

Anagrelide Modulates Proplatelet Formation Resulting in Decreased Number and Increased Size of Platelets

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Abstract

We retrospectively evaluated 48 essential thrombocythemia (ET) patients who were treated in our institute (male/female, 14/34, median age, 61.5 years). In 14 patients treated with anagrelide (ANA), the degree of platelet count reduction (median, -56.6%) was strongly correlated with increase of mean platelet volume (MPV) (median, +11.7%) ($R=0.777$). This correlation was not observed in ET patients treated with hydroxycarbamide alone ($R=0.245$). The change in size of platelets strongly suggested that ANA affected the final process of platelet production. Thus, we hypothesized that ANA modifies the process by which platelets are released from proplatelets. To verify the association in an in vitro setting, we compared MEG-01 cells treated with PMA ± ANA. The number of platelet-like particles (PLPs) was decreased ($P < 0.05$) and the size of PLPs estimated by using flow cytometry was significantly increased when MEG-01 cells were treated with PMA + ANA ($P < 0.05$ vs PMA alone), recapitulating the clinical findings. The cytoplasmic protrusions extending from MEG-01 cells were shorter and thicker and the number of proplatelets was decreased when MEG-01 cells were treated with PMA + ANA ($P < 0.01$ vs PMA alone). Western blotting analysis showed that ANA treatment resulted in increased phosphorylation of MLC2 and reduced phosphorylation of focal adhesion kinase (FAK). The morphological change of proplatelets were reversed by blebbistatin, a specific inhibitor of myosin II. These findings indicated that ANA modulates the FAK-RhoA-ROCK-MLC2-myosine IIA pathway and suppresses proplatelet maturation, leading to a decrease in platelet count and increase in MPV.

Introduction

Essential thrombocythemia (ET) is a chronic myeloproliferative neoplasm (MPN) characterized by an increased number of platelets in the blood. ET is a clonal stem cell disorder mainly induced by gene mutations such as *JAK2*, *MPL*, and *CALR*

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mutations. Major complications due to clonal expansion of platelets include thrombosis and bleeding. In the later stages, ET can transform into myelofibrosis or acute leukemia. The main goal of treatment for patients with ET is prevention of thrombotic events. Age over 60 years, a previous thrombotic event, existence of *JAK2* mutation, cardiovascular risk factors and platelet count over $1500 \times 10^9/L$ have been reported to be risk factors for thrombosis in patients with ET.¹⁻³ In ET patients with high risk factors of thrombotic events, administration of an antiplatelet agent such as aspirin and cytoreductive therapy with hydroxycarbamide (HC), interferons (IFNs) or anagrelide (ANA) are treatment options.^{4,5} The ELN guideline recommends HC or IFN α as first-line treatment and recommend the use of ANA in high-risk patients who are intolerant or resistant to HC treatment.⁴ The NCCN guideline recommends HC, IFN α or ANA as first-line therapy for high-risk ET patients. In Japan, both ANA and HC are recommended as first-line therapy for high-risk ET patients based on the results of comparative studies of HC and ANA.⁶⁻⁸ Because of genotoxicity, the potential risk for secondary leukemia remains a major concern of HC treatment, especially for young patients. ANA was initially developed as an antiplatelet agent,⁹ and its thrombocytopenic effect was discovered during preclinical trials.¹⁰ Initially, inhibition of megakaryocyte maturation was focused on as the mechanism underlying the platelet-lowering effect of ANA.^{11,12} In megakaryocytes derived from CD34-positive cells, ANA decreased mRNA levels of transcription factors such as *GATA-1*, *FOG1*, *FLI-1*, and *NF-E2*, which

are associated with megakaryocyte maturation.^{13,14} At the late phase of megakaryocytic differentiation, megakaryocytes form long, branched cytoplasmic extensions named proplatelets, which protrude into the bone marrow sinusoidal lumen where platelets are released from proplatelet tips.¹⁵ Recently, ANA-induced phosphorylation of myosin light chain 2 (MLC2) was detected in megakaryocytes differentiated from CD34+ cord blood, and the phosphorylation leads to the formation of wider proplatelet shafts, which possibly contribute to the platelet-lowering effect of ANA.¹⁶ Modulation of proplatelets might be a direct mechanism underlying platelet reduction by ANA. Proplatelet maturation is known to be controlled by the focal adhesion kinase (FAK)-RhoA-ROCK-MLC2 pathway; however, the upstream region of the MLC2 pathway has not been explored.

In this report, we report interesting finding that platelet-lowering effect of ANA associated with increased platelet size in ET patients. Due to morphological change of platelets, we hypothesized that ANA affect formation of proplatelet, the final process of platelet release.

Patients and Methods

Patients

We retrospectively evaluated 48 patients who were diagnosed with ET in Hokkaido University Hospital during the period from 2010 to 2018 (male/female: 14/34, ages: 18 to 87 years, median age: 61.5 years). Medical records of the patients were reviewed and we checked age, sex, *JAK2* V617F mutation status, platelet count at the start of administration of ANA or HC, platelet count at the time of maximum response to ANA or HC, MPV at the start of administration of ANA or HC, MPV at the time of maximum response to ANA or HC, and time to maximum response. Three patients were treated with ANA alone. Eleven patients were administered ANA due to an insufficient decrease in platelets after treatment with HC or intolerance to HC. Eighteen patients were treated solely with HC. Sixteen patients were observed without ANA or HC treatment. We analyzed the changes in platelet count and MPV after administration of each medicine. All the patients treated with ANA had at least one of the risk factors of thrombosis including *JAK2* mutation, age over 60 years, history of thrombosis, high platelet count ($\geq 1500 \times 10^9/L$), and cardiovascular risk factors.¹⁻³ Eleven of the 14 ET patients treated with ANA were over 60 years of age. Three of the 14 ET patients treated with ANA had a history of thrombosis. Platelet count was over $1500 \times 10^9/L$ in 5 of the 14 ET patients treated with ANA (Table S1, Supplemental Digital Content 1, <http://links.lww.com/HS/A38>). *JAK2* V617F mutation status was checked in 12 patients and *JAK2* V617F mutation was positive in 8 patients (66.7%, Fig. S1A, Supplemental Digital Content 2, <http://links.lww.com/HS/A39>). ANA was administered in 7 of the 8 ET patients with *JAK2* V617F mutation in this study. WBC count was significantly higher in *JAK2* V617F mutation-positive patients than in *JAK2* V617F mutation-negative patients in this study (Fig. S1B, Supplemental Digital Content 2, <http://links.lww.com/HS/A39>). This study was approved by the Institutional Review Board of Hokkaido University Hospital.

Methods

Cell line and megakaryocytic differentiation. MEG-01 is a human megakaryocytic leukemia cell line and is widely used as an experimental model to study the process of platelet production

from megakaryocytes.¹⁷⁻¹⁹ MEG-01 cells can differentiate into megakaryocytes by stimulation with phorbol-12-myristate-13-acetate (PMA), recombinant human thrombopoietin, or low-concentrated fetal bovine serum.¹⁷⁻¹⁹ MEG-01 cells extend cytoplasmic protrusions similar to those of megakaryocyte proplatelets and release platelet-like particles (PLPs), which express platelet-specific glycoproteins such as CD61 ($\beta 3$) and CD41 (αIIb).¹⁷⁻¹⁹ In this study, MEG-01 cells were cultured in Roswell Park Memorial Institute 1640 medium with 10% fetal bovine serum and 1% penicillin/streptomycin with or without 10 nM PMA (Wako, Osaka, Japan). After 2-day incubation at 37°C with 5% CO₂, the cells were retrieved and lysed with SDS lysis buffer containing 1.7% SDS, 60mM Tris-HCl, pH 6.8, 0.85% 2-mercaptoethanol, and proteinase inhibitor cocktail (Roche, Basel, Switzerland). To examine the expression levels of integrin αIIb and integrin $\beta 3$, which are megakaryocytic markers, Western blotting was performed using an anti-integrin αIIb antibody (B-9, Santa Cruz Biotechnology, Santa Cruz, CA) and an anti-integrin $\beta 3$ antibody (AB2984, Millipore, Bedford, MA), respectively. Protein loading of each well was verified by an anti- β -actin antibody (AC-15; Sigma-Aldrich, St. Louis, MO).

Proplatelet formation and platelet-like particles. MEG-01 cells were seeded in 35 mm glass-based dishes (IWAKI, Chiba, Japan) at a density of 2.5×10^4 cells/ml and cultured with 10 nM PMA alone, 10 nM PMA with 100 μ M HC, 10 nM PMA with 5 μ M ANA (SIGMA-ALDRICH, St. Louis, MO), or 10 nM PMA with 5 μ M ANA and 20 μ M blebbistatin (FUJIFILM Wako Pure Chemical, Osaka, Japan) for 2 days. MEG-01 cells were incubated with 20 μ M blebbistatin for 30 minutes followed by treatment with 5 μ M ANA. To evaluate morphological changes, the cells were stained with May-Grünwald stain followed by Giemsa stain on day 2. Then the number, length and maximum shaft thickness of protrusions extending from MEG-01 cells were determined as previously reported.²⁰ One hundred MEG-01 cells were analyzed in each sample.

To determine the number of PLPs, MEG-01 cells were seeded in 35 mm glass-based dishes at a density of 2.5×10^4 cells/ml and cultured with 10 nM PMA alone or 10 nM PMA with 5 μ M ANA for 2 days. The cells were fixed with 3% paraformaldehyde and permeabilized with 0.1% Triton-X-100. Then the cells were stained with AB2984 and Hoechst 33342 (Dojindo Laboratories, Kumamoto, Japan) followed by Alexa 594-conjugated secondary antibody (Thermo Fisher Scientific, Waltham, MA) staining on day 2. The number of PLPs per high-power field (HPF $\times 200$) was counted. Images were obtained using a Keyence BZ-X700 all-in-one fluorescence microscope (Keyence, Osaka, Japan).

To determine the size of PLPs, PLPs were stained with an anti-human CD41 (αIIb) antibody (5B12; Dako, Glostrup, Denmark) and detected using flow cytometry (FCM). PLP is a low forward scatter (FS) particle expressing CD41 and we considered a CD41-positive particle in the gate for the peripheral blood platelet as a PLP in this study. Mean fluorescent intensity (MFI) of FS was measured to determine the size of PLPs.

Expression of FAK, pFAK, and pMLC2. MEG-01 cells treated with 10 nM PMA alone or 10 nM PMA with 1 μ M or 5 μ M ANA for 2 days were peeled off, and suspended cells were collected after incubation for 1 hour at 37°C with 5% CO₂. The suspended cells were lysed with SDS lysis buffer containing 1.7% SDS, 60mM Tris-HCl, pH 6.8, 0.85% 2-mercaptoethanol, and proteinase inhibitor cocktail to examine the expression of FAK, tyrosine-phosphorylated FAK (pFAK), and phosphorylated

MLC2 (pMLC2) by Western blotting. FAK, pFAK, and pMLC2 were detected with an anti-FAK antibody (EP695Y, Abcam, Cambridge, UK), anti-phospho-FAK (Tyr397) antibody (3283, Cell Signaling, Danvers, MA), and anti-phospho-MLC2 antibody (3675S, Cell Signaling, Danvers, MA), respectively. Protein loading of each well was assessed by an anti- β -actin antibody. The phosphorylation state of FAK was quantified as the density ratio of protein bands pFAK/FAK, and the phosphorylation state of MLC2 was quantified as the density ratio of protein bands pMLC2/ β -actin.

Statistical analysis. The association between rate of change in MPV and rate of change in platelet count after administration of ANA was evaluated by Pearson product moment correlation coefficient. The percentages of patients treated with ANA were compared by Fisher exact test. White blood cell (WBC) count and platelet count were compared by the *t* test. Total expression levels of α IIb, β 3, pFAK, and pMLC2 in MEG-01 cells treated with PMA alone and those in MEG-01 cells treated with PMA and ANA were compared by the *t* test. The number of protrusions, length of protrusions, maximum shaft thickness of protrusions, number of PLPs, and FS intensity of PLPs in MEG-01 cells treated with PMA alone and those in MEG-01 cells treated with PMA and ANA were also compared by the *t* test. *p* values < 0.05 were considered to be statistically significant. All statistical analyses were performed with EZR.²¹

Results

The characteristics of patients treated with ANA are shown in Table S1 (Supplemental Digital Content 1, <http://links.lww.com/HS/A38>). In 11 of the 29 patients treated with HC, treatment with ANA was initiated due to a lack of efficacy of HC ($n=3$, 27.3%) or intolerance to HC ($n=8$, 72.7%). Intolerance to HC was indicated by leg ulcers ($n=4$), skin eruption ($n=1$) and/or progressive anemia ($n=4$). Treatment with ANA alone was initiated in 3 patients. In the 14 patients treated with ANA, platelet counts at the initiation of ANA treatment were 424 to $1810 \times 10^9/L$ (median, $960 \times 10^9/L$). A reduction in platelet count of more than 30% was achieved in 13 patients (92.9%) after ANA treatment. In ET patients treated with ANA, the mean of MPV was significantly higher at the maximum response to ANA than at the induction of ANA (Fig. 1A). In ET patients treated with HC, there was no difference between the mean MPV at the maximum response to HC and the mean MPV at the induction of HC. Then, we analyzed association between change of MPV and the degree of platelet count reduction in each patient (Fig. 1B, C). The plots in Figure 1B, C show the data obtained at the time of maximum response to ANA or HC. Interestingly, the degree of platelet count reduction (-83.4% to -27.1% ; median, -56.6%) was strongly correlated with increase of MPV (-6.6% to $+28.7\%$; median, $+16.7\%$) ($R=0.777$, $P=0.001$, Fig. 1B). In the 29 patients treated with HC, platelet counts at initiation of

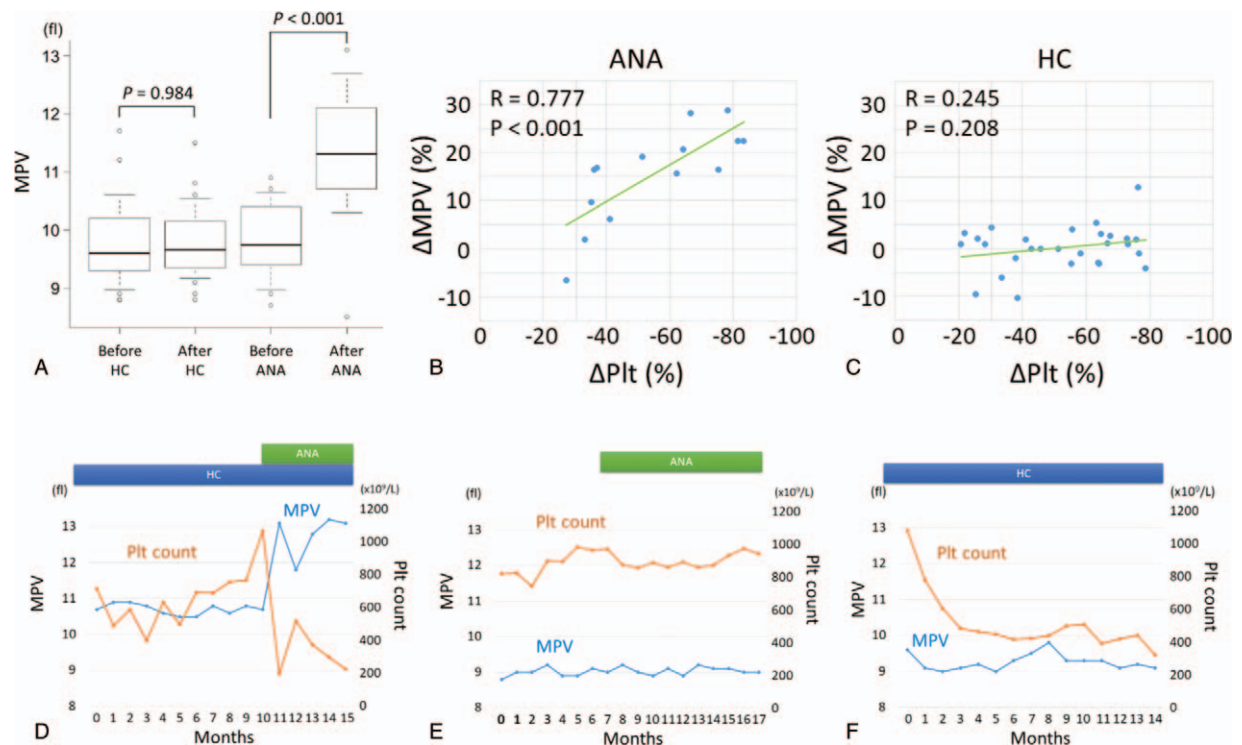


Figure 1. (A) Box plot shows the change of MPV after treatment with HC or ANA. Treatment with ANA increased MPV although treatment with HC did not affect MPV. (B) Scatter plot shows the rate of decrease (%) in platelet count and the rate of change (%) in MPV at the time of maximum response to ANA. The data were obtained from 14 patients who underwent ANA treatment. Pearson product moment correlation coefficient = 0.777, $P < 0.001$. (C) Scatter plot shows the rate of decrease (%) in blood platelet count and the rate of change (%) in MPV at the time of maximum response to HC. The data were obtained from 28 patients who underwent HC treatment only without ANA. Pearson product moment correlation coefficient = 0.245, $P = 0.208$. (D) Clinical course of a representative case that showed a clinical response to ANA. Platelet count (orange line) and MPV (blue line) are shown. (E) Clinical course of a representative case that did not show a clinical response to ANA. Platelet count (orange line) and MPV (blue line) are shown. (F) Clinical course of a representative case that showed a clinical response to HC. Platelet count (orange line) and MPV (blue line) are shown. ANA = anagrelide; HC = hydroxycarbamide; MPV = mean platelet volume; Plt = platelet.

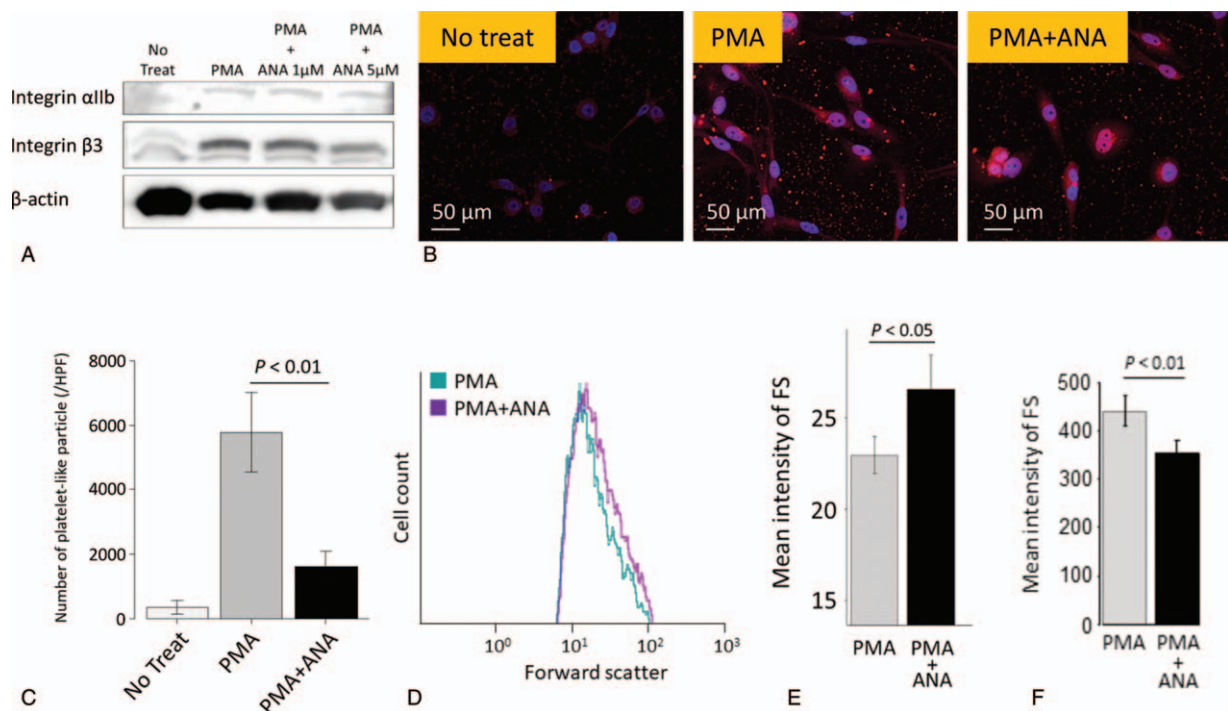


Figure 2. (A) Expression of integrin α IIB and integrin β 3 in MEG-01 cells. PMA increased the expression of integrin α IIB and integrin β 3. Results are representative of 3 independent experiments. (B) Immunofluorescence staining of MEG-01 cells and platelet-like particles (PLPs). PMA promote production of PLPs (center). The number of PLPs was decreased in the PMA + ANA treated MEG-01 cells (right). Red: integrin β 3, Blue: Hoechst 33342. Left: Non-treated MEG-01 cells, Center: 10 nM PMA-treated MEG-01 cells, Right: 10 nM PMA and 5 μ M ANA-treated MEG-01 cells. (C) The number of platelet-like particles stained with anti-CD61 (β 3) antibody per high-power field (HPF, $\times 200$) was counted. PMA increase the number of PLPs. The number of PLPs was decreased in the PMA + ANA treated MEG-01 cells. Results are means plus or minus SD from 3 independent experiments. (D) Evaluation of the size of PLPs produced from MEG-01 cells. PLPs are low forward scatter (FS) particles expressing CD41 (α IIB) and are detected by using flow cytometry. With the addition of ANA, the graph shifted to the right, which meant increased size of PLPs. (E) Mean FS intensity of PLPs was measured to estimate the size of PLPs. FS intensity of platelet-like particles was significantly higher in the PMA + ANA treated MEG-01 cells compared to the cells treated with PMA alone. Results are means plus or minus SD from 3 independent experiments. (F) Mean FS intensity of MEG-01 cells was measured to estimate the size of MEG-01 cells. FS intensity of MEG-01 cells was significantly lower in the PMA + ANA treated MEG-01 cells compared to the cells treated with PMA alone. Results are means plus or minus SD from 3 independent experiments. ANA = anagrelide; FS = forward scatter; PMA = phorbol-12-myristate-13-acetate.

HC treatment were 211 to $1809 \times 10^9/L$ (median, $824 \times 10^9/L$). A reduction in platelet count of more than 30% was achieved in 23 patients (79.3%) after HC treatment. The degree of platelet count reduction (-78.7% to -20.8% ; median, -55.5%) was not correlated with change of MPV (-10.3% to $+12.9\%$; median, $+1.0\%$) ($R=0.245$, $P=0.21$, Fig. 1C). Time to maximum response was shorter in patients treated with ANA (0.5 to 29.4 months; median, 3.7 months, $P < 0.01$ vs HC) than in those treated with HC (1.9 to 102 months; median, 12.0 months). A peripheral blood smear at the time of maximum response to ANA and the clinical course of a representative case responding to ANA are shown in Figure S1D (Supplemental Digital Content 2, <http://links.lww.com/HS/A39>) and Figure 1D, respectively. MPV increased in accordance with the reduction of blood platelet count, and giant platelets were observed in the peripheral blood smear. The clinical courses of a case refractory to ANA and a case responding to HC are shown in Figure 1E and F, respectively. In the case of ANA failure, MPV remained unchanged (Fig. 1E). In contrast, HC treatment did not change MPV despite a reduction in platelet count (Fig. 1F).

In order to determine how ANA affects the process of megakaryocyte differentiation, proplatelet formation, and PLP production, we performed an *in vitro* assay using MEG-01 cells. PMA-induced megakaryocytic differentiation was confirmed by detection of integrin α IIB and β 3 expression using Western blotting (Fig. 2A). MEG-01 cells treated with PMA were stained

with an anti-human CD61 (β 3) antibody (Fig. 2B) for immunofluorescence staining. Following incubation of MEG-01 cells with PMA, the number of PLPs was decreased in the PMA + ANA treated MEG-01 cells ($P < 0.01$ vs PMA alone, Fig. 2B and C). PLP size was estimated by mean intensity of FS in PLPs. For PLP gating, a region was designated by the loading of human peripheral platelets following staining with a fluorescence-labeled anti-human CD41 (α IIB) antibody (data not shown). In the absence of PMA, slight staining of PLPs was detected in the region. The size of PLPs estimated by the intensity of FS using FCM was significantly increased in the PMA + ANA treated MEG-01 cells compared to the cells treated with PMA alone ($P < 0.05$, Fig. 2D and E). These results were consistent with the clinical findings in ET patients treated with ANA. The size of MEG-01 cells estimated by the intensity of FS using FCM was significantly decreased in the PMA + ANA treated MEG-01 cells compared to the cells treated with PMA alone ($P < 0.01$, Fig. 2F). We then focused on morphological changes of proplatelets induced by treatment with ANA. May-Grünwald-Giemsa staining of MEG-01 cells showed that the addition of PMA led to extension of cytoplasmic protrusions and that the addition of ANA with PMA inhibited the extension of cytoplasmic protrusions although the addition of HC with PMA had no influence on the morphology of cytoplasmic protrusions (Fig. 3A). Moreover, the addition of blebbistatin with PMA and ANA canceled the effect of ANA on cytoplasmic protrusions.

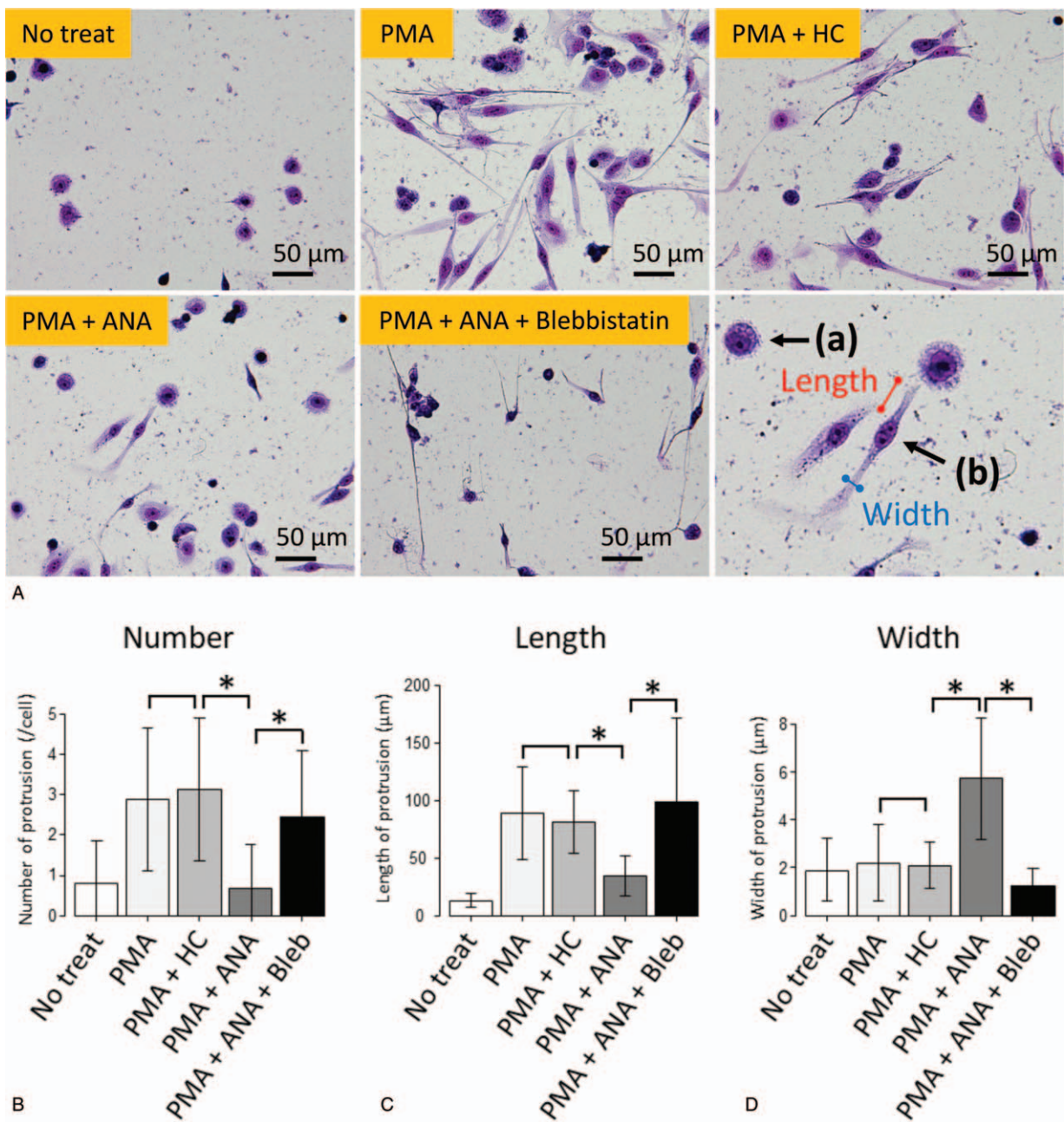


Figure 3. (A) May-Grünwald-Giemsa staining of MEG-01 cells. Upper left: non-treated MEG-01 cells, Upper middle: 10 nM PMA-treated MEG-01 cells, Upper right: 10 nM PMA and 100 μM HC-treated MEG-01 cells, Lower left: 10 nM PMA and 5 μM ANA-treated MEG-01 cells, Lower middle: 10 nM PMA, 5 μM ANA and 20 μM blebbistatin-treated MEG-01 cells, Lower right: example of count of morphological parameters. The addition of PMA led to extension of cytoplasmic protrusions (upper middle), the addition of ANA with PMA inhibited the extension of cytoplasmic protrusions (lower left), and the addition of HC with PMA did not cause any changes about cytoplasmic protrusions (upper right). The addition of blebbistatin with PMA + ANA led to extension of cytoplasmic protrusions (lower middle). The number of protrusions is 0 in cell (a) and the number of protrusions is 2 in cell (b). Red bar shows the length of protrusions and blue bar shows the width of protrusions (lower right). ANA = anagrelide; HC = hydroxycarbamide; PMA = phorbol-12-myristate-13-acetate. (B) The number of protrusions extending from MEG-01 cells was counted. The number of proplatelets was decreased when MEG-01 cells were treated with PMA + ANA than treated with PMA alone. The change was reversed when MEG-01 cells were treated with PMA + ANA + blebbistatin. (C) The length of cytoplasmic protrusions was measured. The cytoplasmic protrusions were shorter when MEG-01 cells were treated with PMA + ANA than treated with PMA alone. The change was reversed when MEG-01 cells were treated with PMA + ANA + blebbistatin. (D) The maximum shaft width of cytoplasmic protrusions was measured. The cytoplasmic protrusions were thicker when MEG-01 cells were treated with PMA+ANA than treated with PMA alone. The change was reversed when MEG-01 cells were treated with PMA + ANA + blebbistatin. For (B) to (D), 100 MEG-01 cells were analyzed in each sample. Results are means plus or minus SD from 3 independent experiments. * $P < 0.001$. ANA = anagrelide; HC = hydroxycarbamide; PMA = phorbol-12-myristate-13-acetate.

To verify the morphological change, we compared morphological parameters including the number, length, and width of cytoplasmic protrusions as shown in Figure 3A (lower right). There was no difference in the number, length, and width of

cytoplasmic protrusions between MEG-01 cells treated with PMA alone and the cells treated with PMA + HC ($P = 0.612$, $P = 0.496$, $P = 0.722$, respectively, Fig. 3B–D). The cytoplasmic protrusions were shorter and thicker and the number of proplatelets was

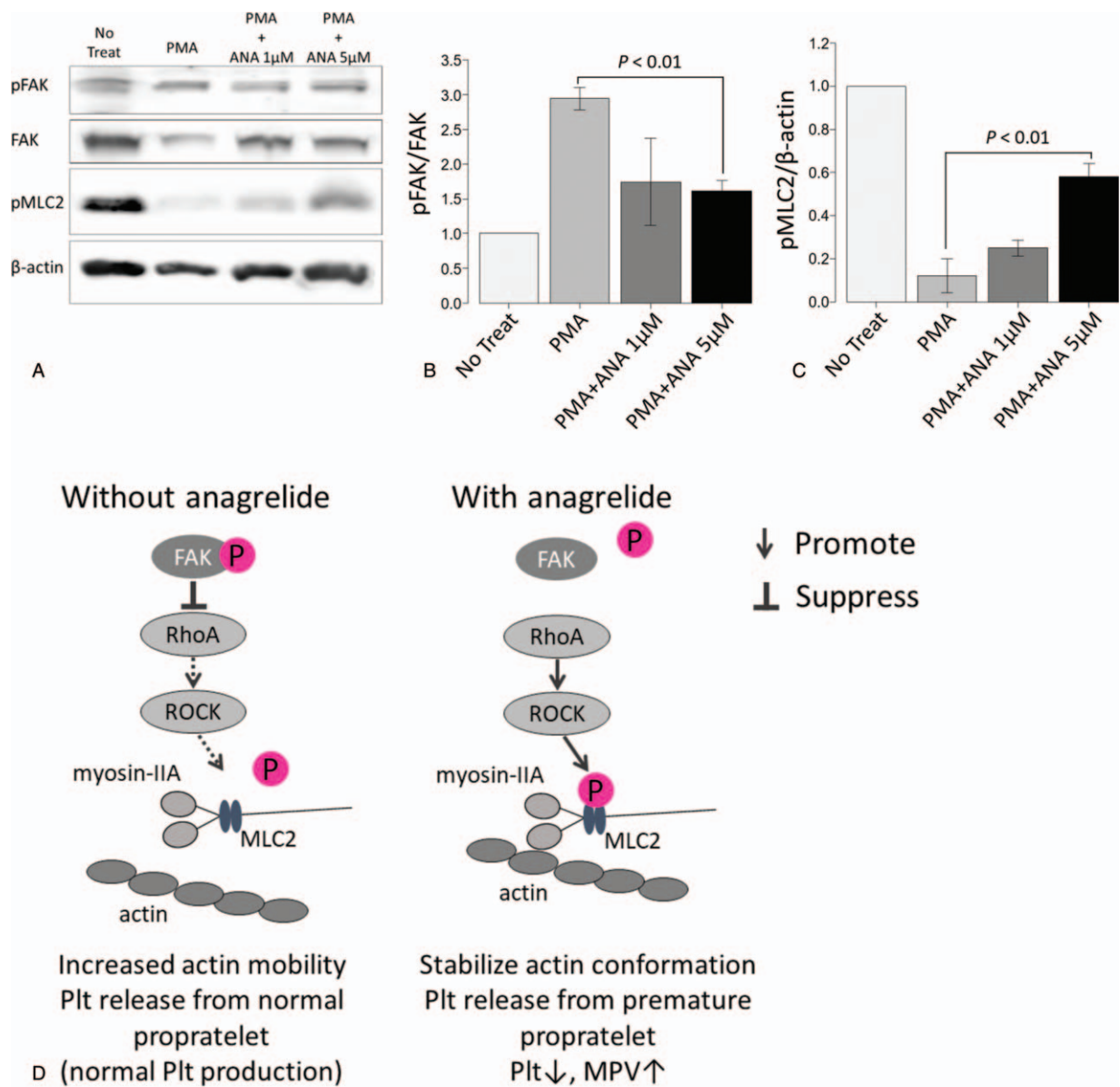


Figure 4. (A) Expression of pFAK, FAK, pMLC2, and β -actin in MEG-01 cells. Results are representative of 3 independent experiments for MEG-01 cells. (B) Relative quantification of the phosphorylation state of FAK. The phosphorylation state of FAK was quantified as a density ratio of protein bands, pFAK/FAK. Values of the samples were calibrated to that of a non-treated sample. Results are means plus or minus SD from 3 independent experiments. (C) Quantification of the phosphorylation state of MLC2. The phosphorylation state of MLC2 was quantified as a density ratio of protein bands, pMLC2/ β -actin. Values of the samples were calibrated to that of a non-treated sample. Results are means plus or minus SD from 3 independent experiments. (D) Possible mechanism of the platelet-lowering effect of ANA. ANA suppresses phosphorylation of FAK and promotes phosphorylation of MLC2. As a result, proplatelet formation is interfered and platelet production is suppressed. ANA = anagrelide; FAK = focal adhesion kinase; pFAK = phosphorylated focal adhesion kinase; PMA = phorbol-12-myristate-13-acetate; pMLC2 = phosphorylated myosin light chain 2; Plt = platelet.

decreased when MEG-01 cells were treated with PMA+ANA ($P < 0.001$ vs PMA alone, Fig. 3B–D). The cytoplasmic protrusions were longer and thinner and the number of proplatelets was increased when MEG-01 cells were treated with PMA+ANA+blebbistatin ($P < 0.001$ vs PMA+ANA, Fig. 3B–D). The FAK-RhoA-ROCK-MLC2-myosin IIA pathway is an important pathway for the formation of proplatelets and production of platelets from megakaryocytes.^{22–26} We evaluated the effect of ANA on phosphorylation of FAK and MLC2. Western blotting analysis showed that ANA induced increased phosphorylation of MLC2 and reduced phosphorylation of FAK (PMA vs PMA+ANA 5 μ M $P < 0.01$, Fig. 4A–C).

Discussion

We found an interesting correlation between platelet reduction and MPV increase in patients treated with ANA. Although a previous study suggested that MPV was higher in ET patients treated with ANA than in those not treated with ANA,²⁷ MPV change after ANA treatment was not analyzed. Our data clearly showed that successful platelet reduction by ANA correlated to increased MPV. This finding might be a clue for clarifying the mechanisms underlying the platelet-lowering effect of ANA. Cytoreduction by ANA is platelet-specific, and ANA does not suppress myeloid and erythroid lineages. In contrast, HC inhibits

DNA synthesis and cytochrome reduction by HC is not lineage-specific, and thrombocyte reduction effect does not correlate to increase of MPV. Unanticipated anemia could be a major indicator of intolerance to HC in ET patients. ANA is known to inhibit PDEIII, but the levels of PDEIII inhibition and elevation of cAMP have no association with the platelet-lowering effect of ANA.^{16,28} ANA decreased platelet count without altering platelet function or thrombospondin content in case with ET.²⁹ The change in size of platelets strongly suggested that ANA affected the final process of platelet production. Thus, we hypothesized that ANA modifies the process by which platelets are released from proplatelets. Since the platelet-lowering effect of ANA is a human-specific phenomenon, we verified the finding using the human megakaryocytic cell line MEG-01, which can be differentiated to megakaryocytes by PMA treatment. ANA reduced PLP production and the size of PLPs was confirmed to be increased, recapitulating clinical findings. The morphological change of cells after ANA treatment was striking. MEG-01 cells form proplatelets and release PLPs after megakaryocytic differentiation induced by PMA. ANA suppressed proplatelet formation, resulting in decreased number and shortened length of proplatelet. Short and thick protrusions may contribute to the decreased number and increased size of PLPs. A previous study showed that ANA suppressed transcription factors responsible for megakaryocytic differentiation such as *FLI-1*, *FOG-1*, and *GATA-1*^{13,14} that resulted in smaller megakaryocytes.¹¹ Platelet size change could not be explained just by suppression of megakaryopoiesis. Besides blocking megakaryopoiesis, our study showed that ANA exerted a direct inhibitory effect on thrombopoiesis.

Proplatelet formation process depends on actin and microtubule dynamics. While microtubules are used to propel proplatelet elongation, an actin-dependent reaction is used to bifurcate the proplatelet shaft, thereby increasing the number of proplatelet tips available to participate in platelet formation.³⁰ The FAK-RhoA-ROCK-MLC2-myosin IIA pathway plays an important role controlling actin dynamics in the process of proplatelet formation.^{22,23,25} An association between decreased platelet count and increased platelet size was reported in congenital macrothrombocytopenia, in which the FAK-RhoA-ROCK-MLC2-myosin IIA pathway was dysregulated.^{23–26} Heterozygous mutation at the membrane proximal region of *ITGA2B* or *ITGB3* causes constitutional partial activation of the integrin α IIb β 3 complex and premature release of platelets, resulting in production of giant platelets. We show a possible mechanism of the platelet-lowering effect of ANA in Figure 4D. Forced expression of the mutated integrin resulted in abnormal cytoplasmic protrusions similar to those in ANA-treated MEG-01 cells.²⁵ In megakaryocytes, phosphorylation of FAK negatively controls the RhoA-ROCK-MLC2-myosin IIA pathway and leads to proplatelet formation and platelet production.^{22–25} In this study, we revealed decreased phosphorylation of FAK and increased phosphorylation of MLC2 in MEG-01 cells treated with ANA. These findings indicated that ANA modulated the FAK-RhoA-ROCK-MLC2-myosin IIA pathway, leading to abnormal proplatelet formation that resulted in a decrease of platelet count and increase of MPV. A decreased level of phosphorylated FAK promotes the RhoA-ROCK-MLC2-myosin IIA pathway and phosphorylated MLC2 stabilizes actin conformation, resulting in poor mobility of the cell surface and reduced platelet production. Some studies in patients with macrothrombocytopenia showed that insufficient activation of integrin α IIb β 3 and platelet production from immature

proplatelets led to the production of larger platelets.^{25,26} Based on the similar change in the number and size of platelets in macrothrombocytopenia, we hypothesize that ANA targets the FAK-RhoA-ROCK-MLC2-myosin IIA pathway as well. Based on ANA's rapid onset of action, besides blocking megakaryopoiesis, repression of proplatelet formation might be the main mechanism underlying its platelet-lowering effect. Other possibility is the action mediated by microtubules. Direct inhibitor of microtubule, such as colchicine, prevent proplatelet formation. Change of microtubule bundling or elastic bending could potentially affect platelet size.³¹ We found morphological change induced by ANA was reversed by blebbistatin, a specific myosin II inhibitor. So, we speculated that RhoA-ROCK-MLC2-myosin IIA-Actin pathway were responsible for clinical effect of ANA treatment. Our study revealed that ANA affects the process of proplatelet formation through modification of FAK-RhoA-ROCK-MLC2-myosin IIA pathway.

Conclusion

ANA reduces platelet counts and increases platelet size by suppressing proplatelet formation through modulation of the FAK-RhoA-ROCK-MLC2-myosin IIA pathway. Morphological observation suggests that ANA induces premature release of platelets, resulting in increased platelet size. Further work on the mechanisms underlying the thrombocytopenic effects of ANA may contribute to elucidation of the pathways regulating platelet biogenesis.

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