All-trans retinoic acid modulates microvascular endothelial cell hemostatic properties

MARINA MARCETTI, ALFONSO VIGNOLI, MARIA ROSA BANI, DONATELLA BALDUCCI, TIZIANO BARBUI, ANNA FALANGA

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 TNF-α-induced TF expression in HMEC-1 as well as HUVEC. ATRA increased t-PA antigen without significantly affecting PAI-1 expression, and counteracted the TNF-α-induced t-PA decrease in both types of EC. Accordingly, t-PA activity was significantly increased by ATRA, even in the presence of TNF-α. Finally, ATRA upregulated TM, and prevented TNF-α-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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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, 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 proinflammatory 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, 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 Rol41-5253 (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 transected human dermal microvascular EC line: 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, 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 x 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), scraped-harvested into 0.4 mL of PBS and, after 1:10
dilution in trypsin blue dye, cells were counted under a microscope in a Burrek chamber. Then EC aliquots were centrifuged and suspended at the concentration of 0.5 × 10⁶ 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 (i.e. t-PA antigen, t-PA activity, and PAI-1 antigen) for number of those that produced the corresponding CM.

**EC procoagulant activity**

Procoagulant activity (PCA) assay. TF-PCA was evaluated in EC lysates (0.5 × 10⁶ 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. 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 exposing (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 10⁻⁹) 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 × 10⁶ cells/mL) in Heps buffer (10 mM Heps, 137 mM NaCl, 4 mM KCl, 5 mg/mL ovalbumin, 2.5 mM CaCl₂, 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.

**TF antigen assay.** TF antigen levels were measured in EC extracted in TRIS buffer (0.5 × 10⁶ 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. Northern analysis was performed as already described. 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% dextran sulfate, 5x SSPE (1x SSPE: 0.15 M NaCl, 0.01 M NaH₂PO₄, 1 mM EDTA pH 7.4), 1x Denhardt's (Ficoll, polyvinylpyrrolidone and BSA, 0.2 mg/mL each), 0.8% SDS, 200 µg/mL denatured salmon sperm DNA, and 32P-labeled specific cDNA probe spanning the complete human TF protein coding region. 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 0.1x 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 32P-end labeled oligonucleotide specific for 18S rRNA as already described. 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/10⁵ cells.

The specific activity was measured in the CM by a commercial chromogenic assay (Coasert 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/10⁶ cells.

**Thrombomodulin (TM)**

TM antigen was measured in cells extracted in TRIS buffer (0.5 × 10⁶ 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/10⁵ 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/10⁵ 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/10⁵ cells; p<0.01 vs TNFα), the RARβ agonist CD2019 (35.6±3.3 RBT units/10⁵ cells; p<0.01 vs TNFα), and the RARγ agonist CD437 (31.5±4.3 RBT units/10⁵ cells; p<0.01 vs TNFα) (Figure 1, left panel). In the same experiments, ATRA and the three RAR-selective agonists significantly decreased 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,
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 RAR β agonist, CD2019, and the RAR γ 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-
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 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.

<table>
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<th>HMEC-1</th>
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<td>Yes</td>
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<tr>
<td>Control</td>
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<td>+ATRA</td>
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<td>2.8±0.2*</td>
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<td>+Am580</td>
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<td>1.25±0.2*</td>
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<tr>
<td>TNFα + Am580</td>
<td>14.2±1.9*</td>
<td>1.15±0.15*</td>
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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.
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-

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**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).** *

**Figure 5. ATRA and Am580 counteracted the anti-fibrinolytic activity of TNFα on EC.**

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.

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**Table 2.**

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<th>tPA:Ag (ng/10⁵cells)</th>
<th>PAI-1:Ag (ng/10⁵cells)</th>
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**Figure 5A.** ATRA and Am580 counteracted the anti-fibrinolytic activity of TNFα on EC. HMEC-1 (A - C) and HUVEC (D-F) were incubated for 24 hours with various concentrations of ATRA (black circles), Am580 (open circles), CD2019 (squares) or CD437 (triangles). * = p<0.05 vs control.
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).

**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 anti-tumor 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 *vivo* and *in vitro* studies have shown that ATRA reduces the procoagulant potential of malignant cells5,10 and modulates a number of hemostatic properties of normal endothelium12–16 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 functions15,16 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 model;26 and (iii) the expres-
A co-operative role in TF downregulation by the authors have proposed that RAR agonist, ATRA as well as by all three synthetic retinoids, experiments inhibition of TF expression occurred in human monocytes. It is known that many of the effects of ATRA are mediated via nuclear receptors, which belong to the large superfamily of ligand-dependent transcription factors, thus inhibiting TNF-induced expression. ATRA selectively inhibits TF gene expression in normal human endothelial cells. 

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 ATRA receptors, which belong to the large superfamily of ligand-dependent transcription factors, thus inhibiting TNF-induced expression. ATRA selectively inhibits TF gene expression in normal human endothelial cells. It is known that many of the effects of ATRA are mediated via nuclear receptors, which belong to the large superfamily of ligand-dependent transcription factors. The retinoic acid receptors subfamily consists of two groups of receptors, the RARs and the RXRs. These groups are each composed of three different members, the RARα, RARβ and RARγ. ATRA is a pan-RAR agonist, which binds to all these subtypes of RARs. 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. Some authors have proposed that RARα and RARβ play a co-operative role in TF downregulation by retinoids in leukemic cells and HUVEC. 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. 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. 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. 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 potential 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, 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.

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. 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-
circulating protein C, which in turn functions as a cofactor for thrombin. The TM-thrombin complex activates a selective agonist in modulating the expression of TF.

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 vitro and in vivo, 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. 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. This increase is mediated by the interaction of activated RAR to RARE. In addition, Ishii et al. 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. 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.

References


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.