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Original

Autophagosome-rich platelets are increased in immune thrombocytopenia

Akihiro OTSU [1], Shugo KOWATA [1], Yuki SEKI [1], Kazunori MURAI [2], Kazuki KIYOHARA [1], Shinri MIYAJIMA [1], Wataru IZUMITA [1], Jiro HITOMI [3], Yoji ISHIDA [1] and Shigeki ITO [1]

[1] Division of Hematology and Oncology, Department of Internal Medicine, School of Medicine, Iwate Medical University, Yahaba, Japan
[2] Division of Hematology, Iwate Prefecture Central Hospital, Morioka, Japan
[3] Division of Human Embryology, Department of Anatomy, School of Medicine, Iwate Medical University, Yahaba, Japan

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Abstract

Immune thrombocytopenia (ITP) is characterized by increased platelet destruction and reduced platelet production, mediated by antiplatelet autoantibodies. Owing to the absence of definitive tests, diagnosis of ITP mainly involves exclusion of other thrombocytopenic disorders. Accumulating evidence has implicated enhanced autophagy in thrombopoiesis and ITP. However, it remains unknown whether platelet autophagy is enhanced in patients with ITP and has diagnostic potential.

To determine this, we measured the proportion of autophagosome-rich platelets (ARPs%) in patients with thrombocytopenia, including ITP. We examined 34 patients with ITP and 52 patients with other types of thrombocytopenic disorders. To detect and quantify autophagosomes within platelets, we used flow cytometry with a cationic fluorescence dye, Cyto-ID, which specifically labels autophagic compartments.

Among thrombocytopenic patients, ARPs% was significantly elevated only in ITP patients ($p < 0.01$) and was found to be significantly correlated with the proportion of reticulated platelets (RPs%) ($r = 0.58$) and mean platelet volume ($r = 0.55$). ARPs% showed a greater sensitivity but reduced specificity (88.2% and 71.2%, respectively) compared with those of RPs% (85.3% and 90.4%, respectively).

These data suggest that enhanced autophagy of platelets is a characteristic finding in patients with ITP and shows diagnostic potential in thrombocytopenia.

Key words : *Immune thrombocytopenia, autophagy, platelet*

I. Introduction

Immune thrombocytopenia (ITP) is characterized by increased platelet destruction in the reticuloendothelial system or reduced platelet production. These events are mediated by IgG antiplatelet autoantibodies, resulting in enhanced platelet turnover and thrombocytopenia [1]. Despite recent advances in understanding the pathophysiology of ITP,

Corresponding author: Shugo Kowata
skowata@iwate-med.ac.jp

diagnosis of ITP mainly involves exclusion of other thrombocytopenic disorders and assessment of therapeutic responses [2, 3]. Definitive tests with clinically sufficient sensitivity and specificity for the diagnosis of ITP have not been established [4].

Thus, it is crucial to evaluate the activity of thrombopoiesis in patients with thrombocytopenia. Reticulated platelets (RPs) and the immature platelet fraction (IPF) represent young platelets with higher contents of dense granules, increased residual megakaryocyte-derived mRNA, and increased mean volume compared with those of older circulating platelets [5]. In addition, they also exhibit higher metabolic activity and thrombotic potential than mature platelets [6]. Whole-blood flow cytometry has been developed for the identification of young platelets (RPs) that contain mRNA via staining with thiazole orange (TO) [7]. Recently, the IPF metric was developed, which is measured by an auto-matched hematology analyzer [4]. The proportions of RPs (RPs%) and IPFs (IPFs%) are used as markers of platelet production activity and are increased in conditions of enhanced platelet turnover, especially in ITP [2, 4, 5].

Accumulating evidence shows that enhanced autophagy is implicated in thrombopoiesis and the pathophysiology of ITP. Autophagy plays many essential roles in cell growth, development, homeostasis, and recycling of cellular components [8]. Megakaryocytes and platelets also possess the autophagy machinery [9]. Autophagy is constitutively active in resting platelets and is enhanced by platelet activation [9]. However, it is unknown what is digested by autophagosomes and how autophagic flux changes over the platelet life span. We recently demonstrated that autophagosome-rich platelets (ARPs), which possess autophagosomes detectable by the cationic fluorescent dye Cyto-ID, are increased in ITP model mice [10]. The dynamics of the proportion of ARPs (ARPs%) parallels that of RPs%, implying that ARPs are young platelets or platelets altered by autoantibodies. In addition, it has been reported that the plasma obtained from ITP patients induces enhanced autophagy of megakaryocytes *in vitro* [11]. However, it remains unknown whether platelet autophagy is enhanced in patients with ITP and whether ARPs% is a novel indicator in the diagnosis of ITP. In this study, to elucidate these aspects, we measured ARPs% in patients with thrombocytopenia, including ITP.

II. Materials and methods

1. Subjects

A total of 86 patients with thrombocytopenia (platelet count $< 1 \times 10^5/\mu L$) at Iwate Medical University Hospital (Morioka, Japan) and 20 healthy individuals were enrolled in this study. The diagnosis of ITP was based on previously described criteria [12]. We excluded patients who had received blood transfusion or medication for infection. The samples from healthy individuals were used to determine the baseline range of RPs% and ARPs% evaluated by flowcytometry. This study was approved by the Ethics Committee of Iwate Medical University School of Medicine (H29-114), and all the patients and healthy individuals provided written informed consent.

2. Blood samples

Blood samples for determination of platelet

count, red blood cell count, white blood cell count, and mean platelet volume (MPV) were collected, anticoagulated with EDTA, and assessed using a hematological analyzer (ADVIA 2120i, Siemens, Munich, Germany). Because EDTA had interfered with platelet autophagosomes in our previous experiments, sodium citrate 3.2% tubes (Terumo, Japan) were used for flow cytometry analysis of RPs% and ARPs%. All blood was drawn into citrate tubes and processed within 2 hours after withdrawal so that the samples were fresh and not altered by transport or storage conditions.

3. Measurement of RPs% and ARPs%

RPs% was measured as previously described with a slight modification [7]. We used anti-CD235a (glycophorin A) antibody to remove the population of red blood cells and their fragments. This procedure enables more specific measurement of the platelet population than a previous method, which allowed the possibility of contamination with red blood cells or their fragments, which bind to platelets. In brief, 5 μL aliquots of whole blood anti-coagulated with citrate were incubated with 50 μL of Retic-COUNT Reagent (BD Pharmingen, Tokyo, Japan), 5 μL of phycoerythrin-conjugated anti-CD41 monoclonal antibody (BD Pharmingen), and 10 μL of PC7-conjugated anti-CD235 monoclonal antibody (Beckman Coulter, Tokyo, Japan). The samples were mixed gently, incubated for 30 minutes at room temperature, and fixed by adding 1 mL of paraformaldehyde (1% w/v in PBS).

To detect autophagosomes, we used a cationic fluorescence dye, Cyto-ID (Enzo Life Sciences Inc., Farmingdale, NY, USA), which specifically labels autophagic compartments [13, 14]. ARPs% was measured according to the product manual with minor modification. In brief, 5 μL aliquots of whole blood anti-coagulated with citrate were incubated with 50 μL of stain solution, which was prepared by diluting 1 μL Cyto-ID Green Detection Reagent in 1 mL of Iscove's modified Dulbecco's medium (IMDM), 5 μL of phycoerythrin-conjugated anti-CD41 monoclonal antibody (BD Pharmingen), and 10 μL of PC7-conjugated anti-CD235 monoclonal antibody (Beckman Coulter). The samples were mixed gently, incubated for 30 min at room temperature, and diluted by adding 1 mL of PBS. Data acquisition was performed on a FACSCanto II flow cytometer (BD Biosciences, Franklin Lakes, NJ, USA). Data were analyzed by FACS Diva version 8.0 software (BD Biosciences).

4. Statistical analysis

Data are presented as means ± standard deviations (SD). Differences between mean values were evaluated using the Tukey-Kramer method, and a p-value less than 0.01 was considered statistically significant. Correlation analysis was performed using a Pearson's correlation test, and a p-value less than 0.01 was considered statistically significant. All data analysis was performed using EZR (Saitama Medical Center, Jichii Medical University, Saitama, Japan), a graphical user interface for R (The R Foundation for Statistical Computing, Vienna, Austria). More precisely, this is a modified version of R Commander, which was designed to add statistical functions frequently used in Biostatistics [15].

Table 1. Characteristics of 86 patients with thrombocytopenia

Variables		Total	ITP	MDS	AA+CIT	LIT
n (%)		86	34 (39.5)	25 (29.1)	815 (17.4)	12 (14.0)
Age, mean ± SD (Years)		60 ± 18	62 ± 16	60 ± 19	46 ± 16	70 ± 13
Sex, n (%)	Male	36 (41.9)	8 (23.5)	8 (32.0)	10 (66.7)	10 (83.3%)
	Female	50 (58.1)	26 (76.5)	17 (68.0)	5 (33.3)	2 (16.7)
Pltelet count, mean ± SD ($\times 10^4/\mu$L)		4.8 ± 2.8	4.3 ± 3.1	4.3 ± 2.4	5.1 ± 2.4	6.5 ± 2.4
MPV, mean ± SD (fl)		9.4 ± 2.6	10.2 ± 3.2	9.4 ± 2.6	8.7 ± 1.6	8.3 ± 0.8
Therapy (%)			PSL only 3(8.8)			
			TPORA only 4 (11.8)			
			TPORA, Danazol 1 (2.9)			
			TPORA, PSL 1 (2.9)			

ITP, immune thrombocytopenia; MDS, myelodysplastic syndrome; AA, aplastic anemia; CIT, chemotherapy-induced thrombocytopenia; LIT, leukemia-induced thrombocytopenia; PLT, platelet count; MPV, mean platelet volume; PSL, prednisolone; TPORA, thrombopoietin receptor agonist.

III. Results

We examined 86 patients with thrombocytopenia, including 34 patients with ITP, 25 patients with myelodysplastic syndrome (MDS), 15 patients with aplastic anemia (AA) and chemotherapy-induced thrombocytopenia (CIT), and 12 patients with leukemia-induced thrombocytopenia (LIT) (Table 1).

1. Measurement of ARPs% by flow cytometry

To quantify platelets undergoing autophagy, we used a simple method to detect autophagosomes within the platelet cytoplasm that involves using a cell-permeable fluorescent dye, Cyto-ID, and flow cytometry. Figure 1 shows the results of flow cytometric analysis of RPs (Fig. 1A) and ARPs (Fig. 1B). Platelets and red blood cells were gated by forward and side scatter (upper panels). To isolate platelets, CD235a-positive populations that contained red blood cells and their fragments were removed (middle panels). CD41-positive cells were gated (middle panels), and the frequencies of TO- and Cyto-ID-positive cells (lower panel) were measured to determine RPs% and ARPs%, respectively. The baseline ranges of proportions of TO- or Cyto-ID-positive platelets in healthy controls were determined to both be 10% ± 1.0% (horizontal lines in lower panels). RPs% and ARPs% were increased in ITP patients.

2. Significant increase in ARPs% in ITP patients

Figure 2 shows the RPs% and ARPs% values in ITP patients and those with other types of thrombocytopenia. Significant increases in RPs% and ARPs% were observed only in ITP patients. Elevated values of both RPs% and ARPs% were detected in 88.2% of ITP patients. The mean RPs% and ARPs% among ITP patients were 29.1 ± 17.8% and 23.8 ± 13.8%, respectively. For the other disorders, no significant increases in RPs% or ARPs% were observed, with mean values of 8.3 ± 3.5% and 12.4 ± 8.4% among MDS patients, 6.6 ± 2.1% and 7.1 ± 3.2% among AA + CIT patients, and 8.0 ± 4.1% and 9.7 ± 5.5% among LIT patients.

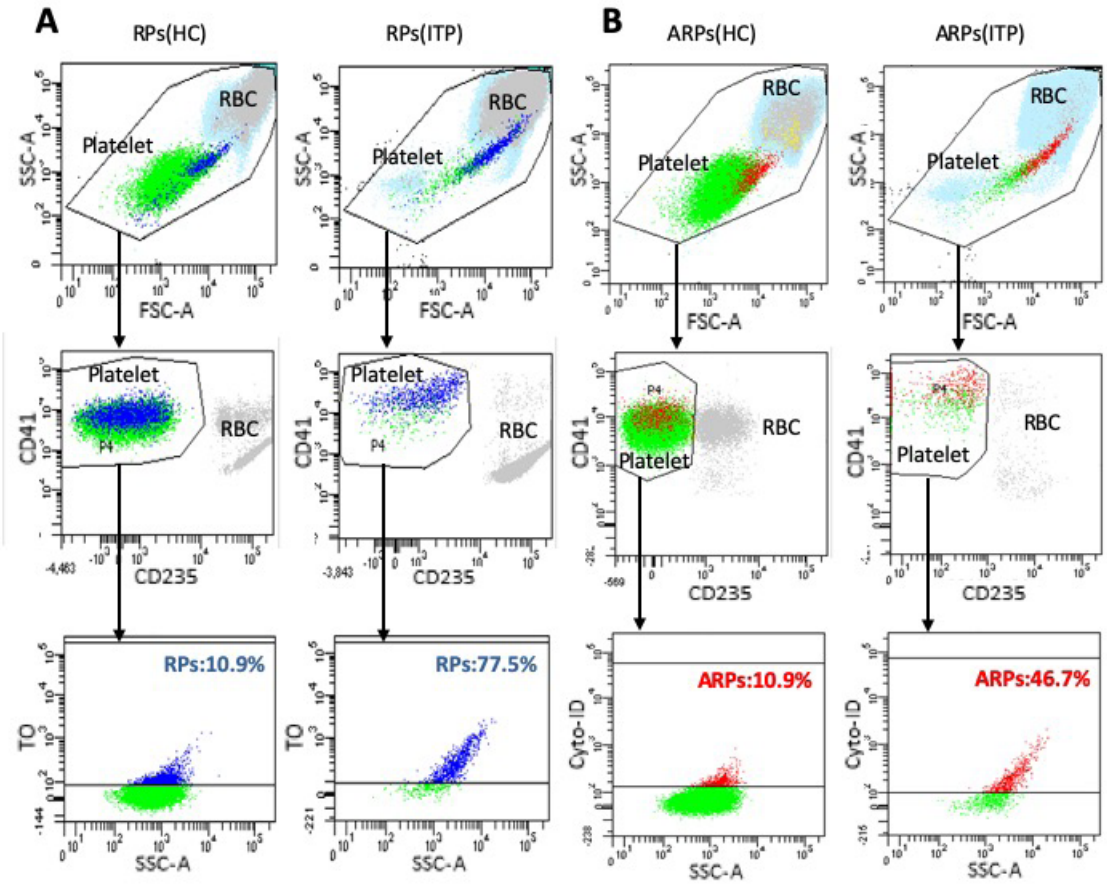

Fig. 1. Flow cytometry analysis of RPs and ARPs. (A) Representative data from RPs (blue population). (B) Representative data from ARPs (red population). Left panels show a healthy control. Right panels show an ITP patient. Middle panels: Staining for CD41 identifies the total platelet population, and staining for CD235a identifies red blood cells and their fragments. Lower panels: Staining for TO identifies mRNA, and staining for Cyto-ID identifies autophagosomes. ARPs% and RPs% from 20 healthy controls were determined to both be 10% ± 1.0% (horizontal lines). RPs, reticulated platelets; ARPs, autophagosome-rich platelets; TO, thiazole orange; RBC, red blood cells; HC, healthy control; ITP, immune thrombocytopenia.

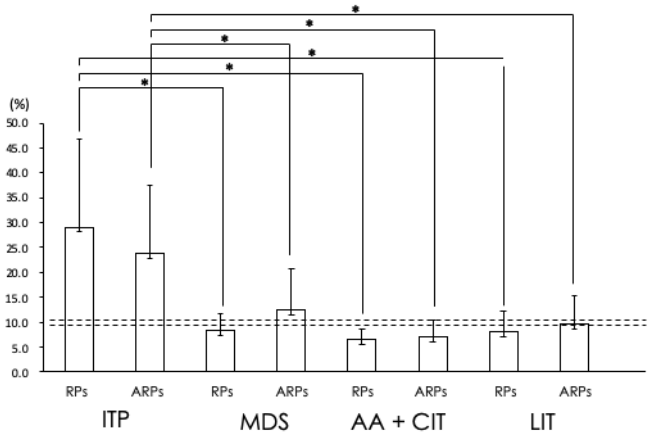

Fig. 2. Median and range of RPs% and ARPs% in all patient groups.
Dotted lines indicate ranges in healthy controls (10% ± 1.0%). *p <0.01. ITP, immune thrombocytopenia; MDS, myelodysplastic syndrome; AA, aplastic anemia; CIT, chemotherapy-induced thrombocytopenia; LIT, leukemia-induced thrombocytopenia; RPs, reticulated platelets; ARPs, autophagosome-rich platelets.

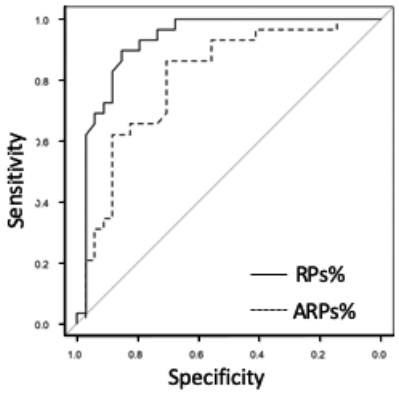

Fig. 3. Receiver operating characteristic (ROC) analysis.
ROC curves were constructed to determine the sensitivity and specificity of differential diagnosis of ITP from other forms of thrombocytopenia, including MDS, AA + CIT, and LIT. RPs%, proportion of reticulated platelets; ARPs%, frequency of autophagosome-rich platelets.

Receiver operating characteristic (ROC) curves were used to determine the sensitivity and specificity of ARPs% and RPs% in the differential diagnosis of ITP from other types of thrombocytopenia (Fig. 3). ROC curves for ARPs% and RPs% showed areas under the curves (AUCs) of 0.83 and 0.93, respectively, which were significantly different from each other ($p = 0.003$). ARPs% showed a higher sensitivity but reduced specificity (cut off value 11.0%, 88.2% and 71.2%, respectively) compared with those of RPs% (cut off value 11.9%, 85.3% and 90.4%, respectively), owing to a slight increase in ARPs% in some MDS patients. These data suggest that enhanced autophagy of platelets is a characteristic finding

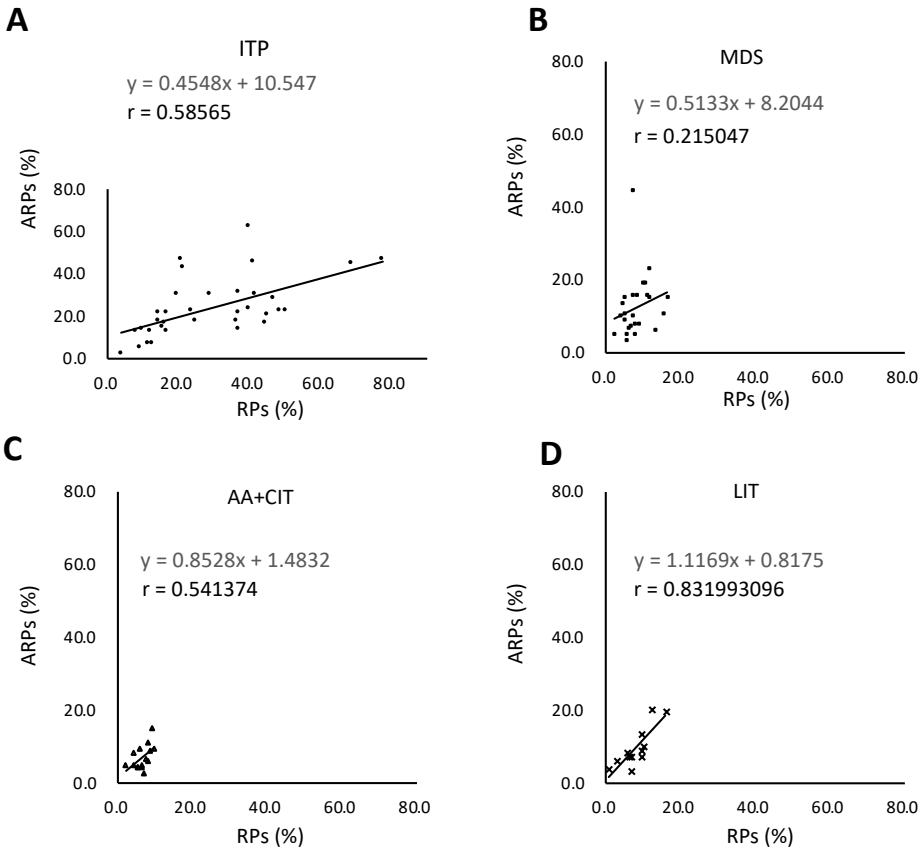

Fig. 4. Correlations between RPs% and ARPs%.
(A) Immune thrombocytopenia (ITP) patients. (B) Myelodysplastic syndrome (MDS) patients. (C) Aplastic anemia and chemotherapy-induced thrombocytopenia (AA + CIT) patients. (D) Leukemia-induced thrombocytopenia (LIT) patients. RPs, reticulated platelets; ARPs, autophagosome-rich platelets.

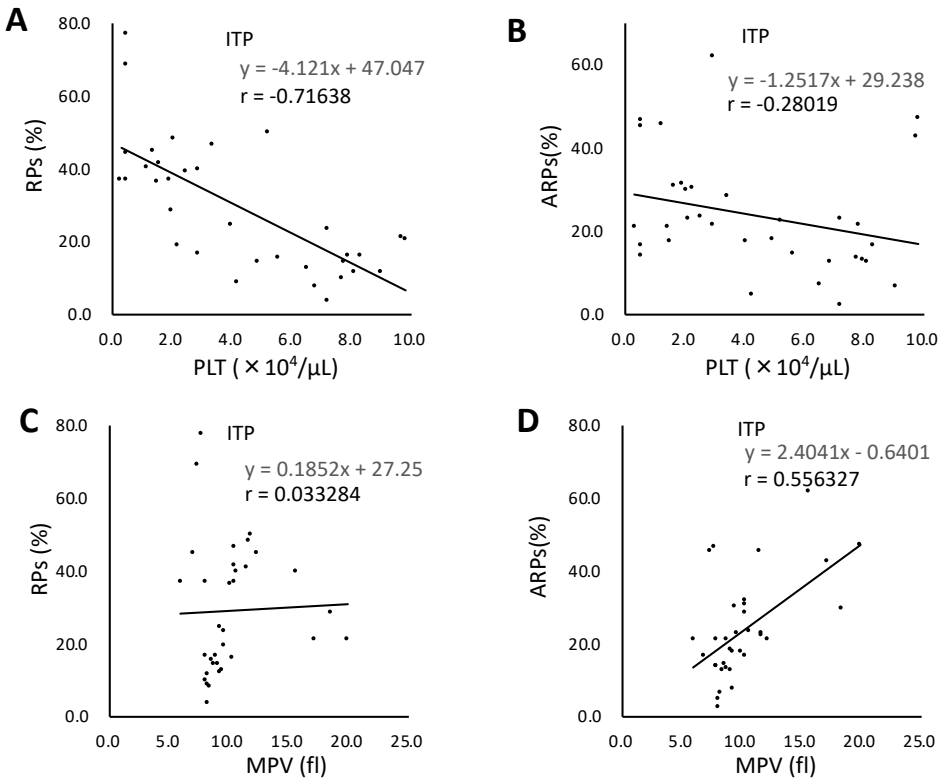

Fig. 5. Correlations between RPs% or ARPs% and platelet parameters in ITP patients.
(A) RPs% and PLT. (B) ARPs% and PLT. (C) RPs% and MPV. (D) ARPs% and MPV.
MPV, mean platelet volume; RPs%, frequency of reticulated platelets; ARPs%, proportion of autophagosome-rich platelets; ITP, immune thrombocytopenia; PLT, platelet count.

in patients with ITP, although an increase in ARPs% is also detected in a small population of patients with MDS.

3. Relationship between ARPs% and platelet parameters

To evaluate the relationship between ARPs% and RPs%, we analyzed the correlation. In ITP, a significant correlation was observed (r = 0.58, Fig. 4A). In other types of thrombocytopenia, there was a weak correlation in MDS (Fig. 4B), but significant correlation in AA + CIT and LIT (Fig. 4C, D). Next, we evaluated the influence of platelet count and MPV on these parameters in ITP patients. As expected, there was a significant correlation between RPs% and platelet count (r = −0.71) (Fig. 5A), and no correlation between RPs% and MPV (r = 0.03, Fig. 5C). However, for ARPs%, there was not a significant correlation with platelet count (r = −0.28, Fig. 5B) and a significant correlation with MPV (r = 0.55, Fig. 5D). In fig.5A-D, patients with outliers did not have any characteristic clinical information including treatment, age, sex, time from diagnosis, and complications.

These data suggest that ARPs% is more affected by platelet volume than by platelet count, in comparison with RPs%.

IV. Discussion

This study showed that enhanced autophagy in circulating platelets is characteristic among ITP patients and has diagnostic potential for ITP.

Autophagy has recently been reported to be essential for the development of the megakaryocyte-platelet lineage. Megakaryocytopoiesis depends on the proliferation and differentiation of hematopoietic cells for commitment to the megakaryocyte lineage and their maturation into large megakaryocytes [16]. A mature megakaryocyte produces several thousands of platelets via a complex process [17]. Mice with conditional deletion of the autophagy-related gene *Atg7* in hematopoietic stem cells fail to maintain normal hematopoiesis, including megakaryocytopoiesis [18]. However, mice with a specific deletion of *Atg7* in megakaryocytes exhibit impaired platelet function but preserve normal platelet counts [9]. These data suggest that while the regulation of megakaryocytopoiesis depends on autophagy, platelet production can occur even in the presence of impaired autophagy. In patients with ITP, bone marrow megakaryocytes are well maintained, but autoantibodies impair platelet production [19]. Recently, it has become clear that megakaryocytes exhibit enhanced autophagy in the presence of autoantibodies against the megakaryocyte-platelet lineage [11, 20]. Moreover, we found that a certain number of platelets also show enhanced autophagy in patients with ITP. Interestingly, it has been reported that the autophagic flux inhibitor chloroquine may be used as a treatment for ITP [21–23]. Chloroquine was shown to improve platelet count in adults and children with refractory ITP. These data suggest that excessive autophagy induced by autoantibodies has little impact on megakaryocytopoiesis but a major impact on platelet production and/or platelet lifespan.

It has remained unclear whether enhanced autophagy of ITP platelets leads to cell survival or apoptotic cell death. Autophagy can be a double-edged sword, with abnormally enhanced autophagy potentially inducing apoptosis and cell death [24]. It has been reported that enhanced autophagy of megakaryocytes protects against apoptosis induced by auto-antibodies [11]. Besides, autophagy of platelets removes damaged mitochondria resulting from oxidative stress to avoid apoptotic cell death [25]. Consistent with these reports, we demonstrated that ARPs% was positively correlated with RPs%, implying that enhanced autophagy of ITP platelets may lead to platelet survival. In contrast, as mentioned above, the autophagy inhibitor chloroquine was shown to improve platelet counts in refractory ITP [21–23]. The relationship between autophagy and apoptosis is thus complicated and context-dependent [24]. These data indicate that enhanced autophagy may considerably affect the survival of cells in the megakaryocyte-platelet lineage depending on the condition. Further studies are needed to clarify the relationship between autophagy and apoptosis in the pathogenesis of ITP.

There are several limitations in the present study. First, there is a possibility that treatment for ITP affects the proportion of RPs and ARPs, because 73% of 34 ITP patients in this small study had not received any medications including prednisolone, thrombopoietin receptor agonist. Furthermore, morphological assays of the autophagy process are required in future studies.

In conclusion, the present study shows that enhanced autophagy of platelets is a characteristic finding in patients with ITP and shows diagnostic potential in thrombocytopenia.

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Conflict of interest: The authors have no conflict of interest to declare.

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ITP 患者では autophagosome 形成血小板比率が高値である

大津瑛裕[1)]，古和田周吾[1)]，関　裕葵[1)]，
村井一範[2)]，清原千貴[1)]，宮島真理[1)]，泉田　亘[1)]，
人見次郎[3)]，石田陽治[1)]，伊藤薫樹[1)]

[1)] 岩手医科大学医学部，内科学講座血液・腫瘍内科分野
[2)] 岩手県立中央病院，血液内科
[3)] 岩手医科大学医学部，解剖学講座人体発生学分野

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要旨

免疫性血小板減少性紫斑病（immune thrombocytopenia, ITP）は，抗血小板自己抗体を介した血小板の破壊の亢進と，血小板産生の減少による血小板減少によって特徴付けられる．確定的な検査方法を欠くことが診断を難しくしており，診断はその他の血小板減少疾患の除外によって成される．今回我々は，ITP を含む血小板減少症の患者で autophagosome 形成血小板比率（autophagosome rich platelets%, ARPs%）を測定し，ITP 診断における有用性を検討した．

血小板減少症患者 86 名の末梢血血小板を CytoID® で染色しフローサイトメトリーで CytoID® 陽性血小板割合を測定し ARPs% とした．ITP 患者では他疾患と比較して，ARPs% は有意に高値を示した（$p < 0.01$）．ITP 患者において，ARPs% と網状血小板比率（reticulated platelets%, RPs%）は正の相関があった（$r = 0.58$）．また，RPs% は血小板数と強い負の相関を示し（$r = -0.71$），ARPs% は平均血小板容積と正の相関示した（$r = 0.55$）．

血小板減少症患者における autophagy の亢進は ITP 患者で特徴的であり，autophagosome 形成血小板比率の測定が血小板減少症の鑑別の一助となる可能性が示唆された．