Suppression of P-glycoprotein gene expression in Hs578T/Dox by the overexpression of caveolin-1

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Abstract Caveolin-1, the principal component of caveolae, is a 21–24 kDa integral membrane protein. The interaction of the caveolin-1 scaffolding domain with signaling molecules can functionally inhibit the activity of these signaling proteins. Little is known about how caveolin-1 influences the expression of P-glycoprotein (P-gp), an ABC transporter encoded by multi-drug resistance (MDR1) gene. To elucidate the possible mechanism between caveolin-1 and P-gp expression, in the present study, we overexpressed caveolin-1 in the Hs578T/Dox breast adenocarcinoma cells, a multidrug resistant line, and then selected single clone cells highly expressing caveolin-1 level. Both Western blot and confocal microscopy analyses showed that caveolin-1 was markedly overexpressed in the transfectants, while P-gp protein was almost abolished. Reverse transcription polymerase chain reaction also showed that the expression of P-gp mRNA was significantly suppressed in the transfectants. It was confirmed further by Northern blot analysis. Moreover, through measuring the changes of drug resistance and P-gp transport activity in the transfectants, we found that overexpression of caveolin-1 reversed drug resistance of transfectants and lowered their P-gp transport activity to the level of Hs578T/S. Taken together, our results indicate that such suppression of P-gp in the transfectants overexpressing caveolin-1 may occur at the transcriptional level.

Keywords: P-glycoprotein; Caveolin-1; Drug resistance; Doxorubicin

1. Introduction

Multi-drug resistance (MDR) is a major pitfall of chemotherapy in human cancer patients. MDR is often caused by overexpression of P-glycoprotein (P-gp) encoded by the MDR1 gene. P-gp, an ATP-binding cassette (ABC) transporter, is responsible for MDR phenotype and is believed to result in increased outward transport of active drugs and reduced the effective concentration of them in the cytosol and their cytotoxic efficacy [1–4].

Caveolae are invaginations of the plasma membrane that are enriched in cholesterol and sphingolipids. Caveolin-1, a 21-kDa integral membrane protein, is a major caveolae coat protein [5]. There is a growing body of evidence that a number of proteins, such as H-ras, protein kinase C_{α}, G protein, eNOS, src family tyrosine kinases and EGFR can directly interact with a specific scaffolding domain of caveolin-1, which consequently result in functional inhibitions of the activity of these molecules, suggesting that caveolin-1 binding plays a negative regulatory role in signal transduction [6–10]. Altering caveolin-1 expression is thought to be involved in several important human diseases, including cancer, diabetes, Alzheimer's disease, cardiovascular disease and muscular dystrophy [11–15].

Reports about the effect of caveolins on the development of MDR are controversial. On the one hand, caveolin-1 expression was upregulated in MDR phenotypes in a number of human cell lines [4]. On the other hand, expression of caveolin-1 and caveolin-2 was not detected in several MDR cell lines that express high levels of P-gp [16,17], i.e., caveolin-1 expression is not associated with that of P-gp protein or MDR1 gene [18]. However, a physical interaction between caveolin-1 and P-gp in the caveolae membrane was observed in the Hs578T cell and brain capillaries [19,20]. Caveolin-1 might render the cells from drug-resistance to drug-sensitive by inhibiting the P-gp transport activity [19]. To further probe the molecular mechanism on correlation between caveolin-1 and P-gp, in the present study, we overexpressed caveolin-1 in the Hs578T/Dox breast adenocarcinoma cells, a multidrug resistant line, and then selected single clone cells highly expressing caveolin-1 level. We examined the changes of P-gp and MDR1 gene in these single clones and compared the drug resistance and P-gp transport activity in these cells. These results show that the single clones overexpressing caveolin-1 decrease the drug-resistance by suppression of the MDR1 gene expression.

2. Materials and methods

2.1. Materials

Monoclonal antibody (mAb) against caveolin-1 was purchased from Zymed Laboratories Inc. Anti-human P-gp mAb was obtained from Signet Laboratories. FITC-labeled goat anti-mouse IgG and Texas Red-labeled goat anti-mouse IgG were purchased from Molecular Probe Inc. Cell culture supplies (RPMI 1640 medium, geneticin, fetal bovine serum, L-glutamine, Trypsin-EDTA, LipofectAMIME 2000, and Penicillin/streptomycin) and SuperScript One-Step reverse transcription polymerase chain reaction (RT-PCR) with Platinum® Taq

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Abbreviations: Cav-1, caveolin-1; P-gp, P-glycoprotein; MDR, multi-drug resistance; Rh-123, rhodamine-123; Dox, doxorubicin; DMSO, dimethyl sulfoxide

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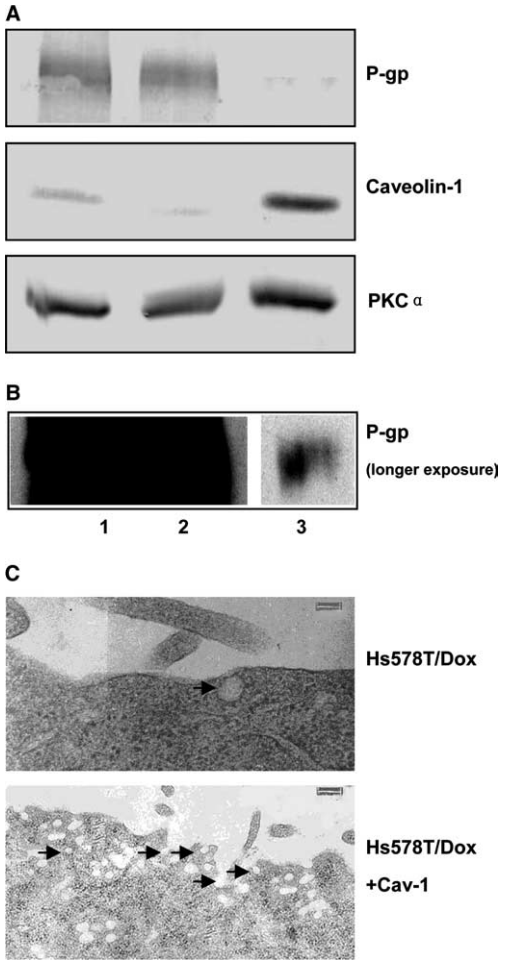

Fig. 1. Caveolin-1, P-gp and PKC$_\alpha$ expression in various Hs578T cells. Western blot analysis of Hs578T/Dox+vector (lane 1), Hs578T/Dox (lane 2) and Hs578T/Dox+cav-1 (lane 3). The detection was performed with the BCIP/NBT reagents (A), and ECL with longer exposure for P-gp (B). Representative electron micrographs (C), showing a large increase in the number of caveolae organelles in cells overexpressing caveolin-1 (bottom panel). Bar = 100 nm.

and TRIzol® Reagent were all from Life Technologies Inc. BCIP/NBT reagents were purchased from Promega. DIG-11-dUTP, DIG Easy Hyb Granules, DIG Wash and Block Buffer Set, Anti-Digoxigenin-AP, Fab fragments and CDP-Star were obtained from Roche. Verapamil, cyclosporine A and rhodamine-123 (Rh-123) were purchased from Sigma. The pCI-neo, pCI-neo-cav-1 plasmide and Hs5780T cell lines were generous gifts of Dr. Eric J. Smart (Kentucky Medical School, USA) [21] and Dr. Kjell Grankvist (Umea University, Sweden) [22], respectively. All other reagents were obtained from regular commercial sources with highest purity.

2.2. Cell lines, plasmids and transfections

The parental human breast cancer cell line Hs578T/S was cultured at 37 °C, 5% CO_2 in RPMI 1640 medium supplemented with 10% fetal bovine serum, 2 mM glutamine, 100 units/ml penicillin, and 100 μg/ml streptomycin. Its doxorubicin (Dox)-resistant subclone Hs578T/Dox was propagated in a culture medium containing 1 μg/ml Dox. The entire cDNA sequence of caveolin-1 was cloned into the *Eco*RI site of pCI-neo, to generate pCI-neo-cav-1. pCI-neo-cav-1 and its empty vector were transfected into Hs578T cell lines as described [21]. After ensuring the Cav-1 expression by Western blot, we counted cells, diluted them at 1 cell/200 μl and plated 100 μl per well in a 96-well plate. Then, we picked up the well which contained only monoclonal population for propagation. Several Hs578T/Dox clones transfected with either the vector alone or vector containing the Cav-1 cDNA were established and analyzed for Cav-1 expression by Western blot. Three clones, Hs578T/Dox (wild-type), Hs578T/Dox+vector and Hs578T/Dox+cav-1 were chosen for further work.

2.3. Western blot

Proteins were separated by SDS–PAGE with a standard reducing condition protocol and transferred to a nitrocellulose membrane. Immunoblotting was performed with designated antibodies and detection was performed with the BCIP/NBT reagents according to the manufacturer's instructions. Or horseradish peroxidase-conjugated secondary antibodies (1:5000 dilutions, Pierce) were used to visualize bound primary antibodies with the Supersignal chemiluminescence substrate (ECL method).

2.4. Cell survival MTT assay

The 3-(4,5-dimethylthiazol-2-yl)-2,5-di-phenyltetrazolium bromide colorimetric assay (MTT) was used to assess the sensitivity of the cells to agents in vitro as described [23]. The survival percent was calculated by the following equation: cell survival percent = (mean OD of one grade)/(mean OD of the blank control) × 100%. The IC_{50} for Dox and cisplatin were defined as such a Dox and cisplatin concentration in which the survival rate was 50%.

2.5. Efflux of rhodamine-123

To examine the transport activity of P-gp, efflux of Rh-123 was measured [24,25]. Briefly, the cells were harvested, then incubated with 2 μM of Rh-123 for 30 min at 37 °C. After three times of washing to remove the extracellular free dye, the cells were kept in dye-free media or media containing 10 μM verapamil or 5 μM cyclosporine A at 37 °C. The fluorescence of remaining Rh-123 in the cells was monitored by Becton–Dikinson FACS-420 flow cytometer.

2.6. Confocal microscopy imaging

Confocal analysis was performed as described [7]. Immunostaining was performed by incubation for 1 h in 1:200 diluted FITC-labeled goat anti-mouse IgG or Texas Red-labeled goat anti-rabbit IgG. After extensive PBS washes, immunostained cells were examined with a Bio-Rad Radiant-2100 confocal microscope. More than 100 cells were inspected per experiment and the results displayed are typical.

2.7. Reverse transcription-polymerase chain reaction

RNA from Hs578T cells was isolated with TRIzol Reagent®. To determine if MDR1 and caveolin-1 gene were expressed in Hs578T cells, RT-PCR was performed using One-Step RT-PCR with Platinum® by using specific human MDR1 primers [26] and caveolin-1 primers [27], respectively. 8 μl of each PCR product was electrophoresed in 1× Tris/acetate/EDTA (TAE) electrophoresis buffer on a 1.2% agarose gel, stained with ethidium bromide and detected by UV. To ensure that RNA load was kept constant in all lanes, β-actin gene was also amplified in the same condition, using following pair of primers: 5′-GAT TAC TGC TCT GGC TCC TA-3′ and 5′-CAG TAA CAG TCC GCC TAG AA-3′.

2.8. Northern blot

10 μg of total RNA was separated on a 1% agarose/2.2 M formaldehyde gel and transferred to a Hybond-N membrane by vacuum blotting. The nylon membrane was subjected to hybridization with a digoxigenin (DIG)-labeled partial PCR products of β-actin, caveolin-1 and P-gp gene. Probe labeling, hybridization, washing and signal detection were carried out according to manufacturer's instructions.

3. Results

3.1. Caveolin-1 and P-gp and PKC$_\alpha$ content in Hs578T/Dox

Dox-resistant cells were transfected with pCI+neo-vector or pCI+neo-caveolin-1. The single clone cells were selected and proliferated. We have obtained three stable single clone cells: Hs578T/Dox as wild-type, Hs578T/Dox+vector as vector control cells, and Hs578T/Dox+cav-1 as transfectant cells overexpressing caveolin-1 and parental cells Hs578T/S as drug sensitive lines. Fig. 1 showed a result of detection by BCIP/

NBT reagents, the expression levels of caveolin-1 in the transfectant cells were about 6-fold higher than wild-type and vector control cells as detected by densitometry (Fig. 1A, middle panels). Although P-gp was observed clearly in wild-type and vector control cells, P-gp protein was almost abolished in the transfectant cells (Fig. 1A, top panels). We used higher sensitive ECL to detect P-gp in the transfectants. Longer exposure was shown to better illustrate the P-gp band (Fig. 1B). We also measured PKC_{α} content in these cells, as a positive control, and not found that a significant change in PKC_{α} in the wild-type, vector control cells and in the transfectant cells (Fig. 1A, bottom panels). We studied the impact of overexpressing caveolin-1 on the caveolae organelles using electron microscopy. As shown in Fig. 1C, the number of caveolae increased greatly in transfectant cells. These caveolae appeared as attached omega-shaped flasks and bunches like clusters of grapes.

3.2. Localization of caveolin-1 and P-gp in Hs578T/Dox

To identify the results as described above about the expression of caveolin-1 and P-gp in the transfectants, we applied confocal microscopy to locate caveolin-1 and P-gp in which various stable single clone cells were stained with FITC-labeled goat anti-mouse IgG (Fig. 2A) or Taxes Red-labeled goat anti-rabbit IgG (Fig. 2B) for 1 h. We observed localization of P-gp (Fig. 2A, a, green color) and caveolin-1 (Fig. 2B, b, red color) in vector control cells (Fig. 2, top panel). Both chromophores superimposed in the same image, showing yellow color (Fig. 2C, c). In contrast, the transfectants (middle panel) and Hs578/S (bottom panel), a drug sensitive cells subjected to the same confocal analysis did not show positive staining with green color (Fig. 2A, d and g), although caveolin-1 with red color could be observed in both of the cell lines (Fig. 2B, e and h). The confocal result is consistent with that of Western blot.

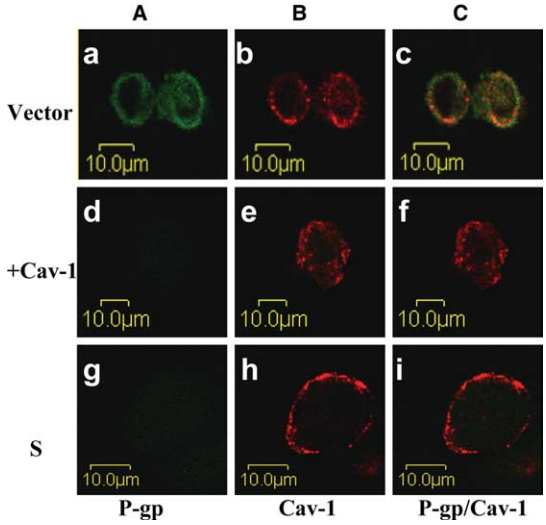

Fig. 2. Confocal analysis of P-gp and caveolin-1 localization in various Hs578Tcells. Immunofluorescence staining of Hs578T/Dox+vector (top panels), Hs578T/Dox+cav-1 (middle panels) and Hs578T/S (bottom panels) cells with anti-P-gp antibody (A, green color) and anti-caveolin-1 antibody (B, red color) is shown. The both staining were superimposed to indicate the colocalization of P-gp and caveolin-1 (C, yellow color). Bar = 10 μm.

3.3. Expression of caveolin-1 and P-gp mRNA in the transfectants

In order to elucidate the expression mechanism of P-gp in the transfectant cells, the mRNA of P-gp was determined by means of RT-PCR. To ensure equal loading, the expression of β-actin mRNA was used as control. As shown in Fig. 3A, P-gp mRNA was not observed in the transfectants and in the drug sensitive cells (Fig. 3A, lanes 7 and 8), although it was found in wild-type and vector control cells (Fig. 3A, lanes 5 and 6). However, the expression of caveolin-1 mRNA can be observed clearly in the four cell lines (Fig. 3A, lanes 1–4). The suppression of P-gp mRNA expression in caveolin-1 transfectant cells was supported further by Northern blot analysis. As shown in Fig. 3B, P-gp mRNA expression was only detected in the wild-type and vector control, however, it was almost abolished in the transfectants and drug sensitive cells (Fig. 3B, top panel). Similarly, caveolin-1 can be detected in the four cell lines (Fig. 3B, middle panel). The disappearance of P-gp mRNA suggests that the endogenous transcription of P-gp gene may be suppressed by transfected caveolin-1 gene.

3.4. Multidrug resistance in Hs578T/Dox

A line of evidence showed that many cases of MDR are due to overexpression of P-gp. It is very likely that the drug resistance is reduced once P-gp disappears in the transfectants. To test this possibility, we selected a specific substrate of P-gp, Dox, and a non-specific substrate, cisplatin, to study the changes of drug resistance in the transfectants. As shown in Fig. 4, the vector control (Fig. 4A) appeared to be much more drug resistant, while caveolin-1 transfectants (Fig. 4B) could

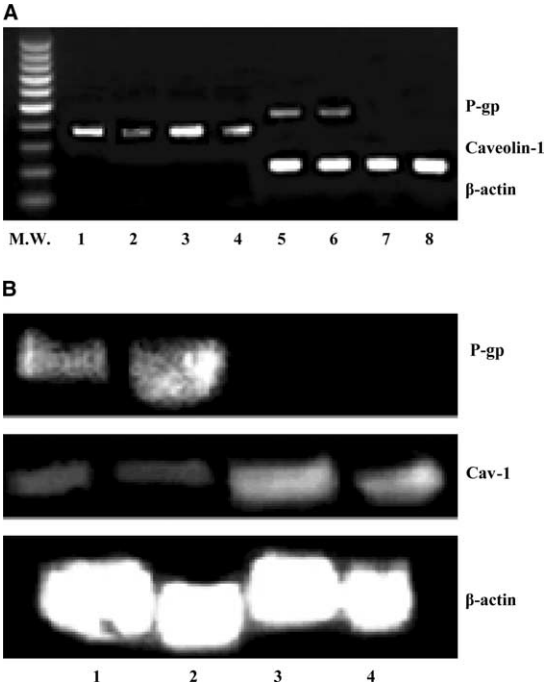

Fig. 3. Analysis of caveolin-1 mRNA and MDR1 mRNA expression in various Hs578T cells. (A) RT-PCR, Hs578T/Dox cells (lanes 1 and 5), Hs578T/Dox+vector cells (lanes 2 and 6), Hs578T/Dox+cav-1 cells (lanes 3 and 7) and Hs578T/S cells (lanes 4 and 8). β-actin was used as the internal control. (B) Northern blot, Hs578T/Dox+vector cells (lane 1), Hs578T/Dox cells (lane 2), Hs578T/Dox+cav-1 cells (lane 3) and Hs578T/S cells (lane 4).

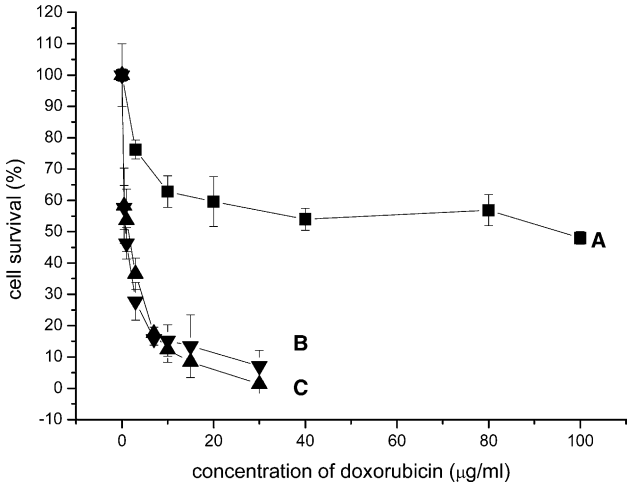

Fig. 4. Multi-drug resistance in various Hs578T cells. Dox was used to study the changes of drug resistance in Hs578T/Dox+vector cells (A), Hs578T/Dox+cav-1 (B) and Hs578T/S cells (C). The data are the mean values (standard error) of three independent experiments.

be observed more sensitive to the drug and close upon drug sensitive line cells (Fig. 4C). The IC_{50} values for Dox in the transfectants and drug sensitive cells were about 1.3 and 0.3 μg/ml, respectively, while the IC_{50} in the vector controls was 95 μg/ml. This equates to a decrease in drug resistance in terms of the IC_{50} values of 98% in the transfectants expressing caveolin-1. The IC_{50} for cisplatin in the transfectants, drug sensitive cells and the vector controls were 8.3, 6.9 and 19.8 μg/ml, respectively. These data demonstrate that overexpression of caveolin-1 cells markedly reduces the multidrug resistance in Hs578T/Dox cells.

3.5. P-gp transport activity in Hs578T/Dox

The reduced drug resistance in the transfectants is likely caused by the suppression of P-gp gene associated with caveolin-1 overexpression. We measured P-gp mediated transport activity by decreasing the intracellular fluorescence of Rh-123, a P-gp substrate [25,28], via flow cytometry. The higher the fluorescence intensity of remaining Rh-123 in the cells, the lower P-gp transport activity. As shown in Fig. 5, the Rh-123 fluorescence intensities were different in the three cell lines: Hs578/S > the caveolin-1 transfectants ≫ Hs578T/Dox+vector, i.e., 669 ± 40, 279 ± 25 and 37 ± 10, respectively (Fig. 5A). This result indicates that P-gp transport activity is lower in caveolin-1 transfectant cells than in vector control cells, consistent with the relative expression levels of P-gp. To confirm the specificity of the Rh-123 efflux from these cells, we employed two P-gp blockers, verapamil and cyclosporin A. Both the inhibitors were able to significantly enhance Rh-123 accumulation (decrease P-gp transport activity) in vector control cells. The fluorescence intensity increased 21- and 51-folds for the cells by using 10 μM verapamil and 5 μM cyclosporine A, respectively (Fig. 5B). However, the fluorescence intensity increased only about 4- and 5-folds for transfectant cells by using same blocker concentration (Fig. 5C). The low sensitivity of P-gp to blockers indicates that P-gp content in the transfectants drastically decrease due to suppression of MDR1 gene by overexpressing caveolin-1. The result is consistent with that of Western blot, RT-PCR and Northern blot experiments.

4. Discussion

The effectiveness of chemotherapy to human tumors has been largely limited by the development of drug resistance. Numerous studies demonstrated that major mechanism underlying drug resistance is overexpression of the MDR-1 gene encoding for the transmembrane efflux pump, P-gp. For instance, verapamil and carvedilol reversed cellular drug resistance by inhibiting P-gp drug efflux in vitro as well as in vivo [22,29]. These results have led to clinical trial of the agents in refractory cancer patients.

Recent studies suggested that a role of P-gp in drug efflux is regulated by protein phosphorylation/dephosphorylation. The incubation of MDR cells with PKC activator and the transfection of MCF-7 cells expressing MDR1 with PKC_{α}, caused P-gp phosphorylation, reduced drug accumulation, and enhanced drug resistance [30–32]. The expression of PKC_{α} antisense cDNA in same cells decreased P-gp phosphorylation and its transport activity, and reversed drug resistance [33].

Caveolin-1, a major scaffolding protein in caveolae, is another protein relevant to MDR and P-gp. Caveolin-1 is colo-

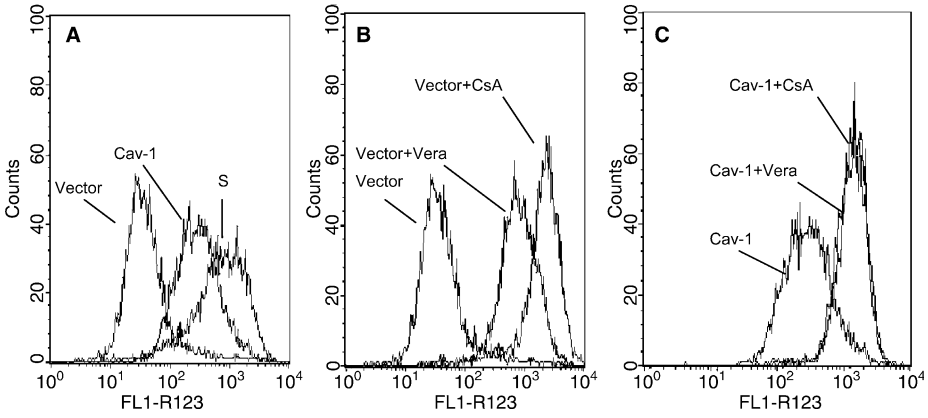

Fig. 5. P-gp transport activity in various Hs578T/Dox. (A) The Rh-123 fluorescence intensity distribution in Hs578T/Dox+vector (Vector), Hs578T/Dox+cav-1 (Cav-1) and Hs578T/S (S), respectively. (B) Effect of P-gp blockers, cyclosporine A (CsA) and verapamil (Vera), on the Rh-123 fluorescence intensity distribution in Hs578T/Dox+vector (Vector). (C) Effect of both P-gp blockers on the Rh-123 fluorescence intensity distribution in Hs578T/Dox+cav-1 (Cav-1).

calized and physically associated with P-gp in MDR cells [19,20]. The interaction of caveolin-1 scaffolding domain with a consensus caveolin-binding motif of signaling molecules can functionally inhibit the activity of these molecules. This caveolin-binding motif is also present in human P-gp between amino acid residues 36 and 44 [20]. It is noteworthy that most of the investigations on relationship between caveolin-1 and P-gp/MDR1 measured either their mRNA or their protein expression in cancer cells. Few of these studies simultaneously analyzed functionality of caveolin-1 and P-gp. It may not be sufficient to understand the biological significance. For example, a high MDR1 mRNA and P-gp expression was not accompanied by low cellular drugs accumulation or sensitivity to efflux blockers, in other words, P-gp expression may not be correlated with the enhanced drug accumulation and cytotoxicity [34–36].

In order to gain insight into the possible mechanism between caveolin-1 and P-gp expression, we have selected single clones of high caveolin-1 expression levels from Hs578T/Dox overexpressing caveolin-1 and employed multiple complementary approaches to analysis the expression levels of both proteins and mRNA of caveolin-1 and P-gp. At the same time, we examined the changes of drug resistance and P-gp transport activity in stable single-clone cells. It is believed that the development of MDR in cancer cells is due to the increased expression of P-gp. We demonstrate in this study that the expression of P-gp protein and mRNA is almost abolished in the transfectants overexpressing caveolin-1 by using Western blot, confocal microscopy, RT-PCR and Northern blot analysis. Moreover, the overexpression reverse cellular drug resistance of the transfectants and decrease P-gp transport activity to the level of Hs578T/S. A low sensitivity to P-gp blockers also indicates that P-gp content in the transfectant cells drastically decrease due to the suppression of MDR1 gene by overexpressing caveolin-1. In order to make our results to be convinced, we detected PKC_{α}, which is localized in caveolae too (6, 20), as a positive control. PKC_{α} content was not significantly changed either in wild-type, vector control cells or in the transfectant cells. It was possible, at least from our study, that the suppression of P-gp mRNA by overexpressing caveolin-1 in Hs578T/Dox is specific. Although we do not know the mechanism underlying P-gp suppression in transfectants, our results imply that the suppression of P-gp in the transfectants may occur at the level of transcriptional control. Analogously, a recent study showed that the overexpression of regucalcin in cloned rat hepatoma H4-11-E cells induced complete abolishment of Ha-ras mRNA expression in the transfectants [37].

It was demonstrated that caveolin-1 expressed in most normal cells, but was downregulated in human cancer cells [38–40]. Working on embryonic fibroblasts from knockout mice supports the idea that cell proliferation increases when caveolin-1 is absent [41]. This indicates that the downregulation of caveolin-1 expression may be relevant to the development of human tumors. Caveolin-1 gene in human is mapped to a common location in chromosome 7q31.1, and is possible candidates for tumor suppressor gene postulated in the region [42]. Caveolin-1, as a "tumor suppressor", interacts with some signaling molecules and inhibits their activity. Caveolin-1 had no sooner downregulated than the cells began to proliferate highly (such as, cancer cells) due to getting rid of this caveolin-1 inhibition on signaling molecules. However, the role of caveolin-1 in multidrug resistance is still unclear. It is possible that caveolin-1 plays a key role in signaling transduction in multiple mechanisms. The results from our and other study groups demonstrated that a direct interaction between caveolin-1 and P-gp, and the functional inhibition of P-gp transport activity were observed in Hs578T/Dox and brain capillaries [19,20]. And a number of evidences showed that the alterations of the physical state (for example, fluidization) and cholesterol content in the plasma membrane of multidrug resistant cancer may indirectly effect P-gp transport activity and reverse MDR phenotype [43,44]. We have also demonstrated that overexpression of caveolin-1 in Hs578T/Dox cells could decrease the plasma membrane cholesterol level, i.e., increase membrane fluidity and indirectly inhibit P-gp transport activity [45]. Other modulation mechanism by the results presented here is that the overexpression of caveolin-1 in single clone cells of Hs578T/Dox may suppress MDR1 gene expression and reverse multidrug resistant.

In order to enhance the efficacy of chemotherapy and decrease multidrug resistance in cancer therapies, we bring an insight into overcoming drug resistance, in which caveolin-1 improve the efficacy of cancer therapy by raising the accumulation of chemotherapeutic drugs in cancer cells through blocking the expression of MDR1 gene. The capability of caveolin-1 in the reversal of multidrug resistant is consistent with its role called "tumor suppressor". Further study elucidating the detailed mechanism for caveolin-1 suppression of P-gp expression should be performed.

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