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P2 Receptor Subtypes in Human Hematopoietic Cells of Peripheral and Cord Blood

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Abstract: P2 receptors have been found in several blood cells and their progenitors. However, most studies lack data about receptor subtypes and of receptors expression time in the process of cell differentiation. The aim of our study was to identify the subtypes of P2Y and P2X receptors on human CD34+ cells, c-kit+ cells, monocytes, lymphocytes of cord and peripheral blood. Expression of P2Y1, P2Y4, and P2Y6 receptors was the uniform in the cord and peripheral blood of all studied cells with the prevalence of monocytes expressing P2Y-receptors (up to 71%). At the same time, a significant difference was found between cells of cord and peripheral blood expressing subtypes of P2X2, P2X3, P2X4, P2X5, P2X6, P2X7 receptors. Cord blood lymphocytes contained a higher percentage of P2X receptors than peripheral blood lymphocytes. Similarly, the percentage of the peripheral blood monocytes, containing P2X receptors was significantly higher than the monocytes of cord blood.

Keywords: P2X receptors; P2Y receptors; CD34+ cells; c-kit; monocytes; lymphocytes.

1. Introduction

It is known that several subtypes of P2 receptors could be involved in the process of maturation and differentiation of blood cells [1,2], but still, there is a lack of data about the expression of particular subtypes of P2-receptors on blood cell surfaces [3–5].

It was shown that $P2Y_1$ receptors are expressed from the early stages of myeloid progenitors differentiation, $P2Y_2$ receptors are expressed from the stage of myeloblast till the stage of maturated neutrophils [6], and $P2Y_{11}$ receptors are involved in the process of differentiation of promyelocytes, granulocytes and dendritic cells [7,8]. ATP, a primary agonist of the P2 receptor, increases the permeability for cations and large molecules in lymphocytes [9] and controls proliferation and cell death via P2X7 receptors [10,11].

Another P2 receptor agonist, ADP, mobilizes Ca2+ ions from an intracellular store and change platelet's shape by activation of $P2Y_1$ receptors [12,13] and reduces platelet aggregation by activation of $P2Y_{12}$ receptors [14]. The latter has been found to be the mechanism of action of thienopyridines (clopidogrel, ticagrelor, and prasugrel), a group of effective antiplatelet drugs [15–17].

Maturation and differentiation of any blood cell begin from the hematopoietic cell [18,19]. CD34 is a transmembrane glycoprotein expressed on hematopoietic cells, including human endothelial progenitor cells, progenitors of peripheral and cord blood cells [20], and is

widely used for their identification. It has been shown that stimulation of $CD34^+$ cells of peripheral blood and bone marrow by extracellular nucleotides cause a fast release of Ca^{2+} ions from intracellular stores and an increase of ion influxes across the plasma membrane, enhancing the proliferation of human hematopoietic progenitors [21,22]. It has been found that human $CD34^+$ cells of peripheral blood and bone marrow express P2X7 and $P2Y_1$ receptors [21] and mRNA transcripts of most P2 receptors has been detected in human monocytes, lymphocytes and $CD34^+$ cells of bone marrow [23,24].

Another marker of hematopoietic cells, c-kit, also known as CD117, is a transmembrane receptor for protein tyrosine kinase and stem cell factor [25,26]. CD117 upon interaction with its ligand stem cell factor stimulates hematopoiesis [27] presents in mast cells, melanocytes of the skin, renal tubular epithelial cells, interstitial cells of Cajal in the gastrointestinal tract, and different cell types in reproductive organs, melanocytes maturation [28] and causes a vast majority of biological effects in mammalian cells [29].

The aim of our study was to identify the subtypes of P2Y and P2X receptors on human $CD34^+$ cells, c-kit^+ cells, monocytes, lymphocytes of cord and peripheral blood.

2. Materials and Methods

The cord blood was obtained during the normal timed labor, while the peripheral blood was taken from the healthy volunteers. The blood was collected into tubes with sodium citrate and immediately delivered to the laboratory. The mononuclear cells (monocytes, lymphocytes, and $CD34^+$ cells) were separated by the centrifugation (BIOSAN LMC-3000, Latvia) (20 min at 1500 rpm) on a Ficoll-Paque density gradient (Sigma) [30].

The immunomagnetic separation of $CD34^+$ cells from the fraction of the mononuclear cells was performed on a magnetic particle concentrator (Dynal MPS) using magnetic antibodies (Invitrogen). The viability of the cells was controlled by the flow cytometry (Becton Dickinson, USA).

A $CD34^+$cells were stained with allophycocyanin-conjugated antibodies (Abcam) for the flow cytometry. P2X and P2Y receptor subtypes were evaluated by the indirect immunofluorescence reaction with primary antibodies against P2X2, P2X3, P2X4, P2X5, P2X6, P2X7, $P2Y_1$, $P2Y_4$, $P2Y_6$ receptors (Alomone, Abcam). Secondary antibodies were conjugated with a fluorescein isothiocyanate (FITC) (Sigma). A c-kit was used as a marker of hematopoietic stem cells to confirm the hematopoietic line of differentiation of studied cells. A c-kit receptor was verified with a phycoerythrin-conjugated antibody (Abcam) [31].

The results of the immunofluorescence study were obtained by a FACSCanto II flow cytometer (Becton Dickinson, USA) with a FACSDiva data acquisition system. The results were analyzed using an unpaired Student's t-test. A probability of less than 0.05 was considered significant.

3. Results and Discussion

3.1. Results.

$P2Y_1$, $P2Y_4$, $P2Y_6$ receptors were identified on the $CD34^+$/c-kit^+ cells, $CD34^+$ cells, the monocytes, the lymphocytes of the cord, and peripheral blood (Figure 1, Table 1, 2). There were no differences between the expression of studied subtypes of P2Y receptors on the $CD34^+$ cells of the cord (Table 1) and the peripheral blood (Table 2). The lowest level of expression in the lymphocytes and the monocytes of the cord and peripheral blood was observed for $P2Y_1$

receptors, while the highest level was for $P2Y_4$ receptors. The ratio of P2Y receptors expressed on monocytes of cord and peripheral blood were higher compared with lymphocytes.

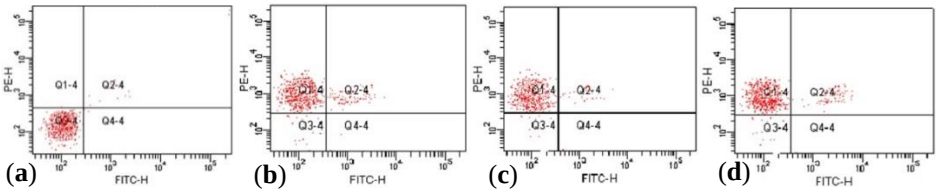

Figure 1. The ratio of CD34+ cells expressing P2Y receptor subtypes. Negative control (the cells without primary antibodies are to evaluate the specificity of monoclonal antibody binding). Horizontal scale indicate fluorescence of FITC-conjugated secondary antibodies to $P2Y_1$(**b**), $P2Y_4$(**c**), $P2Y_6$ (**d**) receptors; (**a**) Control; Vertical scale indicate fluorescence of PE- conjugated anti-c-kit-antibody (Legend: Q1, P2Y - / c-kit$^+$ cells; Q2, P2Y+ / c-kit$^+$ cells; Q3, P2Y - / c-kit$^-$ cells; Q4, P2Y+ / c-kit$^-$ cells).

Table 1. The ratio of expression of subtypes of P2Y receptors on CD34$^+$/ckit$^+$, CD34$^+$cells, lymphocytes, and monocytes (mean ± SEM, %) obtained from the cord blood. Data are shown as a percentage of the total number of cells, *- p< 0,05 comparing with P2Y1 of CD34$^+$ cells.

Receptor subtype	CD34$^+$/ckit$^+$ (n=3)	CD34$^+$ (n=8-11)	Lymphocytes (n=10-11)	Monocytes (n=6)
$P2Y_1$	3,3 ± 0,8	5,1 ± 1,5	0,5 ± 0,3	21,2 ± 7,3
$P2Y_4$	7,0 ± 1,1	9,0 ± 1,9	17,6 ± 4,6	71,7 ± 9,4*
$P2Y_6$	3,0 ± 1,1	4,3 ± 1,3	5,5 ± 1,8	30,4 ± 11,7

Table 2. The ratio of expression of subtypes of P2Y receptors on CD34$^+$cells, lymphocytes, and monocytes obtained from the peripheral blood. Data are shown as a percentage of the total number of cells, *- p< 0,05 comparing with $P2Y_1$ of CD34$^+$ cells.

Receptor subtype	CD34$^+$ (n=9-10)	Lymphocytes (n=8-9)	Monocytes (n=6-9)
$P2Y_1$	2,8 ± 1,4	2,1 ± 0,3	24,64 ± 8,07
$P2Y_4$	15,2 ± 6,2	16,1 ± 2,6*	62,56 ± 11,65*
$P2Y_6$	11,0 ± 5,5	12,7 ± 2,8*	53,98 ± 12,49*

There was no difference between the ratio of peripheral blood CD34$^+$cells containing P2X receptors and the ratio of cord blood CD34$^+$cells containing the same type of receptors (Figure 2(**a**)). It's interesting that lymphocytes of cord blood contained the higher percentage of P2X receptors (Figure 1(**b**)), while the percentage of the monocytes of peripheral blood containing P2X receptors was significantly higher than the cord blood monocytes (Figure 2(**c**)).

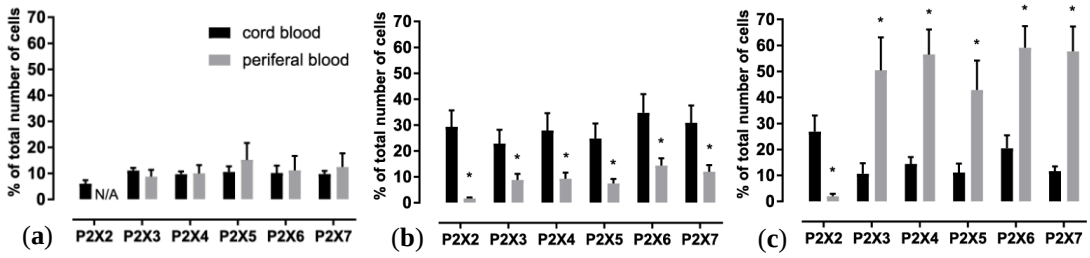

Figure 2. The expression of subtypes of P2X receptors on CD34$^+$ cells (**a**), lymphocytes (**b**), and monocytes (**c**) obtained from the cord blood and peripheral blood. Data presented as a percentage of the total number of cells. Data shown are means, and vertical bars indicate S.E.M., n=5-12, *- p < 0,05 with the same receptor subtype in the cord blood; N/A – not available, P2X2 subtype receptor expression was not evaluated in the peripheral blood.

P2X2, P2X3, P2X4, P2X5, P2X6, P2X7 receptors were expressed on a CD34$^+$/c-kit$^+$ stem cells of the cord blood (Table 3) without any significant difference with the percent of P2X receptors on CD34$^+$ cells of cord and peripheral blood.

Table 3. The ratio of expression of subtypes of P2X receptors on $CD34^+/ckit^-$ cells and $CD34^+/ckit^+$ cells of the cord blood. Data are shown as a percentage of the total number of cells, n=3, * - p< 0,05 comparing with P2X5.

Receptor subtype	Cord blood	
	$CD34^+/ckit^-$	$CD34^+/ckit^+$
P2X2	1,7±1,2*	5,8 ± 1,2
P2X3	2,7±0,9*	7,6 ± 0,8
P2X4	2,2±0,9*	7,2 ± 1,1
P2X5	0,4±0,4	7,4 ± 0,9
P2X6	5,0±2,1*	8,4 ± 1,9
P2X7	1,7±1,7*	8,2 ± 1,1

3.2. Discussion.

The presence of mRNA for the majority of P2 receptors in different cell types has been studied for many years [23,24]. However, there was no much data accumulated on the expression of P2X and P2Y receptors subtypes on the $CD34^+$ cells of human peripheral and cord blood cells. It is known that mRNA presence is not a sufficient requirement for protein expression [32], that is, gene expression does not always correspond to the level of protein expression, and a protein availability should be confirmed experimentally. The occurrence of mRNA of $P2Y_1$, $P2Y_2$, $P2Y_4$, and $P2Y_6$ receptors was shown on lymphocytes and monocytes, but confirmation of their presence is required by specific antibodies [33].

Extracellular nicotinamide adenine dinucleotide is known to increase the intracellular calcium concentration in human lymphocytes. Using P2 receptor-selective agonists and antagonists, the authors demonstrate that $P2Y_1$ and $P2Y_{11}$ receptors play a role in this process [34]. In our study, we identified the expression of $P2Y_1$, $P2Y_4$, and $P2Y_6$ receptors on $CD34^+$ cells of human peripheral and cord blood, as well as on monocytes and lymphocytes. Our study shows almost the same expression of $P2Y_6$ receptors as in the Montano's laboratory, whereas expression for $P2Y_1$ was lower and for $P2Y_4$ were higher in our study [34].

mRNA expression of P2-receptors on lymphocytes, monocytes of peripheral blood, and $CD34^+$cells of bone marrow have been shown earlier [23]. Interestingly, that $P2X_1$, $P2X_4$, $P2X_7$ gene expression were at much higher levels in monocytes than in lymphocytes [23] like in our study, suggesting that they may have an important role in monocyte chemotaxis and activation. The authors indicated that mRNA expression of P2X4 receptors on $CD34^+$cells was three times higher than the expression of mRNA of P2X7 receptors [23]. According to our data, the ratio of P2X7-positive cells does not distinguish between P2X4-positive cells that corroborate the difference between the presence of mRNA and the receptor's protein.

Wang [23] demonstrated that expression of mRNA of $P2Y_1$ receptors on $CD34^+$cells of bone marrow was 10 times higher than the mRNA of $P2Y_4$- and $P2Y_6$-cells. In our study, we did not find any significant difference in the expression of P2Y1, P2Y4, and P2Y6 receptors in $CD34^+$cells of peripheral and cord blood. In the same Wang's study on lymphocyte and monocytes of peripheral blood, it had been shown more quantity of gene of $P2Y_6$ receptors, than of $P2Y_4$ receptors and the less than $P2Y_1$ subtype [23].

Our study showed the highest expression for $P2Y_4$ receptors on lymphocytes and monocytes, so apparently gene amount and receptor expression is not connected.

We have shown that the ratio of lymphocytes of the cord blood that expresses P2X2, P2X3, P2X4, P2X5, P2X6, P2X7 subtypes of receptors was significantly higher than the ratio of lymphocytes of the peripheral blood and $CD34^+$ cells of cord and peripheral blood. This can be explained by the presence of the lymphocytes of the cord blood in the different (activated) form rather than the lymphocytes of the peripheral blood of adult volunteers and $CD34^+$ cells.

Under normal physiological conditions, ATP is localized in the intracellular compartment, where concentrations vary from 1 to 10 mM [35]. The level of the extracellular ATP increases in response to hypoxia and ischemia, shear-stress [36,37]. Since childbirth is stressful for the baby, it is possible that during the birth, there is an increase in the amount of ATP, which is associated with an increase in the percentage of expression of P2X receptors on cord blood lymphocytes.

There is evidence of that 2-MeS-ATP might antagonize P2X7 receptor stimulation by locally secreted ATP and reduce lipopolysaccharide-dependent tumor necrosis factor α and IL-1b release [38].

Our data suggest that the ratio of peripheral blood monocytes expressing P2X3, P2X4, P2X5, P2X6, P2X7 receptors' subtypes were much higher than all other studied cells type (P2X2-receptors were an exception). Probably, that corresponds to the ability of an adult organism to activate host defenses: our observation indicates the expression of P2X7 receptors on monocytes, which consistent with the earlier findings of Ferrari [39], who demonstrated that irreversible P2X blocker completely inhibited extracellular ATP-induced IL-1 beta release *in vitro*. There are some data indicating that the transfection of P2X7 cDNA into lymphoid cells that lack this receptor sustains their proliferation in serum-free medium, and increased proliferation of serum-starved P2X7 transfectants is abolished by the P2X7 receptor blocker oxidized ATP or by the ATP hydrolase apyrase [40].

Our experiments revealed that the quantity of P2X positive lymphocytes in the cord blood was up to three times higher than that in the peripheral blood, including P2X7 receptors that are involved in lymphocyte proliferation.

Current protocols assume that stem cell investigation using one parameter is not acceptable; that is why we used c-kit receptor identification to the stem cells growth factor that is involved in hemogenesis. It has been shown that in humans, c-kit$^{<low}$ pluripotent hemopoietic stem cells can differentiate into c-kit^{low} cells, then c-kit^{+} cells *in vitro*. The expression of c-kit on c-kit$^{<low}$ cells is the first maturational step of hematopoiesis [41,42].

Our results indicate that CD34^{+}/c-kit^{-} cells lack of P2X receptors expression compared to CD34^{+}/c-kit^{+} cells (Table 3). Other studies indicate that ATP and, to a greater extent, UTP acted as potent early acting growth factors for hematopoietic stem cells, in vitro, because they strongly enhanced the stimulatory activity of several cytokines on clonogenic CD34^{+} and lineage-negative CD34^{-} progenitors and expanded more primitive CD34^{+}-derived long-term culture-initiating cells [21].

Probably, a proliferation steps begin after cells activation and expression of c-kit^{+} proliferation marker on them, and P2X receptors are involved in these steps. This data supports the involvement of P2X receptors in the steps of maturation of hemopoietic cells, and it can be one more confirmation of a well-known fact that P2 receptors play a significant role in the growing organism compared to an adult one.

Our method does not distinguish whether all subtypes of P2X receptors are expressed on the same cell or one subtype on one and another subtype on the other cell. Nevertheless, the high level of expression of P2 receptors on the blood cells allows us to suppose the involvement of P2 receptors in the blood cells maturation and organism growth. It is possible that ATP modifies the way of blood cells differentiation by influence on different P2 receptors because ATP is a nonselective agonist of almost all subtypes of P2 receptors that were identified on blood cells.

4. Conclusions

Our study fills the knowledge gap in P2Y and P2X receptors subtype expression on human $CD34^+$ cells, c-kit^+ cells, monocytes, lymphocytes of cord, and peripheral blood. Our results confirm the proposed participation of P2 receptors in the process of the blood cell differentiation [43] and make a background for further investigation of ATP involvement in that process. Currently, it is hard to predict preciously if any functional properties of the cells are controlled with the participation of P2 receptors. Different subtypes of that receptors are detected on cells that have heterogeneous descent population, morphology, and functions [4,44,45]. At the same time, we can suppose that for the studied cells, P2 receptors are connected with the process of differentiation, in one way or another.

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This research has no acknowledgment.

Conflicts of Interest

The authors declare no conflict of interest.

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