

Activated protein C suppresses tissue factor expression on U937 cells in the endothelial protein C receptor-dependent manner

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Abstract The new functional role of activated protein C (APC) in the regulation of tissue factor (TF) expression was investigated using the cultured human monoblastic leukemia U937 cell line. A flow cytometric analysis demonstrated that treatment with APC resulted in time- and dose-dependent decrease in TF expression in unstimulated and phorbol ester-stimulated cells. The effect was antagonized by the monoclonal antibody (mAb) to endothelial protein C/APC receptor (EPCR), 252, which strongly inhibited the interaction between APC and EPCR. In contrast, mAbs 49 and 379, which bind to EPCR without blocking APC binding, had no or only a modest effect. It is concluded that culturing U937 cells in the presence of APC caused down-regulation of TF expression through the EPCR-dependent mechanism, independent of whether induction was triggered by phorbol ester. © 2000 Federation of European Biochemical Societies. Published by Elsevier Science B.V. All rights reserved.

Key words: Activated protein C; Endothelial protein C receptor; Tissue factor

1. Introduction

Tissue factor (TF) is the integral membrane protein that may initiate the blood clotting cascade in hemostasis and many thrombotic disorders [1,2], unstable angina [3], atherosclerotic plaque formation [4], and pre-eclampsia [5]. There is evidence that local fibrin deposition mediated by the expression of the procoagulant TF is one of the initial steps in pre-eclampsia-mediated vasculopathy [5,6]. It has also been reported that macrophages are activated in patients with pre-eclampsia [7], and that monocytes and macrophages can be induced to express procoagulant activity or cell surface TF by several inflammatory mediators [8]. It is therefore important to understand how TF expression is controlled in activated monocytes or macrophages.

Activation of protein C to its active serine protease, activated protein C (APC), initiates a series of events that play a key role in the regulation of blood coagulation [9–14]. APC functions as an anticoagulant in plasma by inactivating FVa and FVIIIa on membrane surfaces, a process that is potentiated by the plasma vitamin K-dependent factor, protein S [15,16]. At present, APC is considered to be one of the anticoagulant agents of choice for pre-eclampsia. The introduction of APC has led to an improvement in severe pre-eclamp-

sia-induced DIC. However, despite its successful use in patients with pre-eclampsia, the precise mechanism of the inhibitory action of APC is still poorly understood.

It has been established that endothelial cells play a critical role in the PC pathway in that they express two of the known receptors responsible for efficient PC activation: thrombomodulin [9,17] and the endothelial protein C receptor (EPCR) [18–22]. EPCR is a recently identified receptor with significant homology to the CD1/MHC class I family. EPCR binds both PC and APC with similar affinity ($K_d = \sim 30$ nM), facilitates PC activation by presenting the PC substrate to the thrombin–thrombomodulin complex.

In attempting to understand the mechanism through which APC regulates procoagulant activity, we have carried out experiments to test the effects of APC on the TF expression of cells of monocyte/macrophage lineage. In the present study, we investigated whether APC influences the TF expression in unstimulated and phorbol ester-stimulated U937 cells, and whether its effect is conducted through the EPCR-dependent mechanism.

2. Materials and methods

2.1. Production of monoclonal antibodies (mAbs) raised against EPCR

mAbs raised against EPCR were prepared as described previously [23]. The antibodies were designated 49, 252 and 379. These mAbs were used for flow cytometric analyses. A purified preparation of each antibody was biotinylated according to the method of Guesdon et al. [24], using *N*-hydroxysuccinimidyl biotinamidocaproate (Sigma Chemical Co., St. Louis, MO, USA) using the manufacturer's suggested procedures.

2.2. Cells and culture conditions

Human promyeloid monoblastic leukemia cell line, U937, was grown and cultured as previously described [25]. U937 cells were grown at a density of $3\text{--}5 \times 10^5$ cells/ml in RPMI 1640 supplemented with 10% fetal calf serum (Life Technologies, Grand Island, NY, USA), 100 U/ml penicillin and 100 µg/ml streptomycin in an atmosphere of 5% CO₂, 95% air. Differentiated (stimulated) U937 cells were obtained by incubation in the above medium containing 1 µM phorbol-12-myristate-13-acetate 4-*O*-methyl ester (PMA; Sigma). A 2 day incubation period resulted in approximately >85% adherent cells. Adherent cells were detached by pipetting, harvested by centrifugation, washed with binding buffer (see below) and used for the binding experiments.

2.3. Preparation of fluorescein isothiocyanate (FITC)-labeled APC

APC purified from human serum was a generous gift of Kaketsuken (The Chemo-Sero-Therapeutic Research Institute, Kumamoto, Japan). 0.5 mg of the fluorescent dye FITC (Sigma) in 0.5 ml dimethyl sulfoxide was added to the purified APC (5 mg/5 ml 0.1 M carbonate buffer, pH 9.2) and incubated for 3 h at 23°C. Resulting FITC-labeled APC was purified by Sephadex G-25 gel filtration (PD-10 column;

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Pharmacia, Uppsala, Sweden). Incorporation of FITC into APC was verified by exposing sodium dodecyl sulfate–polyacrylamide gels to long wave ultraviolet light.

2.4. Binding of FITC-labeled APC to the EPCR on U937 cells

Single cell-associated fluorescence was quantified by flow cytometry with the EPICS XL flow cytometer (Beckman-Coulter, Co., Fullerton, CA, USA; low-power argon laser excitation at 488 nm). Binding of FITC-labeled APC to unstimulated and PMA-stimulated U937 cells was performed essentially as described earlier [25,26]. The cell pellet was washed twice and resuspended in 1 ml phosphate-buffered saline, 0.1% bovine serum albumin, 2 mM CaCl₂, pH 7.3 (binding buffer) containing 0–20 µg/ml FITC-labeled APC (1–2 h at 4°C). After incubation, the cells were centrifuged, resuspended in 1 ml binding buffer, and then cell-associated fluorescence was quantified by flow cytometry. Non-specific binding of FITC-labeled APC to U937 cells was determined in the presence of excess of parent APC. The capacity of mAbs against EPCR (49, 252 and 379) to block binding of FITC-labeled APC to EPCR on U937 cells was tested as follows: inhibition of FITC-labeled APC binding by anti-EPCR mAbs was measured by preincubation of the cells (10⁶ cells/ml) with anti-EPCR mAbs or non-immune IgG (50 µg/ml) in binding buffer for 1 h at 4°C. Subsequently, a saturating concentration of FITC-labeled APC (10 µg/ml, final concentration) was added and incubated for 1 h at 4°C. Bound ligand was quantified by flow cytometry. Between each incubation step, the cells were washed three times with binding buffer.

In addition, the capacity of EPCR-specific mAbs to detect cell surface EPCR was tested. Washed cells were incubated in binding buffer with and without excess of unlabeled APC (20 µg/ml) to saturate the EPCR (2 h, 4°C). Cells were subsequently washed to remove unbound APC and then biotinylated mAbs against EPCR (49, 252 and 379) were added (5 µg/10⁶ cells/ml; 1 h, 4°C). After another washing, 3 µl of FITC-labeled avidin (Dako, Copenhagen, Denmark) was added (1 h, 4°C). Cells were washed to remove excess free fluorescent probe and then assessed for cell-bound fluorescence by flow cytometry.

2.5. Effect of APC on TF expression on plasma membrane of U937 cells

Cell-associated TF expression was determined by flow cytometry. Unstimulated and PMA-stimulated U937 cells were incubated with APC (0–20 µg/ml) or vehicle for 1, 3, 6, 12 and 24 h in RPMI 1640 containing binding buffer. In a parallel experiment, U937 cells were coincubated with the mAbs against EPCR in the presence of APC or vehicle as indicated above. MAb were used at a final concentration of 50 µg/ml. Rat preimmune IgG was diluted at the same

concentration and used as a control. Cells were subsequently washed and then mAb against human TF (American Diagnostica, Greenwich, CT, USA) was added (5 µg/10⁶ cells/ml; 1 h, 4°C). After another washing, 3 µl of FITC anti-mouse IgG (Dako) was added (1 h, 4°C). Cells were washed to remove excess free fluorescent probe and then assessed for cell-bound fluorescence by flow cytometry. All experiments were performed using at least two different cell preparations. The binding data presented are the mean of duplicate determinations in one representative experiment of two or three independent studies.

Each reagent is tested for endotoxin contamination. The levels of endotoxin have been below the detection limit of 30 pg/ml.

3. Results

3.1. Assessment of binding of APC to its specific binding sites on living U937 cells by flow cytometry

Binding of FITC-labeled APC to its receptor (EPCR) on unstimulated and PMA-stimulated U937 cells was quantified by flow cytometry (Fig. 1). Binding of FITC-APC to EPCR on U937 cells depends on the concentration of FITC-APC and time of incubation. Apparent saturation of binding was achieved at approximately 10 µg/ml FITC-APC for both unstimulated and PMA-stimulated U937 cells for 1 h at 4°C. Binding was higher with PMA-stimulated cells. The fluorescence intensity obtained within 1 h at 4°C remained unchanged up to 2 h of incubation. Binding of FITC-APC is inhibited by excess of parent APC. 50% displacement is obtained at a molar ratio of 1:2 for APC and FITC-APC (data not shown). Binding of FITC-APC to unstimulated and stimulated cells was almost completely inhibited by a 20-fold molar excess of unlabeled APC.

3.2. Inhibition of binding of FITC-labeled APC by mAbs directed to various epitopes of EPCR

Unstimulated and PMA-stimulated U937 cells were preincubated with 50 µg/ml of mAbs against EPCR (49, 252 or 379) or non-immune IgG and then binding of FITC-APC (10 µg/ml) to U937 cells quantified by flow cytometry. All three antibodies bound essentially equivalently to U937

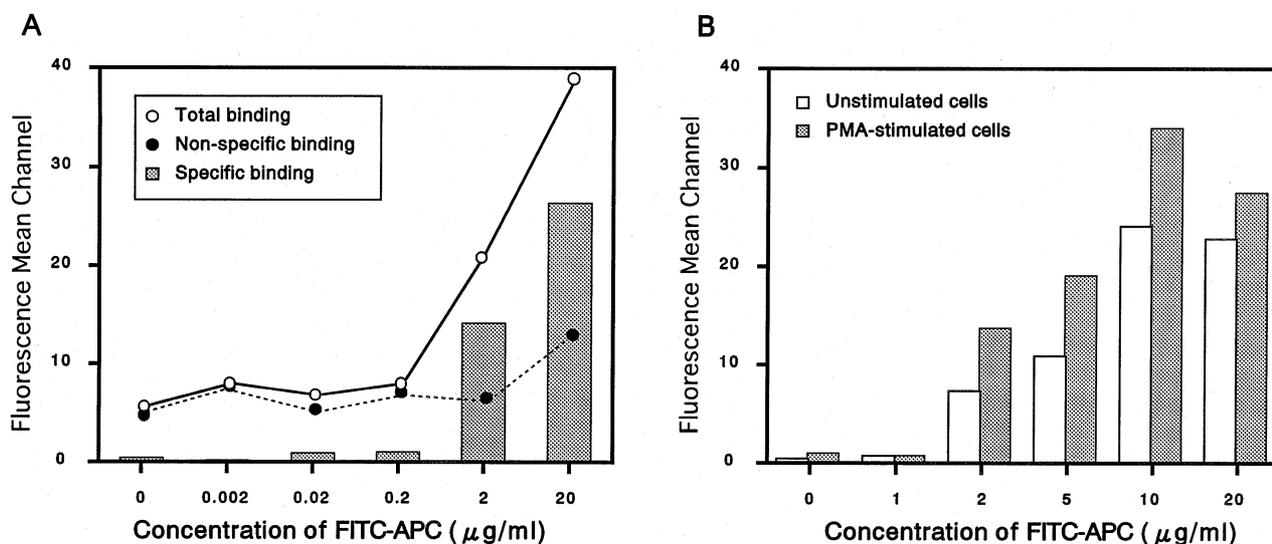


Fig. 1. The binding of increasing concentrations of FITC-labeled APC by unstimulated and PMA-stimulated U937 cells was determined by flow cytometric analysis. A: The PMA-stimulated cells were incubated with FITC-labeled APC at increasing doses in the presence or absence of an excess amount of unlabeled APC. B: The specific binding of FITC-labeled APC by unstimulated (□) and PMA-stimulated (gray) U937 cells. The representative results of two independent experiments are shown.

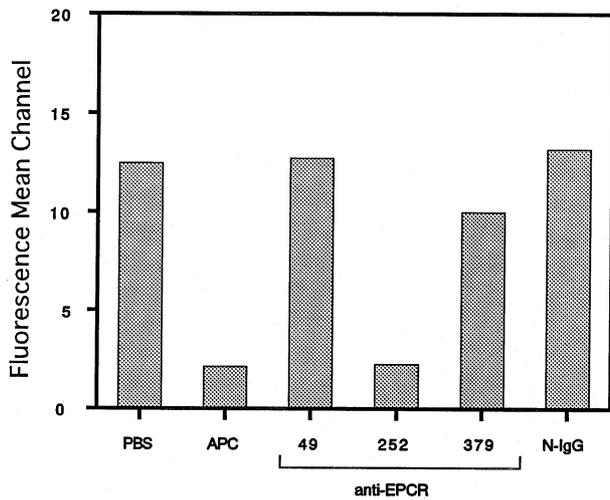


Fig. 2. The binding of FITC-labeled APC (10 $\mu\text{g/ml}$) by PMA-stimulated U937 cells was determined by flow cytofluorometric analysis after preincubation in the absence or presence of anti-EPCR antibodies (49, 252 and 379; 50 $\mu\text{g/ml}$), non-immune IgG (N-IgG; 50 $\mu\text{g/ml}$) or APC (50 $\mu\text{g/ml}$). Characterization was performed in unstimulated U937 cells, demonstrating almost the same inhibition. The representative results of two independent experiments are shown.

cells (data not shown). MAb inhibition of FITC-APC binding to the EPCR on U937 cells is shown in Fig. 2. Even at high concentrations (50 $\mu\text{g/ml}$) of mAb 49, no inhibition was observed. MAb 379 showed only partial inhibition of FITC-APC binding. Preincubation of the mAb 252 to the cells, however, considerably reduced subsequent binding of FITC-APC. More than 90% inhibition was seen when the mAb 252 was applied to the cells at a concentration of 50 $\mu\text{g/ml}$. These results indicate that mAb 252 specifically reacts with peptide sequence representing the ligand binding epitope of EPCR.

In a parallel experiment, U937 cells were preincubated with APC (20 $\mu\text{g/ml}$) and then binding of mAbs against EPCR to

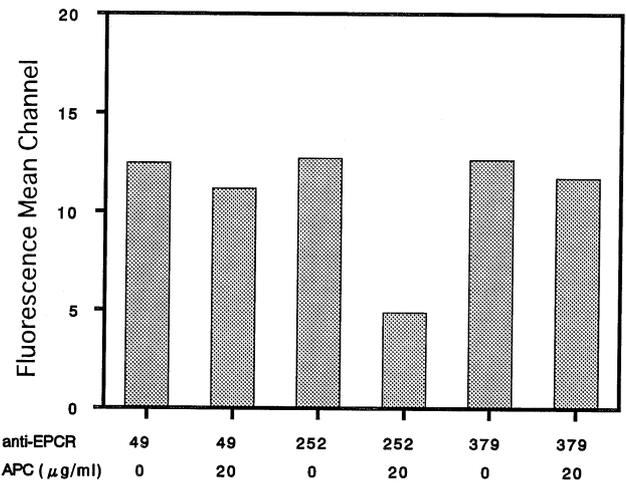


Fig. 3. Biotinylated anti-EPCR antibody binding to PMA-stimulated U937 cells in the absence or presence of APC was assessed by flow cytofluorometry. The cells were incubated with 5 $\mu\text{g/ml}$ of biotinylated mAb after preincubation in the presence of unlabeled APC (20 $\mu\text{g/ml}$). Biotin mAb binding was detected by incubation with FITC-labeled streptavidin. This experiment is representative of two similar observations.

U937 cells quantified by flow cytofluorometry (Fig. 3). Preincubation of APC to the cells considerably reduced subsequent binding of mAb 252. Little inhibition of binding of mAbs 49 and 379 (about <20%) was seen by preincubation of APC. Treatment with varying concentrations of APC resulted in dose-dependent decrease in mAb 252 binding in unstimulated and phorbol ester-stimulated cells (data not shown). These results also support the hypothesis that mAb 252 reacts with peptide sequence representing the APC binding epitope of EPCR. Thus, mAb 252 blocks APC binding to cellular EPCR by steric hindrance, but other antibodies bind to EPCR without blocking APC binding.

It has been established that not only EPCR but also throm-

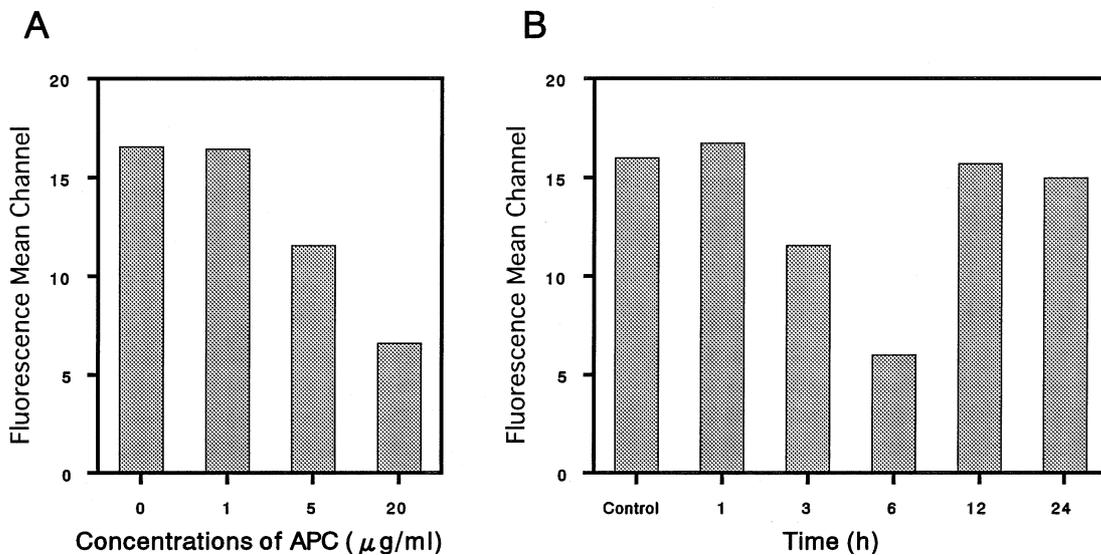


Fig. 4. Effect of APC on the TF expression in PMA-stimulated U937 cells. A: Dose-response study: TF expression was measured on the cell surface after incubating the PMA-stimulated U937 cells with increasing doses of APC (0–20 $\mu\text{g/ml}$) for 6 h by flow cytofluorometry. B: Time-course study: TF expression was measured on the cell surface after incubating the cells with APC (10 $\mu\text{g/ml}$) for the indicated times by flow cytofluorometry. The representative results of two independent experiments are shown. Characterization was performed in unstimulated U937 cells, demonstrating almost the same inhibition.

bomodulin are expressed on the cell surface of U937 cells by flow cytometry (unpublished data; Tsuneyoshi and Fukudome, 1999). MAb 252 does not react with thrombomodulin by solid-phase binding assay (data not shown). When considered together, these data suggest that it is unlikely that cell-associated thrombomodulin is responsible for APC binding sites.

3.3. Effect of APC on TF expression on U937 cell surface

Dose–response and time-course studies: to better characterize the properties of the APC action of U937 cells, we performed both dose–response and time-course studies on APC-mediated regulation of TF expression in unstimulated and PMA-stimulated cells. When the control cells were stimulated by PMA, they demonstrated a 1.5-fold increase in TF expression (data not shown). As shown in Fig. 4A, TF expression was dose-dependently down-regulated when the stimulated cells were treated with APC for 6 h, and the effective concentration of APC necessary to inhibit TF expression by 50% (IC_{50}) was $\sim 5 \mu\text{g/ml}$ for unstimulated and PMA-stimulated cells. The presence of $20 \mu\text{g/ml}$ APC in the conditioned medium of unstimulated and stimulated cells reduced the basal expression of TF to $\sim 45\%$ of that in the control cells 6 h after the treatment. Thus, APC significantly reduced the basal TF expression, independent of whether stimulation was triggered by PMA.

Fig. 4B illustrates the time course of APC inhibition of TF expression, using an APC concentration of $20 \mu\text{g/ml}$ for the stimulated cells. Significant down-regulation of TF expression on the surface of stimulated cells was observed after 3 h and reached a nadir after 6 h. The cells cultured for 12 h showed a higher level of TF expression than those cultured for 6 h. In a parallel experiment, down-regulation of the TF expression on the surface of unstimulated cells was also significant by 3 h and maximal after 6 h. Levels of TF expression then returned to control levels after 12 h (data not shown).

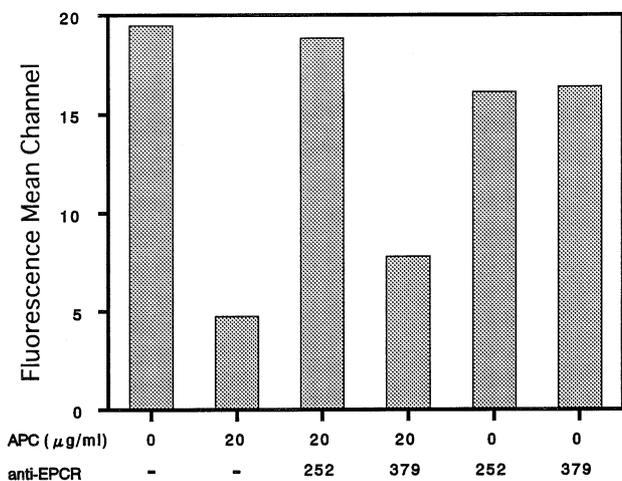


Fig. 5. Monoclonal anti-EPCR antibody abrogates APC-dependent down-regulation of TF expression. TF antigen expression on PMA-stimulated U937 cells was assessed by flow cytometry. MAb 252 ($50 \mu\text{g/ml}$) almost completely reversed the suppressive effects of APC in terms of TF expression, while mAbs 379 and 49 (data not shown) were not efficacious in this regard. The representative results of two independent experiments are shown.

3.4. Effect of mAbs against EPCR on APC-dependent down-regulation of TF expression

It is likely that the effect of APC on TF expression may function via EPCR on U937 cells. MAb 252 inhibits the binding of APC to cellular EPCR, whereas mAbs 49 and 379 do not. Therefore, in order to determine whether binding of APC to EPCR is involved in the down-regulation of TF in U937 cells, we conducted experiments in the presence of each mAb against EPCR. Unlike cells treated with APC alone, when PMA-stimulated cells (Fig. 5) were treated with a combination of mAb 252 ($50 \mu\text{g/ml}$) and APC ($20 \mu\text{g/ml}$), they expressed TF at levels that were essentially the same as cells treated without APC. Thus, mAb 252 completely abrogated the effect of APC on TF levels in U937 cells. On the other hand, mAbs 49 and 379 failed to block the ability of APC to down-regulate TF expression. Characterization was also performed in unstimulated U937 cells, in which almost the same results were obtained (data not shown). These results indicate that APC is required to bind to EPCR in order to down-regulate TF expression in U937 cells.

4. Discussion

APC has been considered to decrease procoagulant activity directly via inactivation of FVa/VIIIa [9,10,27]. Furthermore, we have previously reported that APC enhances the cell-associated fibrinolytic activity [28]. Thus, other beneficial anti-thrombotic effects ascribed to APC include enhanced profibrinolytic activity via an increase in plasminogen activator activity.

TF is known to be transiently inducible in vascular endothelial cells and monocytes/macrophages by a variety of stimuli (LPS, phorbol ester and cytokines including IL- 1β , TNF- α and IFN- γ) [29–31]. Recently, it was shown by Murakami et al. [32] that induction of cytokines in vascular endothelial cells or macrophage activated by LPS can be down-regulated by APC. These findings led us to investigate the effect of APC on monocytes/macrophage TF expression. To study the mechanisms through which APC induces an anti-thrombotic effect, it is often necessary to place the target cells of interest in culture or to use cancer cells instead of normal cells. Here we have used a commercially available human monoclonal promyeloid leukemia cell line U937 to determine whether APC affects the expression of cell-associated TF in vitro, since we and others [23] found that these cells have cell-associated binding sites for APC.

In the present study, we found that there is a specific and functional binding site for APC (EPCR) located on the plasma membrane of the U937 cells. Treatment with APC resulted in dose-dependent decrease in TF expression in unstimulated and phorbol ester-stimulated cells, saturable with an EC_{50} of $\sim 5 \mu\text{g/ml}$ for APC. Treatment of the cells with APC resulted in a time-dependent inhibition of TF expression that was significant by 3 h and maximal after 6 h. Levels of TF expression then returned to control levels after 12 h. Therefore, we conclude that APC induced a consistent, statistically significant decrease in TF expression in a dose- and time-dependent fashion, the decrease was relatively slow and the effect was transient, and that APC significantly down-regulated U937 TF expression, independent of whether induction was triggered by PMA. Furthermore, we have investigated whether APC influences the expression of TF via direct binding of APC

to EPCR on the U937 cells. We have previously developed a protocol to generate mAbs. By this approach, domain-specific antibodies were produced [23]. The present study showed that mAb 252 did not react with cellular EPCR occupied by APC. Preincubation of cellular EPCR with mAb 252 strongly inhibited interaction of APC with EPCR. Therefore, mAb 252 blocks APC binding to the EPCR, while mAbs 49 and 379 reacted with cellular EPCR without blocking APC binding. This feature points to that these mAbs recognize conformation-dependent and domain-specific epitopes. Thus, the region recognized by mAb 252 seems to be critical for binding of APC. Our experiments using mAbs to EPCR strongly indicated that its inhibitory effect on TF expression appears to be conducted through the EPCR mechanism, since a monoclonal anti-EPCR antibody 252 efficiently blocked the APC-dependent down-regulation of TF expression.

Inhibition of TF expression by APC was observed at concentrations neither affecting total protein synthesis nor being directly cytotoxic to the cells (data not shown). To our knowledge, this is the first report showing that APC directly decreases U937 cell TF expression through the EPCR mechanism. Our present results support the hypothesis that APC-induced down-regulation of TF expression may contribute to its successful use in anti-pre-eclamptic medicine.

It is possible that certain cytokines are responsible for the APC's effect shown here. This statement is based on the events reported for cytokine-induced up-regulation of TF expression [31,33] and APC-mediated down-regulation of cytokine release [32]. We thus speculate that APC may block TF expression by down-regulating cytokine release. We have been examining whether neutralizing antibodies to several types of cytokine modulate TF expression by APC. Further studies will be required to determine whether APC reduces the transcription of the TF gene or whether APC causes down-regulation of TF expression via reducing its half-life or enhancing internalization and degradation.

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