Annexin A5 inhibits diffuse large B-cell lymphoma cell invasion and chemoresistance through phosphatidylinositol 3-kinase signaling

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Abstract. Diffuse large B-cell lymphoma (DLBCL) is the most common type of non-Hodgkin's lymphoma worldwide. Although patient outcomes have significantly improved to a greater than 40% cure rate by the combinatorial cyclophosphamide, doxorubicin, vincristine and prednisone (CHOP) chemotherapy, which is widely used, resistance to the CHOP regimen continues to pose a problem in managing or curing DLBCL. While it promotes the malignancy and chemoresistance in certain types of cancer, Annexin A5 is negatively correlated with those in other cancers, including DLBCL. In the present study, we explored the effects of Annexin A5 on DLBCL cell invasion and chemoresistance to CHOP. Stable overexpression and knockdown of Annexin A5 were performed in Toledo and Pfeiffer human DLBCL cell lines. Overexpression of Annexin A5 in both cell lines significantly decreased cell invasion, matrix metalloproteinase-9 (MMP-9) expression/activity, phosphatidylinositol 3-kinase (PI3K) activity/Akt phosphorylation, and cell survival against CHOP-induced apoptosis. On the other hand, knockdown of Annexin A5 markedly increased cell invasion, MMP-9 expression/activity, PI3K activity/Akt phosphorylation, and CHOP-induced apoptosis in the DLBCL cell lines, which was abolished by selective PI3K inhibitor BKM120. In conclusion, our study provides the first in vitro evidence that Annexin A5 inhibits DLBCL cell invasion, MMP-9 expression/activity, and chemoresistance to CHOP through a PI3K-dependent mechanism; it provides new insight not only into the biological function of Annexin A5, but also into the molecular mechanisms underlying DLBCL progression and chemoresistance.

Introduction

Diffuse large B-cell lymphoma (DLBCL) is the most common type of non-Hodgkin's lymphoma (NHL) worldwide (1). DLBCL represents a heterogeneous group of tumors with a high variance of genetic abnormalities, clinical features, response to treatment and prognosis (2). Combinatorial cyclophosphamide, doxorubicin, vincristine and prednisone (CHOP) chemotherapy has been a systemic therapy for DLBCL with a cure rate of 40-50% (3) and is widely used in China. Although a subset of DLBCL patients is cured with CHOP regimens, many succumb to chemorefractory disease (4). Resistance to the CHOP anthracycline-based regimen continues to be a serious challenge in the cure of DLBCL (3). The molecular basis for development of the multi-drug chemoresistance in DLBCL remains unclear.

Annexins are predominantly cytosolic soluble proteins that can reversibly bind to negatively charged phospholipids in a Ca²⁺-mediated manner. Twelve Annexins common to vertebrates are known as Annexins A1-A11 and A13 (5,6). Annexin A5, also known as placental anticoagulant protein I, thromboplastin inhibitor V, endonexin II, calphobindin I and lipocortin V, was first described functionally as a vascular anticoagulant in 1985 (7,8). Annexin A5 deregulations were observed as causative phenomena in a range of physiological and pathological processes. Annexin A5 reportedly enhances chemoresistance in gastric cancer and nasopharyngeal carcinoma (10,11). However, a recent study showed that Annexin A5 was upregulated in CHOP-sensitive DLBCL tissues, suggesting that Annexin A5 may inhibit chemoresistance in DLBCL (12).

Keywords: diffuse large B-cell lymphoma, Annexin A5, invasion, chemoresistance, CHOP chemotherapy, phosphatidylinositol 3-kinase
Our pilot studies suggested that Annexin A5 could increase chemosensitivity in DLBCL cells. In the present study, we explored the effects of Annexin A5 on DLBCL cell invasion and chemoresistance to CHOP.

Materials and methods

Cells lines and reagents. Toledo (CRL-2631) and Pfeiffer (CRL-2632) human DLBCL cell lines were purchased from the American Type Culture Collection (Manassas, VA, USA). Annexin A5 shRNA lentiviral particles (sc-29686-V), control shRNA lentiviral particles-A (sc-108080), selective phosphatidylinositol 3-kinase (PI3K) inhibitor BKM120 (sc-364437A), anti-Annexin A5 antibody (sc-74438), anti-matrix metalloproteinase-9 (MMP-9) antibody (sc-21733), and anti-Akt (5C10) (sc-81434) and anti-P-Akt (ser473) (sc-101629) antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). The SensoLyte® 520 MMP-9 Assay kit (71155) was purchased from AnaSpec (Fremont, CA, USA). The QCM ECMatrix 24-well (8 µM) Fluorimetric Cell Invasion assay kit (ECM554) was purchased from Chemicon (Millipore, Billerica, MA, USA). The TiterTACS in situ apoptosis detection kit (4822-96-K) was purchased from R&D Systems (Minneapolis, MN, USA). The PI3K Activity ELISA kit (K-1000s) was purchased from Echelon Biosciences (Salt Lake City, UT, USA). Superfect™ transfection reagent was purchased from Qiagen (Valencia, CA, USA). The Annexin A5 expression vector (RC205619), in which the full-length human Annexin A5 cDNA was subcloned into the pCMV6-entry vector, was purchased from Origene (Beijing, China). Puromycin, G418, cyclophosphamide, doxorubicin, vincristine, prednisone and methothrexate in 96-well tissue culture plates and incubated at 37°C for 24 or 48 h under CHOP treatment. The composition of CHOP consisted of cyclophosphoramide, doxorubicin, vincristine and prednisone at the clinical ratio of 80/5.5/0.16/11.1 (3), with the combined CHOP concentration set at 80 ng/ml. Cell apoptosis was measured at 24 and 48 h with a microplate reader-based TiterTACS in situ apoptosis detection kit (R&D Systems) as described by the manufacturer. Each experiment was repeated three times in duplicates.

Cell apoptosis assay. Cells were cultured at 9x10^4 cells/well in 96-well tissue culture plates and incubated at 37°C for 24 or 48 h under CHOP treatment. The composition of CHOP consisted of cyclophosphoramide, doxorubicin, vincristine and prednisone at the clinical ratio of 80/5.5/0.16/11.1 (3), with the combined CHOP concentration set at 80 ng/ml. Cell apoptosis was measured at 24 and 48 h with a microplate reader-based TiterTACS in situ apoptosis detection kit (R&D Systems) as described by the manufacturer. Each experiment was repeated three times in duplicates.

Statistical analysis. Statistical analyses were performed with SPSS for Windows 10.0. All data values are expressed as means ± SD. Comparisons of means among multiple groups were performed with one-way ANOVA followed by post hoc pairwise comparisons using Tukey's tests. A two-tailed p<0.05 was considered to indicate statistically significant differences.

Results

Overexpression and knockdown of Annexin A5 in human DLBCL cells. We used Toledo and Pfeiffer human DLBCL cells as cell models in this study. The Toledo and the Pfeiffer
cell lines were established from peripheral blood leukocytes and metastatic site (pleural effusion) of patients with DLBCL, respectively. Western blot analyses revealed that Toledo cells had higher constitutive Annexin A5 expression than Pfeiffer (Fig. 1). We overexpressed and knocked down Annexin A5 in both cell lines by stable transfection of an Annexin A5 expression vector and lentiviral transduction of Annexin A5-shRNA, respectively. As shown in Fig. 1, compared with the controls, Annexin A5 was overexpressed 3.4- and 2.8-fold in Toledo and Pfeiffer cells, respectively. On the other hand, the endogenous Annexin A5 level was knocked down 81 and 89% in Toledo and Pfeiffer cells, respectively (Fig. 1). As our pilot studies suggested that Annexin A5 would regulate DLBCL cell invasion and chemosensitivity through a PI3K-dependent mechanism (data not shown), we included a selective PI3K inhibitor BKM120 (50 µM) in all experiments in this study. As shown in Fig. 1, the PI3K inhibitor had no significant effect on Annexin A5 expression in both Toledo and Pfeiffer cells.

**Effects of Annexin A5 on DLBCL cell invasion and MMP-9 expression/activity.** To examine the effect of Annexin A5 expression vector and lentiviral transduction of Annexin A5-shRNA, respectively. As shown in Fig. 1, compared with the controls, Annexin A5 was overexpressed 3.4- and 2.8-fold in Toledo and Pfeiffer cells, respectively. On the other hand, the endogenous Annexin A5 level was knocked down 81 and 89% in Toledo and Pfeiffer cells, respectively (Fig. 1). As our pilot studies suggested that Annexin A5 would regulate DLBCL cell invasion and chemosensitivity through a PI3K-dependent mechanism (data not shown), we included a selective PI3K inhibitor BKM120 (50 µM) in all experiments in this study. As shown in Fig. 1, the PI3K inhibitor had no significant effect on Annexin A5 expression in both Toledo and Pfeiffer cells.

**Figure 1.** Annexin A5 expression in human diffuse large B-cell lymphoma (DLBCL) cells with overexpression or knockdown of Annexin A5. In (A) Toledo and (B) Pfeiffer human DLBCL cells, expression of Annexin A5 in normal control cells (NC, lane 1), cells stably transfected with the empty pCMV6-entry vector (VC, lane 2), cells stably transfected with Annexin A5 with or without phosphatidylinositol 3-kinase (PI3K) inhibitor BKM120 (50 µM) treatment (A5, lane 3; A5+PI3K-I, lane 4), cells stably transduced with scramble control shRNA (SC, lane 5), cells stably transduced with Annexin A5-shRNA with or without BKM120 (50 µM) treatment (A5-shRNA, lane 6; A5-shRNA+PI3K-I, lane 7), and cells treated with BKM120 (50 µM) (PI3K-I, lane 8) was analyzed with western blot analysis. β-actin blotting was used as a loading control. Density of the Annexin A5 blot was normalized against that of the β-actin blot to obtain a relative Annexin A5 blot density, which was expressed as fold changes to that of NC (designated as 1). Three independent experiments were performed for each western blot analysis. Data values are expressed as mean ± SD. *p<0.05 compared with NC and VC; **p<0.05 compared with A5; ***p<0.05 compared with A5+PI3K-I; ****p<0.05 compared with SC; *****p<0.05 compared with A5-shRNA; ******p<0.05 compared with A5-shRNA+PI3K-I.

**Figure 2.** Effects of Annexin A5 on diffuse large B-cell lymphoma (DLBCL) cell invasion. *In vitro* cell invasion assays were performed with a Fluorimetric Cell Invasion Assay kit (Chemicon; Millipore) in (A) Toledo and (B) Pfeiffer DLBCL cells. Cell invasion in normal control cells (NC), cells stably transfected with the empty pCMV6-entry vector (VC), cells stably transfected with Annexin A5 with or without phosphatidylinositol 3-kinase (PI3K) inhibitor BKM120 (50 µM) treatment (A5; A5+PI3K-I), cells stably transduced with scramble control shRNA (SC), cells stably transduced with Annexin A5-shRNA with or without BKM120 (50 µM) treatment (A5-shRNA; A5-shRNA+PI3K-I), and cells treated with BKM120 (50 µM) (PI3K-I) was determined by fluorescence and shown as fold changes to that of NC (designated as 1). Each experiment was repeated three times in duplicates. Data values are expressed as mean ± SD. *p<0.05 compared with NC and VC; **p<0.05 compared with A5; ***p<0.05 compared with A5+PI3K-I; ****p<0.05 compared with SC; *****p<0.05 compared with A5-shRNA.
on DLBCL cell invasion, we performed in vitro cell invasion assays. Compared with the controls, overexpression of Annexin A5 decreased cell invasion by >60% in both Toledo and Pfeiffer cells (Fig. 2). On the other hand, knockdown of

Annexin A5 respectively increased cell invasion by 2.1-fold in Toledo cells and by 2.6-fold in Pfeiffer cells, which was abolished by BKM120 (50 µM) (Fig. 2).

MMPs play a critical role in cancer cell invasion (19). Among different MMPs tested, we found that the MMP-9 expression was significantly altered by Annexin A5 in DLBCL cells. As shown in Fig. 3, compared with the controls, overexpression of Annexin A5 decreased MMP-9 expression by >70% in both Toledo and Pfeiffer cells. In contrast, knockdown of Annexin A5 respectively increased MMP-9 expression by
2.5-fold in Toledo cells and by 2.9-fold in Pfeiffer cells, which was abolished by BKM120 (50 µM) (Fig. 3). Similar data trend was observed with the MMP-9 activity (Fig. 4).

Effects of Annexin A5 on DLBCL cell chemoresistance to CHOP. To explore the effect of Annexin A5 on DLBCL chemoresistance, we examined cell apoptosis in DLBCL cells treated with CHOP in vitro. Overexpression or knockdown of Annexin A5 did not significantly alter cell apoptosis in either Toledo or Pfeiffer cells under normal culture conditions (data not shown). However, overexpression of Annexin A5 significantly increased CHOP-induced DLBCL cell apoptosis compared with the controls (Fig. 5). On the other hand, knockdown of Annexin A5 markedly decreased DLBCL cell apoptosis during CHOP treatment, which was abolished by BKM120 (50 µM) (Fig. 5).

Effects of Annexin A5 on PI3K activity and phosphorylation of Akt in DLBCL cells. The above results suggested that
Annexin A5 inhibited DLBCL cell chemoresistance to CHOP through a PI3K-dependent mechanism. Indeed, the PI3K/Akt pathway is reportedly important for cancer cell chemoresistance (20-23). Thus, we next examined the effects of Annexin A5 on the PI3K activity and phosphorylation of Akt in DLBCL cells. As shown in Fig. 6, compared with the controls, overexpression of Annexin A5 decreased the PI3K activity by ~65% in Toledo cells and by ~75% in Pfeiffer cells, respectively. In contrast, knockdown of Annexin A5 respectively increased the PI3K activity by ~2-fold in Toledo cells and by 2.3-fold in Pfeiffer cells, which was abolished by BKM120 (50 µM) (Fig. 6). Similar data trend was observed with phosphorylation at serine 473 (ser473) of Akt (Fig. 7), which is required for full activation of Akt by PI3K.

**Discussion**

CHOP is widely used in China to treat DLBCL. Although patient outcomes have significantly improved to a >40% cure rate by CHOP chemotherapy, resistance to the CHOP regimen continues to pose a challenge in managing or curing DLBCL (3). Understanding the molecular basis for development of multi-drug chemoresistance in DLBCL may serve as a basis for identification of novel therapeutic targets and biomarkers involved in DLBCL resistance to CHOP. Previous studies have suggested that Annexin A5, a calcium-dependent phospholipid-binding protein, while promoting the malignancy and chemoresistance in certain types of cancer, is negatively correlated with those in other cancers, including DLBCL (9-12). In the present study, we demonstrated an important inhibitory role for Annexin A5 in DLBCL cell invasion and chemoresistance to CHOP.

DLBCL is a heterogeneous group of tumors with a high variance of genetic abnormalities, clinical features, response to treatment and prognosis (2). Thus, we used two DLBCL cell lines with considerable background differences (respectively established from peripheral blood leukocytes and metastatic site of DLBCL patients) as cell models in this study to demonstrate a generalizable role of Annexin A5 in DLBCL cell invasion and chemoresistance to CHOP. In both cell lines, while overexpression of Annexin A5 significantly decreased cell invasion and cell survival against CHOP-induced apoptosis, knockdown of Annexin A5 markedly increased cell invasion and CHOP-induced apoptosis, which was abolished by a selective PI3K inhibitor. The results suggest that Annexin A5 inhibited DLBCL cell invasion and chemoresistance to CHOP through a PI3K-dependent mechanism. This was corroborated by the results that knockdown of Annexin A5 increased the PI3K activity and Akt phosphorylation in DLBCL cells. The findings are also in line with previous studies that the PI3K/Akt pathway plays a critical role in cancer cell invasiveness and chemoresistance (20-23).

MMPs play a critical role in cancer cell invasion (19). Among different MMPs tested, we found that the MMP-9 expression/activity was significantly altered by Annexin A5, which showed similar data trend as that in DLBCL cell invasion. Previous studies have shown that PI3K signaling can stimulate MMP-9 expression (24,25). In our study, overexpression of Annexin A5 in DLBCL cells significantly decreased the PI3K activity, MMP-9 expression/activity, and cell invasion. On the other hand, knockdown of Annexin A5 markedly increased the PI3K activity as well as MMP-9 expression/activity and cell invasion, which was abolished.
by a selective PI3K inhibitor. The findings suggest that Annexin A5 could inhibit DLBCL cell invasion by downregulating the MMP-9 expression/activity through inhibiting PI3K activity/signaling.

It has been reported that Annexin A5 dysexpression may lead to deregulated activation of PKC and abnormality of cellular signal transduction, which may be involved in carcinogenesis (9). In our study, however, altered expression of Annexin A5 in DLBCL cells only showed major regulatory effects on PI3K activity/signaling, which subsequently led to significant changes in cell invasion and chemoresistance to CHOP. Future studies are required to examine the mechanism by which Annexin A5 inhibits PI3K activity/signaling in DLBCL cells.

Annexin A5 is negatively correlated with the malignancy and chemoresistance in some cancers, while it enhances those in other cancers (9-12). Our study demonstrated an inhibitory effect of Annexin A5 on DLBCL chemoresistance, while previous studies have shown that Annexin A5 enhances chemoresistance in gastric cancer and nasopharyngeal carcinoma (10,11). Thus, Annexin A5 likely plays a dual role in cancer cell malignancy and chemoresistance, depending on tissue specificity. The functional role of Annexin A5 in DLBCL invasiveness and chemoresistance will be verified in more DLBCL cell lines and in animal models in our future studies.

Twelve Annexins common to vertebrates are known as Annexins A1-A11 and A13 (1,2). Annexin A1, A2, A4 and A5 play important roles in breast, pancreatic and laryngeal carcinoma, alone and/or synergistically, which has made them potential therapeutic targets for malignant tumors (26). Based on our findings in the present study, it is noteworthy to investigate whether other Annexin family members play a role, alone or in combination with Annexin A5, in DLBCL cell invasion and chemoresistance.

In conclusion, our study provides the first in vitro evidence that Annexin A5 inhibits DLBCL cell invasion, MMP-9 expression/activity, and chemoresistance to CHOP through a PI3K-dependent mechanism. It provides new insight not only into the biological function of Annexin A5, but also into the molecular mechanisms underlying DLBCL progression and chemoresistance.

References