Expression and Characterization of Purinergic Receptor, $P2Y_{10}$ in Hematopoietic Stem Cells

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ABSTRACT

Hematopoietic stem cells (HSC) are multipotent cells that reside in the bone marrow and replenish all adult hematopoietic lineages throughoutthe lifetime. In this study, we analyzed the expression of receptors of $P2Y_{10}$, purinergic receptor families in murine hematopoietic stem cells, hematopoietic progenitor cells. In addition, the biological activity of $P2Y_{10}$ was investigated with B lymphocyte cell line, Ba/F3 in effect to cell growth and cell cycle. From the analysis of expression in hematopoieticstem cell and progenitor with RT-PCR, $P2Y_{10}$ was strongly expressed in murine hematopoieticstem cells (c-kit+ Sca-1+ Lin-) and progenitor cell population, such as c-kit- Sca-1+ Lin-, c-kit+ Sca-1- Lin- and c-kit- Sca-1- Lin-. To investigate the biological effects by $P2Y_{10}$, retroviral vector from subcloned murine $P2Y_{10}$ cDNA was used for gene introduction into Ba/F3 cells, and stable transfectant cells were obtained by flow cytometry sorting. In cell proliferation assay, the proliferation ability of $P2Y_{10}$ receptor gene-transfected cells was strongly inhibited, and the cell cycle was arrested at G1 phase. These result suggest that the $P2Y_{10}$ may be involved the biological activity in hematopoietic stem cells and immature B lymphocytes.

(Key words : Hematopoietic stem cells, $P2Y_{10}$, Ba/F3, Cell proliferation, Cell cycle)

INTRODUCTION

Stem cells hold great promise for regenerative medicine and cell-based therapies (Lagasse et al., 2001). Many somatic tissues, such as liver, intestine, skin and muscle are replenished from their respective stem cell compartments, but the best characterized stem cells are those responsible for hematopoiesis(Morrison et al., 1997; Ema et al., 2000). Hematopoietic stem cells (HSCs) are multipotent cells that reside in the bone marrow and give rise to all adult hematopoietic lineages cells throughout the lifetime of the animal and are self-renewal cells that regenerates HSC cells it self for long time(Ross et al., 1982; Anderson et al., 2001). HSC was used for transplantation of the treatment of congenital, malignant, and degenerative diseases so far (Blau et al., 2001).

Extracellular nucleotides, adenosin triphosphate(ATP) or uridine triphosphate(UTP) was made from cell lysis, exocytosis, or membrane transport proteins(Ryten et al., 2002; Ryu et al., 2003). The biological effects of nucleotides were reported as cell proliferation, differentiation, chemotaxis, cytokine secretion, generation of reactive oxygen or nitrogen species, as well as induction of cell death in various cell types(Sak et al., 2003; McCloskey et al., 1999; Honda et al., 2001; Idzko et al., 2003; Idzko et al., 2003). The cell membrane protein against purines and pyrimidines for introduction into inside of cells was consisted of two main families, P1 receptor, and P2 receptors, recognizing primarily ATP, ADP, UTP, and UDP. From difference of molecular structure and signal transduction mechanism, P2 receptors were divided with two subfamilies of ligand gated ion channels and G protein-coupled receptors, P2X and P2Y receptors, representatively(Von et al., 2000; Dubyak et al., 1993).

P2Y receptor families were consisted with typical G protein-coupled receptor with seven transmembrane domains. The transduction of signaling into intracellular by P2Y receptor was conducted by activation of phopholiphase C and stimulation or inhibition of adnylate cyclase by stimulation of ATP and UTP agonist. In P2Y subunits, there are three ADP-specific receptors ($P2Y_1$, $P2Y_{12}$, and $P2Y_{13}$), one subtype of $P2Y_{11}$ for specific to ATP, as well as equipotent agonists of ATP and UTP against $P2Y_2$(Ralevic et al., 1998; North et al., 2000).

P2X subfamilies were constitute with seven subunits clone so far and expressed on various mature hematopoietic cells. In special, the function of $P2X_7$ receptor in blood cells was shown that correlation with severity of disease in chronic lymphocytic leukemia (CLL) cells, suggesting that

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novel therapeutic target molecules of chronic leukemia. Among P2Y families, the $P2Y_2$ receptor is expressed in the myeloid lineage from myeloblast to mature neutrophils with involving the control of chemotaxis, superoxide anion production. Furthermore, $P2Y_2$ receptor is also active in eosinophils by ATP and UTP stimuli, with production of reactive oxygen metabolites and chemotaxis, as well as $P2Y_{11}$ was expressed in myeloid precursor and play a role in granulocytic differentiation using human leukemic cell lines, HL-60 and NB4, and also regulated the maturation of human monocyte-derived dendritic cells(DC) which has potent antigen presenting cells in immune system(Di et al., 2001; Flzoni et al., 1995; Suh et al., 2001; Ferrari et al., 2001).

Recently, the expression of P2 receptor families were investigated by purified hematopoietic stem cells from human using CD34 sorted cells and tried to define the physiological function with treatment of ATP/UTP *in vitro* cell culture, showing the several P2X subfamilies ($P2X_1$, $P2X_2$, $P2X_3$, $P2X_4$, $P2X_5$, $P2X_6$, $P2X_7$) were expressed, and also $P2Y_1$ and $P2Y_2$ subtypes were expressed, but not $P2Y_4$,$P2Y_6$, $P2Y_{11}$ subtypes in CD34+ human hematopoietic stem cell lineage, by RT-PCR analysis. In addition of extracellular nucleotides into human hematopoietic stem cell in vitro, the several cytokines was induced and enhanced the biological effects as stimulatory activator in human CD34+ hematopoietic stem cells (Roberto et al., 2004).

$P2Y_{10}$ was identified as PU.1/Spi-B target genes by subtractive hybridization in B cells, restricted to lymphoid cells, having the promoter region binding with PU.1/SP-B functionally required for efficient transcription in B cells. However, the expression of $P2Y_{10}$ in hematopoietic stem cells was not known, even its biological character too. Furthermore, in process of maturation and differentiation of hematopoietic stem cells, little was known the biological activity by extracellular nucleotides.

In this report, we analyzed the expression of $P2Y_{10}$ receptor in hematopoietic stem cells and hematopoietic progenitor by RT-PCR analysis, as well as the physiological function was investigated with overexpression of $P2Y_{10}$ receptor using retroviral gene introduction in B lymphocyte cell line, Ba/F3 using cell proliferation assay and cell cycle analyses.

METHODS AND MATERIALS

Cloning of Murine $P2Y_{10}$ cDNA

Total RNA was isolated from bone marrow cells of C57BL/6 mice using TRIzol reagent (GIBCO BRL, Rockville, MD, USA). cDNA synthesis from total RNA of bone marrow was conducted by following the protocol of superscript pre-amplification system. Briefly, after incubation of 5ug total RNA with 0.5ug Oligo(dT)12-18 primer at 70℃ for 10min, the reaction was carried out in 5X first strand buffer, 10mM DTT and 0.5mM dNTP containing a final volume of 20ul mixture. Mixed contents of the tube were incubated at 42℃ for 2min. And then, Superscript III(GIBCO) was added and incubated for 50min at 42℃ and 15min at 70℃ to synthesize cDNA. The primers for subcloning of murine $P2Y_{10}$ cDNA with PCR reaction was used as sense 5′ GGA TCC ATG GCC TGG GAG CCC ACA TAC, anti-sense 5′ GAA TTC TCA CTG CCC TGG GAG CTC AGC. And the PCR products eluded and cloning into pGEM-T subcloning vector, cloned pGEM-T easy-$P2Y_{10}$ verified by sequencing.

RT-PCR Analyses

For RT-PCR, total RNA was isolated from total 10_4 ~10_5 cells of c-kit+ Sca-1+Lin-, c-kit- Sca-1+Lin-, c-kit+ Sca-1-Lin-, c-kit- Sca-1-Lin- hematopoietic stem cell and progenitor cells from bone marrow by FACSvantage and used the cell-to-cDNA kit(Ambion) with following the protocol of the manufacturer for confirmation of gene expression. For RT-PCR, 2$\mu\ell$ cDNA was amplified using murine $P2Y_{10}$ sense primer was 5′ GGA TCC ATG GGC AGC AAC AGT ACC AGC A and anti-sense primer was 5′ GGA TTC CAA GGT TCA GTT AAA GAA ATT ATT T in a total volume of 50$\mu\ell$ using pfu polymerase (Strategen, USA). As negative control, RNA minus reverse transcriptase (RT)-prepared cDNA was used in PCR reactions. The aliquots of PCR reaction were used for confirmation of expression with agarose gels. The condition of RT-PCR were following; 35 cycles of 45s denaturation (94℃), 30s annealing (55℃), and 60s extension(72℃). The amount of cDNA for RT-PCR each sample was normalized with β-actin primer, sense : 5′ AGG CTG TGC TGT CCC TGT ATG C-3′, antisense: 5′-ACC CAA GAA GGA AGG CTG GAA A-3′. Condition of PCR for β -actin is 35 cycles of 30s at 94℃, 30s of annealing(50℃), 30s extension(72℃).

Cell Culture

To investigate the physiologic function of $P2Y_{10}$, pre-B cell lines, Ba/F3, were cultured in DMEM (GIBCO BRL) supplemented with 10% FBS (GIBCO BRL), penicillin and streptomycin, and 5ng/ml rmIL-3 (R&D system, USA). Retroviral package cell line, Plat-E were grown in DMEM supplemented with 10% FBS, penicillin and streptomycin, 10ug/ml blasticidin S (GIBCO BRL) and 2ug/ml puromycin (Sigma)

Flow Cytometry Analysis and Cell Sorting

Flow cytometer, FACS VantageSE, were used for cell analysis and sorting. Antibody conjugates and matched isotype controls obtained from PharMingen or eBioscience; lineage markers CD3, CD4, CD8, Gr-1, Ter-119, Nk1.1, CD11b, CD45R and B220 (all APC); CD117 (c-Kit, FITC), Ly6/E (Sca-1, PE). For isolation of CD4+, CD8+ and B220+ lymphocytes from lymph node, cells was stained with CD4-FITC, CD8-PE, B220-APC conjugated antibodies. All

antibodies used were obtained from BD Biosciences or eBioscience. Cells for analysis and sorting from lymph node and bone marrow were incubated with indicated antibodies at 4℃ for 30min in staining media(SM), PBS containing with 3% FCS and 0.05% sodium azide. After antibody reaction, cells was washed 2 times with staining media for removing non-binding antibodies from reaction mixture. Finally, antibodies stained cells were applied the FACS vantage sorter for purification of hematopoietic stem cell and progenitor cells, and 5ug/ml of propidium iodide (PI) solution was added to remove death cells in process of n analysis and sorting. For analysis of data, 10,000 to 30,000 cells were stocked for further FACS analysis. All data from FACS for analyses and sorting was analyzed using CellQuest (BD) program.

To confirm the expression of $P2Y_{10}$ in retroviral infected Ba/F3 cell line, antibodies against FLAG obtained from Sigma were used for cell surface staining at dilution of 1:2,000 and then conducted FACS analysis, whether expressed or not on cell membrane at 48 hour after infection.

Construction of Retroviral Vectors and Gene Introduction

For all retroviral transduction experiments, the murine stem cell virus retroviral expression vector, pGCDN-IRESEGFP used, which contained an internal ribosomal entry site (IRES) in front of the enhanced GFP (EGFP). The pGEM-T easy $P2Y_{10}$ was subcloned from pCDNA 3.1-Hygro-FLAG (Invitrogen, USA) into the EcoRI-XhoI site. Subsequently, the PmeI-XhoI fragment from pCDNA 3.1-Hygro-FLAG-m$P2Y_{10}$ was subcloned into the SnaBI-XhoI site from the pGCDN-IRESEGFP. All constructs were verified by sequencing. Stable high titer retroviral producer cell lines were generated by transiently transfected Plat-E cells using 3μg DNA and the Exgen500 (Fermentas, St. Leon-Rot, Germany). High titer retroviral producer Plat-E cell lines were selected by stringent GFP^+ cell sorting. Retroviral supernatants were harvested from stable Plat-E cell line and then concentrated by centrifugation with 15,000rpm, for over night at room temperature. After suspended at RPMI culture media, retrovirus was introduced the Ba/F3 cell lines for 2 days, and then conducted the sorting of GFP^+ cells with FACSvantage to obtain the stable cell line and maintain for cell proliferation experiment and cell cycle analysis.

Cell Proliferation Assay

Cell proliferation was performed using a cell proliferation kit I (Roche) according to the manufacturer's instructions. The cell were plated in 96 well tissue culture plates at a density of 5×10^4 cells/well and cultured. At the culture period from day 1 to 4, 10ul MTT labeling reagent added. Incubate the plate for 4h in a 37.5% CO_2. After allowing the plate to stand overnight, absorbance in immunoreader was measured at 545nm.

Cell Cycle Analysis

Cells was maintained at the presence of rmIL-3(5ug/ml) with normal and $P2Y_{10}$ tranduced Ba/F3 cell lines. For cell cycle analysis, cells were harvested at 2 days after culture, and then fixed with ethanol and conducted RNase digestion. Propidium iodide(5ug/ml, Sigma) was stained for 30min and then analyzed cell cycle with using BD ModiFit LT software.

RESULTS

Characterization of Murine $P2Y_{10}$ Receptor

To obtain the full length of cDNA of murine $P2Y_{10}$ receptor, we preformed RT-PCR with total bone marrow cells from mouse. To characterize the genetic properties of mouse $P2Y_{10}$ gene, the complete amino acid sequences of human, mouse and rat protein was compared the properties of similarities at level of amino acid. Deduced amino acid sequences were analyzed with the multiple alignment programs, t-coffee. The amino acid of $P2Y_{10}$ receptor from human, mouse and rat was consisted of 339, 328 and 328 amino acid(Fig. 1). The amino acids similarities were analyzed by cluster W, multiple alignment programs. The homology of amino acid in $P2Y_{10}$ receptor between human and mouse has shown almost 85%, and 92% of homology between mouse and rat. $P2Y_{10}$ receptor was composed with seven transmembrane domains.

Expression of Murine $P2Y_{10}$ Receptor in Hematopoietic Stem Cells

At the previous report, expression of $P2Y_{10}$ in various murine tissues was investigated with Northern blot analysis. From this analysis, $P2Y_{10}$ mRNA was mainly expressed in spleen and thymus organs and very low levels of expression detected in bone marrow, but not other organs, lung, liver, brain and kidney. In addition, when analysis with murine cell line, $P2Y_{10}$ was expressed exclusively in immature and mature T cells, immature and mature B cells. However, it was not expressed in macrophage, erythrocyte, or fibroblast cells line. Interestingly, the analysis of RT-PCR of murine $P2Y_{10}$ specific primer with murine organs, we observed the high expression in bone marrow, brain, kidney and also relative expression in spleen and liver (data not shown). For further detail analysis of its expression in hematopoietic cell lineage, hematopoietic stem cells and progenitor cells from bone marrow was sorted by staining with c-kit, Sca-I antibodies and lineage antibodies (CD3, CD11b, B220, GP-A) using FACSvantage. From analysis of RT-PCR with adult bone marrow, the $P2Y_{10}$ was expressed very strongly in hematopoietic stem cells (c-kit+ sca-1+ Lin-) and progenitor cell population, c-kit+ sca-1-Lin-, c-kit- sca-1+ Lin- and c-kit- sca-1-Lin-

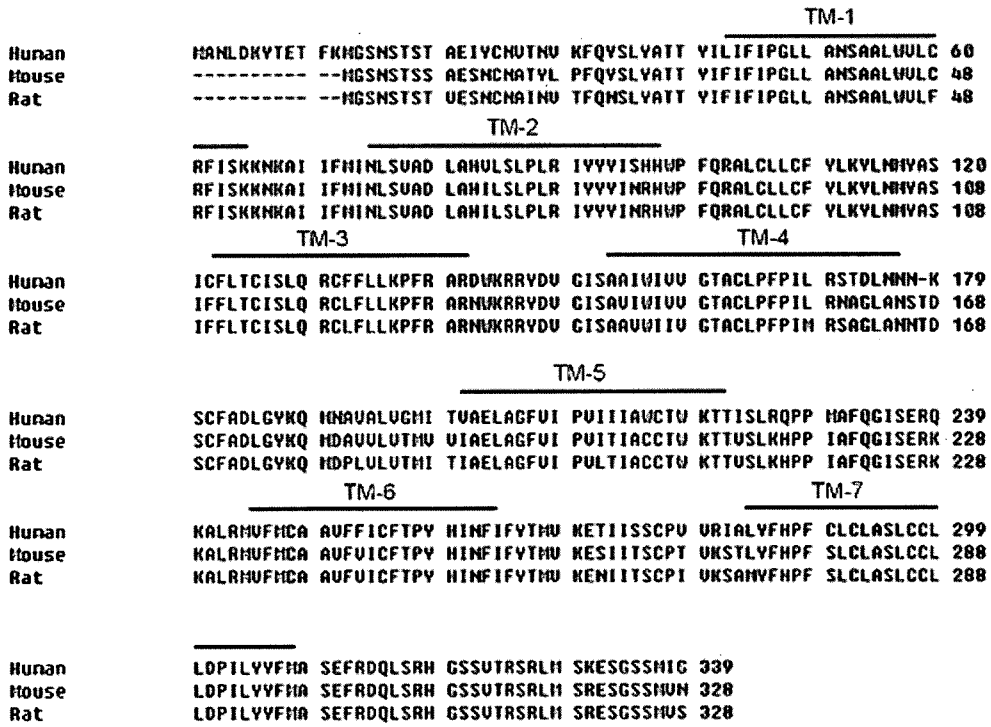


Fig. 1. Deduced amino acid sequence of the $mP2Y_{10}$ proteins and its alignment with other homologous receptor sequences, murine, human and rat, using t-coffee analyses. The putative membrane-spanning domains are designated with bar above the sequence.

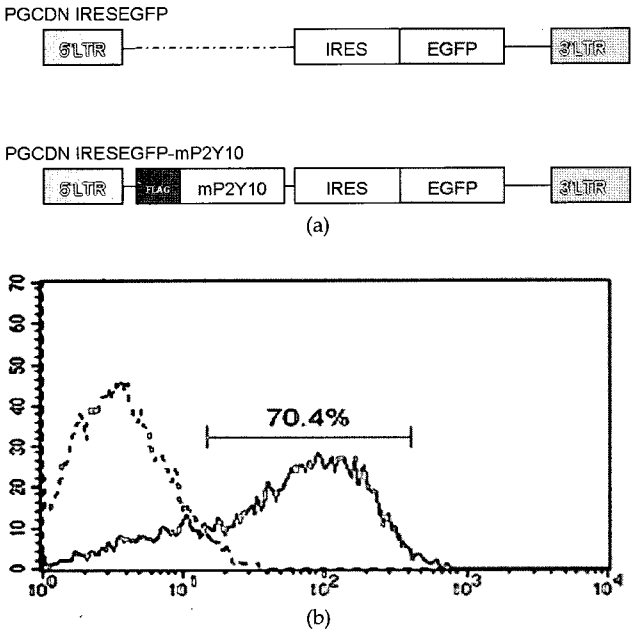


Fig. 2. $P2Y_{10}$ expression in the hematopoietic stem/progenitor cells. RT-PCR expression analysis of $P2Y_{10}$ in sorted hematopoietic stem cells(Lin-c-kit+Sac-1+) and progenitor cell population(Lin-c-kit+ Sac-1-, Lin-c-kit+Sac-1-, Lin-c-kit-Sac-1-) isolated from the mouse bone marrow.

(Fig. 2). This result shown that the $P2Y_{10}$ receptor genes might be involved the hematopoietic development from hematopoietic stem cells in adult bone marrow.

Retroviral Introduction of Murine $P2Y_{10}$ Receptor into Ba/F3 Cell Line

To confirm the physiological function of $P2Y_{10}$ receptor in pro-B cell line, Ba/F3, we generated the retroviral vector which was expressed $P2Y_{10}$-IRES-EGFP tagged with Flag and IRES-EGFP to induce constitute expression in cell (Fig. 3A). The efficiency of retroviral transduction was determined by flow cytometric detection of GFP and sorted the GFP positive cells to purify for assay of cell proliferation and cell cycle analysis in presence of rmIL-3, because cytokine

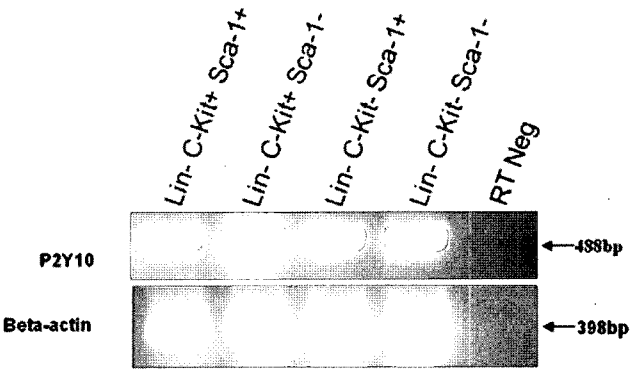


Fig. 3. Construction of $P2Y_{10}$ retrovirus vectors and confirmation of membrane expression. (A) Schematic diagram of retrovirus vector with murine $P2Y_{10}$ receptor cDNA gene. (B) Expression of $P2Y_{10}$ receptor in stable transfected Ba/F3 cells with FACS analysis.

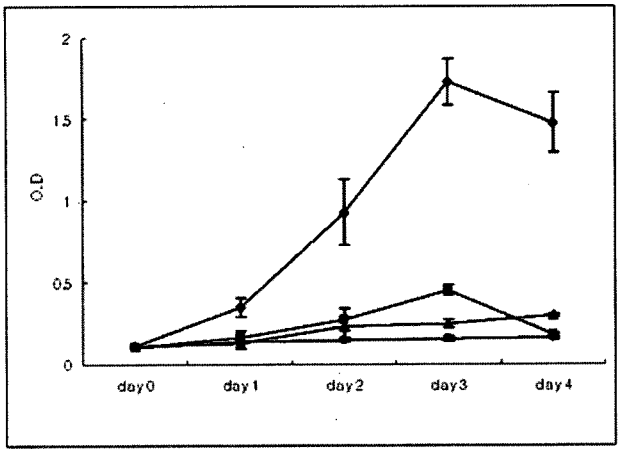


Fig. 4. $P2Y_{10}$ inhibited Ba/F3 cell proliferation. $P2Y_{10}$ transfected Ba/F3 were cultured for 4days and conducted cell proliferation activity with MTT assay (◆: Ba/F3+IL-3, ■ : Ba/F3-$P2Y_{10}$+IL3 ▲ : Ba/F3, ● :Ba/F3-$P2Y_{10}$).

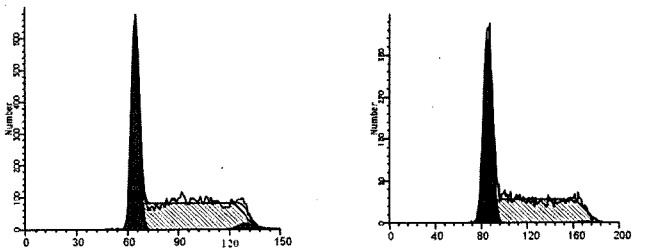
of IL-3 was maintain and sustain the proliferation and growth of Ba/F3 cell line *in vitro*. To identify expression of $P2Y_{10}$ on surface of cell, anti-Flag antibody was used for analysis of flow cytometry with Ba/F3 cell line, and then sorted for culture. As shown, cells transfected with $P2Y_{10}$ receptor were detected up 70% positive on surface of cells (Fig. 3B), which was obtained same result from morphological analysis with single cells (data not shown).

$P2Y_{10}$ Overexpression Inhibits Proliferation for Ba/F3 Cells

To define the biological function of $P2Y_{10}$ receptor in B cell line, $P2Y_{10}$ over-expressed Ba/F3 cells were sorted to obtain complete $P2Y_{10}$ introduced cells by flow cytometry and cultured in the absence or presence of rmIL-3 at indicated time. As shown in Fig. 4, control Ba/F3 cells was shown a normal proliferation with stimulation of rhIL-3. However, stable over-expression of $P2Y_{10}$ receptor on cell surface made the growth retardation in spite of the presence of rhIL-3. The difference of ability of cell growth between $P2Y_{10}$ expressed cells and normal cells, was shown a very high level at day 3 from culture start in $P2Y_{10}$ over- expressing cell line. This result implies that $P2Y_{10}$ receptor might be possessed the anti-proliferation activity in pro-B cells.

$P2Y_{10}$ Gene Regulates Cell Cycle in Ba/F3 Cells

To survey the reason of growth retardation by $P2Y_{10}$ overexpression on Ba/F3 cell, we tried cell cycle analysis whether $P2Y_{10}$ involved the cell cycle arrest; result in a very low cell proliferation activity. We analyzed the cell cycle analyses with $P2Y_{10}$ over-expressing cells and control cells using flow cytometry by PI staining. As shown in Fig. 5, $P2Y_{10}$ over-expressing cells were revealed a relatively high cell cycle arrest at the stage of G0/G1 (45.6%) and a low level at G2 phase (0.02%) stage, as well as S phase (54.4%),



Phase	G1	G2	S
Control	37.84%	2.29%	59.87%
P2Y10	45.62%	0.02%	54.36%

Fig. 5. Cell cycle analysis of $P2Y_{10}$ transfected Ba/F3 cell line. (A) control cells (B) $P2Y_{10}$ gene transfected cells.

compared with control cells, G0/G1 of 37.8%, G2 phase of 2.3% and S phase of 59.9%, at day 2 of culture. These data implicates that $P2Y_{10}$ receptor might regulate the cell proliferation with cell cycle adjustment in pro-B cell line, Ba/F3.

DISCUSSION

Extracellular effects of nucleotides were initially recognized in smooth muscle contraction, neurotransmission, regulation of cardiac function, and platelet aggregation. In addition, blood cells have emerged as one of their most interesting targets for investigating the biological activity of nucleotides through this transmembrane receptor to introduce the intracellular signals, such as cell activation, proliferation and apoptosis(Di et al., 2001; Falzoni et al., 1995). The receptors against extracellular nucleotides, ATP and UTP were divided into two subfamilies, ligand-gated ion channels (P2X) and G protein-coupled (P2Y) receptor. P2Y receptors are seven membrane spanning proteins and signal transduction occurs via the classical pathways triggered by receptor as the pathway of activation of phospholipase C and stimulation or inhibition of adenylate cyclase. ATP induces the activation of all of P2Y receptors, but $P2Y_4$ and $P2Y_6$ receptor is more susceptible to UTP than ATP potent.

Functional P2Rs for extracellular nucleotides have been found in many mature blood cells and their activation has been associated with stimulation of responses as their activation has been associated with stimulation of responses as different as chemotaxis, cytokine secretion, proliferation, and cell death. More recently $P2X_7R$ has also been identified on B leukemic cells and its expression was found to correlate with an aggressive course of the disease. Although the interest for nucleotides as potential regulators of blood cell functions has steadily increased, so far no studies have addressed the issue of whether P2Rs are expressed on hematopoietic stem cells and whether their ligation induces the functional activation of hematopoietic stem cells.

In this study, we investigated the expression characteristic in hematopoietic stem cells and also hematopoietic precursor cells from mouse bone marrow. Cells isolated with flow cytometry using hematopoietic stem cells specific maker, c-kit and Sca-1 in lineage negative cell population in bone marrow, was used for determination of expression of $P2Y_{10}$ receptor gene. From RT-PCR result, $P2Y_{10}$ receptor gene was highly expressed in hematopoietic stem cells and also progenitor cells. This result imply that $P2Y_{10}$ might be involved the biological function in early hematopoietic development from stem cells to mature hematopoietic cells. For the analyses the biological activity of $P2Y_{10}$ receptor gene, Ba/F3 cells, B lymphocyte progenitor cell line, was used by introduction of $P2Y_{10}$ gene with retroviral gene delivery to induce the overexpression. $P2Y_{10}$ over-expressed cells was shown the dramatically growth retardation in presence with IL-3 compared with that of control cells by detection with cell proliferation assay. Together, $P2Y_{10}$ gene transfected cells withdraw of cell survival factor, IL-3 were shown severely deceased their survival rate with non-transfected cells. This result mentioned that $P2Y_{10}$ gene regulated cell growth and survival in Ba/F3 cell lines directly. To confirm the mechanism how to regulate the cell growth, cell cycle analyses was conducted in $P2Y_{10}$ gene transfected cells and $P2Y_{10}$ non-transfected cells. Interestingly, $P2Y_{10}$ overexpression cells were shown high cell cycle arrest at G0 phase and G2 phase cells was eventually shown low cells population, but S phase cells was not shown severe difference compared with control cells.

These data implicates that $P2Y_{10}$ receptor may be a very strong candidate to modify and regulate the cell proliferation and large expansion of hematopoietic stem cells and also to cure of B lymphoid malignance disease for clinical application by gene therapy or expression regulation.

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