Stimulation of Tissue Plasminogen Activator Production by Retinoic Acid: Synergistic Effect on Protein Kinase C–Mediated Activation

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Trans retinoic acid (t-RA) stimulated the production of tissue plasminogen activator (tPA) in HeLa-S_3 and human umbilical vein endothelial cells (huvecs) in a dose-dependent manner with maximal release (four to five times control) at 40 nmol/L and 40 μmol/L, respectively. In endothelial cells, the stimulation of tPA production by phorbol 12-myristate 13-acetate (PMA) was potentiated 1.9-fold by 10 μmol/L t-RA, or 1.8 times the additive effect. In HeLa cells, total tPA secretion with 10 nmol/L PMA was increased from 43 ng/mL to 96 ng/mL by 40 nmol/L t-RA, which was two times the additive effect. Higher concentrations of t-RA (400 nmol/L) depressed tPA secretion by itself and also suppressed PMA-induced tPA production by 50%. Histamine and thrombin also synergized with t-RA. t-RA (40 nmol/L) and 10 μg/mL histamine or 10 U/mL thrombin combined to induce tPA production 3.4 and 1.3 times the additive effect in HeLa cells. Cyclic adenosine monophosphate (cAMP) levels were not significantly affected by 10 nmol/L to 10 μmol/L t-RA. Nor did 10 nmol/L PMA and 40 nmol/L t-RA together affect cAMP levels, suggesting that t-RA–mediated potentiation of PMA-induced tPA production occurred via a mechanism that was independent of cAMP levels. Downregulation of protein kinase C (PKC) by pretreatment of huvecs with 100 nmol/L PMA completely blocked a secondary response to PMA, but did not have a significant effect on t-RA induction. Pretreatment with 10 μmol/L t-RA, on the other hand, did not significantly affect a secondary stimulus by 100 nmol/L PMA, but completely suppressed a secondary stimulation by 10 μmol/L t-RA alone. These studies suggest that the mechanism mediating t-RA stimulation of tPA production interacts with the PKC pathway, resulting in synergism.

FIBRINOLYSIS is a complex process that results in the dissolution of a fibrin clot by the proteolytic enzyme plasmin. Plasmin is formed from its inactive proenzyme, plasminogen, after specific cleavage of a single peptide bond by plasminogen activators. The two physiologically relevant forms of plasminogen activators are tissue plasminogen activator (tPA) and urinary plasminogen activator (uPA). Because of its affinity for and activation by fibrin, tPA is considered to be the plasminogen activator primarily responsible for blood clot lysis.[1,2] The production of tPA in vitro is regulated by several factors including thrombin, histamine, tumor-promoting phorbol esters, hormones, and growth factors.[3-5] In human endothelial cells and HeLa cells, the elevation of tPA mRNA precedes the increase in tPA antigen secretion[6,7] and is associated with the activation of protein kinase C (PKC).[6,7] Phorbol ester–stimulated tPA production can be further regulated by cyclic adenosine monophosphate (cAMP), which, while ineffective by itself, potentiates the phorbol ester–dependent stimulation another fivefold.[8] Thus, an interaction between the cAMP and PKC pathways leads to a synergistic effect on tPA production. Similar interactions between PKC and other signaling molecules may also be important for the regulation of tPA production under various physiologic or pathologic states.

Vitamin A and its derivatives are known to affect cellular morphology, development, and differentiation by mechanisms that are not clearly defined.[9-12] Several reports suggest that vitamin A and other retinoids also stimulate tPA production.[13-16] However, unlike other known agonists of tPA production, retinoids act via intracellular receptors that belong to the family of nuclear receptors that includes the receptors for steroid and thyroid hormones.[17-20] In contrast to cell surface receptors, which initiate specific signaling pathways leading to the activation of gene expression, these receptors bind directly to specific nucleotide sequences on target genes.[21-23] Therefore, regulation of tPA production by retinoids may involve a pathway distinct from those used by the other known tPA agonists. An analysis of the mechanisms that mediate retinoid response and how they influence response to other agonists can contribute to our understanding of the complex series of events that precede stimulated tPA production. A complete understanding of the events leading to tPA production and the factors affecting it can enable the development of a system that can potentially be used to manipulate the levels of fibrinolytic activity in vivo.

Using HeLa and endothelial cells as model systems, we have evaluated various aspects of the effect of retinoids on tPA production. We report that (1) the stimulation of tPA secretion by *trans* retinoic acid (t-RA) in both cell types is dose-dependent with kinetics of release similar to that for other agonists; (2) t-RA potentiates phorbol 12-myristate 13-acetate (PMA)-, histamine-, and thrombin-induced tPA production; and (3) t-RA pretreatment results in desensitization to the homologous agonist, but does not affect heterologous agonist action significantly. These studies suggest a distinct mechanism for t-RA–mediated stimulation of tPA secretion, with possible interaction with the PKC pathway.

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MATERIALS AND METHODS

Cell culture. HeLa-S_3 cells obtained from ATCC (Rockville, MD) were grown in RPMI 1640 containing 10% newborn calf serum, 200 U/mL penicillin, 200 μg/mL streptomycin, 500 ng/mL fungizone, and 2 mmol/L glutamine (all from Whittaker Bioproducts, Walkersville, MD). For treatment with various agonists, cells were cultured into 24-well tissue culture dishes (Corning, NY) until confluent, washed twice with RPMI 1640, and incubated at 37°C in 500 μL of 5% NuSerum (Collaborative Research, Waltham, MA; final concentration, 1.25% newborn calf serum) in RPMI 1640 containing t-RA (Sigma Chemical, St Louis, MO), PMA (Calbiochem, La Jolla, CA), histamine (Calbiochem), forskolin (Calbiochem), or thrombin (a gift from Dr J.W. Fenton, II, Department of Health, Albany, NY). Stock solutions of retinoic acids were prepared in DMSO at 10^{-2} mol/L and stored at −20°C. Before use, the retinoic acid was diluted into culture medium. Control cultures were exposed to an identical final concentration of DMSO that never exceeded 0.1%.

Endothelial cells were isolated from human umbilical cord veins as described previously[4] and cultured into 20 mg/mL gelatin-(Eastman Kodak, Rochester, NY) coated tissue culture flasks (Corning) in RPMI 1640 containing 10% fetal calf serum, 200 U/mL penicillin, 200 μg/mL streptomycin, 500 ng/mL fungizone, 10 μg/mL endothelial cell growth factor (ECGF) (Biomedical Technologies, Stoughton, MA), 90 μg/mL heparin (Sigma), and 2 mmol/L glutamine. Studies were performed on secondary cultures grown to confluence in 12-well dishes (Corning) under the same conditions as primary cultures, except that 50 μg/mL ECGF was used. Cells were washed twice with RPMI 1640 and incubated at 37°C with 500 μL of t-RA, PMA, histamine, forskolin, or thrombin in RPMI 1640 containing 5% NuSerum, 50 μg/mL ECGF, and 90 μg/mL heparin.

The culture supernatants were collected after the appropriate incubation period, centrifuged at 15,000*g* to remove cell debris, made to 0.01% Tween-80 (Sigma), and frozen at −70°C until used. For experiments involving downregulation, cells were treated with the primary agonist for 16 hours, after which they were washed three times with RPMI 1640. Cells were then incubated with the secondary agonist for 24 hours and supernatants collected for tPA measurements by enzyme-linked immunoadsorbent assay (ELISA).

ELISA. Ninety-six-well microtiter plates (Dynatech Laboratories, Chantilly, VA) were coated with polyclonal rabbit anti-human tPA IgG[4] by incubating 150 μL/well of a solution of 10 μg/mL IgG in 50 mmol/L sodium borate, pH 9.0, overnight at 4°C. The coated plates were washed three times with wash buffer (phosphate-buffered saline [PBS], 0.05% Tween-20 [Mallinkrodt, Paris, KY], 0.1% albumin [Calbiochem], 0.01% thimerosal [Sigma]), and stored at −20°C until used. For assays, standards (25 ng to 0.2 ng/mL), as well as samples, in a final volume of 100 μL of 5% NuSerum in RPMI 1640 were added to antibody-coated wells. Twenty microliters of horseradish peroxidase–conjugated monoclonal antibody to human tPA (Corvas Pharmaceuticals, La Jolla, CA) diluted 1:50 into PBS-1% albumin was added to each well, and plates were incubated for 1.5 hours on a tilting table at room temperature. The samples were removed and the plates were washed three times with distilled water to remove excess antibody. One hundred microliters of 0.12 mg/mL 3,3′,5,5′-tetramethylbenzidine (Sigma) and 0.012% H_2O_2 (Mallinkrodt) in 0.1 mol/L sodium acetate, pH 4.5, was added to each well for the chromogenic reaction to occur. Plates were covered with aluminum foil for 10 minutes. The reaction was terminated by the addition of 100 μL of 1N H_2SO_4 per well. The absorbance was measured on an ELISA plate reader (Molecular Devices, Menlo Park, CA) at 450 nm and standard curves were analyzed by logit transformation and linear regression analysis of logit percent band counts versus log tPA concentration.

The extent of stimulation of tPA secretion by various agonists was calculated either in terms of fold stimulation or net increase in tPA secretion. To determine the fold increase, tPA concentrations measured after agonist treatment were divided by the value obtained from control samples (5% NuSerum treatments). To calculate net increase in tPA secretion, tPA levels in control cultures were deducted from those obtained after agonist treatment under identical conditions. The "expected additive effect" of two different agonists was determined by adding the net amount of tPA secreted in cultures treated with each agonist individually. Synergism is defined as occurring when tPA values, on cotreatment with two agonists, exceed the additive value.

In desensitization experiments, where sequential agonist treatment was required, the residual effect of a primary agonist on tPA secretion was determined using 5% NuSerum for the secondary treatment. The residual response was used as a correction factor when interpreting the effect of the second agonist during sequential treatments. Again, the actual ng tPA secreted in response to each agonist was determined by deducting the ng tPA secreted in response to 5% NuSerum alone. Statistical analysis was performed using Wilcoxon's two-tailed rank test.

cAMP assays. Confluent cultures in 75-cm^2 flasks were incubated with the appropriate agonist for 10 minutes, the medium was aspirated, and the cells washed once with ice-cold PBS. The cells were extracted for 2 hours with 3 mL of chilled *n*-propyl alcohol at 4°C. The extracts were transferred to tubes, centrifuged at 12,000*g* to remove cell debris, evaporated under a stream of N_2 at 55°C, and stored at −70°C until used. The extract was reconstituted in 200 μL of 50 mmol/L Tris-HCl, pH 7.4, containing 4 mmol/L EDTA, centrifuged to remove particulate matter, and supernatant used for radiometric assays using a cAMP assay kit from Amersham International (Arlington Heights, IL). Results were calculated as picomoles cAMP per flask of cells, and are represented as fold increase over control flasks, which were treated with 5% NuSerum alone.

RESULTS

Effect of t-RA on tPA secretion. The effect of increasing concentrations of t-RA on the ability of HeLa and human endothelial cells to secrete tPA was evaluated. Picomolar concentrations did not significantly affect tPA secretion in either cell type. At higher concentrations (≥40 nmol/L), there was a steady dose-dependent increase in tPA secretion in endothelial cells (Fig 1), with an average maximum increase of 3.9 times (range, twofold to fivefold) at 40 μmol/L. HeLa cells showed a dose-dependent increase in tPA secretion up to 4.7 times control cultures at 40 nmol/L t-RA (range, 2- to 10-fold). At higher concentrations, there was a rapid decline in the amount of tPA antigen secreted. The viability of both cell types, as evident by trypan blue exclusion, was not significantly affected at any of the t-RA concentrations used, suggesting that the decrease in tPA secretion in HeLa cells at higher concentrations was not due to cell death. At concentrations greater than 40 μmol/L, there was significant cell detachment, restricting the feasibility of using higher concentrations. Thus, both HeLa cells and endothelial cells respond to various concentrations of retinoic acids by increasing tPA production. However, while the effective concentrations observed in the HeLa cell experiments were physiologic, the doses needed

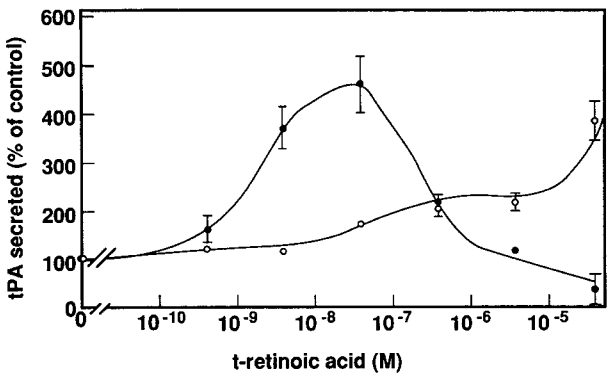

Fig 1. Dose-dependent stimulation of tPA secretion by t-RA. Confluent cultures of once-passaged human umbilical vein endothelial cells (○) or HeLa cells (●) were incubated in 5% NuSerum containing the indicated concentration of t-RA for 20 hours. Control cells were treated with 5% NuSerum alone. tPA antigen levels in supernatants were determined by ELISA. Each data point represents the mean ± SD of triplicate wells assayed in duplicate.

to promote tPA production in endothelial cells exceeded that range.

Effect of t-RA on agonist-induced tPA secretion. We have previously demonstrated that the elevation of cAMP potentiates the phorbol ester–stimulated production of tPA[8] and proposed that tPA production may be regulated by the interaction of different signaling pathways. To determine whether the mechanism of t-RA induction may also interact with other agonists of tPA production, endothelial and HeLa cells were treated with 10 μg/mL histamine or 100 nmol/L PMA in the presence and absence of increasing concentrations of t-RA. In endothelial cells (Fig 2), the addition of 10 μmol/L t-RA with 100 nmol/L PMA increased the level of tPA secreted from 70.1 to 135.4 ng/mL, a concentration 1.8 times the expected additive

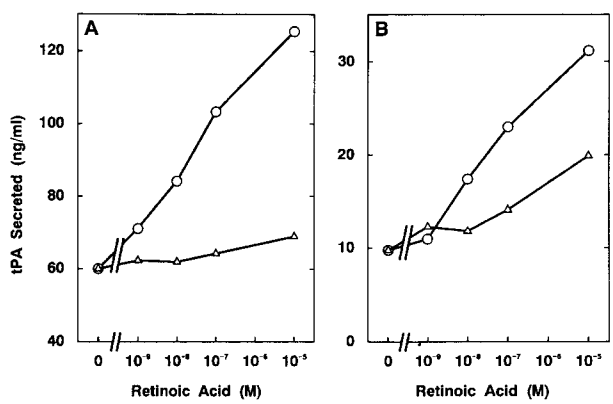

Fig 2. Synergistic effect between retinoic acid and PMA/histamine. Endothelial cells were treated with t-RA (4 nmol/L to 40 μmol/L) in the absence or presence of (A) 100 nmol/L PMA or (B) 10 ng/mL histamine (○). Expected additive tPA secretion (△) is the sum of tPA produced by treatment with PMA/histamine alone and the indicated t-RA concentration. Cells were treated with the appropriate agonist for 20 hours and tPA production measured by assaying culture supernatants by ELISA. Each data point represents the average of duplicate wells assayed in duplicate.

level. This synergistic effect was dose-dependent and was observed with as little as 1×10^{-8} mol/L t-RA (1.4 times additive), despite the fact that this concentration itself had minimal effect on tPA secretion. Thus, it appears that endothelial cells were more sensitive to lower doses of t-RA during activation with phorbol esters than by themselves. This same effect was also observed when PMA was replaced with 10 μg/mL histamine. Combined treatment with 1×10^{-8} mol/L t-RA and histamine induced secretion of tPA which was 1.5 times the expected additive effect, and which increased to 1.7 times at 1×10^{-5} mol/L t-RA.

The effect of t-RA along the entire PMA dose-response curve was examined in HeLa cells. PMA induces tPA secretion in HeLa cells in a dose-dependent manner, with maximal secretion of 48 ng/mL at 100 nmol/L PMA (Fig 3). Addition of t-RA (40 nmol/L) with each PMA concentration enhanced tPA secretion in a synergistic manner, but only within the effective PMA dose range. No effect was observed at substimulatory levels. The lowest PMA concentration that was significantly affected by t-RA was 1 nmol/L. At 100 nmol/L PMA, addition of 40 nmol/L t-RA caused a 1.7-fold increase in PMA-induced tPA secretion, which was 1.5 times the expected additive values. However, t-RA had the greatest effect at a PMA concentration of 10 nmol/L (96 ng/mL, 2.1 times the expected additive effect). Thus, peak tPA secretion occurs at a lower concentration of PMA (10 times less) in the presence of retinoic acid. In the presence of 400 nmol/L t-RA, there was a suppression of PMA-induced tPA secretion to approximately 20% to 50% of that caused by PMA alone (Fig 3). This is the t-RA concentration at which tPA secretion starts declining in the t-RA dose-response curve (Fig 1).

The effect of histamine and thrombin on the t-RA dose-response curve was also examined in HeLa cells (Fig 4). The synergistic effect was observed at all concentrations of t-RA (400 pmol/L to 4 μmol/L), although the degree of synergism increased with histamine from 1.7 times the expected additive effect at 400 pmol/L to 3.4 times the

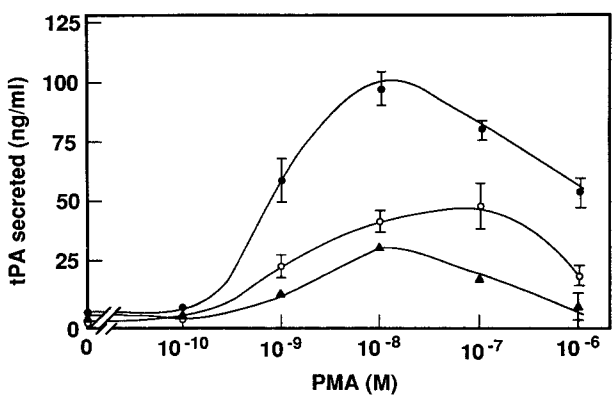

Fig 3. Effect of t-RA on PMA-induced tPA secretion. Confluent cultures of HeLa cells were incubated in 5% NuSerum containing the indicated concentrations of PMA in the absence (○) or presence of 40 nmol/L (●) or 400 nmol/L (▲) t-RA for 20 hours. Supernatants were collected and tPA antigen levels were determined by ELISA. Each data point represents the mean ± SD of duplicate assays of triplicate wells.

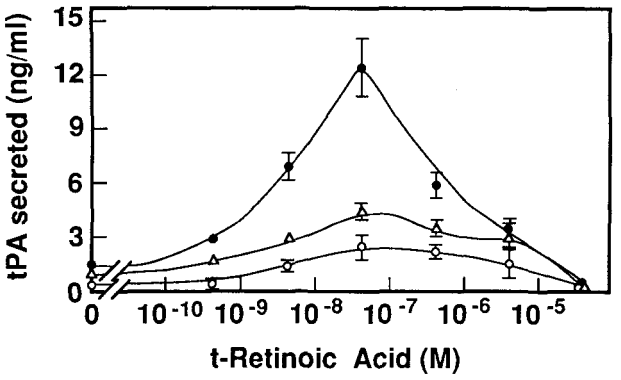

Fig 4. Dose-dependent potentiation of agonist-induced tPA secretion by t-RA. HeLa cells were incubated in 5% NuSerum containing the indicated concentrations of t-RA in the absence (○) or presence of 10 μg/mL histamine (●) or 10 U/mL thrombin (△). Culture supernatants were estimated for the level of tPA antigen by ELISA. Each data point is the mean ± SD of triplicate sets of wells assayed in duplicate.

additive values at the peak of the t-RA dose-titration curve (40 nmol/L). Synergism between t-RA and thrombin was consistent with t-RA plus thrombin, producing a 1.3-fold increase over additive values along the entire curve.

Effect of t-RA on cAMP levels. Previous studies have shown that cAMP acts synergistically with PMA in the production of tPA.[8] To determine if the effect of t-RA on PMA-induced tPA production was mediated by the elevation of cAMP, HeLa cells were incubated with various concentrations of t-RA or forskolin, or t-RA + forskolin for 10 minutes, and the cAMP levels determined. Treatment with forskolin, a known cAMP-elevating compound, resulted in a dose-dependent increase in cAMP (Fig 5A), with 100 μmol/L stimulating a 10-fold increase (4.32 to 45.36 pmol). Treatment with 10 nmol/L to 100 μmol/L t-RA, on the other hand, did not result in cAMP levels significantly different from control levels (Fig 5A). Increasing the duration of incubation in the presence of t-RA up to 6 hours also did not significantly affect cAMP levels (data not presented), which ruled out the possibility of a delayed response. In fact, when cells were incubated with 10 or 100 μmol/L forskolin in the presence of 100 nmol/L t-RA, there was a 40% suppression of the forskolin-mediated elevation of cAMP (Fig 5A). As expected, 10 nmol/L PMA alone did not affect cAMP levels; however, its coincubation with 100 μmol/L forskolin potentiated the forskolin-mediated elevation of cAMP 1.8-fold (Fig 5B). These are the concentrations of PMA and forskolin that synergistically stimulate maximum tPA secretion. When 10 nmol/L PMA and 40 nmol/L t-RA, concentrations that cause synergistic tPA stimulation, were added simultaneously, there was no effect on cAMP levels (Fig 5B). These results suggest that t-RA–mediated increase in tPA production is independent of cAMP levels.

Time course of tPA secretion. The kinetics of tPA release in response to t-RA, PMA, t-RA + PMA, and PMA + forskolin were analyzed (Fig 6). The time courses of tPA secretion in treated cultures were similar and showed a 8-hour lag period, followed by increased secretion that persisted throughout the 24-hour monitoring period. At 24 hours after incubation, 40 nmol/L t-RA, 10 nmol/L PMA, 40 nmol/L t-RA + 10 nmol/L PMA, and 10 nmol/L PMA + 100 μmol/L forskolin-treated cells secreted 2.7-, 78.6-, 143.9-, and 144.4-fold higher tPA, respectively, as compared with 5% NuSerum-treated cells.

Homologous downregulation of tPA secretion. PKC antigen and activity can be downregulated after prolonged exposure to tumor-promoting phorbol esters.[24] Previous studies have determined that PMA, histamine, and thrombin pretreatment of endothelial cells results in desensitization to a homologous secondary stimulus.[24] This property was used to compare the mechanisms of PMA-, histamine-, and thrombin-induced tPA secretion versus t-RA–induced tPA secretion. Endothelial cells were treated with primary agonist for 16 hours, the medium removed, and a second agonist added for an additional 16 hours (agonist > agonist; Table 1). tPA accumulation during the second phase was measured. Pretreatment with NuSerum followed by secondary treatment with t-RA or PMA induced net tPA secretion of 14.7 and 45.6 ng/mL, ie, 2.2 and 4.7 times control, respectively. After pretreatment with t-RA or PMA followed by NuSerum, residual secretion from the primary response was 5.7 and 18.53 ng/mL or 1.5 and 2.5 times

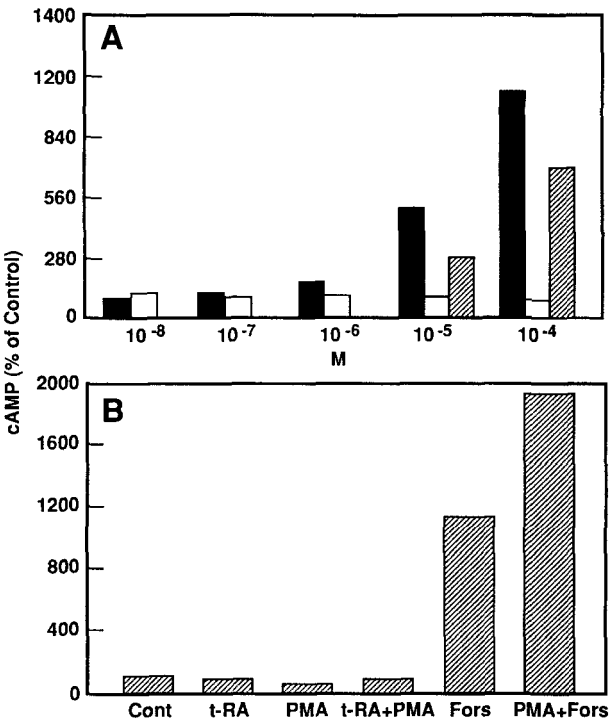

Fig 5. cAMP levels in cells treated with various agonists. Cultures of HeLa cells in 75-cm² flasks were incubated with 5% NuSerum containing the indicated concentrations of (A) forskolin (■), t-RA (□), or 100 nmol/L t-RA + forskolin (▨); or (B) 10 nmol/L PMA, 10 nmol/L PMA + 10 nmol/L t-RA, 100 μmol/L forskolin, or 10 μmol/L PMA + 100 μmol/L forskolin for 10 minutes at 37°C. The cells were washed twice with ice-cold PBS and extracted with *n*-propanol for 1 hour at 4°C. The extracts were dried and the residue assayed for cAMP as described in Materials and Methods. All values represent the mean of two determinations.

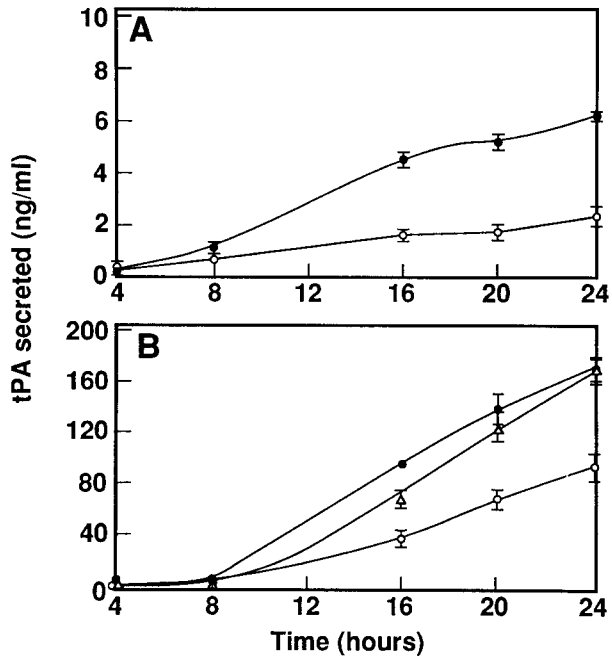

Fig 6. Time course of tPA secretion in response to various agonists. Cultures of HeLa cells were incubated at 37°C in 5% NuSerum containing the appropriate agonist for the indicated period of time. Supernatants were collected and assayed for tPA antigen levels by ELISA. (A) Control (○), 40 nmol/L t-RA (●); (B) 10 nmol/L PMA (○), 10 nmol/L PMA + 40 nmol/L t-RA (●), 10 nmol/L PMA + 100 μmol/L forskolin (△). All values represent the mean ± SD of quadruplicate sets assayed in duplicate.

control. When both primary and secondary treatments were with the same agonist (PMA > PMA or t-RA > t-RA), the level of tPA secreted was only 90% of the level secreted if no secondary addition was performed (agonist > NuSerum),

Table 1. Homologous Downregulation of tPA Secretion in Endothelial Cells

First Treatment (16 h)	Second Treatment (24 h)	tPA (ng/mL)
1. 5% NuSerum	5% NuSerum	12.32 ± 1.16
2. 5% NuSerum	10^{-5} mol/L t-RA	27.06 ± 5.18
3. 5% NuSerum	10^{-7} mol/L PMA	57.98 ± 10.96
4. 10^{-5} mol/L t-RA	5% NuSerum	17.99 ± 5.05
5. 10^{-7} mol/L PMA	5% NuSerum	30.85 ± 4.21
6. 10^{-5} mol/L t-RA	10^{-5} mol/L t-RA	16.90 ± 1.06
7. 10^{-7} mol/L PMA	10^{-7} mol/L PMA	27.59 ± 3.19
8. 10^{-5} mol/L t-RA	10^{-7} mol/L PMA	79.69 ± 2.97
9. 10^{-7} mol/L PMA	10^{-5} mol/L t-RA	45.11 ± 8.18

Confluent cultures of endothelial cells were pretreated with the indicated agonist in 5% NuSerum for 16 hours. Culture supernatants were collected and cells were washed three times with RPMI 1640 to remove residual agonist. This was followed by incubation with the indicated secondary agonist for 24 hours. Culture supernatants following secondary treatment were collected and assayed for tPA antigen levels by ELISA. Values represent data from three experiments performed in duplicate. The actual ng/mL tPA levels from individual experiments were normalized to values from one experiment to correct for variability in the efficiency of basal tPA secretion from different endothelial cell preparations.

indicating that the cells were not responsive to secondary treatment of the same agonist. When t-RA pretreatment was followed by secondary treatment with PMA, net tPA secretion was 67.4 ng/mL or 6.5 times control. This value is 1.3 times that expected from an additive effect of residual tPA release after pretreatment with t-RA (t-RA > 5% NuSerum, net tPA = 5.7 ng/mL) plus secondary PMA treatment (5% NuSerum > PMA, net tPA = 45.7), ie, 51.4. This difference was statistically significant ($P = .002$), indicating that t-RA pretreatment does not desensitize cells to a secondary stimulus with PMA and may continue to have a synergistic effect. If the cells were first treated with PMA and then t-RA, net tPA secretion was 32.8 ng/mL. This was comparable to the expected additive value (33.3 ng/mL) of PMA > 5% NuSerum (net tPA = 18.5 ng/mL) and t-RA > 5% NuSerum (net tPA = 14.7 ng/mL). The small difference was not significant ($P > .5$) and it appears that PMA does not downregulate endothelial cells to t-RA–induced tPA production.

DISCUSSION

This report demonstrates that t-RA promotes enhanced tPA production and potentiates the effects of PMA, histamine and thrombin in a synergistic manner. This synergism does not appear to be mediated by the activation of the cAMP-dependent pathway, which has been previously shown to potentiate PMA-induced tPA production. Pretreatment with t-RA does not affect response to a secondary stimulus by PMA, and vice versa. These results suggest that induction of tPA production by t-RA occurs via a separate mechanism that interacts with the PKC-dependent activation of tPA production following PMA, histamine, or thrombin treatment.

Several reports have suggested that retinoids affect fibrinolytic activity in vitro.[14,15,25-27] Some of these studies have concentrated on correlating this effect to other known changes induced by retinoids, such as cartilage resorption,[26] tissue remodeling,[14] and differentiation.[27] For example, Meats et al have shown that increased plasminogen activator activity in response to retinoid treatment may mediate cartilage resorption in chondrocytes,[26] while Hamilton[14] reported that retinoids, especially t-RA, induce plasminogen activator activity in synovial fibroblasts, which may contribute toward basement membrane degradation, inflammatory lesions, and cartilage damage. Differentiation of F9 teratocarcinoma stem cells into primitive endoderm-like cells and parietal endoderm cells occurs in response to t-RA and dibutyryl cAMP. These changes are also associated with a two-step elevation of plasminogen activator activity[27] and tPA antigen and mRNA levels.[15] Kooistra et al have studied the effects of various retinoids on tPA antigen levels in endothelial cells and have shown that analogues with a terminal carboxyl group were most potent in stimulating tPA production. Retinols or retinol esters were less effective.[13] They also reported that plasminogen activator inhibitor (PAI-1) activity was not significantly affected by any of the retinoids tested.

Our studies have also demonstrated that retinoids promote enhanced tPA production in a dose- and time-

dependent manner. Enhancement of tPA production by t-RA occurs in both endothelial and HeLa cells, although maximum stimulation occurs at different concentrations (40 μmol/L *v* 40 nmol/L, respectively). While optimum t-RA levels inducing tPA production in HeLa cells are within physiologic range, the concentrations of t-RA needed to induce a twofold induction in endothelial cells are quite high, reaching supraphysiologic concentrations. These data are consistent with other reports of t-RA–induced tPA production.[13] These differences may be due to the availability of the required intermediates in the tPA response pathway or the efficiency with which each cell type is capable of targeting t-RA toward induction of tPA secretion (eg, through the number or type of retinoic acid receptors), that endothelial cells in culture are less sensitive to t-RA than in vivo, or that retinoic acid is not the physiologic retinoid responsible for this activity.

However, much lower concentrations of t-RA are more effective at synergizing with PMA and histamine (to 1×10^{-8}) than inducing tPA production by themselves, and it is also possible that retinoic acid may play a different role in tPA regulation than that of a primary stimulator, ie, a secondary modulator of other pathways. The synergy between t-RA and the other agonists employed suggests multiple mechanisms of tPA stimulation. Previous studies of histamine- and thrombin-induced tPA production suggest that both act through a single pathway, since coincubation stimulates tPA secretion to levels no greater than that for each agonist added separately.[24] In these same studies, we proposed that pathway was related to or the same as that activated by phorbol esters, ie, the PKC pathway. PMA is known to directly activate PKC, while histamine and thrombin act via cell surface receptors which trigger signal transduction events leading to the activation of PKC. The fact that the effect of t-RA and any one of these agonists on tPA secretion is not merely additive, but also synergistic, suggests an interaction between the two pathways. Earlier studies have indicated an interaction between the PKC and cAMP-dependent pathways in the production of tPA antigen and mRNA, despite the fact that cAMP elevation had no positive effect on tPA itself. To determine whether t-RA– and PMA-induced synergism was also a result of the activation of the cAMP pathway by t-RA, we determined cAMP levels after t-RA treatment. t-RA, in the concentration range of 10 nmol/L to 100 μmol/L, did not affect cAMP levels. Instead, 100 nmol/L t-RA suppressed forskolin-induced cAMP elevation. Coincubation of cells with 100 μmol/L forskolin and various concentrations of t-RA did not alter the t-RA dose-response for tPA production (data not shown). This is in contrast to the observation in F9 teratocarcinoma cells, where cAMP potentiates the effect of t-RA on tPA mRNA and antigen levels.[15]

Previous studies with endothelial cells have demonstrated the onset of a desensitized state on pretreatment with PKC-activating agonists such as PMA, thrombin, and histamine.[24] During this state, secondary treatment with the same agonist after tPA production has returned to normal does not further stimulate tPA production. However, pretreatment with histamine or thrombin desensitizes the cells to a secondary treatment with PMA; fully 100% of the PMA effect is observed after 16 hours of treatment with either agonist. On the other hand, prolonged treatment with PMA, which downregulates PKC, reduces both histamine and thrombin effects by 75%, suggesting that both of these agonists stimulate tPA production via the PKC pathway. With respect to t-RA, we found that this agonist also desensitizes the cells to itself, but not to PMA. However, PMA pretreatment does not have the same effect on t-RA as thrombin and histamine; the t-RA response is not affected by PMA pretreatment. We thus conclude that t-RA induction of tPA production is independent of PKC activation and that the observed synergistic mechanism occurs downstream of PKC activation.

It has been reported that t-RA acts via retinoic acid receptors,[17-20] which bind to specific sequences called retinoic acid response elements (RARE) on genes that are responsive to it.[21-23] RARE have been identified on the retinoic acid receptor β,[22] alcohol dehydrogenase 3,[23] and laminin B1 genes,[28] and constitute a 6-nucleotide direct repeat flanked by five nucleotides (GTTCAC NNNNN GTTCAC). tPA production by t-RA is probably stimulated by a similar mechanism, although there are no data as yet to substantiate this hypothesis. In mouse F9 cells, Rickles et al[29] have mapped the tPA gene promoter for regions responsible for conferring t-RA and cAMP responses, and both are within 190 bp of the 5′ flanking region. While a cAMP-responsive element exists within 100 bp of the 5′ flanking region of the human tPA gene,[30] the region conferring t-RA response has not been mapped. The human and mouse tPA genes, especially the 5′ flanking regions, have significant differences, with approximately 62% homology.[29,31] This might explain the differential responses to t-RA and cAMP by the mouse and human genes.

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