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Mitochondrial localization of P-glycoprotein in the human breast cancer cell line MCF-7/ADM and its functional characterization

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Abstract. The current view of multidrug resisitance is that overexpression of membrane P-glycoprotein (P-gp) is a major causative factor. However, the controversial presence of subcellular P-gp may also participate in the drug resistance. In this study, we sought to investigate the localization and functional characterization of P-gp in mitochondria isolated from MCF-7 and doxorubicin-resistant MCF-7 (MCF-7/ADM) cells. Mitochondria were isolated and purified from the MCF-7 cell line and its resistant cells MCF-7/ADM. We used electron microscopy, western blot analysis and confocal microscopy to demonstrate the localization of P-gp in the mitochondria of MCF-7/ADM cells. Flow cytometry was used to evaluated the efflux function of mitochondrial P-gp in the presence or absence of the P-gp inhibitor cyclosporine A (CsA). Mitochondria were isolated and purified successfully and were analyzed by electron microscopy. Western blotting demonstrated the expression of P-gp in the cell membrane and purified mitochondria from MCF-7/ADM cells but not from sensitive MCF-7 cells. Immunofluorescence analysis using confocal microscopy demonstrated the localization of P-gp [labeled with green fluorescence (FITC)] to the mitochondria [labeled with red fluorescence (Mitotracker Deep Red 633)] of MCF-7/ADM cells and that was absent in MCF-7 cells. Rho123 (a mitochondrial fluorescent probe) accumulation was largely reduced and efflux was strongly increased in the mitochondria of MCF-7/ADM cells compared to those of MCF-7 cells ($P<0.01$), and these were completely reversed in the presence of the P-gp inhibitor CsA ($P<0.01$). No significant changes were observed in the mitochondria of MCF-7 cells ($P>0.05$). P-gp is expressed in the mitochondria of doxorubicin-resistant MCF-7 cells and has an efflux function. It could be involved in multidrug resistance at the subcellular site by pumping out anticancer drugs from mitochondria to protect the function of mitochondria.

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Introduction

Drug resistance to chemotherapeutic agents is a common problem leading to chemotherapy failure. The current view is that overexpression of a membrane associated efflux transporter called P-glycoprotein (P-gp; 170 kDa), a member of the ATP-binding cassette (ABC) superfamily of transporters, is the primary mechanism involved in multidrug resistance (MDR) formation (1). Previous studies of P-gp have mainly focused on the expression of plasma membrane P-gp. However, recent studies have shown that P-gp expression is observed at the intracellular membranes in tumor-resistant cells, including the nucleus, Golgi apparatus, and the some unconfirmed cytoplasmic vesicles such as lysosomes and inclusion bodies (2-4). Furthermore, intracellular levels of P-gp are roughly equivalent to that of the plasma membrane P-gp. Like the plasma membrane P-gp, the cytoplamic localization of P-gp may play an important role in tumor resistance (5).

Mitochondria are cellular power plants that generate most of the cell's chemical energy. Mitochondria also have a very close relationship with apoptosis and tumor drug resistance. Research has revealed that mitochondria might be the primary compartment for sequestration of antineoplastic drugs in MDR cells (5,6). However, studies are inconclusive as to whether or not the mitochondrial sequestration of drugs is associated with mitochondrial localization of P-gp and its functional properties (7-9).

The objective of this study was to investigate the localization and the expression of P-gp in mitochondria by immunofluorescence confocal microscopy and western blot analysis. Furthermore, this study aimed to verify whether mitochondrial P-gp was involved in drug sequestration by testing the accumulation of rhodamine 123 (Rho123), a mitochondrial fluorescent probe, via flow cytometry. The results have suggested a new avenue of research for exploring the mechanism of tumor resistance.

Materials and methods

Cell culture. The human breast cancer cell line MCF-7 (sensitive cells) and the Adriamycin (ADM; doxorubicin)-resistant

cell line MCF-7/ADM were both purchased from Wuhan University Type Culture Collection Center. MCF-7 cells were cultured in RPMI-1640 medium containing 10% fetal bovine serum, 100 U/ml penicillin and 100 μg/ml streptomycin. Long-term culture was performed in an incubator at 37˚C with 5% CO_2 and saturated humidity. MCF-7/ADM cells were continuously cultured in the above medium containing 1.0 μg/ml ADM to maintain the cell's drug resistance. Two weeks prior to the experiment, MCF-7/ADM cells were placed in non-ADM medium for culture.

Mitochondria isolation, purification, and identification. Mitochondria were extracted from a total of $2x10^8$ cells according to the method provided by Frossi *et al* (10). Supernatant extracts were taken for the determination of protein content employing the Bradford method. The mitochondrial fraction obtained was fixed with 1 ml 2.5% glutaraldehyde and then washed once with PBS. Osmium tetroxide (0.5%) was then used for fixation over 15 min; dehydration by acetone gradient was followed by resin embedding. Ultrathin sections were cut and placed for observation under an electron microscope (Tecnai G212; FEI, The Netherlands).

Western blot analysis of P-gp expression in mitochondria. Total cellular protein extraction and concentration determination were performed as previously described (7). A total of 80 μg of protein were separated by SDS-PAGE and transferred onto a cellulose nitrate membrane. After a 2 h of incubation with blocking reagent at room temperature, TBS buffer containing 0.05% Tween-20 (TBST) was used to rinse the membrane three times. The C219 murine anti-human P-gp monoclonal antibody (1:500; Abcam, Cambridge, UK) was added and incubated at 4˚C overnight. The horseradish peroxidase labeled secondary antibody, FITC-tag II (1:5000; Santa Cruz Biotechnology, Santa Cruz, CA, USA), was added according to the manufacturer's instructions. Finally, autoradiography was completed using an ECL system. β-actin levels were used as the control, and each sample was repeated at least three times.

In situ analysis of P-gp expression by confocal laser scanning microscopy. Two staining protocols were used to identify the intracellular localization of P-gp. Mitochondria were first stained with MitoTracker Deep Red 633 (Molecular Probes, Eugene, OR, USA), and P-gp was then labeled with an anti-Pgp antibody (ab3364; Abcam, Cambridge, UK) and a FITC-labeled secondary antibody (Santa Cruz Biotechnology). The MCF-7 and MCF-7/ADM cells were cultured and prepared on slides. MitoTracker Deep Red 633 in fresh DMEM was added, and the cells were further incubated for 30 min. After washing, the cells were fixed with 3.7% formaldehyde. Following washing and blocking for 10 min, cells were incubated with the P-gp antibody (1:20) and then FITC-labeled rabbit anti-mouse IgG (1:200) at room temperature for 60 min. The prepared cells were then observed using a laser scanning microscope (Fluoview 500; Olympus, Tokyo, Japan) with untreated cells as the control. FITC and MitoTracker Deep Red were stimulated using a blue and green laser emitting at 525 nm and 570 nm laser lines, respectively. Image processing was performed with computer software LSCM 510.

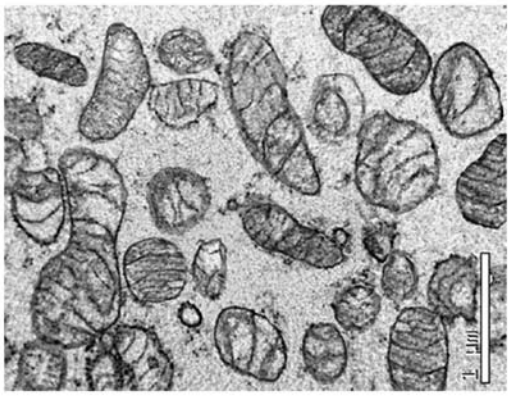

Figure 1. Isolated mitochondria. Image of the integrated isolated mitochondria from doxorubicin-resistant MCF-7 cells under transmission electron microscope.

Function of mitochondrial P-gp by flow cytometry. The prepared MCF-7 and MCF-7/ADM mitochondria were resuspended in buffer A solution (pH 7.5, containing 1.5 nmol/l $MgCl_2$, 10 mmol/l KCl, 0.1 mmol/l PMSF) and Rho123 fluorescence intensity was detected. The mitochondrial sample from each cell line was divided into two groups: Rho123 intake assay and Rho123 efflux assay groups. Each group was subsequently divided into control and intervention groups. In the intervention groups, P-gp inhibitor CsA (5 μM) was added at room temperature for a pre-incubation of 30 min; the control group received an equal volume of buffer A. A mitochondrial sample from each tube was added to Rho123 solution (5 μg/ml) for 4 min at room temperature in the dark (1). The Rho123 intake assay: after addition of Rho123 and a 4-min incubation, the samples were resuspended in 500 μl buffer A. Flow cytometry (LSRII flow cytometer; BD Biosciences, San Diego, CA, USA) was used to detect Rho123 fluorescence intensity in the mitochondria (2). The Rho123 efflux assay: after addition of Rho123 and a 4-min incubation, the mitochondria were centrifuged at 450 x g at 4˚C for 5 min. After precipitation, the pellet was resuspended in 500 μl buffer A and incubated at room temperature for another 6 min, allowing Rho123 efflux from the mitochondria. Subsequently, mitochondria were washed twice with 2 ml buffer A and resuspended in 500 μl buffer A. Subsequently, Rho123 fluorescence intensity in the mitochondria was determined. Each experiment was repeated at least three times. The measured value of the mean fluorescence intensity was determined relative to the intensity of Rho123 in the mitochondria of MCF-7 cells (100%).

Statistical analysis. The experimental data for Rho123 fluorescence intensity are presented as the mean ± standard deviation (mean ± SD). An independent-samples t-test was performed using the statistical software SPSS13.0.

Results

Identification of mitochondrial extracts. Mitochondrial extracts were obtained by differential and sucrose density-gradient centrifugation. Electron microscopy analysis

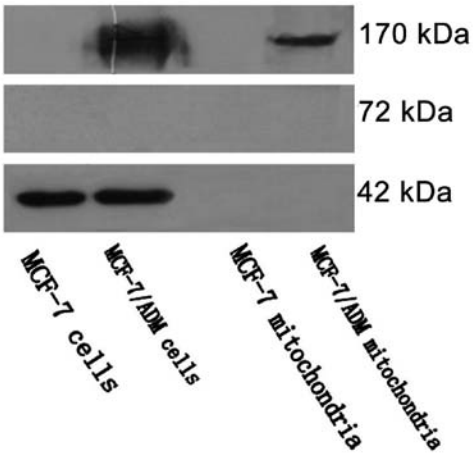

Figure 2. Western blot analysis. The expression of P-gp in the total cells and isolated mitochondria from cell lines MCF-7 and MCF-7/ADM.

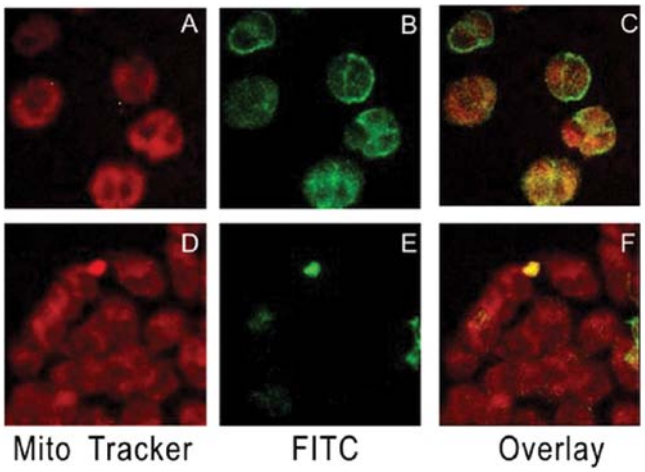

Figure 3. *In situ* ananlysis of P-gp expression on mitochondria isolated from the MCF-7 and MCF-7/ADM cell lines by laser scanning confocal microscope. (A) In MCF-7/ADM cell lines, mitonchondria marked with MitoTracker Deep Red, showing granular red fluorescence were found in the cytoplasm around the nucleus (original magnification, x400). (B) The localization of P-gp was demonstrated by green fluorescence. P-gp expression is strongly positive in MCF-7/ADM, shown as a green ring and punctate fluorescence, mainly distributed in the cell membrane (original magnification, x400). (C) After dual-channel excitation, red fluorescence showed the localization of mitochondria and green fluorescence showed the expression of P-gp are overlaid to form fluorescent orange, with granular distribution in the cytoplasm (original magnification, x400). (D) In MCF-7 cell lines, mitonchondria marked with MitoTracker Deep Red, showing granular red fluorescence were found in the cytoplasm around the nucleus (original magnification, x400). (E) In MCF-7 cell lines, there is no expression of P-gp with negative green fluorescence (original magnification, x400). (F) After dual-channel excitation, there is only red fluorescence showing the expression of mitochondria without the colocalization of P-gp (original magnification, x400).

indicated that the major components of the sediment were extracted, mitochondria, without Golgi, lysosomes, or other cellular organelle contamination. The mitochondria had a round, oval, or spindle shape and the majority had a complete mode structure and maintained a complete ridge structure. A small number of mitochondria were slightly swollen with a sparse ridge (Fig. 1).

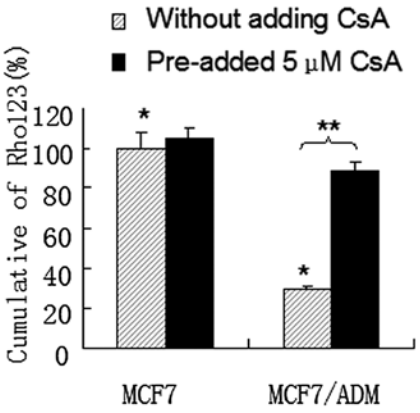

Figure 4. Two cell lines (MCF-7 and MCF-7/ADM) were compared on the uptake of Rho123 of mitochondria before and after CsA addition. In the MCF-7 cell line the mean fluorescence intensity of the cumulative Rho123 has no significant change following addition of CsA (100 vs. 105%, P>0.05). In the MCF-7/ADM cell line the mean fluorescence intensity of the cumulative Rho123 was significantly lower than that of MCF-7 group without adding CsA (29 vs. 100%, P<0.01). It was also significantly lower than the same cell line with addition of CsA (29 vs. 89%, P<0.01). *Indicates MCF-7/ADM compared to MCF-7 as the group without adding CsA, P<0.01; **refers to that group with pre-added 5 μM CsA of MCF-7/ADM compared with the group without adding CsA of MCF-7/ADM, P<0.01.

Western blot analysis of P-gp protein expression in tumor cells and mitochondria fractions. Protein was extracted from MCF-7 and MCF-7/ADM cells and their mitochondria, followed by western blot analysis using an anti-P-gp monoclonal antibody, C219. The results showed that there were specific bands at molecular weight 170 kDa in the MCF-7/ADM cells and their extracted mitochondria; no band was present between 65-94 kDa. MCF-7 cells and their extracted mitochondria did not display any P-gp protein expression.

In situ analysis of P-gp localization via confocal laser scanning microscopy. As seen in Fig. 3A-C, MCF-7/ADM cells displayed the red fluorescence of MitoTracker Deep Red 633, with granules mainly localized around the nucleus; green fluorescence indicating P-gp expression was observed in a ring shape, mainly in the cell membrane, and a small number of points distributed in the cytoplasm. Dual-channel excitation of both fluorophores was used to observe P-gp and mitochondrial colocalization, as indicated by fluorescent orange. In contrast, almost no expression of P-gp was found in the mitochondria of MCF-7 cells (Fig. 3D-F).

Flow cytometry to detect the function of mitochondrial P-gp. Flow cytometry was used to examine the intensity of Rho123 in the cellular mitochondria, for comparing Rho123 accumulation and efflux from the mitochondria before and after CsA addition, thus reflecting P-gp pump function. Fig. 4 shows Rho123 accumulation in mitochondria of MCF-7 and MCF-7/ADM after a 4-min Rho123 incubation. In the group without addition of CsA, Rho123 accumulation in mitochondria of MCF-7/ADM mitochondria was significantly lower than that of MCF-7, with an average fluorescence intensity of 29% and 100%, respectively (P=0.003). However, after a 30-min pre-incubation with 5 μM CsA (intervention group), Rho123 accumulation in MCF-7/ADM mitochondria demonstrated

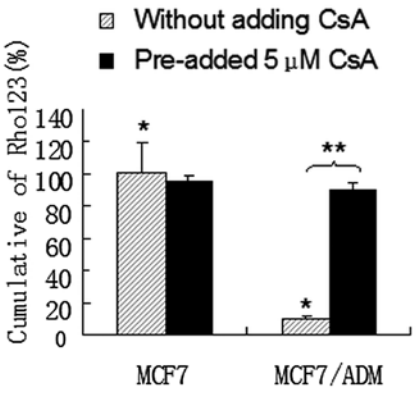

Figure 5. After incubated with the non-Rho123, two cell lines (MCF-7 and MCF-7/ADM) were compared on the efflux of Rho123 of mitochondria before and after CsA addition. In the MCF-7 cell line the mean fluorescence intensity of the retended Rho123 has no significant change following addition of CsA (100 vs. 95%, P>0.05). In the MCF-7/ADM cell line the mean fluorescence intensity of the retended Rho123 was significantly lower than that of MCF-7 group without adding CsA (10 vs. 100%). Compared with the MCF7/ADM with addition of CsA, the mean fluorescence intensity of the retented Rho123 of mitochondria of MCF7/ADM without adding CsA was significantly lower (10 vs. 90%). *Indicates MCF-7/ADM compared with MCF-7 as the group without adding CsA, P<0.01; **refers to that group of MCF-7/ADM with addition of CsA compared with the group of MCF-7/ADM without adding CsA, P<0.01.

significant recovery (89%) compared to the control cells (29%; P=0.000). However, no significant change was observed following addition of CsA in the MCF-7 mitochondria (105%) compared to the control cells (100%; P>0.05).

In the MCF-7/ADM mitochondria, Rho123 rapidly accumulated. Efflux analysis is shown in Fig. 5. In the group without addition of CsA, after a 6-min incubation in buffer A (without Rho123) there was a significantly lower retention of Rho123 in MCF-7/ADM mitochondria (10%) compared with the MCF-7 mitochondria (100%; P=0.000), indicating an increased efflux in MCF-7/ADM mitochondria. However, after a 30-min pre-incubation with 5 μM CsA, Rho123 efflux from MCF-7/ADM mitochondria was completely inhibited (90%; P=0.001). In contrast, no significant change in Rho123 retention was observed following addition of CsA in the MCF-7 mitochondria (95%) compared to the cells without adding CsA (100%; P>0.05).

Discusion

P-gp is an ATP-dependent phospholipid transmembrane protein encoded by the MDR1 gene. It is composed of 1280 amino acids and has a molecular weight of 170 kDa. The traditional view is that P-gp is located at the plasma membrane and functions to transport drugs across the lipid bilayer; P-gp molecules can be combined with drugs, and after ATP hydrolysis, the release of energy can transfer drugs to the outside of cells. As such, the drug concentration within cells is always maintained at a relatively low level, resulting in cells being drug resistant. Overexpression of P-gp is the most important molecular mechanism underlying MDR (11). In-depth study of drug resistance mechanisms found that P-gp is not only distributed at the cell membrane surface, but that it is also expressed at sub-cellular membranes of resistant cells, such as the nucleus, Golgi body, lysosomes, and small vesicles (4,12,13). In a study exploring high expression of P-gp in drug resistant leukemia cells (K562), Bennis *et al* (7) found that the addition of P-gp inhibitors did not fully restore the sensitivity of tumor cells to Adriamycin. Furthermore, the drug was observed to demonstrate a redistribution within the cells. This discovery prompted the idea that changes in intracellular drug distribution may be another mechanism underlying cell resistance. Research by Calcabrini *et al* (14) proposed that P-gp localization at the nuclear envelope prevented the drug from directly gaining access to the nucleus. In studies of Adriamycin distribution in leukemia cells (U937), Hurwitz *et al* (12) confirmed the lysosome as the primary point of localization preventing chemotherapy drugs from accessing their target, the nucleus, and, thus, mediating resistance. But there are also studies pointed out that intracellular organs such as Golgi and endoplasmic reticulum play the separation and retention roles (13). Retention in these organs prevented the drug from getting into the nucleus, resulting in resistance. The addition of the P-gp inhibitor verapamil can restore the diffuse distribution of drugs in drug-resistant cells, indicating that P-gp plays a fundamental role in this process (12,13). Different from the Golgi apparatus, lysosomes, and other organelles, the mitochondria have independent transcriptional machinery and act as the main production center for ATP. In the course of cell growth and metabolism, this organelle can play a decisive role. Nevertheless, the functional expression of P-gp in mitochondria membranes is still controversial.

Purification of mitochondria is difficult due to the variety of sub-cellular organelles and their close contact within the cytoplasm. As such, the issue of cross-contamination is often a problem, and this has become a stumbling block for the isolation of mitochondria and their membrane-targeted P-gp (10). We used differential centrifugation in combination with sucrose density gradient centrifugation with increasing centrifugal speeds to obtain purified mitochondria. This method successfully solved the problem of low recovery rate. Results from our electron microscopy analysis confirmed that the sediments mainly consisted of mitochondria and lacked contamination from other organelles such as the Golgi, lysosomes, etc. Furthermore, the mitochondria retained their basic integrity and structure. Western blot analysis of mitochondrial protein extracts from MCF-7/ADM cells indicated a specific band at 170 kDa. It was kown that the mitochondrial ABC transporter of dimer, which is regarded as 'half-transporter' was the homologue of the P-gp with half of molecular weight of P-gp (65-94 kDa) (15), and it was not detected in our experiment. This further confirmed the successful extraction of mitochondrial proteins. In the isolated mitochondria derived from the MCF-7 cells, P-gp protein expression was not detected, and this was consistent with our *in situ* results utilizing double immunofluorescent staining.

Using a laser scanning confocal microscope, P-gp expression was strongly localized to the plasma membrane of drug-resistant cells. At the same time, the use of dual fluorescent detection provided a more accurate observation of the P-gp distribution within the cells. In the drug-sensitive cell line, MCF-7, the green fluorescence indicating P-gp expression was not visible and orange fluorescence indicating the colocalization of P-gp and mitochondria was also

not observed. In contrast, the drug-resistant MCF-7/ADM cells displayed a strong green fluorescence, indicating P-gp expression, as well as clear orange fluorescence indicating the colocalization of P-gp and mitochondria. This is consistent with the observations of Munteanu *et al* (5) in the drug-resistant cell line K562/ADR and with the findings of Solazzo *et al* (16) in the drug-resistant cell line HCC. Furthermore, the confocal laser scanning results also showed that in MCF-7/ADM cells some green fluorescence did not localize to the mitochondria, indicating P-gp localization at other sub-cellular locations, such as the Golgi apparatus, lysosomes, and inclusion bodies. This is partially consistent with previous research findings (12,13,17).

However, the role in tumor resistance which cytoplamic especially mitochondrial localization of P-gp may play remains unknown. Some researchers believe that some intracellular mechanisms such as cell-mediated changes in pH value of the 'ion trapping' or changes in lipid membrane composition may contribute to drug sequestration (18). However, Shapiro *et al* (6) and Ferrao *et al* (19) have proposed a novel hypothesis, suggesting that P-gp expression at sub-cellular membranes may serve as the mechanism by which the drugs find their way into the cellular compartment. Gong *et al* (20) added the P-gp inhibitor verapamil to the K562/ADR cell line and observed the distribution of danuorubicin within the cells. The results showed that verapamil could restore the diffuse distribution of DNA in drug-resistant cells, and they suggested that this process might be mediated by P-gp. As such, researchers (5) have pointed out that mitochondrial P-gp may function to pump drugs into the mitochondria, excluding them from the nucleus, thus promoting the formation of resistance. However, once anti-cancer drugs enter the mitochondria, they are likely to produce oxidative damage to mitochondrial components. Cells have a very low ability to repair oxidative damage, and oxygen free radicals can induce lipid peroxidation, cellular energy failure, DNA mutation imbalance, and mitochondrial membrane dysfunction. Drugs may also induce mitochondrial release of pro-apoptotic factors, causing irreversible cell apoptosis. This is in contradiction to the current views concerning the role of mitochondrial P-gp in drug resistance. The transport of drug indicated by the P-gp at the mitochondrial membrane remains still controversial.

The results of this study indicated that the role of P-gp at the mitochondrial membrane was not to transport drugs into the mitochondria, but to promote efflux of the drugs out of the mitochondria as shown in Figs. 4 and 5. We selected Rho123 and CsA as sensitive mitochondrial marker and P-gp inhibitor in order to test the efflux of the drugs and the change of the efflux of the drug under the influence of the P-gp inhibitor CsA. Rho123 is a cationic fluorescent dye used as a mitochondria marker. Research has shown that cumulative differences in Rho123 in sensitive and resistant cells are mediated by P-gp, and Rho123 has been widely used as an indicator of P-gp function (21). CsA is an immunosuppressive agent that can inhibit the function of P-gp (8). After addition of P-gp inhibitors, such as CsA, and verapamil, it is possible to observe the changes in accumulation of Rho123 in the mitochondria so as to evaluate P-gp function. We detected the fluorescence intensity of Rho123 in MCF-7 and MCF-7/ADM mitochondria and compared the changes in Rho123 accumulation and efflux before and after addition of CsA. Without the addition of CsA, Rho123 accumulation in MCF-7/ADM mitochondria was significantly lower than that of MCF-7 due to the localization of P-gp at the membrane of mitochondria in MCF-7/ADM and its pump function. After pre-incubation with CsA, the function of P-gp was inhibited, a marked increase in the accumulation of Rho123 was observed in MCF-7/ADM mitochondria; however, no significant change was found in MCF-7 mitochondria following the addition of CsA. Such differences may result from P-gp-mediated Rho123 efflux which is a active transport process against concentration gradient. To confirm that the lower accumulation of Rho123 in MCF-7/ADM mitochondria was derived from the outward transport of P-gp, the mitochondria were placed in solution without Rho123, followed by detection of fluorescence intensity of the remaining Rho123 in the mitochondria. Without addition of CsA, the remaining Rho123 in the MCF-7/ADM mitochondria was significantly lower than that in the MCF-7 mitochondria. Following pre-incubation with CsA, this Rho123 export was inhibited in the MCF-7/ADM mitochondria; however, no significant changes were observed in the MCF-7 mitochondria. Therefore, it confirmed that in the mitochondria of drug resistant cells the reduced accumulation of Rho123 was in fact derived from P-gp-mediated export. Based on the results of this study, we made the following conclusions: P-gp is expressed at the mitochondrial membrane in MDR cells, and has transport activity. Furthermore, P-gp can function to export drugs out of the mitochondria. In combination with the plasma membrane P-gp, mitochondrial P-gp can eventually transport anticancer drugs out of cell, thus mediating drug resistance. The presence of P-gp inhibitors can inhibit the transport function of P-gp at the plasma and mitochondrial membranes, allowing anticancer drugs to enter the mitochondria, resulting in mitochondrial DNA damage, increased apoptosis, and reversal of drug resistance.

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