Annexin-I expression modulates drug resistance in tumor cells

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Abstract

The use of anti-cancer chemotherapy often leads to the rise of multidrug-resistant (MDR) tumors. We have previously reported the overexpression of a 40 kDa protein (P-40) in several MDR tumor cell lines. In this report we describe the cloning of a 1.4 kb cDNA with an open reading frame of 344 amino acids that encodes the P-40 protein. Analysis of the P-40 amino acid sequence showed it is identical to the human annexin I (Anx-I) protein. The identity of the isolated P-40 cDNA as Anx-I was confirmed by the specific binding of IPM96 mAb to a 40 kDa protein following the in vitro expression of P-40 full-length cDNA. Northern blot analysis of total RNA from drug-sensitive and -resistant cells revealed an increase in P-40 (or Anx-I) mRNA in drug-resistant cells relative to drug-sensitive cells. Transfection of Anx-I cDNA into drug-sensitive MCF-7 cells was carried out without further drug selection and showed 2- to 5-fold increase in resistance of transfected cells to adriamycin, melphalan, and etoposide. Conversely, transfection of reverse Anx-I cDNA into SKOV-3 cells decreased the expression of Anx-I without affecting the expression of other members of the annexin family and showed a 3- to 8-fold increase in sensitivity to these drugs. Of interest was the correlation between the presence of Anx-I and MDR in MDA-MB-231 cells when compared to MCF-7 cells. MDA-MB-231 cells show 3- to 20-fold increase in resistance to adriamycin, melphalan, and etoposide in the absence of detectable levels of P-glycoprotein (P-gp1), the multidrug resistance protein (MRP1) or the breast cancer resistance protein (BCRP). Taken together, these results provide the first direct evidence for the role of Anx-I in MDR of tumor cells.

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The ability of malignant cells to develop multidrug resistance (MDR) to cytotoxic drugs is a major impediment in the chemotherapeutic treatment of cancer patients. Evidence from studies using in vitro selected MDR tumor cells has led to the identification of several proteins that can confer resistance to a variety of chemotherapeutic drugs [1]. The overexpression of P-glycoprotein (P-gp1) and the multidrug resistance protein (MRP1) in tumor cells has been shown to confer MDR onto otherwise drug-sensitive cells [2,3]. Both P-gp1 and MRP1 belong to a large family of ATP trafficking proteins that mediate the transport of numerous ligands [4]. Although the molecular mechanisms by which P-gp1 or MRP1 confer drug resistance are not entirely clear, the function of both proteins results in reduced intracellular drug accumulation through an energy-dependent drug efflux [5,6].

P-gp1 and MRP1 are expressed to variable levels in normal tissues and are thought to mediate the transport of normal cell metabolites, hormones, and xenobiotics [7,8]. High levels of P-gp1 expression has been detected in more than 50% of tumors including those derived from tissues in which P-gp1 gene is consecutively activated [9,10]. A correlation between the overexpression of P-gp1 and failure of chemotherapy or poor survival rates have been established for some hematopoietic tumors and childhood malignancies [11]. However, the
lack of P-gp1 expression in other MDR tumors indicates that additional cellular changes confer resistance to anti-cancer drugs [12–15]. Indeed, MRP1 expression in retinoblastoma was shown to correlate with the rare failure of chemotherapy in spite of cyclosporine A reversal of P-gp1-mediated MDR [16]. Other cellular changes, including reduction in topoisomerase II levels or activity [17], overexpression of the lung resistance protein (LRP; a component of human vaults [18]), or overexpression of the breast cancer resistance protein (BCRP) [19] have been observed in MDR tumor cells and may confer resistance to anti-cancer drugs.

We have previously described the overexpression of a 40 kDa protein (P-40) in several MDR tumor cells with or without P-gp1 or MRPI expression [20]. In this study it was of interest to determine the identity of P-40 and to examine its role in drug resistance of tumor cells. Our results show that P-40 is identical to annexin I (Anx-I also known as lipocortin I), member of a large family of Ca$$^{++}$$-dependent phospholipid binding proteins with several suggested functions including intracellular membrane vesicular trafficking and exocytosis [21]. However, Anx-I has not been previously implicated in drug resistance. To assess the role of Anx-I in MDR, we examined the effects of overexpression and inhibition of Anx-I expression on the sensitivity of tumor cells to several anti-cancer drugs.

Materials and methods

Cell culture and metabolic labeling. Cells were grown in RPMI or α-MEM, containing 10–15% fetal calf serum (Hyclon). Drug-sensitive and/or P-gp1-containing 10–15% fetal calf serum (Hyclon). Drug-sensitive and their nucleotide sequence was analyzed using the Blast search at Sheldon Biotech Centre at McGill University. Both strands of two different clones were completely sequenced using the lipofectAMINE 2000 reagent as outlined by manufacturer’s protocol (Gibco, Burlington, Ont., Canada). Stable transfectants were cultured continuously for another 2 to 3 weeks where MCF-7 or SKOV-3 cells were expanded and tested for P-40 expression by Western blotting [26] and immunofluorescence as previously described [20]. P-40 enforced (MCF-7/P-40) or depleted (SKOV-3/pIPM96) clones were tested for drug sensitivity without further selection with anti-cancer drugs as previously described [27]. The effects of drugs on the viability of cells are expressed as means ± SD of triplicate within the same experiment.

Results

In a previous report by Wang et al. [20], we had demonstrated the overexpression of a 40 kDa protein (P-40) in MDR cells without or together with P-gp1 (MCF7/AR) or MRPI (H69/AR) (Fig. 1A). To evaluate the role of P-40 in tumor drug resistance, it was of interest to isolate the gene encoding P-40 and to overexpress it in tumor cells. Using a previously characterized P-40-specific mAb (IPM96), a CDNA expression library prepared from HeLa cells was screened. A total of 5.0 × 10$$^5$$ plaques from λgt11 phage library were screened and several positive plaques were identified following the initial screening. Of the latter positive plaques, two positive clones were isolated following sequential plaque purification. These two positive clones of 1.4 kb were subcloned into PCRII vector and their nucleotide sequence was determined using standard dideoxynucleotide sequencing. Analysis of P-40 cDNA sequence revealed an open reading frame of 344 amino acids, consistent with the expected molecular mass of the protein (38.2 versus 40 kDa). Comparison of P-40 nucleotide and amino acid sequence to sequences in databases data bank showed P-40 sequence to be identical to Annexin I (Anx-I) [28]. Accession No. emb/x059801/HSLIPCR.

To confirm the identity of the isolated P-40 cDNA as that of Anx-I, a pCDNA3 construct containing 1.4 kb fragment encoding the full length of P-40 was expressed in vitro using T7 promoter directed transcription–translation reticulocyte lysate with [35S]methionine. Fig. 1B shows the immunoprecipitation of proteins with IPM96 mAb from an in vitro expression reaction with vector only or vector plus 1.4 kb cDNA insert. An irrelevant IgG2b was used as negative control to immunoprecipitate proteins from a reaction with vector plus...
the 1.4 kb insert (Fig. 1B). The results of Fig. 1B show a 40 kDa [35S]methionine-labeled protein immunoprecipitated with IPM96 mAb but not with an irrelevant IgG 2b. Western blot analysis of the same protein lysates as in Fig. 1B showed a 40 kDa protein that was recognized by the IPM96 mAb but not by an irrelevant IgG2b (Fig. 1C). Previously [20], higher levels of P-40 were shown in MDR cells relative to their parental drug-sensitive cells. To determine if the overexpression of P-40 is due to transcriptional increase, Northern blot analysis was performed with RNA extracted from drug-sensitive (MCF-7, SKOV-3, and H69) and -resistant human MDR cells (MCF-7/AR, SKOV/VLB1.0, and H69/AR) were fractionated on SDS–PAGE and transferred to nitrocellulose membrane. The membrane was probed with P-40-specific monoclonal antibody, IPM96. In vitro expression of P-40 was performed using the T7 promoter directed transcription–translation system. (B) In vitro transcribed and translated mixes containing PCRII vector only or PCRII plus 1.4 kb insert immunoprecipitated with IPM96 mAb or an irrelevant IgG2b. (C) The same samples as in (B) but transferred to nitrocellulose membrane and probed with a specific antibody to P-40 (IPM96 mAb) or an irrelevant IgG2b, respectively. For mRNA levels in the same cell lines, total RNAs were resolved on agarose gel, transferred to nylon membrane, and probed with 32P-labeled 1.4 kb fragment encoding P-40 (or Anx-I) and actin (D).

Fig. 1. Identification of P-40 as Annexin I and its expression in drug-sensitive and -resistant cells. (A) Total cell extracts from drug-sensitive (MCF-7, SKOV-3, and H69) and -resistant human MDR cells (MCF-7/AR, SKOV/VLB1.0, and H69/AR) were fractionated on SDS–PAGE and transferred to nitrocellulose membrane. The membrane was probed with P-40-specific monoclonal antibody, IPM96. In vitro expression of P-40 was performed using the T7 promoter directed transcription–translation system. (B) In vitro transcribed and translated mixes containing PCRII vector only or PCRII plus 1.4 kb insert immunoprecipitated with IPM96 mAb or an irrelevant IgG2b. (C) The same samples as in (B) but transferred to nitrocellulose membrane and probed with a specific antibody to P-40 (IPM96 mAb) or an irrelevant IgG2b, respectively. For mRNA levels in the same cell lines, total RNAs were resolved on agarose gel, transferred to nylon membrane, and probed with 32P-labeled 1.4 kb fragment encoding P-40 (or Anx-I) and actin (D). The results of Fig. 1B show a 40 kDa [35S]methionine-labeled protein immunoprecipitated with IPM96 mAb but not with an irrelevant IgG2b. Western blot analysis of the same protein lysates as in Fig. 1B showed a 40 kDa protein that was recognized by the IPM96 mAb but not by an irrelevant IgG2b (Fig. 1C). Previously [20], higher levels of P-40 were shown in MDR cells relative to their parental drug-sensitive cells. To determine if the overexpression of P-40 is due to transcriptional increase, Northern blot analysis was performed with RNA extracted from drug-sensitive (MCF-7, SKOV-3, and H69) and -resistant (MCF-7/AR, SKOV/VLB1.0, and H69/AR) cells and the membrane was probed with 32P-labeled 1.4 kb fragment. The results in Fig. 1D show a 1.6 kb mRNA in MCF-7/AR, H69/AR, and SKOV/VLB1.0 drug-resistant cell lines but not in their drug-sensitive parental cells with the exception of SKOV-3 cells. However, in comparison with SKOV/VLB1.0, a 4-fold decrease in P-40 transcript levels was observed in SKOV-3 drug-sensitive cells (Fig. 1D). Consistent with the latter results, analysis of P-40 (Anx-I) protein levels by Western blotting with IPM96 mAb showed comparable levels of P-40 protein expression to its mRNA (Fig. 1A versus D). The low level of P-40 (Anx-I) in SKOV-3 cells is interesting in light of the fact that these cells were originally derived from a patient with an ovarian tumor that was clinically resistant to cisplatin and adriamycin [29]. To determine if similar correlation between drug resistance and P-40 overexpression can be observed in other tumor derived cell lines, P-40 expression was evaluated in several unselected tumor cell lines derived from untreated (MCF-7) and drug treated (MDA-MB-231) patients. Fig. 2A shows a Western blot analysis of total cell extracts from MDA-MB-231 and MCF-7 probed with IPM96 mAb. As evidenced, only MDA-MB-231 cells contained P-40, while P-gp1, MRPl, and BCRP were absent in both cell lines (Figs. 2B–D). The sensitivities of these two cell lines to various anti-cancer drugs were tested in an MTT-based assay. As shown in Fig. 2E, the MDA-MB-231/Anx-I-expressing cells displayed greater resistance to adriamycin, melphalan, and etoposide than MCF-7 cells which do not express Anx-I. The presence of Anx-I (P-40) in MDA-MB-231 cells is interesting in light of the fact that these cells were originally derived from a patient with a breast tumor that was clinically resistant to
several chemotherapeutic drugs and in particular to adriamycin [30]. To determine if P-40 (Anx-I) alone confers resistance to anti-cancer drugs, full-length cDNA of P-40 (Anx-I) was cloned into pCIN4 eukaryotic expression vector and transfected into MCF-7 drug-sensitive cells. Stable transfectants of P-40 (Anx-I) MCF-7 cells were selected in the presence of lethal concentrations of G418. Fig. 3A shows a Western blot analysis of total cell extracts from MCF-7/P-40 clone E probed with IPM96 mAb. Fig. 3B shows immunofluorescence staining of MCF-7 cells transfected with vector alone or P-40 (Anx-I). The results in Fig. 3B show similar subcellular localization of P-40 (or Anx-I) to the inner leaflet of cell membrane of MCF-7/AR MDR selected cells and MCF-7/P40 clone E. As expected, no detectable levels of P-40 were observed in cells transfected with vector alone. To determine if the overexpression of P-40 (or Anx-I) affects the sensitivity of MCF-7 cells to anti-cancer drugs, the effects of increasing concentration of anti-cancer drugs on the growth of MCF-7 cells transfected with vector alone or P-40 were assessed in an MTT-based assay (Fig. 3C).

Interestingly, clone E showed a 2- to 5-fold increase in its EC50 to adriamycin, melphalan, and etoposide relative to cells transfected with vector alone. It should be stated that although the observed increase in drug resistance to these drugs is lower than that shown with P-gp1 or MRP1 transfectants, it was observed consistently. Thus,

![Image](image1.png)

**Fig. 3. Analysis of P-40 MCF7 transfectants.** MCF-7 cells transfected with pCIN4 vector without or with P-40 (Anx-I) full-length cDNA. (A) A Western blot analysis of total cell lysates from vector alone and clone E P-40 transfectant probed with anti-P-40 (Anx-I) mAb. (B) An immunofluorescence staining of MCF-7/P-40 transfectant cells, drug-sensitive MCF-7, and MDR selected MCF-7/AR cells probed with anti-P-40 (or IPM96 mAb). Cells were viewed at 400× to 600× magnification. (C) shows the relative viability of MCF-7 cells transfected with vector alone (■) or P-40 (Anx-I) cDNA (▲) in the presence of increasing concentrations of adriamycin, melphalan, and etoposide. The cell viability results represent the average of three independent experiments done in triplicate.

![Image](image2.png)

**Fig. 4. Effects of reduced Anx-I expression on the drug sensitivity of SKOV-3 ovarian cells.** SKOV-3 cells which express P-40 (or Anx-I) were transfected with vector alone or P-40 cDNA in the reverse orientation (clones 16 and 17). Total cell lysates from SKOV-3, vector transfected, and reverse-P-40 transfected cells were resolved on SDS-PAGE and transferred to nitrocellulose membrane. The nitrocellulose membranes were then probed with anti-Annexins I, II or IV mAbs (A). (B) The relative viability of SKOV-3-vector (■) and reverse-P-40 transfectant clones 16 (▲) and 17 (▼) in the presence of increasing concentrations of adriamycin, melphalan, and etoposide after 72 h exposure to drugs. The cell viability results represent the average of three independent experiments done in triplicate.
overexpression of P-40 (Anx-I) confers low level drug resistance to structurally dissimilar anti-cancer drugs. Given the relatively low levels of drug resistance, it was of interest to examine the effects of inhibition of P-40 expression on drug resistance. P-40-expressing SKOV-3 cell line was transfected with the full-length reverse cDNA of Anx-I cloned into pcDNA4 eukaryotic expression vector. Stable transfectants of reverse P-40 SKOV-3 cells were selected in the presence of lethal concentrations of G418. Fig. 4A shows a Western blot analysis of total cell extracts from SKOV-3 clones 16 and 17 probed with IPM96 mAb. As evidenced, clone 16 showed reduced expression of P-40 while clone 17 was completely depleted of the protein. Although Anx-I has no significant similarity with its family members at the nucleotide level, all annexins have a core domain that contains four homologous conserved repeats [21]. It was therefore of interest to verify that other members of the annexin family were not depleted simultaneously with Anx-I. Fig. 4A shows that neither Anx-II nor Anx-IV protein levels were affected in clones 16 and 17. To determine if the depletion of Anx-I affects the sensitivity of SKOV-3 cells to anti-cancer drugs, increasing concentrations of adriamycin, melphalan, and etoposide were assessed on cells to anti-cancer drugs, increasing concentrations of adriamycin, melphalan, and etoposide relative to cells transfected with vector alone. In addition, inhibition of Anx-I expression in SKOV-3 cells transfected with an antisense P-40 cDNA showed 2.5- to 8-fold increased sensitivity to several anti-cancer drugs. Together, these results suggest that Anx-I confers low level resistance by contrast to P-gp1 and MRP1 drug transport mechanisms. Thus, overexpression of Anx-I in tumor cells could represent first level defense similar to heat shock response that protects tumor cells from low levels of cytotoxic drugs, while P-gp1 and MRP1 are overexpressed in tumor cells exposed to higher drug concentrations and are capable of protecting tumor cells from much higher levels of drugs. Consistent with this speculation, tumor cells (SKOV-3 and MDA-MB-231) derived from ovarian and breast cancer patients with clinically resistant tumors showed high levels of Anx-I and reduced sensitivity to anti-cancer drugs. Moreover, neither SKOV-3 nor MDA-MB-231 cells expressed detectable levels of P-gp1, MRPI or BCRP (Fig. 2).

The mechanism by which P-40 (Anx-I) confers drug resistance to anti-cancer drugs is presently unknown. Drug transport studies, using [3H]adriamycin, did not show significant drug transport in Anx-I transfectant cells (data not shown). However, given the role of annexins in promoting aggregation of membrane vesicles through calcium-dependent phospholipid binding, we speculate that Anx-I confers drug resistance through enhanced vesicle aggregation and exocytosis of drug filled vesicles [31]. Consistent with the latter speculation, MDR cells expressing P-40 have been shown to contain higher membrane vacuoles than their drug sensitive parental cells [34]. Furthermore, P-gp1 and MRPI have been detected in the endosomal membranes of drug-resistant cells [35]. Thus, P-40 (Anx-I) could function together with P-gp1 or MRPI to cause the aggregation and possibly exocytosis of drug-filled vesicles. Alternatively Anx-I, which has been suggested to be a stress protein [36], may be cytoprotective to cells exposed to stress signals and cytotoxic agents. In conclusion, the results of this study provide the first evidence for the role of Anx-I in drug resistance in...
tumor cells. These findings are important to our understanding of the drug resistance phenotype and can shed light on the functions of annexins.

References


