Use of annexin-V to demonstrate the role of phosphatidylserine exposure in the maintenance of haemostatic balance by endothelial cells

Catherine RAVANAT, Gisèle ARCHIPOFF, Alain BERETZ, Geneviève FREUND, Jean-Pierre CAZENAVE and Jean-Marie FREYSSINET*
INSERM U.311, Centre Régional de Transfusion Sanguine, 10 rue Spielmann, 67085 Strasbourg Cédex, France

Annexin-V (PAP-I, lipocortin-V) acts as a potent anticoagulant *in vitro* by binding to negatively charged phospholipids with higher affinity than vitamin K-dependent proteins, with a K_d in the 10^{-10} M range. The purpose of the present study was to use annexin-V as a probe to assess the catalytic potential of phospholipids in pro- and anti-coagulant reactions in purified systems and at the surface of endothelial cells in culture after stimulation. Procoagulant tissue factor and anticoagulant thrombomodulin activities were compared by using specific two-stage amidolytic assays performed with purified proteins. Procoagulant activity was estimated by the generation of Factor Xa by the Factor VII(a)–tissue factor complex. Anticoagulant activity was estimated by the generation of activated protein C by either the thrombin–thrombomodulin complex or Factor Xa. Annexin-V induced a decrease of 70% of thrombomodulin activity when thrombomodulin (5.4–214 nM) was reconstituted into phosphatidylcholine/phosphatidylserine (1:1, mol/mol) vesicles at 37.5 or 75 μM-phospholipid concentration, the apparent K_i being 0.5 μM at 75 μM-lipid. The saturating concentration of annexin-V was dependent on phospholipid concentration, but was independent of the phospholipid/thrombomodulin ratio. By contrast, when thrombomodulin was not reconstituted in vesicles, annexin-V had no effect. At 2 μM, annexin-V totally inhibited the generation of activated protein C by Factor Xa in the presence of 75 μM-lipid, the saturating inhibitory concentration being dependent on phospholipid concentration. At 0.1 μM, annexin-V totally inhibited tissue-factor activity present in crude brain thromboplastin. In the absence of stimulation, human endothelial cells in culture expressed significant thrombomodulin activity and no detectable tissue-factor activity. Basal thrombomodulin activity was only slightly inhibited (less than 15%) by 0.5 μM-annexin-V. Phorbol myristate acetate (PMA) induced the expression of tissue-factor activity and decreased thrombomodulin activity at the endothelial-cell surface. Annexin-V, at a concentration of 16 μM, caused an 80% decrease of tissue-factor activity induced by PMA at 10 ng/ml, whereas it inhibited thrombomodulin activity by only 15% on the same stimulated cells. Our results confirm that annexin-V inhibits, *in vitro*, procoagulant tissue-factor activity and anticoagulant activities (activation of protein C by the thrombin–thrombomodulin complex and by Factor Xa), through phospholipid-dependent mechanisms. They provide evidence that phospholipid exposure occurs during activation of human endothelial cells in culture by PMA and that this exposure could be involved in the expression of tissue-factor activity. However anionic phospholipids are of restricted accessibility in the vicinity of cellular tissue factor. The difference in the inhibition of thrombomodulin activity in purified systems and on endothelial cells suggests that the thrombomodulin conformation could be different under these two conditions and/or that vesicles do not reproduce the exact phospholipid environment of cellular thrombomodulin. The different extent of inhibition of tissue-factor and thrombomodulin activities on stimulated endothelial cells suggests that the cofactor environments differ for the optimal expression of these opposite cellular activities.

INTRODUCTION

In the absence of stimulation, phosphatidylserine (PtdSer) is asymmetrically distributed between the two leaflets of blood and vascular cell membranes; it is almost exclusively located on the cytoplasmic side of the plasma membrane. By contrast, after stimulation, PtdSer becomes exposed on the outer side of the plasma membrane at a proportion depending on the nature of the stimuli (Op den Kamp, 1979; Alberts *et al.*, 1983; Zwaal *et al.*, 1977, 1986, 1989). Different mechanisms responsible for the loss of the original asymmetry have been proposed (Seigneuret & Devaux, 1984; Sims *et al.*, 1989; Hamilton *et al.*, 1990; Verhallen, 1988; Comfurius *et al.*, 1990). There is ample evidence that PtdSer exposure is of critical importance in cellular physiology (Zwaal & Hemker, 1982; Allen *et al.*, 1988; Connor *et al.*, 1989). In particular, it is involved in the regulation of haemostatic mechanisms by providing a procoagulant surface on activated platelets, monocytes and endothelial cells where procoagulant complexes can assemble and concentrate (Rodgers & Shuman, 1983; Tracy *et al.*, 1985; Zwaal *et al.*, 1986; Bach & Rifkin, 1990; Hamilton *et al.*, 1990; Komiyama *et al.*, 1990). PtdSer exposure is also required in the anticoagulant protein C pathway, leading to the inactivation of Factors Va and VIIIa at the membrane interface, which prevents them from participating in further

Abbreviations used: PBS, phosphate-buffered saline (137 mM-NaCl/2.7 mM-KCl/0.9 mM-$CaCl_2$/0.5 mM-$MgCl_2$/1.5 mM-KH_2PO_4/6.5 mM-Na_2HPO_4, pH 7.4; 0.295 osM); HSA, human serum albumin; PtdCho, phosphatidylcholine; PtdSer, phosphatidylserine; PMA, phorbol 12-myristate 13-acetate; HSVEC, human saphenous-vein endothelial cells in culture; APC, activated protein C; TM, thrombomodulin; S2366, L-pyroglutamyl-L-prolyl-L-arginine *p*-nitroanilide hydrochloride; S2765, *N*-α-benzyloxycarbonyl-D-arginyl-L-glycyl-L-arginine *p*-nitroanilide hydrochloride; Dns-Glu-Gly-Arg-CH_2Cl, dansyl-L-glutamylglycyl-L-arginylchloromethane; a.t.u., antithrombin unit.

* Present address and address for correspondence: Institut d'Hématologie et d'Immunologie, Faculté de Médecine, 4 rue Kirschleger, 67085 Strasbourg Cédex, France.

amplification of thrombin generation. In this latter step, protein S acts as a cofactor of activated protein C (APC). The proteins assemble at the negatively charged phospholipid interface, thus accelerating the degradation of Factors Va and VIIIa (Solymoss *et al.*, 1988).

Under physiological circumstances, the endothelial cell is basically non-thrombogenic, owing to its ability to maintain a delicate balance between procoagulant and anticoagulant tendencies. However, upon appropriate stimulation, it can acquire an active procoagulant character (Preissner, 1988; Stern *et al.*, 1988). The ability of endothelial cells to trigger or inhibit the generation of thrombin depends on the expression of two essential glycoproteins: an inducible one, tissue factor, and a constitutive one, thrombomodulin (TM). It has been proposed that PtdSer exposure is essential for the expression of tissue-factor activity (Drake *et al.*, 1989; Bach & Rifkin, 1990; Komiyama *et al.*, 1990). By contrast, the involvement of negatively charged phospholipids in the activation of protein C at the surface of endothelial cells has not yet been clearly established, although they have been shown to enhance the activation of protein C by the purified thrombin–TM complex (Freyssinet *et al.*, 1986, 1988; Galvin *et al.*, 1987).

A better knowledge of the involvement of phospholipids in modulation of the thromboresistance of endothelium is necessary to elucidate the physiological and pharmacological regulation of haemostasis and thrombosis. PtdSer exposure has been extensively studied in platelets by using enzymic probes such as phospholipase A_2 or chemical probes (Op den Kamp, 1979). Recently, radiolabelled annexin-V was used as a non-degradative probe to provide evidence that phospholipid exposure is involved in the assembly of the prothrombinase complex at the platelet surface (Thiagarajan & Tait, 1990).

Annexin-V, also referred to as PAP-I, VACα, IBC, PP4 or endonexin II (Funakoshi *et al.*, 1987; Iwasaki *et al.*, 1987; Reutelingsperger *et al.*, 1988; Römish & Heimburger, 1990) is a member of the lipocortin/calpactin/annexin family (Crumpton & Dedman, 1990). The physiological roles of these proteins are still poorly understood. There is evidence that annexin-V interacts with phospholipid vesicles (Tait & Gibson, 1990) and activated human platelets (Thiagarajan & Tait, 1990) in a Ca^{2+}-dependent interaction with a K_d in the 10^{-10} M range. It can thus compete with vitamin K-dependent proteins for binding to procoagulant surfaces. Such competition was shown to be responsible for inhibition of the generation of thrombin *in vitro* (Funakoshi *et al.*, 1987), Factor IXa and Factor Xa (Kondo *et al.*, 1987). As yet, interference of annexin-V in the protein C anticoagulant pathway has not been described.

The purpose of the present study was to use annexin-V as a probe for phospholipids to assess their catalytic potential in pro- and anti-coagulant reactions in purified systems and at the surface of stimulated endothelial cells, all of human origin. Anticoagulant activity was evaluated by the generation of APC by either the thrombin–TM complex or Factor Xa. Procoagulant activity was measured by the generation of Factor Xa by the Factor VIIa–tissue-factor complex.

Annexin-V was shown to be involved in the anticoagulant mechanisms and to inhibit the generation of protein C at the surface of phospholipid vesicles. At the surface of endothelial cells, annexin-V interfered moderately in the anticoagulant mechanism, whereas it behaved as a potent inhibitor of tissue-factor activity, but at high concentration. The differences in extent of inhibition are discussed with respect to phospholipid requirement. These results suggest that annexin-V can be used for assessment of the catalytic role of phospholipids in maintenance of the haemostatic balance at the surface of endothelial cells.

MATERIALS AND METHODS

Reagents and materials

Human serum albumin (HSA) was from the Centre Régional de Transfusion Sanguine de Strasbourg, Strasbourg, France, and essentially fatty-acid-free HSA was from Sigma, St. Louis, MO, U.S.A. Highly purified hirudin [10000–12500 antithrombin units (a.t.u.)/mg] and cephalin, a brain phospholipid extract, were purchased from Diagnostica Stago, Asnières, France. Rabbit brain thromboplastin was provided by Baxter, Dade Division, Chicago, IL, U.S.A. Phosphatidylcholine (PtdCho) and PtdSer from bovine spinal cord were purchased from Lipid Products, Redhill, Surrey, U.K. DEAE-Sepharose CL-6B and Blue-Sepharose were from Pharmacia, Uppsala, Sweden. Ultrogel AcA 54 was from IBF, Villeneuve-la-Garenne, France, n-octyl glucoside was from Boehringer, Mannheim, Germany, and dansyl-L-glutamylglycyl-L-arginylchloromethane (Dns-Glu-Gly-Arg-CH_2Cl) from Calbiochem, La Jolla, CA, U.S.A. Chromogenic substrates L-pyroglutamyl-L-prolyl-L-arginine *p*-nitroanilide hydrochloride (S2366) and *N*-d-benzyloxycarbonyl-D-arginyl-L-glycyl-L-arginine *p*-nitroanilide hydrochloride (S2765) were from Kabi Vitrum, Stockholm, Sweden. Amidolytic activities were measured in multi-well plates using a kinetic microplate reader coupled to a microcomputer (Molecular Devices, Palo Alto, CA, U.S.A.).

Proteins

Annexin-V was purified according to the method of Funakoshi *et al.* (1987) from an EDTA extract of human placenta, followed by precipitation steps using 40 and 80% saturation with $(NH_4)_2SO_4$. The procedure was slightly modified with respect to chromatographic media. DEAE-Sepharose CL-6B was the medium for ion-exchange chromatography, and Ultrogel AcA 54 was used instead of Sephadex G-75 for gel filtration. The Mono S step was omitted, but contaminant traces of albumin were retained on Blue-Sepharose (annexin-V was recovered in flow-through fractions). The anticoagulant activity of the fractions was assayed as follows. A 100 μl portion of plasma, 100 μl of test sample and 100 μl of a kaolin suspension (50 mg/ml) were incubated for 3 min at 37 °C; then 200 μl of $CaCl_2$/cephalin solution was added, giving a final $CaCl_2$ concentration of 3 mM, and the clotting time was recorded. After each chromatographic step, fractions were concentrated by means of a micro-concentrator (Amicon, Paris, France) equipped with PLGC membranes (Millipore, Saint Quentin-en-Yvelines, France). Annexin-V was further characterized by SDS/PAGE (the M_r was 35000) and isoelectric focusing (the pI was 4.8). Annexin-V was stained with Coomassie Blue and was judged to be 98% pure. These preparations were found (C. Ravanat, J. Torbet & J.-M. Freyssinet, unpublished work) to be monodisperse as regards M_r by neutron low-angle scattering using contrast variation (Zaccai, 1983). Human placenta TM was extracted at pH 10.5 and purified by the method of Jakubowski *et al.* (1986). Human protein C, thrombin [3000 National Institute of Health (NIH) units/mg], Factor X and Factor VII were purified from plasma as previously described (Freyssinet *et al.*, 1988). Purified human fibronectin was prepared as described by Ruoslahti *et al.* (1982).

M_r values and absorption coefficients of proteins

M_r values and absorption coefficients at 280 nm ($A^{0.1\%}_{1\,cm,\,280}$) used in calculating protein concentrations were as follows: Factor Xa, 45300 and 1.24 (Jackson, 1972); protein C, 58000 and 1.37 (Kisiel *et al.*, 1976); APC, 54200 and 1.37 (Kisiel *et al.*, 1976); thrombin, 37400 and 1.95 (Owen *et al.*, 1974); TM, 74000 and 0.88 (Esmon *et al.*, 1982); Factor VII, 53000 and 1.29 (Radcliffe

& Nemerson, 1976); annexin-V, 35000 and 0.6 (Funakoshi *et al.*, 1987); fibronectin, 440000 and 1.3 (Ruoslahti *et al.*, 1982).

Phospholipid vesicles

Phospholipid vesicles containing a mixture (1:1, mol/mol) of phosphatidylcholine/PtdCho and PtdSer were prepared either by sonication or by dialysis as follows.

Sonication. Stock solutions of lipid in chloroform were mixed at the indicated molar ratios, dried under N_2 and resuspended at a final concentration of 7 mg/ml in 50 mM-Tris buffer, pH 7.5, containing 0.1 M-NaCl. The lipid suspension was continuously sonicated for 45 min under a nitrogen stream at 4 °C with a 300 W titanium probe sonicator tuned at half maximum power. The suspension was then centrifuged at 25000 *g* for 30 min at 4 °C to remove fragments of the probe. These conditions were shown not to be destructive by Huang & Thompson (1974), as verified by Torbet & Freyssinet (1987) and ourselves (C. Ravanat, J. Torbet, J.-P. Cazenave & J.-M. Freyssinet, unpublished work).

Dialysis. Stock solutions of lipid in chloroform were mixed at the indicated molar ratio, dried under N_2 and resuspended at a final concentration of 7 mg/ml in 50 mM-Tris buffer, pH 7.5, containing 0.1 M-NaCl and 2% (w/v) n-octyl glucoside. The lipid solutions were then dialysed to eliminate the non-ionic detergent against 3 × 100 ml of the above buffer without n-octyl glucoside, in dialysis tubing of 12000–14000-M_r cut-off, for 12 h at room temperature. Assay for phosphorus was performed by the Service Central d'Analyses (CNRS, Vernaison, France), and the phospholipid concentration was estimated by assuming an M_r of 780 for the phospholipid.

For unknown reasons, sonicated vesicles were slightly more efficient in promoting the activation of protein C by the complex TM–thrombin, whereas vesicles prepared by dialysis better accelerated the activation of protein C by Factor Xa.

Methods

Coating of the 96-well plates for studies *in vitro*. The '*in vitro*' assays were performed in 96-well plates precoated with HSA as follows. A 350 µl portion of 50 mM-Tris buffer, pH 7.5, containing 0.1 M-NaCl and 75 µM-HSA (essentially fatty-acid-free) was incubated in the plates for 30 min at 37 °C. The plates were then rinsed three times with buffer without albumin.

Effect of annexin-V on protein C activation by the thrombin–TM complex. TM (final concn. 5.4 to 214 nM) was reconstituted by dilution into phospholipid vesicles prepared by sonication (PtdCho/PtdSer, 1:1, mol/mol) at a final lipid concentration of 37.5 µM or 75 µM in the presence of $CaCl_2$ (final concn. 2 mM), over a 30 min period at room temperature as described by Freyssinet *et al.* (1988). A 60 µl portion of this solution, or blanks without lipid or without TM, were incubated in 96-well plates precoated as described above with 9.7 nM (2.1 NIH units/ml)-thrombin and 0.1 µM-, 0.2 µM- or 0.3 µM-protein C. The effect of annexin-V on this activity was assessed at concentrations ranging between 0 and 7 µM. The incubation medium was 50 mM-Tris buffer, pH 7.5, containing 0.1 M-NaCl and 45 µM-HSA (essentially fatty-acid-free) and incubation was for 15 min at 37 °C in a volume of 140 µl. Thrombin was neutralized with 2.5 a.t.u. of hirudin in 10 µl. A 50 µl portion of S2366 (final concn. 0.5 mM) was then added and the rate of hydrolysis was recorded at 405 nm for 2 min at 37 °C. Concentrations of APC formed were determined by reference to a standard curve established with known amounts of APC.

Effect of annexin-V on protein C activation by Factor Xa. Activation of 0.3 µM-protein C was carried out as described by Freyssinet *et al.* (1989) in the presence of 2 mM-$CaCl_2$, 47 nM-Factor Xa and phospholipid vesicles prepared by dialysis (PtdCho/PtdSer, 1:1, mol/mol) at lipid concentrations of 37.5 µM, 75 µM or 150 µM. The incubation medium was 50 mM-Tris, pH 7.5, containing 0.1 M-NaCl, 45 µM-HSA (essentially fatty-acid-free) and 1 a.t.u. of hirudin; the final volume was 140 µl and incubation was for 30 min at 37 °C. The effect of annexin-V on this activity was assessed at concentrations ranging between 0 and 6 µM. Addition of Dns-Glu-Gly-Arg-CH_2Cl terminated the reaction, giving a final concentration of this inhibitor of Factor Xa of 1.4 µM. A 50 µl portion of S2366 (final concn. 0.5 mM) was then added, and the rate of hydrolysis was recorded at 405 nm for 2 min at 37 °C. Concentrations of APC formed were determined as described above.

Effect of annexin-V on the activation of Factor X by the Factor VII/thromboplastin complex. Thromboplastin from rabbit brain extract (0.1 g/l or 0.2 g/l) in 2 mM-$CaCl_2$ was incubated for 15 min at 37 °C with 10 nM-Factor VII, 0.8 µM-Factor X and annexin-V at concentrations ranging between 0 and 0.28 µM. The incubation medium was 50 mM-Tris buffer, pH 7.5, containing 0.1 M-NaCl and 45 µM-HSA (essentially fatty-acid-free), the incubation volume was 140 µl and the reaction was blocked by addition of 5 mM-EDTA. A 50 µl portion of S2765 (0.5 mM final concn.) was then added, and the rate of hydrolysis was recorded at 405 nm for 2 min at 37 °C. Concentrations of Factor Xa formed were determined by reference to a standard curve established with known amounts of Factor Xa. It was verified that the generation of Factor Xa was linearly dependent on thromboplastin dilution under these conditions of excess Factor Xa and Factor VII.

Cell culture. Human saphenous-vein endothelial cells (HSVEC) were collected from fragments of saphenous vein obtained during coronary-by-pass surgery (Beretz *et al.*, 1989) and cultured by a previously described method (Klein-Soyer *et al.*, 1986). The culture medium was RPMI/M199 (1:1, v/v) containing 10 mM-Hepes, 2 mM-L-glutamine, antibiotics (100 units of penicillin and 100 µg of streptomycin/ml), fungizone (0.25 mg/ml) and 30% (v/v) human serum. It was verified by a *Limulus* chromogenic assay (Coatest; Kabi Vitrum, Mölndal, Sweden) that all media and buffers had an endotoxin content of less than 0.02 ng/ml. Cells were frozen at the second passage and used in experiments at passage 3–10. For experiments, the cells were subcultured in 96-well plates precoated with purified human fibronectin at 0.5 mg/ml, at an initial cell density of 10^4 cells/cm^2.

Stimulation of cells. Assays were performed on confluent cell monolayers (3×10^4 cells/cm^2), the culture medium being replaced 24 h before assays as described by Archipoff *et al.* (1991). The cells were rinsed three times with 200 µl of RPMI/M199 (1:1, v/v) and then incubated with 200 µl of RPMI/M199 containing 150 µM-HSA and 16 nM-phorbol myristate acetate (PMA). After 4 h of incubation at 37 °C, the cells were rinsed three times in PBS. Tissue-factor and TM activities were then measured simultaneously, according to methods published by Archipoff *et al.* (1991).

Effect of annexin-V on tissue-factor activity of HSVEC. For tissue-factor assay, 0–16 µM-annexin-V, 5 nM purified human Factor VII and 400 nM purified Factor X were added to each well. All dilutions were in RPMI/M199 without Phenol Red containing 45 µM-HSA and the final volume was 200 µl. Removal of the supernatant from the cells was sufficient to block the activation of Factor Xa; this was verified by comparing the level of Factor Xa generated under these conditions with the level obtained after addition of excess EDTA. At the end of the incubation period, 20 min at 37 °C, 150 µl of the supernatant was removed and the amidolytic activity of generated Factor Xa was measured by the method of Archipoff *et al.* (1991). Concentrations of Factor Xa formed were determined by reference to standard curves established with known amounts of Factor Xa. Tissue-factor activity was normalized/10^4 cells.

Effect of annexin-V on thrombomodulin activity of HSVEC. For TM assay, 0 to 16 μM-annexin-V, 2.2 nM (0.25 NIH unit/ml) thrombin and 65 nM-protein C were added to each well and the reaction was blocked by the addition of 1.25 a.t.u. of hirudin. All dilutions were in RPMI/M199 without Phenol Red containing 40 μM-HSA and the final volume was 200 μl. At the end of the incubation period, 2 h at 37 °C, 150 μl of the supernatant was removed and the amidolytic activity of generated APC was measured as described by Archipoff *et al.* (1991). Concentrations of APC formed were determined by reference to a standard curve established with known amounts of APC. TM activity was normalized per 10^4 cells.

Controls. Each experimental condition was assayed in three different wells in the vesicle system and in six different wells in the presence of HSVEC. It was verified that activation of protein C or Factor X was linear with time and the number of cells. Substrates were present in excess in order to ensure that phospholipids were rate-limiting in the vesicle system and that tissue factor or TM was rate-limiting in the HSVEC assay. Incubation times were adjusted so that less than 20 % of the total protein substrate, either Factor X or protein C, was converted into its activated form. It was verified that the activity of controls with only one factor added (Factor VII, Factor X, thrombin or protein C) was always less than 2 % of the amidolytic activity measured under the same conditions with the complete assay system.

RESULTS

Effect of annexin-V on protein C activation by the thrombin–TM complex

When 108 nM-TM was reconstituted into PtdCho/PtdSer vesicles (1:1, mol/mol, 75 μM-phospholipids), the rate of activation of 0.3 μM-protein C was enhanced 4-fold compared with the control without phospholipids, as shown in Fig. 1 (1.14 ± 0.2 nM-APC generated without lipids·min^{-1}; 4.5 ± 0.6 nM·min^{-1} with phospholipids). These results were in agreement with those already published (Freyssinet *et al.*, 1986). Annexin-V, between 0 and 7 μM, was responsible for a concentration-dependent limitation of this enhancement, due to the presence of phospholipids, whereas it did not affect the activation of protein C by the thrombin–TM complex in the absence of phospholipids (Fig. 1). At saturating concentrations of annexin-V, inhibition reached a maximum of 70 ± 2 %, with TM reconstituted into vesicles composed of PtdCho/PtdSer (1:1 molar ratio at a lipid concentration of 75 μM). For a lipid concentration of 75 μM, half-maximum inhibition was reached at 0.5 μM-annexin-V (Fig. 1); for a lipid concentration of 37.5 μM, half-maximum inhibition was reached at 0.35 μM annexin-V (results not shown). However, the half-maximum inhibition by annexin-V was not dependent on the phospholipid/TM molar ratio between 350 and 14000 (results not shown).

In order to characterize further the mechanism of inhibition, the substrate-dependence of protein C (0.1 μM, 0.2 μM and 0.3 μM) activation by the thrombin–TM complex (10 nM-thrombin and 108 nM-TM) was examined. These experiments were performed in the presence and absence of annexin-V (maximum 3 μM) with PtdCho/PtdSer vesicles (1:1, mol/mol, 75 μM-lipids). The Dixon plot of the data (Fig. 2) suggested that annexin-V behaved as an apparent non-competitive inhibitor, with a K_i value of 0.5 μM.

Effect of annexin-V on protein C activation by Factor Xa

Protein C at 0.3 μM could be activated by 47 nM-Factor Xa in the presence of 2 mM-$CaCl_2$ and PtdCho/PtdSer vesicles (1:1, mol/mol) (Freyssinet *et al.*, 1989). Annexin-V, between 0 and 6 μM, behaved as a concentration-dependent inhibitor of this activation in the presence of 75 μM-lipids (Fig. 3). This inhibition was total at 2 μM-annexin-V, but half-maximum inhibition was dependent on phospholipid concentration between 37.5 μM and 150 μM. Half-maximum inhibition was achieved at 0.2 μM, 0.9 μM and 1.8 μM respectively with 37.5 μM-, 75 μM- and 150 μM-lipids.

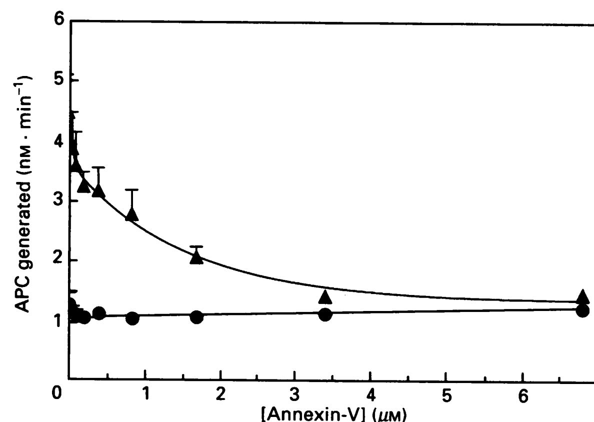

Fig. 1. Neutralization by annexin-V of the enhancing effect of anionic phospholipids on TM activity

Protein C (0.3 μM) was activated in the presence of 2 mM-$CaCl_2$ by the thrombin–TM complex (10 nM/108 nM), after reconstitution of TM into PtdCho/PtdSer (1:1, mol/mol) vesicles at a lipid concentration of 75 μM (▲) or with detergent-solubilized TM (●). Incubation was for 15 min at 37 °C. Values correspond to the means ± S.E.M. of two independent determinations, and for all experiments each condition was assayed in duplicate. When no error bar is visible the S.E.M. was less than 4 % of the mean value. The control without annexin-V gave 4.5 ± 0.6 nM-APC generated·min^{-1} with 75 μM-lipids and 108 nM-TM, and 1.14 ± 0.2 nM-APC generated·min^{-1} with TM only.

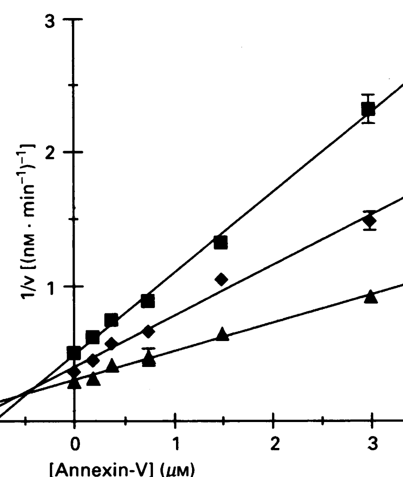

Fig. 2. Dixon plot of the data for inhibition by annexin-V of the enhancing effect of anionic phospholipids on TM activity

Protein C at 0.1 μM (■), 0.2 μM (◆) or 0.3 μM (▲) was activated in the presence of $CaCl_2$ (2 mM) by the thrombin–TM complex (10 nM/108 nM), after reconstitution of TM into vesicles composed of PtdCho/PtdSer (1:1, mol/mol) at a lipid concentration of 75 μM. Incubation was for 15 min at 37 °C. Each condition was assayed in triplicate. Values correspond to the mean ± S.E.M., and when no error bar is visible the S.E.M. was less than 5 % of the mean value. The controls without annexin-V were 1.98 ± 0.03 nM, 2.72 ± 0.05 nM and 3.57 ± 0.15 nM of APC generated·min^{-1} respectively with 0.1 μM, 0.2 μM and 0.3 μM-protein C.

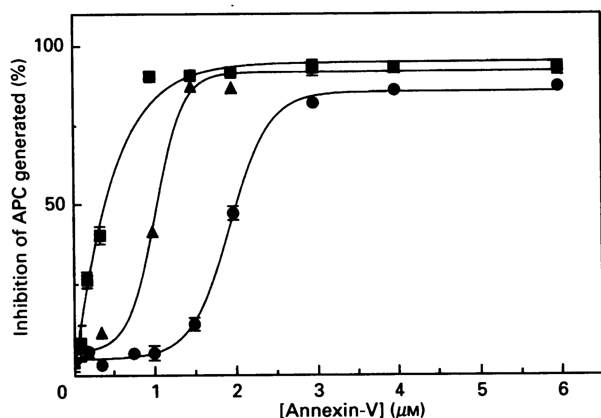

Fig. 3. Inhibition by annexin-V of the phospholipid-dependent activation of protein C by Factor Xa

Protein C (0.3 μM) was activated by Factor Xa (47 nM) in the presence of $CaCl_2$ (2 mM) and phospholipid vesicles composed of PtdCho/PtdSer (1:1, mol/mol) at a lipid concentration of 37.5 μM (■), 75 μM (▲) or 150 μM (●). Incubation was for 30 min at 37 °C. Values correspond to the mean±S.E.M. of two independent determinations, and for all experiments each condition was assayed in triplicate. When no error bar is visible, the S.E.M. was less than 3% of the mean value. The controls without annexin-V were 1.08±0.07 nM, 0.90±0.08 nM and 0.91±0.026 nM-APC generated·min^{-1} respectively with 37.5 μM-, 75 μM- and 150 μM-lipids.

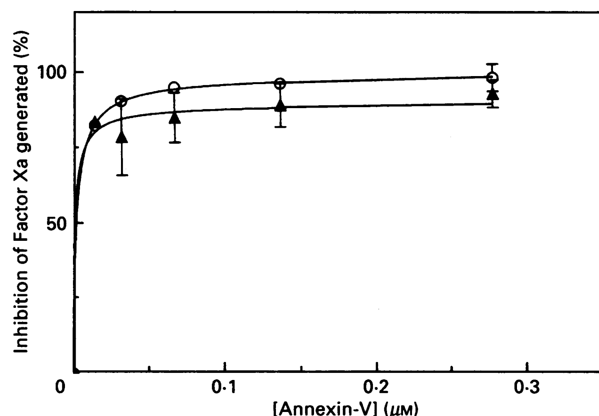

Fig. 4. Inhibition by annexin-V of the activation of Factor X by the Factor VII–thromboplastin complex

Factor X (800 nM) was activated by Factor VII (10 nM) and thromboplastin at 0.1 g/l (▲) or 0.2 g/l (○). Activation was allowed to proceed for 15 min at 37 °C. Values represent the mean±S.E.M. of two experiments, and for all experiments each condition was performed in triplicate. When no error bar is visible, the S.E.M. was less than 4% of the mean value. The controls without annexin-V were 0.37±0.1 nM and 1.05±0.04 nM-Factor Xa generated·min^{-1} with thromboplastin at 0.1 g/l and 0.2 g/l respectively.

Control activities without annexin-V were 1.08±0.07 nM-, 0.90±0.08 nM- and 0.91±0.03 nM-APC generated/min respectively at 37.5 μM-, 75 μM- and 150 μM-lipids.

Effect of annexin-V on Factor X activation by the Factor VII–thromboplastin complex

Annexin-V, between 0 and 0.05 μM, was responsible for a concentration-dependent inhibition of the activation of 800 nM-Factor X by the complex formed between human Factor VII

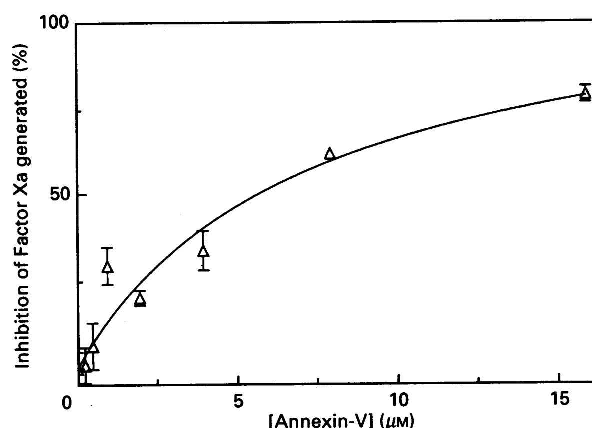

Fig. 5. Inhibition by annexin-V of tissue-factor activity expressed at the surface of PMA-stimulated HSVEC

PMA (16 nM) caused the expression of tissue-factor activity resulting in 2±0.1 nM-Factor Xa generated/min per 10^4 cells. Factor X (400 nM) was activated by Factor VII (5 nM) at the surface of stimulated cells for 20 min at 37 °C. Each condition was assayed in sextuplate. Points correspond to the mean±S.E.M., and when no error bar is visible, the S.E.M. was less than 8% of the mean value.

(10 nM) and rabbit brain thromboplastin (0.1 or 0.2 g/l). Inhibition was total above 0.05 μM-annexin-V (Fig. 4). Control activities without annexin-V were 0.37±0.1 nM-Factor Xa generated/min with 0.1 g of thromboplastin/litre and 1.05±0.04 nM-Factor Xa generated/min with 0.2 g of thromboplastin/litre.

Effect of annexin-V on protein C activation by thrombin at the surface of endothelial cells in culture

Unstimulated endothelial cells expressed significant TM activity (23±0.7 pM-APC generated/min per 10^4 cells). PMA (16 nM for 4 h) caused a 60% decrease of this activity (9.5±0.6 pM-APC generated/min per 10^4 cells) (Archipoff *et al.*, 1991). Whereas annexin-V at 0.5 μM inhibited by 15% the activation of protein C at the surface of unstimulated cells, this concentration did not affect the activity of PMA-stimulated cells. Annexin-V (0.12–16 μM) caused a moderate concentration-dependent decrease in TM activity on PMA-stimulated cells, which also reached 15% (results not shown).

Effect of annexin-V on Factor X activation by Factor VII at the surface of endothelial cells in culture

Unstimulated HSVEC do not express tissue-factor activity. By contrast, addition of 16 nM-PMA for 4 h induced a strong tissue-factor activity at the surface of HSVEC, reaching 2±0.1 nM-Factor Xa generated/min per 10^4 cells (Archipoff *et al.*, 1991). Annexin-V, between 0 and 16 μM, was responsible for a concentration-dependent decrease in tissue-factor activity at the surface of PMA-stimulated cells, 80% inhibition being achieved at 16 μM-annexin-V (Fig. 5).

DISCUSSION

Previous studies have suggested that annexin-V is a potent inhibitor *in vitro* of phospholipid procoagulant mechanisms such as activation of prothrombin by Factor Xa (Reutelingsperger *et al.*, 1985, 1988; Funakoshi *et al.*, 1987) and activation of Factor IX and Factor X by Factor VII and tissue factor (Kondo *et al.*, 1987; Römish & Heimburger, 1990). Annexin-V binds very tightly to anionic phospholipids with a K_d of ~ 10^{-10} M (Andree

et al., 1990) as compared with a K_d of 10^{-6}–10^{-7} M for vitamin K-dependent proteins (Nelsestuen *et al.*, 1978); hence it has been concluded that annexin-V interferes in the Ca^{2+}-dependent assembly of procoagulant complexes on phospholipid surfaces containing phosphatidylserine (Funakoshi *et al.*, 1987; Tait & Gibson, 1990). The present study demonstrates that annexin-V can also be a potent inhibitor of the anticoagulant protein C pathway *in vitro*. Both types of protein C activation, by Factor Xa (Freyssinet *et al.*, 1989) and by the thrombin–TM complex (Freyssinet *et al.*, 1986, 1988; Galvin *et al.*, 1987) have been shown to be, at least *in vitro*, phospholipid-dependent. In addition, the extent of inhibition by annexin-V is dependent on both its concentration and that of phospholipids. This suggests that in purified systems the mechanism of inhibition of the protein C-dependent anticoagulant process by annexin-V can also be explained by competition for binding to phospholipids. Confirmation has been obtained in another study where expression of the anticoagulant activity of activated protein C in the presence of protein S, phospholipids and activated platelets was observed to be inhibited by annexin-V (Freyssinet *et al.*, 1991).

In the present study it could be confirmed that annexin-V is able to inhibit, in a concentration-dependent manner, activation of Factor X by the Factor VII–thromboplastin complex. Inhibition was total at saturating concentrations of annexin-V. These results confirm that annexin-V interacts with the acidic phospholipid component of the thromboplastin preparation (Kondo *et al.*, 1987; Römish & Heimburger, 1990).

It has been demonstrated that, in the presence of hirudin, the most potent known inhibitor of thrombin, human protein C can be activated by human Factor Xa through a TM-independent mechanism, requiring only the presence of $CaCl_2$ and phospholipid vesicles bearing a relatively high proportion of negative charges (Freyssinet *et al.*, 1989). Again, annexin-V behaved as a potent inhibitor in this system. The concentration of annexin-V which produced half-maximum inhibition was dependent on phospholipid concentration, suggesting that it is the rate-limiting factor. The catalytic potential of phospholipids in this anticoagulant process was assessed between 37.5 μM and 150 μM-lipids. This range is close to the equivalent phospholipid concentration of the physiological platelet count (60 μM equivalent to 300000 platelets/μl; Zwaal *et al.*, 1986). However, the local phospholipid concentration at a site of platelet aggregation is expected to be the higher.

The sigmoidal shape of the curves (Fig. 3) obtained at high phospholipid concentrations can be simply explained by a saturation process. At low annexin-V concentration, there are enough phospholipid-binding sites to accommodate annexin-V, Factor Xa and protein C without interference. However, upon increasing annexin-V concentration, competition occurs.

The effects of annexin-V *in vitro* may be attributed to comparable requirements in phospholipids for the expression of these pro- and anti-coagulant activities. Thus annexin-V could be used as a probe to assess the catalytic potential of PtdSer in both activities. This hypothesis was further verified in a purified system by measuring the activation of protein C by the thrombin–thrombomodulin complex in the presence and absence of phospholipids. When thrombomodulin was reconstituted into vesicles having a net negative charge (PtdCho/PtdSer, 1:1, mol/mol), activation of protein C was enhanced 4-fold. Annexin-V suppressed the enhancing effect of phospholipids in a concentration-dependent manner, but had almost no effect on activation of protein C by thrombin in the presence of detergent-solubilized thrombomodulin or when thrombomodulin was reconstituted into 100% PtdCho vesicles (results not shown). Half-maximum inhibition was dependent on phospholipid concentration, but independent of the phospholipid–TM ratio. The inhibitory effect of annexin-V on the potentiation of protein C activation by phospholipids had an apparent non-competitive character, with a K_i of 0.5 μM at 75 μM-phospholipids. A similar type of inhibition has also been observed for prothrombin fragment 1, another phospholipid antagonist, in the activation of protein C by TM reconstituted into phospholipid vesicles (Freyssinet *et al.*, 1988) and for annexin-V as an antagonist of the prothrombinase complex (Reutelingsperger *et al.*, 1988).

Tissue factor is generally considered to be the major cellular initiator of coagulation. However, purified detergent-solubilized tissue factor or tissue factor reconstituted into PtdCho vesicles has very low procoagulant activity (Bach *et al.*, 1986). Therefore phosphatidylserine exposed at the outer leaflet of the plasma membrane is believed to play an essential role in the modulation of tissue-factor activity (Carson & Ross, 1988; Drake *et al.*, 1989; Gramzinski *et al.*, 1989; Bach & Rifkin, 1990; Komiyama *et al.*, 1990). Under normal conditions, tissue factor is not exposed at the surface of cultured endothelial cells, whereas TM is constitutively expressed. Stimulation of the cells with PMA leads to the expression of tissue-factor activity, whereas TM activity is simultaneously slightly lowered (Archipoff *et al.*, 1991). In agreement with the promoting effect of PtdSer on tissue-factor activity, we observed that annexin-V could inhibit tissue-factor activity by 80% at the surface of endothelial cells after stimulation with PMA. These results provide evidence that PtdSer exposure occurs after stimulation of human endothelial cells in culture and that it could be involved in the expression of tissue-factor activity. However, the observation that 0.1 μM-annexin-V was able to achieve 100% inhibition of isolated tissue factor, whereas 16 μM annexin-V was responsible for 80% inhibition of cell-bound tissue factor, suggests restricted accessibility to the lipid patch on the cell surface. This is consistent with the view expressed by Forman & Nemerson (1984) that the tissue-factor–Factor VII(a) complex would preferentially cleave free Factor X instead of phospholipid-bound Factor X. By contrast, membrane phospholipids do not seem to be involved to a significant extent in the thrombin–TM interaction, since annexin-V inhibits TM activity by only 15% either on stimulated or unstimulated cells. Furthermore, this inhibitory effect of annexin-V was comparable between resting cells or cells exposed to PMA, despite the fact that the stimulated cells should have an increased amount of PtdSer exposed on their outer membrane. The differences in the inhibition of TM activity in purified systems and on endothelial cells suggest that the TM conformation could be different under these two circumstances and/or that vesicles do not represent the lipid environment of cellular TM (Galvin *et al.*, 1987; Esmon & Esmon, 1988). This discrepancy between purified systems and endothelial cells in culture could also be explained by differences in composition and physical organization of the PtdCho/PtdSer catalytic patch. The different influence of annexin-V on tissue-factor and TM activities at the surface of stimulated HSVEC also suggests that cofactor environments vary for the optimal expression of these opposite cellular activities.

Annexin-V should be of value as a probe for phospholipid structures bearing a proportion of anionic species. Another field of investigation would be the characterization of endothelial-cell particles that would disseminate a hypercoagulable tendency. After disruption (Drake *et al.*, 1989) or stimulation with endotoxin, tissue factor was found to be transported into the shed membrane of certain cells (Bona *et al.*, 1987). In addition, Hamilton *et al.* (1990) showed that assembly of the terminal complement protein complex C5b–9 on human endothelial cells induced membrane vesiculation. The resulting microparticles exposed binding sites for Factor Va and therefore supported

prothrombinase activity. However, no significant change in TM status was observed during the short exposure (30 min) to C5b–9 (Hamilton *et al.*, 1990). Some mediators of the inflammatory response, such as tumour necrosis factor, interleukin-1 or endotoxin, were found to down-regulate TM activity at the surface of endothelial cells. The effect was significant only at 24 h after exposure, whereas tissue-factor activity was up-regulated as early as 2 h after contact (Moore *et al.*, 1984; Stern *et al.*, 1985; Nawroth *et al.*, 1986; Archipoff *et al.*, 1991). Thus it is conceivable that the early inflammatory response could account for the disruption of the endothelial haemostatic balance reflected in opposite regulation of tissue factor and TM, with further impairment resulting from later vesiculation leading to the formation of microparticles with highly procoagulant phospholipid-dependent activity. Annexin-V could be a powerful tool to sort and characterize such particles under prothrombotic circumstances.

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