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REVIEW

RT-PCR Study of Purinergic P2 Receptors in Hematopoietic Cell Lines

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Abstract—Seven P2X and fifteen P2Y receptors have been identified to date, partly on the basis of amino acid sequence homologies. The expression of all cloned human purinergic P2 receptors was investigated on the messenger RNA level in promonocytic U937 cells, erythroblastic K562 cells, and undifferentiated, dimethyl sulfoxide-differentiated granulocytic, and phorbol-12-myristate-13-acetate-differentiated monocytic HL60 cells. RT-PCR assays showed expression of several P2X receptors, whereas all P2Y receptors were found in at least some of the analyzed cells lines. Granulocytic and monocytic differentiation of HL60 cells lead to a partly dramatic up- or downregulation of receptor transcripts. The number of different P2 receptors expressed in each cell type showed a significant rise from U937 cells via K562 cells, undifferentiated and granulocytic, to monocytic HL60 cells. The total mRNA amounts being normalized to the glyceraldehyde-3-phosphate dehydrogenase levels demonstrated an even more distinct variability of absolute transcript levels. An increased number of different P2 receptors expressed were associated with an increased total average P2 receptor mRNA amount in each cell. This phenomenon of overexpression suggests self-inductive effects of purinergic signaling indicating its involvement in hematopoiesis and possibly in immunoreactive mediation.

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The nucleotide ATP is one of the most important substances in organisms. Intracellular ATP catalyzes energy transmission in mammalians as well as in microorganisms. The biological effects of extracellular ATP and other extracellular nucleotides were first recognized by Drury and Szent-Gyorgyi in 1929. The existence of specific nucleotide receptors was postulated by Burnstock in 1978, which were named purinergic receptors [1].

They mediate diverse biological effects such as contraction of smooth muscles as well as rapid depolarization of neurons and stimulation of the inflammatory system.

Today, we know two main classes of purinergic receptors—the P1 and the P2 receptors. While the P1 receptors are specific to adenosine, the P2 receptors are activated especially by ATP, ADP, UTP, UDP, and adenosine. P2 receptors are divided into two main classes, P2X and P2Y receptors. P2X receptors are ATP-gated ion channels, which mediate rapid and selective permeability to cations. In contrast, P2Y receptors are coupled to G proteins. Activation results in mobilization of inositol-1,4,5-trisphosphate-sensitive Ca^{2+} stores, activation of plasma membrane influx pathways, and stimulation of diacylglyceride-dependant protein kinase C enzymes [1, 2]. Several P2X and P2Y receptor subtypes have been cloned and characterized. Seven mammalian P2X receptor proteins (P2X1-7) are currently known. Fifteen P2Y receptors have been identified to date, partly classified as members of the nucleotide receptor family only on the basis of amino acid sequence homologies. It remains unclear whether all of them represent functional P2 receptors. Within the P2Y receptor subtypes, P2Y2, P2Y5, P2Y8, and P2Y11 are recognized by the triphosphate ATP [2]. The diphosphate ADP activates mainly the P2Y1 receptor, only partly P2Y4 and P2Y5. Within the recently identified P2Y receptors, P2Y12 and P2Y13 are similarly sensitive to ADP [3, 4]. P2Y14 has specifici-

Abbreviations: DMSO) dimethyl sulfoxide; GAPDH) glyceraldehyde-3-phosphate dehydrogenase; PMA) phorbol-12-myristate-13-acetate; RT-PCR) reverse transcriptase polymerase chain reaction.

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ty for UDP-conjugated sugars [5, 6], whereas the P2Y15 receptor responds to both AMP and adenosine [7].

Purinergic receptor expression has been found in virtually every cell and tissue studied. Several articles outline the distinct and extended expression patterns of P2 receptors in hematopoietic cell lines [1, 2, 8-10]. However, a complete study on all P2Y receptors in different hematopoietic cell lines is still lacking. We analyzed semiquantitatively mRNA expression of all known P2X and P2Y receptors in undifferentiated promyelocytic HL60 cells, dimethyl sulfoxide (DMSO)-differentiated granulocytic HL60 cells, phorbol-12-myristate-13-acetate (PMA)-differentiated monocytic HL60 cells, erythroblastic K562 cells, and promonocytic U937 cells.

MATERIALS AND METHODS

Cell culture. All chemicals were obtained from Sigma (Germany) except where otherwise stated. The following cell lines were used: HL60 cells are promyelocytic leukemic cells of lymphoblastic morphology of a 36-year-old Caucasian woman. K562 cells were derived of a 53-year-old Caucasian woman who suffered from a chronic myeloid leukemia. The cells show also a lymphoblastic morphology. Contrastingly, U937 cells were obtained from a monocytes-like histocytic lymphoma of a 37-year-old Caucasian man. All cell lines were obtained from the American Type Culture Center (Rockville, USA) and were grown in RPMI 1640 containing 10% fetal calf serum (Boehringer, Germany), 2 mM glutamine, and antibiotics (100 U/ml penicillin and 50 μg/ml streptomycin). Cultures were kept at 37°C in humidified atmosphere with 5% CO_2, and the cells were diluted every 4-5 days to maintain densities between $5 \cdot 10^4$ and 10^6 cells/ml. Growth was determined from triplicate counts of the cell number after exclusion of trypan blue-stained cells.

Differentiation of HL60 cells into granulocyte-type cells was achieved by culturing in medium containing 1.25% DMSO for four days [11]. Differentiation into monocyte-type cells was induced by treatment for 15 h with 20 nM PMA [12].

Undifferentiated as well as DMSO-treated HL60 cells grow as single cell suspension cultures, whereas PMA-treated cells become adherent. For experimental purposes, these cells were removed from the tissue culture dish by gentle scraping.

Reverse transcription-coupled polymerase chain reaction (RT-PCR). Total RNA was isolated from culture cells using a Qiagen RNeasy Mini Kit (Qiagen, Germany). The quantity of RNA was determined photometrically at 260 nm. Five micrograms of RNA were reverse transcribed to cDNA in a 20 μl reaction volume containing 300 ng random hexamers, 0.5 mM dNTPs, 10 mM dithiothreitol, 40 U RNasin, and 400 U Superscript II reverse transcriptase dissolved in the supplied buffer (all reagents from Gibco-BRL Life Technologies, Germany). The reactions were allowed to proceed for 2 h at 42°C, stopped by heating at 70°C for 15 min, and were finally diluted to 100 μl.

Primers and annealing temperatures for P2X1-7 and P2Y1-11 were used as described in our work [8], except for P2X2 [13]. Primer and probe sequences for P2Y12 and P2Y13 have previously been reported in [14], for P2Y14 in [6], and for P2Y15 in [10]. The PCR reactions were performed in 50 μl final volumes containing 2 μl cDNA (diluted 1 : 5), 7.5 pmol of each primer, 250 μM dNTPs, 2.0 U of Taq DNA polymerase, and 2 mM $MgCl_2$ dissolved in the supplied buffer (all reagents from Boehringer). After initial denaturation for 2 min at 94°C, amplifications were carried out for 35 cycles (for glyceraldehyde-3-phosphate dehydrogenase (GAPDH) 30 cycles) as follows: denaturation at 94°C for 45 sec, annealing at primer-specific temperature for 45 sec, and extension at 72°C for 90 sec. After the final PCR cycle, extension was allowed to proceed for 15 min at 72°C.

As a control for contaminations of the RNA preparation with genomic DNA, the crude product of the RNA extraction procedure without any reverse transcription reaction was used as template for a PCR reaction. No signal was detected in these RNA samples without cDNA synthesis indicating that the samples were free of genomic DNA contamination.

For quantification, ethidium bromide-stained gels of the PCR products were analyzed by densitometric scanning with a FluorImager (Molecular Dynamics, USA) using Image Quant software (Molecular Dynamics). Sequence analysis of both strands of the PCR products was performed on an ABI377 Automatic Sequencer (Perkin Elmer, Germany).

RESULTS

Expression levels of all 23 currently known cloned human P2X and P2Y receptors were analyzed in U937, K562, and HL60 cells by a semiquantitative RT-PCR assay using specific primers. To determine the effects of granulocytic differentiation on the mRNA expression, HL60 cells were treated with 1.25% DMSO for four days. Stimulation with 20 nM PMA over a time course of 15 h induced a differentiation to a monocytic/macrophage phenotype. Stimulation times were optimized on the basis of the results of our previous work [8].

RT-PCR products were eluted from the gels and analyzed by sequencing. The relative amounts of the amplified RT-PCR products were quantified by FluorImager analysis and normalized to levels of GAPDH RT-PCR products in the same cells. As we previously showed with dot-blot analysis of the mRNA levels, the RT-PCR technique provides sufficient accuracy within the linear range of amplification to compare rela-

Expression pattern of P2X and P2Y receptors and GAPDH in HL60, K562, and U937 cell lines

	U937	K562	HL60, undifferentiated	HL60, DMSO-differentiated	HL60, PMA-differentiated
P2Y1	+	++	++	++	+++
P2Y2	+	—	+++	++	+
P2Y4	+	++	+++	+++	+++
P2Y5	—	—	—	+	++
P2Y6	+++	+	+++	+++	+++
P2Y8	+++	++	+++	—	++
P2Y9	—	+	++	+	++
P2Y10	—	+	+	+	+
P2Y11	+	+++	+	+++	+++
P2Y12	+	++	++	++	++
P2Y13	—	—	—	+	++
P2Y14	+	+	+	++	+++
P2Y15	—	—	+	—	++
P2X1	+	+	+	+	++
P2X2	—	—	—	—	+
P2X3	—	—	—	—	—
P2X4	—	—	+	+	+++
P2X5 long splicing variant	++	++	+	++	+++
P2X5 short splicing variant	—	—	+	+	++
P2X7	+	—	+++	++	+++
GAPDH	+++	+++	+++	+++	+++

Note: (+) denotes minimal, (++) moderate, (+++) strong, and (–) absent signal intensity corresponding to the amount of RT-PCR product.

tively mRNA amounts of different receptors [8]. All quantification experiments were repeated three times, using three independent batches of cell preparation. Triplicate determinations were performed for each set of measurement.

We observed a widespread distribution pattern for the expression of most P2X and P2Y receptor mRNA in U937, in K562, and in undifferentiated as well as in differentiated HL60 cells (table). GAPDH mRNA was equally expressed in all samples analyzed.

Within the P2X receptors, only P2X3 mRNA was not detected in any of these cells. Undifferentiated and differentiated HL60 cells expressed P2X1, P2X4, both known splicing variants of P2X5, and P2X7, but clearly showing variable mRNA amounts dependent on the stage of differentiation. P2X4 and the long splicing variant of P2X5 were not detectable in U937 and K562 cells. P2X1 mRNA in U937 cells and K562 cells as well as P2X7 mRNA in U937 cells were only observed at low transcript levels.

All P2Y receptors were found in at least some of the analyzed cells lines. Differentiation of HL60 cells lead to dramatic up- or downregulation of some receptor transcripts. While monocytic HL60 cells expressed all P2Y receptors, P2Y5 and P2Y13 receptor mRNA was not detectable in undifferentiated cells and P2Y8 and P2Y15 receptors mRNA was not found in granulocytic HL60 cells. As in undifferentiated HL60 cells no P2Y5 and P2Y13 mRNA was seen in U937 and in K562 cells. The amount of most P2Y receptor transcripts in U937 cells differed widely from those in K562 cells.

Comparison of the number of different P2 receptor expressed in each cell type showed a significant increase from U937 cells via K562 cells, undifferentiated and granulocytic to monocytic HL60 cells. P2X receptors were predominant in HL60 cells; however, there was no change during granulocytic or monocytic differentiation (Fig. 1).

The total mRNA amounts being normalized to the GAPDH levels in each cell line demonstrated an even more distinct variability of absolute transcript levels (Fig. 2). Both total P2X and P2Y mRNA amounts were similar in K562 and U937 cells. HL60 cells expressed twice as much P2X transcripts as U937 cells. Although there was

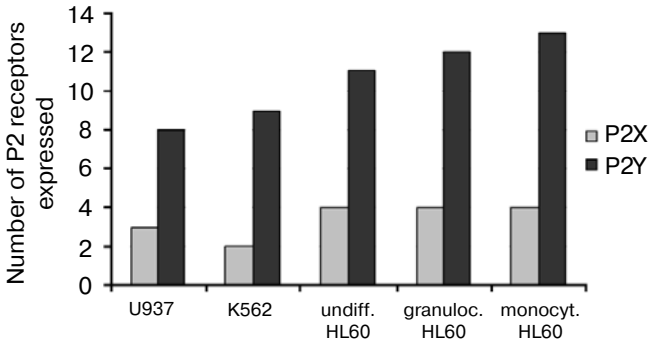

Fig. 1. Number of different P2X and P2Y receptors expressed in U937, K562, and undifferentiated, granulocytic, and monocytic HL60 cell lines.

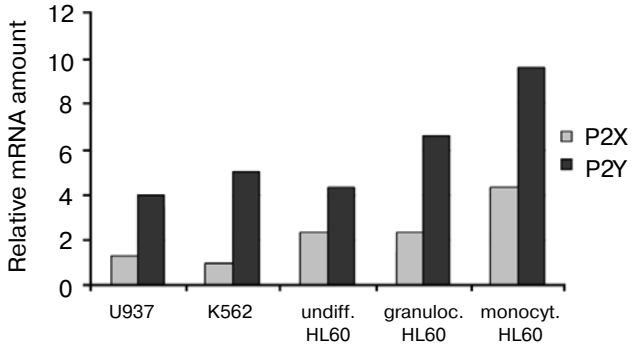

Fig. 2. Relative P2X and P2Y receptor mRNA amount in U937, K562, and undifferentiated, granulocytic, and monocytic HL60 cell lines. The level of GAPDH mRNA is defined as unity.

no change of the number of P2X receptors expressed during granulocytic and monocytic differentiation, the mRNA amount nearly doubled from undifferentiated and granulocytic to monocytic HL60 cells. Within the P2Y receptors, transcript levels of P2Y receptors were comparable in U937 cells and undifferentiated HL60 cells. K592 cells showed a slight increase, whereas total P2Y mRNA raised more than twice via granulocytic to monocytic HL60 cell differentiation. In general, the phenomenon of overexpression was observed in all cell types analyzed: an increased number of different receptors expressed were associated with an increased average transcript level of each receptor itself.

DISCUSSION

In this study, we analyzed the mRNA expression levels of all currently known P2X and P2Y receptors in K562, in U937, and in undifferentiated, granulocytic, and monocytic HL60 cells.

Our data confirm previous studies of the expression of single purinergic receptors in hematopoietic cell lines. P2X1, P2X7, P2Y1, P2Y2, and P2Y4 receptor expression in U937 and in K562 cell lines has also been detected by other researchers [15-17]. Expression of the recently cloned P2Y12, P2Y13, P2Y14, and P2Y15 receptors was found in leucocytes, spleen, bone-marrow hematopoietic cells, or platelets [3, 6, 7, 18]. Clifford et al. were the first who analyzed stage-specific changes in P2Y2 mRNA levels during myeloid differentiation of HL60 cells [19]. Similar data has been published by Martin et al. [20]. Our group extended these studies to an investigation of the expression of several purinergic P2X and P2Y receptors during myeloid differentiation of HL60 cells. Most receptors revealed a distinct regulation during the course of differentiation [8].

For this study, stimulation times were changed and optimized on the basis of the previous data. Interestingly, even slight changes resulted in partly dramatic changes in expression levels. This indicates a highly sensitive regulation mechanism during myeloid differentiation.

It has recently been shown that the pharmacological profiles of P2Y receptors change also during differentiation of myeloid, megakaryoid, erythroid, and lymphoid cells [21].

Comparison of the number of purinergic receptors expressed and the average P2 receptor messenger RNA amount of each cell reveals positive synergism. The total number of P2 receptors expressed at a certain stage of hematopoietic differentiation correlates with an increase in transcript amount of each receptor in the cells. We call this phenomenon overexpression.

The complex function and regulation of the purinergic system is still little understood. Schulze-Gahmen et al. [22] demonstrated for HL60 cells that extracellular ATP, the purinergic ligand itself, induces differentiation and suppresses growth, which leads consequently again to modification of purinergic signaling. Differentiation-inducing activity of cytokines is closely associated with the synthesis of their nucleotides in HL60 cells. However, addition of ATP or AMP has only additive effects on cytokine-induced differentiation, suggesting that cytokine-induced differentiation is not directly associated with signal transduction mediated by P2 receptors. Interpretation of such results is aggravated by the fact that not only the receptor-mediated response to purinergic ligands but the signal itself is modified through extracellular enzymes. These enzymes underlie a partly strong up- or downregulation during differentiation as well [8].

Little is yet known about the intrinsic function of such a large pattern of seven P2X and fourteen P2Y receptors in organisms. For the P2Y2, P2Y4, and P2Y6 receptors, ATP and UTP have been demonstrated to stimulate the airway mucociliary escalator, representing potential targets for the therapy of cystic fibrosis and other pulmonary diseases [23]. For instance, adenosine receptor antagonists such as theophylline or caffeine are

used in obstructive respiratory diseases as bronchodilators [24]. Low plasma concentrations of caffeine relieve histamine-induced bronchoconstriction [25]. Therefore, direct pathways or at least reaction cascades between extracellular nucleotides, purinergic receptors, and immune reactions must exist.

The phenomenon of overexpression might partly explain for example the observation of self-inductive effects in the development of pulmonary obstructive diseases or even late reaction of anaphylaxis. The dramatic changes in expression levels of a large bunch of receptors stimulated by extracellular nucleotides within hours enables the purinergic system to contribute largely to the main streams of physiological regulation mechanisms and therefore to the clinical manifestation of diseases.

Further investigations of these regulations will hopefully give more knowledge about the purinergic system as a target for pharmacological therapies for in our days still untreatable diseases.

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