**Effect of all-trans Retinoic Acid on Procoagulant and Fibrinolytic Activities of Cultured Blast Cells From Patients With Acute Promyelocytic Leukemia**

By V. De Stefano, L. Teofili, S. Sica, S. Mastrangelo, A. Di Mario, S. Rutella, P. Salutari, C. Rumi, G. d’Onofrio, and G. Leone

The mechanisms underlying acute promyelocytic leukemia (APL) coagulopathy and its reversal by administration of all-trans retinoic acid (ATRA) have been investigated. Bone marrow promyelocytic blasts from nine patients with APL were cultured with or without ATRA 1 µmol/L. Cultured blasts (days 0, 3, 6, and 9) were washed, resuspended in phosphate buffer, lysed by freezing and thawing, and then assayed for procoagulant activity (PCA), elastase activity, tissue factor (TF) antigen, tissue-type plasminogen activator (t-PA) antigen, and urokinase-type plasminogen activator (u-PA) antigen. PCA was determined by a recalcification assay. Elastase was measured by an amidolytic assay (S-2484). TF, t-PA, and u-PA antigens were measured by an enzyme-linked immunosorbent assay (ELISA). Malignant promyelocytes isolated from the patients had increased levels of PCA and TF as compared with the control polymorphonucleates, and low levels of elastase, t-PA, and u-PA; the patient blast PCA level was significantly related to the degree of hypofibrinogenemia. In this system, blast PCA depended on the tissue factor and was significantly correlated to the TF antigen values. In the cultures without ATRA, PCA, TF, and u-PA progressively increased, whereas elastase and t-PA levels remained essentially unchanged. In the presence of ATRA, all parameters (except u-PA) decreased during the culture time. Thus, a major role of the promyelocytic blast cell PCA in the pathogenesis of M3-related coagulopathy is suggested; the ATRA effect on coagulopathy seems mainly mediated by a downregulation of the PCA.

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**Patients and Methods**

Patients. Promyelocytic blasts from bone marrow of nine patients were investigated. The main clinical and laboratory features of the patients at the moment of the bone marrow drawing are reported in Table 1. All patients had a diagnosis of APL according to the French-American-British (FAB) classification (M3); in two of them (patients 6 and 9), the M3 leukemia was defined as variant subtype. Bone marrow blast infiltration ranged from 85% to 98%; cytofluorimetric analysis was performed as previously reported using the following fluorescein isothiocyanate (FITC)- or phycoerythrin (PE)-conjugated monoclonal antibodies: CD34 (8G12; Becton Dickinson, Mountain View, CA), CD33 (WM/54; Ylem, Avezzano, Italy), CD13 (S.JD1; Immunotech, Marseille, France), HLA-DR (IOT2a; Immunotech), and CD14 (MOP9; Becton Dickinson). All patients showed a typical immunophenotypic pattern consisting of a strong expression of CD33 and CD13 and lack of reactivity with CD14, CD34, and HLA-DR. Cytogenetic examination revealed the (15;17) translocation in all of them.

Six patients (patients 1 through 4, 8, and 9) were studied at diagnosis; two (patients 5 and 6) at first relapse; and one (patient 7) at second relapse. All patients were studied before starting chemotherapy or treatment with ATRA; one patient (patient 8) was studied both at diagnosis and during a treatment schedule (LAP 0493 Gruppo Italiano Malattie Ematologiche Maligne dell’Adulto [GIMEMA] protocol) including ATRA 45 mg/m² all days and Idarubicin (Farmitalia-Carlo Erba, Milan, Italy) 12 mg/m² on days 2, 4, 6, and 8.

Coagulopathy, defined by the presence of fibrinogen level below 150 mg/dL, fibrinogen degradation products (FDPs) level higher than 20 µg/mL, or both, was present in all patients: eight had a fibrinogen level less than 150 mg/dL, and three less than 100 mg/dL. Routine coagulation tests (prothrombin time, activated partial thromboplastin time [aPTT], fibrinogen, Godel test) were performed by standard methods; FDPs were determined by a latex agglutination test.

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Blast cells were obtained by density-gradient separation of 111; Kabi, Stockholm, Sweden). Samples promyelocytic blasts were more than 95%. After Trypan blue dye exclusion test, viable cells were suspended at a concentration of 5 X 10^6/mL in RPMI 1640 supplemented with 15% fetal calf serum (Biological Industries, Kibbutz Beit Haemek, Israel) with or without ATRA 1 pmol/L (Hoffmann-La Roche, Basel, Switzerland). The cell suspension was examined by phase microscopy after May-Grunwald-Giemsa staining, and in all samples promyelocytic blasts were more than 95%. After Trypan blue dye exclusion test, viable cells were suspended at a concentration of 1 X 10^9/mL in RPMI 1640 supplemented with 15% fetal calf serum (Biological Industries, Kibbutz Beit Haemek, Israel) with or without ATRA 1 µmol/L (Hoffmann-La Roche, Basel, Switzerland).

Cell aliquots were taken soon after drawing (day 0) and after 3, 6, and 9 days of culture, washed three times in PBS, suspended in PBS at a concentration of 5 X 10^6/mL, and frozen in liquid nitrogen until assayed. Cells were then lysed by freezing and thawing three times. The cell lysate was assayed for PCA.

The control sample was a preparation of normal polymorphonuclear clear cells suspended in PBS at a concentration of 5 X 10^9/mL and lysed as were the patient samples. In patient 8, a bone marrow sample was also drawn 7 days after the start of treatment. Blast cell lysate was obtained as described above testing for CD33, CD13, CD11b (44IgG1, Ylem), and HLA-DR.

Morphologic and phenotypic analysis of promyelocytic blasts. Cytospin slides were made after 9 days of culture and stained with May-Grünwald-Giemsa. Cytofluorimetric analysis of cell aliquots at day 0 and after 6 and 9 days of culture was performed as described above for CD33, CD13, CD11b (44IgG1, Ylem), and HLA-DR.

PCA. The PCA was determined in all patients on a coagulometer Koagulab K-60 (Ortho Diagnostic Systems, Milan, Italy) using recalibration time. Normal plasma (0.1 mL; Ortho Diagnostic Systems) was incubated with 0.1 mL of cell lysate for 5 minutes at 37°C. Then 0.1 mL of calcium chloride 0.025 mol/L was added, and the clotting time was recorded. PCA was expressed as arbitrary units obtained by interpolation with a reference curve of serial dilutions of a commercial repurposed recombinant tissue factor (Recombiplastin, lot R11 132; Ortho Diagnostic Systems). The PCA of Recombiplastin depended on the tissue factor concentration and the lipid composition, which were proprietary and were not disclosed by the manufacturer; the dilution 1:16 in imidazole buffer, pH 7.35 of the commercial preparation, yielding a clotting time of 23 seconds after mixing with the control plasma and calcium chloride, was arbitrarily assumed as 1,000 U of tissue factor. The addition of ATRA 1 µmol/L to the Recombiplastin suspensions did not modify the clotting time values of the curve.

The PCA of the cell lysate obtained from four different patients at different culture times was assayed also on plasmas selectively deficient in factors VII, IX, and X (Organon Teknika, Durham, NC).

Inhibition studies. The effect of iodoacetamide (Sigma, St. Louis, MO), a cysteine proteinase inhibitor, and concanavalin A (Sigma), an inhibitor of tissue factor, on the PCA was investigated according to Falanga et al. In brief, PCA was assayed after preincubation of the cell lysate for 30 minutes at 25°C with iodoacetamide (final concentration, 2 mmol/L) or for 1 hour at 37°C with concanavalin A (final concentration, 100 µg/mL). PCA of the cell lysate was assayed also in the presence of aprotinin (Trasylo1 Bayer, Leverkusen, Germany): 0.08 mL of the control plasma was added to 0.02 mL of aprotinin, 20,000 U/mL. After addition of 0.1 mL of cell lysate, the final concentration of aprotinin was 2,000 U/mL. In this case, the reference curve to calculate the PCA units was obtained by addition of serial dilutions of Recombiplastin on a control plasma diluted 4:5 with PBS.

TF antigen concentration. TF antigen concentration in the cell lysate was determined in five of the patients (patients 1 through 4 and 6) using an enzyme-linked immunosorbent assay (ELISA; Imubind Tissue Factor Kit, American Diagnostica, Greenwich, CT) according to the manufacturer’s instructions. Cell lysate (0.1 mL) diluted in phosphate buffer with bovine serum albumin 1% (wt/vol), pH 7.4 (ratio 1:10), was incubated overnight at 4°C; wells were then washed four times with phosphate buffer and filled with 0.1 mL of biotinylated murine antihuman TF monoclonal antibody. After incubation,

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Table 1. Clinical and Laboratory Features of the Patients Investigated

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<th>Patient No.</th>
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Abbreviations: BM, bone marrow; Hb, hemoglobin; WBC, white blood cell; Plt, platelet; PT, prothrombin time; INR, international normalized ratio; AT III, antithrombin III.
for 1 hour at room temperature, wells were washed again and filled with 0.1 mL of streptavidine-horseradish peroxidase provided in the kit. After 1 hour at room temperature, wells were washed again, and 0.1 mL of tetramethylbenzidine was added; the reaction was stopped after 20 minutes at room temperature by adding 0.05 mL of sulfuric acid 0.5 mol/L. Absorbance values were read at 450 nm. The standard curve was obtained by different TF standards (with concentrations from 50 to 1,000 pg/mL) provided in the kit.

Elastase activity. Elastase activity of the cell lysate was determined in five of the patients (patients 1 through 4 and 6) by an amidolytic method using a chromogenic substrate highly specific for granulocytic elastase (S-2484; KabiVitrum, Stockholm, Sweden). The substrate (8 mmol/L in dimethyl sulfoxide [DMSO]) was diluted 1:4 with H2O before use; 25 μL of cell lysate was mixed with 75 μL of buffer (Tris 0.1 mol/L, NaCl 0.96 mol/L, pH 8.3). After incubation for 3 minutes at 37°C with 50 μL of substrate, the reaction was stopped by the addition of acetic acid 50%, and the absorbance was measured at 405 nm.

The elastase concentration (μg/mL) was expressed by interpolating the absorbance values with a reference curve obtained by serial dilutions of purified human granulocytic elastase (EC 3.4.21.37, E-8140; Sigma).

t-PA antigen concentration. The concentration of t-PA antigen in the cell lysate was measured in five of the patients (patients 1 through 4 and 6) using an ELISA (Imutest t-PA; Innogenetics, Antwerp, Belgium). The assay was performed according to the manufacturer’s instructions. In brief, 0.05 mL of cell lysate was mixed with 0.15 mL of phosphate buffer and incubated for 1 hour at 37°C. Incubation wells were washed three times with phosphate buffer and filled with 0.2 mL of mouse monoclonal anti-t-PA labeled with horseradish peroxidase. After 1 hour at 37°C, wells were washed again and filled with 0.2 mL of tetramethylbenzidine dissolved in DMSO with 0.006% hydrogen peroxide. After 30 minutes at 25°C, the reaction was stopped with addition of 0.05 mL of sulphuric acid, 1 mol/L, and absorbance values were read at 450 nm. The standard curve was obtained by different concentrations (from 20 ng/mL to 1 ng/mL) of recombinant t-PA provided with the kit.

u-PA antigen concentration. The concentration of u-PA antigen in the cell lysate was determined in seven of the patients (patients 1 through 7) by ELISA (Imubind uPA kit; American Diagnostica) and according to the manufacturer’s instructions. Cell lysate (0.1 mL) diluted in phosphate buffer with bovine serum albumin 2% (wt/vol) and 0.1% Triton X-100, pH 7.4 (ratio 1:5), was incubated overnight at 4°C. Wells were then washed four times with phosphate buffer and filled with 0.1 mL of biotinylated murine anti-human u-PA monoclonal antibody. After incubation for 1 hour at room temperature, wells were washed again and filled with 0.1 mL of streptavidine-horseradish peroxidase provided in the kit. After 1 hour at room temperature, wells were washed again, and 0.1 mL of tetramethylbenzidine was added. The reaction was stopped after 20 minutes at room temperature by adding 0.05 mL of sulfuric acid, 0.5 mol/L, and absorbance values were read at 450 nm. The standard curve was obtained by different u-PA standards (with concentrations from 0.1 to 1 ng/mL) provided in the kit.

Statistical analysis. Statistical analysis was performed using the Wilcoxon test for paired data (significance level, P < .05). The Spearman’s rank correlation coefficient (rs) was calculated when indicated.

RESULTS

Morphologic and phenotypic analysis. Cells examined after 9 days of culture with ATRA showed signs of differentiation such as chromatin condensation, nucleus segmentation, and decreased cytoplasmic granules. Most cells were identifiable as metamyelocytes and band cells. Persistence of Auer rods confirmed the origin of the differentiated cells from the promyelocytic blasts.

In one case (patient 5), phenotypic analysis was performed on blast cells at day 0 and after 3, 6, and 9 days of culture. A high expression of CD33 (90%) and CD13 (75%) was found at day 0, with low expression of HLA-DR (6%). This pattern had no substantial variation during the culture time,
either in the absence or in the presence of ATRA. In the cultures with ATRA, a strong increase of the granulocytic differentiation antigen CD11b was evident (from 10% at day 0 to 50% at day 3, 92% at day 6 and 93% at day 9); in the absence of ATRA, this increase was much less evident (25% at day 9).

Assays performed on the cell lysate. The results obtained from the different assays performed on the cell lysates (PCA, TF antigen, elastase activity, t-PA antigen) are shown in Fig. 1. At basal condition in all patients, the PCA of promyelocytic blasts was higher than that of the control polymorphonuclear cells (1 tissue factor unit per million cells [U/10^6 cells]), yet with a large range of values (3 to 64 U/10^6 cells); a progressive increase of PCA was observed during the culture without ATRA up to mean values twofold higher than the basal values (culture day 9, P < .02). In six of the nine patients investigated, the PCA increase in the absence of ATRA was from 2- to 12-fold higher than the basal level after 9 days of culture. However, the remaining patients, who showed no substantial increase during culture (patients 1, 4, and 9), had PCA basal levels 6- to 11-fold higher than the control polymorphonuclear cells (Table 1). In the patients with the highest blast PCA levels (patients 2 and 8), the PCA increase after 9 days of culture without ATRA was 2.1- to 2.5-fold higher than the basal values; in the other cultures with ATRA increase after 9 days of culture without ATRA was with the highest blast PCA levels (patients 2 and 8); in the other patients with relatively lower PCA levels (patients 3 and 5 through 7), the increase was from 3- to 12-fold higher than the basal value (Table 1). In the cultures with ATRA, the PCA was lower than the basal value all through the culture time, with values significantly different at each culture day as compared with the corresponding control without ATRA (Fig 1). A significant downregulation of the blast PCA as compared with the basal value was noticed after 3 days of culture with ATRA (P < .001).

In agreement with these in vitro results, the PCA of the promyelocytic blasts freshly isolated from patient 8 after 8 days of ATRA plus chemotherapy was 3 U/10^6 cells (basal value, 47 U/10^6 cells); at that moment, the bone marrow blast infiltration was still 95%. PT and aPTT were in the normal range, fibrinogen was 106 mg/dL (basal value, 56 mg/dL), and PDFs were no longer detectable. Subsequent bone marrow drawings in the following 2 weeks did not furnish a number of cells sufficient to be assayed; afterwards, the patient achieved complete remission.

In the samples from five patients (patients 1 through 6) tested at diagnosis and throughout the culture time, TF antigen values showed a significant correlation with PCA values (rs = 0.65, n = 35, P < .002), with similar behavior during the culture time. TF antigen increased progressively up to levels twofold higher than the basal levels in the absence of ATRA, whereas in the presence of ATRA, at each culture day, TF levels were lower than the corresponding control (Fig 1).

From a clinical point of view, it is noteworthy that the lowest values of fibrinogen were found in the patients with the highest blast PCA (patients 2 and 8; Table 1); a significant inverse correlation was found between fibrinogen levels (assumed as index of severity of the coagulopathy) and the blast PCA found at day 0 (rs = -0.92, n = 9, P < .002). An inverse correlation (rs = -0.70) was found also between the blast TF antigen basal values and the fibrinogen levels, although it was not significant because of the low number of patients tested (n = 5).

Elastase activity and t-PA antigen concentration of the bone marrow promyelocytic blasts were lower than in the control polymorphonuclear cells at basal condition as well as at each culture day with and without ATRA (Fig 1).

In all patients tested (n = 7), no appreciable amounts of u-PA antigen were detected in bone marrow promyelocytic blasts at basal condition; blasts from three patients (patients 1 through 3) were tested after 9 days of culture with or without ATRA. In the presence of ATRA, u-PA levels after 9 days of culture increased to 0.68 ± 0.41 ng/10^6 cells (mean ± SEM; control without ATRA, 0.18 ± 0.09 ng/10^6 cells).

Characterization of the PCA. The PCA of the blast cell lysate was independent of the intrinsic pathway, because it was found to be substantially unmodified using as substrate either normal human plasma or factor IX-deficient plasma. In the absence of factor X, no PCA was detectable, whereas in the absence of factor VII, the PCA was extremely reduced (Fig 2). These results were independent of the presence or absence of ATRA, as well as of the culture day (data not shown).

Five samples randomly chosen from cultures without
EFFECT OF ATRA ON BLAST CELLS FROM M3 PATIENTS

ATRA (patient 2, days 0 and 9; patient 6, days 3, 6, and 9) were incubated with concanavalin A or iodoacetamide and assayed for PCA. The mean basal PCA of these samples was 30.4 ± 5.3 U/10^6 cells (mean ± SEM); incubation with concanavalin A or iodoacetamide induced a mean decrease of the PCA to 12.8 ± 2.7 U/10^6 cells (−57%) or to 25.6 ± 6.7 U/10^6 cells (−15%).

The effect of aprotinin on the PCA was assayed in three cases (patients 2, 8, and 9): the addition of aprotinin resulted in a marked decrease of the PCA values at both basal condition and each culture day, both in the absence and in the presence of ATRA (Fig 3). The inhibition of the PCA observed in the presence of aprotinin ranged from 14% to 80%, as compared with the values in the absence of aprotinin (mean, 50%; median, 50%; n = 21; Fig 3).

FIG 3. Effect of aprotinin 2,000 U/mL on the PCA (mean ± SEM) of fresh blast cells and after culture in the absence (■) or in the presence (□) of ATRA 1 μmol/L (n = 3 patients). (III), + aprotinin.

DISCUSSION

Coagulopathy occurring in APL has been related to different triggering factors released from malignant cells, such as procoagulant substances, activators of fibrinolysis, and elastases. The presence of a PCA in blast cells from patients with acute nonlymphoid leukemia (ANLL) is well known; such activity is particularly elevated in malignant promyelocytes. Previous studies on intact or lyzed cells characterized PCA as tissue factor; another procoagulant substance has been also demonstrated in extracts from ANLL blast cells and has been characterized as cancer procoagulant, a cysteine proteinase with direct activation of factor X.

A contribution of fibrinolytic mechanisms has been claimed in the pathogenesis of APL coagulopathy, but investigations of the fibrinolytic and elastase activity of APL blasts have yielded conflicting results, sometimes based on reports of isolated cases.

The introduction of ATRA as a differentiating agent in the induction treatment of APL achieved a high rate of complete remission, with a rapid disappearance of the coagulopathy. The efficacy of this therapy is related to differentiation of promyelocytic cells, as demonstrated by studies performed on cells isolated from patients treated with ATRA or cells incubated in vitro with ATRA. Prospective studies in APL patients treated with ATRA showed a very early correction of the rough hemostatic alterations (hypofibrinogeminemia), yet sensitive laboratory indices of hypercoagulability persisted even after 2 weeks of treatment. The mechanisms underlying correction of APL-induced coagulopathy have been extensively investigated in vitro only on cell lines; incubation with ATRA induces in the NB4 cell line a down-regulation of the PCA, either tissue factor-linked or cancer procoagulant-linked, an upregulation of thrombomodulin, and an increase in u-PA, subsequently downregulated by an increased production of plasminogen activator inhibitors.

We studied the effect of ATRA on fresh promyelocytic blasts isolated from nine different patients and cultured for 9 days. A control experiment confirmed in our culture system a differentiating action of ATRA on blast cells, as shown by morphologic examination and by the high expression of CD11b antigen after incubation with ATRA, as previously reported. The persistence of early myeloid antigens such as CD33 is in agreement with the development during ATRA incubation of intermediate cells with both mature and immature characteristics.

A high PCA was demonstrated in fresh blast cells, but with a wide range of results. In our system (cell lysate), PCA was represented mostly by tissue factor, which is factor VII-dependent and depressed after incubation with concanavalin A. Moreover, immunologic techniques using a specific monoclonal antibody confirmed the presence of relevant amounts of tissue factor. Our system assay was not designed to investigate cancer procoagulant, which is usually detected using cell extracts; however, the persistence of detectable clotting times (even though extremely prolonged) induced by cell lysate even in the absence of factor VII and the slight decrease of PCA after incubation with iodoacetamide (an inhibitor of the cysteine proteinases) suggested the presence of minimal amounts of cancer procoagulant also in this system.

We did not find relevant amounts of elastase, t-PA, and u-PA in the blast cells studied before culture, in agreement with Wijermans et al, who reported elastase levels lower than in control polymorphonucleates in blasts from three of four patients with APL investigated. The discrepancy with the results of Bennett et al and Tapiovaara et al, who reported high levels of t-PA and u-PA in APL blasts, could be explained by a higher specificity of our assays using monoclonal antibodies.

In control cultures without ATRA, PCA and TF antigen levels progressively increased, which could be related to a selection of cells with higher expression of procoagulant substance. During cultures with ATRA, PCA and TF antigen levels were significantly reduced. A major decrease in these parameters was observed after 3 days of incubation with ATRA, without any relevant modification during the remaining culture time; however, the amounts of PCA and TF even in the presence of ATRA were constantly higher than in the control polymorphonucleates. This behavior during in
vitro culture with ATRA was confirmed by the evident PCA downregulation observed in the promyelocytic blasts isolated from a single patient after 8 days of treatment with ATRA and chemotherapy.

In cultures with or without ATRA, a slight decrease in elastase and t-PA antigen levels was noticed as compared with the basal values. In the samples we tested, u-PA antigen increased in both culture conditions; the most relevant increase occurred after incubation with ATRA, in agreement with previous observations.24

The data obtained on the marrow blasts suggests that the main mechanism underlying APL coagulopathy is related to a high expression of PCA, with no relevant contribution of primary fibrinolysis due to release of plasminogen activators or elastase. Moreover, the severity of the coagulopathy as roughly indicated by the fibrinogen levels seems related to the amount of PCA present in the patient blasts. Thus, the evidences of excess fibrinolysis reported in plasma of patients with APL24,44 should be regarded as mainly related to a secondary response; a pathogenetic role could be hypothetically played by plasminogen activators released from sources other than blast cells, such as endothelium.

The inhibition of the PCA by aprotinin confirmed previous observations obtained with APL blasts.26 Tissue factor-induced coagulation has been reported to be susceptible to inhibition by aprotinin, yet this action should be considered to be nonspecific, as aprotinin is a known inhibitor of trypsin, plasmin, and kallikrein.27 However, the inhibitory action on the blast PCA could contribute to the efficacy of aprotinin administration in the management of APL coagulopathy in a manner not significantly different from other approaches.28

A specific downregulation of PCA and tissue factor by ATRA was evident, confirming in blast cells from patients with APL the data obtained in investigations with cell lines; the rapid correction of coagulopathy during ATRA treatment seems to be mainly mediated by this effect. However, incubation with ATRA did not induce a complete normalization of the PCA present in the blast cells, because after 9 days of culture, PCA and TF persisted at slightly higher levels than in the control polymorphonucleates. That could be in accordance with the intermediate-cell nature (ie, having both immature and mature markers) of the promyelocytes exposed to ATRA. Moreover, the presence in the differentiated cells of a low pathologic PCA could explain the low-rate hypercoagulability persistent in patients treated with ATRA44 and the occurrence of thrombotic complications observed after ATRA administration.29,30

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Effect of all-trans retinoic acid on procoagulant and fibrinolytic activities of cultured blast cells from patients with acute promyelocytic leukemia

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