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Original article

Nuclear localization of P-glycoprotein is responsible for protection of the nucleus from doxorubicin in the resistant LoVo cell line

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ABSTRACT

The high expression of P-glycoprotein (P-gp) belongs to one of the most important factors causing multidrug-resistant (MDR) of cancer cells. P-gp is primarily associated with plasma membrane; however, small fraction of that protein is present in the nuclear envelope. Such phenomenon is observed in cancer cells and may result in the selection of MDR cells as the secondary tumor and/or resistant metastasis that significantly shorten patient survival rate. Here, we confirmed nuclear localization of P-gp in resistant LoVo cells and demonstrated its impact on doxorubicin efflux from the nucleus to cytoplasm. Furthermore, we showed that P-gp located at the nuclear envelope might have a different glycoside chain when compared to the form located in the cytoplasm. It suggests that the glycoside chain plays a role in the intracellular trafficking of P-gp and may decide about the destination place in the cell.

1. Introduction

P-glycoprotein (P-gp) is an ATP-binding efflux transporter associated with biological membranes [1]. It is encoded by *MDR1* and *MDR3* genes in humans but some additional homologous were found in animals (*mdr1a*, *mdr1b*, *mdr2*) [2]. Human mature P-gp, encoded by *MDR1*, yields a large 170 kDa protein, comprised of 1280 amino acids with some phosphorylation and/or glycosylation [3]. Glycosylation is an important step required for the maturation of P-gp. It takes place in the Golgi apparatus as part of post-translational modification processes usually associated with secretory or membrane proteins (reviewed in [4]). Here, the sugar residues (polysaccharides) can be covalently bound to the asparagine residues (*N*-glycosylation) or at the serine/threonine residues (*O*-glycosilation). P-gp undergoes *N*-glycosylation. So far, there is no known detailed pathway demonstrating P-gp transport from ER to either cell membrane or nucleus via the Golgi apparatus.

P-gp is expressed in the apical membrane of many secretory cells of such organs as kidney, liver, intestine and adrenal glands. Furthermore, it participates in the correct function of blood-brain barrier [5]. Its inherited function was detoxification and therefore, P-gp plays a critical role in drugs disposition. In consequence, P-gp activity is of great clinical importance in both non-cancer and cancer-related drug therapies due to its broad-ranging effects on the absorption and excretion of a variety of drugs. P-gp is able to transport many different types of substrates ranging from small molecules, such as organic cations, carbohydrates, amino acids, some antibiotics to macromolecules, such as polysaccharides and proteins [6,7].

P-gp was first described by Juliano and Ling in 1976 as a highly overexpressed glycoprotein associated with membranes in the drug-resistant Chinese hamster ovary cell line [8]. Overexpression of P-gp is associated with the most frequently observed molecular mechanism, which confers multidrug resistance (MDR) [9]. The MDR phenotype may have either inherited or acquired character as a consequence of its application during chemotherapy. Furthermore, developed MDR quite often showed cross-resistance to a variety of structurally and functionally unrelated drugs [10].

P-gp was found to be localized not only in the plasma membrane but also in the intracellular membranes. Several cellular molecular compartments may be a potential site for P-gp functional activity. It was found in intracytoplasmic vesicles [11] and in Golgi apparatus [12,13]. Furthermore, the nuclear envelope is a place for P-gp localization [14,15]. The nucleus is the main target for various anticancer drugs, including anthracyclines, such as doxorubicin (DX). So far, the best-known and widely accepted mechanisms of these drug actions are based on the inhibition of DNA replication, transcription and repair processes, which occur in the nucleus. The inhibition is mediated by the drug intercalation into DNA [16]. Therefore, nuclear P-gp may

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participate in the elimination of cytotoxic drugs from their place of action, i.e. nucleus. Furthermore, P-gp located in the Golgi apparatus as well as in the intacytoplasmatic membranes could be involved in the drug transport from the perinuclear region to the cell periphery.

2. Materials and methods

2.1. Cell lines and culture conditions

Colon adenocarcinoma cell lines LoVo and LoVo/Dx cells were cultured in MEM medium (Sigma–Aldrich, USA) supplemented with 2 mM L-glutamine, 10% (v/v) fetal bovine serum, and antibiotics (penicillin, streptomycin and amphotericin B). The drug-resistant cell line LoVo/Dx was complemented with DX in concentration 0,2 μg/mL. Cells were plated on tissue culture flasks and maintained at 37 °C in an incubator with 5% CO_2 for 7–10 days until confluancy was attained.

2.2. Immunofluorescence staining and fluorescence microscopy

For fixation and permeabilization, cells were washed in ice-cold acetone/methanol for 10 min at −20 °C and rinsed three times with PBS. To detect P-gp, cells were treated with mouse monoclonal antibody in 1:25 (Alexis, US) for 1 h at RT. Cells were subsequently rinsed five times with PBS and incubated for 1 h (RT, darkness) with anti-mouse antibody, labeled with MFP488 dye (MoBiTec). To visualize the nuclei of cells, they were mounted in medium with DAPI (Santa Cruz Biotechnology, CA). Double immunofluorescence simultaneous protocol was performed on isolated nuclei to detect P-gp and nuclear lamin B1. As previously anti-P-gp and additional rabbit polyclonal anti-human to lamin B1 antibody (1:400, Abcam, Cambrige, UK) were used as the primary antibodies. Afterwards, the nuclei were treated with anti-mouse MFP488 and anti-rabbit MFP590 (MoBiTec), respectively. Fluorescent images were collected on Zeiss Axio Imager Z1 microscope with 63× oil-immersion objective and monochrome CCD camera (Carl Zeiss, Germany). The presence of P-gp and lamin B1 was analyzed by pseudo-color representation of fluorescence intensity. To show the sensitive and the resistant pattern of DX uptake LoVo and LoVoDx, cells were incubated for 60 min with 2 μg/mL DX. Subsequently, cells were rinsed with PBS twice, for 5 min each and mounted in DAPI medium. Cells were viewed using Zeiss Axio Imager Z1 microscope. The excitation and emission wavelengths for DX fluorescence were 546 and 590 nm, respectively. DAPI fluorescence was excited by a 365 nm wave and an emission wavelength of 420 nm (blue) was detected.

2.3. Isolation of nuclei from LoVo and LoVo/DX cells

Nuclei from LoVo and LoVo/Dx cells were isolated according to Sigma Nuclei EZ Prep Kit protocol (Sigma–Aldrich, USA). Briefly, cells from the flasks (75 cm^2) were harvested using trypsin solution, centrifugated at 200 × *g* for 5 min, next, we added 4 mL of ice-cold Nuclei EZ buffer to each cells, vortexed them briefly and set on ice for 5 min. Nuclei were collected by centrifugation at 500 × *g* for 5 min at 4 °C. Clear supernatant (cytoplasmic components) was aspirated to new tube, nuclei pellet was washed in 4 mL of ice-cold Nuclei EZ lysis buffer as follows. Clear nuclei pellet was mixed with Nuclei EZ lysis buffer and set on ice for 5 min, thend collected by centrifugation, next we added RIPA buffer to obtain the protein solution necessary for western blot analysis. Cytoplasmic components were collected by centrifugation at 13000 × *g* for 5 min at 4 °C, pellet was also treated with RIPA buffer as follows.

2.4. Determination of P-gp mRNA expression using qRT-PCR

Reverse transcription was carried out on approximately 1.0 μg of the total RNA following the manufacturer's protocol (First Strand cDNA Synthesis Kit, Fermentas). Template RNA, random hexamer primers (1 μL) and DEPC-treated water were mixed together to a total volume of 11 μL and pre-incubated at 65 °C for 5 min in the Biometra thermocycler. Then, the sample were chilled on ice, centrifugated to spin it down and moved to a fresh PCR tube in order to prevent the permeability of the cup. 5X Reaction buffer (4 μL), RiboLock™ RNase Inhibitor 20 U/μL (1 μL), 10 mM dNTP Mix (2 μL) and M-MuLV Reverse Transcriptase 20 U/μL (2 μL) were added to the pre-incubated solution, mixed by pipeting and incubated at 42 °C for 60 min (Biometra thermocycler). Finally, cDNA was stored in H_2O at −20 °C or immediately used for subsequent amplification reactions.

One microliter of a given cDNA or DNA was added to the reaction mixture, composed of 12.5 μL 2× Maxima® SYBR Green/ROX qPCR Maxter Mix (Fermentas), 1 μL specific pair of primer (f.c. 0.3 μM; F: 5′-TGACAGCTACAGCACGGAAG-3′; R: 5′-TCTTCACCTC-CAGGCTCAGT-3′) and 10.5 μL of H_2O. The reactions were driven in twin.tec real-time PCR plates with PCR Film (Eppendorf®) using Mastercycler® ep-realplex^2 (Eppendorf®). The PCR program was as follows:

- initial denaturation, 95 °C, 10 min;
- denaturation, 95 °C, 15 s;
- annealing 60 °C, 30 s;
- extension 72 °C, 30 s.

The number of cycles was 40–50. Melting curves were made and 2% agarose gel electrophoresis was used to verify the amplification product specificity and size, respectively. All the samples were amplified in duplicate or triplicate and in case the results varied more than 15%, the reactions were repeated.

Absolute quantification method was used to quantify the P-gp mRNA copy number as described previously [17]. Absolute quantification determines the exact copy concentration of a target gene by relating the C_t value to a standard curve. Prior to absolute quantification, the C_t values were normalized by comparison to the average of C_t's obtained from five housekeeping genes (*β-actin*, *B2 M, GAPDH, HPRT1, MRLP19*; for primer sequences see [18]). Primers for our studies on expression were purchased from the Laboratory of DNA Sequencing and Oligonucleotide Synthesis, Institute of Biochemistry and Biophysics, Polish Academy of Sciences, Warsaw.

2.5. Western blot analysis technique

Protein solutions from LoVo, LoVo/Dx and their cytoplasmic and nuclei fractions were obtained by using RIPA buffer. Cells/fractions were lysed using RIPA buffer containing protease inhibitor cocktail (Sigma–Aldrich) for 20 min at 4 °C and collected by centrifugation at 12000 × *g* for 15 min at 4 °C. Protein concentration was measured according to Bradford's Protein Assay method. For immunoblotting, 35 μg of each protein's fraction were used and resolved on 7% polyacrylamide gel, and then gel was electrotransferred onto a PVDF membrane. The membrane was blocked for 1 h at room temperature in Tris buffer containing 0.1% (v/v) Tween-20 (TBS-T) and 5% (m/v) non-fat dry milk. After washes with TBS-T, the membrane was incubated overnight at 4 °C with monoclonal P-gp antibody (Alexis) in 1% non-fat dry milk (dilution 1:500). Following washes, the membrane was incubated for 1 h at room temperature with anti-mouse horseradish peroxidase-conjugated secondary antibody (Santa Cruz Biotechnology), dilution 1:2000. Protein bands were detected by chemiluminescence.