All-Trans-Retinoic Acid Counteracts Endothelial Cell Procoagulant Activity Induced by a Human Promyelocytic Leukemia-Derived Cell Line (NB4)

By Anna Falanga, Marina Marchetti, Silvia Giovanelli, and Tiziano Barbui

Therapy with all-trans-retinoic acid (ATRA) can rapidly improve the coagulopathy of acute promyelocytic leukemia (APL). This study was designed to evaluate whether the APL cell line NB4 induces the procoagulant activity (PCA) of human endothelial cells (ECs) in vitro, and whether this property is modified after ATRA-induced NB4 maturation. EC monolayers were incubated for 4 hours at 37°C with the conditioned media (CM) of NB4 treated with 1 μmol/L ATRA (ATRA-NB4-CM) or the vehicle (control-NB4-CM). EC lysates were tested for PCA. ATRA-NB4-CM induced significantly more PCA (TF) than control-NB4-CM (P < 0.01). To identify the cause of TF induction, interleukin (IL)-1β antigen levels were measured in CM samples. ATRA-NB4-CM contained significantly more IL-1β than control-NB4-CM. EC PCA was significantly inhibited by an anti-IL-1β antibody. The addition to the media of 10 μmol/L ATRA counteracted the EC TF expression induced by NB4-CM. These data indicate that ATRA increases the procoagulant stimulus of leukemic cells.

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MATERIALS AND METHODS

ECs. ECs from human umbilical veins were harvested from 5 to 10 cords after exposure to 0.1% collagenase in Hank's salt solution for 25 minutes at 37°C, according to the method of Jaffe et al.1 Pooled ECs were resuspended in RPMI 1640 medium (GIBCO Laboratories, Grand Island, NY) supplemented with 20% fetal calf serum (FCS; GIBCO), penicillin, streptomycin, and glucose (GIBCO). They were grown to confluence in plastic flasks (Falcon; Becton Dickinson, Meylan, France) under an atmosphere consisting of 95% air and 5% CO2. Cells were confirmed as endothelial based on their typical cobblestone morphology and the characteristic granular staining pattern for von Willebrand factor (vWF) by indirect immunofluorescence.

Confluent primary cultures were passaged using trypsin (0.25%) and EDTA (0.02%) (Sigma, St. Louis, MO), seeded in 24-well tissue culture plates (Falcon) coated with 1% gelatin (Sigma), and grown to confluence under the same conditions. ECs were used for experiments at the second or third passage.

NB4-CM. The NB4 cell line, recently established in vitro from an APL patient, was provided by Dr Lanotte (Hôpital St. Louis, Paris, France). Cells were grown in RPMI 1640 medium plus 10% FCS, as described.2 Conditioned medium (NB4-CM) was prepared from NB4 cells (2 × 10^6/mL) cultured in the presence of 1 μmol/L ATRA, final concentration (fC), dissolved in 0.01% dimethyl sulfoxide (DMSO) in RPMI 1640 (ATRA-NB4-CM) or the vehicle (control-NB4-CM) for 24, 72, and 120 hours; 1 μmol/L ATRA is the dose that induces NB4 maturation to granulocytes.3 At each interval, the NB4-CMs were removed by centrifugation at 1,100 g for 10 minutes filtered, and then tested for their ability to induce PCA and TF antigen (Ag) expression by EC.

IL-1β Ag. The concentration of IL-1β Ag in the NB4-CMs was measured by enzyme-linked immunosorbent assay (ELISA; sand-
wich principle) using a commercially available kit (Research and Diagnostics Systems, Minneapolis, MN).

**ATRA levels.** The concentration of ATRA in the NB4-CMs was measured by a high-performance liquid chromatography method, according to Guiso et al.\textsuperscript{20} The procedure involves one-step extraction of ATRA from the culture medium, isocratic elution from a reversed-phase column (LiChrosorb RP-18, 5-\textmu m particle size) and ultraviolet (UV) detection at 340 nm.

**Experimental procedures.** EC confluent monolayers (24-well microplates, 2 \times 10^5 per well) were washed twice with RPMI 1640 (time 0) and incubated for 4 hours at 37°C with (1) fresh medium (resting EC control); (2) fresh medium containing IL-1\beta, 25 U/L (stimulated EC control); or (3) control--NB4-CM or ATRA--NB4-CM to a final concentration of 50% (vol/vol). In some experiments, TF activ-

**PCA assay.** The PCA of EC lysate was measured by the one-stage recalcification assay of normal human plasma, according to a previously described procedure.\textsuperscript{22,27} Briefly, 0.1 mL cell lysate was added to 0.1 mL citrated normal human plasma and warmed at 37°C for 1 minute; 0.1 mL prewarmed CaCl\textsubscript{2} (25 mMol/L) was added, and the clotting time was recorded with a coagulometer (Mechrolab dual channel; Carlo Erba, Milan, Italy). EC PCA was identified and characterized as TF by the clotting assay of factor VII-, VIII-, or X-deficient human plasmas (FVII-D, FXIII-D, FX-D, respectively; Behringwerke, Marburg, Germany). In some experiments; TF activ-

**TF:Ag detection.** TF:Ag levels of EC extracts were measured by an ELISA method (sandwich principle) using a commercial kit (Imubind Tissue Factor ELISA Kit; American Diagnostica Inc, Greenwich, CT).

**Statistical analysis.** The following statistical tests were used: (1) the two tailed Student's t-test and (2) the linear regression analysis by the least squares method. A P value less than .05 was considered significant.

**RESULTS**

Figure 1 shows the effects of control--NB4-CM and ATRA--NB4-CM on TF:Act and TF:Ag expression by ECs. Results are means \pm SD of at least 10 experiments. Both control-- and ATRA--NB4-CMs were able to induce EC PCA, which showed the characteristics of TF because it failed to shorten the coagulation time of FVII-D or X-D plasmas, while being equally active in the presence and absence of FVIII. In addition, preincubation of EC lysates with an anti-TF polyclonal Ab significantly reduced the clotting activity from 90 \pm 12.3 U/L \times 10^5 cells (mean \pm SD) to 3 \pm 1.1 U/L \times 10^5 cells (mean \pm SD; n = 4; P < .001). The negative control antibody did not alter the clotting time. The capacity of NB4-CM to induce TF:Act and TF:Ag of ECs increased with the culture time (Fig 1). The control--NB4-
ATRA PREVENTS HUVEC ACTIVATION BY APL CELLS

Fig 2. IL-1β concentration of ATRA-NB4-CM and control-NB4-CM. The levels of IL-1β were measured by ELISA at different treatment intervals (24, 72, and 120 hours). Results are means ± SD of at least 10 experiments.

CM collected after 120 hours of culture induced significantly more TF than the corresponding 24-hour sample (P < .01). Likewise, the ATRA–NB4-CM collected after 72 and 120 hours induced more TF:Act and Ag than the 24-hour treatment sample (P < .05). However, as shown in Fig 1A, the ATRA–NB4-CMs induced significantly more EC PCA than the control–NB4-CMs (P < .001) after 72 hours (68.2 ± 15.4 U/1 × 10^5 cells vs 17.8 ± 3.5 U/1 × 10^5 cells) and 120 hours of culture (95.6 ± 7.8 U/1 × 10^5 cells vs 36.2 ± 6.6 U/1 × 10^5 cells). The levels of EC TF:Ag (Fig 1B) induced by the untreated and treated media were significantly different as well and paralleled the TF:Act results. In the same experiments, little or no TF:Act was detectable in the resting control EC, whereas the IL-1β–stimulated (25 U) control had 95.5 ± 10 U/1 × 10^5 cells (1.2 ± 0.1 ng/mL TF:Ag).

To characterize factor(s) possibly responsible for the NB4-CM–induced EC TF, the level of IL-1β, a known endothelial TF inducer, was measured in the control– and ATRA–NB4-CMs. As shown in Fig 2, increasing IL-1β was found in the 24-, 72-, and 120-hour incubations of both CMs. However, the levels in ATRA–NB4-CM after 72 and 120 hours were significantly higher than the control-CMs (P < .001). A significant correlation was found (P < .001) between the levels of IL-1β in the NB4-CMs and EC TF:Act induced by the same samples (Fig 3).

To define whether IL-1β release by NB4 cells is involved in the EC TF increment, we conducted an inhibition study with an anti–IL-1β Ab. The 120-hour ATRA–NB4-CM sample, containing the highest IL-1β level (116.7 ± 32.5 pg/mL), was incubated with different Ab dilutions 1 hour before starting the experimental procedure. There was a dose-dependent EC TF:Act decrease, which was paralleled by a reduced expression of TF:Ag (Fig 4). The 1:300 dilution reduced both the TF:Act and the TF:Ag expression by 50% to 60%. The same Ab dilution completely neutralized the EC TF induced by 10 U standard IL-1β. ATRA was measured in the ATRA–NB4-CMs. After 120 hours cell growth, it was 85% lower than at 24 hour (Table 1).

To verify whether ATRA counteracted the EC TF induction by IL-1β, as described for TF induced by TNF-α, a series of experiments was performed in the presence of ATRA in the experimental system. Increasing concentrations from 0 to 10 μmol/L ATRA counteracted the induction of EC TF:Act elicited by 25 U standard IL-1β in a dose-dependent manner (Fig 5). In agreement with this finding, exposure of EC to 10 μmol/L ATRA during incubation with NB4-CM

Fig 3. Correlation between the EC TF:Act (U/10^5 cells) induced by NB4-CMs and the IL-1β Ag content. The correlation was obtained by plotting the mean IL-1β concentration of both ATRA (n = 7) and control–NB4-CM (n = 7) from all the treatment time samples (independent variable) against the mean EC TF:Act induced by the media from the same samples (dependent variable). Linear least-squares regression analysis yielded a correlation coefficient of .982, with a significant P value of less than .001.

ATRA in the experimental system. Increasing concentrations from 0 to 10 μmol/L ATRA counteracted the induction of EC TF:Act elicited by 25 U standard IL-1β in a dose-dependent manner (Fig 5). In agreement with this finding, exposure of EC to 10 μmol/L ATRA during incubation with NB4-CM

Fig 4. EC TF:Act and TF:Ag expression induced by ATRA–NB4-CM: inhibitory effect of an anti–IL-1β Ab. CM collected from NB4 after 120 hours of treatment with 1 μmol/L ATRA was incubated for 1 hour at 37°C with increasing dilutions of a purified anti–IL-1β rabbit polyclonal Ab before incubation with EC. Bars represent the EC TF:Act induced by samples after exposure to different Ab dilutions. Black triangles represent the EC TF:Ag induced by the same samples. Results (mean of three experiments) are expressed as percent activity and antigen of the control samples.
significantly affected the EC TF:Act and TF:Ag expression induced by 120-hour ATRA–NB4-CM samples (Fig 6).

Multiple regression analysis of PCA (y) versus IL-1β (x1), ATRA (x2); and time (x3) of six experiments showed a significant P value (<.05) and r value (.98). However, due to the fact that all the variables were highly correlated to each other (PCA v IL-1β: P < .001, r = .97; PCA v ATRA: P < .001, r = .89; PCA v time: P < .05, r = .82; IL-1β v ATRA: P < .01, r = .88; IL-1β v time: P < .01, r = .95; ATRA v time: P < .01, r = .90), the independent effect of each variable was not assessable.

**DISCUSSION**

We have previously demonstrated that ATRA downregulates the PCA of the promyelocytic NB4 cells, suggesting that this may be one of the possible mechanisms of its beneficial effect on the coagulopathy associated with APL.10,11,13 We have now investigated whether the same treatment with ATRA reduces the NB4 cell stimulus (if present) on the PCA of the vascular endothelium.

We first demonstrated that the CM from NB4 cells is able to induce EC TF expression. PCA had the characteristics of TF, as it was FVII- and X-dependent and FVIII-independent in the clotting assay of selectively deficient human plasmas and was sensitive to an anti-TF specific polyclonal Ab. However, the treatment with ATRA, while reducing the promyelocytic blast cell procoagulant potential,10,11,13 increased the capacity of NB4-CM to elicit EC TF. Therefore, an inverse relation with the NB4-intrinsic PCA is present.

Among the products of leukemic cell origin that could promote TF expression by EC, we chose IL-1β for various reasons. First, cells from patients with AML can secrete IL-1β,24 and this is potentially involved in triggering leukemia-associated DIC through the modulation of EC TF expression.25 Second, IL-1β is upregulated by ATRA in human promyelocytic cells, as recently described by Dubois et al.,14 who also observed a good correlation between this property and cell proliferation. In agreement with this, we found measurable amounts of IL-1β in the NB4-CMs. The IL-1β levels increased with the culture time, and more interestingly, they were significantly higher in the ATRA-treated samples than the controls. There was also a strong correlation between the levels of IL-1β and the EC expression of TF. The direct involvement of IL-1β in the NB4-CM–induced endothelial TF was further demonstrated by the dose-dependent inhibition of this effect by an anti–IL-1β polyclonal Ab, even though this antibody did not completely abolish the EC PCA. This suggests the presence of other EC stimulating factor(s) in the NB4-CMs. Blasts from APL patients can produce many endothelium-affecting substances, such as TNF-α, IL-6, IL-8.15 Preliminary data from our laboratory (not shown) show our samples contained TNF-α, which elicits EC TF as well as IL-1β. This could partly explain the residual EC TF activity after NB4-CM exposure to an anti–IL-1β Ab.

The enhanced EC procoagulant potential by ATRA-treated NB4 promyelocytes indicated a prothrombotic effect, in contrast with the rapid improvement of the coagulopathy reported in patients with APL. Therefore, as ATRA is able to counteract the EC TF induction by TNF-α,13 we explored the possibility that it might also prevent the effect of IL-1β on the endothelium. ATRA added to the medium during incubation with EC significantly counteracted the induction of EC TF by both standard IL-1β and ATRA–NB4-CMs.

### Table 1. ATRA Concentration in ATRA–NB4-CM

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<th>ATRA–NB4-CM Culture</th>
<th>ATRA (μmol/L)</th>
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<tr>
<td>24 h</td>
<td>0.389 ± 0.096</td>
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<tr>
<td>120 h</td>
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ATRA (1 μmol/L) was added to the cell cultures at time 0. After 24 and 120 hours of culture, the ATRA level was measured by an HPLC method (see Materials and Methods).
Therefore, ATRA can protect the EC from the TF expression induced by different cytokines.

Conceivably, at the beginning of induction treatment in patients with APL, ATRA may protect the vascular endothelium from the procoagulant stimulus of limited amounts of blast cell-derived cytokines, thus contributing to the overall antithrombotic action of therapy. As the therapy proceeds and the cells proliferate, excessive amounts of IL-1β may be released compared with the levels of circulating ATRA; thus, the IL-1β clot-promoting effect on the vascular endothelium may prevail at this time. The thrombotic events, respiratory distress symptoms, and organ failures (the retinoic acid syndrome) described in the course of long-term ATRA treatment may be the clinical expression of cytokine-promoted fibrin formation.

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REFERENCES

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