All-trans Retinoic Acid Upregulates Thrombomodulin and Downregulates Tissue-Factor Expression in Acute Promyelocytic Leukemia Cells: Distinct Expression of Thrombomodulin and Tissue Factor in Human Leukemic Cells

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The expressions of thrombomodulin (TM) and tissue factor (TF) by all-trans retinoic acid (ATRA) were studied in human leukemic cell lines including NB4 (acute promyelocytic leukemia) and U937 (monoblastic leukemia). ATRA remarkably upregulated TM antigen expression in cell lysates as well as TM cofactor activity on the cell surfaces of NB4. The level of TM mRNA in NB4 cells was increased by ATRA. Inherently procoagulant NB4 cells contained markedly higher content of TF, which was efficiently reduced by ATRA. Modest increase of TM and decrease of TF were observed when NB4 cells were treated with dibutyryl cyclic adenosine monophosphate (dbcAMP). On the other hand, both ATRA and dbcAMP showed dramatic increase of TM antigen level and modest decrease of TF antigen in U937 cells. These results suggest that ATRA regulates expressions of TM and TF antigens and activity in NB4 and U937 cell lines, and provide evidence for a potential efficiency of ATRA as a preventive and therapeutic agent for disseminated intravascular coagulation in promyelocytic and monoblastic leukemia.

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man TM IgGs, KA-3 and KA-4, were obtained by established hybridoma techniques as previously reported.\textsuperscript{13} KA-3 and KA-4 recognize different epitopes of TM. KA-3 binds to N-terminal part of the epidermal growth factor homology domain, and KA-4 recognizes the \textquoteleft scTissue/thrombin-rich\textquoteright polypeptide core near the primary thrombin binding site of TM molecule.\textsuperscript{14} Recombinant TF standard\textsuperscript{5} and monoclonal mouse antithrombin TF IgGs, 6B4, and 5G9,\textsuperscript{16} were provided by Corvas International Inc (San Diego, CA). Polyclonal rabbit antiserum against human TF was purchased from American Diagnostica Inc (Greenwich, CT). All other chemicals were reagent-grade products, and reagents were purchased from Wako Pure Chemicals (Osaka, Japan), unless otherwise indicated. [\textsuperscript{32}P]DCTP (6,000 Ci/mmol, 1 Ci = 37 GBq) was purchased from Amersham (Arlington Heights, IL).

Cell culture. The following human leukemic cell lines were used in this study: NB4; APL, AML (M3),\textsuperscript{17} HL60; AML (M2),\textsuperscript{18} NB4; monoblastic leukemia,\textsuperscript{19} K562; erythroleukemia,\textsuperscript{20} UT7; acute megakaryoblastic leukemia. AML (M7),\textsuperscript{21} TMD2; B-lymphocytic leukemia;\textsuperscript{22} NB4 was kindly provided by Dr M. Lanotte, Hôpital Saint Louis, Paris, France. NB4 is characterized by the presence of 15; 17 chromosomal translocation t(15;17) and the expression of PML-RARa fusion protein. UT7 was a gift from Dr N. Komatsu, Jichi Medical School, Tochigi, Japan. TMD2 was established in our department. The other cell lines were provided by the Japanese Cancer Research Bank, Tokyo, Japan. Each cell line was cultured as already described. Only a patient from whom NB4 was established suffered from DIC at diagnosis.\textsuperscript{17} Cell counts and viability were determined in a duplicate by the trypan blue exclusion method. All treatments described below affected neither cell viability nor cell number as monitored by phase-contrast microscopy. ATRA was 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%. Ethanol of 0.1% level has no effect on cell growth of each cell line as well as on differentiation of NB4\textsuperscript{17} and HL60 cells. For the untreated control the same amount of ethanol was added in the culture medium. All the procedures involving ATRA were performed in subdued light, and the tubes and culture plates containing ATRA were covered with aluminum foil. All antigens and activity assays described below were repeated independently more than three times. The results were expressed as means \pm standard deviation (SD).

Measurement of TM antigen in cell lysates and cell surface TM cofactor activity. Each leukemic cell was incubated with agents for the time indicated, and washed with phosphate-buffered saline (PBS) three times, and cell numbers were counted and adjusted. Cells were then extracted for 30 minutes with 250 \muL of 0.5% Triton X-100 in PBS. Cell debris was removed by centrifugation at 12,000 g for 20 minutes. Cell lysates were stored at \textminus80°C until the assay. Total TM antigen in cell lysates and soluble TM antigen in the conditioned media were measured by enzyme-linked immunosorbent assay (ELISA) using anti-human TM monoclonal antibodies, KA-3 and KA-4, as reported before.\textsuperscript{21} TM cofactor activity on cell surfaces was also measured as described previously.\textsuperscript{14,22} Surface TM cofactor activity was expressed as percent 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.

Measurement of TF antigen in cell lysates and cell surface TF activity. Total TF antigen in cell lysates and soluble TF antigen in the conditioned media were measured by ELISA method using antithrombin TF monoclonal antibodies, 6B4 and 5G9, as already reported.\textsuperscript{15,23} Measurement of TF activity was based on the procedure of Hische et al.\textsuperscript{25,26} After incubation, cells were washed with PBS three times. TF activity was determined using suspended cells (10\textsuperscript{6} cells/tube). Cells were suspended in 0.2 mL TBS, pH 7.3, containing 7.5 mmol/L CaCl\textsubscript{2} and incubated for 10 minutes at 37°C with PPSB (final factor IX activity was 2.5 times larger than in plasma). At intervals of 10 minutes, the incubation mixture and 0.7 mmol/L TRIS buffer, pH 8.3, containing 2.1 mmol/L (final concentration) EDTA were mixed. Factor Xa activity developed in the mixture was determined spectrophotometrically by adding 0.5 mmol/L (final concentration) S2222 for 3 minutes. The absorbance at 405 nm was recorded. Surface TF activity was expressed as percentage of the control basal level. Controls without PPSB in the presence of cells were treated similarly and no activation of factor X was observed.

Analysis of procoagulant activity in clotting assay. Leukemic cell suspensions were adjusted to 1 x 10\textsuperscript{7}/mL in PBS. 0.1 mL (1 x 10\textsuperscript{7} cells) was added to 0.1 mL of pooled human normal plasma. Following incubation at 37°C for 2 minutes, 0.1 mL of 25 mmol/L calcium chloride was added and plasma recalcification time was determined with a semi-automatic coagulometer CA-100 (Sysmex, Kobe, Japan). When procoagulant activity was markedly intense, cell suspensions were further diluted with PBS.

Neutralization test. One-half milliliter of each leukemic cell suspension (2 x 10\textsuperscript{7}/mL) and the same volume of the polyclonal anti-TF serum were mixed and incubated at 25°C for 1 hour. The clotting time of each mixture was measured by the recalcification method as described above. Each cell suspension was mixed and incubated with an inhibitory monoclonal anti-TF antibody 6B4 or an anti-TM antibody KA-4 at a concentration of 500 mmol/L. Control assays were performed using irrelevant polyclonal antiserum and a monoclonal antibody of the same isotype.

Analysis of surface TM and TF antigens by flow cytometric analysis. After incubation for 24 hours with or without 1 \mumol/L ATRA, NB4 cells were washed with PBS and 10\textsuperscript{6} cells were suspended in 1 mL PBS. The suspensions were incubated with preimmune mouse IgGs, anti-TM antibodies of KA-3 and KA-4 or an anti-TF antibody of 6B4, at a concentration of 10 \mug/mL, 500 mmol/L calcium chloride was added and incubated for 3 minutes at 37°C and standard indirect immunofluorescence techniques were used.\textsuperscript{21}

Immunoblotting analysis. Western blot analysis of TM and TF in cell lysates were performed essentially as described previously.\textsuperscript{27} KA-3 and horseradish peroxidase-conjugated rabbit-antimouse IgG antibody were used for the detection of TM. Polyclonal rabbit antibody against TF and horseradish peroxidase-conjugated goat-antirabbit IgG antibody were used for the detection of TF. The same amount of lysate from 10\textsuperscript{6} cells was applied in each lane.

Northern blotting. Total cytoplasmic RNA was prepared from NB4 cells after treatment with ATRA, dbcAMP, or IL-1\alpha for 5 hours by acid guanidium thiocyanate-phenol-chloroform method. Isolated RNA was analyzed in Northern blotting by standard techniques.\textsuperscript{20} A 5' fragment in the coding region of human TM\textsuperscript{27} was kindly provided by Dr K. Nawa, the Molecular Biology Research Laboratory, Daiichi Pharmaceutical Co (Tokyo, Japan) and used as a hybridization probe. The probe was random-primer labeled to a specific radioactivity of >10\textsuperscript{6} dpm/\mug. A \textsuperscript{32}P-labeled cDNA probe for human \beta-actin (Nippon Gene, Tokyo, Japan) was hybridized to estimate the amount of blotted RNAs to be equal.

RESULTS

Effects of ATRA on total TM and TF antigens in NB4 cell lysates (dose- and time-dependency). NB4 cells were incubated with ATRA as indicated in Figs 1 and 2. ATRA increased TM antigen level while decreasing TF antigen level dose-dependently (up to 0.1 \mumol/L) and time-dependently (up to 24 hours). The optimal ATRA concentration for enhancement of TM expression and reduction of TF expression was 0.1 to 1.0 \mumol/L (Figs 1A and 2A). There-
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Fig 1. Effects of ATRA on total TM antigen in NB4 cell lysates. (A) Concentration dependency of ATRA on the TM expression. NB4 cells (10⁶ cells/mL) were incubated with various concentrations of ATRA (0.01 to 5 μmol/L) for 24 hours. (B) Time dependency of ATRA on the TM expression. NB4 cells were treated with 1 μmol/L ATRA for indicated times (0 to 48 hours).

Therefore, cells were incubated with 1.0 μmol/L ATRA for 24 hours otherwise indicated. All NB4 cells still kept morphologically promyelocytic appearance even after treatment with ATRA for 24 hours.

Effects of ATRA, dbcAMP, and IL-1α on total TM and TF antigens in NB4 cell lysates and the conditioned media. Untreated leukemic cells, NB4, U937 and UT7, contained comparable TM protein per 10⁷ cells as shown in Fig 3 (closed columns). In contrast, NB4 cells contained markedly higher content of TF protein (7.4 ± 1.9 ng/10⁷ cells, n = 3) than the other cells (Fig 3, striped columns). Previous studies showed that dbcAMP (5 mmol/L) upregulated TM in HUVECs⁶ and UT7, and that IL-1 downregulated TM in HUVECs.³⁰ So we compared effects of dbcAMP and IL-1α on TM and TF expression with those of ATRA. dbcAMP induced only a modest increase of TM antigen in NB4 cells (Fig 4A). In contrast, in U937 cells ATRA and dbcAMP markedly increased TM antigen levels (Fig 4B). In UT7 cells dbcAMP induced a marked increase of TM as reported before, while ATRA increased TM only slightly (data not shown). ATRA and dbcAMP did not induce detectable TM antigen expression in HL60, K562, and TMD2 cells within 24 hours. Incubation with IL-1α (0.6 μmol/L) for 24 hours did not significantly affect TM antigen levels in NB4 and

Fig 2. Effects of ATRA on total TF antigen in NB4 cell lysates. (A) Concentration dependency of ATRA on the TF expression. NB4 cells (10⁶ cells/mL) were incubated with various concentrations of ATRA (0.01 to 5 μmol/L) for 24 hours. (B) Time dependency of ATRA on the TF expression. NB4 cells were treated with 1 μmol/L ATRA for indicated times (0 to 48 hours).
U937 cells, although incubation for 5 hours reduced TM antigen levels by about 30%. While in NB4 cells ATRA decreased TF expression, dbcAMP did not significantly affect TF antigen levels (Fig 5A). In contrast, ATRA and dbcAMP decreased TF antigen levels in U937 cells (Fig 5B). Incubation with IL-1α for 24 hours did not significantly affect TF antigen levels in NB4 and U937 cells (Fig 5), although incubation for 5 hours increased TF antigen levels by about 30%. The change in soluble TM and TF antigen levels in the conditioned media was not detected at any experiments in this study.

Enhancement of surface TM activity and reduction of surface TF activity in NB4 cells by ATRA. Surface TM activity was determined by the acceleration of thrombin-catalyzed protein C activation. Surface TF activity was measured by the generation of factor Xa. ATRA increased the rate of protein C activation to about threefold, while suppressing surface TF activity to 37 ± 13% of basal levels (basal level; ΔOD_{405nm}/min/10^6 cells = 0.32 ± 0.043/min/10^6 cells) (Fig 6). dbcAMP increased surface TM activity to about 150% of basal levels (basal level; ΔOD_{405nm}/min/10^6 cells = 0.017 ± 0.0017/min/10^6 cells) while suppressing TF activity only modestly. IL-1α did not significantly affect surface TM activity but suppressed TF activity only slightly by incubation for 24 hours.

Procoagulant activity of NB4 cells and neutralization test. Viable NB4 cells dramatically reduced the recalcification time as compared with other leukemic cells (K562 and TMD2) (Fig 7A). U937 cells was also procoagulant but the intensity was less than NB4 cells. Those procoagulant activities were neutralized by preincubation of those cells with an anti-TF polyclonal antibody and a monoclonal antibody 6B4 (Fig 7B), which confirms that cell-surface TF contributes to the procoagulant status of those cells. When NB4 cells were
pretreated with ATRA for 24 hours, the procoagulant activity was reduced and preincubation of cells with an anti-TM monoclonal antibody KA-4 reduced the protecting effect, which means cell-surface TM is effective. However, anticoagulant effect of TM might not be expressed in this assay system, since the system was based on cell surface TF-dependent clotting time.

**Monitoring of surface TM and TF antigen expressions in NB4 cells by flow cytometric analysis.** NB4 cells were incubated with ATRA (1 μmol/L) for 24 hours and the expression of cell surface TM and TF antigens were examined directly by flow cytometry using KA-3, KA-4, and 6B4. When untreated, 64.2% (Fig 8A) and 45.2% of NB4 cells were found positive for KA-3 and KA-4, respectively. When treated with ATRA, 94.5% (Fig 8B) and 89.6% of NB4 cells were positive for KA-3 and KA-4, respectively. In contrast, when NB4 cells were untreated, 60.0% (Fig 8C) of the cells were positive for 6B4, while identifying 30.9% (Fig 8D) of positive cells when treated with ATRA.

**Western blot analysis of NB4 cell lysates with anti-TM or anti-TF antibody.** The presence of TM and TF in NB4 cells was determined by immunoblotting with the antibodies specific for human TM and TF, respectively. The monoclonal antibody KA-3 identified the non-reduced form of TM as a prominent band at approximately 75 kD (Fig 9). This was similar to placental and HUVEC-derived TM.

After treatment with ATRA, this band was markedly increased in NB4 (Fig 9A, lane 2) and U937 (Fig 9B, lane 2) cells, while only modestly increased in UT7 (Fig 9C, lane 2) cells. In contrast, after treatment with dbcAMP, the band was markedly increased in UT7 (Fig 9C, lane 3) and U937 (Fig 9B, lane 3) cells, while only modestly increased in NB4 (Fig 9A, lane 3) cells. The polyclonal antibody against human TF identified the non-reduced form of TF in NB4 cells as a doublet at approximately 45 kD and 41 kD and a minor at 29 kD (Fig 10, lane 2). A 45 kD band was similar to placental TF (Fig 10, lane 1). After treatment with ATRA, the densities of the bands were markedly decreased (Fig 10, lane 3).

**Northern blot analysis of cytoplasmic RNA from NB4 cells.** To further examine whether ATRA is capable of upreg-
Fig 8. Flow cytometric analysis of surface TM and TF antigens in NB4 cells. NB4 cells were untreated (A and C) or treated (B and D) with ATRA (1 μmol/L) for 24 hours. The flow-cytometric analysis of both untreated and treated cells, incubated with KA-3 (anti-TM) (A and B) or 6B4 (anti-TF) (C and D) monoclonal antibody followed by goat FITC-antimouse IgG, were shown. Abscissa shows fluorescence intensities and ordinate shows number of cells.

Fig 9. Western blot analysis of leukemic cell preparations with anti-TM antibody. 10⁶ leukemic cells were lysed in detergent and the preparations were subjected to SDS-PAGE under nonreducing conditions. After transfer to Immobilon (Millipore Corp., MA), immunoblotting was accomplished with a monoclonal anti-TM antibody KA-3. Distinct induction of TM expression in NB4 (A), U937 (B), and UT7 (C) cells after ATRA (lane 2) and dbcAMP (lane 3) treatment were shown. The prominent band at approximately 75 kD represents TM. Molecular mass markers were given along the left margin. Lane 1 represents untreated control.

DISCUSSION

In the present study we have demonstrated upregulation of TM expression and downregulation of TF expression in inherently procoagulant APL cells by ATRA. The increase of TM expression in APL cells by ATRA occurred within 24 hours and was associated with the increase of the amount of relative TM mRNA and anticoagulant activity expression. The downregulation of TF expression in APL cells by ATRA was also observed. Plasma-based clotting assays and neutralization tests have shown that cell-surface TF contributes to the procoagulant status of APL cells, and that prior treatment of APL cells with ATRA reduces the procoagulant activity. These effects were found in monoblastic leukemic cells as well.

It is generally recognized that hemorrhages in APL patients result, at least in part, from the release of a tissue procoagulant activity present in the azurophilic granules of APL cells. Since plasma ATRA levels between 0.1 and 1 μmol/L are easily attainable continuously in patients being treated with ATRA, changes in TM and TF expression induced within 24 hours after treatment with ATRA may explain rapid amelioration of DIC and reduction of hemorrhagic complications in APL patients treated with ATRA.

Our present study further provides data for different expressions of TM and TF in leukemic cells. Regarding TM in particular, a remarkable induction of TM expression by ATRA was observed in NB4 (promyelocytic cell) and U937 (monoblastic cell) cells but not in K562 (erythroblastic cell), UT7 (megakaryoblastic cell), TMD2 (B lymphoblastic cell), and HL60 (myeloblastic cell) cells. On the other hand, marked induction of TM expression by dbcAMP was observed in UT7 and U937 cells but not in the other cell lines. TM was initially isolated from macrovascular and microvascular endothelial cells. However, the cell surface receptor has recently been identified on synovial cells, mesothelium, activated smooth muscle cells, monocytes, neutrophils, and platelets as well as on various transformed cell lines. TM

(A)NB4 : TM  (B)U937 : TM  (C)UT7 : TM

![Image of Western blot analysis with different cell lines and treatments showing TM expression levels.](image-url)
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Fig 10. Western blot analysis of NB4 cells preparation with anti-TF antibody. Immunoblotting was done with a polyclonal anti-TF antibody. Lane 1 is placental TF as a control. Lane 2 and lane 3 were cell lysates from NB4 cells untreated or treated with ATRA, respectively. A doublet of 45 kD and 41 kD and a minor band at 29 kD were detected in untreated NB4 cells.

is also synthesized during formation of the extra-embryonic parietal endoderm just before the appearance of the receptor on yolk sac endothelial cells. Although NB4 and HL60 have similar inducibility of differentiation in a myeloid direction, inducibility of TM by ATRA was remarkably different. Cell differentiation in a myeloid direction alone may not induce upregulation of TM. The presence of rearrangement of RA receptor α gene alone may not induce upregulation of TM by ATRA, because ATRA is also capable of upregulating TM expression in U937 cells, which does not possess rearrangement of the gene. The regulation of RA target genes is believed to be largely caused by the mediation of nuclear receptors that are capable of binding to particular DNA sequences known as RA-responsive elements. Because the human TM gene does not include a binding site for AP-1, a particular transcription factor, the upregulation of TM expression by ATRA is probably the consequence of an activation of RA-responsive element. In fact, an RA-responsive element has been recently reported in the 5′ flanking region of human TM gene. A functional cAMP-responsive element in the TM gene has been identified in the 3′ untranslated region of the TM gene in our laboratory. The accessibility of the RA-responsive and cAMP-responsive elements to the binding proteins induced in different blood cell types may control the expression of the TM and TF genes. The analysis of such an interaction is an intriguing subject for future investigation. The molecular mechanisms underlying the putative cross-talk between RA and cAMP should also be determined. Further studies using leukemic cell lines described in this communication may be useful for determining drug response element in the TM gene, for investigating mechanism of the regulation of TM expression, and for exploring certain roles of TM in biologic processes including regulation of cell-cell or protein-protein interactions and cell differentiation.

A previous report suggested that TM was synthesized in neutrophils under nonstimulated conditions and the protein synthesized was largely excluded from the membrane surface and lacks the ability to promote protein C activation by thrombin. In NB4 cells under nonstimulated conditions, TM

Fig 11. Northern blot analysis. Twenty micrograms of total RNA extracted from cultured NB4 cells after exposure to 1 μmol/L ATRA (lane 2), 5 mmol/L dbcAMP (lane 3), and 0.6 μmol/L IL-1α (lane 4) for 5 hours were applied to each lane and electrophoresed on a 1% agarose/formaldehyde gel. The blot was probed with a 32P-labeled cDNA fragment specifically encoding TM. Lane 1 represents untreated control. Locations of ribosomal RNAs are indicated on the left margin. A β-actin probe was hybridized to indicate the equal amount of blotted RNAs.
cells had TF antigen positivity suggesting that TF antigen release of TF by the breakage of cells initiates coagulation by decreasing the amount of relative mRNA as reported by events. It has been reported that HL60 cell differentiation functional assay and the procoagulant assay in clotting time, NB4 cells. The functional TF expression on the surface of may not be predominantly expressed on the surface of APL be not representative of APL cells but reclassified as AML; procoagulant activity, while not affecting the fibrinolytic or proteases have been proposed as important associated in a myeloid direction induced by RA led to a diminished marked TF activity could be measured on the surface of APL cells as well as in endothelium may appreciably contribute to the increase of anticoagulant activity in the circulation. On the other hand, TF expression was downregulated by ATRA in APL cells. RA receptor α has been reported to form a nonproductive complex with c-Jun (products of the nuclear proto-oncogene c-jun) and represses AP-1 (Jun/Fos) transcriptional activity. Because an AP-1 binding site exists in the 5′ flanking region of the human TF gene, RARα bound with ATRA may reduce the expression of TF mRNA by making a complex with c-Jun. The downregulation of TF expression in APL cells by ATRA may be induced by decreasing the amount of relative mRNA as reported by another group. Flow cytometry showed that 60% of NB4 cells had TF antigen positivity suggesting that TF antigen may not be predominantly expressed on the surface of APL cells. This finding may be consistent with the fact that the release of TF by the breakage of cells initiates coagulation in APL patients and the aggravation of DIC in the patients on initiation of chemotherapy. However, using the chromogenic functional assay and the procoagulant assay in clotting time, marked TF activity could be measured on the surface of NB4 cells. The functional TF expression on the surface of intact cells may also be indicative for DIC in APL patients as suggested before.

DIC is not the sole etiology of APL-associated coagulation abnormalities and the severe clinical bleedings in APL patients. Fibrinolytic and proteolytic activities due to leukocyte proteases have been proposed as important associated events. It has been reported that HL60 cell differentiation in a myeloid direction induced by RA led to a diminished procoagulant activity, while not affecting the fibrinolytic or elastase activity, although HL60 cells have been proven to be not representative of APL cells but reclassified as AML; M2. It has been reported that the proteolysis syndrome was more rapidly corrected in APL patients treated with ATRA than the procoagulant activity, and that DIC persisted especially in patients who developed hyperleukocytosis during ATRA therapy. A recent study has shown downregulation of urokinase activity associated with plasminogen activator inhibitor production in NB4 cells treated with ATRA, which implicates reduced fibrinolytic activity in APL cells treated with ATRA. Hyperleukocytosis undergoing differentiation therapy should be avoided because it may induce thromboembolic events.

In conclusion, we suggest that ATRA regulates expression of TM and TF in certain types of leukemic cells probably at transcriptional level, and that ATRA is not only an efficient differentiating drug but also a preventive and therapeutic agent for DIC in APL. Furthermore, ATRA may be an efficient agent against DIC in monocytic leukemia. We expect that prevention and treatment of DIC at transcriptional levels of TM and TF genes may be possible in the near future.

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