Upregulation of caveolin-1 and caveolae organelles in Taxol-resistant A549 cells

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Abstract Caveolin is a principal component of caveolae membranes. It has been demonstrated that the interaction of the caveolin scaffolding domain with signaling molecules can functionally inhibit the activity of these molecules. Taxol is an antitumor agent that suppresses microtubule dynamics and binds to microtubules thereby stabilizing them against depolymerization. The drug also has been implicated in the induction of apoptosis through activation of components in signal transduction cascades. Here we have investigated the role of caveolin in the development of drug resistance by examining the expression of caveolins in low- and high-level drug-resistant cell lines. Caveolin-1, but not caveolin-2, was upregulated in highly multidrug resistant SKVLB1 cells that express high levels of P-glycoprotein, and in low-level Taxol-resistant A549 cell lines that express low amounts of P-glycoprotein. Two drug-resistant A549 cell lines (one 9-fold resistant to Taxol and the other 1.5-fold resistant to epothilone B), both of which express no P-glycoprotein, demonstrate a significant increase in the expression of caveolin-1. These results indicate that in low-level epothilone B- or Taxol-resistant A549 cells, increased caveolin-1 expression occurs independently of P-glycoprotein expression. Electron microscopic studies clearly demonstrate the upregulation of caveolae organelles in Taxol-resistant A549 cells. Upregulation of caveolin-1 expression in drug-sensitive A549 cells was observed acutely beginning 48 h after incubation with 10 nM Taxol. Thus, caveolin-1 may play a role in the development of Taxol resistance in A549 cells.

Key words: Taxol; Drug resistance; P-glycoprotein; Caveolin; Caveolae

1. Introduction

The development of resistance to Taxol that may occur by a variety of mechanisms is a significant problem in cancer chemotherapy. It is known that overexpression of P-glycoprotein, an energy-dependent drug efflux pump, confers multidrug resistance (MDR). Taxol as well as other hydrophobic antitumor drugs are substrates for P-glycoprotein, the product of the *MDR*1 gene [1]. In addition to the overexpression of P-glycoprotein, other potential mechanisms that have been implicated include overexpression of c-erbB2/neu in breast cancer cells [2], alterations in β-tubulin isotypes [3–6], mutations in tubulin [6], and specific changes in various components of signal transduction pathways [7,8]. Alterations in molecules involved in signal transduction cascades, such as activation of Raf-1 and mitogen-activated protein kinase (MAP kinase), have been reported after treatment of drug-sensitive cells with Taxol [7–12].

Caveolins, a family of 21–24 kDa integral membrane proteins, are the principal protein components of caveolae membranes. Three distinct caveolin genes, caveolin-1, caveolin-2, and caveolin-3, have been identified [13]. Two isoforms of caveolin-1 (Cav-1α and Cav-1β) are derived during translation utilizing alternative initiation sites. Caveolin-1 and -2 are highly expressed in adipocytes, endothelial cells, and fibroblasts, whereas caveolin-3 is specifically expressed in striated muscle cells [13].

It has been suggested that caveolins function as scaffolding proteins to organize and concentrate certain caveolin-interacting signaling molecules within caveolae membranes [13]. Interaction of the caveolin scaffolding domain with signaling molecules functionally suppresses the activity of these molecules, suggesting that caveolin binding plays a negative regulatory role in signal transduction [13,14]. We have investigated the role of caveolins in drug-resistant cells by studying the expression of this protein in Taxol-, vinblastine-, and epothilone- B-selected cells, as well as in sensitive cells treated with Taxol.

In this report, we show that caveolin-1, but not caveolin-2, is upregulated in a variety of drug-resistant cells. In addition, caveolin-1 expression was increased acutely beginning 48 h after drug-sensitive A549 cells were treated with 10 nM Taxol.

2. Materials and methods

2.1. Materials

Anti-caveolin-1 mouse IgG (mAb 2297) and anti-caveolin-2 mouse IgG (mAb 65) were generous gifts from Dr. John R. Glenney, Transduction Laboratories, and have been previously characterized [15,16]. Anti-P-glycoprotein monoclonal antibody, C219, was from Signet Laboratories. Taxol was obtained from the Drug Development Branch, National Cancer Institute, Bethesda, MD. Epothilone B was kindly provided by Professor Samuel J. Danishefsky.

2.2. Cell culture

Human lung carcinoma cells, A549, together with their Taxol-resistant derivatives were grown in RPMI 1640 containing 10% fetal bovine serum (FBS). A549-T12 and A549-T24 [5] were maintained at a final concentration of 12 and 24 nM Taxol, respectively. Two epothilone B-resistant A549 cell lines, A549-EP0.2 and A549-EP0.3, were derived in our laboratory as previously described for other drug-resistant cell lines [17], and maintained in medium containing 0.2 and 0.3 nM epothilone B, respectively. Drug-sensitive (SKOV3) and vinblastine-resistant (SKVLB1) human ovarian carcinoma cell lines were

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Abbreviations: MDR, multidrug resistance; MAP kinase, mitogen-activated protein kinase; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; TNF-α, tumor necrosis factor α

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gifts from Dr. V. Ling and grown in minimum essential α medium containing 15% FBS. SKVLB1 was maintained in medium containing 1 μM vinblastine.

2.3. Preparation of plasma membranes and cell lysates

Plasma membranes from drug-sensitive and -resistant A549 and SKOV3 cells were prepared as described [17]. To prepare cell lysates, cells were washed twice with phosphate buffered saline, lysed in buffer containing 10 mM Tris-HCl, pH 7.5 and 1% SDS using 21 and 26 gauge needles, and centrifuged for 5 min at 13 000 rpm in a microfuge. Protein concentrations of the supernatants were determined by the method of Lowry [18].

2.4. Immunoblot analysis

Plasma membranes or cell lysates were resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), and transferred to nitrocellulose. Immunoblot analysis was performed as described [11] with ECL reagents and densitometry was done to quantitate the levels of caveolin-1 expression.

2.5. Reverse transcription-PCR (RT-PCR) analysis of MDR1

To determine if *MDR*1 was expressed in low-level epothilone B-resistant A549 cells, RT-PCR, using specific human *MDR*1 primers, was performed as described [5].

2.6. Transmission electron microscopy

Samples were fixed with glutaraldehyde, postfixed with osmium tetroxide, and stained with uranyl acetate and lead citrate, as previously detailed [19,20]. Samples were examined under the Philips 410 TEM.

3. Results

3.1. Upregulation of caveolin-1 and caveolae organelles in drug-resistant cell lines

A variety of drug-resistant cell lines were used in this study (see Table 1). Two low-level Taxol-resistant cell lines were derived from the human lung carcinoma cell line A549 [5]. A549-T12 that exhibited a 9-fold resistance to Taxol had a 3.4-fold increase in caveolin-1 levels as compared to parental drug-sensitive A549 cells. No P-glycoprotein was detected in its plasma membrane. A slightly more resistant cell line, A549-T24 (17-fold resistant to Taxol), expressed very low levels of P-glycoprotein and demonstrated a 9.5-fold increase, compared to A549 cells, in caveolin-1 (Fig. 1A,B). An increased level of caveolin-1 expression also was observed in a highly resistant human ovarian MDR cell line SKVLB1, which is approximately 8000- and 2500-fold resistant to Taxol and vinblastine, respectively, as compared to its drug-sensitive counterpart, SKOV3. In contrast, caveolin-2 levels in A549, A549-T12 and A549-T24, and in SKOV3 and SKVLB1 cells demonstrated no significant differences (Fig. 1C).

As viewed by transmission electron microscopy, drug-sensitive parental A549 cells lacked any detectable caveolae. However, A549-T24 cells show a dramatic upregulation of caveolae organelles, in parallel with the upregulation of caveolin-1 expression (Fig. 2). Caveolae appear as 50–100 nm vesicles near or attached to the plasma membrane. A similar observation was made during adipocyte differentiation where upregulation of caveolin-1 expression led to an increased number of caveolae organelles in mature adipocytes, as compared with fibroblastic pre-adipocytes [21].

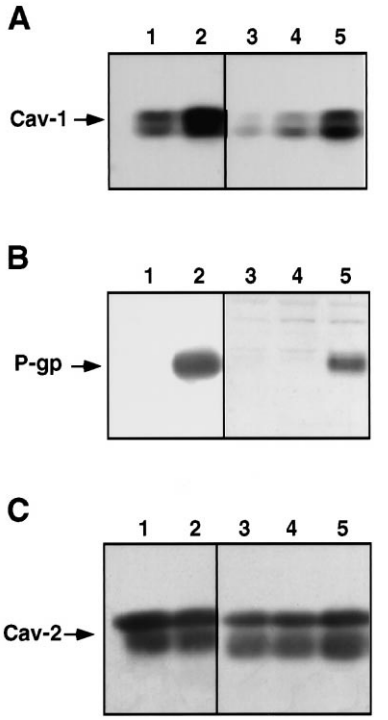

Fig. 1. Expression of caveolin-1 and caveolin-2 in drug-sensitive and -resistant SKOV3 and A549 cells. Lanes 1: SKOV3; lanes 2: SKVLB1; lanes 3: A549; lanes 4: A549-T12 and lanes 5: A549-T24. A and C: Lysates were prepared from vinblastine-sensitive and -resistant SKOV3 cells and from Taxol-sensitive and -resistant A549 cells. After SDS-PAGE on a 12.5% gel and transfer to nitrocellulose, immunoblotting was performed with mono-specific antibody probes that recognize only caveolin-1 (A) or caveolin-2 (C). B: Plasma membranes from these cells were resolved by SDS-PAGE on a 5–13% gradient gel without boiling the samples, transferred to nitrocellulose, and probed with monoclonal antibody C219 against P-glycoprotein. The antibody dilution for the SKOV3 cell lines was 1:2500 and for the A549 series was 1:1000. ECL exposure time was 100 times shorter for the SKOV3 cell lines than for the A549 series.

3.2. Acute effects of Taxol on caveolin-1 expression in A549 cells

Sixty to 70% confluent A549 cells were treated with increasing concentrations of Taxol (20–1000 nM) and the expression of caveolin-1 in these cells was studied 20 h after drug administration. There were no significant differences between un-

Table 1
Drug-resistant cell lines

Cell line	Parental, drug-sensitive cell line	Drug used for selection	Fold resistance	P-gp[a] expression	Cav-1 upregulation[d] (fold)
SKVLB1	human ovarian SKOV3	VBL[b]	2500	yes (strong)	2.0
A549-T12	human lung A549	Taxol	9	no	3.4
A549-T24	human lung A549	Taxol	17	yes (weak)	9.5
A549-EP0.2	human lung A549	EPOB[c]	1.4	no	6.8
A549-EP0.3	human lung A549	EPOB	1.5	no	5.5

[a]P-gp, P-glycoprotein; [b]VBL, vinblastine; [c]EPOB, epothilone B.
[d]Values represent the average of two independent experiments.

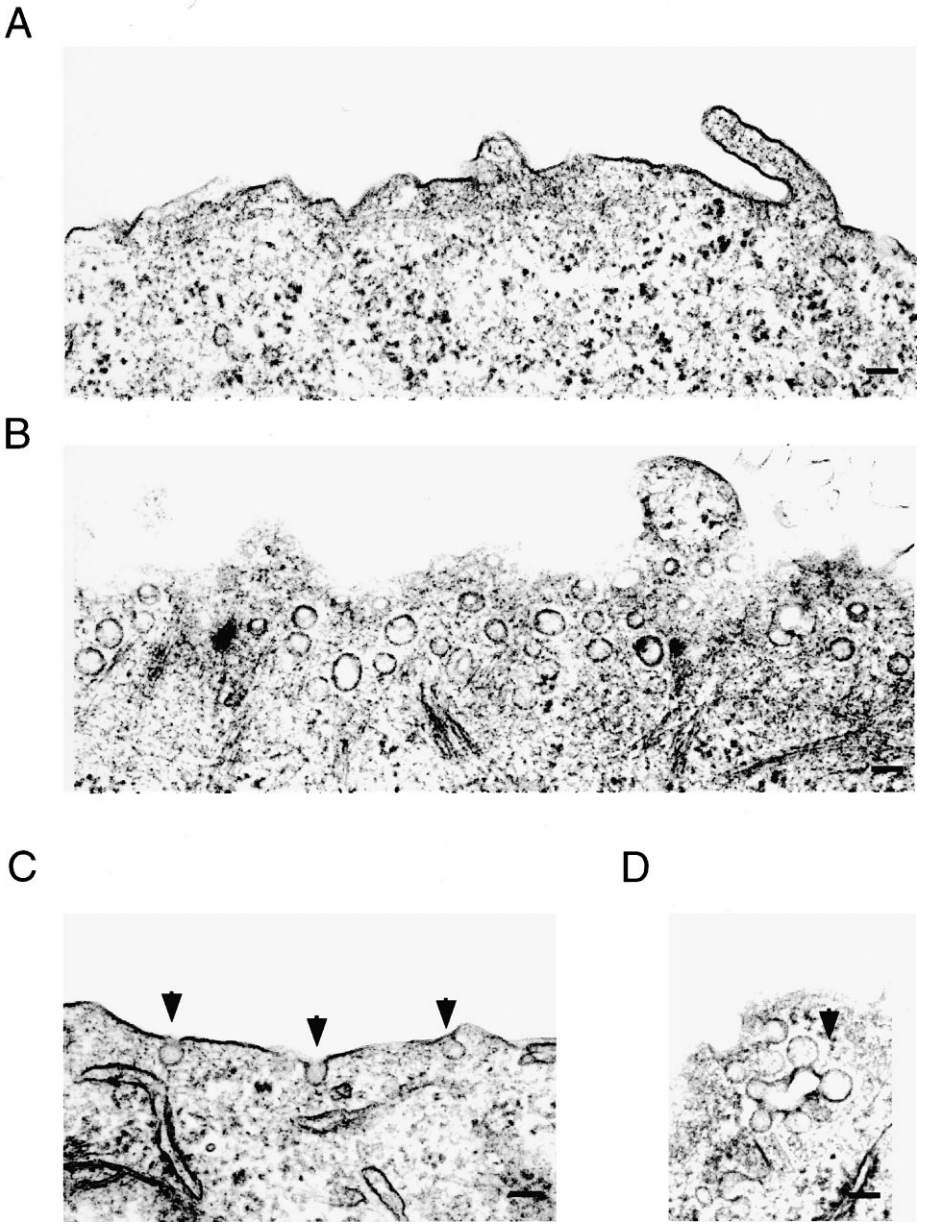

Fig. 2. Upregulation of caveolae organelles in Taxol-resistant A549-T24 cells. Samples were fixed with glutaraldehyde, postfixed with osmium tetroxide, and stained with uranyl acetate and lead citrate. Electron micrographs show views of the plasma membrane. A: Parental drug-sensitive A549 cells. B: A549-T24 cells. C and D: Additional views of A549-T24 cells. Depending on the plane of the section, note that caveolae can appear as free vesicles (B), attached omega-shaped flasks (C), or can be seen in bunches like clusters of grapes (D). Bar = 100 nm.

treated and Taxol-treated A549 cells with respect to caveolin-1 (Fig. 3A). However, when 30–40% confluent A549 cells were treated with 10 nM Taxol and the expression of caveolin-1 was monitored over a longer period of time, it was found that there was an increase in caveolin-1 expression beginning 48 h after drug treatment. This increase continued for at least 4 days (Fig. 3B).

3.3. Expression of caveolins in epothilone B-resistant cells

Epothilone B is extremely cytotoxic to mammalian cells and has a mechanism of action similar to that of Taxol [22]. A549-EP0.2 and A549-EP0.3 were 1.4- and 1.5-fold resistant to epothilone B, respectively. A competitive RT-PCR analysis [5] indicated that there was no *MDR*1 gene expression in these cells. In contrast, very low levels of *MDR*1 were detected in A549-T24 and high levels were present in the very resistant SKVLB1 cells (Fig. 4B). There was an approximate 5–7-fold increase in caveolin-1 levels in A549-EP0.2 and A549-EP0.3 (Fig. 4A, left panel). Caveolin-2 levels remained similar in the sensitive and Epothilone B-resistant A549 cells (Fig. 4A, right panel).

4. Discussion

In this study, we have demonstrated (i) an increase in the level of caveolin-1 in a 2500-fold vinblastine-resistant MDR cell line, SKVLB1, that expresses high levels of P-glycoprotein compared to its parental, drug-sensitive cell line SKOV3; (ii) greater expression of caveolin-1 in 17-fold Taxol-resistant A549 cells that do not express significant levels of P-glycopro-

tein, compared to drug-sensitive A549 cells; (iii) a prominent increase in caveolin-1 expression in a 9-fold Taxol-resistant and in two approximately 1.5-fold epothilone B-resistant A549 cells, all of which did not express any P-glycoprotein, compared to A549 cells; and (iv) increased expression of caveolin-1 48 h after drug-sensitive A549 cells were treated with 10 nM Taxol.

Interestingly, an increase in caveolin-1 levels was observed only in those cell lines that express caveolins endogenously. For example, the expression of caveolin-1 and caveolin-2 was not observed in several MDR cell lines that express high levels of P-glycoprotein, such as J7.V1-1 [23] and J7.T3-1.6 [3] that were derived from murine macrophage-like J774.2 cells, and Caco-V100 that was derived in our laboratory from human colon carcinoma Caco-2 cells (data not shown). Unlike epothilone B-resistant A549 cells, epothilone B-resistant MCF-7 cells derived in our laboratory did not express any caveolins. All of these parental drug-sensitive cells also did not express caveolins. Although upregulation of caveolin-1 may represent one component of resistance, it clearly is not a requirement for the development of drug resistance.

It has been reported that glycosylceramides, the most widely distributed glycosphingolipids in cells, accumulate in various types of MDR cells [24]. Some agents that reverse MDR, such as verapamil and cyclosporin A, block glycosphingolipid metabolism by inhibiting ceramide glycosylation in human cancer cells [25]. Since glycosphingolipids are important constituents of caveolae and caveolins are the principal components of caveolae, the role of caveolins in MDR was investigated. However, our data demonstrate that the induction of caveolin-1 expression does not require the expression of P-glycoprotein. For example, in low level Taxol-resistant A549 cells, the induction of caveolin-1 expression occurs independently of P-glycoprotein expression.

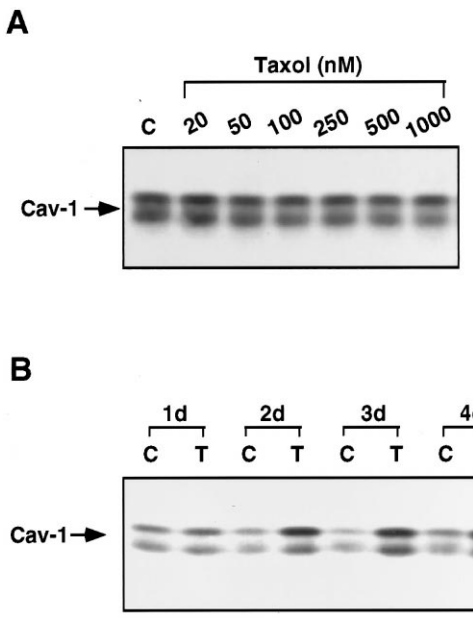

Fig. 3. Acute effects of Taxol treatment on the expression of caveolin-1 in drug-sensitive A549 cells. A549 cells were treated with 20–1000 nM Taxol for 20 h, or treated with 10 nM Taxol for 1–4 days. Lysates were prepared and immunoblot analysis with anti-caveolin-1 IgG (A and B) was performed as described in Section 2 and as in Fig. 1. C: control cells; T: 10 nM Taxol.

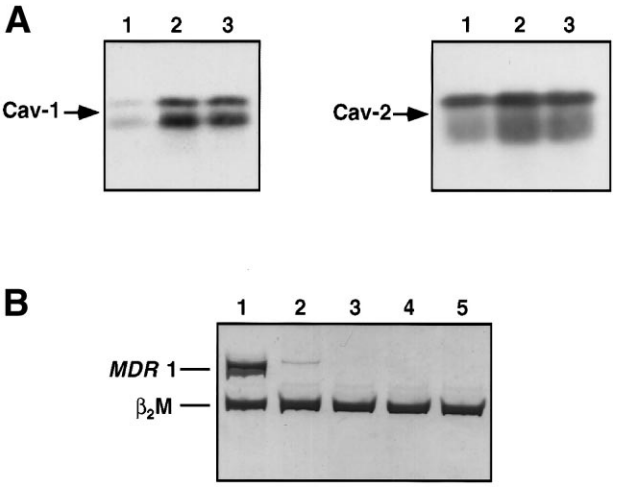

Fig. 4. Expression of caveolins in epothilone B-sensitive and -resistant A549 cells. A: Lysates from drug-sensitive and epothilone B-resistant A549 cells were prepared and immunoblotting was performed with monoclonal antibodies against caveolin-1 (A, left panel) or caveolin-2 (A, right panel). Lanes 1: A549 cells; lanes 2: A549-EP0.2 cells; lanes 3: A549-EP0.3 cells. B: RT-PCR determination of *MDR*1 gene expression was performed on SKVLB1 (lane 1, a highly resistant MDR cell line), A549-T24 (lane 2, a relatively low Taxol-resistant cell line), drug-sensitive A549 (lane 3), epothilone B-selected A549-EP0.2 (lane 4) and A549-EP0.3 (lane 5) cells. Competitive RT-PCR, involving coamplification of *MDR*1 (167 bp) and control β_2-microglobulin (120 bp) gene sequences, was done for 35 cycles and the products were separated on an 11.5% polyacrylamide gel followed by ethidium bromide staining as previously described [5].

It has been proposed that microtubules are necessary for the normal cycling of caveolin-1 between the plasma membrane and the Golgi [26]. The introduction of Taxol, a molecule that stabilizes microtubules [27], may affect the cycling of caveolin-1. The presence of Taxol could result in an upregulation of caveolin-1 as a way for the cell to compensate for the effect of the drug on the microtubule cytoskeleton.

We do not yet know the mechanism by which caveolin-1 levels are upregulated in the Taxol-resistant cells. Such upregulation may occur at the level of transcriptional or translational control. Alternatively, upregulation of caveolin-1 could also occur through gene amplification. However, our results shown in Fig. 3 are more consistent with the former possibility, as caveolin-1 levels were upregulated acutely (within 48 h) in response to Taxol treatment. Interestingly, several recent reports have indicated that caveolin-1 levels also are elevated in hormone-refractory prostate cancers [28–30].

Several lines of evidence indicate that Taxol can activate a variety of signal transduction pathways. For example, (i) Taxol activates c-jun N-terminal kinase (JNK) through both Ras and apoptosis signal-regulating kinase (ASK1) pathways [8]. ASK1, a MAP kinase kinase kinase, causes the activation of JNK and p38 and induces apoptotic cell death upon TNF-α treatment [31]. (ii) Taxol induces activation of extracellular signal-regulated kinases (ERK) [9–11]. Tyrosine phosphorylation of Shc and formation of a Shc/Grb2 complex also have been demonstrated in Taxol-treated RAW 264.7 cells [11]. (iii) Taxol causes tyrosine phosphorylation of p38 [32]. The interaction of signaling molecules with caveolin may influence Taxol-induced signaling cascades that may undergo alterations during the development of drug resistance.

In summary, our data indicate that Taxol induces the upregulation of caveolin-1 and caveolae organelles that could play a role in Taxol-related drug resistance. Thus, caveolin-1 and caveolae organelles may represent novel therapeutic targets for molecules capable of modulating drug resistance in those cells that have upregulated levels of caveolin-1.

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