Effects of annexin A1 on apoptosis and cell cycle arrest in human leukemic cell lines

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Recent studies suggest that annexin A1 (ANXA1) promotes apoptosis in cancerous cells. This study aims to investigate the effects of ANXA1 on apoptosis and cell cycle arrest in K562, Jurkat and U937 cells and peripheral blood mononuclear cells (PBMC). Cells were treated with ANXA1 and cyclophosphamide prior to flow cytometry analysis for apoptosis and cell cycle arrest induction. At 2.5 μ M, ANXA1 induced significant apoptosis in K562 ($p \leq 0.001$) and U937 ($p \leq 0.05$) cells, with EC_{50} values of 3.6 and 3.8 μ M, respectively. In Jurkat cells, induction was not significant (EC_{50} , 17.0 μ M). No significant apoptosis induction was observed in PBMC. ANXA1 caused cycle arrest in the G0/G1 phase in K562 and U937 cells with $p \leq 0.001$ for both, and ($p \leq 0.01$) for Jurkat cells. ANXA1 induced apoptosis and cycle arrest in the G0/G1 phase in K562 and U937 cells, causing only cell cycle arrest in Jurkat cells.

Keywords: annexin A1, apoptosis, cell cycle, flow cytometry, human leukemic cell lines

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Leukemia is a hematopoietic system malignant proliferative disease (1, 2). Like other cancerous diseases, it is characterized by the inability of hematopoietic stem cells (HSC) to undergo apoptosis and cycle arrest, resulting in uncontrollable cell growth (3, 4). Leukemia is difficult to cure since it is a highly complex and multi-targeted disease in which current therapy is associated with severe toxicity and treatment-related death (5) the lack of molecular markers precludes minimal residual disease (MRD).

Annexin A1 (ANXA1) is a 37kDa protein, which belongs to the group of cellular proteins called Annexins. ANXA1 is either located in the cytosol or membrane bound in various types of cells (6). Initially described as glucocorticoid-inducible protein, it was previously known as lipocortin A1. It is a member of the Annexin family proteins and binds to calcium and phospholipids, giving rise to specific biological functions in membrane reorganization and intracellular regulation, such as down-regulation of inflammation (7, 8). As a phospholipid-binding protein, ANXA1 has been implicated in various intra- and extracellular functions, including inhibition of cyclooxygenase 2 (COX-2), phospholipase A2, signal transduc-

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tion, DNA replication, cell transformation, ion channel formation, and mediation of cell proliferation, differentiation and apoptosis (7, 9, 10). Ahmad *et al.* (2014) have shown that production of ANXA1 in PBMC can be induced by curcumin and phytoestrogens (11). ANXA1 has been vastly studied for its anti-inflammatory properties, but little is known about its anticancer effects, including its effect on leukemia. However, ANXA1 has been recently proposed to play a role in cancer cell apoptosis (12, 13). ANXA1 can affect many components of the inflammatory reaction besides the metabolism of arachidonic acid. Recent data have shown that ANXA1 may specifically target cytosolic PLA2 by both direct enzyme inhibition and suppression of cytokine-induced activation of the enzyme. ANXA1 inhibits the expression and/or activity of other inflammatory enzymes like inducible nitric oxide synthase (iNOS). It was suggested that the deregulation and subcellular localization of ANXA1 was involved in the development, invasion, metastasis, progression and drug resistance to a variety of cancers. The pattern of ANXA1 involvement in tumorigenesis, cell differentiation, proliferation and apoptosis was taken to be tissue-specific (14). Its expression has been reported to be increased in certain cancers, such as pancreatic and gastric cancers, but decreased in prostate, breast and esophageal cancers (7, 14).

Endogenous intracellular ANXA1 has been found to promote apoptosis in cells of myelomonocytic derivation (15). Over-expression of ANXA1 was shown to induce caspase-mediated apoptosis in macrophage cells (16). Hirata (2014) stated that ANXA1 has the ability to down-regulate the COX-2 pathways making it a possible molecular target for future anticancer treatment (17). Petrella *et al.* (2008) demonstrated that ANXA1 might also assist in histone deacetylase (HDAC) inhibitors' effect in inhibiting cell proliferation of three human leukemic cell lines, U937, K562 and Jurkat (18). Various studies have shown that the administration of exogenous recombinant ANXA1 plays a role in attenuating several diseases, such as Alzheimer's disease, restores cell polarity, cytoskeleton integrity and paracellular permeability of cerebrovascular endothelial cells, attenuates STZ-induced diabetes, cardiac and renal dysfunction and mimics the improvement effect of mesenchymal stromal cells (MSCs) on islet cells function (19–22).

The present study aims to investigate the role of ANXA1 as an endogenous protein in leukemic cells. The effects of ANXA1 on leukemia were assessed in K562, Jurkat, and U937 leukemic cell lines by determining its effect on apoptosis and cell cycle arrest.

EXPERIMENTAL

Reagents

ANXA1 (Cat. No.: RPE787Hu01, USCNK, China), Cyclophosphamide (Endoxan, Germany), BD Pharmingen™ Annexin V-FITC (BD Bioscience, USA), Propidium iodide, PI (Sigma, USA), BD Cycletest™ Plus DNA Reagent Kit (Becton Dickinson, USA), methyl thiazolyltetrazolium, MTT (Sigma, USA), dimethyl sulfoxide, DMSO (Merck, Germany).

Cell lines

Chronic myelogenous leukemia (K562), acute lymphocytic leukemia (Jurkat) and acute myelogenous leukemia (U937) cell lines were purchased from the American Type Culture Collection (ATCC, USA). K562 cells were cultured in Iscove's Modified Dulbecco's

Medium, IMDM (Gibco, USA) supplemented with 10 % fetal bovine serum (FBS), 100 IU mL⁻¹ penicillin and 100 µg mL⁻¹ streptomycin. U937 and Jurkat cells were cultured in RPMI 1640 (Gibco, USA) supplemented with 10 % FBS (Gibco), 100 IU mL⁻¹ penicillin and 100 µg mL⁻¹ streptomycin (Amresco, USA).

Isolation of peripheral blood mononuclear cells (PBMC)

Blood was obtained from healthy donors ($n = 3$), aged 18 to 50 years, who participated in the study. The donors satisfied the inclusion criteria: were non-smokers, fasted overnight and did not take any medication or supplements. Written and informed consent was obtained from the participants prior to blood collection. The experimental protocol was approved by the UKM Human Ethical Committee (Approval No. UKM 1.5.3.5/244/NF-050-15) and followed the principles outlined in the Declaration of Helsinki.

Blood samples were diluted with the same volume of RPMI 1640 media. Separation of blood cells was performed using density centrifugation. Briefly, the diluted blood sample was carefully layered on Lymphoprep™ (Axis-Shield, Norway) and the mixture was later centrifuged at 600× *g* for 20 min at 20 °C. The mononuclear cell layer was carefully separated. The PBMCs were washed twice with RPMI 1640 media and resuspended in RPMI-1640 media supplemented with 2 mM L-glutamine, 100 IU mL⁻¹ penicillin, 100 µg mL⁻¹ streptomycin and 10 % FBS. The cell suspension was adjusted to approximately 5 × 10⁵ cells mL⁻¹.

Enzyme-linked immunosorbent assay (ELISA) and quantification of ANXA1

The cells were seeded at 5 × 10⁵ cells mL⁻¹ and incubated at 37 °C for 24 h. The extracellular ANXA1 were collected by centrifugation at 130× *g*, at 25 °C for 7 min for leukemic cell lines and at 300× *g*, 4 °C for 10 min for PBMC. The cell pellet was resuspended and incubated in 200 µL of phosphate-buffered saline (PBS) with 2 mM ethylenediaminetetraacetic acid (EDTA) for 5 min at room temperature to collect the membrane-bound ANXA1. The cells were then centrifuged and the supernatants containing the membrane-bound ANXA1 were collected. RIPA lysis buffer (200 µL) containing protease inhibitor was added to the cell pellet and the mixture was vortexed for 30 s before centrifugation at 13,000× *g* for 15 min at 4 °C to collect the intracellular ANXA1. ANXA1 quantification was performed using an ANXA1 ELISA kit according to the supplied instructions.

Cell viability assay

Cell viability was assessed using the MTT assay according to the established procedure with minor modifications (23). Cells were seeded at a density of 2 × 10⁵ cells mL⁻¹ in a 96-well plate. The cells were then treated with ANXA1 and cyclophosphamide at concentrations ranging from 0–5 µM and 0–28700 µM, respectively, and incubated for 24 h at 37 °C. Following incubation, MTT (5 mg mL⁻¹) was added into each well. Insoluble formazan was then dissolved in DMSO and absorbance was recorded at 570 nm using a microtiter plate reader. Cell viability was determined using the following formula:

$$\text{Cell viability (\%)} = \frac{A_{\text{treatment}} - A_{\text{blank}}}{A_{\text{control}} - A_{\text{blank}}} \times 100$$

Apoptosis assay

The apoptosis assay was carried out based on the procedures described by Maioral *et al.* (2013) with modifications (23). The K562, U937, Jurkat cells and PBMC were seeded as 1×10^6 cells in 6-well plates and exposed to cyclophosphamide at a concentration of 28700 μM and ANXA1 at concentrations of 0.5, 2.5 and 5 μM , with an additional concentration of 25 μM for Jurkat cells, and incubated at 37 °C for 24 h. The cells were washed with cold PBS and re-suspended in annexin V binding buffer. AnnexinV-FITC dye and PI were added to the cells and analyzed using a flow cytometer (BD FACS Canto II, CA, USA).

Cell cycle analysis

The cell cycle analysis assay was performed by flow cytometry following the method described by Maioral *et al.* (2013) with minor modifications (23). Briefly, the cells were seeded at 1×10^6 cells in 6-well plates and were exposed to cyclophosphamide and ANXA1 at concentrations of 28700 μM and 2.5 μM , respectively, and incubated at 37 °C for 24 h. Cell cycle profiles were evaluated by staining DNA with PI using a BD Cycletest™ Plus DNA Reagent Kit according to the supplied protocol. Samples were analyzed with a flow cytometer (BD FACS Canto II, CA, USA) and ModFit LT cell cycle analysis software (ModFit LT, ME, USA).

Statistical analysis

Results were presented as mean $\pm$ S.E.M. from three independent experiments. Data were analyzed using One Way ANOVA followed by Bonferroni's post-hoc test employing the GraphPad Prism 5 software (CA, USA). Differences between groups were considered significant if $p \leq 0.05$ and were marked as follows: *** ($p \leq 0.001$), ** ($p \leq 0.01$) and * ($p \leq 0.05$).

RESULTS AND DISCUSSION

Concentration of total endogenous ANXA1 in all three cell lines and PBMC, extracellular, membrane-bound and intracellular ANXA1, was quantified using the ELISA meth-

Fig. 1. Quantification of total endogenous ANXA1 in the three tested cell lines and PBMCs. * $p \leq 0.05$, *** $p \leq 0.001$ significant difference compared to PBMC.

od. Total endogenous ANXA1 levels in all three cell lines and PBMC are given in Fig. 1. In the current study, the levels of total endogenous ANXA1 were shown to be significantly higher in the three tested leukemic cell lines compared to PBMC. Concentrations of total endogenous ANXA1 concentrations in K562 ($8.15 \pm 0.04 \text{ ng mL}^{-1}$) and Jurkat ($4.37 \pm 0.25 \text{ ng mL}^{-1}$) cells were significantly higher ($p \leq 0.001$) compared to the PBMC ($2.57 \pm 0.13 \text{ ng mL}^{-1}$). U937 cells also had a significantly higher ANXA1 concentration ($3.34 \pm 0.11 \text{ ng mL}^{-1}$, $p \leq 0.05$) compared to the PBMCs, but significantly lower than the K562 ($p \leq 0.001$) and Jurkat ($p \leq 0.01$) cells. This is in accord with the proteomic study by Luczak *et al.* (2012), which

Fig. 2. Effect of a) ANXA1 and b) cyclophosphamide treatment on cell viability in K562, Jurkat and U937 cell lines. a) K562, Jurkat and U937 were treated with ANXA1 in the 0–5 μM concentration range, b) K562, Jurkat and U937 were treated with cyclophosphamide in the concentration range 0–28700 μM . * $p \leq 0.05$, *** $p \leq 0.001$ significant difference compared to the negative control (untreated cells).

showed that ANXA1 can be used as a biomarker to differentiate between acute myeloid leukemia and healthy individuals (24).

Viability of the K562, Jurkat and U937 cells against ANXA1 was evaluated using MTT after a 24-h treatment. Fig. 2 shows the gradual decline in cell viability in the presence of increasing concentrations of ANXA1 (Fig. 2a) and cyclophosphamide (Fig. 2b). Sharp reductions were observed from concentrations 0.5 to 5 μM in each cell line tested. At 5 μM of ANXA1 treatment, all cell lines showed less than 50 % viability. U937 cells showed the highest sensitivity to ANXA1 treatment with cell viability decreased to 7.51 ± 2.74 % post administration. K562 cells also showed remarkable sensitivity towards ANXA1 treatment (12.17 ± 2.45 % viable cells) compared to Jurkat cells, which showed the highest ($p \leq 0.05$) resistance to ANXA1 treatment (39.85 ± 5.94 % viable cells). Cyclophosphamide was used in different ranges from ANXA1 based on the sensitivity of cells. Treatment with cyclophosphamide resulted in a significant decrease in viability of all cell lines at a concentration of 28700 μM . However, the IC_{50} value of cyclophosphamide did not differ significantly between the cell lines (Table I). Therefore, cyclophosphamide, at the 28700 μM concentration, was used as a positive control in the assays below.

Apoptosis is an essential, highly conserved, and tightly regulated cellular process of cell death, which is important for the development, host defense, and suppression of malignant transformation and inflammatory processes (25). Flow cytometric analysis by dual staining with Annexin V-FITC and propidium iodide of the cell lines and PBMC treated with ANXA1 was used to determine the percentage of apoptotic and necrotic cells. The results demonstrated that ANXA1 treatment enhanced apoptosis in leukemic cell lines. Percentage of apoptosis by ANXA1 in leukemic cell lines increased in a dose-dependent

Fig. 3. Effect of ANXA1 treatment on apoptosis and necrosis of K562, Jurkat, U937 cells and PBMC. Cells were stained with Annexin V-FITC and propidium iodide before being quantified by flow cytometry. a) Percentage of apoptotic and b) necrotic cells of leukemic cell lines after a 24-h treatment with positive control (cyclophosphamide at 28700 μM concentration) and ANXA1 at concentrations of 0.5, 2.5 and 5 μM with additional 25 μM for Jurkat cells, c) dot plots of forward-angle light scatter (FSC) *vs.* side-angle light scatter (SSC) of leukemic cell lines treated with positive control and ANXA1 at 2.5 μM concentration for 24 h. Dot plots were divided into four quadrants to indicate necrotic (Q1), late apoptotic (Q2), viable (Q3) and early apoptotic (Q4). * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$ significant difference compared to the negative control. # $p \leq 0.05$, ## $p \leq 0.01$, ### $p \leq 0.001$ significant difference compared to the positive control.

Fig. 3. Continued.

manner where the optimal apoptosis was detected at 2.5 μ M of ANXA1 onwards (Fig. 3). Apoptosis by cyclophosphamide in the positive control was best at the concentration of 28700 μ M.

The level of significance in apoptosis due to ANXA1 treatment reached up to $p \leq 0.001$ for K562 and $p \leq 0.05$ for U937 cells compared to their respective negative controls, while no significant apoptosis induction was observed in either Jurkat cells or PBMC. These results were in accord with the MTT results, which demonstrated that Jurkat cells were more resistant to ANXA1 treatment. Significant necrosis ($p \leq 0.05$) was observed only in K562 cell lines. The EC_{50} value of ANXA1 apoptosis induction in Jurkat cells, 17.0 μM , was also higher ($p \leq 0.05$) compared to K562 (3.6 μM) and U937 cells (3.8 μM). It has been reported that enhanced ANXA1 level influences caspase 3 and caspase 9 signaling, which is of importance for the apoptosis pathway (26, 27). Caspases are members of cysteine protease where their activation will induce apoptosis in diverse cell types, including cancer cells (28). Increase in ANXA1 level results in a decrease in the bcl-2 level, a protein that functions to promote cell survival and prevent apoptosis (26, 27). COX-2 over-expression, on the other hand, can lead to an increase in bcl-2 expression, preventing the mitochondria from releasing cytochrome c and subsequently inhibiting apoptosis (29). COX-2 also decreases the caspase activation, preventing apoptosis. Previous studies have shown that ANXA1 was able to inhibit COX-2 activities (17). A study by Ferreira *et al.* (1997) showed

Fig. 4. Flow cytometric analysis of cell cycle distribution. Tested leukemic cell lines were treated with 2.5 μM ANXA1 and positive control (cyclophosphamide 28700 μM). Each histogram depicts the cell cycle distribution of each cell line with the percentage of cells in G0/G1, S and G2/M phases. Data were gated to exclude apoptotic cells for these calculations. * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$ significant difference compared to the negative control.

that ANXA1 inhibited COX-2 activities in the J774 murine macrophage-like cell line (30). In addition, COX-2 has also been shown to play a role in inhibiting leukemic cell apoptosis (30, 31). Taken together, these previous findings suggested that it is possible for ANXA1 to induce apoptosis in leukemic cells by decreasing COX-2 expression, leading to a decrease in bcl-2 expression and activating apoptosis, either *via* its intrinsic or extrinsic pathway. However, the exact mechanism by which ANXA1 induces apoptosis needs to be further explored. Our study showed that the Jurkat cell was more resistant to ANXA1 treatment compared to K562 and U937 cells. Differences in cell type because of which Jurkat cells are of lymphoblastic origin and K562 and U937 cells are of myeloid origin could be the possible factor.

Significant apoptosis ($p \leq 0.001$) was detected in cyclophosphamide treated cells at a concentration of 28700 μM for both K562 and U937 cell lines compared to their respective negative controls. In contrast to ANXA1, cyclophosphamide induced significant apoptosis ($p \leq 0.01$) in PBMC. However, at this concentration, no significant apoptosis was detected in Jurkat cells. In addition, no significant necrosis was observed at this concentration in any of the cell lines or PBMC. Among all the cell lines, U937 showed the highest sensitivity to cyclophosphamide treatment, followed by K562 and Jurkat cells. Cyclophosphamide treatment induced U937 cells to enter a late apoptotic stage, whereas in PBMC, K562 and Jurkat cells, no significant difference was observed in early and late apoptotic cell percentages. The EC_{50} value of cyclophosphamide could only be determined in U937 cells (40660 μM).

One of the main characteristics of cancer cells is the deregulated cell cycle that produces uncontrollable cell growth (3, 4, 32). The eukaryotic cell cycle is regulated through activation and inactivation of cyclin-dependent kinases (CDKs), cyclin, and tumor suppressor protein (32, 33). Numerous chemotherapeutic agents target the cell cycle by interfering with the signal transduction pathways, affecting the DNA synthesis and causing the cell cycle to halt at certain stages (1).

To investigate the influence of ANXA1 on the cell cycle distribution of K562, Jurkat and U937 cells, flow cytometric analyses based on propidium iodide stained nuclei cells were performed. Representative flow histograms depicting cell cycle distribution of K562, Jurkat and U937 cells following a 24 h exposure to media (negative control), ANXA1 (2.5 μM) and cyclophosphamide (positive control) are shown in Fig. 4. The results demonstrated that ANXA1 caused significant cell cycle arrest in the G0/G1 phase in Jurkat cells ($p \leq 0.01$) and U937 ($p \leq 0.001$), with significant reduction of G2/M phase ($p \leq 0.001$) for both cell lines. In K562, the G0/G1 phase arrest ($p \leq 0.001$) was compensated by a significant reduction in both S ($p \leq 0.01$) and G2/M phases ($p \leq 0.001$) compared to their respective negative controls. Thus, ANXA1-mediated apoptosis induction in both K562 and U937 cells was suggested to be correlated with the G0/G1 phase cell cycle arrest. Cell cycle progression from the G1 phase to S phase requires CDK-cyclin complexes between CDK and cyclin D, E and A (32, 33). Growth arrest in the G0/G1 phase may be due to inhibition of CDK-cyclin complex activity (31). This inhibition prevents the phosphorylation of retinoblastoma tumor suppressor protein (pRB), which inhibits the binding of pRB to transcription factors E2F, halting the cell cycle progression into the S phase and thus causing arrest in the G0/G1 phase (31–33). Other than pRB, the tumor suppressor protein, p53, also plays a major role in regulating the cycle progression from the G0/G1 phase to the S phase. Previous studies have shown that accumulation of this protein increases expression of the cyclin-

CDK complex inhibitor protein, p21. According to Weinberg (2014), the increase in p53 expression induces both apoptosis and cell cycle arrest (34). Hence, it may be assumed that ANXA1 induces apoptosis and cycle arrest by increasing the p53 expression.

The positive control, cyclophosphamide treatment, showed a significant ($p \leq 0.001$) cycle arrest in the G2/M phase in K562 and Jurkat cells. No significant reduction was detected in either G0/G1 or S phase in K562, while significant reduction ($p \leq 0.05$) of cell percentage in the S phase was observed in Jurkat cells compared to their negative control. Cyclophosphamide significantly increased cell percentage of U937 in both S phase ($p \leq 0.001$) and G2/M phase ($p \leq 0.01$), with a significant reduction ($p \leq 0.001$) in the G0/G1 phase. The PBMC cell cycle stages could not be analyzed *in vitro* as the samples were of peripheral location and PBMC could only proliferate unstimulated in the bone marrow.

CONCLUSIONS

In conclusion, ANXA1 at 2.5 μM could induce both apoptosis and cell cycle arrests in the G0/G1 phase in K562 and U937 cell lines. However, a higher concentration of ANXA1 is required to achieve both effects in Jurkat cells. Based on the EC_{50} values, U937 cells were the most sensitive to ANXA1 treatment, followed by K562 and Jurkat. PBMC was not affected by ANXA1 at this concentration. Cyclophosphamide could only show a similar pattern to the ANXA1 in K562 and Jurkat cells but required a significantly higher concentration compared to ANXA1.

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