Anticoagulant Effects of Retinoic Acids on Leukemia Cells

By Takako Saito, Takatoshi Koyama, Kaoru Nagata, Ryuichi Kamiyama, and Shinsaku Hirosawa

We have recently found that all-trans retinoic acid (ATRA) upregulates thrombomodulin (TM) and downregulates tissue factor (TF) expression in acute myelogenous leukemia (AML) M3 cells (NB4) and acute monoblastic leukemia cells (U937) (Koyama et al., Blood 84:3001, 1994). We have further investigated the effects of ATRA on leukemic cells freshly isolated from patients at diagnosis. Increase of TM antigen was documented in all AML cells: M0 (n = 1), M2 (n = 5), M3 (n = 3), M4 (n = 3), M5 (n = 3), and M6 (n = 1). Decrease of TF antigen was observed in 4 M2, 1 M4, and all M3 and M5 patients. However, no TM and TF antigens were detected in all chronic lymphocytic leukemia cells (n = 3) with or without ATRA treatment. Changes of TM and TF antigen levels were associated with those of TM and TF cofactor levels on the cell surface. A stereoisomer of RA, 9-cis RA, is a high-affinity ligand for the RA receptors (RARs) and the retinoid X receptors, although ATRA and another isomer, 13-cis RA, solely bind to RARs. We have also studied the effects of 9-cis RA and 13-cis RA on the expressions of TM and TF in NB4 and U937 cells. A relatively wide range of 9-cis RA concentrations (0.01 to 1 μmol/L) compared with ATRA was optimal for prolongation of normal plasma-based calcification time (reduction of cell surface TF activity), decrease of TF antigen, and increase of TM antigen on the surface and in the lysates of NB4 and U937 cells. Western blot analysis under nonreducing conditions showed that both ATRA and 9-cis RA markedly induced the prominent band at 75 kD of TM and reduced the band at 46 kD of TF. Northern blot analysis has shown similar changes of mRNA levels, which indicates that RAs regulate TM and TF expression in leukemic cells at transcriptional levels. Anticoagulant effects of ATRA, i.e., upregulation of TM expression and downregulation of TF expression, are applied not only to established cell lines of specific subtypes (M3 and M5) but also to more universal AML (most cases of M3 and M5 and a part of the other types of AML) cells freshly isolated from patients. 9-cis RA may be more effective than ATRA as an inducer of differentiation of AML M3 cells and as an anticoagulant agent for patients with certain types of AML as well.

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Acute Promyelocytic leukemia (APL), acute myelogenous leukemia (AML) M3 according to the French-American-British classification, leads to disseminated intravascular coagulation syndrome (DIC) with considerably high frequency. DIC is often exacerbated by treatment with cytotoxic drugs. Recently, all-trans retinoic acid (ATRA) has been introduced for treatment of APL and has become practically and consistently effective in inducing complete remission. ATRA induces not only terminal differentiation of malignant cells but also rapid amelioration of DIC associated with marked hyperfibrinolysis. We have recently found that ATRA upregulates thrombomodulin (TM) and downregulates tissue factor (TF) expression in APL (NB4) and monoblastic leukemia (U937) cell lines. Furthermore, distinct expressions of TM and TF in AML patients. 9-cis RA may be more effective than ATRA as an inducer of differentiation of AML M3 cells and as an anticoagulant agent for patients with certain types of AML as well.
and reagents were purchased from Wako Pure Chemicals (Osaka, Japan), unless otherwise indicated.

Cell culture. The following human leukemic cell lines were used in this study. NB4 (APL; AML M3) was kindly provided by Dr M. Lanotte (Hôpitaux Saint Louis, Paris, France). NB4 is characterized by the presence of chromosomal translocation t(15;17) with the rearrangements of RARα and promyelocytic leukemia (PML) genes as shown in the most cases with APL and the presence of PML-RARα fusion protein.7-9 A patient from whom NB4 was established suffered from DIC at diagnosis.9 U937 was provided by the Japanese Cancer Research Bank (Tokyo, Japan). ATRA, 9-cis RA, and 13-cis RA (RAs) were dissolved in absolute ethanol to a concentration of 8.32 mmol/L and further diluted into growth medium at the desired concentrations so that the final ethanol concentration in the culture media was less than 0.1%. The control leukemia cells (those incubated without RAs) were exposed to an appropriate amount of ethanol in the culture medium. Whether primary leukemia cells were analyzed immediately after isolation or after 24 hours of culture, no significant differences of TM and TF expressions were generally observed. All the procedures involving RAs were performed in subdued lights.

Leukemic cells freshly isolated from patients. Peripheral blood was drawn from 16 patients with AML (M0; M2-1, -2, -3, -4, and -5; M3-1, -2, and -3; M4-1, -2, and -3; M5a; M5b-1 and -2; and M6), 2 patients with acute lymphoblastic leukemia (ALL; ALL-1 and -2) and 3 patients with chronic lymphocytic leukemia (CLL; CLL-1, -2, and -3). Peripheral blood samples were obtained at diagnosis before the start of chemotherapy and the mononuclear fraction was isolated by sedimentation on Ficoll-Hypaque density gradients. Blood samples were selected for study only when they were shown to contain more than 85% blasts. Mononuclear cells were then washed three times in phosphate-buffered saline (PBS) and suspended in RPMI 1640 medium (Nissui Pharmaceutical Co, Tokyo, Japan) supplemented with 10% fetal calf serum.

Measurement of TM and TF antigens. Leukemic cells were incubated with agents for the times indicated and washed with PBS three times. The cell numbers were counted and adjusted. Cells were then extracted for 30 minutes with 0.5% Triton X-100 in PBS at 4°C. Cell debris was removed by centrifugation at 12,000g for 20 minutes. Cell lysates were stored at −80°C until the assay. Total TM antigen in cell lysates was measured by enzyme-linked immunosorbent assay (ELISA) using an antihuman TM monoclonal antibody U-3 and a polyclonal antihuman TM antibody as reported before.8 Total TF antigen in cell lysates was also measured by ELISA using antihuman TF monoclonal antibodies 6B4 and 5G9 as already reported.9

Cell surface TM cofactor activity. TM cofactor activity on cell surface was measured as described previously.8 Surface TM cofactor activity was determined by the hydrolysis of chromogenic substrate.
Fig 2. Western blot analysis of patients’ leukemic cell preparations with anti-TM and anti-TF antibodies. Leukemic cells (10⁶) were lysed in detergent and the preparations were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) under nonreducing conditions. After transfer to Immobilon (Millipore Corp, Bedford, MA), immunoblotting was accomplished with a monoclonal anti-TM antibody KA-3 (A) and a monoclonal anti-TF antibody 5G9 (B). Each set of leukemic cells was treated with 1 μmol/L ATRA for 24 hours. The prominent band at approximately 75 kD represents TM (A). As a control, soluble recombinant TM was used (A, lane 11). A monoclonal anti-TF antibody 5G9 identified the nonreduced form of TF as a band at approximately 45 kD (B). As a control, placenta TF was used (B, lane 3).

S-2266. The results were expressed as ΔmOD₄₀₅/min/minute or as a percentage of the initial velocity of activated protein C formation (100% for the formation with the cell surface TM under basal conditions). Controls of the cells in the absence of thrombin and protein C were treated similarly and no activation of protein C was observed.

Cell surface TF cofactor activity: analysis of procoagulant activity in clotting assay. Leukemic cell suspensions were adjusted to 1 x 10⁷/mL in PBS. Cells (1 x 10⁶) were added to 0.1 mL of pooled human normal plasma. After incubation at 37°C for 3 minutes, 0.1 mL of 25 mmol/L calcium chloride was added and plasma recalcification time was determined with a semiautomatic coagulometer CA-100 (Sysmex, Kobe, Japan). Because our previous report has already shown that the procoagulant activities on the surface of NB4 and U937 cells determined as described above are deduced from TF expression, prolongation of recalcification time is mainly due to downregulation of TF expression by RAs. TF cofactor activity was quantitated by reference to standard curves (log-log plot) constructed with human placenta TF and TF activity, that yielded a 50-second recalcification time was defined as 1 U/mL.

Analysis of surface TM and TF antigens by flow cytometric analysis. After incubation for 24 hours with or without 1 μmol/L ATRA, 9-cis RA, and 13-cis RA, NB4 cells and U937 cells were washed with PBS and 10⁶ cells were resuspended in 1 mL PBS. The suspensions were incubated with an inhibitory monoclonal anti-TF antibody 6B4 or an anti-TM antibody KA-4 at a concentration of 10 μg/mL for 30 minutes at 4°C, and standard indirect immunofluorescence
techniques were used for the analysis of surface TM and TF antigens.

**Immunoblotting analysis.** Western blot analyses of TM and TF in cell lysates were performed essentially as described previously. KA-3 and horseradish peroxidase-conjugated rabbit antimouse IgG antibody were used for the detection of TM. Horseradish peroxidase-conjugated anti-TF monoclonal antibody 5G9 was used for the detection of TF. The same amount of lysates from 10⁶ cells was applied to each lane.

**Northern blotting.** Total cytoplasmic RNA was prepared from NB4 and U937 cells after treatment with ATRA, 9-cis RA, or 13-cis RA for 5 hours by the acid guanidium thiocyanate-phenol-chloroform method. Isolated RNA was analyzed in Northern blotting using Digoxigenin Luminescent Detection Kit (Boehringer Mannheim, Mannheim, Germany). A Thl111 I-Nhe I fragment in the coding region of the human TM gene and a TF cDNA probe were kind gifts from Dr K. Nawa and Dr K. Wakita, respectively (Molecular Biology Research Laboratory, Daiichi Pharmaceutical Co, Tokyo, Japan), and were used as hybridization probes. The TF cDNA probe was prepared by a polymerase chain reaction (PCR) method from a cDNA library of tumor necrosis factor α-stimulated human umbilical vein endothelial cells.

**RESULTS**

**Effects of ATRA on TM and TF antigens in leukemic cells freshly isolated from patients.** When untreated, TM and TF antigen levels in freshly isolated leukemic cells were varied in the range of 0 to 865 ng TM protein and 0 to 9.24 ng TF protein per 10⁶ cells. After incubation with ATRA, total TM antigen was increased in all 16 AML cells (Fig 1A). In contrast, ATRA decreased TF antigen levels in 11 of 16 AML cells (M2-1, -2, -4, and -5; M3-1, -2, and -3; M4-3; M5a; and M5b-1 and -2; Fig 1B). Both TM and TF expression in all CLL cells was not detected before and after ATRA treatment. In 2 patients with ALL, TM expression in the leukemic cells was not detected before and after treatment, whereas TF expression was documented in 1 patient. The cases in which DIC was documented or suspected are marked with an asterisk in Fig 1B.

**Western blot analysis for TM and TF in leukemic cells from patients.** The presence of TM and TF molecules in the patients' leukemic cells was determined by immunoblotting with the antibodies specific for human TM and TF. The monoclonal antibody KA-3 identified the nonreduced form of TM as a prominent band at approximately 75 kD. After treatment of the leukemic cells with ATRA, TM markedly increased in all AML cells (M3-1, M4-3, and all M5 [M5a and M5b-1 and -2] cells) examined (Fig 2A, lanes 2, 4, 6, 8, and 10). The monoclonal antibody against human TF 5G9 identified the nonreduced form of TF in M3-1 cells as a band at approximately 45 kD (Fig 2B, lane 1). TF of the leukemic cells was markedly decreased after treatment with ATRA (Fig 2B, lane 2).

**Cell surface TM and TF activities of patients' leukemic cells modulated by ATRA.** Effects of ATRA on TM and TF activities on the surface of leukemic cell samples freshly obtained from several patients are shown in Fig 3. After treatment with ATRA, TM activities were increased in all 16 AML cells (Fig 4. Effects of RAs on total TM and TF antigens in NB4 cell lysates and cell surface TF activity of NB4 and U937 cells. Concentration dependency of 9-cis RA on the TM (A) and TF (B) antigen expression in NB4 cell lysates and cell surface TF activity of NB4 cells were determined. NB4 cells were incubated with various concentrations of 9-cis RA (0.0001 to 5 μmol/L) for 24 hours. Furthermore, the effects of ATRA, 9-cis RA, and 13-cis RA on cell surface TF activity were compared (D). NB4 and U937 cells were incubated with 1 μmol/L RAs (ATRA, 9-cis RA, and 13-cis RA) for 24 hours.

**Fig 4.**
ANTICOAGULANT EFFECTS OF RETINOIC ACID

Fig 5. Effects of RA on total TM and TF antigens in NB4 and U937 cells. NB4 (A and C) and U937 (B and D) cells were incubated with 1 μmol/L ATRA (lane 2), 9-cis RA (lane 3), and 13-cis RA (lane 4) for 24 hours. Lane 1 represents the control. Total TM (A and B) and TF (C and D) antigen levels are shown on the ordinates.

Effects of ATRA, 9-cis RA, and 13-cis RA on TM antigen in NB4 and U937 cells. In NB4 cells, ATRA, 9-cis RA, and 13-cis RA at a concentration of 1 μmol/L increased TM antigen to similar levels (Fig 5A). The TM antigen levels in U937 cells were increased by treatment with ATRA, 9-cis RA, and 13-cis RA. 9-cis RA was most effective for increase of the TM antigen level (Fig 5B).

Effects of ATRA, 9-cis RA, and 13-cis RA on TF antigen in NB4 and U937 cells. ATRA, 9-cis RA, and 13-cis RA (1 μmol/L) reduced TF antigen levels in NB4 and U937 cells (Fig 5C and D). In both NB4 and U937 cells, 9-cis RA was slightly more potent than ATRA at 1 μmol/L, the concentration at which ATRA decreases expression of TF most efficiently.

FCM analysis of TM and TF antigens on the cell surface of NB4 and U937 modulated by RAs. We found 40.8% of the NB4 cells positive for the anti-TM antibody KA-4 by FCM analysis (Fig 6A). The percentage of positive cells for KA-4 was increased to 94.1%, 92.8%, and 94.1% by treatment with ATRA, 9-cis RA, and 13-cis RA, respectively (Fig 6B, C, and D). We found 66.4% of the NB4 cells to
be positive for the anti-TF antibody 6B4 (Fig 6E), whereas the percentage of positive cells decreased to 9.5%, 9.0%, and 7.4% when treated with ATRA, 9-cis RA, and 13-cis RA, respectively (Fig 6F, G, and H). We also found 79.1% of the untreated U937 cells to be positive for KA-4. When treated with ATRA, 9-cis RA, and 13-cis RA, 99.2%, 99.5%, and 98.7% of U937 cells were positive for KA-4, respectively. Of the untreated U937 cells, 18.0% were positive for 6B4, whereas the percentage of the positive cells decreased to 3.1%, 1.1%, and 3.7% when treated with ATRA, 9-cis RA, and 13-cis RA, respectively.

Enhancement of surface TM activity in NB4 and U937 cells by 9-cis RA. Surface TM activity induced by 9-cis RA was determined by the acceleration of thrombin-catalyzed protein C activation. 9-cis RA increased the rate of protein C activation to about threefold in NB4 cells and to about 2.5-fold in U937 cells (Fig 7A and B). Basal $\Delta$OD$_{405 nm}$/min levels were 0.036 ± 0.003/min/5 × 10$^6$ NB4 cells (34 ± 2 ng activated protein C/10$^6$ cells) and 0.108 ± 0.004/min/5 × 10$^6$ U937 cells (87.6 ± 3 ng activated protein C/10$^6$ cells).

Western blot for TM: effects of RAs. After treatment of NB4 and U937 cells with 9-cis RA and 13-cis RA, a prominent band of TM at 75 kD was markedly increased to the level as high as when the cells were treated with ATRA (data not shown).

Northern blot for TM and TF: effects of RAs. Increased expression of specific mRNA for TM was detected as a single band at 3.7 kb when NB4 and U937 cells were treated with RAs (Fig 8A, lanes 2, 3, and 4). Expression of specific mRNA for TF found as a single band at 2.3 kb was markedly decreased when NB4 cells were treated with RAs (Fig 8B, lanes 2, 3, and 4). In leukemic cells freshly isolated from patients, changes of TM and TF mRNAs by ATRA were similar to those of NB4 and U937 cells (data not shown).

DISCUSSION

In the present study, we have shown that anticoagulant effects of RAs are inducible not only to specific subtypes (M3 and M5) of the established cell lines but also to certain AML subtypes (most cases of M3 and M5 and a part of the other types of AML) of the leukemic cells freshly isolated from patients.

Constitutive expression levels of TM and TF were varied in untreated leukemic cells that were freshly isolated. Although a rough correlation between relatively high TF antigen expression and the development of DIC seems to be present, as shown in Fig 1, anticoagulant glycoprotein TM is also expressed variably in leukemic cells. Thus, development of DIC can be estimated by evaluating overall procoagulant and anticoagulant activities. Although overall procoag-
Fig 7. Changes in TM cofactor activity for protein C activation on NB4 and U937 cell surfaces after exposure to 9-cis RA (1 μmol/L). Surface TM activity was determined for 5 × 10⁶ suspended cells as described in the Materials and Methods. Results were expressed as the percentage of initial velocity of activated protein C (lane 2) compared with the control (lane 1) that was not treated with the reagent.

Fig 8. Northern blot analysis: effects of RAs. Twenty micrograms of total RNA was extracted from cultured NB4 and U937 cells after exposure to 1 μmol/L ATRA (lane 2), 9-cis RA (lane 3), and 13-cis RA (lane 4) for 5 hours and electrophoresed on a 1% agarose/formaldehyde gel. The blot was probed with a digoxigenin-labeled cDNA fragment specifically encoding TM (A) or TF (B). Lane 1 represents untreated control. RNA molecular weight markers are given along the left margin.
TM expression and downregulation of TF expression in NB4 and U937 cell lines by stereoisomer RA. 9-cis RA was slightly more effective than other types of RAs at 1 μmol/L, the concentration at which ATRA has its most optimal effect. In the experiment of RAs for the effects on total TM and TF antigens in the cell lysates and procoagulant activities on the surface of NB4 cells, 9-cis RA was especially more effective in a relatively wide range of concentrations (0.01 to 1 μmol/L) than was ATRA. Such concentrations are easily attainable in patients treated with 9-cis RA. In human umbilical vein endothelial cells, NB4 cells, and U937 cells, ATRA increases transcription of TM gene without changing TM mRNA stability, whereas cAMP increases TM mRNA stability (our unpublished observations). RAs thus regulate TM expression in leukemia cells at transcriptional level, but it is not yet known whether RAs change TF mRNA stability. Because ATRA upregulates TM expression in all primary AML cells, TM inducibility may not depend on cell differentiation by ATRA. The TM antigen level induced by ATRA in NB4 cells reaches a maximum level within a few days. Furthermore, a recent report has shown that TM induction in AML M2 cell line HL60 cells cultured with ATRA reflecting TM biosynthesis levels is independent of HL60 differentiation into neutrophilic cells. However, according to a recent report using RA-resistant NB4 cells, NB4 cells’ sensitivity to maturation by ATRA may be crucial to the loss of their procoagulant activity, including TF and C5b-9. TM mRNA stability, whereas cyclic AMP increases TM mRNA stability (our unpublished observations). RAs thus regulate TM expression in leukemia cells at transcriptional level, but it is not yet known whether RAs change TF mRNA stability. Because ATRA upregulates TM expression in all primary AML cells, TM inducibility may not depend on cell differentiation by ATRA. The TM antigen level induced by ATRA in NB4 cells reaches a maximum level within a few days. Furthermore, a recent report has shown that TM induction in AML M2 cell line HL60 cells cultured with ATRA reflecting TM biosynthesis levels is independent of HL60 differentiation into neutrophilic cells. However, according to a recent report using RA-resistant NB4 cells, NB4 cells’ sensitivity to maturation by ATRA may be crucial to the loss of their procoagulant activity, including TF and cancer procoagulant activities. More detailed analysis of cell differentiation and TM/TF expressions may be necessary.

Retinoids derived from retinol (vitamin A) are critical regulators of vision, cell proliferation, differentiation, and embryonal morphogenesis. Intracellular isomerases further convert some intermediates to 9-cis, 11-cis, and 13-cis RAs. 13-cis RA and 9-cis RA are naturally occurring ligands of the nuclear RARs. The regulation of RA-target genes is caused by the mediation of the nuclear receptors that are capable of binding to RA-responsive elements (RAREs) of a direct repetition of two 5'-PuGG(G/T)TCACA-3'. High-affinity binding of RARs to RAREs takes place only if a separate retinoid receptor family, RXRs, is present. ATRA binds to RARs with high affinity, but ATRA and 13-cis RA bind to RXRs with very low affinity. On the other hand, 9-cis RA has high binding affinity to both RARs and RXRs. This may be one of the reasons why 9-cis RA is more effective than ATRA and 13-cis RA in the regulation of TM and TF in the leukemic cells.

9-cis RA may be slightly more effective than ATRA not only as an inducer of differentiation of AML M3 cells but also as an anticoagulant agent for treatment of patients with certain types of AML. Identification of specific pathways responsible for the anticoagulant effects of RAs, including ATRA and 9-cis RA, are intriguing and indispensable subjects for further investigation.

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