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TITLE: Purinergic Receptors in Quiescence and Localization of Leukemic Stem Cells

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A small subpopulation of drug- and radiation-resistant leukemia is an immediate concern for leukemia patients as this subtype remains the actual cause of morbidity and mortality. Our preliminary results showed that P2Y14 functions in bone marrow to preserve stem progenitor cells from premature senescence/cell death induced by genotoxic stress. As P2Y14 is highly expressed in differentiation resistant leukemia cells, P2Y14 expression in leukemia cells may function in preserving regenerative capacity by constraining cell death/senescence induction. We also found that P2Y14 axis is associated with stem cell mobilization. By providing a mechanistic insight for the roles of P2Y14 in the genotoxic stress-induced injury, directly documenting the effects of P2Y14/UDP-Glc axis on stem cell mobilization, our preliminary results are expected to provide the foundation for an effective treatment to destroy therapy resistant leukemia cells.
# Table of Contents

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>Introduction</td>
<td>2</td>
</tr>
<tr>
<td>Body</td>
<td>3</td>
</tr>
<tr>
<td>Key Research Accomplishments</td>
<td>12</td>
</tr>
<tr>
<td>Reportable Outcomes</td>
<td>12</td>
</tr>
<tr>
<td>Conclusion</td>
<td>12</td>
</tr>
<tr>
<td>References</td>
<td>13</td>
</tr>
<tr>
<td>Appendices</td>
<td>14</td>
</tr>
</tbody>
</table>
Introduction

Although conventional therapy temporarily lessens the burden of the disease, a lingering subpopulation of drug- and radiation-resistant leukemia may regenerate. This small subpopulation of drug- and radiation-resistant leukemia is an immediate concern for leukemia patients as this subtype remains the actual cause of morbidity and mortality.

Nucleotides, once recognized as mere sources of energy, are now recognized as key extracellular messengers that regulate diverse aspects of homeostasis in various physiological and pathophysiological conditions (1). Extracellular nucleotides exert their actions through interaction with their cognate receptors, purinergic receptors. Purinergic receptors are classified into P1 and P2 receptors, based on their ligand binding and function (2). The role of P2 receptors as regulators of hematopoiesis has become more evident in recent years (3-4). A wide variety of P2 receptors are expressed in blood and inflammatory cells, and their physiological significance has been demonstrated (5).

Recent results defined molecular signatures predicting the drug resistance of leukemia cells (6). P2Y14 expression has been shown to be highly upregulated in differentiation-resistant acute myeloid leukemia (AML) cases in 28 freshly isolated AML blast populations, making the P2Y14 gene a prime suspect of incurable leukemia. P2Y14 is also listed as a target gene of the Wnt3A, whose aberrant regulation is closely associated with hematological malignancies and several types of other cancers (7). In this report, when leukemia cells are treated with Wnt3A, P2Y14 was the gene most strongly upregulated. More recently, a comprehensive mutational analysis of human cancer identified P2Y14 as one of the candidate cancer genes that is mutated at a significant frequency in a large fraction of colorectal cancers (8). It is believed that a similar set of genes controls both normal and cancer stem cells. Therefore, if the genes expressed by normal stem cells are found to be mutated or used differently in cancer cells, it is very likely that those genes play a role in the development of cancer stem cells. Taken together, these studies provide strong evidence that P2Y14 is functionally and molecularly linked to drug-resistant leukemia and possibly other types of cancers. Following our preliminary results also lead to the likelihood that P2Y14 is closely associated not only with maintenance of the normal HSC but also with the dysfunction of the LSC.

Therefore, we hypothesized that the P2Y14 receptor is an important local regulatory molecule that leads to leukemic stem cell migration and quiescence and we aim to investigate
whether manipulating P2Y_{14}/UDP-Glc signaling axis can sensitize leukemic stem cells to conventional cancer therapy.

**Body**

**Specific Aim 1: To investigate how P2Y14 signaling axis regulates the quiescence of LSC**

In our previous annual report, we demonstrated that the \( P2ry14 \) deficiency confers hypersusceptibility to irradiation (IR) and/or chemotherapy drug, such as 5-flurouracil (5-FU). Since it is hypothesized that leukemic stem cells arise either from normal stem cells or from progenitor cells, we further investigated the mechanisms by which P2Y_{14} mediates the resistance to radiation using \( P2ry14 \) knock-out (KO) mouse. We previously showed that P2Y_{14} KO LSK cells were preferentially decreased upon total body irradiation (TBI) compared to WT counterparts. Preferential diminution of P2Y_{14} KO LSK cells under radiation stress could be the result of increased susceptibility of P2Y_{14} KO LSK cells to IR-induced cell death or increased senescence, or both. Radiation induces both apoptotic and non-apoptotic cell death \( \{\text{Zhuang, 2009 \#535}\} \). When we analyzed radiation-induced cell death, there was a trend toward increased IR-induced apoptosis (Annexin V+) in P2Y_{14} KO LSK cells (Fig. 1, left panel). Meanwhile, P2Y_{14} deficient LSK cells exhibited a significantly higher rate of Annexin V-, DAPI+ cells (\( P=0.047 \)) (Fig. 1, lower), suggesting that P2Y_{14} KO HSPCs die through non-apoptotic rather than apoptotic pathways.
Since sublethal radiation has been reported to selectively induce HSC senescence (9), we used this modality to investigate the role of P2Y14 in IR-induced senescence. LSK cells lacking P2Y14 showed a markedly increased level of SA-β-Gal activity (p=0.003), indicating that P2Y14 KO HSPCs are more susceptible to IR-induced senescence (Fig. 2) than WT HSPCs.

The formation of reactive oxygen species (ROS) by radiation is one of the major direct causes for cellular injury. Hematopoietic stem cells (HSCs) are especially sensitive to oxidative stress (10), and elevated level of ROS has been reported to be a major contributing factor toward loss or functional impairment of HSCs in other settings (10-12). Following IR, significantly higher levels of mitochondrial superoxide were detected in KO Hematopoietic stem progenitor cells (HSPCs) both in vivo (Fig. 3A) and in vitro (Fig. 3B). The increased ROS level is inversely correlated with mitochondrial membrane potential (Δψm) and mitochondrial dysfunction is closely associated with impaired HSC function (12). As shown in Fig. 3C, the increased ROS level in P2Y14 KO LSK cells coincided with a low mitochondrial membrane potential (Δψm).
To further test whether IR-induced ROS are indeed responsible for the observed preferential reduction of BM cellularity and LSK cells in P2Y14 KO mice, WT and KO mice were treated with the ROS scavenger N-acetyl-cysteine (NAC). NAC treatment reduced levels of superoxide in LSK cells (Fig. 3A) and alleviated the IR-induced cell death (Fig. 1) and
senescence (Fig. 2) in P2Y$_{14}$ deficient LSK cells. This was accompanied with restoration of BM cellularity and LSK cell number (data not shown).

Hyper-radiosensitivity of P2Y$_{14}$ KO HSCs may relate to their differential DNA repair capacity. Therefore, we assessed levels of $\gamma$-H2AX in WT and P2Y$_{14}$ deficient LSK cells before and after IR. Similar levels of DNA damage (at 30 min post-IR) and repair (at 6h post-IR) were detected in P2Y14 KO HSPCs in comparison to WT counterparts (Fig. 4). This indicates that P2Y14 KO HSPCs respond normally to DNA double-strand breaks (DSB) and repair DNA damage.

![Figure 4](image)

**Figure 4.** Mice (n=2) of the indicated genotypes were exposed to 6Gy of TBI. The levels of H2AX phosphorylation on gated LSK cells were quantified using anti–phospho-histone H2AX (Ser139) antibody. Numbers denote mean fluorescent intensity (MFI).

Taken together, our preliminary results strongly suggest that the presence of P2Y$_{14}$ in stem/progenitor cells may determine radio and chemo-resistance of cells. Based on these results, we will continue to investigate whether P2Y$_{14}$ axis is associated with therapy resistant leukemia phenotypes using the chimeric mice that we established in Specific Aim 2.
Specific Aim 2: To investigate functional correlation between P2Y14 signaling and therapy-resistant leukemia

p38 MAPK has been shown to act downstream of ROS and its activation in HSC can lead to a defect in HSCs (10-11). We thus determined whether P2Y14 KO HSPCs display differential activation of p38 MAPK under genotoxic stress. Since ROS generation occurs within seconds of radiation exposure and persists for 2–5 min post-IR {Leach, 2002 #531}, the activation of p38 MAPK was analyzed at an early time point immediately after TBI. Whereas there was no detectable difference in p38 MAPK activity between WT and KO HSPCs under homeostatic conditions, p38MAPK activity was notably higher in P2Y14 deficient LSK and SLAM LSK cells following radiation (6 Gy, TBI) (Fig. 5, left). Of note, p38 MAPK activation was transient as its level returned to near basal control levels about 5h after TBI (data not shown). NAC treatment almost completely abolished the p38 MAPK activation (Fig 5, right panel). Taken together, our data demonstrate that P2Y14 deficiency confers increased susceptibility to genotoxic stress-induced cell death and senescence in HSPC compartments via the excessive activation of p38 MAPK triggered by IR-induced ROS.

Figure 5. WT (red line) and P2ry14−/− (blue line) mice were exposed to TBI (6Gy). Mice were sacrificed immediately after IR and their LSK (upper) and SLAM LSK (lower) cells were analyzed for phosphorylated p38 MAPK by flow cytometry. NAC was treated as described. Note that p38 MAPK activity was completely abrogated upon NAC treatment. The accompanying graphs (right) show the mean fluorescence intensity of p38 MAPK in LSK (upper) and SLAM (lower) LSK cells. Data are represented as absolute numbers of cells ± s.d. of at least two independent experiments. The graph on the right displays the mean±s.d. fluorescence intensity. Mice were analyzed individually (n >4 mice/each genotype).
p38 MAPK and JNK are critical regulators for the cell survival, apoptosis and cell cycle progression and the aberrant activation of these pathways are potential causes of hematologic malignancies. Therefore, we investigated whether P2Y14/UDP-Glc axis can trigger p38 MAPK and/or JNK signaling pathway in leukemia cells as shown in normal HSPCs. KG-1 cells, an immature leukemia cell line, were treated with UDP-Glc. As shown in Fig. 6, UDP-Glc triggered the activation of both p38 MAPK and JNK pathway in KG-1 leukemia cells starting as early as 5 min after UDP-Glc treatment. For JNK proteins, the activation peaked at 30 min post-treatment and decreased thereafter. Interestingly, p38 MAPK activity reached a peak after 5 min and then sustained its activity over prolonged time. We are currently assessing the impact of these pathways on cell death and senescence in leukemia cells.

Figure 6: UDP-Glc triggers the activation of p38 MAPK and JNK in a P2Y14 expressing immature leukemia cells, KG1. Cells were treated with 100µM UDP-Glc and analyzed for the activation of p38 MAPK and JNK at the indicated times.

We proposed to investigate whether P2Y14/UDP-Glc axis is functionally associated with differentiation-resistant leukemia. For this purpose, we have obtained bone marrow samples from 41 unidentified leukemia patients. Among these samples, five human acute leukemia samples (AML) (M0 or M1) were chosen based on FAB classification, cytogenetic abnormalities, CD34 and CD14 expression (Table 1).
Two of five AML samples failed to engraft in NOD-scid \( IL2R\gamma^{null} \) mice. Three AML samples showed detectable human leukemia cells 8 weeks post-transplantation (Fig. 7). Currently, approximately 20 NOD-scid \( IL2R\gamma^{null} \) mice have been transplanted. To assess whether we indeed transplanted leukemia initiating cells, BM cells from these engrafted recipients were subjected to secondary transplantation.

**Figure 7.** Bone marrow cells from AML patients (M0 or M1) were transplanted into NOD/LtSz-scid/IL-2Rgamma null mice. Eight to nine weeks after transplantation, peripheral blood cells from recipient mice were incubated with human-specific antibodies directed to human CD45. Numbers in histograms indicate the percentage of human AML-derived cells.
Specific Aim 3: Examine whether the activation of P2Y14/UDP-Glucose signaling axis mobilizes leukemic stem cells from recipient’s bone marrow.

As shown in specific aim 2, while we are in the process of establishing mouse xenograft model system in which primary human leukemic stem cells are engrafted into NOD-scid IL2Rγnull mice, we further examined whether activation of P2Y14/UDP-Glc signaling axis mobilizes normal stem cells from recipient’s bone marrow. UDP-Glc is known to be released into the extracellular space under stresses (13). To investigate whether the administration of UDP-Glc may result in HSPC mobilization, we injected UDP-Glc into mice and assessed for its ability to mobilize CFU-Cs and LSKs into the blood circulation. In determining optimal dose, UDP-Glc exerted its maximal mobilizing effects at a dose of 200mg/kg body weight (data not shown). Although i.v. administration was superior to s.c. or i.p. in mobilizing CFCs (data not shown), s.c. injection was chosen for further studies because of its simplicity and to minimize potential side effects of i.v. injection. There was a noticeable increase in CFU-C (Fig. 8a) and circulating LSKs (Fig. 8b) after 6 daily single UDP-Glc injections.
Importantly, the frequency of LSKs in the blood of mice treated with UDP-Glc was similar to that obtained with G-CSF treatment (Fig. 8c). Furthermore, UDP-Glc acted in synergy with G-CSF to induce LSK mobilization (Fig. 8c). No animals transplanted with control blood cells (PBS injected) survived lethal irradiation (data not shown). In contrast, UDP-Glc mobilized cells not only displayed the ability to rescue lethally irradiated recipients but also were far superior to control blood cells in a competitive repopulation assay (Fig. 8d). UDP-Glc-induced mobilization was not the strain-specific phenomenon, since similar results were obtained using Balb/c mice (data not shown). To determine whether UDP-Glc triggers HSPC mobilization through P2Y14 receptor, P2Y14 KO mice were treated with UDP-Glc. UDP-Glc treatment did not result in LSK cell mobilization in KO mice (data not shown), suggesting that UDP-Glc mobilizes LSK cells through P2Y14 signaling axis. Because RANKL induces HSPC mobilization (14), we examined whether UDP-Glc modulates RANKL expression. Indeed, UDP-Glc increased expression of RANKL (Fig. 8e). RANKL promotes HSPC mobilization in part via MMP-9 mRNA upregulation (14). WT LSKs, but not KO LSKs, showed increased expression (1.7-2.5 fold) of MMP-9 upon UDP-Glc treatment (Fig. 8f), which is comparable to the fold increase in RANKL treated mice (14).

As normal HSCs and AML initiating cells are known to share many surface molecule and are supposed to use common mechanisms for their mobilization, these results support our hypothesis leukemia stem cells may use P2Y14/UDP-Glc axis for their localization in bone marrow. Using chimeric recipient mice that established in Specific Aim 2, we will continue to investigate the mobilization of leukemia cells.
Key Research Accomplishments

1. Successfully established xenograft mouse model system.
2. Identify potential mechanisms by which $P2Y_{14}$ signaling axis mediates radiation and chemoreagent resistance.
3. Identify potential mechanisms by which $P2Y_{14}$ signaling mediates HSC and LSC mobilization.

Reportable Outcomes


Conclusion

How leukemia stem cells gained resistance to radiation and chemotherapy is poorly defined, yet critically determines how leukemia cells tolerate conventional leukemia therapy. Our preliminary results showed that $P2Y_{14}$ functions in bone marrow to preserve stem progenitor cells from premature senescence/cell death induced by genotoxic stress.

As $P2Y_{14}$ is highly expressed in differentiation resistant leukemia cells, $P2Y_{14}$ expression in leukemia cells may function in preserving regenerative capacity by constraining cell death/senescence induction. We also found that $P2Y_{14}$ axis is associated with stem cell mobilization. By providing a mechanistic insight for the roles of $P2Y_{14}$ in the stress-induced injury, directly documenting the effects of $P2Y_{14}$/UDP-Glc axis on stem cell mobilization, our preliminary results are expected to provide the foundation for an effective treatment to destroy therapy resistant leukemia cells.
References


**Appendices**

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