British Journal of Pharmacology (2001) 133, 217–228

www.nature.com/bjp

Transfection of annexin 1 in monocytic cells produces a high degree of spontaneous and stimulated apoptosis associated with caspase-3 activation

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1 Transfection of the pre-monomyelocytic U937 cell line with a plasmid coding for full-length annexin 1 (ANX1, 347 amino acid) leads to cell death by promoting apoptosis. In addition, over-expression of the N-terminal and the first domain of the protein (144 amino acids, clone ANX1-S), which does not contain the Ca^{2+} binding sites, gives susceptibility to cell apoptosis following activation by either 5 ng ml^{-1} tumour necrosis factor (TNF)-α or 1–40 μg ml^{-1} etoposide. This was demonstrated by using the fluorescent labelled annexin V, cell cycle and nuclear staining analyses.

2 Transfection with an empty plasmid (clone CMV) or with a plasmid carrying the cDNA antisense for ANX1 (clone ANX1-AS) did not alter U937 cells to the degree of apoptosis promoted by either stimulant.

3 Treatment of CMV U937 cells with TNF-α increased ANX1 mRNA and protein expression in a time-dependent manner, with maximal increases at 3 and 6 h, respectively.

4 Clone ANX1-S showed higher constitutive (more than 2 fold) and activated caspase-3 activity, associated with higher phospholipase A_2 (PLA_2) activity (in the region of +50–100%), whereas expression of cytosolic PLA_2 Bax and Bcl-2 were similar in all cell clones, as determined by Western blotting.

5 In conclusion, this study demonstrates a complex regulatory role of cell apoptosis for ANX1, at least with regards to cells of the myelo-monocytic lineage.
British Journal of Pharmacology (2001) **133,** 217–228

Keywords: Human; monocytes; apoptosis; lipocortin

Abbreviations: ANX1, annexin 1; ANX1-S, annexin 1 sense clone; ANX1-AS, annexin 1 antisense clone; CMV; cytomegalovirus (empty clone); EDTA, ethylene diamine-tetraacetic acid; EGTA, ethylene glycol-tetraacetic acid; FCS, foetal calf serum; FDG, fluorescein di-β-D-galactopyranoside; FITC, fluoresceine isothiocyanate; ICAM-1, intercellular adhesion molecule-1; M 2-3-4, ANX1 mutant in the Ca^{2+} binding sites in repeat 2, 3 and 4; PBS, phosphate-buffered saline; PECAM-1, platelet endothelial cell adhesion molecule-1; PI, propidium iodide; PLA_2 phospholipase A_2; RT–PCR, reverse transcriptase polymerase chain reaction; TNF, tumour necrosis factor; WT, wild type

Introduction

The term apoptosis describes a series of changes that cells undergo during programmed or physiological cell death. During apoptosis, cells shrink, rapidly display an altered plasma membrane, suffer cytoplasm damage and undergo nuclear condensation prior to endonuclease-dependent chromatin fragmentation. Early changes in the cell surface are particularly important as they signal macrophages to rapidly phagocytose moribund cells before toxic breakdown products or contents can injure the surrounding tissue (Anderson, 1996). The ability to mount a rapid and effective response to an acute threat such as invasive infection or tissue injury is essential to survival. Yet the inflammatory response is nonselective in its targets, causing injury to affect invading organisms and host tissues alike. Inflammatory cell apoptosis is an important mechanism leading to the termination of inflammation; conversely, disorders of apoptosis result in diseases characterized by inappropriately expressed inflammation (Marshall & Watson, 1997).

Members of the annexin super-family of Ca^{2+}- and phospholipid-binding proteins have been found in all eukaryotes examined to date except *S. cerevisiae* (Morgan & Fernandez, 1995), suggesting that these proteins have a fundamental cellular role, both in the cytosolic and plasma membrane compartments. Annexins have a partial structural similarity in that they contain four or eight homologous repeats (of 70–80 amino acids each). This core contains the sequences responsible for the common biochemical features of these proteins, that is the capacity to bind acidic phospholipid and calcium ions. Much less conserved is the area corresponding to the N-terminus, and it has been proposed that this portion of amino acids (which may vary from 5–113 amino acids) confers the specific biological function to each member of the super-family (Raynal & Pollard, 1994).

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Annexin 1 (ANX1, previously known as lipocortin 1) contains a 49 amino acid long N-terminus that is responsible for the anti-inflammatory effects of the protein (Perretti *et al*., 1993). The expression of ANX1 is under the control of circulating glucocorticoid hormones (Vishwanath *et al*., 1992). Steroid induction of the protein as been demonstrated in several cell types (De Caterina *et al*., 1993; Raynal & Pollard, 1994), and supported by recent studies on the ANX1 promoter region (Solito *et al*., 1998a). Together these findings support the notion of ANX1 as a mediator of anti-inflammatory glucocorticoids, particularly with regard to leukocyte trafficking and to regulation of pituitary hormone release (Flower, 1988; Lim *et al*., 1998).

A novel aspect of the biology of ANX1 has been recently reported, and this is the distinct and selective expression of the protein in rat mammaries during the regression phase (McKanna, 1995), a phenomenon that relies heavily on apoptosis of mammary cells. More recently we have corroborated this observation by testing the susceptibility to TNF-α-induced apoptosis of U937 cells stable transfected with a plasmid containing a cDNA coding the first 144 amino acids of ANX1 (Canaider *et al*., 2000; Solito *et al*., 1998c). In the present study we have extended these preliminary observations and investigated the link between ANX1 and U937 cell apoptosis in much more detail. In particular here we have: (i) analysed U937 cells transfected with two novel constructs (which produce full length ANX1, and an ANX1 mutant site directed mutagenized in calcium binding sites); (ii) shed light on the biochemical pathways which are modified in cells over-expressing ANX1; and (iii) used two distinct pro-apoptotic agents. We conclude that over-expression, but not under-expression, of ANX1 in U937 cells is associated with elevated caspase-3 activity and a higher susceptibility to apoptosis.

Methods

Cell culture and transfection

Wild type U937 cells and the clones transfected with the plasmids reported in Figure 1A were maintained in culture with RPMI medium supplemented with 10% of foetal calf serum at 37°C under a 5% CO_2 atmosphere.

U937 cells transfected with full-length ANX1 cDNA were plated at a density of 5×10^5 cells per plate (10 cm diameter) the day before transfection. pRC/CMV plasmid containing the ANX1 cDNA full length of 1.3 kilobase, and even the sequence mutagenized on the calcium binding (M 2-3-4) sites according to Kunkel *et al*. (1987) (Figure 1A) were transfected in dextran (1 mg ml^{-1} in TBS buffer) as described (Ausubel *et al*., 1995). Transfection of cDNA coding for the first 147 amino acids of ANX1, or with an antisense cDNA to the same portion of the protein was performed as described (Solito *et al*., 1998c) and led to the production of the sense clone, termed ANX1-S, and of the antisense clone, termed ANX1-AS. Transfection with the empty plasmid led to the control clone CMV. Figure 1A shows also these cDNAs in a schematic manner, and indicates the point mutations in repeats 2, 3 and 4 of the protein for the clone M 2-3-4.

Forty-eight hours after cell transfection, cell sorting was performed as already described (Nolan *et al*., 1988). Briefly, 10^7 cells ml^{-1} in staining medium containing 300 μM chloroquine were distributed into flow cytometric analysis (FACS) tubes and placed for 20 min in a 37°C water-bath. One hundred μl of pre-warmed 2 mM fluorescein di-β-D-galactopyranoside (FDG, by Molecular Probes, Eugene, OR, U.S.A.) in H_2O were mixed and placed back at 37°C. Cells found positive for Lac-Z expression had also retained the pRC/CMV vector. They were sorted using an Epics-Elite (Coultronics) flow cytometer and then maintained in a selection medium containing 400 μg ml^{-1} geneticin (Sigma Chemical Co., Poole, U.K.). Geneticin was removed from the cell culture medium at least 3 weeks prior to experimentation.

Western blotting (Figure 1B) monitored ANX1 expression. In line with a previous study (Solito *et al*., 1998c) the U937 clone transfected with the antisense cDNA (ANX1-AS) showed approximate 60% reduction in protein expression with respect to the cells transfected with empty plasmid (clone CMV). Several clones were produced and tested for ANX1 expression. The following clones were selected and used for most of the experiments, however the biological effects observed were shared by each group of clones. Transfection with a construct coding for the first 144 amino acids of ANX1 (clone ANX1-S) expressed $\sim$20% more protein in basal conditions. Whereas ANX1-S was used throughout the entire study, clones ANX1-B6, ANX1-F1, ANX1-G8 and ANX1-F9 (over-expressing 20–40% more of the protein) were used in selected experiments. U937 cells transfected with full length ANX1 (clone ANX1 full length) had 80% more protein, and the cells which over-expressed the full length protein mutagenized on the calcium binding sites 50% more than the CMV clone. It is noteworthy that a band of 24 kDa was seen in the clone ANX1-S that is clearly due to the effect of the transfection with the truncated form of ANX1 (shown schematically in Figure 1B).

U937 cell activation

The different clones (U937: WT cells, CMV empty plasmid, ANX1-AS and ANX1-S clones) were activated by incubation with TNF-α or with etoposide. The concentration of the cytokine (5 ng ml^{-1}) was chosen from our previous study (Canaider *et al*., 2000), whereas etoposide was used at concentrations ranging from 10–40 μg ml^{-1}, with 20 μg ml^{-1} giving the optimal degree of apoptosis over the incubation times used. In particular, cells were stimulated with TNF-α (5 ng ml^{-1}) for 3 or 16 h, whereas etoposide was added for 3 or 6 h. At the end of these incubation periods, cells were washed and the extent of apoptosis determined as described below. For the assay of cell activation as well as for determination of caspase-3 activity, shorter incubation times were used, as specified below for each distinct experimental condition.

FITC-annexin V binding for quantifying apoptosis

Cells were washed twice with cold PBS and resuspended in buffer at a concentration of 10^6 ml^{-1}: 10^5 cells were mixed with 10 μl of fluoresceine isothiocyanate (FITC)-conjugated annexin V reagent (R&D, Abingdon, U.K.) and 10 μl of 3 mM propidium iodide (PI). After a 15-min incubation at room temperature in the dark and further washings, samples were analysed by flow cytometry. Flow cytometry was

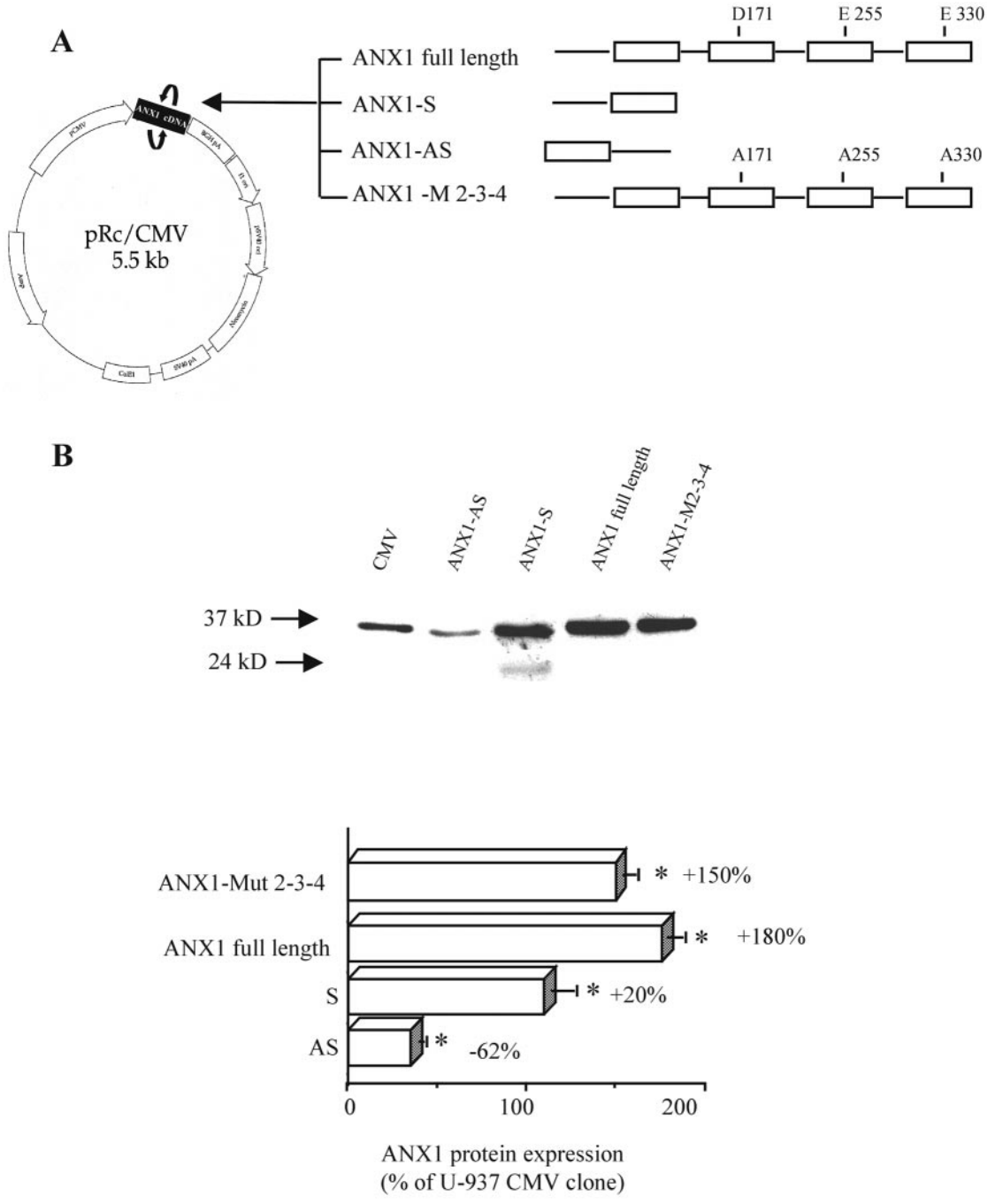

Figure 1 Successful ANX1 transfection in U937 cells. (A) Plasmid used for the different constructions were ANX1-full length, ANX1-S containing the N terminal domain and the first repeat of the protein (144 amino acid, 20 kDa), and the calcium binding site mutagenized cDNA (M2-3-4). (B) Western blot analysis of ANX1 expression in the different U937 transfected cells. The histogram shows values normalized on the internal control obtained by immunodetection of α-tubulin. $*P<0.05$ *vs* value of CMV clone. Values are mean ± s.e.mean of $n=6$ independent experiments.

performed with a FACScan II analyzer (Becton Dickinson) with 15 mW argon ion laser (488 nm) and a Consort 32 computer running Lysis II software. Annexin V staining was detected in the FL1 (green) channel, whereas PI staining was monitored in the FL2 (red) channel: appropriate quadrants were set and the percentage of cells negative for both stains (viable cells), positive for annexin V (apoptotic cells) and positive for PI (dead cells) were acquired. For reasons of clarity the data referring to FITC-annexin V binding only are reported.

Cell cycle analysis

Because activation of endonucleases in apoptotic cells leads to DNA fragmentation and subsequently to extensive DNA loss, apoptosis can be determined by simple measurement of

DNA content using intercalating DNA dyes, such as propidium iodide (PI) (Telford *et al*., 1992). The most rapid method for measuring apoptosis is DNA staining with a hypotonic PI solution (Nicoletti *et al*., 1991). Briefly resting or treated cells were washed twice with cold-phosphate buffered saline (PBS), and fixed in cold 80% ethanol for 30 min. Subsequently, cells were rinsed with PBS, treated with propidium iodide buffer (PI, PBS pH 7.4, 0.1 mM EDTA, 0.1% Triton X-100) containing 5% RNAse T1 and 1 mg ml^{-1} RNAse A, and stained with PI 100 μg ml^{-1} (final concentration). Distribution of cell cycle phases with different DNA contents was determined using a FACScan flow cytometer (MRC-1000, BioRad, Hercules, CA, U.S.A.). A pass filter of 585 nm was used to collect PI fluorescence, acquiring 10,000 events for each sample.

Morphological analysis with Hoechst H33342

U937 cells were plated in 24-well plates and treated with 20 μg ml^{-1} etoposide. After a 3 or 6 h incubation, cells were resuspended in 50 μl at the concentration of 10^6 cells ml^{-1}, and 5 μl of a solution of Hoechst H33342 (10 μg ml^{-1}) were added. Cells were then incubated for further 20 min at 37°C, and subsequently fixed with 10 μl of cold formaldehyde (40% w v^{-1}). Cells were then analysed by fluorescence microscopy with an Olympus BH-2-RFCA. Penetration of Hoechst 33342 into the nucleus was evident, as it was possible to distinguish between the nuclei of healthy and apoptotic cells in view of the chromatin structure and condensation. An operator unaware of the treatments counted at least 200 cells per slide.

Arachidonic acid release

[^{3}H]-arachidonic acid from pre-labelled cells was performed as already described (Solito *et al*., 1998c). Cells were incubated overnight in the presence of 0.1 μCi ml^{-1} of [^{3}H]-arachidonic acid in culture medium containing 10% FCS. After measuring the incorporation of labelled arachidonic acid, cells were washed three times with PBS containing calcium and magnesium. At the end of the incubation period, the reaction was stopped by adding a cold solution containing 5 mM ethylene glycol-tetraacetic acid (EGTA), 150 mM NaCl and 1% free fatty acid-BSA. The medium was removed after centrifugation and radioactivity was measured in a β counter.

Adhesion molecule expression

To exclude the possibility that differences in TNF-α-induced apoptosis among the clones could be simply due to a non-specific increase in cell sensitivity to the cytokine, TNF-α induction of cell surface adhesion molecules was monitored. U937 WT cells, or the clones CMV, ANX1-AS or ANX1-S were left untreated or were activated with 5 ng ml^{-1} TNF-α for 24 h. At the end intercellular adhesion molecule-1 (ICAM-1) and platelet endothelial cell adhesion molecule-1 (PECAM-1) levels on the plasma membrane were determined by FACS analysis as recently described (Perretti *et al*., 1996b). Primary antibodies were mouse anti-human ICAM-1 (clone B-C14; Serotec, Oxford, U.K.) or mouse anti-human PECAM-1 (clone HC1/6; Serotec). FACS analysis was performed within 5 days using a FACScan II analyzer (Becton Dickinson, Mountain View, CA, U.S.A.) with air-cooled 100 mW argon ion laser tuned to 488 nm and Consort 32 computer running Lysis II software (Becton Dickinson). At least 5000 events were analysed for each labelling. Data were analysed as units of fluorescence measured in the FL1 channel.

Quantification of caspase-3 protease activity

Caspase-3 activity was quantified using a fluorometric assay kit purchased from R&D Systems (Abingdon, U.K.) and following the instructions provided by the manufacturer. Briefly, cells were incubated with or without etoposide (20 μg ml^{-1}) for 30 min, 1 or 3 h, or with 5 ng ml^{-1} TNF-α up to 6 h, prior to lysis in cell lysis buffer (1 ml per 2×10^6 cells). Aliquots (50 μl) of cell lysates were transferred into a 96-well plate, and 200 μl of reaction buffer plus 5 μl of substrate (Ac-DEVD-7-amino-4-trifluromethyl coumarin). Negative controls for the enzymatic reaction were wells without reaction mixture or with lysates and no substrate. After 1 h incubation at 37°C in a shaking water bath, plates were read in a Cytofluor II fluorescence microplate reader using an excitation light of 360 nm and emission light of 530 nm.

The effect of a known caspase-3 inhibitor Z-DVED fluoromethylketon (Fahy *et al*., 1999) was also tested. The compound (purchased from Bachem, Saffron Walden, U.K.) was added to cell clones at 100 μM for 30 min prior to addition of the stimuli.

Determination of protein expression in resting and TNF-α-stimulated cells

Protein aliquots (30 μg) were separated by SDS–PAGE according to Laemmli (1970) and electroblotted onto nitrocellulose membranes (Biorad, Hercules, CA, U.S.A.). Immunoreactive proteins were detected after immunoblotting with either: (i) a polyclonal anti-ANX1 antibody (a rabbit serum directed against the N-terminal domain, 1 : 1000 final dilution) (Becherucci *et al*., 1993); (ii) a monoclonal mouse IgG1 anti-human Bcl-2; or (iii) a polyclonal goat IgG fraction raised against human Bax (Santa Cruz Biotechnology, San Diego, CA, U.S.A.; 1 : 1000 final dilution in both cases); (iv) a polyclonal antibody raised against cytosolic PLA_2 (1 : 1000; Santa Cruz Biotechnology). A monoclonal anti-human α-tubulin antibody (1 : 1000, Amersham, France) was used as internal control. For immunoprecipitation experiments, 100 μg of cell lysates were incubated overnight with protein A sepharose (Sigma Chemical Co, St. Louis, MO, U.S.A.) and rabbit polyclonal anti-ANX1. The samples were loaded on SDS–PAGE, blotted onto nitrocellulose membranes and probed with a monoclonal anti-phosphotyrosine antibody (clone 4G10; 1 : 250 final dilution, Euromedex, Souffelweyersheim, France) to detect tyrosine-phosphorylated ANX1.

Intracellular ANX1 levels were also measured by flow cytometry using a validated protocol, and a selective monoclonal antibody (Perretti *et al*., 1996a).

Determination of ANX1 mRNA by RT–PCR in resting and TNF-α-stimulated cells

The total RNA was extracted from U937 cells using the RNAeasy Qiagen Kit (TEBU-France), following the manufacturer's instructions. For first-strand cDNA synthesis, 3 μg

of total RNA was reversed transcribed according to (Ausubel *et al*., 1995). PCR reactions were performed on cDNA aliquots in presence of specific oligonucleotides for ANX1 and GAPDH (Genset SA-Paris, France). Analysis of RT–PCR was performed under u.v. light using a camera image analysis setup (Herolab GmBH, Germany). Densitometric analysis was carried out using an Ultrascan XL Laser Densitometer (Agfa).

Statistics

All results are shown as mean ± s.e.mean. Statistical differences were analysed by non-parametric tests on raw data. The Mann–Whitney *U*-test was used to detect statistical differences with a P value less than 0.05 considered being significant.

Results

TNF-α-induces ANX1 expression and mobilization in U937 cells

To demonstrate that the increased susceptibility to apoptosis in U937 clone ANX1-S (Canaider *et al*., 2000) was a reflection of the role played by the protein in the normal life of this cell type, we initially monitored ANX1 levels in WT U937 cells following induction of apoptosis by TNF-α. Figure 2A illustrates the RT–PCR analysis showing a significant increase of the messenger between 30 min and 3 h after TNF-α stimulation, after that the level decreases. Immunoblotting data (Figure 2B) confirmed the marked increase in ANX1 protein level at 3 h post-TNF-α treatment. This pool of ANX1 was not phosphorylated on tyrosine (data not shown). Similar increases in cell-associated ANX1 were also measured by flow cytometry and are shown in Figure 2C: maximal induction was apparent at 6 h, with a profile essentially similar to that obtained by Western blotting.

U937 cells transfected with full length ANX1 did not require activation to enter into apoptosis. When U937 cells were transfected with the full length ANX1 cDNA, cells entered into spontaneous apoptosis. Figure 3 shows the combined measurement of cellular apoptosis (double staining PI and FITC-annexin V) (Figure 3A) and the cell cycle on the different clones (Figure 3B). Wild type (not shown) as well as the U937 cells transfected with empty plasmid (CMV) and the clone ANX1-AS did not show any significant degree of apoptosis in these culture conditions (data refer to day 0, calculated from 24 h post-cell thawing, and day 4 of culture). The clone ANX1-S showed a slightly higher degree of apoptosis, which could rise during the time, but essentially 80% of the cells were viable even after 1 week of culture. In contrast, a remarkably high degree of apoptosis was measured in the clone ANX1 full-length (Figure 3A, B). Since ANX1 is a calcium binding protein, and this cation plays an important role also in promoting the process of apoptosis (Berridge *et al*., 1998), the spontaneous apoptosis of the clone expressing the mutant M 2-3-4 (point mutations are shown in Figure 1A) was determined. A pronounced degree of apoptosis was seen in U937 clone M 2-3-4 (Figure 3). Table 1 summarizes the data regarding spontaneous apoptosis as measured with three distinct protocols in U937

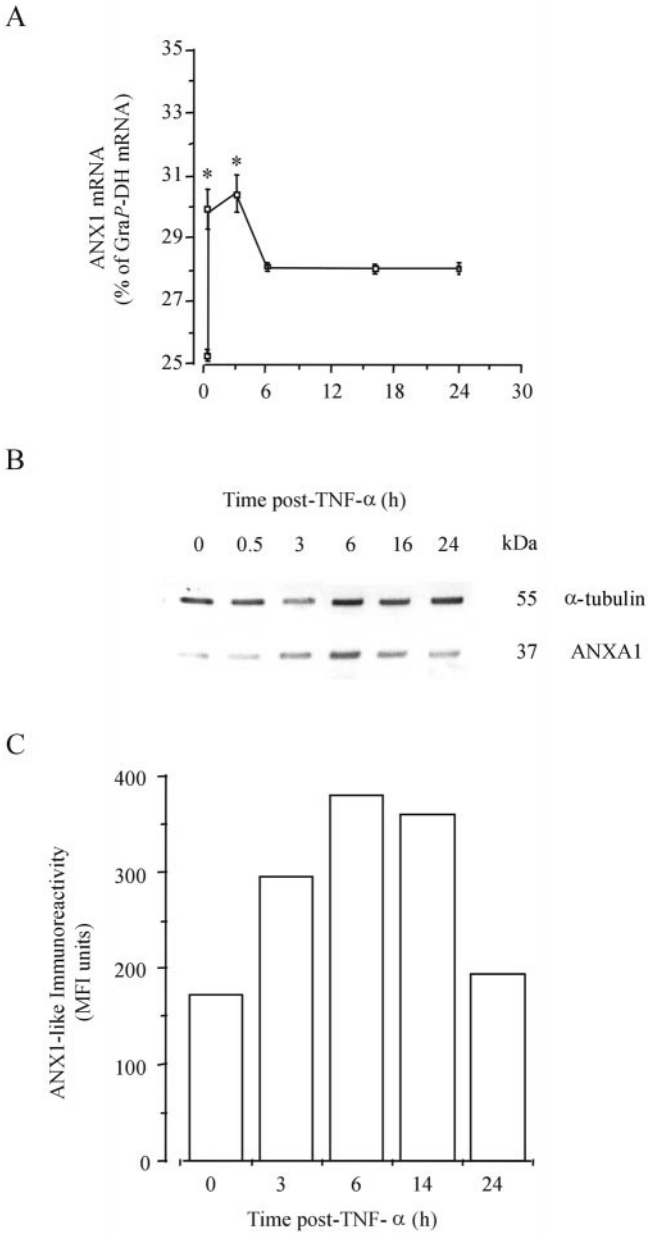

Figure 2 TNF-α induces ANX1 expression. (A) RT–PCR analysis of ANX1 expression after TNF-α (5 ng ml^{-1}) treatment occurring over time. (B) Western blot analysis of ANX1 expression after TNF-α treatment was performed as described in Methods. The blot was reprobed with an anti-tyrosine antibody to detect the level of ANX1 phosphorylation on Tyr-21. (C) FACS analysis of intracellular ANX1 expression in U937 cells. Data are representative of two experiments producing similar results.

ANX1 full-length clone. Since a large majority of the cell population was not viable even after 3 days of culture, most of the remaining experiments were performed with U937 ANX1-AS, ANX1-S and CMV clones.

U937 ANX1-S clone is more sensitive to stimulated apoptosis

The effect of the reported alterations in biochemical pathways on the process of stimulated apoptosis was then studied. In

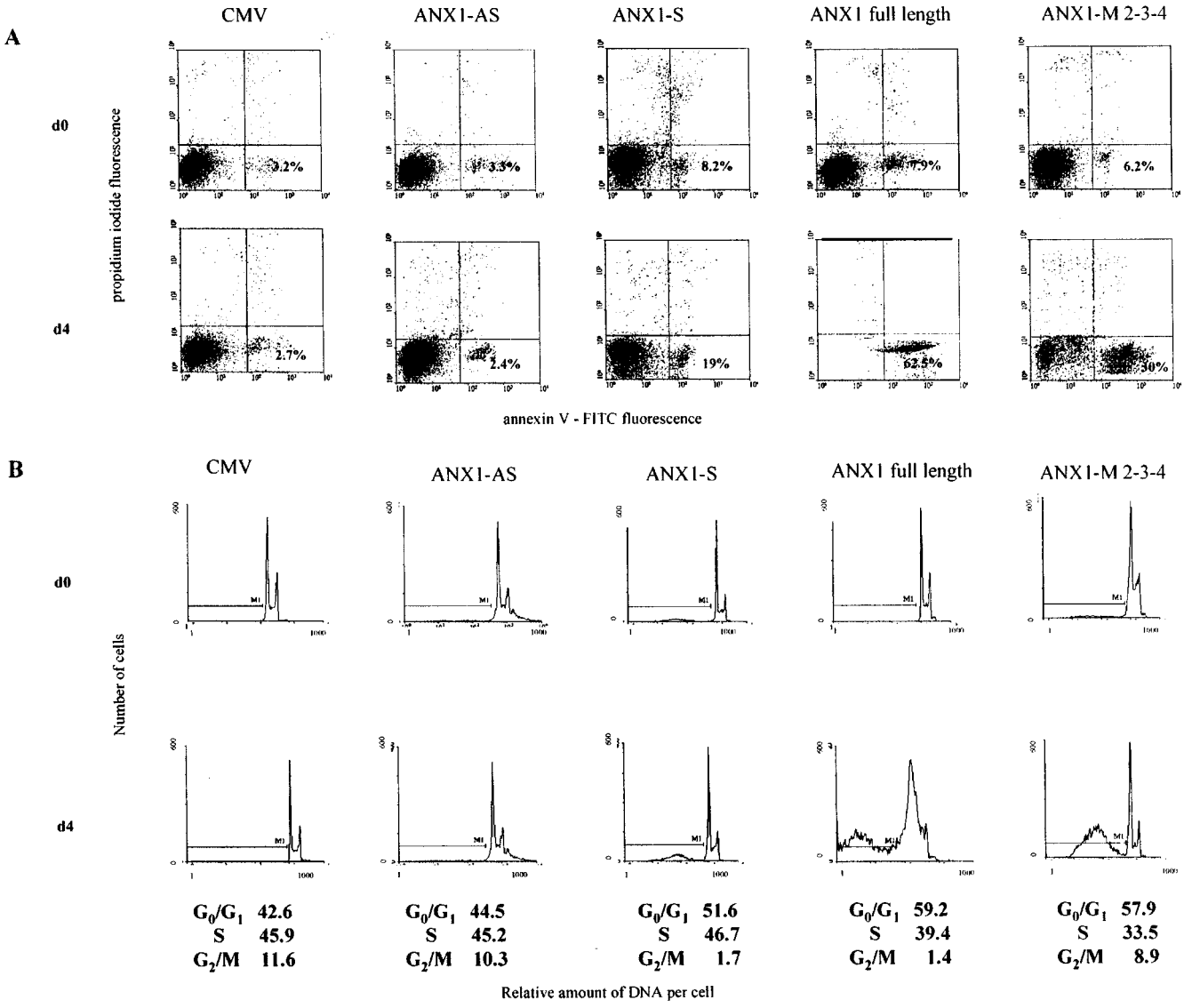

Figure 3 Spontaneous apoptosis of U937 ANX1 transfected cells. (A) Flow cytometric analysis of annexin V binding in U937 clones: CMV, ANX1-AS, ANX1-S, ANX1-full length, ANX1-M2-3-4 transfected cells. The percentage of cells binding annexin V at day 0 (see Table 1) and day 4 is indicated. (B) The same clones were stained with PI before analysis by flow cytometry for DNA content. The fractions of cells with G1, S and G2/M DNA content are shown. Graphs are representative of five independent experiments.

Table 1 Spontaneous apoptosis in U937 cells transfected with ANX1-full length cDNA

Time (days)	*Per cent of cell apoptosis*		
	Morphology	*Cell cycle*	*FITC-Annexin V*
0	1.6±1.2	3.8±0.9	3.2±1.1
2	27±1.3*	28.5±2.3*	19.5±2.3*
3	43±1.6*	46.3±2.2*	49.5±1.2*
5	66±1.9*	73.1±2.4*	75.5±1.5*

U937 cells transfected with ANX1 full-length were evaluated for morphology, PI staining and binding of FITC-annexin V as outlined in Methods. Data are shown as mean±s.e.mean of three distinct experiments. Day 0 is considered after the first 24 h post-cell thawing. WT U937 cells always displayed less than 5% apoptosis during the course of these experiments as measured with either methodology. *$P<0.05$ *vs* value at time 0.

agreement with our initial study (Canaider *et al*., 2000), a higher incidence of TNF-α-induced apoptosis was seen in the U937 ANX1-S clone as measured by binding of FITC-annexin V (Figure 4A). This increased sensitivity to TNF-α actions was specific for apoptosis since, for instance, no differences were seen among the clones under study in terms of ICAM-1 induction (Table 2). In addition, this phenomenon was not restricted to the clone ANX1-S, and it was shared by four distinct clones over-expressing ANX1 first 144 amino acids. At day 0, clones ANX1-B6, ANX1-F1, ANX1-G8 and ANX1-F9 showed a degree of apoptosis ranging between 7 and 11% (data from $n=4$ replicates).

U937 ANX1-S clone displayed higher sensitivity to apoptosis also in response to etoposide application. Figure 4B and C illustrates a significantly higher incidence of etoposide-induced apoptosis in U937 ANX1-S, but not in

CMV or U937 ANX1-AS, clones. U937 ANX1-S cells were more sensitive to etoposide effects both in terms of time-dependency and concentration sensitivity as measured by binding of FITC-annexin V (Figure 4B,C). A similar finding was also obtained when cell apoptosis was quantified with the Hoechst H33342 staining. Figure 5A shows a representative field in which apoptotic cells were identified by the nuclear fragmentation at variance from intact cells. Figure 5B reports the incidence of apoptosis as measured with this staining in the four cell types (data being shown in a cumulative manner).

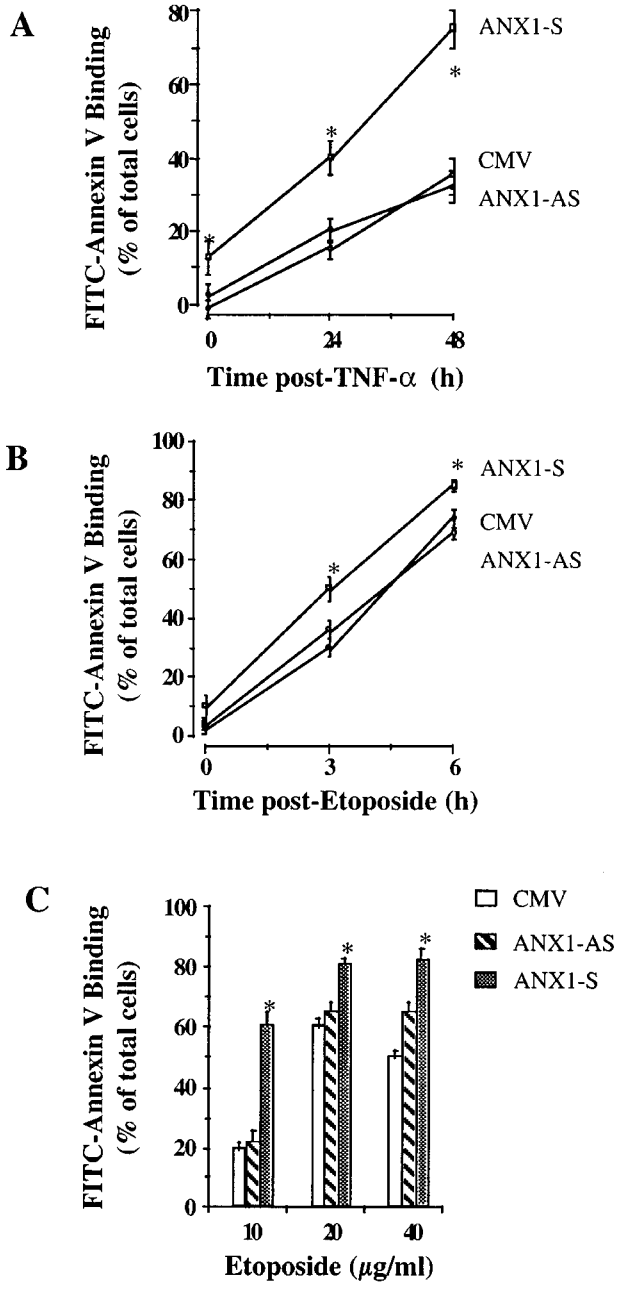

Figure 4 U937 ANX1-S clone is sensitive to TNF-α or etoposide-induced apoptosis. Apoptosis was measured by double staining with FITC-annexin V and PI on the three different U937 clones: CMV, ANX1-AS, and ANX1-S. (A) Time course over 48 h after TNF-α (5 ng ml^{-1}) treatment. (B) Time course over 6 h following 20 μg ml^{-1} etoposide. (C) Concentration dependency of etoposide-induced apoptosis. Data are mean ± s.e.mean of $n = 3-6$ experiments performed in triplicate. *$P < 0.05$ *vs* CMV clone.

Alteration of some 'executioners' of apoptosis in the clone over-expressing ANX1

Initially the expression of Bcl-2 and Bax was determined by Western blotting since an elevated ratio of Bcl-2 to Bax, may protect the cells from apoptosis (Hale *et al*., 1996; Harrington *et al*., 1994). However, no particular differences were found among the three clones either in basal conditions (Figure 6) or after cell activation with TNF-α (data not shown).

Activation of cPLA$_2$ was monitored, by using the gel shift assay, finding no variations among the different cell clones (Figure 7A). In contrast, when PLA$_2$ activity was measured, the clone ANX1-S showed higher basal values of release of arachidonic acid. In addition, TNF-α stimulated this release in the early hours post addition to U937 cells, with higher activity in the ANX1-S clone (Figure 7B). Etoposide, however, did not modify PLA$_2$ activity in either cell type (Figure 7C).

Next, caspase-3 activity was measured in etoposide- or TNF-α-activated cells. Figure 8A and B show the profiles of caspase-3 activity as measured following cell activation with TNF-α or etoposide. The clone U937 ANX1-S had a higher starting point also in this set of experiments (see also Figure 4C), and these cells were more responsive to TNF-α in a time-dependent fashion compared to U937 cells CMV or ANX1-AS. Significantly higher caspase-3 levels were measured not only at time 0 but also 6 h post-TNF-α stimulation (a time-point in which this enzymatic activity was not augmented in the other cells; Figure 8A). A slightly different data was seen following cell stimulation with etoposide: by the 6 h time-point caspase-3 activity was maximally activated in all cell types. The U937 ANX1-S clone, though, had significantly higher values within the first hour of activation (Figure 8B). Finally the constitutive caspase-3 activity was also measured in the clones transfected with the ANX1-full length or ANX1 M 2-3-4 (Figure 8C). As shown, caspase-3 activity increased during the period of selection after transfection, which corresponded to the enrichment in the cells over-expressing ANX1.

Addition of the caspase inhibitor Z-DEVD (100 μM) suppressed the increase in caspase-3 activity measured in the CMV and ANX1-S clone after addition of TNF-α or etoposide and significantly reduced cell apoptosis (Table 3).

Discussion

We have recently demonstrated that U937 cells over-expressing ANX-1 are more susceptible to TNF-α induced apoptosis (Canaider *et al*., 2000). The present study was undertaken to extend this finding and to gain information on the molecular mechanism(s) and structural requirements associated with this observation. The data obtained support the existence of a functional link between ANX1 and apoptosis of mono-myelocytic cells. This was shown by the fact that: (i) U937 cells transfected with full length ANX1 entered spontaneously into programmed cell death; (ii) TNF-α increased ANX1 mRNA and protein synthesis before cellular apoptosis was detectable; and (iii) U937 cells

Table 2 Adhesion molecule expression in U937 clones following activation with TNF-α

	ICAM-1 levels		*PECAM-1 levels*	
Cell type	*Basal*	*TNF-α-stimulated*	*Basal*	*TNF-α-stimulated*
CMV	290±20	790±10	240±10	180±15
ANX1-AS	279±12	720±30	215±15	175±15
ANX1-S	330±14	820±60	305±30	240±20

The three U937 cell clones were incubated with or without 5 ng m^{-1} TNF-α for 24 h prior to quantification of ICAM-1 and PECAM-1 expression by flow cytometry. Data are median fluorescence intensity units (mean±s.e.mean of triplicate determinations) as measured in the FL1 channel. TNF-α was equally active in all three-cell types to cause significant ICAM-1 up-regulation and PECAM-1 down-regulation.

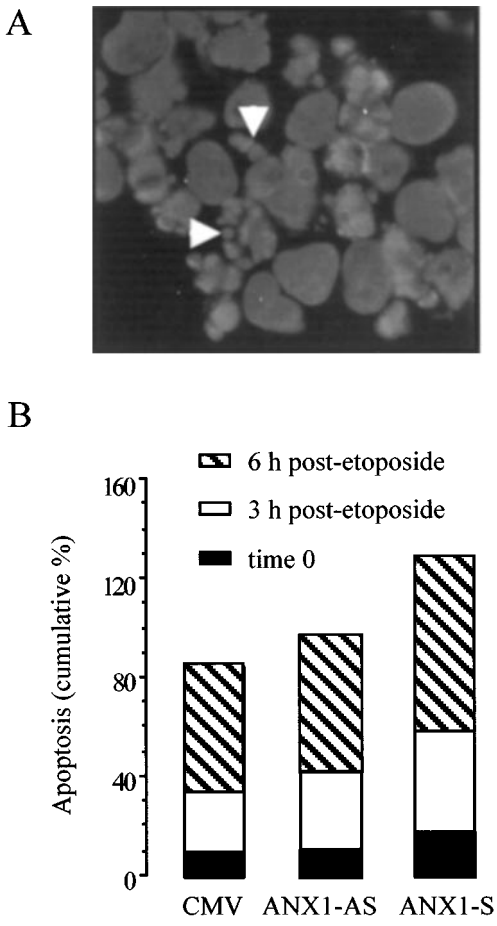

Figure 5 Etoposide-induced apoptosis as detected by morphology after staining with Hoechst H33342. (A) Representative photograph showing nuclei from alive and apoptotic (arrows) cells. Profiles of apoptosis obtained with Hoechst staining (representative photograph). (B) Histogram of cumulative data ($n=4$ experiments).

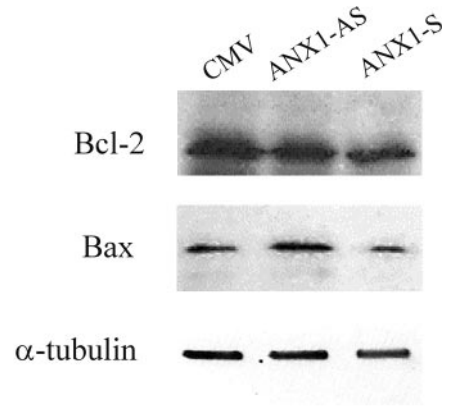

Figure 6 Measurement of apoptotic executioners: Bcl-2 and Bax. Proteins from U937 clones (CMV, ANX1-AS and ANX1-S) were immunoblotted-using antibodies that specifically recognize Bcl-2 or Bax. As control, the same blot was tested for α-tubulin expression. Data are representative of four independent experiments.

transfected with a fragment of ANX1 were in large majority viable but were more responsive to both TNF-α and etoposide-induced apoptosis. Mechanistically, higher ANX1 levels were associated with activation of caspase-3.

Transfection of U937 cells with full length ANX1 almost doubled the cellular levels of this calcium-binding protein. Surprisingly, these cells were difficult to maintain in culture because they spontaneously entered into apoptosis. These *in vitro* data supports the macroscopic observations made during rat mammary regression. McKanna (1995) demonstrated a surge in ANX1 protein levels prior to atrophy of mammary gland in adult rats. Two other studies have shown a link between ANX1 and cell apoptosis. Using rat thymocytes, Sakamoto *et al*. (1996) reported that ANX1 increased H_2O_2-induced apoptosis. We have also been able to demonstrate a higher susceptibility to TNF-α-induced apoptosis in U937 ANX1-S clone (Canaider *et al*., 2000). In the present study, the functional relation between ANX1 and apoptosis was investigated in a systematic and more complete manner using transfection strategies, different pro-apoptotic agents, and analysing several biochemical pathways to shed light on this biological property of the protein.

The pro-apoptotic effect of ANX1 in U937 cells was optimally obtained when the intact protein was transfected. Site mutagenesis of the three calcium binding sites present on the domain 2, 3 and 4 (Chen *et al*., 1993; Weng *et al*., 1993) did not reverse the pro-apoptotic function of ANX1: a similar although delayed pattern of apoptosis compared to the wild type protein was obtained. U937 cell transfection with an ANX1 fragment which contains an intact N-terminus but it is deprived of the last three repeats resulted compatible with cell survival except for a modest incidence of spontaneous degree (between 10 and 20%) of apoptosis. The latter effect was genuine and not clone specific. In line with other structural studies on ANX1, and more generally on annexins, the current model for their functioning is that the N-terminal region confers the specific property to each annexin, whereas the core formed by four or eight repeats, which contains the area of calcium binding and phosphatidyl

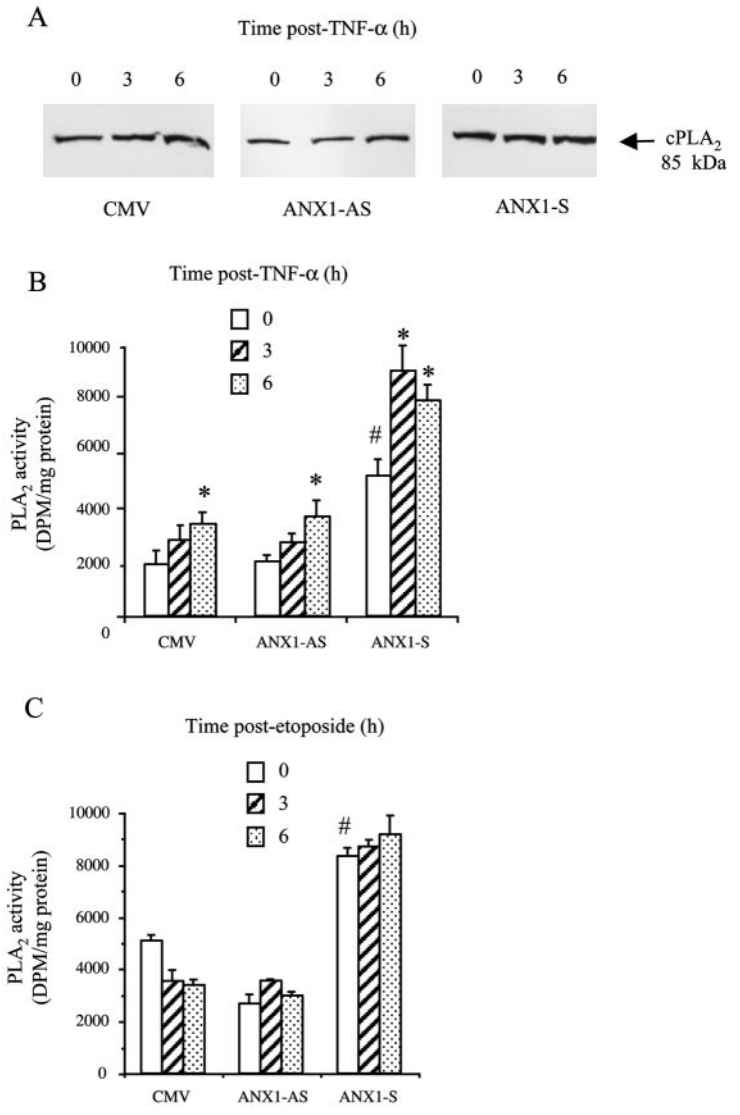

Figure 7 PLA_2 expression and activity in U937 cell clone after TNF-α and/or etoposide treatments. (A) Western blot analysis of cytosolic extracts for $cPLA_2$ (bands correspond to the phosphorylated isoform; see also Frey *et al.* 1999). (B) Time course for PLA_2 activity as measured in the three clones after TNF-α (5 ng ml^{-1}). (C) Time course for PLA_2 activity as measured in the three clones after etoposide (20 μg ml^{-1}) treatment. Data are mean ± s.e.mean from three experiments performed in triplicate. $^*P<0.05$ *vs* time 0 (B) or $\#P<0.05$ *vs* CMV clone (C).

serine binding, is crucial to achieve the exact location of the protein in the intracellular milieu (Moss, 1997)

TNF-α stimulation of WT U937 cells was associated with a fast alteration in intracellular ANX1 levels. ANX1 mRNA increased within 2 h, as demonstrated by PCR analysis, whereas the cell associated protein content was significantly above control values by 4–6 h. The prompt induction of ANX1 by TNF-α merits further discussion, since this is the first time that it is reported. The ANX1 gene can be switched on by glucocorticoid hormones which exert a tonic control on it: in fact, surgical or chemical removal of these hormones reduces ANX1 levels in circulating leukocytes as well as in specific organs by ⩾50% (Perretti & Flower, 1996; Vishwanath *et al.*, 1992). More recently we have demonstrated that interleukin-6 is able to increase ANX1 levels by *de novo* synthesis *in vitro* in an extra-hepatic system (Solito *et al.*, 1998b) as well as *in vivo* in interleukin-6 knock-out liver mice (unpublished data). Therefore the ANX1 gene is finely regulated and its promoter region responds to several factors besides those associated with cell differentiation (Solito *et al.*, 1998b). For instance, NF-IL-6 also activates this gene (Solito *et al.*, 1998a). Our latest observation with TNF-α (which is also corroborated by experiments *in vivo*; E. Solito and M. Perretti, unpublished data) seems to confirm the hypothesis of ANX1 acting as an acute phase protein: its synthesis is partially under the control of several pivotal pro-inflammatory agents, and increased levels are clearly associated to several anti-inflammatory effects both in the periphery and in the central nervous system (Perretti, 1997; Buckingham, 1996).

U937 cells transfected with the ANX1 fragment (clone ANX1-S) survived in cultured conditions and represented a useful tool to investigate potential alterations in biochemical pathways activated during the process of apoptosis. In

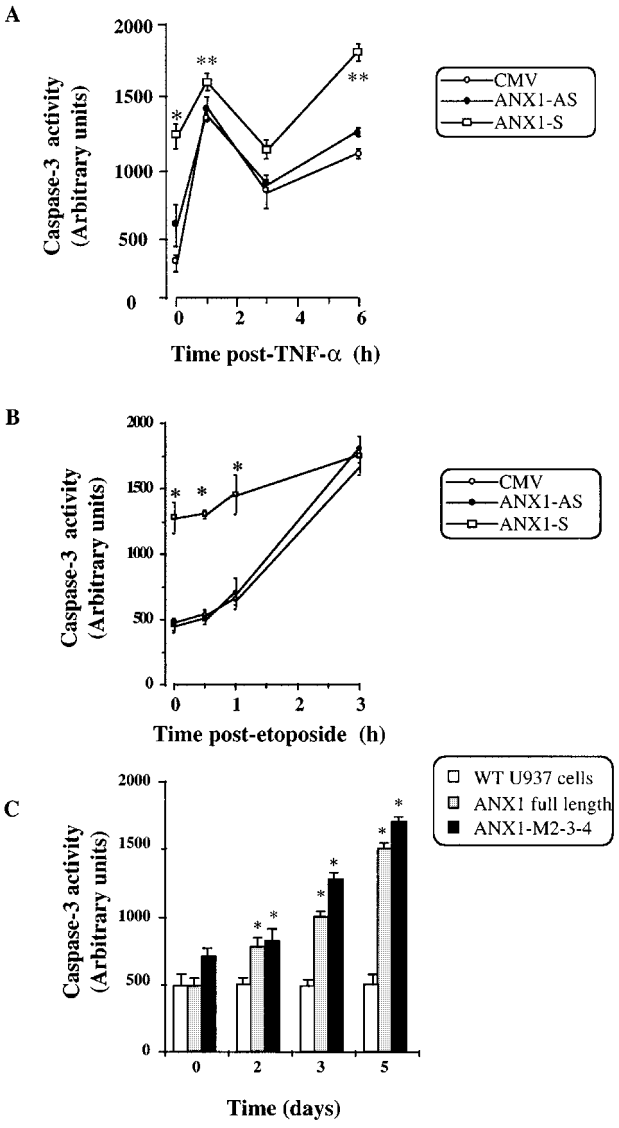

Figure 8 Caspase-3 activity in U937 clones treated with TNF-α or etoposide. (A) Caspase-3 induction in U937 clones after TNF-α (5 ng ml^{-1}) treatment. (B) Etoposide treatment (20 μg ml^{-1}) activates caspase-3 activity over the time. (C) Constitutive caspase 3 activity of ANX1-full length and ANX1-M 2-3-4 clones during the 4 days of culture. Data are mean±s.e.mean of $n=3$ independent experiments performed in triplicate. *$P<0.05$ and **$P<0.01$ *vs* CMV and ANX1-AS clone.

Table 3 Z-DVED effect on TNF-α-induced apoptosis

	FITC-annexin V (Per cent of cell apoptosis)		
Cell type	*Basal*	*TNF-α-stimulated*	*TNF-α+Z-DEVD*
CMV	4.0±0.4	15.7±0.5	3.2±0.3*
ANX1-S	10.5±1.2	22.0±1.2	11.8±1.4*

The two U937 cell clones were incubated with or without 100 μM Z-DVED for 30 min prior to addition of 5 ng ml^{-1} TNF-α for 24 h prior. Apoptosis was determined by the binding of FITC-annexin V as outlined in Methods. Data are shown as mean±s.e.mean of two distinct experiments, performed in triplicate. *$P<0.05$ *vs* value of TNF-α-stimulated cells.

preliminary experiments, U937 ANX1-S cells, but not clone CMV and ANX1-AS, were found to be more susceptible to TNF-α-induced apoptosis. This data was confirmed and extended using an unrelated pro-apoptotic stimulus, etoposide. In U937 cells, TNF-α-induced apoptosis has been shown to involve the activation of cytosolic phospholipase A_2 (Wissing *et al*., 1997; Wu *et al*., 1998). Etoposide, a topoisomerase II inhibitor, is a potent and widely used antineoplastic agent able to induce apoptosis in most cell types with a mechanism of action which is still controversial (Garcia-Bermejo *et al*., 1998; Stefanelli *et al*., 1998).

In the subsequent series of experiments we focused on the molecular pathways which could be altered in U937 ANX1-S clone and therefore potentially associated with the higher degree of apoptosis. For instance, alteration of the ratio between the Bcl-2 and Bax proteins is another feature of the apoptotic process (Reed, 1994), however no differences amongst the three cell types were seen either in basal conditions or following stimulation with TNF-α (data not shown). TNF-α-induced apoptosis of U937 cells has been clearly associated with PLA_2 activation (Canaider *et al*., 2000; O'Brien *et al*., 1998). We have also reported alterations in PLA_2 activity in the U937 clones (CMV, ANX1-S and ANX1-AS) following differentiation with phorbol esters, a process associated with marked changes in phenotypes, including ANX1 expression (Solito *et al*., 1998c) and adhesion molecule levels on the cell surface (Perretti *et al*., 1996b). Here we could detect increases in PLA_2 activity following addition of TNF-α, but not of etoposide. Importantly, basal and TNF-α-induced PLA_2 activity was also increased selectively in the ANX1-S clone. Since U937 cells contain both isoforms of PLA_2 (Marshall & Roshak, 1993; Solito *et al*., 1998c) our data do not discriminate if secretory, or cytosolic, or both PLA_2 enzymatic activities are increased following cell transfection with the ANX1 cDNA fragment. We cannot exclude that a secretory PLA_2 isoform(s) may account for the differences in arachidonate release detected in U937 ANX1-S clone, though further studies will clarify this aspect.

At variance from PLA_2, caspase-3 was activated after U937 cell stimulation with either TNF-α or etoposide. This enzyme is a widespread executioner of apoptosis, which activation can be produced upstream by proteolytic cleavage. For example caspase-3 appears to amplify caspase-8 and caspase-9 signals into fully-fledged commitment to disassembly (Thornberry, 1997). Both caspase-8 and caspase-9 can activate caspase-3 (Slee *et al*., 1999). It is now clear that caspase-1-independent pathways also operate to activate caspase-3, as recently demonstrated in human monocytes (Fahy *et al*., 1999). In view of this central role that caspase-3 plays in the apoptotic process, it may not be surprising that we found higher activity of this enzyme in cells over-expressing ANX1. This finding was selective because higher basal levels of caspase-3 were measured in U937 ANX1-S clone, but not in CMV and ANX1-AS cells. Among these clones, a good relationship between caspase-3 activity prior to detectable cell apoptosis was obtained (e.g. ANX1-S clone displayed high caspase-3 activity at 30 and 60 min, and this was likely related to the higher degree of apoptosis measured 3 and 6 h post-etoposide). However, in some cases caspase-3 activation did not match with the difference in the extent of apoptosis (for instance, the mutant ANX1-M-2-3-4 displayed

similar caspase-3 activity but a reduced degree of apoptosis compared to ANX1 full length). This suggests that, besides caspase-3 activation, other biochemical and/or molecular mechanism(s) contribute to the higher susceptibility to U937 cell apoptosis after ANX1 transfection. Future studies may address these aspects.

Using TNF-α as a stimulus, higher ANX1 expression is associated to increased PLA_2 activity, probably induced by a pathway involving caspase-3 (Wissing *et al*., 1997). This positive loop between ANX1 and PLA_2 is an apparent paradox, but we have recently shown in murine hepatocytes (De Coupade *et al*., 2000) that ANX1 phosphorylated on tyrosine[21] could participate in the activation of $cPLA_2$. In the present experimental conditions, ANX1 exists mainly in a non-phosphorylated form, except for a modest incidence of phosphorylation on serine-threonine, which does not change following treatment with TNF-α (data not shown). TNF-α-induced apoptosis of U937 cells requires the internalization of the receptor into caveolae (Ko *et al*., 1999) and some annexins have been found to interact with coated vesicles (Turpin *et al*., 1998), so it may well be that most of these enzymatic interactions may occur at the level of caveolae formation and/or functioning. In contrast to TNF-α, etoposide-induced apoptosis was not associated with activation of PLA_2: nonetheless the process was magnified in U937 cells over-expressing ANX1 and associated with caspase-3 activation. The biochemical pathways operating in these cells upstream caspase-3 activation are currently a matter of speculation. Recently it has been shown that cells over-expressing ANX1 have higher intracellular calcium levels (Frey *et al*., 1999) and MAP/ERK activity (Alldridge *et al*., 1999) following appropriate cell stimulation. Future studies will clarify whether one or both of these signal transduction pathways are up-regulated in our U937 clones, and are responsible for the higher constitutive caspase-3 activity.

In conclusion, in this study we provide the first mechanistic reason to explain the pro-apoptotic effect of ANX1, a phenomenon that has been observed macroscopically during mammary regression. Monocytes up-regulate ANX1 expression during extravasation into inflamed organs, such as during a DTH reaction in human volunteers (Perretti *et al*., 1999). It remains to be seen if such a regulated mechanism also operates in other white blood cells including lymphocytes and neutrophils. Interestingly, recent data using *in situ* hybridization demonstrated that extravasated neutrophils up-regulate ANX1 synthesis (Oliani *et al*., 2001), and this phenomenon may well be part of an anti-inflammatory mechanism aiming at altering the fate of neutrophils at the site of inflammation.

This study was supported by the Arthritis Research Campaign UK (ARC; grant P0567). The Authors wish to thank the laboratory of INSERM, U332 (Paris, France) for the facilities used at the beginning of this work. M. Perretti is a post-doctoral fellow of the ARC.

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(Received November 20, 2000
Revised February 1, 2001
Accepted February 22, 2001)