

Adenosine A₁, A_{2a}, A_{2b}, and A₃ Receptors in Hematopoiesis. 1. Expression of Receptor mRNA in Four Mouse Hematopoietic Precursor Cells

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Received November 24, 2008

Accepted January 22, 2009

On-line February 27, 2009

Summary

Four mouse bone marrow or thymus cell populations, namely granulopoietic/monocytopoietic, erythropoietic, B-lymphopoietic, and T-lymphopoietic precursor cells have been assayed by RT-PCR technique for the presence and relative amounts of adenosine A₁, A_{2a}, A_{2b}, and A₃ receptor mRNA. It has been found that (i) all four populations studied express all four adenosine receptor subtypes, (ii) the A₁ receptor is the least expressed in all populations studied, (iii) the A₃ receptor is markedly expressed in the populations of granulopoietic/monocytopoietic and erythropoietic cells, (iv) the A_{2a} receptor is markedly expressed in the populations of B-lymphopoietic and T-lymphopoietic cells, and v) the A_{2b} receptor does not predominate in any of the precursor cells studied. Our data offer a new possibility for the assessment of the readiness of these cells to respond, by receptor-mediated mechanisms, to adenosine or its analogs present in the tissues as a result of endogenous processes and/or following their administration.

Key words

Adenosine receptors • mRNA expression • Hematopoiesis

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Hematopoiesis is under the control of regulatory factors acting on hematopoietic stem, progenitor, and

precursor cells. Their action is often pleiotropic and final effects result from interactions in the regulatory network. To elucidate the role of particular active substances in the regulation of hematopoiesis, two basic experimental approaches are possible. First, it can be investigated whether and how particular cells respond to the presence of an active substance. Second, studies can be targeted at revealing whether the given cells possess the ability to respond to an active substance; studies concerning the expression of the cell surface receptors are an example.

Adenosine acts as a paracrine regulator of many cellular functions including proliferation and differentiation. The regulatory role of extracellular adenosine is based on the activation of cell surface receptors, namely A₁, A_{2a}, A_{2b}, and A₃. Receptor activation can be achieved either non-selectively, by adenosine, or selectively, using various adenosine analogs (Jacobson 2002).

A number of hematological studies have been published revealing the significant role of non-selective activation of adenosine receptors in stimulating hematopoiesis (Pospíšil *et al.* 1993, Hofer *et al.* 2001). Recently it has been shown that synthetic selective adenosine receptor agonists stimulate or suppress proliferation of important cells of the hematopoietic system (Pospíšil *et al.* 2004, 2005, Hofer *et al.* 2006, 2009).

Far less attention has been paid to attempts to obtain data on the expression of individual adenosine

Table 1. Sequence of primers used in RT-PCR.

Gene	Accession No.	Primer	5' → 3' sequence	Amplicon size (bp)
<i>Adora 1</i>	AJ555877	Forward	ATCCCTCTCCGGTACAAGACAGT	120
		Reverse	ACTCAGGTTGTTCCAGCCAAAC	
<i>Adora 2A</i>	Y13346	Forward	CCGAATCCACTCCGGTACA	120
		Reverse	CAGTTGTTCCAGCCCAGCAT	
<i>Adora 2B</i>	NM_007413	Forward	TCTTCCTCGCCTGCTTCGT	121
		Reverse	CCAGTGACCAAACCTTTATACCTGA	
<i>Adora 3</i>	XM_131085	Forward	ACTTCTATGCCTGCCTTTTCATGT	128
		Reverse	AACCGTTCTATATCTGACTGTCAGCTT	
<i>β-actin</i>	NT_081055	Forward	CAATAGTGATGACCTGGCCGT	148
		Reverse	AGAGGGAAATCGTGCGTGAC	

Adora 1 – gene for adenosine A₁ receptor; *Adora 2A* – gene for adenosine A_{2A} receptor; *Adora 2B* – gene for adenosine A_{2B} receptor; *Adora 3* – gene for adenosine A₃ receptor.

receptor subtypes in relevant cells with the aim of judging whether these cells can respond to the pertinent stimulation. In particular, no information exists on the expression of individual adenosine receptor subtypes in differentiating and proliferating hematopoietic cell compartments. This communication represents an attempt to fill at least partially this gap. Four mouse *in vivo* bone marrow or thymus hematopoietic cell populations, namely granulopoietic/monocytopoietic, erythropoietic, B-lymphopoietic, and T-lymphopoietic precursor cells were assayed by quantitative RT-PCR technique for the presence and relative amounts of adenosine A₁, A_{2a}, A_{2b}, and A₃ receptor mRNA.

Specific pathogen free C57Bl/6 female mice (AnLab, Czech Republic), 3 months old, 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 performed with the approval of the Institute's Ethics Committee.

Cell sorting: Following subpopulations were sorted from mouse bone marrow: granulopoietic/monocytopoietic cells: Gr-1+, Mac-1+, CD45+; erythropoietic cells: Ter119+; B-lymphopoietic cells: B220+, CD45+, or thymus: T-lymphopoietic cells: CD45+, Gr-1-, Mac-1-. Bone marrow was flushed from femurs into PBS supplemented with 1 % of BSA (PBS-BSA). Thymus was minced in PBS-BSA using loose-fitting glass tissue homogenizer. Pooled samples from three donors were used for each sorting. Erythrocytes were briefly lysed with ammonium chloride solution, the

cells were washed twice, stained with fluorescently labeled antibodies (BioLegend, USA) and sorted according to presence/absence of specific surface antigens on FACS Aria sorter (Becton Dickinson, USA) using Purity Mode. Cells were sorted directly to RNeasy Protect Cell Reagent (Qiagen, Germany). The purity was measured on duplicate sorting into PBS and was higher than 98 % in all samples.

RNA isolation was done by TRIzol (Invitrogen, USA) method. RNA concentration and purity were measured on Nano-Drop ND1000 Spectrophotometer (Thermo Scientific, USA). The 260/280 ratio was not less than 1.8 for each RNA sample. cDNA was prepared by iScript cDNA Synthesis Kit (Bio-Rad, USA). One µg of RNA template was used per each RNA-to-cDNA reaction.

Quantitative real-time RT-PCR: mRNA levels of selected genes in the sorted populations were measured in triplicate on RotorGene 6000 (Corbett Research, Australia) using SYBR Green Master Mix reagent (Roche Diagnostics, Germany) (for details see Štreitová *et al.* (2010). Primer sequences (see Table 1) were obtained from Ashton *et al.* (2003) and Overbergh *et al.* (1999).

To calculate relative gene expression, we used delta-delta Ct method based on the difference of the threshold cycles (Ct) of target gene and β-actin sequence. We assumed a twofold increase in PCR products per cycle. A receptor was taken for relative quantification if the threshold cycle number was less than 36. If the threshold cycle number was greater than 36, the receptor was considered to be present in minimal quantities and

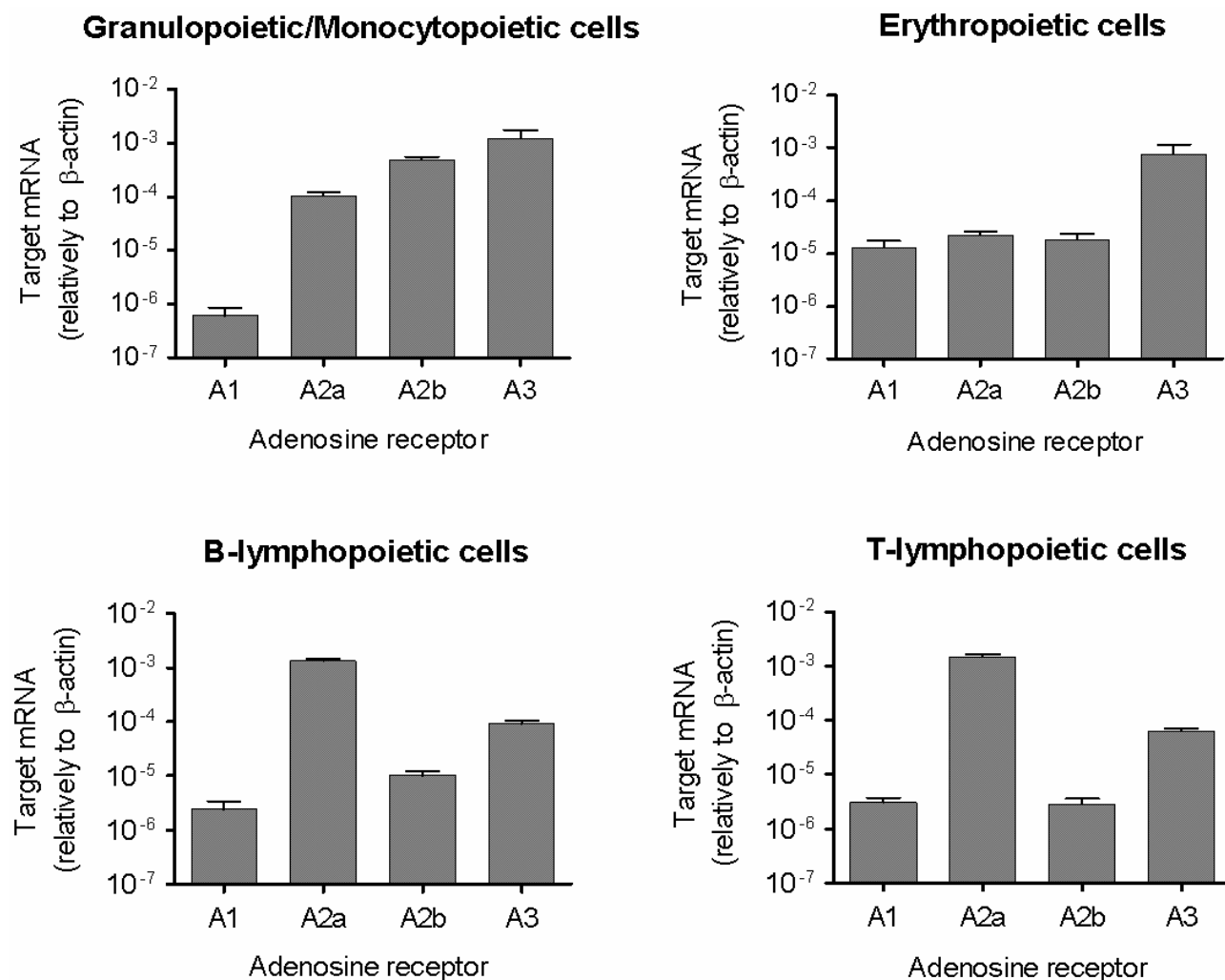


Fig. 1. Relative mRNA gene expression in four mouse hematopoietic cell populations. The experiments were repeated three times. Values are expressed as means \pm S.D. Numeric values ($\times 10^{-7}$): granulopoietic/monocytopoietic cells: 6.1 ± 4.9 , 1038.0 ± 358.8 , 4901.6 ± 1112.3 , and $11,995.2 \pm 5594.8$ for A_1 , A_{2a} , A_{2b} , and A_3 receptors, respectively; erythropoietic cells: 129.8 ± 79.6 , 218.0 ± 73.1 , 180.7 ± 109.3 , and 7607.3 ± 3785.1 for A_1 , A_{2a} , A_{2b} , and A_3 receptors, respectively; B-lymphopoietic cells: 23.8 ± 21.3 , $13,121.3 \pm 1612.9$, 100.7 ± 42.9 , and 911.1 ± 244.7 for A_1 , A_{2a} , A_{2b} , and A_3 receptors, respectively; T-lymphopoietic cells: 30.37 ± 11.0 , $14,710.4 \pm 1924.8$, 28.5 ± 13.6 , and 626.7 ± 125.0 for A_1 , A_{2a} , A_{2b} , and A_3 receptors, respectively.

relative quantification was not performed. A gene was considered not to be expressed if no amplification was detected by cycle 40.

Figure 1 summarizes the results on mRNA expression of adenosine A_1 , A_{2a} , A_{2b} , and A_3 receptor mRNA in four *in vivo* mouse bone marrow and thymus precursor cell populations, namely those of granulopoietic/monocytopoietic and erythropoietic precursor cells, as well as those of B- and T-lymphopoietic cells.

It follows from our findings that (i) all four populations studied express mRNA for all four adenosine receptor subtypes, (ii) A_1 receptor is the least expressed in all populations studied, (iii) A_3 receptor is markedly expressed in the populations of granulopoietic/

monocytopoietic and erythropoietic cells, and iv) A_{2a} receptor is markedly expressed in the populations of B-lymphopoietic and T-lymphopoietic cells. The amount of mRNA does not always correlate with protein levels. Although in our experiments protein levels could not be determined, since antibodies against mouse adenosine receptor proteins, with the exception of A_{2a} , are not available, at least the marked expression of the A_3 receptor mRNA in granulopoietic/monocytopoietic and erythropoietic cells corresponds with the previously observed significant ability of an A_3 receptor agonist to stimulate granulopoiesis and erythropoiesis in mice (Pospíšil *et al.* 2004, Hofer *et al.* 2006). The presence of adenosine A_1 receptor mRNA was demonstrated in all four hematopoietic precursor cell populations studied,

even at low levels, which can be meaningful in view of the findings of inhibitory effects of an A₁ receptor agonist on some hematopoietic cell populations *in vivo* (Pospíšil *et al.* 2004, 2005).

Our results represent the first data on the determination of adenosine A₁, A_{2a}, A_{2b}, and A₃ receptor mRNA in proliferating and differentiating hematopoietic tissues. The up-to-now published findings have only reported the expression of adenosine receptor mRNA in isolated mature human peripheral blood cells: all four subtypes of adenosine receptors have been described in human neutrophils (Fortin *et al.* 2006) and monocytes (Thiele *et al.* 2004), A_{2b} and A₃ receptors have been found in human lymphocytes (Mirabet *et al.* 1999, Gessi *et al.* 2004).

The determination of adenosine receptors in mature blood cells, summarized in above citations, enabled to use this methodological approach in evaluation of the regulation of biological activities of these cells by adenosine and its analogs. However, our data on the occurrence of adenosine receptors mRNA in differentiating and proliferating cells of various precursor cell populations of the bone marrow and thymus offer a new possibility for the assessment of the readiness of

these cells to respond, by a receptor-mediated mechanisms, to adenosine or its analogs. Thus, a possibility appears to assess the feasibility to regulate the processes of hematopoietic cell proliferation and differentiation by activating various adenosine receptors.

Under the conditions *in vivo*, a contribution of an indirect mechanism of hematopoiesis-modulating effects of adenosine receptors agonists cannot be excluded. In that case, the final effects on hematopoietic cells would be achieved by the influence on the hematopoietic microenvironment, as has been suggested by Weiterová *et al.* (2007).

Conflict of Interest

There is no conflict of interest.

Acknowledgements

Supported by grants from the Academy of Sciences of the Czech Republic (AV0Z50040507, AV0Z50040702), the Grant Agency of the Czech Republic (305/06/0015, 305/08/0158), and the Ministry of Education, Youth and Sports of the Czech Republic (MSM 00216220806, LC06044). The authors thank dr. Vladimír Znojil for cooperation in preparation of the manuscript.

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