All-trans retinoic acid modulates microvascular endothelial cell hemostatic properties

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Background and Objectives. All-trans retinoic acid (ATRA) is an anti-tumor agent capable of controlling the hypercoagulable state associated with malignancy. Among hemostasis-regulating functions, ATRA modulates the procoagulant and fibrinolytic properties of endothelial cells (EC) from large vessels (HUVEC). In this study we investigated whether ATRA may affect the same activities of EC derived from microvessels (HMEC-1 cell line).

Design and Methods. We studied the effects of ATRA on procoagulant (i.e. tissue factor, TF), fibrinolytic (i.e. tissue plasminogen activator and inhibitor, t-PA and PAI-1) and anticoagulant (i.e. thrombomodulin, TM) properties of HMEC-1, compared to HUVEC. The type of retinoic acid receptor (RAR) possibly involved was identified by using synthetic retinoid selective agonists or antagonists for RAR α, β or γ. The study was conducted with or without tumor necrosis factor (TNF)α to induce the expression of some endothelial hemostatic properties.

Results. ATRA significantly inhibited TNFα-induced TF expression in HMEC-1 as well as HUVEC. ATRA increased t-PA antigen without significantly affecting PAI-1 expression, and counteracted the TNFα-induced t-PA decrease in both types of EC. Accordingly, t-PA activity was significantly increased by ATRA, even in the presence of TNFα. Finally, ATRA upregulated TM, and prevented TNFα-induced TM downregulation. The study with selective RARs agonists and antagonists indicated that RARα played a major role in t-PA and TM modulation, whereas all three receptors were involved in TF downregulation.

Interpretation and Conclusions. This study provides the first evidence that ATRA increases antithrombotic potential also in microvascular EC, a very relevant compartment for tumor- and/or antitumor therapy-associated vascular complications.

Key words: retinoids, tissue factor, fibrinolysis, thrombomodulin, endothelium.

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All-trans retinoic acid (ATRA), the active metabolite of vitamin A, is a differentiating agent increasingly utilized for the treatment of various malignancies, including acute promyelocytic leukemia (APL)[1] and solid tumors.[2] Besides inhibiting various tumor cell growth and proliferation features, ATRA is unique in that it can modulate tumor-associated prothrombotic mechanisms considered relevant for the pathogenesis of the hypercoagulable state associated with malignant diseases.[3] Indeed, patients with malignancy typically present with an altered hemostatic balance, characterized by abnormalities of laboratory tests, indicating ongoing activation of blood coagulation even without thrombosis. This is a subclinical condition which predisposes to overt clinical thrombotic complications. These manifestations in cancer can vary from localized venous and arterial thrombosis to systemic syndromes, such as disseminated intravascular coagulation (DIC) and thrombotic microangiopathies.[4-6]

An important role in promoting activation of blood coagulation and the thrombotic diathesis in cancer patients is attributed to tumor-associated prothrombotic mechanisms, which include the capacity of tumor cells to activate blood coagulation directly, (e.g. by expressing procoagulant activities), and to produce cytokines i.e. interleukin (IL)-1β and tumor necrosis factor (TNF)-α, which in turn activate the endothelium.[10,11] Of clinical interest is the observation that the administration of ATRA to patients with APL provokes a significant loss of procoagulant activity by bone marrow blast cells *in vivo* and simultaneously resolves the DIC syndrome commonly accompanying this disease.[9] Recent studies demonstrated that ATRA also down-regulates the expression of the procoagulant tissue factor (TF) in human breast cancer cells,[10] and reduces the levels of circulating hypercoagulation markers in patients with breast cancer.[11] Therefore, ATRA represents the first antitumor agent showing anti-thrombotic properties. These properties include ATRA's capacity to affect endothelial cell (EC) procoagulant functions, which are very important for blood clotting activation and can be elicited by tumor tissues. In the *in vitro* model of macrovascular EC (i.e. HUVEC), ATRA protects against the procoagulant stimulus of different cytokines and enhances EC anticoagulant and fibrinolytic functions.[12-15]

No extensive investigations have been conducted so far on the effects of ATRA on the procoagulant, fibrinolytic, and anticoagulant properties of EC derived from the microvasculature, a compartment strongly facili-

tating direct interactions of EC with both malignant and normal cells (i.e. platelets, monocytes, neutrophils). These interactions stimulate EC activities leading to coagulation activation and fibrin deposition in the peripheral blood vessels. Because important biological and functional differences exist between EC derived from large vessels and those from small vessels,[17] the aim of this study was to evaluate the effect of ATRA on the hemostatic properties of EC from the microvascular bed (HMEC-1 cell line). In particular, we investigated the expression of the procoagulant, TF, the fibrinolytic proteins, t-PA and PAI-1, and the endothelial thrombin receptor, TM. The study was conducted in the presence and absence of a pro-inflammatory stimulus (TNFα), and the results were compared with those obtained with EC from large vessels (HUVEC).

Since ATRA is an agonist of all types of retinoic acid receptors (RAR), including RARα, β and γ,[18,19] synthetic retinoids, selective agonists and antagonists of various RARs, were used to identify the type of receptor(s) possibly involved in the regulation of the different hemostatic functions in HMEC-1 and in HUVEC.

Design and Methods

Chemicals

The following retinoids were used: (i) the pan-RAR agonist ATRA (Sigma, St. Louis, MO, USA); (ii) three RAR selective agonists, i.e. the RARα agonist Am580 {[4-(5,6,7,8-tetrahydro-5,5,8,8-tetramethyl-2-naphthalenyl)-carboxamido] benzoic acid}, a kind gift from Hoffman-La Roche, Basel, Switzerland; the RARβ agonist CD2019 {6-[3-(1-methylcyclohexyl)-4-methoxyphenyl]-2-naphthoic acid}, and the RARγ agonist CD437 {6-[3-(1-adamantyl)-4-hydroxyphenyl]-2-naphthoic acid}, both gifts from CIRD Galderma, Sophia Antipolis, Valbonne, France; and (iii) two RAR antagonists: CD3106 (CIRD Galderma), which blocks RARα, β, and γ (pan-RAR antagonist), and Ro41-5253 (Hoffmann-LaRoche), which is a RAR-α specific antagonist. Retinoids were dissolved in dimethylsulfoxide (DMSO, Sigma) at a concentration of 0.01 mol/L and then diluted in culture medium to the required final concentrations. Retinoid stock solutions were kept at -80°C protected from light until use. All procedures with retinoids were performed in dim light.

Cell cultures

For this study, the immortalized human microvascular endothelial cell line, HMEC-1, was used. This cell line is a SV-40 transfected human dermal microvascular EC line:[20] it was a kind gift from Dr. F.J. Candal (CDC, Centers for Disease Control and Prevention, Atlanta, GA, USA). HMEC-1 were grown in RPMI 1640 medium (Gibco, Gaithersburg, MD, USA) supplemented with 10% fetal bovine serum (Gibco), 100 μg/mL streptomycin, 100 U/mL penicillin, 5 μg/mL amphotericin B, 2 mM L-glutamine (Gibco), 10 ng/mL epidermal growth factor (ICN, Costa Mesa, CA, USA) and 1 μg/mL hydrocortisone (ICN). Subcultures were obtained by trypsin/EDTA treatment of confluent monolayers at a split ratio of 1:3. HMEC-1 were serially passaged in T25 flasks and, at passages 8 to 16, were seeded in 24-well cell culture plates and grown to confluence for the experiments.

Human macrovascular endothelial cells were isolated from umbilical cord veins (HUVEC), as described elsewhere,[21] and grown in RPMI 1640 medium supplemented with 20% fetal bovine serum, 100 μg/mL streptomycin, 100 U/mL penicillin, 5 μg/mL amphotericin B and 2 mM L-glutamine. Confluent primary cultures were passed by trypsin treatment (split ratio 1:3) and cultured under the same conditions to confluence in T25 flasks, and then seeded in 24-well cell culture plates pre-coated with 0.5% gelatine (Sigma). These cells were grown to confluence and used for the experiments.

HMEC-1 and HUVEC were grown in a 5% CO2 and 95% air atmosphere in a humidified incubator at 37°C. The cell culture medium was checked for bacterial endotoxin contamination with the limulus amebocyte lysate assay (Chromogenix AB, Molndal, Sweden), and endotoxin concentration was found to be less than 5 pg/mL at working concentrations of the reagents. Trypan blue exclusion dye test was used to determine cell viability. More than 95% of cells were viable in all experiments.

Experimental system

Endothelial cells (HMEC-1 and HUVEC) at confluence on the bottom of 24-well culture plates were incubated for up to 24 hours at 37°C with ATRA, Am580, CD2019 and CD437 at the doses of 0.001, 0.01, 0.1 and 1 μmol/L, or with the vehicle (DMSO). To confirm the role of the three RAR subtypes in modulating the different hemostatic parameters, the RARs were blocked by RAR antagonists: CD3106 (pan-RAR antagonist) and Ro41-5253 (RARα antagonist). Cells were pre-incubated for 60 minutes with 1 μmol/L of the antagonist and subsequently incubated with the agonists. In some experiments, EC were incubated with 100 U/mL TNFα (Sigma), in the presence or absence of the different retinoids.

At the end of each incubation period, EC conditioned media (CM) were collected from each well, centrifuged at 16,000 × g for 5 min at 4°C in a Eppendorf microfuge to eliminate cells debris, and the supernatants stored at -80°C until assayed. The underlying EC monolayer was washed three times with phosphate-buffered saline (PBS, pH 7.49), scrape-harvested into 0.4 mL of PBS and, after 1:10

dilution in trypan blue dye, cells were counted under a microscope in a Burker chamber. Then EC aliquots were centrifuged and suspended at the concentration of 0.5×10^6 cells/mL in the specific buffers for each test (i.e. TF-PCA, TF activity, TF and TM antigen). The fibrinolytic properties of the EC (i.e. t-PA antigen, t-PA activity, and PAI-1 antigen) were evaluated in CM, and the results were adjusted for the respective cell counts, i.e. for the number of cells that *produced* the corresponding CM.

EC procoagulant activity

Procoagulant activity (PCA) assay. TF-PCA was evaluated in EC lysates (0.5×10^6 cells/mL PBS), obtained after three cycles of freezing/thawing, by the one-stage recalcification assay of normal human plasma, according to a previously described procedure.[13] TF-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, DADE Behring, Milan, Italy). In some experiments, TF activity was further characterized by incubating (15 minutes at 37°C) EC samples with a purified polyclonal rabbit IgG antibody (1 mg/mL final concentration) directed against human TF (# 4502, American Diagnostica Inc, Greenwich, CT, USA) before the clotting assay. A normal non-immune rabbit IgG was the negative control in this assay. TF-PCA was referred to a calibration curve constructed with different dilutions (from 10^{-1} to 10^{-6}) of a standard rabbit brain thromboplastin (RBT; Sigma). Results are expressed as RBT units where 1 unit = the activity of 1 mEquiv/mL of RBT in the coagulation assay.

TF chromogenic assay. TF activity was evaluated in EC lysates (0.5×10^6 cells/mL) in Hepes buffer (10 mM Hepes, 137 mM NaCl, 4 mM KCl, 5 mg/mL ovalbumin, 2.5 mM $CaCl_2$, pH 7.45; all reagents from Sigma) produced by three cycles of freezing and thawing. TF activity was measured as the rate of FX hydrolysis using a chromogenic assay for FXa, as described elsewhere.[22]

TF antigen assay. TF antigen levels were measured in EC extracted in TRIS buffer (0.5×10^6 cells/mL of 50 mM TRIS, 100 mM NaCl and 1% Triton X-100, pH 7.5) for 2 hours at 4°C, followed by three cycles of freezing/thawing. The ELISA (Imubind Tissue Factor ELISA Kit; American Diagnostica) was performed according to the manufacturer's instructions.[22] Results are expressed as pg/10^5 cells.

TF mRNA

TF mRNA was evaluated in confluent endothelial cells exposed or not to 100 U/mL of TNFα in the presence or absence of 1μmol/L ATRA or 0.01 μmol/L of one of the three synthetic RAR agonists. After 3 hours' incubation, total cellular RNA was extracted using Trizol reagent (Life Technologies, Paisley, Scotland) according to the manufacturer's instructions. Northern analysis was performed as already described.[23] Briefly, 10 μg total RNA were electrophoresed through an agarose-formaldehyde denaturing gel and transferred overnight onto nylon membranes. RNAs were cross-linked by exposing the filters to UV light. The filters were hybridized overnight at 42°C with 15 mL hybridization mixture containing 50% formamide, 9% dextrane sulfate, 5× SSPE (1× SSPE: 0.15 M NaCl, 0.01 M NaH_2PO_4, 1mM EDTA pH 7.4), 1× Denhardt's (Ficoll, polyvinylpyrrolidone and BSA, 0.2 mg/mL each), 0.8% SDS, 200 μg/mL denatured salmon sperm DNA, and ^{32}P-labeled specific cDNA probe spanning the complete human TF protein coding region.[24] This probe was kindly supplied by Dr. R. Lorenzet (Consorzio M. Negri Sud, Santa Maria Imbaro, Italy). Filters were then washed at 65°C with SSC 0.1× and SDS 0.1% and exposed to X-ray film. To account for the amount of RNA being analyzed the filters were hybridized overnight with a ^{32}P-end labeled oligonucleotide specific for 18s rRNA as already described.[23] The intensity of the bands on the autoradiography was evaluated by densitometric scanning with the aid of GelPro4 software (Media Cybernetics). The ratio between the density of the TF mRNA and the corresponding rRNA was calculated. It was arbitrarily assumed that this ratio in untreated endothelial cells corresponds to 100% TF expression.

EC fibrinolytic activity

t-PA and PAI-1 antigens were measured in the CM from HMEC-1 and HUVEC by ELISA, using commercial kits (Asserachrom t-PA and PAI-1, respectively, Roche Diagnostics, Monza, Italy) according to the manufacturer's instructions. The Asserachrom PAI-1 ELISA measures all the forms of circulating PAI-1, free or in complex with t-PA, bound or not to vitronectin, in an active or inactive state. Results for both antigens are expressed as μg/10^5 cells.

t-PA specific activity was measured in the CM by a commercial chromogenic assay (Coaset t-PA, Instrumentation Laboratory, Monza, Italy). To improve the specificity of the assay, t-PA activity was measured in EC samples before and after the addition of a greater than 98% inhibitory concentration of goat anti-human-tPA IgG (20 μg/mL, American Diagnostica Inc.). Results are expressed as IU/10^5 cells.

Thrombomodulin (TM)

TM antigen was measured in cells extracted in TRIS buffer (0.5×10^6 cells/mL of 50 mM TRIS, 100 mM NaCl and 1% Triton X-100, pH 7.5) for 2 hours at 4°C, followed by three cycles of freezing/thawing. The ELISA (Asserachrom TM, Roche) was carried out according to the manufacturer's instructions. Results are expressed as μg/10^5 cells.

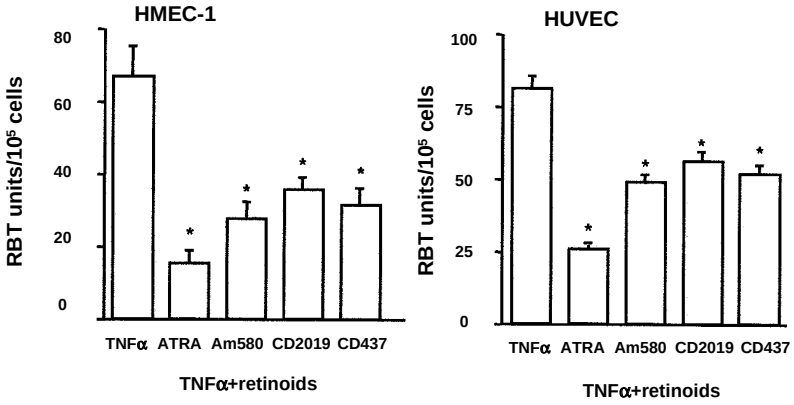

Figure 1. Effect of retinoids on TF procoagulant activity (TF-PCA) expression by HMEC-1 (left panel) and HUVEC (right panel). EC were incubated for 4h with 100 U/mL TNFα in the presence or absence of 1 μmol/L ATRA or 0.01 μmol/L synthetic retinoids; the cells were then tested for TF-PCA. *$p<0.05$ vs TNFα-treated cells.

Statistical analysis

The results are reported as mean ± SD of three independent experiments. Each experiment was conducted separately on cells prepared on different days; duplicate samples were treated with ATRA or synthetic retinoids and determinations of each parameter were performed in duplicate for each sample. At least three independent experiments were conducted. The mean value of each parameter was calculated for each experiment. The final results are the means of all the experiments. Student's paired and unpaired t-tests were used to determine the levels of significance between results of different treatments. Differences were considered statistically significant when $p<0.05$.

Results

ATRA decreases TF expression by HMEC-1

The first series of experiments analyzed the effect of increasing doses of ATRA and RAR-selective agonists on the expression of TF induced by TNFα. Figure 1 (left panel) shows that 1 μmol/L ATRA significantly counteracted the TNFα -induced TF-PCA in HMEC-1 (TNFα+ATRA vs TNFα: 17.5±2.8 vs 66.7±8.1 RBT units/10^5 cells; $p<0.01$). To determine which RAR subtype was responsible for the down-regulation in HMEC-1, we treated the cells with each of the three RAR-selective retinoids in the presence of TNFα. Inhibition of TNFα-induced TF-PCA was observed when HMEC-1 were exposed to increasing concentrations of the three synthetic RAR-selective agonists. At the final concentration of 0.01 μmol/L (the highest concentration of these compounds showing selective binding for the receptors) TF-PCA was significantly inhibited by the RARα agonist Am580 (27.3±4.7 RBT units/10^5 cells; $p<0.01$ vs TNFα), the RARβ agonist CD2019 (35.6±3.3 RBT units/10^5 cells; $p<0.01$ vs TNFα), and the RARγ agonist CD437 (31.5±4.3 RBT units/10^5 cells; $p<0.01$ vs TNFα) (Figure 1, left panel). In the same experiments, ATRA and the three RAR-selective agonists significantly decreased the TNFα-induced TF-PCA expression by HUVEC (Figure 1, right panel). Accordingly, all the retinoids significantly reduced the TF antigen levels induced by TNFα in HMEC-1 as well as in HUVEC (*data not shown*). These results were further confirmed by the measurement of TF activity performed with a specific chromogenic assay. Figure 2 (left panel) shows that ATRA and the RAR agonists counteracted the TNFα-induced TF chromogenic activity of HMEC-1 in a dose-dependent manner. At the highest retinoid concentration utilized, TF activity was inhibited 88±10.2% by ATRA ($p<0.01$), 83±7.3% by Am580 ($p<0.01$), 75±8.7% by CD2019 ($p<0.01$), and 68±7.2% by CD437 ($p<0.01$).

The role of RARs in TF regulation in HMEC-1 and HUVEC was confirmed by blocking the RARs with the pan-RAR antagonist CD3106. Pre-incubation of cells with this antagonist resulted in complete inhibition of the effects induced by ATRA and by each of the three RARs agonists (Table 1).

Northern blot analysis was performed in HMEC-1 to compare TF mRNA levels in cells treated with TNFα with those in cells treated with TNFα + 1 μmol/L ATRA or + 0.01 μmol/L RAR-selective agonists. Incubation with TNFα for 3 hours caused a significant increase in TF mRNA expression; in fact, as shown in Figure 2 (right panel), twice as much TF mRNA was found in cells incubated with TNFα than in untreated cells. This increase was inhibited by co-incubation with ATRA, as well as with the RAR α, β and γ, selective agonists (Figure 2, right panel).

ATRA induces the pro-fibrinolytic potential of HMEC-1

Expression of t-PA antigen. EC were incubated with 1 μmol/L ATRA for up to 24 hours. Thereafter,

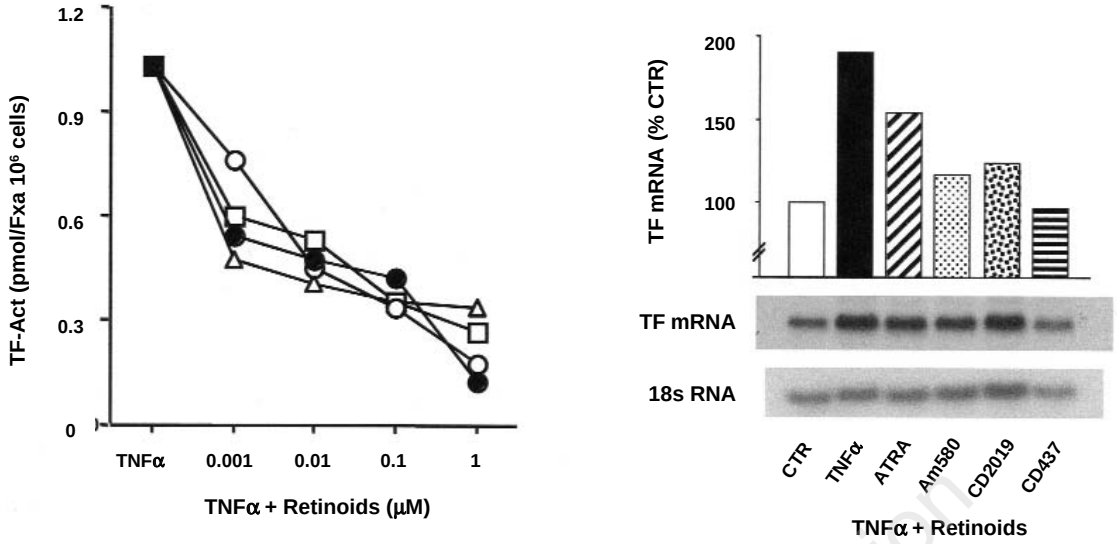

Figure 2. TF specific activity (left) and TF-mRNA (right) of HMEC-1 incubated with retinoids. Left: HMEC-1 were incubated for 4 hours with 100 U/mL TNFα in the presence or absence of increasing concentrations of ATRA (black circles), Am580 (open circles), CD2019 (squares) or CD437 (triangles); the lysates were then tested for TF activity by chromogenic assay. Right: Total RNA was extracted from cultured HMEC-1 after exposure to 100 U/mL TNFα in the presence or absence of 1 μmol/L ATRA or 0.01 μmol/L RAR agonists for 3 hours. Changes in TF-mRNA levels were assessed by Northern blot analysis as described in the Design and Methods section.

Table 1. Effect of the pan-RAR antagonist, CD3106, on TF-PCA expression by EC treated with TNFα ± retinoids.

	CD310	
	No	*Yes*
HMEC-1		
TNFα	74.3±8.7	72.1±7.9
TNFα + ATRA	40.4±3.1*	71.5±9.6§
TNFα + Am580	31.1±3.6*	72.5±8.2§
TNFα + CD2019	39.6±5.7*	73.5±9.1§
TNFα + CD437	33.9±4.8*	74.8±7.5§
HUVEC		
TNFα	82.9±4.7	78.1±6.5
TNFα + ATRA	47.2±3.8*	75.3±6.8§
TNFα + Am580	48.3±4.3*	79.2±7.3§
TNFα + CD2019	51.6±4.2*	76.7±5.6§
TNFα + CD437	50.5±5.1*	74.8±6.4§

*The effect of retinoids (0.01 μM) in reducing the TNFα-induced TF:PCA (RBT units/10^5 cells) expression was completely prevented, in both micro- and macro-vascular EC, by pre-incubating the EC with the pan-RAR antagonist, CD3106 (1 μM). *= p<0.05 vs TNFα; §=p<0.05 vs no CD3106. Data are expressed as mean ± SD.*

t-PA antigen levels were measured in the EC conditioned media (CM). As shown in Figure 3A, ATRA induced a time-dependent increase of t-PA antigen concentration in CM from HMEC-1. Starting from 18 hours' incubation, the levels of t-PA in CM collected from ATRA-treated cells were significantly higher ($p<0.05$) than those from the vehicle-treated control cells. The levels of t-PA antigen in HUVEC were significantly lower than those in HMEC-1.[27] These levels were significantly increased by ATRA treatment (Figure 3B). When increasing concentrations of ATRA (from 0.001 to 1 μmol/L) were added to HMEC-1 cultures, a dose-dependent increase of t-PA was evident at 24 hours' incubation, and was significant ($p<0.01$) at concentrations ≥ 0.001 μmol/L (Figure 3C). At 24 hours, ATRA increased t-PA levels of HUVEC in a dose-dependent way (Figure 3D). The incubation of HMEC-1 with Am580 produced a dose-dependent increase of t-PA expression (Figure 3C), reaching statistical significance from the concentration of 0.01 μmol/L upwards. In contrast, the RARβ agonist, CD2019, and the RARγ agonist, CD437, had virtually no effects. Similar results were obtained with EC from the macrovascular bed. Of the specific RAR agonists, only the RARα agonist, Am580, was able to raise t-PA levels of HUVEC significantly (Figure 3D), although to a lesser extent than ATRA. To confirm the role of RARα in t-PA regulation in HMEC-1 and HUVEC, we blocked RARα activity with the RARα antagonist, Ro 41-5253, 60 min prior to ATRA and Am580 treatment. Incubation with the RARα antagonist completely prevented the up-regulation of t-PA antigen expression induced by ATRA and Am580, indicating that the retinoid-induced t-PA expression relies promi-

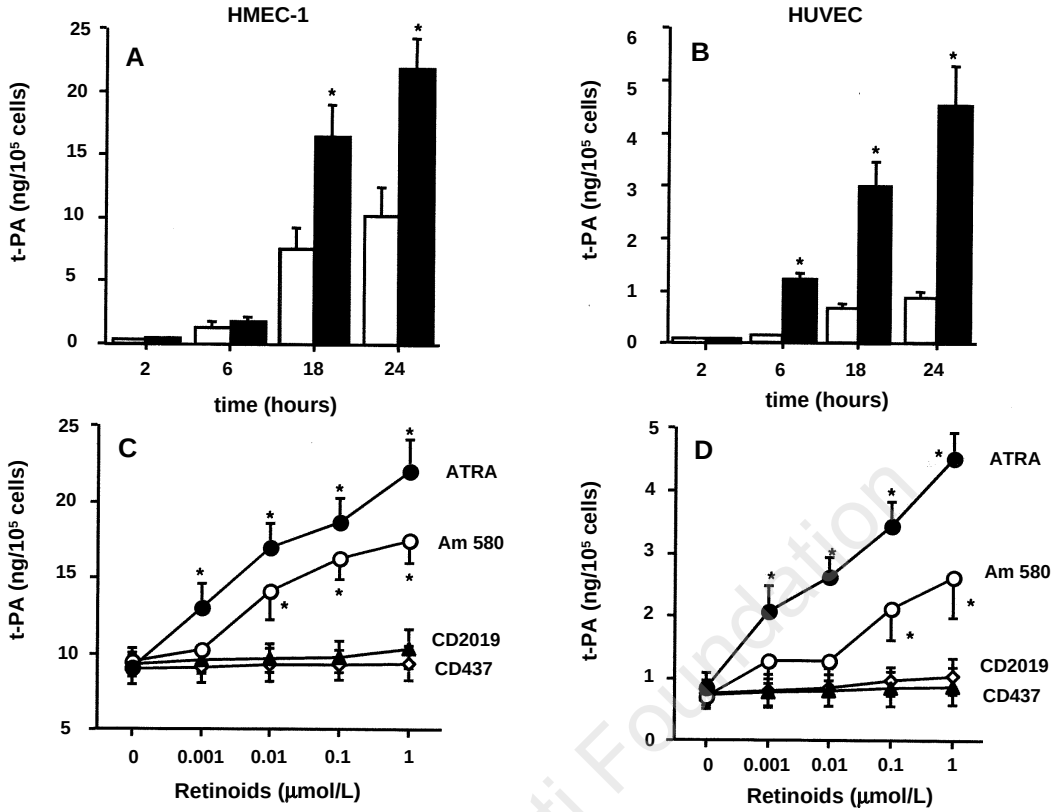

Figure 3. Effects of ATRA and RAR-selective synthetic agonists on t-PA antigen expression by HMEC-1 and HUVEC. Time-dependent induction of t-PA antigen in CM from HMEC-1 (A) and HUVEC (B) incubated for up to 24 hours with 1 μmol/L ATRA (black bars) or with the vehicle (open bars). t-PA antigen levels in CM of HMEC-1 (C) and HUVEC (D) incubated for 24 hours with increasing concentrations of ATRA (black circles), Am580 (open circles), CD2019 (squares) or CD437 (triangles). *=p<0.05 vs control cells.

Table 2. Effect of the RARα antagonist, Ro41-5253, on t-PA antigen expression by EC treated with ATRA or Am580±TNFα.

	RO41-5253	
	No	*Yes*
HMEC-1		
Control	9.0±1.2	9.2±0.9
+ATRA	18.2±1.9*	9.1±1.3§
+Am580	14.8±2.2*	9.3±0.8§
TNFα	9.6±0.9	9.5±0.7
TNFα + ATRA	15.0±2.1°	9.8±1.1§
TNFα+ Am580	14.2±1.9°	10.1±1.0§
HUVEC		
Control	0.85±0.07	0.9±0.05
+ATRA	2.8±0.2*	1.2±0.06§
+Am580	1.25±0.3	0.9±0.1§
TNFα	0.7±0.09	0.8±0.06
TNFα + ATRA	1.25±0.2°	0.75±0.08§
TNFα + Am580	1.15±0.15°	0.7±0.06§

*The increase in t-PA antigen levels (ng/10⁵ cells) induced by both the pan-RAR agonist, ATRA (0.01 μM), and RARα agonist, Am580 (0.01 μM), was significantly counteracted by pre-incubating the cells with the RARα specific antagonist, RO41-5253 (1 μM), both in the absence and presence of TNFα stimulus. *: p<0.05 vs control; °: p<0.05 vs TNFα; §: p<0.05 vs no Ro41-5253. Data are expressed as mean ± SD.*

nently on the activation of RARα (Table 2) in both cell types.

Expression of t-PA activity

To examine whether the ATRA- and Am580-induced increase of t-PA antigenic levels translated into actual increments of t-PA activity, we measured t-PA activity in the CM collected after 24 hours' incubation of EC with increasing concentrations of these two retinoids. As depicted in Figure 4 (left panel), a dose-dependent increment of t-PA activity was observed in CM from HMEC-1 treated with both ATRA and Am580. Similar results were obtained with HUVEC (Figure 4, right panel), confirming that ATRA and Am580 were able to induce dose-dependent increases of t-PA activity. These elevations reached statistical significance for both compounds.

Expression of PAI-1 antigen

The levels of the specific t-PA inhibitor, PAI-1, were measured by ELISA in the CM collected from HMEC-1 and HUVEC treated with 1 μmol/L ATRA

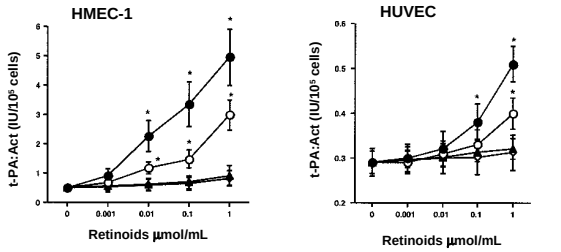

Figure 4. Effects of ATRA and of RAR-selective synthetic agonists on t-PA activity expression by HMEC-1 and HUVEC. t-PA activity in CM of HMEC-1 and HUVEC incubated for 24 hours with different concentrations of ATRA (black circles), Am580 (open circles), CD2019 (squares) or CD437 (triangles). * = $p<0.05$ vs control.

for up to 24 hours. ATRA did not significantly influence PAI-1 antigen levels of HMEC-1 or those of HUVEC (*data not shown*). Furthermore no changes were observed in the levels of this protein after treatment with the selective RAR agonists.

ATRA and Am580 counteracted the anti-fibrinolytic activity of TNFα on EC

To verify whether ATRA and Am580 could act on the endothelium by preventing the anti-fibrinolytic activity of TNFα, t-PA antigen and activity, and PAI-1 antigen were measured in CM after incubation of EC for up to 24 hours with each retinoid in the presence of TNFα. The upper part of Figure 5 shows the results in HMEC-1. TNFα did not significantly affect the levels of t-PA antigen compared to those in unstimulated control cells (Figure 5A). However, when cells were treated with TNFα in the presence of ATRA or Am580, a statistically significant ($p<0.05$) increase of t-PA antigen expression was observed. This t-PA increment was abolished by preincubating the cells with the RARα antagonist (Table 2). TNFα significantly induced PAI-1 antigen release from HMEC-1 ($p<0.05$) in a dose- and time-dependent way; ATRA and Am580 did not affect this increment (Figure 5B). Treatment of HMEC-1 with TNFα produced a significant inhibition of t-PA activity compared to that in control cells; both ATRA and Am580 were able to prevent this TNFα-induced reduction in t-PA activity (Fig-

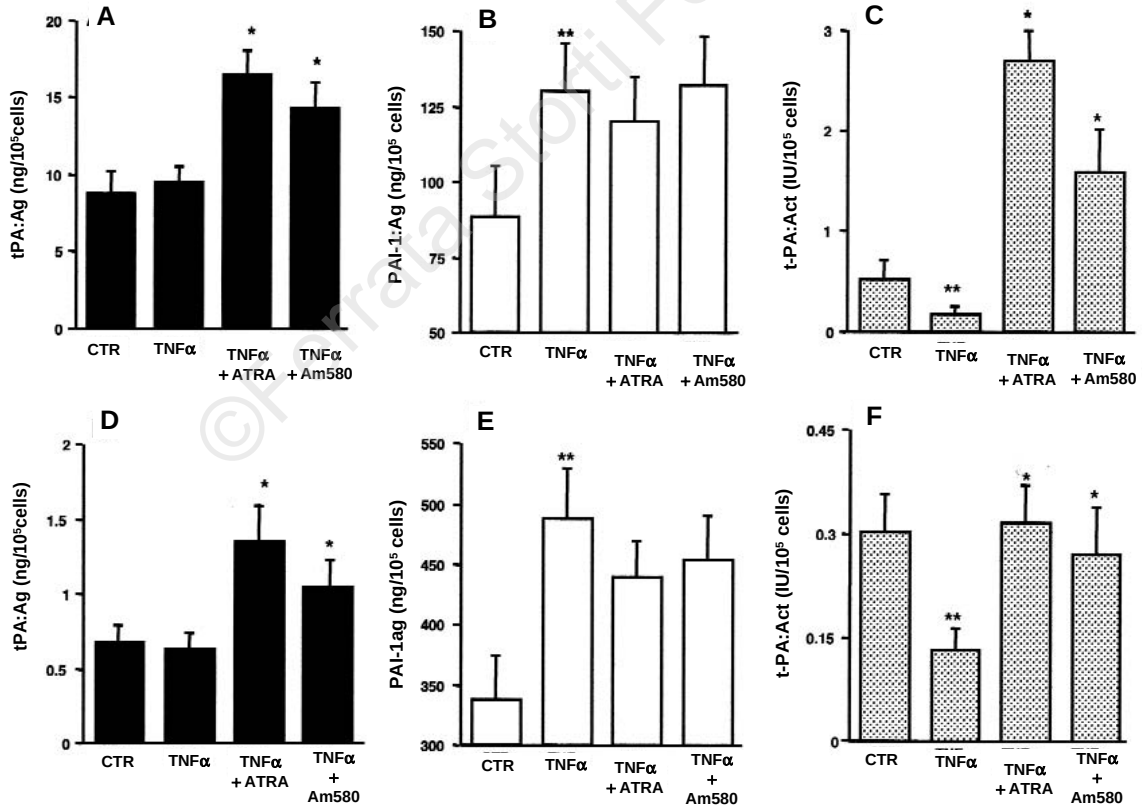

Figure 5. ATRA and Am580 counteracted the anti-fibrinolytic activity of TNFα. HMEC-1 (A - C) and HUVEC (D-F) were incubated for 24 hours with 100 U/mL TNFα in the presence or absence of 1 µmol/L of ATRA or 0.01 µmol/L Am580. The control was untreated (unstimulated) cells. CM were then collected and tested for t-PA antigen, PAI-1 antigen and t-PA activity. *$p<0.05$ vs TNFα, **$p<0.05$ vs control.

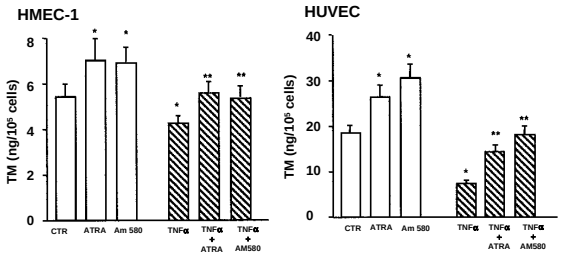

Figure 6. Effect of retinoids on TM expression by HMEC-1 (left) or HUVEC (right). EC were incubated for 24 hours with 1 μmol/L of ATRA or 0.01 μmol/L Am580 or vehicle alone (control), in the presence or absence of 100 U/mL TNFα. EC were then extracted and tested for TM antigen content by ELISA. *= *p*<0.05 vs control, ** = *p*<0.05 vs TNFα.

ure 5C). Results obtained from HUVEC, in the same experimental conditions, were consistent with those observed with HMEC-1 (Figure 5, lower panels). Incubation with TNFα for 24 hours did not influence the CM levels of t-PA antigen, compared to those in the untreated control cells, while the addition of ATRA and Am580 caused a significant ($p<0.05$) increase in t-PA antigen expression (Figure 5D). As observed in HMEC-1, the t-PA increase was prevented by preincubating the HUVEC with the RARα antagonist (Table 2). TNFα induced the release of PAI-1 antigen from HUVEC (in a dose- and time-dependent way), and ATRA and Am580 did not significantly modify this induction (Figure 5E). Analysis of t-PA activity showed that TNFα reduced the fibrinolytic activity of CM from HUVEC, while the presence of ATRA or Am580 significantly counteracted this effect and restored t-PA activity (Figure 5F).

ATRA enhances TM expression by HMEC-1

After 24 hours' incubation with ATRA, the levels of TM antigen in the extracts of both HMEC-1 and HUVEC increased in a dose-dependent manner, compared to the levels in the vehicle-treated cells. Among the RAR-selective agonists used, only Am580 was able to mimic the effect of ATRA to a similar extent (Figure 6), while the other two synthetic retinoids did not have significant effects (*data not shown*). Treating HMEC-1 and HUVEC with 100 U/mL TNFα induced a significant decrease in the levels of TM in both. This effect of TNFα was almost completely counteracted ($p<0.05$) by ATRA and Am580 (Figure 6). Incubation with the RARα antagonist, Ro 41-5253, completely prevented the up-regulation of TM antigen expression induced by ATRA and Am580, both in the absence and presence of TNFα. These data indicate that the retinoid-induced TM expression mainly involves RARα activation (Table 3).

Table 3. Effect of the RARα antagonist, Ro-41-5253, on TM antigen expression by EC treated with ATRA or Am580 ± TNFα.

	Ro-41-5253	
	No	*Yes*
HMEC-1		
Control	5.2±0.3	5.4±0.9
+ATRA	6.2±0.8*	5.3±1.1§
+Am580	6.9±0.7*	5.5±0.8§
TNFα	4.0±0.4*	4.2±0.7
TNFα + ATRA	5.2±0.4°	4.4±0.6§
TNFα + Am580	5.1±0.6°	4.2±0.6§
HUVEC		
Control	18.2±2.1	17.6±1.7
+ATRA	23.5±2.4*	18.5±2.1§
+Am580	28.5±2.8*	18.9±2.0§
TNFα	7.2±0.8*	7.3±0.7*
TNFα + ATRA	12.4±1.8°	7.8±0.4§
TNFα + Am580	16.6±1.7°	7.4±0.6§

*The RARα antagonist, RO41-5253 (1 μM), was able to inhibit significantly the increase of TM antigen levels (ng/10⁵ cells) induced by 0.01 μM ATRA or Am580, both in the absence and presence of TNFα. This effect was observed in both types of EC. *p<0.05 vs control; °p<0.05 vs TNFα; §p<0.05 vs no Ro-41-5253. Data are expressed as mean±SD.*

Discussion

ATRA inhibits proliferation of various tumor cells, but can also affect the hypercoagulable state associated with cancer.[1-3,8] So far ATRA is the only antitumor agent known to inhibit the activation of blood coagulation in these patients. Much understanding in this field has come from clinical and experimental studies of human APL. Indeed ATRA induces complete remission of human APL and a rapid resolution of the associated life-threatening coagulopathy.[1,2] *In vitro* and *in vivo* studies have shown that ATRA reduces the procoagulant potential of malignant cells[9,10] and modulates a number of hemostatic properties of normal endothelium[12-16] and monocytes.[25] Of particular interest is the demonstration that ATRA has a protective role against the procoagulant stimulus induced in vascular EC by tumor cell-derived cytokines.[12,13] In addition, ATRA directly enhances EC pro-fibrinolytic functions[15,16] and TM expression.[14] However, all the studies evaluating the impact of ATRA on endothelial hemostatic properties have involved EC isolated from the macro-vasculature (i.e. HUVEC), while no studies have been conducted on microvascular EC.

In the present study we describe for the first time the effects of ATRA on some hemostatic properties of microvascular endothelial cells. We utilized the HMEC-1, a dermal microvascular immortalized cell line, because: (i) it retains morphologic and functional characteristics of normal human microvacular EC;[20] (ii) we have experience with this specific microvascular cell model;[26] and (iii) the expres-

sion profile of some hemostatic properties by these cells is, at least, in part known.[27,28] In this study we further characterized these properties by quantifying, for the first time, HMEC-1 procoagulant and fibrinolytic activities by functional assays. Evaluating the impact of ATRA on microvascular EC is important because these cells may behave differently from those in large vessels.[17] Furthermore, the process of fibrin formation in the microvascular environment may have implications for both the biology of tumor progression and the thrombotic complications associated with cancer (including DIC and thrombotic microangiopathies).[25]

In addition, the study was performed in two experimental conditions, i.e. in the absence and presence of the pro-inflammatory stimulus, TNFα, because: (i) it has been observed that ATRA can directly induce the expression of t-PA and TM in HUVEC; (ii) ATRA can counteract the induction of TF and the reduction of TM induced by TNFα in HUVEC, and (iii) TNFα is one of the tumor-derived cytokines that can influence endothelial cell hemostatic properties.

The results of our study show that ATRA is able to enhance the antithrombotic potential of microvascular endothelial cells. This effect is achieved by multiple actions of ATRA on both procoagulant, fibrinolytic and anticoagulant properties of the endothelium. Concerning the procogulant activity, our data on HMEC-1 show that ATRA significantly counteracted TNFα-induced TF expression (evaluated as activity, antigen, and mRNA) in a dose-dependent manner. This was confirmed in parallel experiments in HUVEC. TF expression is modulated by ATRA in several cell types, including the NB4[22] and U937[29] leukemic cell lines, the MCF7 and MDA-MB-231 breast cancer cell lines,[7] and cells freshly isolated from leukemic patients.[9,30] In addition, ATRA selectively inhibits TF gene expression in normal human monocytes.[25] It is known that many of the effects of ATRA are mediated via nuclear receptors, which belong to the large superfamily of ligand activated transcription factors. The retinoic receptors sub-family consists of two groups of receptors, the RARs and the RXRs. These groups are each composed of three different members, the receptor subtypes α, β and γ: ATRA is a pan-RAR agonist, which binds to all these subtypes of RARs.[18] The development of synthetic retinoids with varying receptor affinity has provided a valuable tool for identifying the particular receptor or co-operation of receptors involved in a certain biological or pharmacological function.[19] Some authors have proposed that RARα and RARβ play a co-operative role in TF downregulation by retinoids in leukemic cells and HUVEC.[31] In our experiments inhibition of TF expression occurred at the level of transcription and was produced by ATRA as well as by all three synthetic retinoids, selective ligands of RARα, β and γ. The mechanism by which ATRA interferes with cytokine-induced TF expression is not completely understood. Inflammatory cytokines, including TNFα, induce TF expression by affecting TF promoter activity; this in turn activates gene transcription through LPS-response elements (LRE) containing two AP-1 and one NF-κB binding sites.[32] Some studies report that RARs bound to ATRA can inhibit gene expression through their ability to form non-productive complexes with transcription factors, thus inhibiting AP-1-mediated gene transcription.[33] Differently, other studies in human monocytes and in the monocytic THP-1 cell line support the hypothesis that TF inhibition occurs by specific mechanisms that do not involve repression of AP-1 or NF-κB mediated transcription.[25] Our results, indicating that inhibition of TNFα-induced TF expression involves all types of RARs and that blocking RAR activation with the pan-RAR antagonist CD3106 prevents this effect, favor the first hypothesis.

The vascular endothelium also plays an important role in determining plasma fibrinolytic activity by synthesizing both t-PA and its specific inhibitor PAI-1. Circulating t-PA, which is predominantly responsible for plasma fibrinolytic potential, is mainly derived from the vascular wall, where it is localized in the endothelial cells. It has been demonstrated that ATRA induces expression of t-PA in HUVEC,[15,34,36] without having a marked influence on PAI-1 synthesis. The induction of t-PA expression by ATRA in HUVEC depends on direct activation of the gene by ATRA and its receptors via corresponding retinoic acid responsive elements (RARE) in the promoter region.[36]

Our experiments showed that ATRA produced a time- and dose-dependent increase of t-PA antigen levels in CM from HMEC-1 and confirmed the same findings in HUVEC. ATRA did not significantly influence PAI-1 levels, confirming previous evidence from HUVEC.[35,36] The induction of t-PA in the absence of altered PAI-1 synthesis enhances the fibrinolytic potential of the endothelium. Accordingly, in our study the elevation of t-PA antigen in CM of ATRA-treated EC resulted in an increment of t-PA activity in both types of EC. In order to identify the RAR subtype(s) involved in t-PA induction by ATRA in HMEC-1, experiments were performed with the three synthetic RAR agonists. The results showed that the RARα agonist, Am580, determined a dose-dependent increase in t-PA expression, while the RARβ (CD2019), and the RARγ (CD437) agonists, did not have significant effects. Am580 was the only RAR agonist able to raise the t-PA levels significantly in HUVEC as well. Further ATRA and the RAR agonists did not significantly influence the PAI-1 antigen levels of either EC. The ATRA- and Am580-induced modifications of t-PA antigen levels resulted in variations of t-PA activ-

ity. These data are in agreement with findings previously reported by others on the involvement of RARα in ATRA-induced t-PA expression in HUVEC.[34,36] Further evidence of the involvement of RARα in retinoid-induced t-PA expression in both types of EC was provided by an inhibition study with the RARα selective antagonist, Ro41-5253. This compound completely suppressed the t-PA increment induced by ATRA and Am580.

We found that ATRA and the RARα agonist, Am580, could act on HMEC-1 as anti-inflammatory agents, by preventing the anti-fibrinolytic activity of TNFα (induction of PAI-1 production). In fact, when HMEC-1 were incubated with TNFα, PAI-1 levels increased significantly, while t-PA antigen levels were not affected. The increment of PAI-1 resulted in a significant decrease of t-PA activity. Our data show that both ATRA and Am580 were able to counteract this effect by inducing a significant increase in t-PA antigen and activity levels. Neither ATRA nor Am580 affected the TNFα-induced PAI-1 increase. Similar results were obtained in HUVEC. Therefore, this study demonstrates that ATRA increases the fibrinolytic capacity of microvascular endothelium both in the absence and the presence of a pro-inflammatory stimulus. The use of selective RAR agonists and antagonists suggests that RARα plays the predominant role in fibrinolysis modulation.

Finally, we explored the effect of ATRA and RAR-selective agonists in modulating the expression of endothelial TM, the surface high-affinity receptor for thrombin. The TM-thrombin complex activates circulating protein C, which in turn functions as a potent anticoagulant.[37] TF up-regulation and TM down-regulation lead to the vessel wall having a prothrombotic state. ATRA can directly induce the synthesis of TM in HUVEC.[14] This increase is mediated by the interaction of activated RAR to RARE in the TM gene promoter.[38,39] In addition, Ishii *et al.*[11] have demonstrated that ATRA protects the endothelium against the anti-TM effects of TNFα and IL-1β.

Our experiments demonstrated that ATRA dose-dependently increased TM expression also in the microvascular EC, HMEC-1. Among the RAR-selective agonists used, only Am580 was able to mimic the effect of ATRA to a similar extent. In addition, both ATRA and Am580 almost completely counteracted the decrement of TM induced by TNFα. Our data support the belief that RARα activation plays a major role in TM up-regulation, as demonstrated by others in leukemic cells.[31] The fact that ATRA inhibits the effects of cytokines and directly stimulates TM anticoagulant activity of macro- and micro-vascular EC provides further evidence of its likely anti-thrombotic effects in tumors and inflammatory diseases.

In conclusion, our data show that ATRA has a profound impact on the hemostatic properties not only of macrovascular EC but also of microvascular EC. This study with RAR selective agonists and antagonists indicates that RARα has a major role in modulating t-PA and TM expression, whereas all three types of receptors are involved in TF down-regulation. Induction of a biological effect by the agonist and suppression of the same effect by an antagonist provides evidence for active receptor-dependent pathways of regulation of the proteins.

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Pre-publication Report & Outcomes of Peer Review

Contributions

All authors directly participated in the conception, design, experimental work, data analysis, article drafting, critical revision, and final approval of the study.

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In the following paragraphs, Professor Vicente summarizes the peer-review process and its outcomes.

What is already known on this topic

All-trans-retinoic acid (ATRA) inhibits proliferation of various tumor cells. This activity has been associated with the ability to modulate the procoagulant and fibrinolytic properties of endothelial cells (EC) from large vessels.

What this study adds

This study provides evidence that ATRA is able to increase the antithrombotic potential also at the microvascular EC site, a relevant compartment for the tumor and/or antitumor therapy-associated vascular complications.