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Annexin 1 Binds to U937 Monocytic Cells and Inhibits Their Adhesion to Microvascular Endothelium: Involvement of the $\alpha_4\beta_1$ Integrin[1]

Egle Solito,[2]* Ignacio A. Romero,[3]* Stefano Marullo,* Françoise Russo-Marie,* and Babette B. Weksler†

Annexin 1 (ANX1), a calcium-binding protein, participates in the regulation of early inflammatory responses. Whereas some of its effects depend on intracellular interactions, a growing number of observations indicate that ANX1 may also act via autocrine/paracrine functions following externalization to the outer side of the plasma membrane. We studied the effects of ANX1 on leukocyte adhesion to endothelial cells using as a model system the monocytic cell line U937 and human bone marrow microvascular endothelial cells. Exogenous rANX1, as well as endogenous ANX1 externalized by U937 differentiated in vitro, inhibited monocyte firm adhesion to vascular endothelium. Both binding of ANX1 to U937 cells and ANX1-mediated inhibition of cell adhesion involved the short N-terminal domain of the ANX1 molecule. Under experimental conditions in which ANX1 inhibited U937 adhesion to human bone marrow microvascular endothelial cells, this protein specifically colocalized with the α_4 integrin, and a direct interaction between ANX1 and the α_4 integrin could be documented by immunoprecipitation experiments. Moreover, ANX1 competed with the endothelial integrin counterreceptor, VCAM-1, for binding to α_4 integrin. These results indicate that ANX1 plays an important physiological role in modulating monocyte firm adhesion to the endothelium. ***The Journal of Immunology,*** **2000, 165: 1573–1581.**

The release of proinflammatory mediators (cytokines and vasoactive amines) as a consequence of tissue injury induces rapid, dramatic changes in the adhesive properties of endothelial cells (EC)[4] in blood vessels near the injury site. As an immediate consequence of these changes, leukocytes attach to and begin to roll along the activated EC surface. Subsequently, leukocytes are activated by chemoattractants, which cause shedding of L-selectin and activation of adhesion receptors such as the $\alpha_4\beta_1$ integrin (also known as VLA-4 or CD49d/CD29), the $\alpha_M\beta_2$ integrin (Mac-1 or CD11b/CD18), and the $\alpha_L\beta_2$ integrin (LFA-1 or CD11a) (1). The interactions of leukocyte integrins with ICAM-1 and VCAM-1, two Ig-like adhesion molecules expressed by endothelial cells, determine the firm adhesion of activated leukocytes to the EC, leukocyte emigration, and their recruitment into the inflammatory site.

Annexin 1 (ANX1, lipocortin 1) belongs to a family of multifunctional proteins that all bind acidic phospholipids in the presence of calcium ions. The observation that cell-associated ANX1 was up-regulated by glucocorticoids (GCs) suggested that ANX1 itself might participate in the anti-inflammatory effects of these compounds (2). A single administration of dexamethasone in laboratory animals increased ANX1 levels in circulating leukocytes (3). Conversely, lowering endogenous steroids by adrenalectomy or by a sustained treatment with the steroid antagonist mifepristone (RU486) reduced the amount of cell-associated ANX1 protein and mRNA by ~50% (4). In inflammatory models, exogenous ANX1 inhibited recruitment of polymorphonuclear (PMN) leukocytes in vivo (5, 6). Moreover, treatments with Ab to ANX1 blocked the anti-inflammatory effect of dexamethasone in animal models of acute inflammation (7). In these models, the inhibition of cell migration to inflammatory sites appeared to be one of the mechanisms by which GCs exert their anti-inflammatory effect.

Among the different classes of peripheral blood leukocytes, both PMN and monocytes have been shown to contain large amounts of ANX1 (8–11). The monocytic cell line U937 expresses increased amounts of endogenous ANX1 upon cell differentiation into macrophages (12), and GC treatment of differentiated U937 cells produced translocation of the ANX1 to external leaflet of the plasma membrane (13). Adhesion of monocytes to the vascular endothelium during inflammation is a critical step preceding their recruitment into extravascular tissue (14). Therefore, in the present study, we have investigated whether ANX1 modulates monocyte adhesion to human microvascular endothelial cells and have characterized the molecular mechanisms involved. We show that both exogenous rANX1 and endogenous ANX1 inhibit the firm adhesion of U937 cells to EC, most likely through direct interaction with the $\alpha_4\beta_1$ integrin. These data indicate that the ANX1 can mediate

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[4] Abbreviations used in this paper: EC, endothelial cell; ANX1/ANX5, annexin 1/5; ANX1–5, chimeric annexin 1-annexin 5; CXCR, CXC chemokine receptor; GC, glucocorticoid; HBMEC, human bone marrow endothelial cell; ^{125}I-ANX1, ^{125}I-labeled ANX1; IP, immunoprecipitation; PMN, polymorphonuclear; RT, room temperature; SDF, stromal cell-derived factor.

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inhibition of monocyte migration through the endothelium, as part of the anti-inflammatory actions of ANX1.

Materials and Methods

Cells

Wild-type U937 cells, stable clones of U937-expressing antisense ANX1 mRNA (15), and control U937 transfected with the empty expression vector were maintained in culture in RPMI medium (Life Technologies, Gaithersburg, MD) supplemented with 10% FCS, 100 U/ml penicillin, 100 μg/ml streptomycin, and 1% (v/v, 7.7 mg/ml) glutamine. G-418 (200 μg/ml) was used to maintain stable transfected clones. A human microvascular endothelial cell line (HBMEC) from adult human bone marrow (16) was used to study U937 cell adhesion. HBMEC cells display a normal pattern of regulation of adhesion molecules (17); they were maintained in DMEM supplemented with 5% FCS, 10 mM HEPES, and antibiotics at the same concentrations as above. In some experiments, U937 cells were differentiated into macrophages by incubation for 24 h with 6 ng/ml of PMA, as described (12), before use in adhesion studies.

Abs, recombinant proteins, and peptides

The following Abs were used: A polyclonal anti-ANX1 Ab (1:1000) (18) was used for immunofluorescence analysis as well as immunoprecipitation (IP) studies. A mAb, 1-B (5, 19), was used to block biological activities of ANX1. Ab against the α_4 integrin (FITC-conjugated CD49d mAb, diluted 1/20) was from Immunotech (Luminy, France). Ab directed against LFA-1 (CD11a mAb diluted 1/40) and Ab directed against VCAM-1 (1/40) were from R&D Systems (Minneapolis, MN). The Ab against α_4 integrin used for IP (1/100) was from Santa Cruz Biotechnology (Santa Cruz, CA), as was anti-actin Ab (1/100). A goat anti-rabbit Cyanin 3 secondary Ab (diluted 1/300) and a goat anti-mouse FITC (1/300) were both from Boehringer Mannheim (Mannheim, Germany). The Ab against CXCR4 was from PharMingen (San Diego, CA) (monoclonal 12GA5-N, at 0.26 mg/ml).

The ANX1 N-terminal acetylated, 2–25 synthetic peptide was kindly provided by Dr. M. Perretti (The William Harvey Institute, London, U.K.).

Recombinant ANX1, annexin 5 (ANX5), and a chimeric protein ANX1–5, were expressed as GST-fusion proteins in pGEX vectors, as described (6). Fusion proteins were purified from bacterial lysates by affinity chromatography using prepacked glutathione-Sepharose 4B, and the GST moiety was removed by cleavage with thrombin (20). All three recombinant proteins were further purified by fast protein liquid chromatography (Pharmacia). Proteins were >95% pure, as determined by SDS-PAGE, fast protein liquid chromatography profiles, and Western blot analysis. Endotoxin contamination was less than 20 pg/ml, as measured by the *Limulus* amebocyte chromogenic assay (Calbiochem, La Jolla, CA).

Adhesion of U937 cells to endothelial cells

Adhesion of U937 cells to HBMEC was measured, as previously described (21). Briefly, 1×10^6 U937 cells were radiolabeled by incubation in 100 μl of RPMI containing 10% FCS and 20 μCi of ^{51}Cr (as sodium chromate) for 1 h at 37°C. After washing three times with HBSS labeled U937 cells were resuspended in RPMI 1640 medium containing 10% FCS, and were then incubated at 37°C for 30 min with or without human rANX1 (6). Next, 200 μl (2×10^5) labeled U937 cells, treated or not with ANX1, were added to each well of 96-well plates containing confluent HBMEC monolayers. The HBMEC had either been pretreated or not pretreated with 5 ng/ml human rTNF-α for 18 h (Amersham, Arlington Heights, IL).

After incubation of U937 on HBMEC monolayers for 1 h at 37°C, nonadherent U937 cells were removed by four consecutive washes. U937 cells remaining adherent to HBMEC monolayers after washing were then lysed with 1% Triton X-100 and gamma emissions quantified with a LKB (1282-Compugamma-CS) gamma counter. The percentage of adherent U937 was calculated from 6 to 12 independent wells per condition, as the ratio between radioactivity recovered in the cell lysate and the total amount of radioactivity in the initial aliquot of labeled U937 cells.

For analysis of the effects of externalized endogenous ANX1 on U937 adhesion to HBMEC, U937 cells that had been first differentiated with PMA and then treated with dexamethasone to externalize the up-regulated endogenous ANX1 were incubated with unstimulated HBMEC monolayers and further processed to quantitate adhesion, as described above.

Radioiodination of rANX1 and ^{125}I-ANX1-binding assay

rANX1 was radioiodinated using the chloramine T method (22). Small quantities of rANX1 (500 ng in 50 μl of PBS, pH. 7.2) were added to a mixture of 15 μl of chloramine T, according to Lowenthal et al. (23). The radiolabeled protein was applied to a prepacked Sephadex G-50 column prepacked in PBS (with 1% BSA) and purified. Specific radioactivity was further calculated and was ~20 μCi/μg of protein.

Binding studies of ^{125}I-ANX1 to cells were conducted according to Dower et al. (24). Briefly, aliquots of 2×10^5 cells were incubated in RPMI medium in the presence of varying concentrations of ^{125}I-ANX1 at 4°C for 4 h. To avoid any potential internalization of the labeled protein, experiments were conducted at 4°C, a temperature known to inhibit endocytosis. Bound ^{125}I-ANX1 was separated from free by centrifugation through a 30% sucrose cushion. Nonspecific binding was measured in the presence of a 50–100 molar excess of unlabeled ANX1.

Immunofluorescence and confocal imaging

U937 cells (treated or not with PMA and/or dexamethasone) were harvested, and aliquots of 1×10^6 cells were incubated at 37°C in the presence of 100 nM rANX1. Cells were then fixed with 2% paraformaldehyde for 10 min at room temperature (RT) and washed three times in a buffer consisting of 25 mM HEPES, 1 mM $CaCl_2$, 1 mM $MgCl_2$, and 1% BSA. Cells were incubated for 2 h at RT with a polyclonal anti-ANX1 Ab (1:1000), or with mAbs directed against the α_4 integrin (FITC-conjugated CD49d mAb, diluted 1/20) or against LFA-1 (CD11α mAb, diluted 1/40). Anti-actin Ab was used as the control of cellular membrane integrity. PBS/BSA was used as negative control. A goat anti-rabbit Cyanin 3 secondary Ab (diluted 1/300) and a goat anti-mouse FITC secondary Ab (diluted 1/300) were used, as was appropriate, for 30 min at RT. After four washes, samples were mounted in moviol (Sigma, St. Louis, MO). Images were obtained with a laser-scanning confocal microscope (MRC-1000; Bio-Rad, Hercules, CA) mounted on a Nikon Optiphot microscope. Dual detection was performed with separate photomultiplier tubes, and resultant images were merged.

Polarization of U937 cells

U937 cells (10^7) were added to the upper chamber of Transwell-Clear inserts (3 μm diameter pore; Costar, Cambridge, MA). SDF-1 (R&D Systems) was added at a concentration of 10^{-7} M to the lower chamber of the transwell. After 18 h at 37°C, U937 cells that had migrated into the lower chamber were harvested, washed, and incubated with human rANX1 (100 nM). After fixation in paraformaldehyde, cells were processed for immunofluorescence studies, as described above. A mAb against CXCR4 (12GA5-N terminal region 0.26 mg/ml; PharMingen) was used as marker of cell polarization.

Immunoprecipitation

Cells (10^7) were collected after 30-min incubation with human rANX1 (100 nM) or human rVCAM-1 (10 nM; R&D Systems) or both, and lysed in 500 μl of a lysis buffer (10 mM Tris, pH 7.2, 150 mM NaCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$ and 1% Nonidet P-40) for 10 min on ice in presence of protease inhibitors (100 mM PMSF, 1 μg/ml leupeptin, 1 μg/ml aprotinin). Cell lysates were preincubated with protein A-Sepharose and preimmune sera and centrifuged, and the recovered supernatants were incubated at 4°C overnight with protein A-Sepharose and appropriate Abs: either polyclonal anti-ANX1, anti-VCAM-1, or anti-α_4 integrin. After two washes in low salt (150 mM NaCl) and three washes in high salt (500 mM NaCl) buffers, Laemmli sample buffer (25) was added to pellets and samples were boiled for 5 min. Samples were then analyzed by SDS-PAGE, and separated proteins transferred onto nitrocellulose membranes. The membranes were saturated in 5% BSA and then incubated with a polyclonal Ab directed against the α_4 integrin (dilution 1/100) or ANX1 (1/100) or VCAM-1 (1 μg/ml). An anti-rabbit or anti-mouse Ig HRP secondary Ab (1/10,000; Amersham) was employed, and peroxidase reaction was developed using enhanced chemiluminescence (ECL; Amersham).

Statistics

Data, reported as mean ± SEM of n separate observations, were analyzed by a two-tailed Student's t test with p value threshold of 0.05, which was considered significant.

Results

Paracrine inhibition by ANX1 of monocyte adhesion to endothelial cells

ANX1 is externalized by PMN after contact with endothelium (9) and by U937 cells after treatment with dexamethasone (13). To study whether extracellular ANX1 modulates the adhesion of U937 monocytes to endothelial cells, we first evaluated its paracrine effects of rANX1 in a monocyte adhesion assay (21).

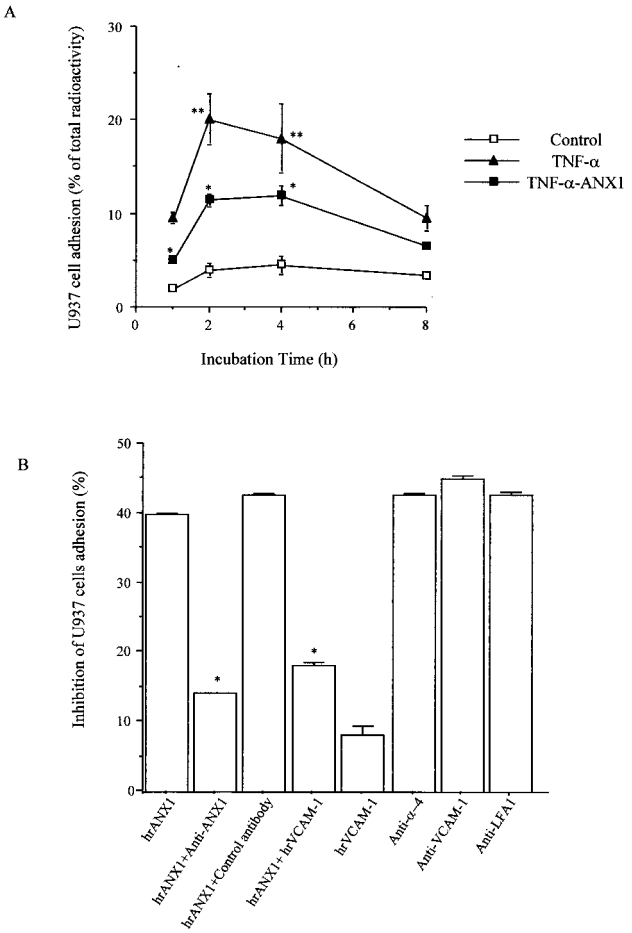

FIGURE 1. Human rANX1 blocks U937 adhesion to TNF-α-treated HBMEC. *A*, ^{51}Cr-labeled U937 cells were incubated for the indicated times on monolayers of HBMEC pretreated (▲) or not (□) with 5 ng/ml TNF-α. U937 cells incubated for 30 min (at 37°C) with 1 μM (50 μg/ml) human rANX1 (■) showed a reduced firm adhesion to HBMEC. U937 cell adhesion was measured, as described in *Materials and Methods*. Bars indicate mean ± SEM of triplicates measurements; data are representative of six independent experiments giving similar results. *, Significant difference between ANX1-treated and control cells, $p < 0.001$; **, significant difference between TNF-α-treated and untreated HBMEC, $p < 0.001$. *B*, U937 cell adhesion was studied in the presence of 100 nM ANX1 plus control Abs or Abs directed against either ANX1 (the 1-B mAb), integrins (α_4, α_L) the adhesion molecule (VCAM-1), or the recombinant extracellular domain of VCAM-1. Bars indicate mean ± SEM of triplicates; data are representative of three experiments giving similar results. *, Significant difference between cells treated with ANX1 alone and cells incubated with ANX1 and inhibitors of ANX1 effect, $p < 0.001$.

HBMEC monolayers were treated with TNF-α to induce expression of E-selectin and VCAM-1, as previously reported (17). Adhesion of U937 cells to TNF-α-activated HBMEC was examined starting 12 h after exposure of the latter to TNF-α. ^{51}Cr-labeled U937 cells were seeded on monolayers of control or stimulated HBMEC for varying time periods, the monolayers were then washed, and the radioactivity associated with cells remaining adherent was measured (Fig. 1*A*). Pretreatment of U937 cells with human rANX1 resulted in a rapid inhibition of U937 cell adhesion to TNF-α-stimulated HBMEC (Fig. 1*A*, closed squares). ANX1 alone produced 40% inhibition of U937 cell adhesion, which represented inhibition of adhesion comparable with that induced by Abs that blocked the α_4 integrin, LFA-1, or VCAM. Maximal inhibition (43%) was observed 2 h after ANX1 treatment of the

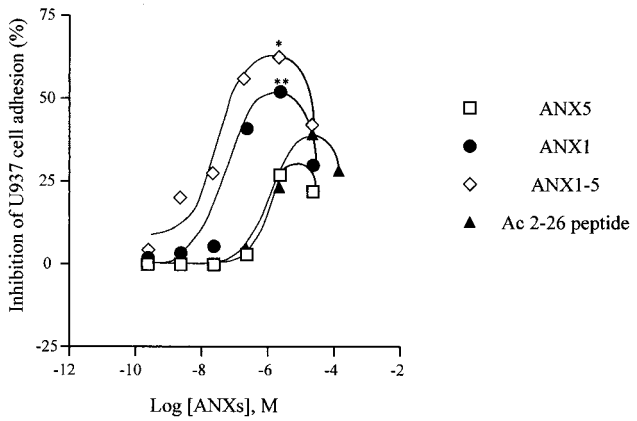

FIGURE 2. Inhibition of U937 cell adhesion to HBMEC by ANX1 and ANX1-related pepetide. ^{51}Cr-labeled U937 cells were preincubated for 30 min at 37°C in the presence of increasing concentrations of rANX1, the ANX1–5 chimera, ANX5, or the ANX1 N-terminal peptide (Ac 2–25), and were then incubated for 2 h with confluent monolayers of HBMEC prestimulated with TNF-α. Inhibition of adhesion corresponds to the loss of radioactivity associated with the HBMEC monolayer after the indicated treatments. It is expressed as the percentage of total radioactivity measured as associated with the HBMEC monolayers after incubation with untreated control U937 cells not pretreated with these peptides. Data represent mean ± SEM of triplicates from one of three independent experiments giving similar results. *, Significant difference between ANX1–5-treated cells and ANX1-treated cells, $p < 0.001$. **, Significant difference between ANX1 and ANX5, $p < 0.001$.

U937. To confirm that inhibition of monocyte adhesion was due to ANX1, U937 cells were seeded on HBMEC monolayers either in the presence or absence of the anti-ANX1 mAb 1-B or in the presence of unrelated control Ab (Fig. 1*B*). The specific anti-ANX1 mAb 1-B reversed most of the ANX1-inhibitory effect on U937 adhesion, whereas control Ab did not reverse the inhibition of adhesion.

We then examined which domain of ANX1 is responsible for inhibiting U397 cell adhesion to HBMEC by comparing the effects of ANX1 with those of the following ANX1-related peptides: 1) ANX5, a closely related protein displaying a core domain similar to that of ANX1, but a completely unrelated N-terminal domain; 2) a chimeric ANX1–5 recombinant protein, consisting of the N-terminal domain of ANX1 fused with the core domain of ANX5; and 3) a 25-aa, acetylated peptide corresponding to the N-terminal region of ANX1 (Ac 2–26). Both ANX1 and chimeric ANX1–5 inhibited U937 adhesion to HBMEC to a similar extent, whereas ANX5 achieved only 50% as much inhibition of adhesion as did ANX1 (Fig. 2). Correspondingly, the calculated for ANX1 was 89 nM, for the ANX1–5 chimera 33 nM, while the EC_{50} for ANX5 was 690 nM, almost 10-fold higher. Indeed, the most potent inhibitor of U937 adhesion was the ANX1–5 chimeric protein. In comparison, the maximal inhibition of adhesion observed with the N-terminal peptide of ANX1 (Ac 2–26) reached 75% of that of ANX1. This maximum inhibition with peptide alone required a molar concentration 20 times higher than that of intact ANX1 ($EC_{50} \sim 1.6$ μM). These data indicate that the inhibitory effect of ANX1 on U937 cell adhesion depends mainly on its N-terminal domain. The bell-shaped pattern of the dose-response curves may relate either to toxic effects at highest concentrations or to additional specific characteristics of ANX1 binding sites, such as dimerization.

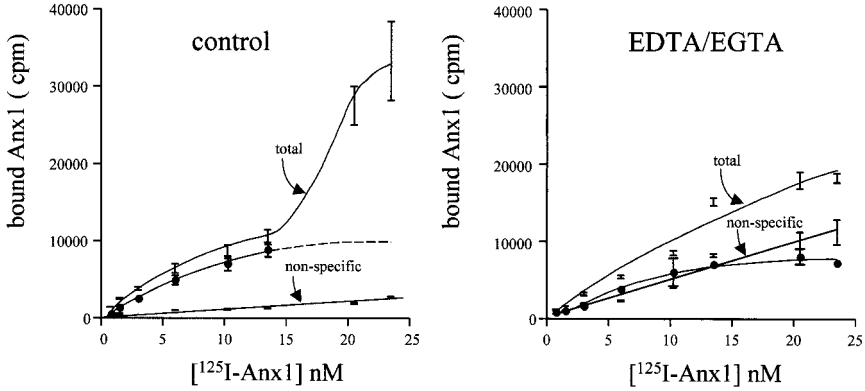

FIGURE 3. Binding studies of ANX1 to U937 cells. Cells were incubated at 4°C in the presence of the indicated concentrations of ^{125}I-ANX1 in the absence (control) or presence (EDTA/EGTA) of 1 mM EDTA and EGTA. Nonspecific binding was determined in the presence of a 50–100-fold excess of cold ANX1. ●, Specific binding values obtained by subtracting nonspecific binding from total binding. Data were analyzed using a nonlinear regression plot. Calculations and curve fitting were performed using the GraphPad Prism (version 2) software. In the control panel, the dotted segment of the specific binding curve corresponds to an extrapolation of the first part of the curve. Bars correspond to mean ± SEM of triplicates. Data are from one experiment representative of three independent experiments with similar results.

Binding studies of ANX1 to U937 cells

ANX1 presumably binds to a surface site on the U937 plasma membrane that blocks adhesion of U937 cells to HBMEC. Binding assays with ^{125}I-ANX1 were performed at 4°C (to avoid endocytosis of bound ligand) to characterize this putative binding site. The binding of ^{125}I-ANX1 to U937 cells was biphasic. Nonlinear regression analysis of binding data showed a saturable binding site characterized by a K_d of 14.3 + 6.1 nM, and a maximal binding of 94,000 + 24,000 sites per cell (Fig. 3). This high affinity ^{125}I-ANX1 binding site was not significantly modified by calcium-chelating compounds: in the presence of EDTA/EGTA, the K_d was 14.8 + 5.9 nM, and maximal binding was 63,000 + 12,600 sites per cell. In contrast, a second, low affinity, binding component, which could not be characterized because saturation was not achieved, was blunted when U937 cells were incubated with ^{125}I-ANX1 in the presence of 1 mM EDTA and EGTA. These findings are consistent with the hypothesis that the N-terminal domain (rather than the Ca^{2+}-binding domain) is principally involved in the inhibitory effects of ANX1 on U937 cell binding to endothelium.

Endogenous ANX1 exerts an autocrine inhibitory effect on monocyte adhesion to endothelial cells

To investigate whether endogenous ANX1 could have an autocrine effect on U937 function, U937 cells were first differentiated in vitro into macrophages by incubating with PMA (13) and then were treated for 12 h with 1 μM dexamethasone to promote the externalization of ANX1 to the outer side of the plasma membrane (13). PMA-differentiated U937 macrophages display more adhesion than do undifferentiated U937 cells, no matter whether the differentiated U937 cells have been treated or have not been treated with dexamethasone. Compared with adhesion of PMA-differentiated U937, adhesion of dexamethasone-treated, PMA-differentiated U937 was decreased by 40% (Fig. 4*A*). This decrease in adhesion was reversed when the dexamethasone-treated, PMA-differentiated U937 macrophages were coincubated with a mAb directed against ANX1 (mAb 1-B at 20 μg/ml), indicating that ANX1 mediates the inhibition of adhesion that is associated with dexamethasone treatment. A control Ab not directed against ANX1 did not modify the inhibition of U937 adhesion induced by dexamethasone treatment.

To confirm this hypothesis, that endogenous ANX1 is responsible for inhibition of monocyte adhesion to EC in this model, we repeated the same type of adhesion studies using stable clones of U937 cells that express antisense mRNA for ANX1. These stable clones consequently contain less ANX1 than do wild-type U937 cells or control clones of U937 cells expressing the empty vector (15). After PMA differentiation and dexamethasone treatment of these U937 clones to externalize ANX1, the inhibition of adhesion to EC of the U937 clones expressing ANX1 antisense mRNA was significantly less than was inhibition of adhesion to EC of U937 wild-type controls or U937 clones expressing vector alone (Fig. 4*B*). Taken together, our results indicate that both exogenous ANX1 acting in a paracrine manner and endogenous ANX1 acting in an autocrine manner serve as potent inhibitors of monocyte adhesion to endothelial cells.

ANX1 colocalizes with $\alpha_4\beta_1$, but not with $\alpha_L\beta_2$ integrins on U937 cells

Because the results described above suggest that ANX1 inhibits monocytic cell adhesion to EC by interacting with or masking an adhesion molecule at the surface of U937 cells, we next characterized the expression of cell surface adhesion molecules by flow cytometry of U937 cells before and after treatment with PMA, or treatment with PMA and then dexamethasone. These cells expressed both $\alpha_4\beta_1$ and $\alpha_L\beta_2$ integrins at their surface (Fig. 5, *upper* and *lower panels*, respectively). Dexamethasone treatment did not decrease and even increased integrin expression compared with untreated cells (not shown). Redistribution of $\alpha_4\beta_1$ and $\alpha_L\beta_2$ integrins was analyzed by confocal microscopy using cells incubated with ANX1 for 30 min at 37°C (Fig. 6). Exogenous ANX1 (Fig. 6*B*, red) was mostly concentrated to one pole. ANX1 did not colocalize with the $\alpha_L\beta_2$ integrin (Fig. 6*B*, upper series, green), whereas it colocalized with the $\alpha_4\beta_1$ integrin (Fig. 6*B*, lower series, green), as indicated by merging confocal images (Fig. 6*B*, lower series I + II, yellow). The plot of fluorescent signals within the cells confirmed the colocalization of the two fluorochromes in cells doubly labeled with anti-ANX1 and anti-α_4 integrin Abs, but not in cells doubly labeled with anti-ANX1 and anti-α_L integrin Abs (Fig. 6*C*). Similar results were also obtained in cells treated with PMA and dexamethasone, in which endogenous externalized ANX1 colocalized with the α_4 integrin, but not with LFA-1 (α_L)

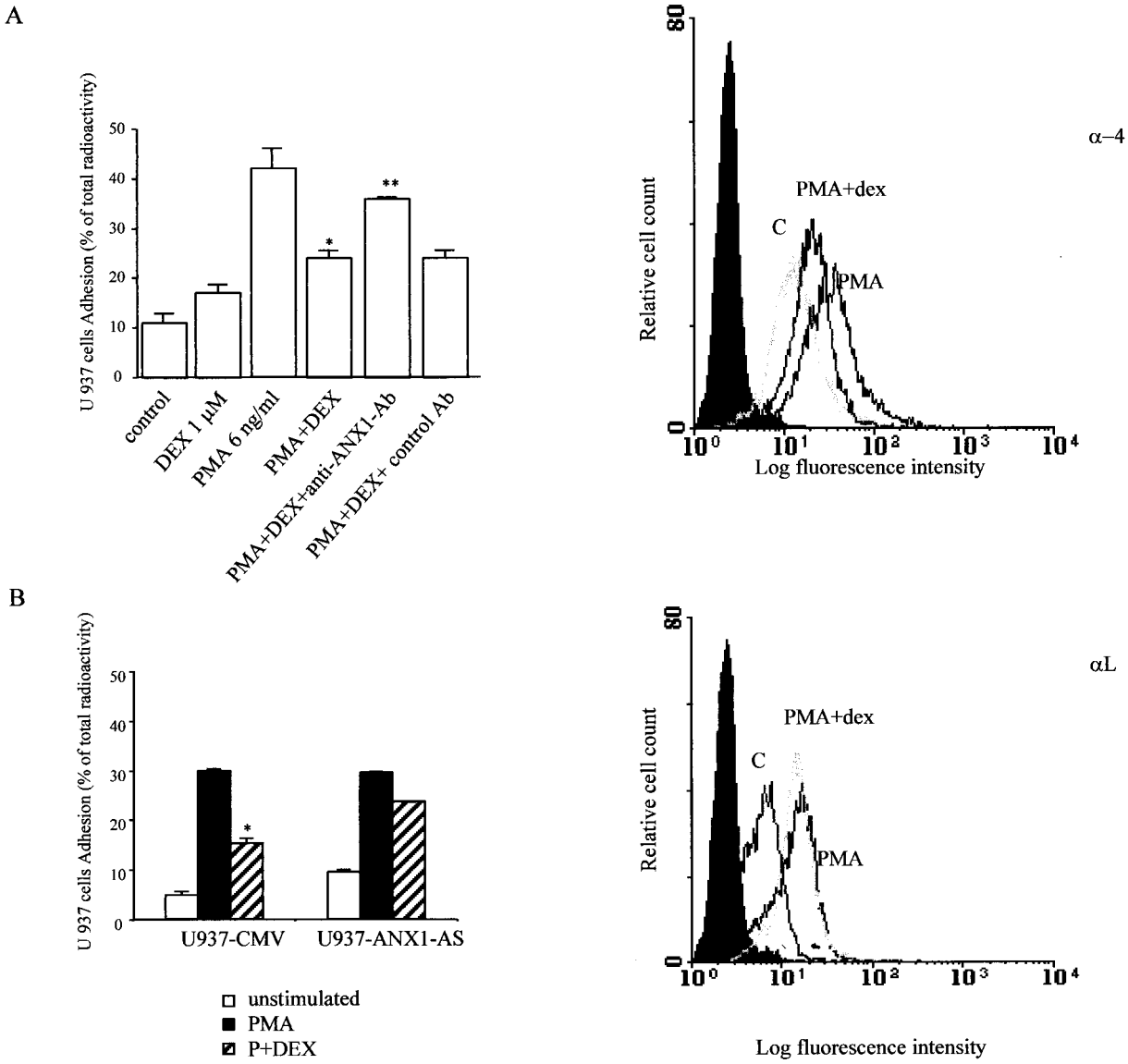

FIGURE 4. Effect of endogenous ANX1 on U937 cell adhesion to HBMEC. *A*, U937 cells were treated (PMA) or not (control) with PMA for 24 h to up-regulate ANX1 synthesis and then were incubated (+DEX) or not (−DEX) with dexamethasone for 16 h to endogenous ANX1. U937 cell adhesion to HBMEC was measured after 2 h, as described in Fig. 1, except that HBMEC were not pretreated with TNF-α. The contribution of ANX1 to the inhibition of adhesion of PMA- and dexamethasone-treated U937 cells was evaluated by coincubating the cells with the specific 1-B anti-ANX1 mAb at 20 μg/ml (+anti-ANX1-Ab). A control Ab (+ control Ab) was used at 30 μg/ml. Bars indicate mean ± SEM of sextuplicates; data are from a single experiment representative of three independent experiments giving similar results. *, Significant difference ($p < 0.001$) between PMA + DEX and PMA. **, Significant difference ($p < 0.01$) between PMA + DEX + anti-ANX1-Ab and PMA + DEX or PMA + DEX + control Ab. *B*, Cells from a U937 clone that expresses antisense ANX1 mRNA (U937-ANX1-AS) and, consequently, contains 40% less ANX1 than control U937 cells (15) were compared with cells from a clone expressing the empty expression vector (U937-CMV) for adhesion to HBMEC. Both clones were pretreated with PMA and dexamethasone to up-regulate ANX1 and to induce ANX1 externalization. Cell adhesion to HBMEC was measured after 2 h, as described in Fig. 1. Adhesion of antisense mRNA-expressing U937 cells to HBMEC was significantly lower than adhesion of cells expressing the empty vector. Bars indicate mean ± SEM of sextuplicates; data are representative of three experiments giving similar results. *, Significant difference ($p < 0.001$) between PMA + DEX and PMA.

FIGURE 5. FACS analysis of α_4 and α_L integrin expression on U937 cells. Anti-CD49d and anti-CD11a Abs conjugated to FITC were used to label the α_4 (*upper panel*) and α_L (*lower panel*) integrins, respectively. Integrin expression was compared between U937 control cells (*C*) and U937 cells treated either with PMA or with PMA and dexamethasone (PMA + DEX). An unrelated FITC-conjugated Ab was used as a control for background fluorescence (filled area).

(Fig. 6, *D–F*). In an effort to characterize more precisely the subcellular colocalization of ANX1 with the α_4 integrin, we took advantage of the expression of the chemokine receptor CXCR4 at the surface of U937 cells. Preliminary unpublished experiments have shown that U937 cells are chemoattracted by the CXCR4 ligand SDF-1. Chemotaxis is associated with particular morphological changes and with redistribution of CXCR4 to the leading edge of the cell (not shown). U937 cells were plated in the upper chamber of transwell plates, and 100 nM SDF-1 was added to the lower chamber. After 18-h incubation, U937 cells that had migrated into the lower chamber were harvested and compared with control resting U937 cells for their distribution of CXCR4 and of ANX1 binding sites (Fig. 7). As expected, CXCR4 displayed a homogeneous distribution at the plasma membrane of resting cells, whereas it concentrated in one pole of SDF-1-activated polarized U937 cells,

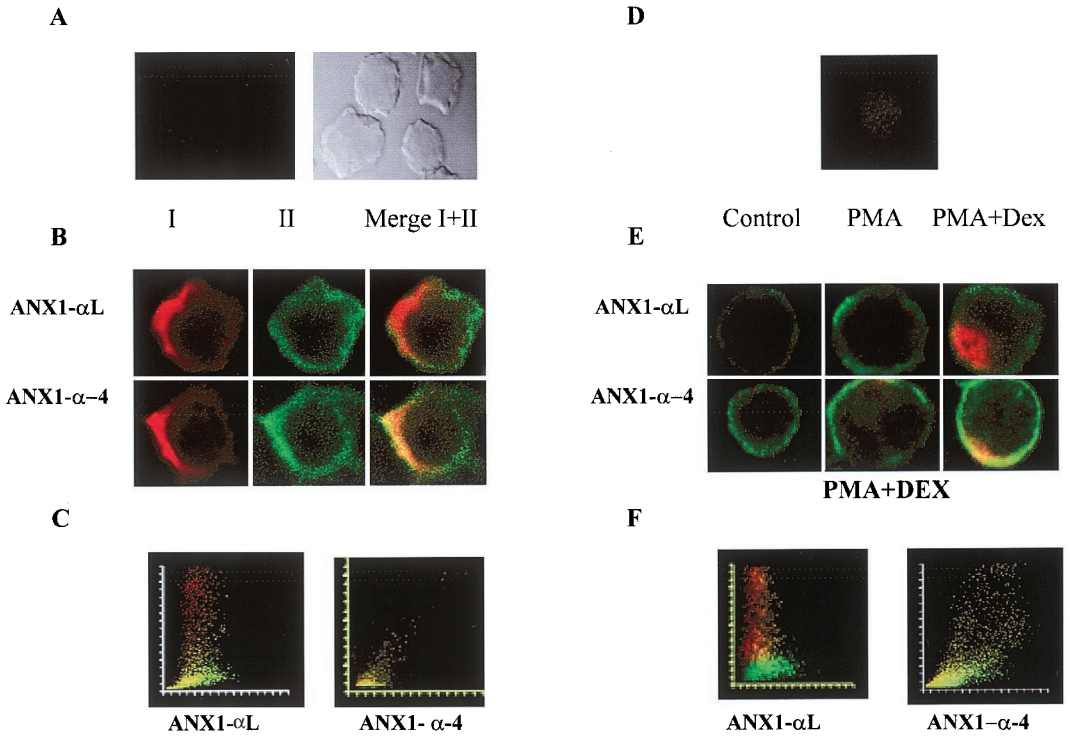

FIGURE 6. Confocal analysis of ANX1 and integrin colocalization. *A*, Fixed U937 cells were stained with goat anti-rabbit Cyanin 3 secondary Ab alone as a control of background; the *right panel* shows the phase contrast image of the same field. *B*, U937 cells were immunostained with a specific rabbit anti-ANX1 polyclonal Ab and/or with mAbs directed against either the α_L integrin (anti-CD11a-FITC) (*upper series*) or the α_4 integrin (anti-CD49-FITC) (II, I + II *lower series*). Column I shows the Cyanin 3 window, column II the FITC window, and column I + II corresponds to the merged images. Images were obtained with a X-60 immersion objective. *C*, Distribution of fluorescence signals in merged images. *D–F*, The same analysis was performed with endogenous ANX1. ANX1 synthesis was stimulated by PMA treatment, whereas translocation of up-regulated endogenous ANX1 to the external plasma membrane was stimulated by incubating PMA-treated cells with dexamethasone (PMA + DEX). Culture conditions were as described in Fig. 4. Anti-actin Abs were used to demonstrate control that the plasma membrane was not permeabilized during immunofluorescence studies, and showed no staining (not shown).

presumably at the leading edge. Exogenous ANX1 was mostly concentrated in one pole of resting cells and only partially colocalized with CXCR4. In SDF-1-activated cells, ANX1 seemed to be more diffusely distributed than in resting cells but, interestingly, it was almost completely excluded from the area of the plasma membrane in which CXCR4 molecules were concentrated.

ANX1 and VCAM-1 compete for the same ligand

The adhesion molecule VCAM-1 expressed by endothelial cells is known to interact with the $\alpha_4\beta_1$ integrin. In our biological assay, ANX1-mediated inhibition of U937 adhesion to EC was blunted by incubation with the extracellular domain of VCAM-1 (Fig. 1*B*). In the same assay, the extracellular domain of VCAM-1 could not block cell adhesion directly. These findings, together with the observations from confocal microscopy experiments, that ANX1 and $\alpha_4\beta_1$ integrin colocalize, suggest that ANX1 and VCAM-1 may compete for binding to the $\alpha_4\beta_1$ integrin. To test this hypothesis, U937 cells were incubated with rANX1 and/or the extracellular domain of VCAM-1 for 30 min at 37°C, then were lysed and the lysates incubated with specific Abs directed against either ANX1 or VCAM-1. Immunoprecipitated proteins were blotted on membranes and probed with an anti-α_4 integrin-specific Ab. Both ANX1 and VCAM-1 coprecipitated with the α_4 integrin (Fig. 8*A*, *lanes 1* and *3*). When U937 cells were incubated with both ANX1 and VCAM-1 (Fig. 8*A*, *lane 2*) no α_4 integrin could be coprecipitated with ANX1, indicating that ANX1 and VCAM-1 compete for binding to the $\alpha_4\beta_1$ integrin on intact cells. Control IP with anti-α_4 Abs, probed with anti-VCAM-1 or anti-ANX1 Abs, confirmed that both ANX1 and VCAM-1 may be found in molecular complexes with α_4 integrin (Fig. 8*B*). A similar observation was made in U937 treated with PMA and dexamethasone to externalize endogenous ANX1 (Fig. 8*C*).

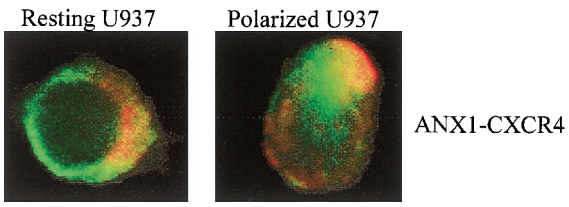

FIGURE 7. Confocal analysis of ANX1 and CXCR4 colocalization. Resting or SDF-1-activated, polarized U937 cells were immunostained with a rabbit polyclonal anti-ANX1 Ab and with the anti-CXCR4 12G5 mAb. After addition of appropriate secondary Abs, cells were subjected to analysis by confocal microscopy analysis. CXCR4 labeling appears in green, ANX1 labeling in red.

Discussion

In the present work, we have demonstrated that ANX1 inhibits monocyte adhesion to endothelium and have identified a potential mechanism for this inhibition. These findings are novel since previous studies of ANX1 had been limited to PMN-EC interaction (5, 6).

The inhibitory effect is mediated by the unique N terminus of the ANX1 molecule. Thus, both rANX1 and a chimeric fusion

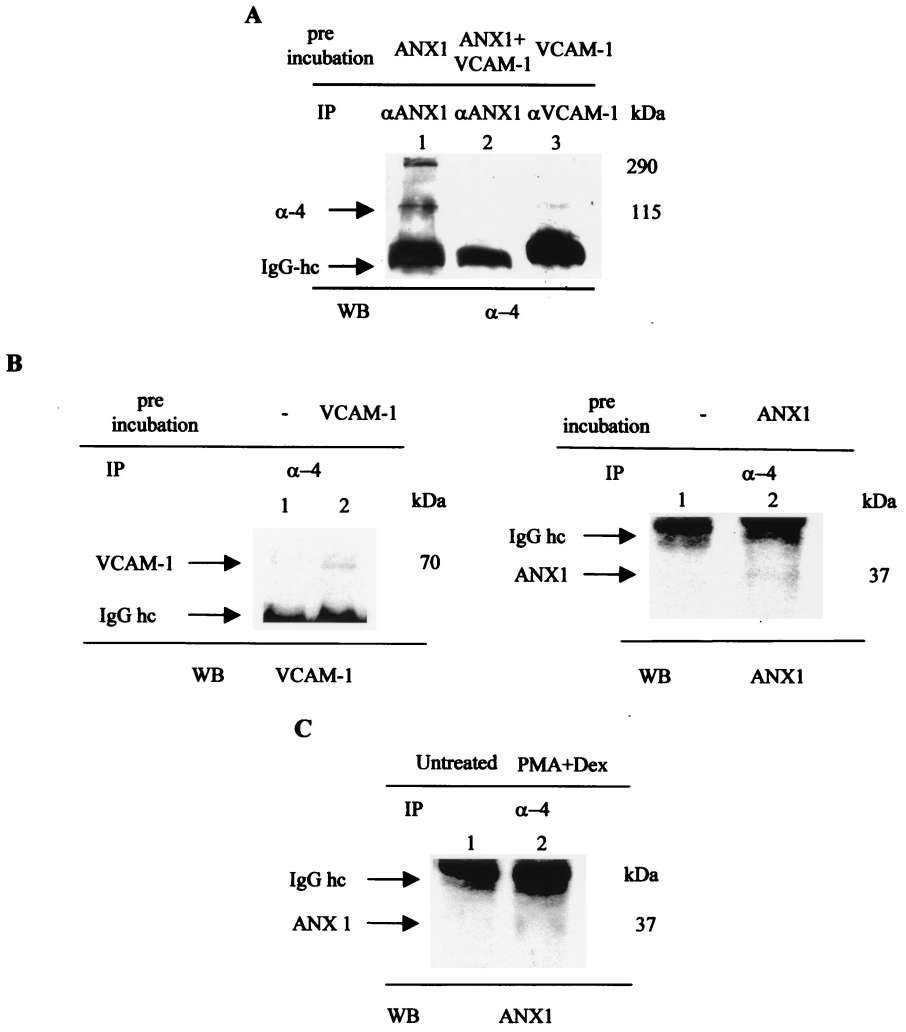

FIGURE 8. Coimmunoprecipitation experiments. *A* and *B*, U937 cells were preincubated with recombinant ANX1, VCAM-1, or both together. Cell extracts, prepared as described in *Materials and Methods*, were immunoprecipitated with indicated Abs (IP). After separation on SDS-PAGE and transfer onto nitrocellulose, the immunoprecipitated proteins were probed with appropriate Abs (WB). *C*, Upregulation and externalization of endogenous ANX1 were induced by sequential treatment with PMA-dexamethasone (PMA + DEX), as described in Fig. 4. IP was then performed as above. Data shown are representative of three independent experiments giving similar results.

protein between the N-terminal domain of ANX1 and the core domain of ANX5, ANX1–5 chimera, which share the same N-terminal segment, inhibited monocyte adhesion to a similar extent when they were incubated with U937 cells, whereas rANX5, which has a different N terminus, displayed only one-hundredth as much inhibition on a molar basis. In a previous report, an N-terminal peptide present in ANX1 that spans aa 2–26 was observed to block PMN adhesion to endothelial cells in vitro (26). We showed in this study that this peptide also blocks U937 cell adhesion; the efficacy of the peptide as an inhibitor is 20-fold less than that of ANX1 itself. This finding, together with the 3-fold greater potency of the ANX1–5 chimera compared with ANX-1, suggests that the annexin core domain does participate in the correct folding of the N-terminal domain. The predominant role of the N-terminal domain in this inhibitory effect of ANX1 is further supported by the binding studies that show the lack effect of Ca^{2+} on inhibition of adhesion: namely, that the high affinity binding of ANX1 to U937 cells is not affected by Ca^{2+} depletion.

Monocytes contain abundant intracellular stores of ANX1. Under physiological conditions of cell migration, monocytes and PMN display marked increases in ANX1 associated with the outer surface of the plasma membrane in vivo (13) (11). We investigated in this study whether or not ANX1 externalization had functional significance for cell adhesion, using U937 cells in which ANX1 production had first been stimulated by PMA, and then ANX1 externalization then effectuated by dexamethasone treatment (13). PMA + dexamethasone-treated U937 cells adhered significantly less to activated HBMEC than did control monocytes. We confirmed that the action of dexamethasone to decrease PMA-induced monocyte adhesion to EC was mediated by externalization of endogenous ANX1, by showing that exposure of the PMA + dexamethasone-treated U937 to an anti-ANX1 Ab reversed the inhibitory effect that dexamethasone exerted on cell adhesion. Furthermore, stable U937 clones expressing antisense ANX1 mRNA contained 20–30% less ANX1 than did the parental U937 cells (15), and adhered better to HBMEC than did stable U937 clones transfected with an empty expression vector. These data confirm that autocrine ANX1 is directly involved in the inhibition of cell adhesion in this model.

Leukocyte adhesion and extravasation, key phenomena in inflammation, depend on a complex array of interactions between adhesion molecules present on leukocytes and those on endothelial cells. For example, the $\alpha_4\beta_1$ integrin, present on monocytes and lymphocytes, interacts with its counter structure, VCAM-1, present on activated endothelium (27, 28). The $\alpha_4\beta_1$ integrin mediates several functions such as tethering, rolling, and firm arrest on VCAM-1 (29–31). Monocytes express multiple integrins in vitro, including the $\alpha_4\beta_1$, $\alpha_M\beta_2$, and $\alpha_L\beta_2$ integrins. In vivo, each of these integrins has a distinct function in cell migration, because only simultaneous blockade of all three integrins at the same time

completely abolishes monocyte accumulation in tissues (32). Previous observations that blocking adhesion molecules hampers inflammatory reactions (33) prompted us to investigate whether ANX1 interacts with one or more adhesion molecules expressed by U937 cells. We first established by flow cytometry that these U937 cells express both the $\alpha_4\beta_1$ and the $\alpha_L\beta_2$ integrins. The bone marrow endothelial cells used in our study express ICAM-1 and VCAM-1 adhesion molecules upon stimulation with TNF-α (17). Using confocal microscopy, we showed that exogenous rANX1 colocalizes on the U937 cell membrane with α_4 integrin, but not with the $\alpha_L\beta_2$ integrin on the U937 cell membrane. Similar results were also obtained with U937 cells in which endogenous ANX1 had been externalized by sequential treatment with PMA and then dexamethasone. IP studies confirmed and extended these findings. Since ANX1 not only coprecipitated with the α_4 integrin, but also competed with VCAM-1 for binding to the α_4 integrin, our results provide a plausible mechanism by which externalized autocrine or paracrine ANX1 may inhibit firm adhesion.

As a result of chemoattractant activation, leukocytes become polarized and migrate in the direction of the chemotactic gradient. During this process, chemoattractant receptors and integrins are redistributed in different areas of the plasma membrane. Whereas in most cases chemoattractant receptors are concentrated at the leading edge of the cells (34), integrins are redistributed at the trailing edge or uropod (35, 36). In SDF-1-activated polarized U937 cells, CXCR4 receptors become concentrated at one pole of the cell, which is almost completely devoid of ANX1. ANX1, in turn, appears to be redistributed at the trailing edge of the cell. The association between ANX1 and integrins thus seems to be maintained in activated polarized cells.

ANX 1 has long been known as a mediator of the anti-inflammatory properties of GCs. Whereas early studies focused on such intracellular effects of cytoplasmic ANX1 as the inhibition of phospholipase A_2 (15, 37, 38), more recent reports indicated that ANX1 may have additional important physiological actions after it becomes externalized at the outer side of the plasma membrane (6, 9, 11). Our data showing that ANX1 inhibits monocyte adhesion to endothelium through an autocrine/paracrine interaction with surface integrins strongly support this hypothesis. Accordingly, the fate of activated leukocytes, which either migrate into tissues or detach from the endothelium, may depend upon a fine balance between leukocyte integrins and other species of regulatory molecules, such as ANX1, that are expressed on the cell surface. To obtain a clearer picture of ANX1 functions affecting leukocyte physiology is an important issue that remains to be solved to understand which are the precise molecular signals and what are the cellular mechanisms that promote ANX1 externalization.

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