Annexin V (vascular anticoagulant alpha)

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Annexin V (vascular anticoagulant alpha). Its mechanism of anticoagulation and its perspectives as a future antithrombotic drug

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Introduction

The vitamin-K dependent coagulation factors and tissue factor require negatively charged phospholipids for their efficient expression of procoagulant activity (1, 2).

Considerable understanding of the function of phospholipids in coagulation has emerged from studies with the prothrombinase complex (3, 4). The catalytic effect evolves from the binding of the coagulation factors to the phospholipid surface and subsequent formation of intermolecular interactions. The basic principle of this architecture is believed to be involved in the other phospholipid dependent reactions in coagulation (1, 5).

Annexin V (formerly vascular anticoagulant alpha) was first discovered in a particular protein fraction of an arterial tissue homogenate as consequence of its pronounced anticoagulant activity (6). Molecular cloning of its cDNA (7) revealed annexin V to be identical to placental anticoagulant protein (8), endonexin II (9), PP4 (10), lipocortin V (11) and calphobindin (12). Its primary structure is organised in a 4-fold repeat of a 67/68 amino acid long conserved sequence, containing a consensus first described for a number of Ca^{2+}-dependent membrane binding proteins (13), which constitute a new family of proteins termed the annexins (14, 15). Up to date ten different annexins have been discovered and their primary structures have been elucidated by means of protein sequencing or cDNA cloning techniques [for refs. of annexin I-VI see Pepinsky 1988 (11), annexin VII (16), annexin VIII (17), and annexin IX and X (18)].

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Despite of the accumulated chemical and biological information on the annexins no clear picture about their physiological functions exists.

The anticoagulant mechanism of annexin V appears to be distinct from those of the well known anticoagulants like antithrombin III/heparin and activated protein C. In contrast to these annexin V shows no direct effect on the coagulation factors per se but interferes with the participation of negatively charged phospholipids in the procoagulant process by its high affinity binding to the phospholipids (19, 20, 21).

In this paper we present further insight in the mechanism through which annexin V inhibits the phospholipid dependent activation of prothrombin by factor Xa and Va. It is shown furthermore that annexin V's potential to inhibit prothrombinase is not confined to the synthetic phospholipid surface but attains at surfaces of activated platelets and surfaces present in plasma of healthy volunteers and major surgery patients.

The implications of our findings to the understanding of annexin V as a physiological relevant anticoagulant and as a potential anticoagulant/antithrombotic drug is discussed.

Materials and Methods

Proteins – Recombinant annexin V was purified from E. coli transformed with plasmid pRH291, containing the complete coding region of human annexin V (7). The coagulation factors were purified from bovine plasma as described in LINDHOUT et al. (22). Factor X and factor V were activated with Russel's Viper Venom (23) and thrombin (22) respectively. Concentrations of factor Xa were determined by active site titration with p-nitrophenyl-p'-guanidinobenzoate hydrochloride. Prothrombin concentrations were determined similarly after activation with Echis carinatus. Factor Va concentration was determined by assessment of prothrombinase activity under factor Va limiting conditions (22). Annexin V concentration was determined by using $E_{280}^{1\%} = 6.0$.

Measurement of prothrombinase activity in a purified system – Prothrombinase activity was determined at 37° C in buffer (50 mM Tris/HCl, pH 7.5, 100 mM NaCl, 3 mM $CaCl_2$ and 20 µM $ZnCl_2$), containing 1 µM phospholipid vesicles (20% PS/80% PC). In an Eppendorf centrifuge tube, 150 µl reaction mixture, containing buffer, vesicles and amounts of factor Va, factor Xa and annexin V as indicated was incubated for 5 minutes at 37° C. The reaction was initiated by addition of prothrombin

and the prothrombin activation was measured by taking sequential samples and adding these to a buffer of 50 mM Tris/HCl, pH 7.5, 100 mM NaCl, 20 mM EDTA and 0.1 g/l soybean trypsin inhibitor. S-2238 was then added and its hydrolysis was measured in a Cobas Bio (Hoffmann-La Roche) autoanalyzer. Within this experimental framework the apparent Kd of factor Xa binding to phospholipid bound factor Va was determined by titration of the reaction mixture with factor Xa. The K_m of prothrombin was determined similarly by titration of the prothrombinase mixture, 10 pM factor Va, 1 nM factor Xa and 1 µM vesicles, with prothrombin.

Measurement of prothrombinase activity in plasma – Platelet free plasma was prepared by differential centrifugation at room temperature and contained 15 mM citrate. Normal plasma pool was prepared from blood collected from at least 30 healthy volunteers. Control patients were at the outpatient's clinic for various reasons without history of thrombosis or bleeding disorders. Plasma samples were also taken from patients immediately after major surgery.

The plasma was diluted 1:10 in buffer A. The diluted plasma was incubated for 20 minutes with a variable amount of annexin V, 2 µM prothrombin and 200 µM S-2238. Thrombin generation was started by addition of 100 pM factor Va and 500 pM Xa. All reactions were limited by the phospholipid concentration. The S-2238 in the reaction mixture prevents the formed thrombin from cleaving factor VIII and V and fibrinogen, and obviates its inhibition by plasma proteinase inhibitors.

The prothrombin activation rate was determined by continuous recording of the hydrolysis of S-2238 in a photometer by means of light absorption at 405 nm. The hydrolysis rate of S-2238 is proportional to the thrombin concentration in our experimental conditions. Hence after attainment of a steady state thrombin formation rate (after 1–2 minutes), when the thrombin concentration increases linearly with time, the light adsorption A(t) as function of time t can be described by the equation:

$$A(t) = A0 + A1 \times t + A2 \times t^2$$

with A2 proportional to the thrombin generation rate:

$$A2 = 1/2 \times k1 \times V_{IIa}.$$

k1 – an apparatus constant proportional to the conversion rate of S-2238 by thrombin and the extinction coefficient of the product.

V_{IIa} – the thrombin formation rate (nM/min).

The coefficients A0, A1 and A2 were determined from a least squares fit to the observed absorption curve and the rate of thrombin formation is then calculated from A2.

Measurement of prothrombinase activity at blood platelets – Human platelets were obtained by wash procedures from platelet rich plasma as described (24). Washed platelets were diluted to 1.2×10^6/ml and incubated with stimulus for 10 minutes at 37° C. Then 10 pM factor Va and 1 µM prothrombin were added and after 5 minutes a mixture of 1 nM factor Xa, 10 nM thrombin inhibitor I-2581 and 1 µM PGE-1 were added. Aliquots were taken in time to determine the thrombin generation rate. Buffer: 25 mM Hepes, pH = 7.5, 136 mM NaCl, 2.68 mM KCl, 2.5 mM $CaCl_2$, 25 mM glucose and 5 g/l bovine serum albumin.

Results

The interference of annexin V with the protein-protein interactions of the prothrombinase complex – The heteromer prothrombinase complex catalyses the conversion of prothrombin as effective as it does by virtue of the specific intermolecular interactions (1). These interactions can be assessed by kinetic analysis, such as titration of fixed amounts of vesicles, factor Va and prothrombin with factor Xa and subsequent determination of the rate of prothrombin activation. The obtained rates offer information on the affinity of factor Xa for phospholipid bound factor Va (22).

Michaelis-Menten analysis by titration with prothrombin obtains the parameters K_m and V_{max} of the prothrombin activation (3).

TABLE 1. Dissociation constants of Xa-phospholipid bound Va.
The rates of thrombin formation as presented in fig. 1 were analyzed by double reciprocal plots, from which Kd values were determined (± S.D., n >3).

Annexin V (nM)	Kd (pM)
0	97 ± 30
10	441 ± 166
100	554 ± 157
1000	790 ± 126

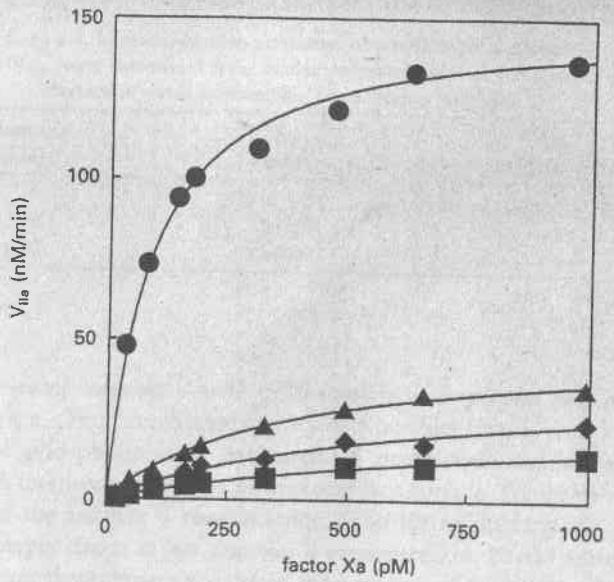

Figure 1. Interference of annexin V with prothrombinase. Titration with factor Xa. The reaction mixture consisted of 100 pM Va, 1 µM PS/PC, 1 µM prothrombin and factor Xa as indicated. Thrombin production was measured in the absence of annexin V (circles) and in the presence of 10 nM (triangles), 100 nM (diamond) and 1 µM annexin V (squares).

When a mixture of 100 pM Va, 1 µM PS/PC vesicles, 1 µM prothrombin and 3 mM Ca^{2+} is titrated with Xa, the analysis of the observed reaction rates reveal a K_d of 0.1 nM for the functional interaction between Xa and phospholipid bound Va. The presence of annexin V in the mixture causes a descrease of this affinity in a dose-dependent manner (fig. 1, table 1). Since a synergistic effect of zinc ions on the binding of annexin V was observed (20) we added 20 µM Zn^{2+} ions to the purified system. Plasma has a physiological amount of about 20 µM Zn^{2+}. This indicates that annexin V interferes with the interaction between Xa and Va either directly or indirectly through interference with the phospholipid binding of these factors.

The affinity of the substrate for the enzyme complex was determined by titration of a mixture, consisting of 10 pM Va, 1 nM Xa, 1 µM PS/PC vesicles and 3 mM Ca^{2+}, with prothrombin. Annexin V induces an increase of the K_m of the reaction to values, which could not be determined

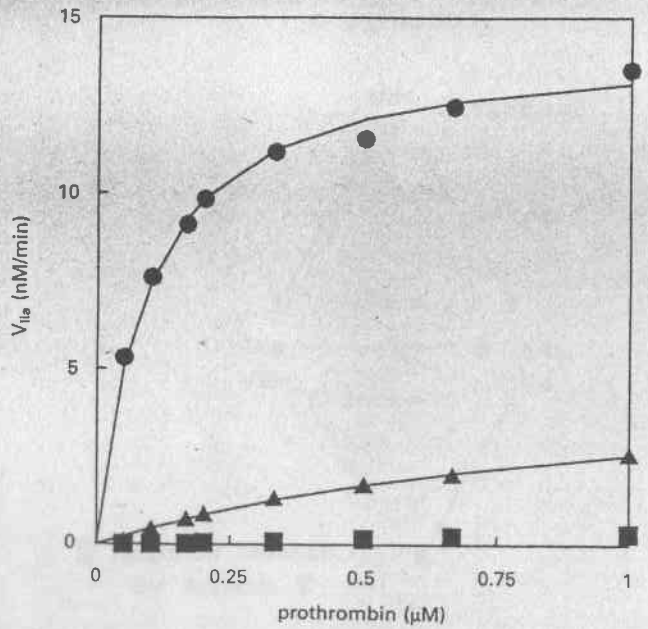

Figure 2. Interference of annexin V with prothrombinase. Titration with prothrombin. The reaction mixture consisted of 10 pM Va, 1 nM Xa, 1 μM PS/PC and prothrombin as indicated. Rate of thrombin formation was measured in the absence of annexin V (circles) and in the presence of 10 nM (triangles) and 1 μM annexin V (squares).

anymore, indicating a strong interference with the enzyme substrate interaction (fig. 2, table 2).

Hence, annexin V has an intricate effect on the prothrombinase complex, by interference through its phospholipid binding with probably all the intermolecular interactions required for optimal enzymatic activity. This mechanism entangles annexin V to be a very effective inhibitor of prothrombinase activity if the reaction is limited by phospholipid surface availability. Implicit to its mechanism of action the efficacy of annexin V to inhibit prothrombinase reflects the availability of procoagulant phospholipid surface.

Analogously, it can be inferred that annexin V interferes with the assembly of the other phospholipid dependent procoagulant complexes (25, 26, and fig. 3).

TABLE 2. Michaelis-Menten parameters of prothrombin activation. The K_m and V_{max} were determined from double reciprocal plots of the rates of thrombin formation versus prothrombin concentration (see fig. 2).

Annexin V (nM)	K_m (nM)	V_{max} (nM/min)
0	141 ± 36	15 ± 4
10	>1000	>8
1000	>5000	>4

Interference of annexin V with prothrombinase in plasma and on the platelet surface – Prothrombinase activity of a purified system with known amounts of phospholipids is compared to prothrombinase activity in plasma with unknown quantities of procoagulant surface. Figure 4a shows the effect of the annexin V concentration upon thrombin formation rate. This rate steeply drops at low annexin V concentration. 20 nM annexin V inhibits the prothrombinase activity in the presence of 1 μM phospholipid vesicles for more than 80%. This is consistent with our earlier report that annexin V binds with a ratio of 1 molecule to 42 molecules phospholipids (20) of the outer leaflet of a membrane. From these latter data it is calculated that 15 nM annexin V is required to cover 1 μM phospholipid vesicles, assuming 66% of the phospholipid molecules in the outer leaflet.

In figure 4b the thrombin generation rate in a normal pool of plasma is plotted as a function of the annexin V concentration. Since no phospholipids were added to the plasma, the observed prothrombinase activity must be associated with procoagulant surfaces present in the plasma. In this situation a concentration of 3 nM annexin V already suffices to reduce the prothrombinase activity by 80%. Thus our pooled plasma had procoagulant surface area available equivalent to about 150 nM PS/PC vesicles.

The platelet membrane presents a more physiological model of a procoagulant surface. The prothrombinase activity of added factor Va (10 pM) and factor Xa (1 nM) in a platelet suspension, activated by various stimuli, was determined in the absence or presence of annexin V. The procoagulant activity of platelets stimulated by thrombin (10 nM) alone or in combination with collagen (10 μg/ml) or by calcium ionophore (A23187, 10 μM) could be almost completely blocked by 1 μM annexin V. Figure 5 shows a titration with annexin V of the procoagulant activity of platelets that were stimulated with 10 μM A23187, which is the strongest stimulus

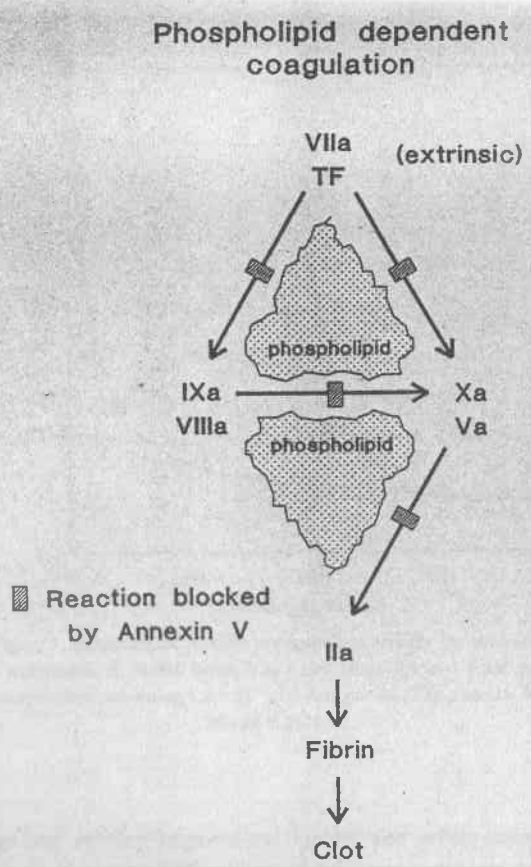

Figure 3. Simplified presentation of the coagulation pathway and the sites where phospholipids play a pivotal role.

Coagulation is initiated by the exposure of tissue factor (TF) to the blood (2). Enzymatic conversions lead to the formation of thrombin, the key enzyme in coagulation. The activations of factor IX and X by TF/VIIa, the activation X by IXa and the activation of prothrombin by Xa are greatly enhanced by phospholipids (3, 32). Annexin V inhibits these reactions through a high affinity binding to the phospholipids (hatched boxes).

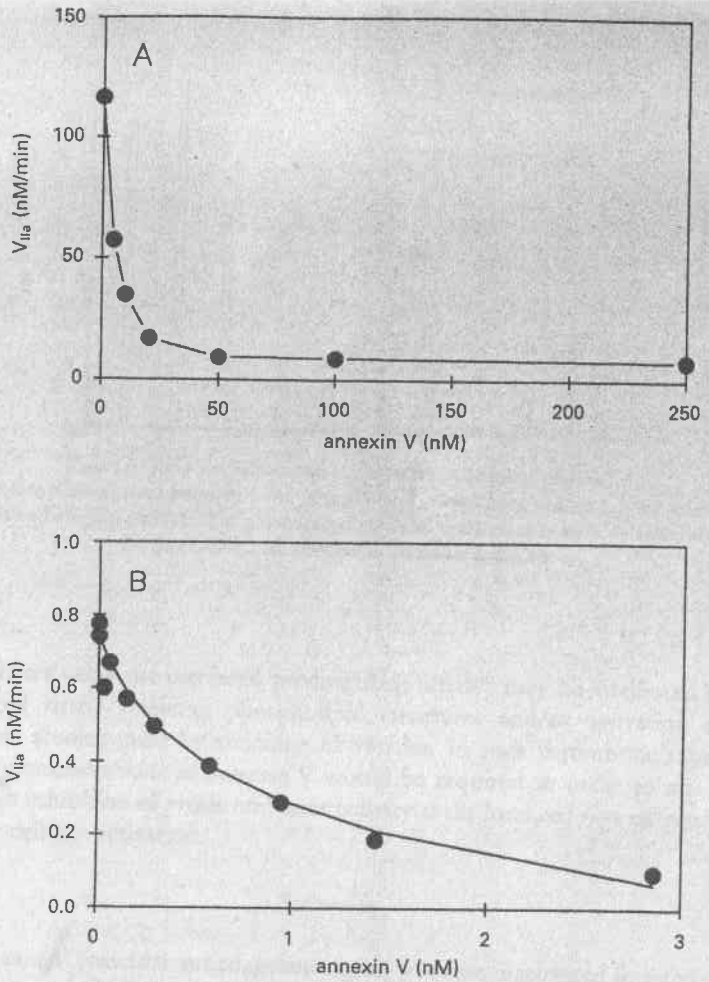

Figure 4. Effect of annexin V concentration on prothrombinase in a purified system and in plasma. A: Annexin V was added to prothrombinase (1 µM vesicles, 100 pM factor Va, 1 nM factor Xa and 1 µM prothrombin) to buffer (50 mM Tris/HCl, pH 7.4, 100 mM NaCl, 3 mM $CaCl_2$, 20 µM $ZnCl_2$, 0.5 mg/ml ovalbumin, T = 37° C). B: Annexin V was added to diluted plasma, followed by measurement of added factor Xa – factor Va activity. 100 pM factor Va, 500 pM factor Xa, 2 µM prothrombin. Buffer: 50 mM Tris/HCl, pH 7.5, 100 mM NaCl, 10 mM $CaCl_2$, 0.5 g/l ovalbumin, T = 37° C.

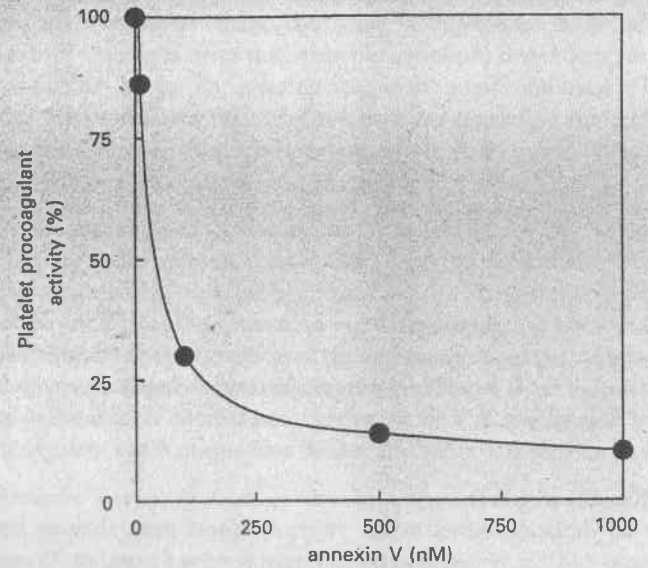

Figure 5. Inhibition of platelet procoagulant activity by annexin V.
The thrombin generation of 10 pM factor Va, 1 nM factor Xa and 1 μM prothrombin wa measured in a suspension containing 1.2×10^6 platelets per ml. The platelets were activated by 10 μM A23187.

for procoagulant activity expression. Under the given conditions about 250 nM annexin V blocks 80% of the platelet procoagulant activity.

Prothrombinase measurements in patients plasma and the susceptibility to annexin V inhibition – The physiological relevance of annexin V as an anticoagulant and its use as an anticoagulant/antithrombotic drug will not only depend on the dose-effect relation of annexin V in plasma as shown in figure 4b but also on the patients plasmas procoagulant status. Figure 5 shows the prothrombinase activity in various plasma samples, determined without the addition of annexin V or phospholipid. The observed activities appeared to range widely. This procoagulant activity could be inhibited by annexin V (Fig. 6). Extrapolation from the results obtained with our pooled plasma, it can be estimated that the amount of annexin V to give 80% inhibition would range from 3 nM plasma concentration in healthy volunteers to up to 30 nM in patients immediately after major surgery. In

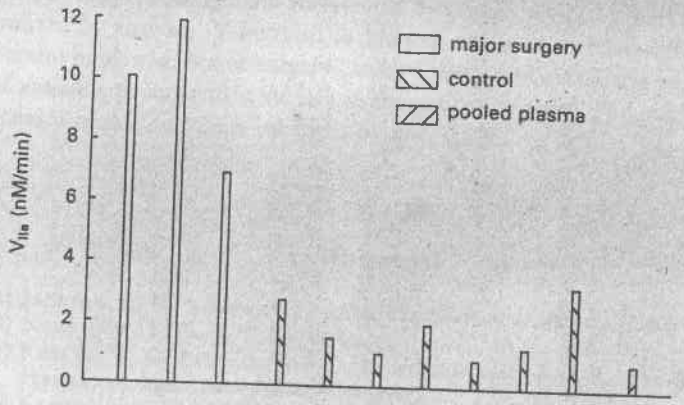

Figure 6. Variation in procoagulant surfaces in different plasmas.
Platelet free plasmas were prepared from citrated blood pooled from various healthy donors, controls and surgery patients. The procoagulant status of these plasmas were determined by the prothrombinase reaction as described in fig. 4b.

these latter cases the increased procoagulant activity may be attributed to damaged tissue releasing phospholipid structures and/or activation of platelets accompanied by shedding of vesicles. In such thrombotic states higher concentrations of annexin V would be required in order to attain effective inhibition of prothrombinase activity at the localised sites of injury and/or cellular activation.

Discussion

Annexin V (vascular anticoagulant alpha) was first discovered in arteries of human umbilical cords due to its strong anticoagulant potency (6). It was then hypothesized that the mechanism of action differed from the ones of the known anticoagulants. Several following studies have confirmed the hypothesized mechanism, that annexin V interferes with the phospholipid binding of the coagulation factors (19, 21). In addition this study demonstrates that annexin V bound to the phospholipid surface also has the potency to interfere with the assembly of prothrombinase and with the interaction of the substrate with the complete prothrombinase. Thus the presence of annexin V at the lipid surface apparently interferes with the protein-protein interactions at the lipid surface required for expression of

prothrombinase activity. Since the different phospholipid dependent procoagulant complexes share the same architecture, it is inferred that annexin V interferes with the other phospholipid dependent reactions in a way similar as to its interference with prothrombinase. This novel mechanism completes the set of mechanisms regulating the activity of the heteromer procoagulant complex, comprising enzyme, substrate, non-enzymatic protein cofactor and phospholipid surface (1, 27).

A special feature of this mechanism is its localized action at sites of vessel wall injury and at plasma membranes of perturbed and activated cells and malignant cells (unpublished observations, 28). This mechanism could be of great physiological importance to regulate coagulation because it not only inhibits locally the progression of the activation of coagulation factors but it also displaces already activated coagulation factors from the surface, which once in the soluble compartment are susceptible to inactivation by the other anticoagulant mechanisms like the antithrombin III/heparin pathway.

Annexin V is synthesized in vascular endothelial cells (29, 30). It has no signal peptide that would support active externalisation via the classic routes (7, 8) but it has been found in extracellular fluids like blood (29, 31). At the moment remains uncertain whether this occurs through cell death or through an as yet unidentified secretory pathway. Despite the considerable amount of accumulated data the physiological function of annexin V has not been clarified so far, while an important role as an anticoagulant has been doubted (29). Regardless its true physiological function annexin V acts as a strong anticoagulant once present in blood. The features of its new anticoagulant mechanism, which has been demonstrated to function *in vivo*, makes it an attractive tool for the treatment of hemostatic disorders. The merits of annexin V as a therapeutic agent, compared to the established ones (heparins and coumarins) remain to be demonstrated by *in vivo* experiments.

Summary

Annexin V is an anticoagulant, that binds with high affinity to procoagulant phospholipids. This binding results in a composite interference with the assembly and function of the prothrombinase complex. Annexin V decreases the affinity of factor Xa for phospholipid bound factor Va and increases the K_m of prothrombin. The anticoagulant efficacy of annexin V is governed by the amount of procoagulant phospholipids.

Not only procoagulant surfaces of synthetic phospholipids are neutralized by annexin V but also surfaces of activated platelets and surfaces present in plasma. Major surgery causes a significant increase in the amount of available procoagulant surface in the patients plasma. The procoagulant activity of this surface is inhibited by annexin V.

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Passagerer Lupus-Inhibitor bei einem sechs Jahre alten Mädchen bei gleichzeitiger passagerer Thrombopenie seines Vaters

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Anhand einer Kasuistik von 1984 möchte ich auf einen Gesichtspunkt hinweisen, den man bei der Frage nach der Ätiologie des Auftretens von Lupus-Inhibitoren mitberücksichtigen sollte.

In unserem Fall handelt es sich um ein damals sechs Jahre altes Mädchen mit bis dahin unauffälliger Familien- und Eigenanamnese. Aus Wohlbefinden heraus waren bei dem Kind zunächst Schmerzen am Fußknöchel aufgetreten und in den darauffolgenden Tagen spontan zahlreiche Hämatome am ganzen Körper, beginnend am schmerzenden Knöchel. Etwa eine Woche vorher war die gesamte Familie an einer Gastroenteritis erkrankt.

Tabelle 1. Gerinnungsparameter bei Lupus-Inhibitor (weiblicher Patient, 6 Jahre).

Pathologische Befunde			
	Aktivität	Antigen	Hemmkörper
PTT	127″	---	+++ (PTV)
Quick	9 %	---	+++ (PTV)
Faktor II	2,5%	0%	neg. (PTV)
Faktor VIII	0,1%	100%	35 BE
Faktor IX	5 %	100%	0 BE
Faktor XI	10 %	---	neg. (PTV)
Faktor XII	3 %	100%	+++ (PTV)
Blutungszeit (subaqual)	10′30″		

Normale Befunde
Thrombozyten (230000/µl)
Faktoren I, V, VII und X
Antithrombin III
Plasminogen, α_2-Antiplasmin
FSP ∅

PTV = Plasmatauschversuch