Annexin A5 Down-regulates Surface Expression of Tissue Factor

A NOVEL MECHANISM OF REGULATING THE MEMBRANE RECEPTOR REPERTOIR*

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Phosphatidylserine (PtdSer) is exposed on the external leaflet of the plasma membrane during apoptosis. The protein annexin A5 (anxA5) shows high affinity for PtdSer. When anxA5 binds to the PtdSer-expressing membranes during apoptosis, it crystallizes as an extended two-dimensional network and activates thereby a novel portal of cell entry that results in the internalization of the PtdSer-expressing membrane patches. This novel pathway of cell entry is potentially involved in the regulation of the surface expression of membrane receptors. In this study we report the regulation of surface expression of the initiator of blood coagulation tissue factor (TF) by this novel pathway of cell entry. AnxA5 induces the internalization of tissue factor expressed on the surface of apoptotic THP-1 macrophages. This down-regulation depends on the abilities of anxA5 to bind to PtdSer and to form a two-dimensional crystal at the membrane. We furthermore show that THP-1 cells produce and externalize anxA5 that cause the internalization of TF in an autocrine type of mechanism. We extended our in vitro work to the in vivo situation and show in a mouse model that anxA5 causes the down-regulation of TF expression by smooth muscle cells of the media of the carotid artery that was mechanically injured. In conclusion, anxA5 down-regulates surface-expressed TF by activating the novel portal of cell entry. This mechanism may be part of a more general autocrine function of anxA5 to regulate the plasma membrane receptor repertoire under stress conditions associated with the surface expression of PtdSer.

The annexin A5 protein (anxA5) belongs to a large family of structurally related proteins, which share the common characteristic of binding strongly to phospholipids in a calcium-dependent manner (1–3). The outer phospholipid leaflet of the plasma membrane of eukaryotic cells in contact with the extracellular environment is usually devoid of negatively charged phospholipids. Under certain conditions such as platelet activation or apoptosis the anionic phospholipid phosphatidylserine (PtdSer) is transported to the outer leaflet of the plasma membrane involving the ABC1 transporter (4, 5). Because of the high affinity for PtdSer, anxA5 has been used as a specific and reliable method to measure platelet activation (6) and apoptosis in several experimental models (7). Although, it has been shown that anxA5 is involved in various intra- and extracellular processes including blood coagulation, signal transduction, anti-inflammatory processes, membrane trafficking, and ion channel activity (1, 8, 9), the exact biological function of the anxA5 remains unknown (10). However, the biological functions of anxA5 are believed to depend primarily on its interactions with lipids in membranes. In this regard, it has been shown that anxA5 enters a proteolipid complex, crystallizing in the form of an extended two-dimensional protein network in contact with the lipid bilayer and stabilized by protein-protein interactions (11). This two-dimensional network is proposed to act as an antithrombotic shield on the surface of the placental syncytium (12). Recently our laboratory discovered that anxA5 and cell surface-expressed PtdSer open a novel portal of cell entry (71). AnxA5 crystallizes in a two-dimensional network on the PtdSer-expressing membrane patch and binds thereby the patch into the cell leading to invagination, budding and endocytic vesicle formation. We hypothesized that the PtdSer-anxA5 portal of cell entry may internalize membrane proteins that are embedded in the PtdSer-expressing membrane patch. A close interaction has been described for PtdSer and the coagulation protein tissue factor (TF) (13, 14). TF, a 47-kDa transmembrane glycoprotein, is a member of the cytokine receptor superfamily that initiates blood coagulation by binding the coagulation factor VII in its non-activated (FVII) and activated form (FVIIa) (15). The binary complex TF-FVII(a) proteolytically activates factors IX and X, triggering the downstream coagulation pathway (16–19). TF is hardly present in the intima and media of healthy blood vessels, whereas it is abundant in the adventitia (20). In animal models of balloon injury, TF is rapidly induced in the smooth muscle cell (SMC) of the media (21) and accumulates in the SMC of the developing neointima (20, 21).

Membrane phospholipids, especially PtdSer, are essential for the assembly of the coagulation complex including TF-FVIIa, factor IXa-factor VIIIa and factor Xa-factor Va, which ultimately leads to thrombin generation (22–24). The exteriorization of PtdSer on the cell surface membrane during apoptosis enhances the procoagulant activities in several cell types (25–27). However, the exposure of PtdSer on the cell surface does...
not only constitute a suitable surface in which the coagulation reactions take place, but it has also been implied in the regulation of TF activity. It is believed that the exteriorization of PtdSer during apoptosis places PtdSer in close proximity to the extracellular domain of TF and enhances its cofactor function (28). In this regard, it has been shown that apoptotic cells show an increased TF activity, which can be inhibited by annexin A5 (29–31). This could be explained by the fact that the crystallization of annexin A5 when bound to PtdSer, provokes shielding of the procoagulant surface reducing the availability for anionic phospholipid-dependent coagulation reactions, such as the activation of factor X and IX by the TF-FVIIa complex (32–35). In the present study we have shown a new mechanism by which annexin A5 plays its anticoagulant role. We have conducted in vitro and in vivo experiments showing that by modifying plasma membrane dynamics, annexin A5 down-regulates the expression of the transmembrane protein TF through internalization in Ptd-Ser-containing patches.

**EXPERIMENTAL PROCEDURES**

**Cell Culture**

THP-1 and Jurkat cell lines were obtained from the American Type Culture Collection and cultured in RPMI 1640 with glutamax and phenol red (Invitrogen, Life Technologies, Inc.), supplemented with 10% (v/v) heat-inactivated fetal calf serum (PAA Laboratories GmbH), 25 mM HEPES, 100 units/ml penicillin, and 50 μg/ml streptomycin. Cells were maintained at 37°C in a humidified 5% CO2 atmosphere. THP-1 monocytes were stimulated with 100 nM PMA for 24 h to differentiate into macrophage-like adherent cells.

**Labeling of Proteins**

Annexin A5 and its mutants M23 and M1234 were labeled with Oregon Green and Alexa 568 as described previously (71). FVIIa was labeled with fluorescein as described (36). Briefly, FVIIa (Novoseven, NovoNordisk) was reacted with N-acetylthioacetylethyl-d-Phe-Pro-Arg-CH2Cl. Then, 1 mg/ml acetylthioacetylethyl-d-Phe-Pro-Arg-FVIIa was mixed with 0.5 mg/ml iodoacetamide fluorescein, to which 50 μl of 2 N NH2OH was added. After 1 h of incubation in the dark, the fluorescein isothiocyanate-labeled FVIIa (FVIIa-F) was separated from free dye on a PD-10 (G25) column equilibrated in sterile PBS. The concentration of the labeled proteins was determined by measurements of the absorbance at 496 nm.

**Cell Stimulation**

To induce apoptosis, THP-1 macrophages were treated with 100 μM etoposide (Sigma), a topoisomerase-II enzyme inhibitor, and Jurkat cells were stimulated with anti-Fas (200 ng/ml), both dissolved in serum-free Medium 199 (Invitrogen, Life Technologies, Inc.) supplemented with 0.5 mM CaCl2. The respective control cultures were incubated in equal volumes of culture media.

**Visualization of PtdSer Expression**

Etoposide-stimulated THP-1 macrophages were rinsed once with PBS. Binding buffer (10 mM HEPES, 150 mM NaCl, 5 mM KCl, 1 mM MgCl2, and 2.5 mM CaCl2) was added, which was supplemented with 1 μg/ml annexin A5 Oregon Green and 2.5 μg/ml propidium iodide (PI). The cells were incubated in the dark for 5 min at room temperature, washed with binding buffer, and fixed with 4% paraformaldehyde (PFA) solution (4% (v/v) PFA, HEPES 10 mM, NaCl 140 mM, CaCl2 2.5 mM, pH 7.4) for 15 min at room temperature. After washing with PBS, cells were mounted in a solution containing glycerol 90%, 0.02% Tri-HCL, pH 8, 0.002% NaN3, and 2% 1,4 diazabicyclo (2,2,2)-octane (DABCO, Merck) also with 0.5 μg/ml DAPI (4′,6-diamidino-2-phenylindole hydrochloride) to stain the nuclei. Confocal scanning laser microscopy (CSLM) (Bio-Rad) was employed to visualize and quantify PtdSer-expressing cells. At least 2000 cells were counted in more than 20 fields. Apoptotic index was calculated as PtdSer-positive/PI-negative cells per 105 of total cells.

**CSLM Analysis of AnxA5 and FVIIa-F Internalization**

THP-1 macrophages or Jurkat cells, that were stimulated to express PtdSer, were incubated in M199 medium with 20 μg/ml annexin A5 Alexa 568, 50 nM FVIIa-F, or 20 μg/ml annexin A5-Alexa 568 + 50 nM FVIIa-F. The cytoskeleton-disrupting agents latrunculin B (Sigma) and colchicine (Sigma), and the anti-annexin A5 monoclonal antibody WAC2a were added if indicated. After the treatment, cells were washed in PBS and then rapidly rinsed in a buffer containing EDTA, 5 mM, HEPES 25 mM, NaCl 140 mM, pH 7.4, and in a low pH glycine buffer containing glycine 0.1 M, pH 2.5 to remove surface-bound annexin A5 Alexa 568 and FVIIa-F, respectively. Cells were fixed and mounted as described above. Slides were examined with CSLM, and the images were recorded and analyzed with NIH image, ImageJ software, and Adobe Photoshop. Control experiments were done with the annexin A5 mutants M23-Alexa 568 and M1234-Alexa 568, which do not form a two-dimensional network on the surface and do not bind to PtdSer, respectively, and with annexin A5 Alexa 568, which binds to PtdSer but does not form a two-dimensional network.

**AnxA5 Antigen Quantification**

AnxA5 antigen was measured in the extracellular medium, and in the cytosolic and membrane fractions of THP-1 monocytes and THP-1 macrophages stimulated or not with etoposide. Cells were collected, centrifuged at 300 × g for 5 min, and resuspended in PBS. Cell suspensions were lysed by five freeze-thaw steps, and soluble (cytosol) and insoluble (membrane) fractions were separated by centrifugation at 14,000 × g. Total protein concentration was determined by a protein determination assay (Micro BCA protein assay, Pierce), and annexin A5 antigen was measured by an annexin A5-specific ELISA (Zymutest Annexin V, Hyphen BioMed) as described previously (37).

**Measurement of TF Activity**

TF activity expressed at cell membrane surfaces was measured through its ability to enhance the FVIIa-catalyzed activation of factor X. THP-1 macrophages were incubated with etoposide in the presence of annexin A5 (20 μg/ml), annexin A1 (20 μg/ml), cytoskeleton-disrupting agents when necessary, and the anti-annexin A5 antibody (10 μg/ml) for 4 h. After washing with PBS and sodium citrate buffer (sodium citrate 1 mg/ml, HEPES 25 mM, NaCl 140 mM, pH 7.4) to remove surface-bound annexin A5 or annexin A1, cells were first washed with rinse buffer (HEPES 10 mM, NaCl 136 mM, MgCl2 2 mM, KCl 2.7 mM, glucose 1 mg/ml, and bovine serum albumin 1 mg/ml, pH 7.5), and second incubated with a reaction mixture containing: 100 nM FX, 10 pM FVIIa, 3 mM CaCl2, and 200 μM Pentfluor FXa substrate (Pentapharm). Fluorescence tracings were recorded in a 96-well plate spectrofluorometer (Spectravmax Gemini XS, Molecular Devices) at 37°C with an excitation and emission wavelength of 350 and 450 nm, respectively. Known amounts of purified factor Xa were used to construct a reference line.

**Animal Model**

To perform the experiments in vivo, we used a mouse model of acute vascular injury (38). Adult male 16–24-week-old Swiss mice weighing between 30 and 45 g were anesthetized with an injection of pentobarbital (70 mg/kg i.p.). A ventral incision was made in the neck area, the bifurcation of the right common carotid artery was exposed carefully, and the accompanying nerve was separated. To control the blood flow temporarily, we put three sutures: one proximally of the bifurcation of the artery and two distally of the bifurcation, one on each external carotid artery. A flexible wire of 0.35-mm diameter was inserted into the external carotid artery to remove the endothelium (3 times). Later, the flow was restored by releasing the sutures on the commune artery and the internal carotid arterial. The external carotid artery was tied off proximally of the incision.

For detection of PtdSer exposure, 4 mg/kg annexin A5-Alexa 568 (two-photon microscopy analysis) or 4 mg/kg annexin A5-biotin (immunohistochemical studies) were injected immediately after injury through the jugular vein. After 30 min, mice were perfused via the left ventricle with 0.9% saline for 3 min. To obtain evidence about the nature of the apoptotic cells, the injured right and uninjured left common carotid arteries from mice perfused with annexin A5-Alexa 568 were immediately removed and frozen to −80°C. Afterward, samples were cut into cryosections of 6 μm, incubated in Syto-13 (green) for nuclei staining, and analyzed using a two-photon microscope (Bio-Rad 2100MP). To perform the immunohistochemical studies, mice that had received annexin A5-biotin were perfused with 2% PFA solution and sacrificed. The injured right and uninjured left common carotid arteries were immediately removed, further fixed in a 1% PFA solution, embedded in paraffin, and cut into 4-μm longitudinal sections.

Surgery was performed using a stereomicroscope (Leica MZ FL III, Leica, Switzerland). The mouse body temperature was maintained at 38.5°C, and an electrocardiogram (ECG) was monitored.

**Tissue Factor Down-regulation by Annexin A5**

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Immunohistochemistry

AnxA5-biotin and TF Staining—4-µm sections from uninjured and injured mouse arterial tissue were dewaxed in xylene, rehydrated, and incubated with 3% H2O2 in methanol for 15 min to block endogenous peroxidase activity. For anxA5-biotin detection, slides were incubated in VECTASTAIN ABC kit PK-6100 for 30 min. For TF staining, sections were first incubated with rabbit anti-mouse TF antibodies (1:200) at 4 °C overnight and later with Swine anti-rabbit peroxidase (1:150) for 45 min. The sections were developed in a freshly prepared developing medium containing: DAB, Tris-HCl, and imidazole (activated with 1.5% H2O2).

Measuring Positivity—TF and anxA5-biotin-stained sections were digitally photographed. The images were used to quantify the staining with Adobe Photoshop software. The TF and anxA5-biotin-positive areas were measured in the intima/media layer and expressed in percentage of the total area.

Statistical Analysis

Results are presented as mean ± S.D. Normal distribution of data was checked by means of the Shapiro-Wilks test. A Levene statistical test was performed to check the homogeneity of variances. The unpaired Student’s test was used to assess statistical differences between data from two groups. Differences between data obtained from more than two experimental conditions were tested by a one-way analysis of variance. Subsequent analysis for significant differences between two groups was performed by means of the multiple comparison Sheffe’s test. Probability values of <0.05 were considered significant.

RESULTS

The PS-anxA5 Portal of Cell Entry Is Present in THP-1 Macrophages—We investigated the internalization of the transmembrane receptor tissue factor through the PtdSer-anxA5 portal of cell entry (71). THP-1 macrophages were selected because they express both the transmembrane receptor tissue factor and PtdSer constitutively as well as inducibly (28). Cell surface expression of PtdSer was up-regulated by activation of apoptosis with etoposide (39). Incubating THP-1 macrophages with etoposide over 4 h increased the number of PtdSer-expressing cells from 6.9 ± 2.9% to 15.1 ± 5% when measured with anxA5-Oregon Green (Fig. 1).

Next, we examined whether PtdSer-expressing THP-1 macrophages internalize anxA5. THP-1 macrophages were incubated with or without etoposide in the presence of anxA5-Alexa 568 and analyzed by CSLM. Fig. 2 shows that 11.3 ± 2.0% of untreated cells (Fig. 2, panel A) and 19.6 ± 1.9% of etoposide-treated cells (Fig. 2B) internalized anxA5-Alexa 568. The cells that had internalized anxA5-Alexa 568 expressed PtdSer at their cell surface, as was demonstrated by washing with EDTA followed by incubation with anxA5-Oregon Green (Fig. 2, panel C). We confirmed that the uptake occurred through the PtdSer-anxA5 portal of cell entry by examining the internalization of the anxA5 mutants M1234-Alexa 568 and M223-Alexa 568. Both mutants were not internalized (data not shown), indicating that the internalization of anxA5-Alexa 568 is dependent on its ability to bind to PtdSer and to organize in a two-dimensional network on the membrane surface (71). The latter dependence was confirmed by experiments with anxA1, which binds to PtdSer without forming a two-dimensional network (40). THP-1 macrophages that were incubated with etoposide and anxA1-Alexa 568 showed a modest internalization of anxA1 (Fig. 3, panel A). It has been suggested that macrophages present an anxA1 receptor (41), making it highly probable that the observed internalization of anxA1-Alexa 568 occurred through the internalization of the ligated anxA1 receptor. This latter type of internalization depends on the polymerization of actin (42) whereas anxA5 internalization does not (71). We inhibited actin polymerization and endocytic vesicle trafficking in THP-1 macrophages with latrunculin B and colchicine, respectively. These cytoskeletal-disrupting agents prevented anxA1-Alexa 568 internalization (Fig. 3, panel C) but not anxA5-Alexa 568 internalization (Fig. 3D).

These results clearly demonstrate that the PS-anxA5 portal of cell entry is present in THP-1 macrophages.

TF Is Internalized through the PS-AnxA5 Portal of Cell Entry—To analyze TF internalization we selected its ligand FVIIa as the TF-imaging probe. For that purpose FVIIa was labeled with fluorescein at its active site yielding a fluorescent FVIIa derivative (FVIIa-F) that blocked TF activity completely (data not shown), indicating that FVIIa-F binds to TF in the same manner as FVIIa.

Incubation of THP-1 macrophages with FVIIa-F in the absence or in the presence of etoposide resulted in a modest internalization of FVIIa-F (Fig. 4, panels A and B). 13.5 ± 2.9% and 18.8 ± 4.4% of the THP-1 macrophages internalized FVIIa-F in the absence and presence of etoposide, respectively (Fig. 4, panel D). The relation of FVIIa-F internalization with etoposide treatment was comparable to that of anxA5-Alexa 568 internalization (Fig. 2, panel D), suggesting an involvement of cell surface-expressed PtdSer. To investigate whether PtdSer and TF are part of the same membrane patch, THP-1 macrophages were incubated with etoposide in the presence of anxA5-Alexa 568 and FVIIa-F. CSLM analysis revealed that both anxA5-Alexa 568 and FVIIa-F were internalized massively and showed co-localization in endocytic vesicles (Fig. 5, panel C). Control experiments in which FVIIa-F was co-incubated with the anxA5 mutants, M23-Alexa 568 and M1234-Alexa 568, were negative for co-localization. To exclude TF-independent internalization of our probe FVIIa-F because of binding to cell surface-expressed PtdSer, we incubated Jurkat cells that lack TF with anti-Fas to induce PtdSer exposure (71) in the presence of anxA5-Alexa 568 and FVIIa-F. Apoptotic Jurkat cells internalized only anxA5-Alexa 568 and not FVIIa-F (data not shown). The above results together demon-
THP-1 Macrophages with Surface-Expressed PtdSer Internalize anxA5. THP-1 macrophages were either untreated or treated with 100 μM etoposide for 4 h in the presence of 20 μg/ml anxA5-Alexa 568. The cells were subsequently analyzed by CSLM. Panel A, THP-1 macrophages that were incubated with anxA5-Alexa 568. Panel B, THP-1 macrophages that were incubated with etoposide and anxA5-Alexa 568. To detect PtdSer exposure in those cells with anxA5 internalized, apoptotic cells were treated with EDTA and counterstained with anxA5-Oregon Green (panel C). Original magnification ×400 for all panels. Panel D, quantification of the number of THF-1 macrophages internalizing anxA5-Alexa 568 in the absence (control, white bars, n = 6) and in the presence of etoposide (gray bars, n = 10). *p < 0.05 compared with untreated cells.

Cell Entry through Distinct Mechanisms. Panels A and B present CSLM analyses of THP-1 macrophages that were co-incubated with 100 μM etoposide and 20 μg/ml anxA1-Alexa 568 (A) or 20 μg/ml anxA5-Alexa 568 (20 μg/ml) (B). Panels C and D present CSLM analyses of THP-1 macrophages that were stimulated with etoposide and the cytoskeleton-disrupting agents latrunculin B and colchicine in the presence of anxA1-Alexa 568 (panel C) or anxA5-Alexa 568 (panel D).

Entry through Endogenously Produced anxA5—THP-1 macrophages were either untreated or treated with 100 μM etoposide for 4 h in the presence of 20 μg/ml anxA5-Alexa 568. The cells were subsequently analyzed by CSLM. Panel A, THP-1 macrophages that were incubated with anxA5-Alexa 568. Panel B, THP-1 macrophages that were incubated with etoposide and anxA5-Alexa 568. To detect PtdSer exposure in those cells with anxA5 internalized, apoptotic cells were treated with EDTA and counterstained with anxA5-Oregon Green (panel C). Original magnification ×400 for all panels. Panel D, quantification of the number of THP-1 macrophages internalizing anxA5-Alexa 568 in the absence (control, white bars, n = 6) and in the presence of etoposide (gray bars, n = 10). *p < 0.05 compared with untreated cells.

THP-1 Macrophages Open their PtdSer-anxA5 Portal of Cell Entry through Endogenously Produced anxA5—The experiments performed so far showed that THP-1 macrophages modestly internalize TF in large endocytic vesicles in the absence of exogenously added anxA5. We reasoned that this could be caused, at least partially, by endogenous anxA5 produced by THP-1 macrophages. Therefore we measured the amount of anxA5 synthesized by THP-1 cells using an anxA5-specific ELISA. THP-1 cells produce anxA5 and up-regulate its levels upon PMA and etoposide treatment (Table 1). Most of the anxA5 resides in the cytosol, part of it associates with the membrane fraction, and part of it is released into the extracellular medium. The latter is increased by PMA and etoposide treatment. The externalized anxA5 could be responsible, at least partially, for the observed TF internalization in the absence of exogenously added anxA5. These data strongly indicate the existence of an autocrine/paracrine-regulated opening of the PtdSer-anxA5 portal of cell entry.

TF Activity Is Down-regulated by the PtdSer-anxA5 Portal of Cell Entry—To determine whether anxA5-induced internalization of TF reduces the TF procoagulant activity of THP-1 macrophages, we set up an assay that measures TF activity as a function of the amount of surface-expressed TF per number of cells. The assay conditions were chosen such that a linear relationship existed between cell density and FXa generation both in the absence or presence of etoposide. Addition of FVIIa-F inhibited completely FXa production (data not shown) demonstrating the dependence of the assay on TF.

THP-1 macrophages, which were incubated with etoposide for 4 h, expressed TF activity. If the incubation with etoposide proceeded in the presence of anxA5, the TF activity was reduced by 25% (Fig. 7). Note that the THP-1 macrophages were treated with sodium citrate after the incubation period of time and prior to the measurement of TF activity. This procedure removed cell surface-bound anxA5 and precluded the direct anticoagulant action of anxA5 at the cell surface. Hence, the observed inhibition of TF activity arises from the internalization of TF. At this point we reasoned that the TF internalized by the PtdSer-anxA5 pathway could recycle back to the cell surface. As demonstrated previously the PtdSer-anxA5 portal of cell entry does not depend on the involvement of the cytoskeleton although intracellular trafficking of the endocytic vesicles does (71). The cytoskeletal-disrupting agents latrunculin B and

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colchicine had no effect on the TF activity of etoposide-treated THP-1 macrophages. These agents did augment the inhibition of TF activity if the cells were incubated with etoposide and anxA5 (Fig. 7). These results demonstrate that part of the TF internalized by the PtdSer-anxA5 pathway recycle back to the plasma membrane of the THP-1 macrophages. anxA1, which does not induce TF internalization (see above), was without effect on the TF activity (Fig. 7).

To explore whether the autocrine/paracrine mechanism of TF internalization (see above) contributes to the regulation of TF activity we incubated THP-1 macrophages with WAC2a. Fig. 8 illustrates that WAC2a causes the increase of TF activity of THP-1 macrophages that were untreated or treated with etoposide. The WAC2a caused an increase in TF activity that was more pronounced if THP-1 macrophages were treated with etoposide, a procedure, which leads to an increase in extracellular anxA5 levels (Table I). A murine isotype-matched control antibody had no effect on the TF activity. Taken together, these results demonstrate that the PtdSer-anxA5 pathway regulates TF activity of the THP-1 cells by internalizing cell surface-expressed TF.

The PtdSer-AnxA5 Pathway Regulates TF Expression in Vivo under Stress Conditions—Having discovered that an anxA5-dependent TF internalization process is taking place if apoptosis proceeds in the presence of anxA5 in vitro, we investigated whether anxA5 is able to regulate TF expression in vivo under stress conditions. Therefore we employed a mouse model in which a carotid artery is mechanically injured. Injury of the own internalization by causing invagination and budding of the membrane patch to which it is bound (71). It is unknown whether the PtdSer-exposing medial SMC expressed TF, we injected anxA5-biotin (30 min prior to collection of the injured carotid artery for immunohistochemical inspection. The medial SMC with exposed PtdSer also expressed TF as was demonstrated by staining anxA5-biotin using avidin-horseradish peroxidase (Fig. 9A, panel ii) and TF using an anti-TF antibody (Fig. 9A, panel iii). The uninjured contralateral carotid artery was devoid of PtdSer-expressing cells and contained only a small amount of TF-expressing cells (Fig. 9A, panel iv). Quantification of the TF-positive cells revealed that injury of the carotid artery resulted in an increase in the number of cells that express TF (uninjured: 3.5 ± 1.3% versus injured: 19.3 ± 9.8%; p < 0.05) (Fig. 9B). If anxA5 was injected intravenously immediately after the injury, the number of cells expressing TF was dramatically decreased compared with the injured situation in the absence of anxA5 (uninjured: 5.8 ± 1.4%; injured and no anxA5: 19.3 ± 9.8%; injured in the presence of anxA5: 5.2 ± 0.9%) (Fig. 9B). These results together with the findings that the (i) anxA5 binds to the PtdSer-exposing medial SMC that express TF and (ii) the PtdSer-anxA5 pathway down-regulates TF strongly suggest that the PtdSer-anxA5 portal of cell entry operates in vivo under stress conditions and regulates TF expression of stressed SMC.

**DISCUSSION**

Results previously obtained in our laboratory showed that anxA5, when bound to the PtdSer-exposed plasma membranes of apoptotic cells, modifies membrane dynamics and induces its own internalization by causing invagination and budding of the membrane patch to which it is bound (71). It is unknown whether this also results in the internalization of membrane receptors embedded in the PtdSer-expressing membrane domain. Here we report that anxA5 down-regulates TF expression from the sur-
of other proteins bound to or in the vicinity of surface-expressed PtdSer. TF being one of these proteins, these results also reveal a way by which anxA5 enters into the cell leads to internalization of the cell membrane, and extracellular medium were extracted, and the anxA5 antigen was quantified by an ELISA. Data represent mean ± S.D. (n = 6).

|                | Control          | PMA             | PMA + etoposide |
|----------------|------------------|-----------------|-----------------|
| Cytosol (ng/µg total protein) | 44.04 ± 11.39 | 67.87 ± 35.01   | 133.78 ± 38.47  |
| Membrane fraction (pg/µg total protein) | 47.38 ± 8.29   | 34.50 ± 2.46    | 30.14 ± 8.06    |
| Extracellular medium (ng/ml) | 2.65 ± 0.70    | 6.70 ± 1.26     | 9.36 ± 0.96     |

* p < 0.05 compared with control and PMA.
† p < 0.01 compared with PMA and PMA + etoposide.
‡ p < 0.05 compared with control.
§ p < 0.01 compared with PMA.

FIG. 6. Endogenous anxA5 contributes to the internalization of TF by THP-1 macrophages. THP-1 macrophages were incubated with 50 nM FVIIa-F alone (control, n = 6), with 50 nM FVIIa-F and 100 µM etoposide (n = 12), or with 50 nM FVIIa-F, 100 µM etoposide, and 10 µg/ml anti-anxA5 antibody WAC2a (n = 10). Cells with internalized TF were enumerated and expressed as percentage of the total cell count. *, p < 0.05 compared with control and etoposide + anti-anxA5.

FIG. 7. AnxA5 inhibits the TF activity of THP-1 macrophages by internalizing TF. THP-1 were treated with 100 µM etoposide in the absence (Control) or presence of 20 µg/ml anxA5, 20 µg/ml anxA5 + latrunculin B + colchicine, or 20 µg/ml anxA1. TF activity of the treated THP-1 macrophages was measured as described and expressed as % of the control (n = 8 for each bar). *, p < 0.05 compared with control and anxA1. §, p < 0.05 compared with anxA5.

face of apoptotic cells through the mechanism of internalization. This is accompanied by a reduction in TF activity more remarkable when intracellular trafficking is disrupted, meaning that the intracellular traffic followed by anxA5 endocytic vesicles leads to the recycle and reexposition of TF on the cell membrane. We also found that cells in apoptosis produce and release anxA5, which once in the extracellular compartment causes down-regulation of surface-expressed TF through this mechanism. We demonstrate furthermore that anxA5 down-regulates TF expression by medial smooth muscle cells in an in vivo mouse model of carotid artery injury. Together our data clearly document that this novel pathway by which anxA5 enters into the cell leads to internalization of other proteins bound to or in the vicinity of surface-expressed PtdSer. TF being one of these proteins, these results also reveal that anxA5 triggers a novel anticoagulant mechanism.

Previous studies show that the TF-FVIIa complex is internalized in different cell lines. In baby hamster kidney (BHK) cells stably transfected with TF, FVIIa is actively internalized by a TF-dependent process (44). In fibroblasts, Iakhiyaev et al. (45) have shown that fibroblasts internalize FVIIa bound to TF through two different pathways: (i) a low density lipoprotein receptor-related protein (LRP)-dependent mechanism, in the presence of the complex TFPI-Xa and (ii) an LRP-independent mechanism in the absence of TFPI-Xa. Only the former pathway was associated with TF down-regulation from the cell surface. This pathway has also been described to be responsible for the down-regulation of TF activity from the cell surface of monocytes (46). Using an optical imaging approach with fluorescent FVIIai we demonstrated that anxA5 induces the internalization of surface-expressed TF of THP-1 macrophages through a different mechanism. We conclude that anxA5 causes TF internalization because TF is embedded in the membrane patch expressing the PtdSer to which anxA5 binds. This conclusion is based on the following results: (i) anxA5 and TF are localized in the same endocytic vesicles, (ii) internalization of both anxA5 and TF is increased per cell and per number of cells if THP-1 macrophages have up-regulated PtdSer expression due to the activation of apoptosis by etoposide, and (iii) both the anxA5 mutant M23 and anxA1 (which lack the ability to crystalize on the PtdSer-expressing surface) and the anxA4 mutant M1234 (which fails to bind to PtdSer) do not induce TF internalization.

We observed that in the absence of exogenously added anxA5 some THP-1 macrophages internalize TF, although this is less...
have previously demonstrated that endogenous anxA5, consistent with the fact that it does not contain a signal peptide, is not secreted. Alternative secretory routes for proteins that do not possess a secretory signal peptide do exist. Annexin A1 and annexin A2, other members of the annexin family that do not have signal peptides, have been detected on cell surfaces and in culture medium (51–53). Proteins other than annexins such as endothelial cell growth factors (54), interleukin-1 (55, 56), and fibroblast growth factor 2 (57) are additionally secreted in the absence of a signal peptide. On this basis we propose that anxA5 is released under stress conditions and acts as an autocrine regulator of the plasma membrane receptor repertoire through its ability to internalize PtdSer-expressing membrane patches and the receptors embedded within them.

AnxA5 incubation during stimulation with etoposide inhibits significantly TF activity of THP-1 macrophages. This effect is dependent on the capacity of anxA5 to form a two-dimensional network on the cell surface since the anxA5 mutant M23 and anxA1 have no influence in TF activity. The inhibition of TF activity by anxA5 is increased when intracellular trafficking is disrupted during the apoptotic stimulation. Also, contribution of endogenous anxA5 to the inhibition of TF was confirmed using the antibody that blocks the binding of anxA5 to PtdSer. Thus, our results show that anxA5 inhibits TF activity involving a new mechanism by which anxA5 internalizes TF in PtdSer-containing patches. Furthermore, our experiments suggest that this process could be reversed because of recycling of the endocytic anxA5 vesicles. In our previous work we had already confirmed that anxA5 endocytic vesicles were conducted from the membrane to the cytosol through a process of intracellular trafficking (71). In this work, we may add that anxA5 endocytic vesicles might also be conducted back to the membrane, reexpressing the membrane proteins cointernalized with anxA5, like TF, on the cell surface.

To investigate the existence of anxA5-induced down-regulation of TF in vivo we tested the effects of anxA5 on TF expression in a mouse model of arterial injury. Previous studies have demonstrated that after endothelial injury, both apoptosis (58, 59) and TF expression (60–63) are induced in SMCs from the media. Corroborating these studies, our results show that in the mouse carotid wall after endothelium scratching, TF expression is highly increased in the SMCs from the media and also a high PtdSer exposure reveals a high incidence of apoptosis. Under these conditions, we examined the effects of anxA5 injected into the carotids just after endothelial injury. Administration of anxA5 decreased the TF expression after the injury significantly. Because PtdSer and TF are localized on the same SMCs we propose that anxA5 binds to PtdSer and induces the internalization of TF. However, we should consider that TF initiates blood coagulation reactions leading to the formation of thrombin, which in turn has been shown to increase TF mRNA levels and TF activity in VSMCs (64–66). Thus, it is possible that in our in vivo model anxA5 reduces TF expression also through the inhibition of thrombin formation. In this regard, Gertz et al. (67) reported that hirudin inhibited TF expression if hirudin was infused for a prolonged period of time (28 days). In our model, a bolus infusion of anxA5 is sufficient to reduce TF expression after 30 min. Hence, these kinetics preclude a significant role for the pathway via inhibition of thrombin formation.

In conclusion, our in vitro and in vivo experiments demonstrate the existence of a novel mechanism to regulate the surface expression of the membrane receptor TF. We have shown that the PtdSer-anxA5 endocytic pathway down-regulates TF with functional consequences. The anticoagulant mechanism of anxA5 is thought to be based on its ability to
bind to PtdSer (68) and to form a two-dimensional network that prevents the lateral diffusion of coagulation factors on the surface (69, 70). This work demonstrates that the anticoagulant mechanism of annexin A5 is more complex on a dynamic cellular surface because it also involves internalization of the procoagulant TF. Because the PtdSer-annexin A5 pathway of down-regulating TF may apply more generally to membrane receptors located in the vicinity of PtdSer and because endogenous annexin A5 can activate this pathway, we postulate that the PtdSer-annexin A5 pathway may be part of a more general autocrine/paracrine function of annexin A5 to regulate the plasma membrane receptor repertoire under stress conditions that lead to the surface expression of PtdSer.

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Annexin A5 Down-regulates Surface Expression of Tissue Factor: A NOVEL MECHANISM OF REGULATING THE MEMBRANE RECEPTOR REPERTOIR
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