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BRIEF COMMUNICATION

Lack of adenosine A_3 receptors causes defects in mouse peripheral blood parameters

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Abstract The role of the adenosine A_3 receptor in hematopoiesis was studied using adenosine A_3 receptor knockout (A_3AR KO) mice. Hematological parameters of peripheral blood and femoral bone marrow of irradiated and untreated A_3AR KO mice and their wild-type (WT) counterparts were investigated. Irradiation of the mice served as a defined hematopoiesis-damaging means enabling us to evaluate contingent differences in the pattern of experimentally induced hematopoietic suppression between the A_3AR KO mice and WT mice. Defects were observed in the counts and/or functional parameters of blood cells in the A_3AR KO mice. These defects include statistically significantly lower values of blood neutrophil and monocyte counts, as well as those of mean erythrocyte volume, mean erythrocyte hemoglobin, blood platelet counts, mean platelet volume, and plateletcrit, and can be considered to bear evidence of the lack of a positive role played by the adenosine A_3 receptor in the hematopoietic system. Statistically significantly increased values of the bone marrow parameters studied in A_3AR KO mice (femoral bone marrow cellularity, granulocyte/macrophage progenitor cells, and erythrocyte progenitor cells) can probably be explained by compensatory mechanisms attempting to offset the disorders in the function of blood elements in these mice. The pattern of the radiation-induced hematopoietic suppression was very similar in A_3AR KO mice and their WT counterparts.

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Introduction

Adenosine membrane receptors and their activation represent a universal regulatory system modulating cellular functions including those of cell proliferation and differentiation [1, 2]. The regulatory function of extracellular adenosine is based on the activation of cell surface receptors, namely A_1, A_{2a}, A_{2b}, and A_3; this activation can be achieved either nonselectively, by adenosine itself, or selectively, using various adenosine analogs [3].

In previous years, attention had been focused on hematological effects of pharmacological stimulation of adenosine receptors. We have shown that pharmacologically induced elevation of extracellular adenosine, i.e., nonselective activation of adenosine receptors, stimulates hematopoiesis in mice (e.g., [4–6], for review, see [7]). In a subsequent study using adenosine receptor agonists selective for individual receptor subtypes, it was revealed that activation of adenosine A_3 receptors by their selective agonist, N^6-(3-iodobenzyl)adenosine-5′-*N*-methyluronadenosine (IB-MECA), stimulated proliferation of hematopoietic progenitor cells for granulocytes/macrophages (granulocyte/macrophage colony-forming cells (GM-CFC)) and erythrocytes (burst-forming unit erythroid (BFU-E)) [8]. Afterwards, in an in vitro study, IB-MECA was shown to potentiate stimulatory effects of interleukin-3 (IL-3), the stem cell factor (SCF), and the granulocyte/macrophage colony-stimulating factor (GM-CSF) on the proliferation of GM-CFC [9]. The results of recent experiments on mice revealed that IB-MECA supports suppressed hematopoiesis in mice exposed to sublethal doses of ionizing radiation [10, 11]. In the latter studies, stimulation by IB-MECA was observed in various

parameters of the bone marrow progenitor and precursor cells and of the peripheral blood. The compartment of hematopoietic stem cells in mice was not found to be influenced by the administration of IB-MECA [12].

The abovementioned summarized results on the significance of adenosine A_3 receptors were obtained when administering the appropriate receptor agonist to genetically normal mice. Another view on the role of adenosine A_3 receptors in hematopoiesis can be obtained when adenosine A_3 receptor knockout (A_3AR KO) mice would be used, and the picture of hematopoiesis in the situation of the lack of the adenosine A_3 receptor would be put together. A_3AR KO mice were indeed constructed, and several articles based on the use of these mice in experiments with nonhematological topics were published (e.g., [13–15]). The first study on A_3AR KO mice aimed at hematological evaluations was performed quite recently in our laboratory, and results were published illustrating disorders in selected parameters of erythropoiesis and thrombopoiesis in these mice [16]. In the present study, we have focused our attention also on other hematopoietic compartments and on the bone marrow. To unveil contingent differences in hematopoiesis between the A_3AR KO mice and their wild-type (WT) controls under the conditions of hematopoietic suppression, this study has been performed in both nontreated and sublethally γ-irradiated mice.

Materials and methods

Mice

A_3AR KO (Adora3^{tm1Jsbn}/Adora3^{tm1Jsbn}) mice, back-crossed onto a C57BL/6 background [17], were obtained from Merck Research Laboratories (West Point, PA, USA) and bred in the Laboratory Animal Breeding and Experimental Facility of the Faculty of Medicine, Masaryk University, Brno, Czech Republic. WT C57BL/6 mice were obtained from the same Laboratory Animal Breeding and Experimental Facility of the Faculty of Medicine, Masaryk University, Brno, Czech Republic. The mice were kept in controlled conditions; standardized pelleted diet and HCl-treated tap water were available ad libitum. For material sampling, 2.5-month-old mice were used. The use and treatment of the animals followed the European Community Guidelines as accepted principles for the use of experimental animals. The experiments were carried out with the approval of the Institute's Ethical Committee.

Irradiation

The mice were whole-body irradiated at a dose rate of 0.5 Gy/min using a γ-ray source (^{60}Co, Chisostat, Chirana, Prague, Czech Republic). A single dose of 4 Gy was used.

Hematological techniques

Sampling of material was performed on day 3 after irradiation. For evaluation of the peripheral blood parameters, the animals were anesthetized with an intraperitoneal (i.p.) injection of 0.07 ml of a Narcamon/Rometar solution (5 % Narcamon and 2 % Rometar [both Spofa, Prague, Czech Republic]) in a ratio of 2.63:1, and the peripheral blood was sampled by cardiac puncture. The numbers of leukocytes, erythrocytes, and platelets per 1 μl of the peripheral blood, as well as the percentages of neutrophils, lymphocytes, monocytes, and eosinophils among total peripheral blood leukocytes, were determined by an Auto Hematology Analyzer Mindray 5300Vet (Shenzhen, China). This analyzer allows us to determine also the numbers of neutrophils, lymphocytes, monocytes, and eosinophils per 1 μl of the peripheral blood but only when the total leukocyte number exceeds the value of 0.5×10^{12}/l. Therefore, these determinations were only performed in untreated mice. The same device was used for determination of the parameters of hemoglobin concentration (HGB), hematocrit (HCT), mean erythrocyte volume (MCV), mean erythrocyte hemoglobin (MCH), the width of the erythrocyte distribution curve (RDW), plateletcrit (PCT), mean platelet volume (MPV), and the width of the platelet distribution curve (PDW). In mice sacrificed by cervical dislocation, the femurs were removed, marrow cells were harvested by standard procedures, and the numbers of nucleated cells of the femoral bone marrow were determined using a Coulter Counter (Model ZF, Coulter Electronics, Luton, UK). Standard procedures were used for the in vitro assays of the femoral clonogenic progenitor cells. GM-CFC were assessed using the MethoCult M3001 medium (StemCell Technologies, Vancouver, Canada). Erythroid progenitor cells (BFU-E) were determined using the MethoCult SF M3436 medium (StemCell Technologies). Femoral marrow cell suspensions were plated (1.5×10^5 and 1×10^5 nucleated bone marrow cells for GM-CFC and BFU-E, respectively) in triplicate for both assays and incubated at 37 °C in a humidified atmosphere containing 95 % air and 5 % CO_2. GM-CFCs were scored after a 7-day incubation as colonies containing 50 or more cells. Hemoglobinized colonies were counted as BFU-E after an 8-day incubation. Considering the differences in the average body weight between the groups of mice studied (24.6, 24.7, 25.7, and 26.5 g for irradiated A_3AR KO mice, untreated A_3AR KO mice, irradiated WT mice, and untreated WT mice, respectively), the values of the bone marrow parameters were expressed as per 1 g of body weight.

Statistics

The values are presented as arithmetic means and a 95 % confidence interval. In each parameter, the global statistical

significance of differences among the groups was assessed using one-way ANOVA. The differences between the individual experimental groups were evaluated using the Mann-Whitney test.

Results

Table 1 shows the values of nine peripheral blood leukocyte parameters in 4 Gy-irradiated and untreated A_3AR KO mice and their WT counterparts. As regards white blood cells, we have not observed any significant differences in total leukocyte counts between A_3AR KO and WT mice, either irradiated or untreated. However, differential blood cell counting, which could be performed in the untreated mice (see "Materials and methods"), revealed significantly lower values of neutrophils and monocytes per unit blood volume in the A_3AR KO mice. Percent values of blood neutrophils and eosinophils among blood leukocytes were significantly lower in the A_3AR KO mice, whereas those found in blood lymphocytes were significantly higher, respectively, in comparison with their WT counterparts.

Table 2 summarizes the values of parameters concerning peripheral blood erythrocyte and platelet parameters. In the erythroid peripheral blood compartment, significantly higher values of blood erythrocytes, HGB, and HCT were found in untreated A_3AR KO mice compared with their WT counterparts. However, a comparison of irradiated A_3AR KO and WT mice did not show any significant differences in these parameters. A significant decrease of the values of MCV, MCH, and RDW in both irradiated and untreated A_3AR KO mice in comparison with their WT counterparts was observed. Significantly lower values in A_3AR KO mice were also found in all the parameters of blood thrombocytes, i.e., in their blood counts and in the values of MPV and PCT.

Table 3 presents the values of femoral bone marrow cellularity, GM-CFC/femur, and BFU-E per femur in the mice studied. In all these parameters, clearly and significantly higher values were observed in the A_3AR KO mice in comparison with the WT mice.

Contingent differences in the behavior of the hematopoietic system under the condition of hematopoietic suppression between the A_3AR KO mice and WT mice were studied by means of inclusion of groups of mice irradiated with a sublethal dose of 4 Gy of γ-rays. With the exception of the differences found in the parameters of blood erythrocyte counts, HGB, and HCT, the response of the cells of the hematopoietic system of both A_3AR KO mice and WT mice to the irradiation was very similar and the rates of radiation-induced decrease of the hematological parameters studied were nearly identical (results not shown).

Discussion

From the point of view of the use of A_3AR KO mice in this study, important findings are those showing that adenosine A_3 receptors are expressed in human neutrophils [18], monocytes [19], and lymphocytes [20], and especially in the differentiating and proliferating cells of various hematopoietic cell compartments, namely in mouse bone marrow granulopoietic/monocytopoietic, erythropoietic, and B-lymphopoietic cells, as well as in the T-lymphopoietic cells of the thymus [21]. Thus, the lack of these receptors in A_3AR KO mice could be expected to be connected with modulation and/or disorders of hematopoiesis. These phenomena could also be anticipated on

Table 1 Values of peripheral blood leukocyte parameters in 4 Gy-irradiated and untreated adenosine A_3 receptor knockout (A_3AR KO) and wild-type (WT) mice

Parameter	Irradiated A_3AR KO mice n=8	Irradiated WT mice n=10	Untreated A_3AR KO mice n=8	Untreated WT mice n=10
Blood leukocyte count ($\times 10^9$/l)	0.22 (0.19; 0.26)	0.23 (0.17; 0.29)	4.39 (3.43; 5.35)	4.90 (3.41; 6.39)
Blood neutrophils (%)	ND	ND	12.6* (10.2; 14.9)	19.6 (14.9; 24.3)
Blood lymphocytes (%)	ND	ND	80.0$^{\#}$ (76.0; 84.0)	70.2 (64.4; 76.0)
Blood monocytes (%)	ND	ND	7.0 (5.3; 8.7)	9.3 (7.5; 11.1)
Blood eosinophils (%)	ND	ND	0.49* (0.19; 0.79)	0.98 (0.63; 1.32)
Blood granulocyte count ($\times 10^9$/l)	ND	ND	0.52** (0.36; 0.68)	1.06 (0.53; 1.58)
Blood lymphocyte count ($\times 10^9$/l)	ND	ND	3.41 (2.35; 4.46)	3.35 (2.50; 4.20)
Blood monocyte count ($\times 10^9$/l)	ND	ND	0.28* (0.21; 0.36)	0.48 (0.28; 0.68)
Blood eosinophil count ($\times 10^9$/l)	ND	ND	0.015 (0.003; 0.027)	0.049 (0.018; 0.080)

The results are presented as arithmetic means. The values of 95 % confidence limits are given in parentheses

n numbers of animals, *ND* not done (see "Materials and methods")

*$P \leq 0.05$; **$P \leq 0.01$—the value in A_3AR KO mice is statistically significantly lower than that in the corresponding WT counterparts. $^{\#}$ $P \leq 0.05$—the value in A_3AR KO mice is statistically significantly higher than that in the corresponding WT counterparts

Table 2 Values of peripheral blood erythrocyte and platelet parameters in 4 Gy-irradiated and untreated adenosine A_3 receptor knockout (A_3AR KO) and wild-type (WT) mice

Parameter	Irradiated A_3AR KO mice n=8	Irradiated WT mice n=10	Untreated A_3AR KO mice n=8	Untreated WT mice n=10
Blood erythrocyte count ($\times 10^{12}$/l)	7.82 (7.51; 8.13)	7.54 (7.22; 7.87)	$9.41^{\#\#\#}$ (9.18; 9.63)	8.49 (8.18; 8.80)
Blood hemoglobin level (HGB) (g/l)	115 (110; 119)	113 (108; 118)	$134^{\#}$ (127; 141)	128 (124; 133)
Hematocrit (HCT) (%)	38.5 (36.9; 40.1)	38.5 (36.8; 40.2)	$46.5^{\#\#}$ (45.2; 47.7)	44.0 (42.5; 45.5)
Mean erythrocyte volume (MCV) (fl)	49.3*** (48.9; 49.6)	51.2 (50.8; 51.6)	49.4*** (49.0; 49.9)	51.9 (51.5; 52.2)
Mean erythrocyte hemoglobin (MCH) (pg)	14.7*** (14.6; 14.8)	15.1 (14.9; 15.3)	14.7*** (14.4; 14.9)	15.1 (15.0; 15.2)
Red cell distribution width (RDW) (%)	11.6** (11.5; 11.7)	12.8 (12.2; 13.3)	12.2*** (12.0; 12.3)	13.6 (13.0; 14.1)
Blood platelet count ($\times 10^9$/l)	1,006*** (939; 1,072)	1,185 (1,119; 1,252)	1,048* (964; 1,132)	1,133 (1,075; 1,192)
Mean platelet volume (MPV) (fl)	4.90*** (4.84; 4.96)	5.33 (5.26; 5.39)	4.92*** (4.85; 4.99)	5.39 (5.31; 5.46)
Plateletcrit (PCT) (%)	0.49*** (0.46; 0.53)	0.63 (0.60; 0.66)	0.52** (0.47; 0.56)	0.61 (0.58; 0.64)
Platelet distribution width (PDW) (%)	14.6*** (14.5; 14.6)	14.8 (14.7; 14.8)	14.6*** (14.5; 14.6)	14.8 (14.7; 14.8)

The results are presented as arithmetic means. The values of 95 % confidence limits are given in parentheses

n numbers of animals, *ND* not done (see "Materials and methods")

*$P \leq 0.05$; **$P \leq 0.01$; ***$P \leq 0.001$—the value in A_3AR KO mice is statistically significantly lower than that in the corresponding WT counterparts. $^{\#}P \leq 0.05$; $^{\#\#}P \leq 0.01$; $^{\#\#\#}P \leq 0.001$—the value in A_3AR KO mice is statistically significantly higher than that in the corresponding WT counterparts

the basis of the results of experiments employing the administration of an adenosine A_3 receptor agonist to normal (WT) mice (see "Introduction").

The findings in the peripheral blood of both untreated and γ-irradiated A_3AR KO mice (see Tables 1 and 2) clearly suggest that these animals exhibit defects in the process of production of these cells and/or in their functional parameters caused by the lack of adenosine A_3 receptors. The values of functional parameters of erythrocytes of A_3AR KO mice (MCV, MCH) are statistically significantly decreased in comparison with those in their WT counterparts. On the other hand, the values of erythrocyte numbers per 1 μl of peripheral blood, of HGB, and of HCT are significantly higher in A_3AR KO mice than in WT mice when untreated mice are investigated; such a difference is, however, lacking in irradiated animals. The latter finding will be discussed later in connection with observations in the progenitor cell compartments. The significant suppression found in A_3AR KO mice in all studied parameters of the peripheral blood thrombocytes, as well as in the values of blood neutrophils and monocytes in untreated mice, also bears evidence of malfunctions in the mature peripheral blood cell compartments.

Of interest are the differences in the parameter of RDW between the A_3AR KO and WT mice, whose values are significantly lower in A_3AR KO mice irrespective of their irradiation status. Since RDW is considered to indicate a pathological status if being increased [22] (increased RDW values reflect anisocytosis of erythrocytes), our finding of lower values of RDW in A_3AR KO mice compared to WT controls cannot be interpreted as a pathological manifestation when considered separately. However, when assessed together with other parameters of blood erythrocytes in the A_3AR KO mice, it suggests the existence of insufficient erythropoiesis in these mice, which deserves further studies.

In contradiction to the insufficiencies observed in the peripheral blood compartments, the findings in the bone marrow of the A_3AR KO mice show a quite different picture (see Table 3). The femoral bone marrow cellularity, as well as the

Table 3 Values of femoral bone marrow parameters in 4 Gy-irradiated and untreated adenosine A_3 receptor knockout (A_3AR KO) and wild-type (WT) mice

Parameter	Irradiated A_3AR KO mice n=8	Irradiated WT mice n=10	Untreated A_3AR KO mice n=8	Untreated WT mice n=10
Femoral bone marrow cellularity/g body weight ($\times 10^3$)	$0.37^{\#\#}$ (0.29; 0.46)	0.28 (0.23; 0.34)	$1.40^{\#\#}$ (1.29; 1.51)	1.16 (1.08; 1.24)
GM-CFC/femur/g body weight	$103^{\#\#\#}$ (88; 117)	47 (32; 63)	$721^{\#\#\#}$ (631; 812)	502 (453; 551)
BFU-E/femur/g body weight	$98.6^{\#\#\#}$ (67.9; 129.4)	52.3 (35.2; 69.5)	$798^{\#\#}$ (700; 896)	628 (562; 693)

The results are presented as arithmetic means. The values of 95 % confidence limits are given in parentheses

n numbers of animals

$^{\#\#}P \leq 0.01$; $^{\#\#\#}P \leq 0.001$—the value in A_3AR KO mice is statistically significantly higher than that in the corresponding WT counterparts

numbers of femoral hematopoietic progenitor cells for granulocytes and macrophages (GM-CFC) and for erythrocytes (BFU-E), were found to be significantly higher in both irradiated and untreated A_3AR KO mice in comparison with their WT counterparts. These observations were somewhat surprising taking into account the previously observed stimulation in these compartments of normal (WT) mice after administration of an adenosine A_3 receptor agonist [10, 11].

Considering the hematological findings as a whole, it can be hypothesized that the stimulated bone marrow hematopoiesis in A_3AR KO mice is the result of processes by which insufficiencies in the peripheral blood compartments in these mice endeavor to be compensated.

There exists a general agreement between the findings reported here and those shown in [16] in the parameters of erythropoiesis and thrombopoiesis of untreated A_3AR KO and WT mice. The only exception is the values of HCT, which were reported in [16] to be significantly higher in WT mice, whereas the opposite is true in the present communication. This difference can be explained by a quantitatively more expressive stimulation of the production of erythrocytes by erythroid progenitor cells (BFU-E) in the experiments whose results are presented here. The differences in the degree of stimulation of the production of mature blood cells from the level of progenitor cells can be influenced by various factors, e.g., by the season of the year in which the experiments were performed. The values of HCT can also be influenced by the actual condition of water intake and excretion.

Though we observed significantly higher values of BFU-E/femur in both irradiated and untreated A_3AR KO mice in comparison with their WT counterparts, this phenomenon in the progenitor cell compartment in the A_3AR KO mice was more intensively reflected in the increase of the values of blood erythrocyte counts, HGB, and HCT in untreated mice, where this increase achieved statistical significance (see Tables 1 and 2). The absence of this behavior of the erythropoietic system in irradiated mice is probably caused by radiation-induced suppression of erythropoiesis, which does not allow reflection of the increase in the femoral BFU-E into the peripheral blood erythroid parameters in a rate corresponding to that in the untreated mice.

As mentioned in the "Results" section, the differences between the behavior of the parameters characterizing blood erythrocytes in the A_3AR KO and WT mice, as discussed in the previous paragraph, represented the only exception from the otherwise nearly identical pattern of radiation-induced decrease of the values of the hematological parameters studied in both A_3AR KO and WT mice. On the basis of these findings, it can be assumed that the observed disturbances in the hematopoiesis of A_3AR KO mice do not reach such an extent as would cause an aggravated ability of the hematopoietic system of these mice to respond to a hematopoiesis-suppressing stimulus.

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

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