Anticoagulant Effects of Retinoic Acids on Leukemia Cells

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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 cell lines, and have examined the regulation of TM and tissue factor (TF) expression in acute myelogenous leukemia patients' leukemic cells and have compared the effects of 9-cis RA for treatment of APL with those of ATRA and 13-cis RA on leukemic cell lines, NB4 and U937. 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 recalcification 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, ie, 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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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 (Hôpital 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 most cases with APL and the presence of PML-RARα fusion protein.7,8 A patient from whom NB4 was established suffered from DIC at diagnosis.9,10 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.9 Total TF antigen in cell lysates was also measured by ELISA using antihuman TF monoclonal antibodies 6B4 and 5G9 as already reported.11

**Cell surface TM cofactor activity.** TM cofactor activity on cell surface was measured as described previously.7 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 (10⁸) 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).

Fig 3. Cell surface TM and TF activities of patients' leukemic cells modulated by ATRA. Leukemic cell samples freshly obtained from several patients were incubated with 1 μmol/L ATRA for 24 hours. Cell surface TM (A) and TF (B) activities were determined for 10⁶ suspended cells as described in the Materials and Methods.
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. \(^1\) 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 \(1 \times 10^6\) 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 Tth111 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 alpha-stimulated human umbilical vein endothelial cells.\(^13\)

**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 \(1 \times 10^7\) 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.

**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 on the
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.

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 9-cis RA on total TM and TF antigens in NB4 cells lysates (dose-dependency). The effects of a stereoisomer, 9-cis RA, on the expression of TM and TF in NB4 cells were examined. 9-cis RA increased TM antigen level while decreasing the TF antigen level dose-dependently. A relatively wide range of 9-cis RA concentrations, ie, 0.01 to 1 μmol/L, which is an easily attainable level in patients treated with 9-cis RA, induced an efficient decrease of TF antigen and an increase of TM antigen (Fig 4A and B).

Cell surface TF activity of NB4 cells modulated by 9-cis RA and ATRA. Effects of 9-cis RA and ATRA on TF activity on the surface of NB4 cells were examined. As shown in Fig 4C, a relatively wide range of 9-cis RA concentrations (0.01 to 1 μmol/L) was optimal for reduction of TF activity when compared with ATRA.

Cell surface TF activity of NB4 and U937 cells modulated by RAs. When NB4 and U937 cells were pretreated with 1 μmol/L of ATRA, 9-cis RA, or 13-cis RA for 24 hours, the TF activities were reduced by all RAs (Fig 4D). 9-cis RA is slightly more effective than ATRA and 13-cis RA.

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. In NB4 cells, ATRA, 9-cis RA, and 13-cis RA at a concentration of 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 cells 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
Control all-trans Qcis 134s

Fig 6. Flow cytometric analysis of surface TM and TF antigens in NB4 and U937 cells modulated by RAs. NB4 and U937 cells were untreated (A and E) or treated with 1 μmol/L ATRA (B and F), 9-cis RA (C and G), and 13-cis RA (D and H). The flow cytometric analyses of both untreated and treated cells incubated with KA-4 (anti-TM; A, B, C, and D) or 6B4 (anti-TF; E, F, G, and H) monoclonal antibody followed by goat FITC-antimouse IgG were shown. The abscissa shows fluorescence intensities and the ordinate shows the number of cells.

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 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 ΔOD405/min levels were 0.036 ± 0.003/min/5 × 10⁶ NB4 cells (34 ± 2 ng activated protein C/10⁶ cells) and 0.108 ± 0.004/min/5 × 10⁶ U937 cells (87.6 ± 3 ng activated protein C/10⁶ 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-
ululant activity in leukemic cells may be controlled mainly by levels of TF and TM expressed, expressions of the other procoagulant and anticoagulant proteins should be considered to be involved in DIC. The presence of a unique procoagulant with cysteine proteinase activity distinct from TF in leukemic cells including NB4 cells has been reported.

Differentiation therapy with RA induces complete remission in APL patients, with a rapid correction of the bleeding tendency. ATRA therapy exerts rapid improvement in coagulopathy and hyperfibrinolysis, with normalization of plasma fibrinogen/fibrin degradation product (fragment E), cross-linked fibrin degradation product (D-dimer fragment), thrombin-antithrombin III complexes, and plasmin-α2-plasmin inhibitor complex. Several possible explanations for marked reduction of hemorrhagic complications by ATRA are proposed. (1) The differentiation process induced by ATRA does not exacerbate the release of procoagulant and fibrinolytic substances from the leukemic cell, as cytotoxic drugs do. (2) Rapid amelioration of DIC is induced by ATRA through downregulation of TF and cancer procoagulant expressions in APL cells and upregulation of TM expression in APL and endothelial cells. (3) Rapid reduction of elevated fibrinolytic and proteolytic activities in APL cells is exerted by ATRA.

Upregulation of TM antigen and activity expression by ATRA was shown in all primary leukemic cells from AML patients studied, although the levels of induction were different in each leukemic cells. ATRA decreased the level of TF antigen expression in 11 of 16 AML cells. Cell surface TF activities measured by recalcification time were reduced in 3 of 4 AML cells. These data suggest that upregulation of TM and downregulation of TF, as shown in NB4 and U937 cells, are major factors in the improvement of DIC. In this context, the past and the future clinical trials of RAs for AML and other malignant cells to induce differentiation should be evaluated from the coagulation and fibrinolysis points of view. However, in ALL and CLL cells, no TM expression was documented in the absence and presence of ATRA. In CLL cells, TF expression was not documented either. In 1 of 2 patients with ALL, TF expression was documented, whereas downregulation of its expression by ATRA was not shown. These observations suggest that differentiation into mature lymphocytic cells implies the loss of TM and TF inducibility but that premature lymphoblastic cells may express TF.

TM may possess not only natural anticoagulant properties but also antifibrinolytic properties by acceleration of thrombin inactivation of single-chain urokinase-type plasminogen activator (u-PA). Upregulation of TM may also reduce hyperfibrinolysis induced by u-PA in patients with APL, which leads to severe hemorrhagic complications. Antifibrinolytic activity of TM should be further evaluated.

In this investigation, we have also shown upregulation of
TM expression and downregulation of TF expression in NB4 and U937 cell lines by steressoris RAs. 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 (0.61 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 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 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 TM expression and downregulation of TF expression in NB4 cells: Distinct expression of thrombomodulin and tissue factor in acute promyelocytic leukemia cells Blood 84:3001, 1994

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