned in the “Introduction” which properly describes the target hematopoietic cell populations functionally interacting with adenosine receptor agonists: the target of the adenosine receptor agonists in the hematopoietic system, leading to distinct modulation of a wide spectrum of hematopoietic parameters, are probably the hematopoietic precursor and committed progenitor cell populations; these cells are further downstream from the less differentiated hematopoietic populations tested in this study [21, 23, 24]. This consideration has been seriously supported by studies conducted at approximately the same time as the long-term study described here. Important findings have been obtained in studies comparing the hematopoiesis-modulating effects of CPA and IB-MECA on murine hematopoiesis during the cell depletion and cell regeneration induced by 5-FU. These results have revealed that the stimulatory effects of the adenosine A_3 receptor agonist, IB-MECA, on granulopoietic and erythroid committed progenitor or precursor cells have been most prominent when the agonist was administered to mice in the phase of hematopoietic cell depletion [17, 18, 25]; on the other hand, CPA, an agonist of adenosine A_1 receptors, was found to be most effective as an inhibitor of cycling in the above cell compartments when administered in the cell regeneration phase [17, 26]. These observations could be hypothetically explained by taking into account possible changes in the expression of adenosine A_1 and A_3 receptors that depend on a physiological need for their activity.

For this reason, further studies were aimed directly at determining of the expression of mRNAs for individual adenosine receptors in the relevant cells. First, it was found that the mRNAs for both the adenosine A_1 and A_3 receptors are expressed in both granulopoietic and erythroid precursor cell populations in murine bone marrow [27], so these cell populations are able to interact with adenosine A_1 and A_3 receptor agonists. In a subsequent study, HL-60 cells served as a model population for precursor (promyelocytic) cells in the bone marrow [28]. This study discovered that there is a distinct correlation between the individual cell cycle phases and the intensity of expression of adenosine A_1 and A_3 receptors. Moreover, while the adenosine A_3 receptor was most expressed in the S-phase of the cell cycle, i.e., when there is an intensive multiplication of the cell population, the adenosine A_1 receptor was most expressed in the G_0/G_1 and G_2/M phases of the cell cycle, i.e., when the cell proliferation is less intensive [28].

Based on the results of this study and those from numerous previous investigations on the hematopoiesis-modulating effects of adenosine A_1 and A_3 receptor agonists, two general summarizing statements can be made. First, the interactions of adenosine A_1 and A_3 receptor

agonists with early undifferentiated HSPCs do not play a significant role, if any, in the modulation of the state of more differentiated hematopoietic cell compartments, as shown by the results of a number of independent experiments presented here. Second, a direct activation of adenosine A_1 and A_3 receptors in the cells of the committed progenitor and precursor hematopoietic cell populations is highly likely to be responsible for the effectiveness of the administration of adenosine A_1 and A_3 receptor agonists at modulating hematopoiesis.

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Conflicts of interest The authors declare that there are no conflicts of interest.

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