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

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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:tissue factor (TF) than control–NB4-CM (P < .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 promyelocyte-induced EC TF, partly through increased IL-1β production. However, ATRA can protect the endothelium from the procoagulant stimulus of leukemic cells.

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ACUTE PROMYELOCYTIC leukemia (APL) is a variety of acute myeloblastic leukemia (AML) distinguished by the balanced chromosomal translocation t(15;17) and by a life-threatening hemorrhagic diathesis. The bleeding complications have been partly attributed to a coagulopathy that shares some biologic features with disseminated intravascular coagulation (DIC) and is responsible for early deaths in this disease.[1,2] Factors related to the blast cells are considered important in the pathogenesis of the coagulopathy, ie, the expression of procoagulant, fibrinolytic and proteolytic proteins, and the release of cytokines.[3] Specifically, the cytokines interleukin-1β (IL-1β) and tumor necrosis factor (TNF-α) can regulate the expression of endothelial cell (EC) products active on the hemostatic system, including tissue factor (TF),[4] adhesive receptors,[5] thrombomodulin (TM),[6] and fibrinolysis proteins.[7]

Differentiating therapy with all-*trans*-retinoic acid (ATRA) for remission induction in APL induces complete remission in up to 90% of patients, accompanied by rapid improvement of the related coagulopathy.[1,8,9] The mechanisms of ATRA/hemostatic system interactions have attracted considerable interest. ATRA can affect the hemostatic properties of different cells, including the promyelocytic blasts and the endothelial cells. Specifically, ATRA reduces the procoagulant and fibrinolytic properties of leukemic cells in vitro[10-12] as well as in vivo[13] and regulates the ability of these cells to produce cytokines.[14] ATRA counteracts both the TM downregulation and the TF upregulation of ECs induced by TNF-α[15] and increases the EC tissue-plasminogen activator (t-PA) production[16] and TM expression.[17]

Because it is not known whether the promyelocytic blasts influence the endothelial procoagulant properties, in this study we investigated (1) the effect of NB4-conditioned medium (CM) on the expression of procoagulant activity (PCA) by human umbilical vein ECs in vitro, (2) how this NB4-CM capacity changed after treating the NB4 cells with ATRA; and (3) the effect of ATRA itself on ECs during the cell/cell interaction.

The results indicate that NB4-CM is able to induce EC PCA, which is identified as TF. Treatment of NB4 cells with ATRA further increases this capacity, which is significantly correlated to the IL-1β content of the CM. ATRA can affect the EC/NB4 interaction in that exposure of ECs to ATRA during cell/cell interaction counteracts the EC TF expression induced by NB4-CM.

MATERIALS AND METHODS

ECs. ECs from human umbilical veins were harvested from 5 to 10 cords after exposure to 0.1% collagenase in Hanks' salt solution for 25 minutes at 37°C, according to the method of Jaffe et al.[18] Pooled ECs were resuspended in RPMI 1640 medium (GIBCO Laboratories, Grand Island, NY) supplemented with 20% fetal calf serum (FCS; GIBCO), penicillin, streptomycin, and glutamine (GIBCO). They were grown to confluence in plastic flasks (Falcon; Becton Dickinson, Meylan, France) under an atmosphere consisting of 95% air and 5% CO_2. 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,[19] was provided by Dr Lanotte (Hôpital St. Louis, Paris, France). Cells were grown in RPMI 1640 medium plus 10% FCS, as described.[10] Conditioned medium (NB4-CM) was prepared from NB4 cells (2×10^5/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.[10] At each interval, the NB4-CMs were recovered by centrifugation at 1,100g 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.[20] The procedure involves one-step extraction of ATRA from the culture medium, isocratic elution from a reversed-phase column (LiChrosorb RP-18, 5-μm particle size) and ultraviolet (UV) detection at 340 nm.

Experimental procedures. EC confluent monolayers (24-well microplates, 2×10^5 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-1β, 25 U fc (stimulated EC control); or (3) control–NB4-CM or ATRA–NB4-CM to a final concentration of 50% (vol/vol). In some experiments; serial dilutions of a purified anti–IL-1β rabbit polyclonal antibody (Ab; 1 mg/mL)[21] were added to the NB4-CM for 1 hour at 25°C before starting incubation with ECs; a nonimmune normal rabbit IgG was used as the negative control. In another series of experiments; ATRA was added to the whole experimental system, after starting incubation of NB4-CMs with ECs.

After the 4-hour incubation with NB4-CMs or controls, ECs were washed three times with phosphate-buffered saline (PBS), pH 7.4, and two types of samples were prepared. (1) For cell lysates for the PCA assay, ECs were lysed by three cycles of freezing/thawing and scrape-harvested into 200 μL PBS. (2) For cell extracts for the TF:Ag determination, ECs were extracted with 200 μL of 50 mmol/L Tris containing 100 mmol/L NaCl and 1% Triton X-100 (pH 7.5) for 2 hours at 4°C.

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.[22,23] 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$_2$ (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, FVIII-D, FX-D, respectively; Behringwerke, Marburg, Germany). In some experiments; TF activity (TF:Act) was further characterized by incubating EC lysates with a purified polyclonal rabbit IgG antibody (1 mg/mL fc) directed against human TF (#4502; American Diagnostica Inc, Greenwich, CT) before the clotting assay. Incubation was performed for 15 minutes at 37°C. A normal nonimmune rabbit IgG was the negative control in this assay.

TF:Act was quantitated by reference to a calibration curve (log-log plot) constructed with different dilutions (from 10^{-1} to 10^{-6}) of a standard rabbit brain thromboplastin (RBT; Sigma, St Louis, MO); $r^2 = .96$. Results were expressed as standard thromboplastin arbitrary units: 1 unit = the activity of 1 mEquiv/mL of RBT in the coagulation assay.

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 ± SD of at least 10 experiments. Both control– and ATRA–NB4-CMs were able to induce EC
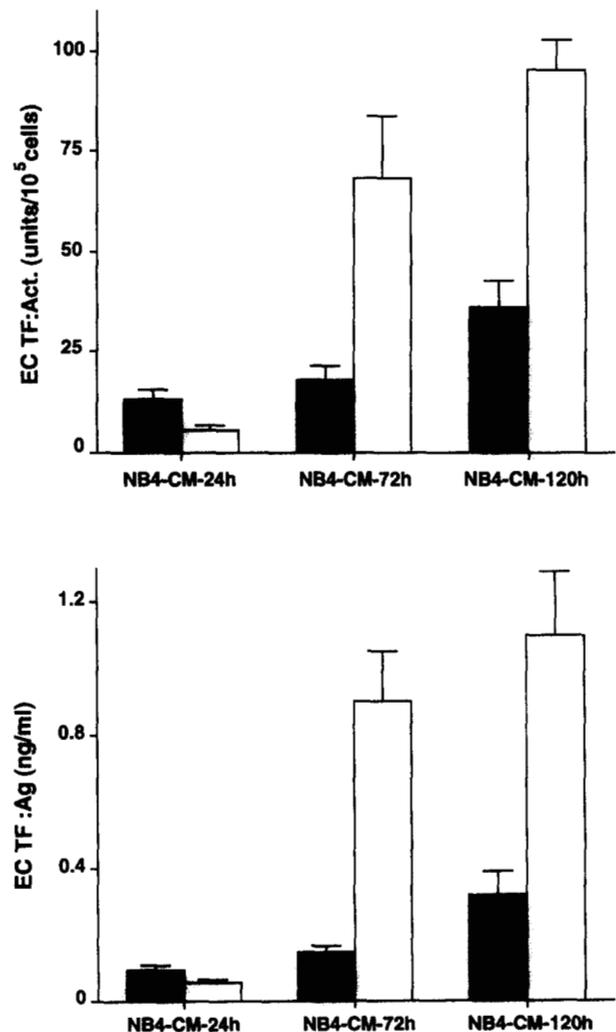
Fig 1. Tissue factor activity (TF:Act) and TF antigen (TF:Ag) of ECs after 4 hours of incubation with ATRA–NB4-CM (open bars) and control–NB4-CM (solid bars). NB4-CMs were obtained from cells cultured for 24, 72, and 120 hours in the presence of 1 μmol/L ATRA or the vehicle before incubation with ECs. TF:Act of EC lysates was determined by the recalcification assay of normal human plasma and expressed as units per 10^5 cells: 1 unit = the activity of 1 mEquiv standard thromboplastin in the clotting assay. The activity was identified as TF because it was FVII- and FX-dependent and FVIII-independent in the clotting assay of factor-deficient plasmas and was 96% reduced by a polyclonal anti-TF Ab. TF:Ag of EC extracts was quantitated by ELISA and expressed as ng/mL. Results are means ± SD of at least 10 experiments.

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 ± 12.3 U/1 × 10^5 cells (mean ± SD) to 3 ± 1.1 U/1 × 10^5 cells (mean ± 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-
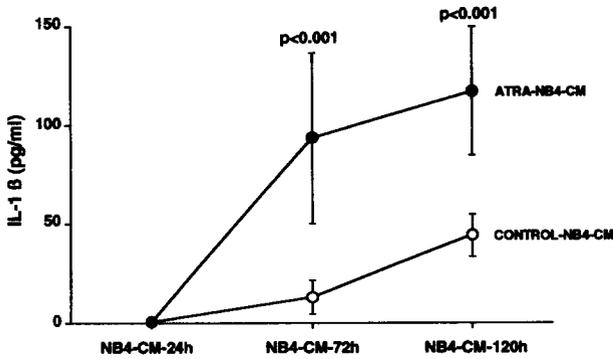
Fig 2. IL1-β concentration of ATRA–NB4-CM and control–NB4-CM. The levels of IL-1β were measured by ELISA at different treatment intervals (24, 72, and 120 hours). Results are means ± SD of at least 10 experiments.
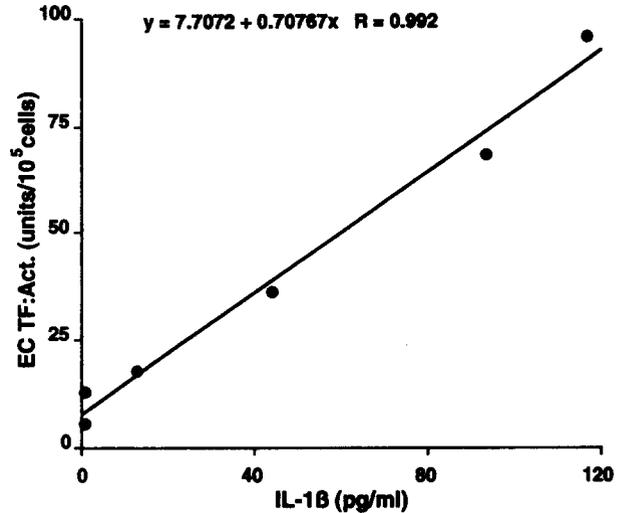

Fig 3. Correlation between the EC TF:Act (U/10⁵ cells) induced by NB4-CMs and the IL-1β Ag content. The correlation was obtained by plotting the mean IL-1β concentration of both ATRA-(n = 7) and control-NB4-CM (n = 7) from all the treatment time samples (independent variable) against the mean EC TF:Act induced by the media from the same samples (dependent variable). Linear least-squares regression analysis yielded a correlation coefficient of .992, with a significant P value of less than .001.

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 × 10⁵ cells v 17.8 ± 3.5 U/1 × 10⁵ cells) and 120 hours of culture (95.6 ± 7.8 U/1 × 10⁵ cells v 36.2 ± 6.6 U/1 × 10⁵ 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 × 10⁵ 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,[15] 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
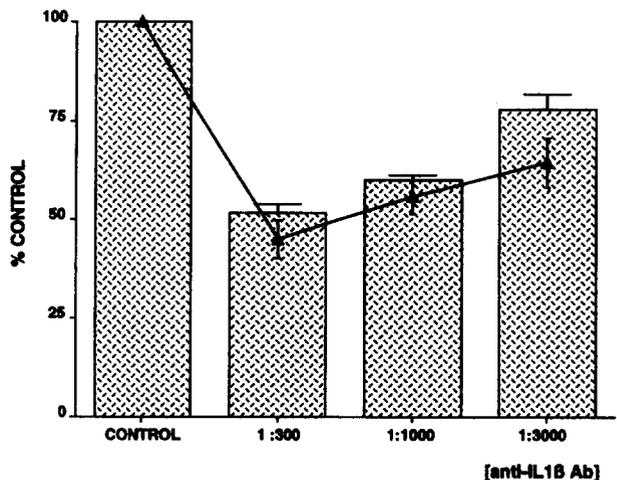

Fig 4. EC TF:Act and TF:Ag expression induced by ATRA–NB4-CM: inhibitory effect of an anti–IL-1β Ab. CM collected from NB4 after 120 hours of treatment with 1 μmol/L ATRA was incubated for 1 hour at 37°C with increasing dilutions of a purified anti–IL-1β rabbit polyclonal Ab before incubation with EC. 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.

Table 1. ATRA Concentration in ATRA-NB4-CM

ATRA-NB4-CM Culture	ATRA (μmol/L)
24 h	0.369 ± 0.096
120 h	0.053 ± 0.008

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).

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 ($\times$1), ATRA ($\times$2); and time ($\times$3) 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.[10,13] 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 PCA 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 pro-
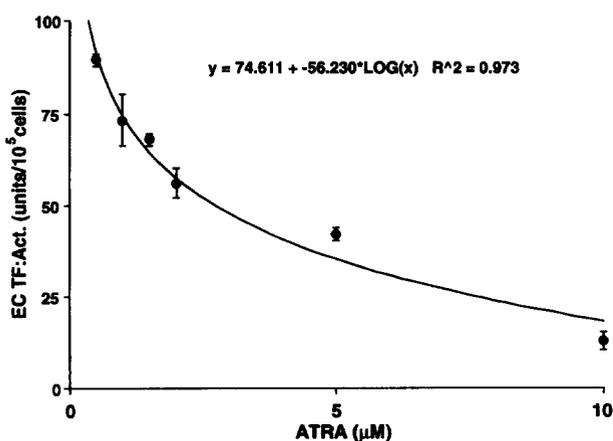
Fig 5. Effect of increasing concentrations of ATRA on EC TF:Act induced by standard IL-1β. ECs were exposed to 25 U standard IL-1β for 4 hours at 37°C in the presence of increasing concentrations of ATRA (from 0 to 10 μmol/L). After incubation, EC lysates were made and tested for TF:Act. Samples were determined in duplicate; results are means of three experiments.
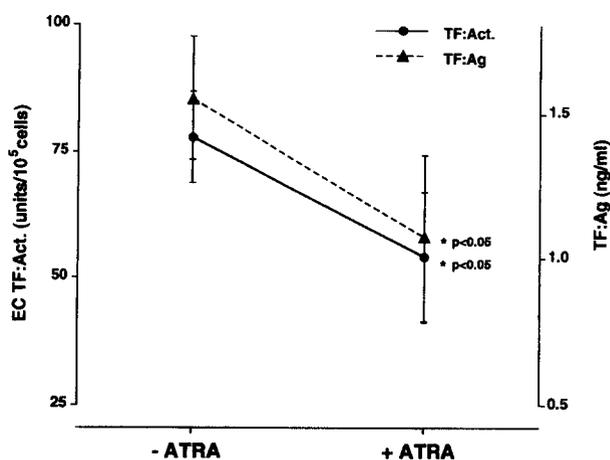
Fig 6. EC TF:Act and TF:Ag expression induced by ATRA-NB4-CM in the presence of 10 μmol/L ATRA. ATRA (10 μmol/L) was added to the ATRA-NB4-CM (120-hour sample) after starting incubation with ECs. After 4 hours, TF:Act (continuous line) and TF:Ag (broken line) were measured. Results are mean ± SD of six experiments.

myelocytic 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.[14] 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,[15] 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.

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,[26] respiratory distress symptoms, and organ failures (the retinoic acid syndrome)[27] described in the course of long-term ATRA treatment may be the clinical expression of cytokine-promoted fibrin formation.

ACKNOWLEDGMENT

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