Adenosine A1, A2a, A2b, and A3 receptors in hematopoiesis. 1. Expression of receptor mRNA in four mouse hematopoietic precursor cells

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Short title: Adenosine receptors and hematopoiesis
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, A2a, A2b, 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 A2a receptor is markedly expressed in the populations of B-lymphopoietic and T-lymphopoietic cells, and v) the A2b 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

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, A2a, A2b, 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 (e.g., 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 (e.g., Pospíšil et al. 2004, 2005, Hofer et al. 2006, 2008, in press).
Far less attention has been paid to attempts to obtain data on the expression of individual adenosine 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 expression of individual adenosine receptor subtypes in differentiating and proliferating hematopoietic cell compartments. This communication represents an attempt to partially fill 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 $A_1$, $A_{2a}$, $A_{2b}$, and $A_3$ 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 RNAprotect 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. (Physiol Res, submitted concommitantly). Primer sequences (see Table 1) were obtained from Ashton et al. (2003) and Overbergh et al. (1999).
Table 1. Sequence of primers used in RT-PCR

<table>
<thead>
<tr>
<th>Gene</th>
<th>Accession No.</th>
<th>Primer 5´→3´</th>
<th>Amplicon size (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Adora 1</td>
<td>AJ555877</td>
<td>Forward</td>
<td>ATCCCTCTCCGGTACAAGACAGT</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Reverse</td>
<td>ACTCAGGTTGTTCCAGCACAAC</td>
</tr>
<tr>
<td>Adora 2A</td>
<td>Y13346</td>
<td>Forward</td>
<td>CCGAATTCCACTCCGGTTACA</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Reverse</td>
<td>CAGTTTGTCCAGCCCCAGCAT</td>
</tr>
<tr>
<td>Adora 2B</td>
<td>NM_007413</td>
<td>Forward</td>
<td>TCTTCTCGCCTCTGCCTTGTT</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Reverse</td>
<td>CCAGTGACCAAACCTTTATACCTGA</td>
</tr>
<tr>
<td>Adora 3</td>
<td>XM_131085</td>
<td>Forward</td>
<td>ACTTCTATGCTGCTTTTCAGCTGTT</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Reverse</td>
<td>AACCCTTACATCTGACTGACGCTT</td>
</tr>
<tr>
<td>β - actin</td>
<td>NT_081055</td>
<td>Forward</td>
<td>CAATAGTGATGACCTGGCCTGAC</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Reverse</td>
<td>AGAGGGAATCGTGCGTGAC</td>
</tr>
</tbody>
</table>


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 2-fold 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 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 A1, A2a, A2b, and A3 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) A1 receptor is the least expressed in all populations studied, (iii) A3 receptor is markedly expressed in the populations of granulopoietic/monocytopoietic and erythropoietic cells, and iv) A2a receptor is markedly expressed in the populations of B-lymphopoietic and T-lymphopoietic cells. The amount of mRNA needs not automatically correlate with protein levels. Although in our experiments protein levels could not been determined since antibodies against mouse adenosine receptor proteins, with the exception of A2a, are not available, at least the marked expression of the A3 receptor mRNA in granulopoietic/monocytopoietic and erythropoietic cells corresponds with the previously observed significant ability of an A3 receptor agonist to stimulate...
granulopoiesis and erythropoiesis in mice (e.g., Pospíšil et al. 2004, Hofer et al. 2006). The presence of adenosine A₁ 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 (e.g., Pospíšil et al. 2004, 2005).

Our results represent the first data on the determination of adenosine A₁, A₂a, A₂b, 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 (e.g., Fortin et al. 2006) and monocytes (Thiele et al. 2004), A₂b and A₃ receptors have been found in human lymphocytes (Mirabet et al. 1999, Gessi et al. 2004).

Determination of adenosine receptors in mature blood cells, summarized in above citations, has made it possible to use this methodological approach in discussions about regulation of actions 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 response, 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.

In 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 a mediation of the cells of the hematopoietic microenvironment, as has been suggested (e.g., Weiterová et al. 2007).

**Acknowledgements**

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POSPÍŠIL M, HOFER M, VACEK A, HOLÁ J, PIPALOVÁ I, ZNOJIL V: N6-
Cyclopentyladenosine inhibits proliferation of murine haematopoietic progenitor cells in vivo.


Legend to Figure 1:

Figure 1. **Relative mRNA gene expression in four mouse hematopoietic cell populations.** The experiments were repeated three times. Values are expressed as means ± S.D. Numeric values (x 10^-7): granulopoietic/monocytopoietic cells: 6.1 ± 4.9, 1038.0 ± 358.8, 4901.6 ± 1112.3, and 11,995.2 ± 5594.8 for A1, A2A, A2B, and A3 receptors, respectively; erythropoietic cells: 129.8 ± 79.6, 218.0 ± 73.1, 180.7 ± 109.3, and 7607.3 ± 3785.1 for A1, A2A, A2B, and A3 receptors, respectively; B-lymphopoietic cells: 23.8 ± 21.3, 13,121.3 ± 1612.9, 100.7 ± 42.9, and 911.1 ± 244.7 for A1, A2A, A2B, and A3 receptors, respectively; T-lymphopoietic cells: 30.37 ± 11.0, 14,710.4 ± 1924.8, 28.5 ± 13.6, and 626.7 ± 125.0 for A1, A2A, A2B, and A3 receptors, respectively.