Identification of annexin 1 as a PU.1 target gene in leukemia cells

Yuko Iseki¹, ¹, Akemi Imoto¹, ¹, Toshio Okazaki², ³, Hideo Harigae³, Shinichiro Takahashi¹, ², ⁴

¹ Division of Molecular Hematology, Kitasato University Graduate School of Medical Sciences, 1-15-1 Kitasato, Sagamihara City, Kanagawa 228-8555, Japan
² Division of Hematology, Kitasato University School of Allied Health Sciences, 1-15-1 Kitasato, Sagamihara City, Kanagawa 228-8555, Japan
³ Department of Hematology and Rheumatology, Tohoku University Graduate School of Medicine, Japan

A R T I C L E   I N F O

Article history:
Received 19 January 2009
Received in revised form 12 March 2009
Accepted 7 April 2009
Available online xxx

Keywords:
PU.1
AML
Annexin 1

A B S T R A C T

To identify PU.1 downstream target genes, we first established PU.1-knockdown K562 (K562PU.1KD) cells expressing reduced levels of PU.1 by stably transfected PU.1 siRNAs. From microarray analysis, we found that several genes including annexin 1 were markedly induced in K562PU.1KD cells. Annexin 1 is a calcium- and phospholipid-binding protein and increased expression leads to the constitutive activation of extracellular signal-regulated kinase (ERK). Consistent with this, we observed constitutive activation of ERK in K562PU.1KD cells. Furthermore, we revealed the mRNA expression of annexin 1 was negatively correlated with PU.1 mRNA expression in 43 primary AML specimens (R = −0.31, p < 0.042).

© 2009 Elsevier Ltd. All rights reserved.

1. Introduction

PU.1 is a member of the Ezb transformation-specific sequence (Ets) family of transcription factors and is expressed in granulocytic, monocytic and B-lymphoid cells [1]. PU.1 expression levels increase during the differentiation of granulocytes [1]. PU.1-deficient mice exhibit defects in the development of neutrophils, macrophages and B cells [2]. Thus, PU.1 is indispensable for myelomonocytic differentiation during normal hematopoiesis. Recently, it was reported that mice carrying hypomorphic PU.1 alleles that reduce PU.1 expression to 20% of its normal levels were reported to develop AML [3]. This finding is extremely important because the authors showed that a single molecular event, namely tightly graded reduction of the lineage-indispensable transcription factor PU.1, could solely induce AML.

In the present study, we first cloned cell lines expressing reduced levels of PU.1 by stable transfection of PU.1 short inhibitory RNAs (siRNAs) to understand the mechanisms of leukemogenesis. Next, we tried to identify the target genes regulated by PU.1 by performing microarray analyses between PU.1-knockdown cells and parental cells. As a result, we found that annexin 1 was induced in PU.1-knockdown cells.

Annexin 1 is a member of the annexin family of calcium and phospholipids-binding proteins. Annexins are ubiquituous and expressed in diverse organisms [4]. Annexins are cytosolic or associated with the membrane or the cytoskeleton in a calcium-dependent manner. Their precise functions are unclear, however, they have been implicated in a broad range of cellular functions, including signal transduction, DNA replication, cell transformation, ion channel formation, and apoptosis [5]. Annexin 1 is a substrate for protein kinase C and protein-tyrosine kinases [6]. This fact, coupled with multiple phosphorylation sites and its calcium- and phospholipid-binding properties, may be indicative of a role for annexin 1 in signal transduction as a means to affecting its pleiotropic physiological roles. Consistent with this, it was reported that increasing the expression of annexin 1 leads to the constitutive activation of extracellular regulated kinase (ERK) in RAW macrophages [7]. Quite interestingly, from the proteomic analysis of AML specimens, annexin 1 (lipocortin 1) protein expression was significantly induced in AML blast cells compared to normal mononuclear cells [8], but the significance remained unknown.

In this study, we found the link between PU.1 and annexin 1. We further revealed aberrant ERK activation in PU.1-knockdown cells. These findings may provide a novel insight for the molecular pathogenesis of leukemia.

2. Materials and methods

2.1 Cell culture and generation of PU.1-knockdown and PU.1-over-expressing cells

K562 and NB4 cells were grown in RPMI (Gibco BRL, Rockville, MD) containing 10% heat-inactivated fetal bovine serum, 100 U/ml penicillin and 100 μg/ml streptomycin. To generate PU.1-knockdown cells, PU.1 siRNA expression vectors and their control vector were transfected into K562 cells by electroporation. Three different
PU.1 siRNA expression vectors (pcPURU6bicassette) were designed by the manufacturer (Takara, Otsu, Japan) and employed for this study to obtain optimal effects. Target sequences of these siRNA expression vectors are, AGAAGAAGCTTCTGTGCTA and GCAAGAAATGACGACTACACC. In addition, we employed pcPURU6Jstop vector (Takara) as a control. To generate PU.1-over-expressing cells, a PU.1 expression vector [9] and its parental pcdNA3.1(+) Myc-His version A vector (Invitrogen, Carlsbad, CA) were transfected by electroporation into K562 and NB4 cells. The electroporation procedure was performed as previously described [10]. K562 clones stably transfected with the PU.1 siRNA vectors and PU.1 expression vector were isolated by limiting dilution by selection with 1 μg/ml puromycin (for siRNA expression vectors) or 400 μg/ml neomycin (for the PU.1 expression vector) for approximately three to four weeks. The clones were isolated and maintained for further analyses.

2.4. mRNA expression analyses

For transient transfection assays, cells were harvested and RNA was prepared two days after transfection as described previously [12]. Total RNA was extracted from transgenic cells by ISOGEN reagent (Nippon gene, Tokyo, Japan) and reverse transcription was performed by Superscript II (Invitrogen, Carlsbad, CA). 35,000 human genes were loaded in each lane and immunoblotted for PU.1 and β-actin. To examine the signaling, anti-phospho-ERK rabbit polyclonal, anti-total-ERK mouse monoclonal, anti-phospho-Akt rabbit monoclonal and anti-total-Akt rabbit monoclonal antibodies were used. To examine the expression of PU.1 and annexin 1, rabbit polyclonal anti-PU.1 monoclonal was obtained from BD Biosciences (San Jose, CA) and employed for this study. For FCM analysis, the cells were incubated with monoclonal antibodies, lysed with FACs lysis solution (Becton Dickinson, Mountain View, CA) and applied to a FACsCalibur (Becton Dickinson) as described previously [16].

2.5. Surface marker expression analysis by flow cytometry (FCM)

For FCM analysis, the cells were incubated with monoclonal antibodies, lysed with FACs lysis solution (Becton Dickinson, Mountain View, CA) and applied to a FACsCalibur (Becton Dickinson) as described previously [16].

2.6. Statistical analysis

Correlations between two continuous variables were calculated by the Spearman rank correlation test. For all analyses, the p values were 2-tailed and values of p < 0.05 were considered statistically significant.

3. Results

3.1. Establishment of PU.1-knockdown K562 cells

RNA interference using siRNAs has been widely explored for the suppression of cellular mRNA levels to investigate the functions of specific genes. In the present study, we used a plasmid vector-based siRNA expression system to silence PU.1 expression. We first generated human chronic myeloid leukemia cell line K562 cells stably expressing PU.1 siRNAs as well as control siRNA-vector-transfected clones as controls. K562 cells were selected for this study because of their relatively high transfection efficiency among hematopoietic cell lines. Among the >50 lines isolated and analyzed, clone 2–10 exhibited sufficient suppression of PU.1 mRNA and protein expressions (Fig. 1A and B). In addition, clone 3–10 had a modest level of PU.1 suppression compared with the vector-transfected clones vec 5 and vec 6 (Fig. 1A and B). Thereafter, clones 2–10 and 3–10 were used to assess the effects of PU.1 suppression, and designated K562 PU.1-knockdown (K562PU.1KD) cells.

3.2. PU.1 suppression results in downregulation of myeloid surface markers

PU.1 is a master regulator, and critical for the development of a common progenitor for lymphoid-myeloid cell lineages in the hematopoietic system. Therefore, we examined whether the reduction of PU.1 expression affected differentiation markers in K562PU.1KD cells. As shown in Fig. 1C, the expression levels of CD13 and CD33 in 2–10 cells (arrows) were markedly reduced compared with control vec6 cells (arrowheads). Other markers, namely CD11b, CD15, CD41, CD34 and glycoporphin A, were not affected by the reduced PU.1 suppression. In 3–10 cells with modest PU.1 suppression, the CD13 and CD33 expression levels were also slightly decreased, and the other markers remained unaffected (data not shown). Taken together, these results suggest that reduction of PU.1 expression leads to impairment of myeloid differentiation of the cells. Therefore, K562PU.1KD cells are useful tools for analyzing the pathology of myeloid leukemia.

3.3. Annexin 1 is a bona fide target of PU.1

To identify PU.1 target genes, microarray analyses between 2–10 cells and vec6 cells were carried out using a human array 35k containing 35,000 human genes. The microarray analyses identified several candidate PU.1 target genes (Fig. 2A). Among the 35,000 genes examined, 10 genes were upregulated by ≥3-fold in 2–10 cells compared with vec6 cells. A further 18 genes, including PU.1, were downregulated in 2–10 cells by ≥3-fold compared with vec6 cells (Table 1). In this study, we focused on the genes with fully characterized functions that are known to play roles in hematopoiesis or oncogenesis, to clarify the roles of PU.1 in leukemogenesis. On these bases, we selected inhibitor of differentiation (ID) 3 [17], CD36 (glycoprotein IIIb) [18] and annexin 1 [8] as candidates for PU.1 target genes for the initial analysis. Next, we checked the expression levels of these genes by quantitative real-time PCR. ID3 (Fig. 2B, upper left panel) suppression was observed in 2–10 cells, but not in 3–10 cells. CD36 (Fig. 2B, upper right panel) induction was not observed.

Table 1

<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>NM_003380</td>
<td>Vimentin</td>
<td>8008.601</td>
<td>903.208</td>
<td>8.867</td>
</tr>
<tr>
<td>NM_000700</td>
<td>Annexin 1</td>
<td>12232.659</td>
<td>1506.719</td>
<td>7.919</td>
</tr>
<tr>
<td>NM_00106667</td>
<td>Neurokinin B precursor</td>
<td>8572.392</td>
<td>1230.540</td>
<td>6.967</td>
</tr>
<tr>
<td>NM_00100547</td>
<td>CD36</td>
<td>3198.542</td>
<td>606.914</td>
<td>5.270</td>
</tr>
<tr>
<td>NM_0009500</td>
<td>Metallothionein 1G</td>
<td>1634.894</td>
<td>321.945</td>
<td>5.078</td>
</tr>
<tr>
<td>NM_002770</td>
<td>Trypsin I precursor (EC 3.4.21.4)</td>
<td>671.460</td>
<td>141.423</td>
<td>4.748</td>
</tr>
<tr>
<td>NM_004445</td>
<td>Ephrin type-B receptor 6 precursor</td>
<td>1177.995</td>
<td>290.761</td>
<td>4.051</td>
</tr>
<tr>
<td>NM_020485</td>
<td>Blood group Rh(CE) polypeptide</td>
<td>2738.603</td>
<td>703.850</td>
<td>3.891</td>
</tr>
<tr>
<td>NM_005415</td>
<td>Solute carrier family 20</td>
<td>6212.998</td>
<td>1850.841</td>
<td>3.357</td>
</tr>
<tr>
<td>NM_138618</td>
<td>Blood group Rh(CE) polypeptide</td>
<td>3153.722</td>
<td>948.176</td>
<td>3.286</td>
</tr>
</tbody>
</table>
in 3–10 cells as well. Induction of annexin 1 (Fig. 2B, lower panel) was observed in both K562PU.1KD clones (2–10 and 3–10), suggesting that this gene is a candidate for PU.1 target gene. To verify this, we established PU.1-over-expressing K562 (K562PU.1OE) cells and examined how the expression level of this gene was affected by expression of PU.1. We generated K562 cells stably expressing a human PU.1 cDNA as well as vector-transfected clones (V1 and V2) as controls. Among the various lines isolated and analyzed, clones A2 and H8 had sufficient levels of PU.1 mRNA and protein expressions (Fig. 3A and B). Next, we checked the expression levels of the annexin 1 in these cells. We found that annexin 1 was suppressed in K562PU.1OE cells (Fig. 3C). Furthermore, the protein expression was verified. The expression of annexin 1 was significantly induced in K562PU.1KD cells, and suppressed in K562PU.1OE cells (Fig. 4). These results suggest that annexin 1 is a bona fide target of PU.1.

3.4. Annexin 1 and PU.1 transcript levels in AML cells

To address the potential relevance of our findings for human AML, we analyzed the gene expression levels in leukemic bone marrow cells from 43 individuals with AML. As expected, we detected a negative correlation between the PU.1 mRNA expression levels and the mRNA expression levels of the annexin 1 in 43 AML specimens \( R = -0.31, p < 0.042 \), Fig. 5A). To further confirm the relations between PU.1 and annexin 1 in myeloid leukemia, we transiently transfected PU.1 expression vector into acute promyelocytic leukemia NB4 cells. Then, the expression of annexin 1 was examined by quantitative real-time PCR. As shown in Fig. 5B, the annexin 1 expression is significantly suppressed to approximately 50% by the over-expression of PU.1. Together with these, the correlations of PU.1 with annexin 1 identified in the PU.1 transgenic cells play roles in human leukemia cells. Diminished PU.1 expression may contribute to the pathogenesis of AML through the induction of annexin 1 expression.

3.5. Constitutive activation of ERK in K562PU.1 KD cells

As over-expression of annexin 1 specifically regulates the proximal signaling components of the ERK kinase signal transduction pathway [7], we next speculated whether the downregulation of PU.1, in concordance with annexin 1 over-expression, affects to the downstream signaling pathways. To test this, we examined the activation of ERK and Akt phosphorylation by Western blotting. As we predicted, although there was no difference of Akt phosphorylation in transgenic cells compared to control cells, ERK was significantly phosphorylated in K562PU.1KD cells (Fig. 6). These suggest that the
downregulation of PU.1 expression is playing a role in the aberrant signal transduction in these cells.

4. Discussion

The mitogen-activated protein kinase (MAPK) pathways are key regulators of cell proliferation, differentiation and survival [19]. Although genetic alterations affecting the functions of transcription factors that regulate myeloid maturation play important roles in leukaemogenesis [20], inappropriate MAPK activation may also play a role in leukaemic transformation. The Ras-MAPK and phosphoinositide 3-kinase (PI3K)-Akt pathways are frequently activated in haematological malignancies [21,22]. Upregulation of the MAPK network in AML has been reported to arise through several mechanisms, including the Fms-like tyrosine 3 (Flt3) kinase receptor, c-KIT and Ras mutations frequently detected in AML [15,23–25]. Our current study sheds light to the fact that the downregulation of PU.1 plays a role in the aberrant induction of ERK pathway, possibly via annexin 1 induction. Recently it was reported that SHP-1, protein tyrosine phosphatase, the key negative regulator of signaling pathways, is directly regulated by PU.1 and downregulated by the PU.1 suppression [26]. The aberrant activation of downstream signaling pathway regulated by these PU.1 target genes, may play a pivotal role in the pathogenesis of PU.1 knockdown induced leukaemia.

It has been reported that PU.1 binds to the corepressor mSin3A and forms a complex with histone deacetylase 1 [27]. They also demonstrated that over-expression of PU.1 induces transcriptional repression of several gene promoters including c-myc [27]. The same research group recently reported that PU.1 forms a complex with DNA methyltransferase (Dnm1) 3a and Dnm1 3b, and clearly revealed that CpG sites in the p16INK4A promoter were methylated by over-expression of PU.1 [28]. We speculate that the inverse correlation between PU.1 and annexin 1 expression may be due to these epigenetic activities of PU.1. In other words, PU.1 might functions as a transcriptional repressor for the regulation of annexin 1 gene. The precise mechanisms of this regulation is now analyzing in our laboratory.

It was reported that upregulation of annexin 1 inhibits tumor necrosis factor induced apoptosis in human leukemic cells [29]. High constitutive levels of annexin 1 in leukemic blasts may protect them against immune mediated killing [29]. In follicular lymphoma, strong PU.1 expression, together with CD20 and CD75 expressions, showed significant associations with longer progression-free survival and overall survival [30]. We recently reported that in 24 AML cases, PU.1 expression was inversely correlated with Flt3 expression [9], whereas strong expression of Flt3 was found to be an unfavorable prognostic factor for overall survival in 91 AML cases without Flt3-internal tandem duplication, which is a prevalent mutation in AML with worse prognosis [31]. Together with the present findings, decreased PU.1 expression, as well as induced annexin 1 expression, may represent a poor prognostic marker for AML or other types of leukemia and hematopoietic malignancies. These important issues need to be addressed in future investigations. Such future analyses may lead to the development of useful prognostic markers in AML.

Conflict of interest

There are no conflicts of interest.

Acknowledgements

We thank for Ms. Asami Takahashi for flow cytometric analysis and Dr. Mitsue Inomata for establishing PU.1 over-expressing cell lines. This work is supported in part by a grant from Japan Leukemia Research Foundation, Foundation from Kitasato University School of Allied Health Sciences (Grant-in-Aid for Research Project, No. 2007-1003, No. 2008-1001) and Foundation from Kitasato University Graduate School of Medical Sciences Research Project.

Contributions. Dr. S. Takahashi contributed to acquired data, the concept and design, interpreted and analyzed the data, provided drafting of the article, provided critical revision of the article for important intellectual content, gave final approval of the article, provided study materials, supplied statistical expertise, obtained a funding source, provided administrative, technical or logistical support, and collected and assembled data. Ms. Y. Iseki acquired data and supplied statistical expertise. Ms. A. Imoto acquired data. Dr. T. Okazaki and Dr. H. Harigae provided study materials.

References


