Erythropoiesis- and Thrombopoiesis-Characterizing Parameters in Adenosine A_3 Receptor Knock-Out Mice

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Summary

Influence of the regulatory system mediated by adenosine A_3 receptors on the functioning of erythropoiesis and thrombopoiesis was studied by means of evaluation of the numbers and attributes of peripheral blood erythrocytes and platelets, as well as of erythroid bone marrow progenitor cells in adenosine A_3 receptor knock-out (Adora3^{tm1Jbsn}/Adora3^{tm1Jbsn}, $A_3AR^{(-/-)}$) mice and their wild-type C57BL/6 counterparts, both males and females. Minor but statistically significant disturbances in the properties of erythrocytes, namely in the parameters of mean erythrocyte volume and mean erythrocyte hemoglobin were observed in $A_3AR^{(-/-)}$ mice. In addition, adenosine A_3 receptor knock-out mice were found to exhibit an expressive, statistically significant decrease of their blood platelet count, amounting to 17 % and 21 % in males and females, respectively. This decrease in platelet levels was accompanied by a significant 17 % decline in the plateletcrit in both sexes. The obtained data can help to define therapeutic applications based on the principle of adenosine receptor signaling.

Key words

Adenosine A_3 receptor • Mice • Erythrocytes • Thrombocytes

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Introduction

Signaling through adenosine membrane receptors was shown to modulate cell proliferation, differentiation, and apoptosis (*e.g.*, Jacobson 1999, Schulte and Fredholm 2003). Adenosine receptors exist in four types, denominated as A_1, A_{2a}, A_{2b}, and A_3 (Olah and Stiles 1995), which can be stimulated either non-selectively (by the natural agonist adenosine) or selectively (by synthetic selective adenosine receptor agonists). The effects of pharmacological interventions into the processes of adenosine receptor signaling were lately studied in many pathological states with the aim to positively influence therapeutic outcomes (*e.g.*, Caruso *et al*. 2009, Williams-Karnesky and Stenzel-Pore 2009, Polosa and Blackburn 2009, Eltzschig 2009, Ohta and Sitkovsky 2009).

The laboratory of the authors was engaged in studies evaluating the effects of pharmacological activation of adenosine receptors on hematopoiesis, which had been a rather neglected field of study (Hofer and Pospíšil 2006, Hofer *et al*. 2011a). In the first instance the topic of the non-selective adenosine receptor activation was followed. Administration of drugs elevating extracellular adenosine before exposure of experimental mice to ionizing radiation was found to have radioprotective effects as a consequence of decreased hematopoietic damage (Hošek *et al*. 1992, Boháček *et al*. 1993), as well as enhanced hematopoietic recovery (Pospíšil *et al.* 1992, 1993a, b, 1995a, Hofer *et al.* 1995, 1997). Thus, pharmacologically induced

elevation of extracellular adenosine was shown to be beneficial under myelosuppressive states (Pospíšil *et al*. 1998, Weiterová *et al*. 2000, Hofer *et al*. 2001, 2002), and it was demonstrated that these effects can be the result of the induction of hematopoietic progenitor cells into cycling (Pospíšil *et al*. 2001). Extracellular adenosine was also observed to mobilize hematopoietic progenitor cells and granulocytes into the peripheral blood of mice (Hofer *et al*. 2003). Moreover, several studies were performed in which mutually potentiating effects of elevated extracellular adenosine and of the granulocyte colony-stimulating factor (G-CSF) in normal mice (Pospíšil *et al*. 1995b) and under the conditions of hematopoiesis suppressed by ionizing radiation and/or cytotoxic chemotherapy (Pospíšil *et al*. 1998, Hofer *et al*. 1999, 2001, 2002, Weiterová *et al*. 2000) were observed.

Recent studies of the authors were aimed at testing the hematopoiesis-modulating action of selective adenosine receptor activation. It was shown that whereas selective activation of A_3 receptors by the pertinent receptor agonist 1-deoxy-1-(6-[([3-iodophenyl]methyl)-amino]-9H-purin-9-yl)-N-methyl-ß-D-ribofuranoamide (IB-MECA) stimulated the cycling of hematopoietic progenitor cells, that of A_1 receptors by the pertinent agonist N^6-cyclopentyladenosine inhibited proliferation of these cell types (Pospíšil *et al*. 2004, 2005, Hofer *et al.* 2006a, 2007, 2008). Pharmacological activation of adenosine A_3 receptors mediated hematopoiesis-stimulating effects which could be employed in the treatment of radiation-induced myelosuppression (Hofer *et al.* 2010, 2011b). Adenosine A_3 receptor agonists were reported to show a low level of undesirable side effects (summarized in Hofer *et al.* 2012).

Concerning the mechanisms of the hematopoiesis-modulating effects of adenosine receptor agonists, it was found that pharmacologically evoked elevation of extracellular adenosine induces serum colony-stimulating activity and interleukin-2 in mice (Weiterová *et al.* 2007). In *in vitro* studies it was observed that adenosine potentiates the stimulatory effects of interleukin-3 (IL-3) and the stem cell factor (SCF) on the proliferation of hematopoietic progenitor cells for granulocytes and macrophages (GM-CFC) (Hofer *et al*. 2006b), and that pharmacological activation of adenosine A_3 receptors by IB-MECA potentiates the stimulatory effects of IL-3, SCF, and of the granulocyte-macrophage colony-stimulating factor (GM-CSF) on this hematopoietic cell compartment (Hofer *et al*. 2009). It was also found that all four adenosine receptor subtypes are expressed in various hematopoietic cells (Štreitová *et al.* 2010a, b). In a model promyelocytic HL-60 cell line, expression of adenosine receptors, including those of the A_3 type, was observed to be dependent on the cell cycle phase (Hofer *et al.* 2011c). Taken together, the above studies have clearly shown that adenosine receptors play an important role in the regulation of hematopoietic processes, a key hematopoiesis-stimulating role being played by the adenosine A_3 receptor subtype.

The use of genetically engineered mice represents a modern and efficient methodological approach for studies on the mechanisms of various physiological and pathophysiological processes, as well as of drug effects in the mammalian organism. Among the numerous genetically modified mice also A_3 adenosine receptor knock-out ($A_3AR^{(-/-)}$) mice were constructed and several articles based on the use of these mice were published (*e.g.* Wu *et al.* 2002, Fedorova *et al.* 2003, Björklund *et al.* 2008). Nevertheless, the hematopoietic status of A_3 adenosine receptor knock-out mice has not been investigated yet. We have focused our attention on the parameters of erythropoiesis and thrombopoiesis of $A_3AR^{(-/-)}$ mice, and we present here the findings obtained.

Material and Methods

Mice

Adenosine A_3 receptor knock-out ($Adora3^{tm1Jbsn}/Adora3^{tm1Jbsn}$, $A_3AR^{(-/-)}$) mice, backcrossed onto a C57BL/6 background (Salvatore *et al.* 2000), 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. Wild-type (WT) C57BL/6 mice were obtained from the Laboratory Animal Breeding and Experimental Facility of the Faculty of Medicine, Masaryk University, Brno, Czech Republic. For material sampling 2.5 months old mice were used. Their body weight was determined and hematological analysis was performed. The findings on erythroid and platelet compartments are reported here.

Analysis of peripheral blood

For determination of erythrocyte and platelet parameters the peripheral blood was sampled from the ocular sinus. The erythrocyte and platelet count, as well as hematocrit, hemoglobin level in the peripheral blood, mean erythrocyte volume, mean red blood cell

Table 1. Values of chosen parameters in $A_3AR^{(-/-)}$ and their wild-type (WT) counterparts.

Parameter	Male $A_3AR^{(-/-)}$ mice (n=11)	Male WT mice (n=10)	Female $A_3AR^{(-/-)}$ mice (n=10)	Female WT mice (n=10)
Mouse body weight (g)	23.5***	26.1	20.4	20.4
	(22.7; 24.2)	(25.3; 26.9)	(19.2; 21.6)	(19.7; 21.1)
Peripheral blood erythrocyte count (x 10^{12}/l)	9.0	8.8	8.8	8.9
	(8.7; 9.3)	(8.5; 9.0)	(8.5; 9.2)	(8.5; 9.3)
Hematocrit (%)	38.2**	40.7	43.2^{+++}	39.8
	(36.7; 39.7)	(39.9; 41.5)	(42.7; 43.7)	(38.5; 41.2)
Hemoglobin level in peripheral blood (g/l)	123.8	124.9	121.9	126.1
	(118.3; 129.3)	(121.7; 128.1)	(115.2; 128.5)	(121.8; 130.4)
Mean erythrocyte volume (fl)	42.4***	46.5	43.2*	44.9
	(41.9; 43.0)	(45.1; 47.9)	(42.7; 43.7)	(44.2; 45.6)
Mean red blood cell hemoglobin (pg)	13.5*	14.2	13.8*	14.2
	(13.3; 13.7)	(14.0; 14.4)	(13.5; 14.0)	(14.0; 14.4)
Red blood cell distribution width (%)	12.6**	14.0	12.7	13.2
	(12.0; 13.2)	(13.5; 14.4)	(12.1; 13.2)	(12.7; 13.8)
Peripheral blood platelets (x 10^9/l)	304.4*	355.3	290.6**	352.6
	(286.6; 322.2)	(325.7; 384.9)	(260.6; 320.5)	(327.7; 377.5)
Mean platelet volume (fl)	5.9	6.0	6.1	5.9
	(5.9; 6.1)	(6.0; 6.1)	(5.9; 6.1)	(5.9; 6.0)
Plateletcrit (%)	0.18**	0.21	0.18**	0.21
	(0.17; 0.19)	(0.20; 0.23)	(0.16; 0.19)	(0.20; 0.23)
Platelet distribution width (%)	14.7	14.8	14.7	14.8
	(14.7; 14.8)	(14.7; 14.9)	(14.7; 14.8)	(14.7; 14.8)
BFU-E/femur /g body weight	408.7	393.5	488.0	481.6
	(349.3; 468.1)	(355.6; 431.3)	(414.1; 561.9)	(417.5; 545.7)

Values are presented as means (95 % confidence limits); n – numbers of mice; $^{+++}$ the value is significantly (P<0.001) higher than that in the corresponding WT counterparts; *, **, *** the value is significantly (P<0.05, P<0.01, P<0.001) lower than that in the corresponding WT counterparts

hemoglobin, red blood cell distribution width, mean platelet volume, plateletcrit, and platelet distribution width were determined using an Auto Hematology Analyzer BC-2800 (Mindray, Shenzen, China).

Determination of hematopoietic progenitor cells for erythrocytes (BFU-E)

The total number of nucleated cells per femur was determined by means of a Coulter Counter (Model ZF, Coulter Electronics, Luton, UK). Erythroid progenitor cells (burst-forming units, BFU-E) were cultivated on methylcellulose. Hemoglobinized colonies were counted as BFU-E after 8-day incubation and the numbers of BFU-E per femur were calculated.

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 particular values of the groups of mice were evaluated using the Tukey's post-hoc test.

Results

The results of our observations on erythroid and thrombocytopoietic parameters in $A_3AR^{(-/-)}$ male and female mice and their WT controls are summarized in Table 1. Since there was a significant difference in body weight between male $A_3AR^{(-/-)}$ mice and their WT

controls leading to the necessity to present the values of BFU-E/femur as per g of body weight, the values of body weight are shown, as well.

The erythrocyte counts are nearly equal in all groups of mice without any statistically significant differences between $A_3AR^{(-/-)}$ and WT mice. The values of hematocrit are significantly lower in male $A_3AR^{(-/-)}$ mice in comparison with their WT counterparts, whereas the opposite was true for the female mice. This finding was difficult to relate to the processes of hematopoiesis, rather it could reflect differences in managing the volume of body fluids between the sexes. The hemoglobin level in the peripheral blood did not show any significant differences between the compared groups of mice. However, both the parameters of the mean erythrocyte volume and mean erythrocyte hemoglobin were significantly lower in $A_3AR^{(-/-)}$ mice when compared with WT animals, both in males and females; the relative size of the differences did not exceed 10 %. The red blood cell distribution width was significantly lower in $A_3AR^{(-/-)}$ males in comparison with their WT controls. The numbers of erythroid progenitor cells BFU-E in the femur were not significantly influenced by the lack of adenosine A_3 receptors.

Statistically significant differences were found between the counts of blood platelets in $A_3AR^{(-/-)}$ mice and their WT counterparts amounting to 17 % and 21 % in males and females, respectively. The mean platelet volumes did not differ significantly between the groups of animals studied. However, the plateletcrit was observed to be lower by 17 % in both sexes of the $A_3AR^{(-/-)}$ mice when compared to WT mice, the differences being always statistically significant. The platelet distribution width was nearly equal in all the groups studied.

Discussion

The occurrence of lower mean erythrocyte volume and mean erythrocyte hemoglobin in $A_3AR^{(-/-)}$ mice suggests a positive role of adenosine A_3 receptors in erythropoiesis. The same holds for peripheral blood platelets. The evidence that the values of erythrocytes and hemoglobin in the peripheral blood do not differ in $A_3AR^{(-/-)}$ mice from those found in WT mice can be due to the regulatory complexity of erythropoiesis. Adenosine A_3 receptors seem to be important regulators of the blood-forming functions here investigated, and agonists of these receptors can act as curative means enabling an optimum operation of these functions.

If the observed significant decrease of blood platelet counts from that in the wild-type controls to that found in the $A_3AR^{(-/-)}$ mice was physiologically relevant to produce manifestations of bleeding, then the observed minor disturbances in some parameters characterizing erythrocytes in the $A_3AR^{(-/-)}$ mice might be the result of not quite a fully-fledged regeneration following an erythrocyte loss. Nevertheless, the possible intensification of erythropoietic processes does not include significant changes in the compartment of marrow erythroid cells BFU-E; their numbers in $A_3AR^{(-/-)}$ mice are comparable to those in their WT counterparts. It can be inferred from this finding that the possible intensified regeneration of erythropoiesis takes place in the more differentiated compartments of the bone marrow precursor cells. These considerations are in agreement with our recent findings that administration of IB-MECA, an adenosine A_3 receptor agonist, to mice does not significantly influence the compartment of hematopoietic stem cells (Hofer *et al.* 2013).

The observed difference in the male body weight of $A_3AR^{(-/-)}$ and WT mice might be theoretically caused by some sex-dependent function of the adenosine A_3 receptor but the finding is not explainable on the basis of the data available now. Further studies are needed to confirm this finding and, as the case may be, to determine its cause.

In conclusion, the results of this study provide new evidence on the role of adenosine A_3 receptors in hematopoiesis. The methodical approach using adenosine A_3 receptor knock-out mice did not only confirm but also extended data on the already known role of adenosine A_3 receptor signaling in erythropoiesis (Pospíšil *et al.* 1998, 2004, Hofer *et al.* 2002, 2007, Weiterová *et al.* 2000). In addition, the role of adenosine A_3 receptors in the regulation of blood platelet levels was demonstrated.

Conflict of Interest

There is no conflict of interest.

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