Characteristics of fibrinolytic disorders in acute promyelocytic leukemia

Ping Wang a,b, Yingmei Zhang a, Huiyuan Yang a, Wenyi Hou a, Bo Jin a, Jinxiao Hou a, Haitao Li a, Hongli Zhao a,c and Jin Zhou a

aCenter for Hematology and Oncology, The First Affiliated Hospital, Harbin Medical University, Harbin, People’s Republic of China; bDepartment of Neonatology, The First Affiliated Hospital, Harbin Medical University, Harbin, People’s Republic of China; cDepartment of Hematology, The Fourth Affiliated Hospital, Harbin Medical University, Harbin, People’s Republic of China

ABSTRACT

Objectives: Catastrophic hemorrhage remains the main cause of acute promyelocytic leukemia (APL) treatment failure. This study was aimed to study the pathogenesis of coagulopathy in patients with APL.

Methods: Multiple procoagulant and profibrinolytic parameters in plasma and peripheral leukocytes from 24 patients with newly diagnosed APL accompanied by coagulopathy before and after arsenic trioxide (ATO) treatment were evaluated.

Results: Prior to the treatment, the patients had elevated D-dimer and decreased fibrinogen levels. Plasma urokinase-type plasminogen activator receptor (uPAR) and plasmin–α2 antiplasmin complexes (PAP) levels, plasmin (Pn) activity, and cell surface levels of urokinase-type plasminogen activator (uPA) and tissue-type plasminogen activator (tPA) were significantly higher; plasma plasminogen activator inhibitor-1 (PAI-1) levels and plasminogen (Pg) activity were significantly decreased; plasma plasminogen activator (PA) activity, uPA and tPA levels; and cell surface levels of uPAR and annexin II were not significantly different from levels in the control group. During ATO treatment, both patients’ plasma PA activity and uPAR on leukocytes gradually increased, annexin II on leukocytes increased initially and decreased afterwards, and tPA and uPA on leukocytes remained consistently higher in the patients than in the controls. Other parameters gradually tended toward normal values.

Conclusions: In APL, activated coagulation system activated fibrinolytic system, and increased uPAR levels could contribute to the hyperfibrinolysis. Annexin II might not be involved in the coagulopathy.

Introduction

Acute promyelocytic leukemia (APL) is a special subtype of acute myeloid leukemia caused by a specific balanced reciprocal translocation t(15;17), which leads to the creation of the PML-RARα fusion gene. Patients with APL often present with coagulopathy, rendering them prone to death by hemorrhage and thrombosis prior to and during treatment. Differentiation therapies such as all-trans retinoic acid and arsenic trioxide (ATO) have greatly improved the prognoses of these patients. Nonetheless, early death rates of APL have not been significantly reduced. Bleeding is still the leading cause of early death in APL, and is attributable to the majority of APL treatment failure [1–4].

Current evidence shows that the pathogenesis of APL coagulopathy is more complicated than that of disseminated intravascular coagulation. At present, at least 3 different yet interrelated potential mechanisms have been proposed: activation of the coagulation system, increased fibrinolytic activity, and increased nonspecific proteolytic activity. The PML-RARα fusion gene in APL cells can induce tissue factor (TF) expression, thereby associating a primary carcinogenic event with coagulation activation [5,6]. Studies have reported that hyperfibrinolysis in APL patients results from increased levels of urokinase-type plasminogen activator (uPA), and uPA receptor (uPAR) [7,8], and decreased levels of fibrinolysis inhibitors such as plasma plasminogen activator inhibitor-1 (PAI-1) and α2-antiplasmin [9,10]. Other studies have shown that annexin II is overexpressed on t(15;17)–positive cells in APL patients [11,12]. As a co-receptor of plasminogen (Pg) and tPA, annexin II promotes fibrinolysis by increasing plasmin (Pn) production and protecting Pn activity by preventing binding to its inhibitors in the plasma [13,14]. To date, the existing studies only focused on one or several profibrinolytic markers, it is necessary to systematically study more fibrinolytic indicators to better define the mechanism of hyperfibrinolysis in APL patients.

As a differentiation therapy, ATO induces APL cell differentiation by degrading the PML-RARα fusion protein. Studies have shown that TF and procoagulant activity were significantly decreased in ATO-treated...
APL cells [15,16]. There are also reports that observed a significant reduction in Pn generation on ATO-treated APL cells [12]. So far, the effect of monotherapy with ATO on coagulopathy has not been completely defined. This study evaluated coagulation- and fibrinolysis-related parameters in APL patients at the time of hospital admission, and explored the mechanism of APL coagulopathy by measuring changes in these parameters prior to and during ATO treatment.

Materials and methods

Patients

Twenty-four patients admitted to the Department of Hematology of The First Affiliated Hospital of Harbin Medical University between July 2014 and June 2016 were included in the study. All patients were at least 18 years old, newly diagnosed with APL accompanied by coagulopathy, and gave their informed consent to participate in this study. Patients were excluded if they refused to continue treatment for personal reasons or died during the induction phase. This study was approved by the Harbin Medical University Ethics Committee, and was conducted according to the ‘Declaration of Helsinki.’ All patients received APL diagnoses based on clinical data, bone marrow imaging, bone marrow genotyping, and bone marrow immunophenotyping. All patients were confirmed to have t(15;17) translocation and the PML-RARA fusion gene through cytogenetic analysis. Twenty-four healthy age- and gender-matched participants to the patients were selected as controls. Healthy participants were excluded if they had coronary heart disease, diabetes, or hypertension, or if they took antiplatelet agents or anticoagulants during the sample collection period.

Coagulopathy was defined as clinical manifestations of bleeding tendency or/and more than 3 laboratory abnormalities as follows: Platelet (PLT) counts < 50 × 10^9/L, Fibrinogen level < 1.8 g/L, Increased levels of D-dimer, a prolonged prothrombin time (PT) ≥5s or activated partial thromboplastin time (APTT) ≥10s.

Hemorrhagic events were classified as described in the literature [17]: grade 0 (no hemorrhage), grade I (minor bleeding), grade II (moderate bleeding not requiring a transfusion), grade III (bleeding requiring transfusion), grade IV (catastrophic bleeding requiring major non-elective intervention), and grade V (death).

Treatment protocol

All patients received remission induction therapy with ATO (0.16 mg/kg/day, with a maximum of 10 mg/day) by continuous slow intravenous infusion. Hydroxyurea was taken orally for patients who had leukocytosis after initiation of therapy. Platelet concentrates and fresh-frozen plasma were transfused to maintain target PLT counts of >30 × 10^9/L and target fibrinogen levels >1.5 g/L. Cryoprecipitate or/and fibrinogen was administered if necessary.

Sample collection

Early morning fasting venous blood was simultaneously collected from all patients and healthy controls. Blood samples were collected once prior to treating the patients, and weekly thereafter until three weeks after treatment. The collected blood samples were mixed with 3.2% sodium citrate at a 1:9 ratio and processed within 1 hour. Blood samples were centrifuged at 1500xg for 15 min to obtain platelet-poor plasma, which was centrifuged at 13,000xg for an additional 2 min to remove the remaining platelets and obtain platelet-free plasma (PPF). The PFP was aliquoted and frozen at −80°C until use. Peripheral leukocytes from nine patients and their controls were obtained for flow cytometry analysis.

Routine laboratory testing

Routine laboratory testing on prothrombin time (PT), APTT, fibrinogen (Fg), D-dimer (DD), white blood cell (WBC) count, and PLT count was performed on all patients.

Markers of fibrinolysis activation

Plasma samples of all patients and controls were assayed using commercial ELISA kits for uPA antigen, tPA antigen, uPAR antigen, PAI-1 antigen (all from R&D, Minneapolis, U.S.A.) and plasmin–α₂ antiplasmin complexes (PAP) antigen (DRG, Springfield, U.S.A.) according to the manufacturers’ instructions. Plasminogen activity in plasma was assayed by chromogenic assay using commercial kit (HYPHEN BioMed, Neuville-sur-Oise, France).

Activity of plasmin and plasminogen activator

Plasma Pn and PA activities were measured using the plasmin substrate D-Val-Leu-Lys 4-nitroanilide dihydrochloride (VALY; Sigma-Aldrich, Saint Louis, U.S.A.) as described in the literature [17,18,19] with minor modifications. Briefly, 5 μL of PFP diluted with phosphate-buffered saline (PBS) was added to 180 μL of preheated (37°C) buffer (9.25 mM potassium phosphate, 64.8 mM sodium phosphate, 92.6 mM l-lysine, pH 7.5 at 37°C) and 50 μL of VALY (1.3 mM) and placed into a thermostatic microplate reader (kept at 37°C). A405nm was detected every 10 min for at least 14 h. VALY was cleaved by Pn and the released p-nitroaniline was detected by measuring A405nm as a function of time. Results were reported as PnA (OD405nm × 10^-3/min). For measuring
plasminogen activator activity in the plasma, 20 μl (2 μM) of human glu-plasminogen (Sekisui Diagnostics, Stamford, U.S.A.) was added to the detection system, and the results were reported as ΔPn (OD$_{405nm}$). When indicated, increasing concentrations of following inhibitors were pre-incubated with the plasma for 1 hour: mouse anti-human tPA, mouse anti-human uPA (both from American Diagnostica GmbH, Pfungstadt, Germany), and control antibody (BD Bioscience, San Jose, U.S.A.).

Flow cytometry

Peripheral leukocytes were obtained by lysing erythrocytes from whole blood samples: 1 × Lysing Buffer (BD Bioscience) was mixed with the whole blood samples, incubated at room temperature for 5 min, centrifuged at 250×g for 5 min. The lysis process was repeated once. Then leukocytes pellet was washed with PBS containing 0.2% bovine serum albumin and 0.1% sodium azide. CD87 and CD33 were used for direct labeling. Briefly, peripheral leukocytes collected from patients and healthy controls were incubated with CD87-PE antibody, CD33-PE-Cy5 antibody, and their isotype controls (all from BD Bioscience) at room temperature for 30 min. uPA, tPA, and annexin II were used for indirect labeling. Briefly, peripheral leukocytes collected from patients and healthy controls were incubated with mouse anti-human tPA, mouse anti-human uPA, and rabbit anti-human annexin II antibodies (Abcam, Cambridge, U.K.), respectively, at room temperature for 30 min. After incubation, the samples were washed once and incubated with PE-conjugated goat anti-mouse IgG (BD Bioscience) or Alexa Fluor 488-conjugated goat anti-rabbit IgG (Abcam) at room temperature for 30 min. Samples without the respective primary antibody were used as controls. Cells were then analyzed by flow cytometry on FASCARia (BD Biosciences). Data were analyzed with FlowJo software (TriStar, Ashland, U.S.A.). Results are expressed as the ratio of the mean fluorescence intensity (MFI) of the fluorescent antibody to the MFI of the isotype antibody (control).

Statistical analysis

Results for each parameter are presented as median (range). The SPSS 19.0 software was used for the following statistical analyses: changes of the results in patients across time were analyzed using a mixed linear model. The difference in each parameter between the patient and the control groups was analyzed using the Mann–Whitney U test. Correlation of each parameter with pre-treatment values was analyzed using Spearman’s correlation (with the Spearman’s rank-order coefficient r). A difference of $P < 0.05$ was deemed statistically significant. Excluding the correlation between the within-subject repeated measures, the correlation between two variables with repeated observation was analyzed using mixed effects model [20] with SAS 9.3 software. Variable and variable*$week$ were included as fixed effects, and variable and week as repeated measures. The correlation coefficient was presented as $\rho$, with $|\rho|>0.4$ deemed equivalent.

Blood samples were collected from healthy controls and patients at the same time points, and relevant indices were measured. The mean of 4 measurements for each control group measurement result was calculated. The mean value of each index in each control was used to compare with the result from blood collected from each patient.

Results

Admission characteristics and treatment outcome of APL patients

Patient characteristics at diagnosis are shown in Table 1. The bleeding severity of the patients at the time of admission was graded from 0 to III (grade 0 in 6 patients, grade I in 1 patients, and grade III in 17 patients). Of the 18 patients that presented with bleeding at the time of admission, the hemorrhagic symptoms disappeared in 14 patients 1 week after treatment, 1 patient 2 weeks after treatment, and 3 patients 3 weeks after treatment (in all 3 patients, bleeding symptoms reappeared during the third week after cessation of clinical bleeding within 1-week treatment). In one of the six patients who did not present with any bleeding symptoms at admission,

| Characteristic | Patient group(n= 24) | Control group(n = 24) |
|---------------|----------------------|-----------------------|
| Sex (Male/Female) | 12/12 | 12/12 |
| Age (Median years [Range]) | 46 (21–66) | 38 (25–63) |
| Physical signs of bleeding (yes/no) | 18/6 | / |
| WBC count (Median × 10$^3$/L [Range]) | 4.18 (0.45–54.11) | / |
| PLT count (Median × 10$^3$/L [Range]) | 29.06 (4.00–126.20) | / |
| Hb (Median g/L [Range]) | 78.29 (52.00–143.90) | / |

Note: WBC: white blood cell; PLT: platelet; Hb: hemoglobin.
bleeding manifestations occurred two weeks after treatment. All patients achieved hematologic complete remission (HCR), and the median time to HCR was 34 days (range, 28–53 days).

Routine laboratory testing results in patients with APL

WBC count of patients with APL increased gradually during treatment. It peaked at the end of week 2, followed by a decrease (Figure 1(A), supplemental material, Table S2). Prior to the treatment, the patients had elevated D-dimer and PT levels and decreased Fg levels. All three gradually tended toward normal values, changes were the most significant at the end of week 1, and the rate of change slowed down afterwards (Figure 1(C–E), Table S2). During treatment, most values of APTT remained within the normal range, however, the levels significantly increased, with the most significant increase observed during the first week (Figure 1(F)).

Markers of fibrinolysis activation

Compared with the control group, the patient group had no significant differences in pretreatment plasma tPA and uPA levels but exhibited significantly higher plasma uPAR and PAP levels, and significantly lower plasma PAI-1 levels and Pg activity (Figure 2(A–F), supplemental material, Table S3). During treatment, plasma levels of all markers except uPA and Pg in the patient group significantly decreased. Changes of PAI-1, uPA, uPAR, and PAP were the most significant at the end of week 1. Plasma tPA levels in the patient
group were significantly decreased at the end of treatment week 3 (Table S2).

### Plasma Pn and PA activities in patients with APL

PnA detected in the absence of glu-plasminogen was due to plasma Pn, and ΔPn detected in the presence of glu-plasminogen was due to PA (See supplemental material, Figure S1). Before treatment, the Pn activity in the patient group was significantly higher compared with that in the control group ($P < .05$) (Figure 2(G)), and it was restored to the normal levels following 1 week of treatment ($P > .05$). Pretreatment plasma PA activity (ΔPn) levels were not significantly different between the patient and control groups (Figure 2(H)). ΔPn was
significantly increased in the patient group during treatment (Table S2).

**Flow cytometry**

Levels of tPA and uPA on the surface of peripheral blood leukemic cells remained significantly higher in the patient group than those on the surface of peripheral blood granulocytes and monocytes in the control group (Figure 3(G, I), Table S3). No significant changes were observed during the three-week treatment (Table S2). Pretreatment uPAR and annexin II levels on the surface of peripheral blood leukocytes were not significantly different between the patient and control groups. uPAR expression levels increased with treatment and were significantly higher at the end of week 3 (Figure 3(H), Table S3). Annexin II levels significantly increased with treatment. Annexin II levels in the

![Figure 3](image_url)

*Figure 3.* Effect of ATO on fibrinolytic markers at the surface of peripheral blood leukocytes in patients with APL (Median with interquartile range). (A) In peripheral blood leukocyte samples of one patient (APL) and the control at diagnosis, gates were set including the granulocytes and monocytes (Control), or the leukemic cell population (APL) in the forward (FSC)/sideward (SSC) scatter projection. Detection of annexin II (B), tPA (C), uPAR (D) and uPA (E) on cells in the defined gates. Fluorescence and concentration-matched isotype controls (Iso) were used. Histograms show overlays of representative fluorescence histograms obtained with isotype controls (red) and annexin II, tPA, uPAR or uPA specific antibodies (blue) on cells in the defined gates. The difference in each parameter (F–I) between the patient and the control groups was analyzed using the independent samples Mann–Whitney U test (P).
patient group reached the highest level at the end of week 2 and were significantly higher than those of the controls, then the levels decreased again at the end of week 3 (Figure 3(F), Table S3).

**Correlations among parameters tested in patients**

The significant pairwise correlations among the patients’ parameters tested before treatment were shown in Figure 4(A), and the significant pairwise correlations among the patients’ parameters repeatedly tested at 4 time points were shown in Figure 4(B). Pre-treatment plasma uPA levels were correlated with WBC count ($r = 0.468$, $P = .024$), pretreatment PLT count was correlated with bleeding severity ($r = -0.405$, $P = .0497$), and plasma tPA levels were correlated with PAI-1 levels during the 4 measurements ($\rho = 0.535$). Plasma uPAR levels were correlated with multiple parameters, it may be the main determinant of hyperfibrinolysis in APL patients.

Patients with bleeding presentations had significantly increased levels of DD, PAI-1, PnA, and decreased levels of PLT count, Fg than patients without bleeding (See supplemental material, Table S4).

**Discussion**

The results reported here showed that bleeding symptoms rapidly disappeared in most patients within one week of ATO treatment initiation. Plasma coagulation- and fibrinolysis-related indices except tPA improved most significantly during the first week of treatment, which was highly consistent with the clinical manifestations of bleeding tendency in APL patients. After that, the improvement rate slowed down, and parameters often did not return to normal at the end of three weeks of treatment, suggesting that the coagulopathy still existed. Therefore, monitoring of coagulation and fibrinolysis parameters was still required.

Significantly increased D-dimer suggested the activation of coagulation and an increase in crosslinked fibrin. TF is considered to be the most important physiological trigger in the coagulation system [21]. Studies have reported increased TF expression in APL cells [5,6,22]. The increased TF could lead to the activation of the extrinsic coagulation pathway, which led to the increased crosslinked fibrin, decreased Fg levels and prolonged PT in patients with APL. The increased APTT during treatment may be due to the release of promyelocytic chromatin from APL cells undergoing ETosis after treatment [23], which may trigger the intrinsic coagulation pathway, cause excessive consumption of intrinsic coagulation factors.

Similar to results of previous reports, patients in this study exhibited significantly higher PAP levels, and significantly lower Pg activity [12,24], suggesting increased fibrinolytic activity in these patients. The finding that pretreatment Pn activity was significantly higher in the patient group and returned to normal after one week of treatment was consistent with bleeding presentations. However, the patient group had no significant differences in pretreatment plasma tPA and uPA levels compared with the control group. The increased fibrinolytic activity may be due to increased plasma uPAR levels. Plasma uPAR levels were significantly higher compared to levels in the control group, with the most significant decrease during the first week, and were correlated with multiple procoagulant and profibrinolytic parameters. Thus, although this study showed no significant change in plasma uPA levels in patient group, the relative effects of the uPA might have been amplified by uPAR in plasma. It remains to be explained why plasma uPA activity ($\Delta$Pn) levels were significantly increased in the patient group during treatment, while the changes of plasma

![Figure 4](image-url)
uPA levels during the three-week treatment period were not significant.

The results for plasma tPA levels in this study were similar to the results from Liu et al. [12] and Tallman et al. [25] but differed from those of Zhu et al [26]. The discrepancies in different studies may be due to differences in the induction therapy, the patients (newly diagnosed or relapsed APL) or the severity of the coagulopathy. All 24 patients in this study had hemorrhagic events of grade 0-III, however, the severity of coagulopathy was not clearly reported in the above-mentioned publications.

Annexin II on the surface of peripheral blood leukocytes was detected in the patients and healthy controls of this study. Before treatment, patients’ annexin II levels on the surface of peripheral blood leukemic cells was not significantly higher than that of the controls, which is in contrast to the data of bone marrow cells in other studies [11,12,27]. Compared with bone marrow cells, peripheral blood cells may have a more direct effect on fibrinolytic activity. Annexin II is also expressed on endothelial cells, peripheral blood monocytes, macrophages and neutrophils in healthy people [28,29]. Results from this study could not conclude that the increased fibrinolytic activity in patients with APL patients was caused by annexin II. Further research will be required to investigate this relationship.

Most parameters did not return to normal at the end of treatment week 3, while the hemorrhagic symptoms disappeared one to two weeks after treatment. The coagulopathy might actually be aggravated if combined with other inducing factors, such as infection. In this study, all four patients whose hemorrhagic symptoms reappeared two to three weeks after treatment initiation had an infection before the rebleeding. Further studies will be required to determine when coagulopathy can fully recover and the key indicators for monitoring coagulopathy after initiation of treatment with ATO.

Conclusions

Results from this study have shown that in patients with APL, activation of coagulation and hyperfibrinolysis occurred simultaneously. Increased plasma uPAR might contribute to abnormally high fibrinolytic activity and bleeding. ATO treatment can significantly improve the bleeding manifestations in patients. However, the coagulopathy has not fully recovered at three weeks after treatment initiation; therefore, prolonged monitoring for coagulopathy is required.

Disclosure statement

No potential conflict of interest was reported by the authors.

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