Ability of a Specific ERK Signal-Pathway Inhibitor to Reverse P-Glycoprotein- Mediated Vincristine Resistance in Colon Cancer Cell Lines

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OBJECTIVE To investigate the effect of a specific inhibitor PD098059 of the extracellular-signal regulated protein kinase (ERK) pathway on the P-glycoprotein (P-gp)-mediated resistance of colon cancer cell lines SW480/VCR and CoLo205/VCR.

METHODS SW480/VCR and CoLo205/VCR cells were generated by exposuring SW480 and CoLo205 cells to vincristine (VCR) (30 ng/ml) for 72 h, which resulted in a comparatively higher level of P-gp expression. Western blotting was used to analyze P-gp, MRP, LRP, GST-π and TOPOII expression after exposuring the SW480 and CoLo205 cells to VCR (30 ng/ml) for 72 hrs. P-gp and pERK1/2 expressions was analyzed in SW480/VCR and CoLo205/VCR cells treated with or without the specific inhibitor of MEK, PD098059. The MTT assay was used to determine the susceptibility of SW480/VCR and CoLo205/VCR cells to VCR, treated with or without PD098059.

RESULTS The results showed that VCR induced a comparatively higher level of P-gp expression in the cell lines, but not that of MRP, LRP, GST-π or TOPOII. P-gp expression levels were depressed significantly in SW480/VCR and COLO205/VCR cells by the specific inhibitor of MEK, PD098059. The IC_{50} (248 ±19.6 and 215 ±10.7 ng/ml) to VCR of SW480/VCR and CoLo205/VCR cells exhibited a 2.16 and 2.03-fold higher resistance compared to the negative control group (SW480 and CoLo205 cells)(115± 15.6 and 106 ±11.9 ng/ml), but a 1.35 and 1.21-fold higher resistance than the group treated with VCR (30 ng/ml)+ PD098059 (184 ± 21.8 and 177± 19.4 ng/ml).

CONCLUSION This study shows that the expression of P-gp can be induced by exposuring cells to VCR, and that this induction can be reversed by inhibiting the ERK signaling pathway at the point of MEK by its specific inhibitor, PD098059. The ERK signal-transduction pathway may play a role in modulating mdr1 expression in colon cancer.

KEYWORDS: P-glycoprotein, ERK, colon cancer.

Multidrug resistance (MDR) is a major factor in the failure of many forms of chemotherapy, and several different molecular mechanisms are activated in MDR cells. The mechanisms most investigated with known clinical significance are: (1) activation of transmembrane proteins, such as P-gp and MRP, effluxing different

chemical substances from the cells; (2) activation of the enzymes of the glutathione detoxification system, such as GST-π; (3) alteration of genes and proteins which control apoptosis (especially p53 and Bcl-2).[1] MDR associated genes are expressed in a large proportion of human tumors, and in several different forms of tumors MDR was shown to be associated with a lack of response to combination chemotherapy. P-glycoprotein (P-gp) expression is usually low or undetectable prior to treatment, but it is frequently increased during the progression of the disease and, most noticeably, after chemotherapy.[2-5] An increase in expression of mdr1 mRNA can be found in some drug-sensitive cancer cells by transient exposure to different chemotherapeutic drugs.[6,7]

The signal-transduction mitogen-activated protein kinase (MAPK) pathway plays a critical role in cell proliferation, differentiation and apoptosis. The ERK1/2 (Ras / Raf-1 / MEK1/2 / ERK1/2) signal transduction pathway is a subfamily of MAPK.[8] The expression of the mdr1 gene and the activation of the MAPK pathway often are up-regulated in cancer cells after treatment with various therapeutic drugs.[9] The specific inhibitor of MEK1/2, PD098058, has been shown to significantly reverse drug resistance in drug-resistant cell lines,[10-12] but the specific mechanism needs to be elucidated. Of particular interest is whether MAPK plays a role in MDR, and whether ERK can regulate expression of MDR associated genes in colon cancer.

Our study was designed to investigate the expression of MDR-associated genes in colon cancer cells SW480 and CoLo205, by exposuring cells to vincristine (VCR) to induce MDR, and to reverse MDR by down-regulating P-gp expression with a specific MEK inhibitor, PD098059.

MATERIALS AND METHODS

Materials

Colon cancer cells SW480 and CoLo205 and a P-gp positive control cell line K562/DOX were obtained from Kyushu University, Japan. A pERK1/2 positive control cell line Soas-2 was obtained from Tokyo Medical and Dental University, Japan. LRP, MRP, GST- π and TOPOII positive clinical samples were from the China Medical University. Standard control of amount of protein loading was marked by non-phosph-ERK2. RPMI 1640 medium was a product of GIBCO (USA). Vincristine (VCR) and PD098059, a specific MEK inhibitor, were purchased from Sigma (St. Louis, MO). Anti P-gp [Mdr (H-241): sc-8313], MRP [MRP1 (C-20): sc-7773], LRP [L RP1 (N20): sc-16166], ERK2 [(D-2): sc-1647], GST- π (glutathione-S-transferase) [GST (Z-5): sc-459] and TOPO II (topoisonerase II, TOPO II) [Topo II(N-16): sc-5349] antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA) and anti-phospho-ERK1/2 (Thr 202/Tyr 204) antibodies were from New England Biolab, MA, USA.

Induction and reversal of MDR

Colon cancer cells SW480 and CoLo205 were grown in RPMI 1640 essential medium supplemented with 10% fetal bovine serum (FBS; Sigma), 1% penicillin G/streptomycin sulfate (Sigma). Vincristine (30 ng/ml) was added directly to the medium with the SW 480 and CoLo205 cells. After 72 h, the SW480/VCR and CoLo 205/VCR cells were harvested. Then the SW480/VCR and CoLo 205/VCR cells were divided into 2 groups, namely VCR (30 ng/ml) or VCR (30 ng/ml) + PD098059 (15 nmol/ml). The cells were then harvested after 48 h.

Gel electrophoresis and immunoblotting

The cells (2×10^7) were lysed in 200 μl RB buffer (20 mM Tris-HCl, pH 7.6, 150 mM NaCl, 0.5% NP-40, 50 mM NaF, 1 mM β-glycerophosphate, 1 mM Na_3VO_4, 5 mM $Na_4P_2O_7$, 5 mM EDTA, 10 ug/ml each of leupeptin and aprotinin), containing a Protease-Inhibitor-Cocktail (Roche, Diagnostics, Tokyo, Japan). Samples were sonicated on ice, held for 40 min at 4℃, then centrifuged (15,000 rpm, 30 min) at 4℃. The supernatant was transferred to a new tube on ice and the protein concentration was determined, using a Bio-Rad DC Protein Assay Kit (Bio-Rad Laboratory, CA, USA). Protein samples (100 ug) were separated by sodium dodecyl sulfate polyacrylmide gel electrophoresis

(SDS/PAGE), then transferred to a PVDF membrane (Nihon Millipore, Tokyo, Japan). The filter was blocked with 1 × TTBS (20 mM Tris-HCl pH7.6, 137 mM NaCl and 0.1% Tween-20) containing 5% skim milk, incubated at 4℃ overnight with either of the following first antibodies: P-gp (1:2,000), MRP (1:2,000), LRP (1:1,500), GST-π (1:1,000), TOPO II (1:1,500), phospho-ERK1/2 (Thr202/ Tyr204, 1:1,000) and ERK2 (1:2,000). Then the filter was incubated with alkaline phosphatase-conjugated second antibody for 1 h. Proteins were detected with the ECL detecting system (Amersham, Tokyo, Japan). The intensity for each protein band after Western blotting was measured by an image analyzer using imaging software of Image-Pro PLUSTM Media Cybenetics Silver Spring, MD, USA.

MTT assay

SW480/VCR and CoLo205/VCR cells (1×10^5/ ml) were plated into a 96-well plate and cultured in 100μl RPMI medium supplemented with 10% fetal bovine serum (FBS; Sigma) and 1% penicillin G/streptomycin sulfate. After incubation for 4 h, the cells were divided into 2 groups: one group was treated with increasing concentrations of VCR (1 ng/ml, 10 ng/ml, 100 ng/ml, 1000 ng/ml); the other group was treated with identical concentration of VCR plus a fixed level of PD098059 (15 nmol/ml); SW480 and CoLo205 cells treated with the same concentrations of VCR served as negative controls. After 72 h, 20 μl of 5 mg/ml MTT [3-(4,4-dimeththiazol-2-yl)2,5-diphenylterazolium bromide] in PBS was added to each well, and the mixtures were incubated for 4 hrs at 37 ℃. The formazan crystals formed were dissolved in 100 μl of DMSO (Sigma). The above procedure was repeated in triplicate. The optical density was recorded at 570 nm on a microplate reader(BIORAD). Sensitivity to VCR was expressed as IC_{50} for cells, which was the concentration of VCR that caused a 50% reduction in the absorbance at 570 nm relative to untreated cells (controls).

RESULTS

VCR mainly induced P-gp overexpression, but not MRP, LRP, GST-π or TOPOII in colon cancer cell lines

Overexpression of MDR associated genes can be induced or stimulated by different anti-tumor drugs. After 72 h of vincristine treatment of the colon cancer cell lines, SW 480 and CoLo205, a 3.53 and 3.06 fold higher P-gp expression compared to non-induced SW480 and CoLo205 cells was seen. However, MRP, LRP, GST-π and TOPOII showed only a slightly higher or equal level of expression after 72 h of vincristine induction, compared to the non-induced SW480 and CoLo205 cells (Fig.1). These results indicated that VCR can predominantly induce mdr1 gene over-expression in colon cancer.

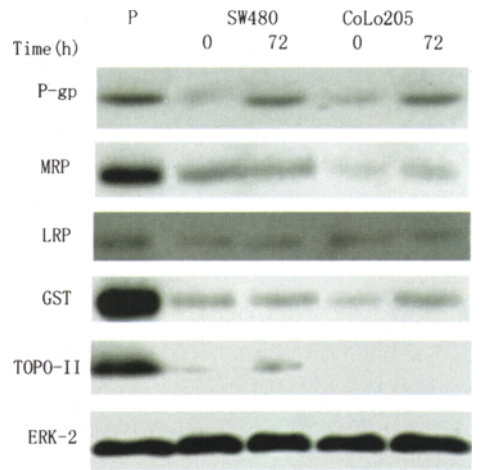

Fig.1. P-gp, MRP, LRP, GST-π and TOPOII expression before and after VCR induction in colon cancer cell lines SW 480 and CoLo 205. P: positive control; SW 480,0: SW 480 cells before exposure to vincristine; SW 480,72: SW 480 cells exposed to vincristine for 72 h; CoLo 205, 0: CoLo 205 cells before exposure to vincristine; CoLo 205,72: CoLo 205 cells exposed to vincristine for 72 h.

Down-regulated mdr1-gene expression by specific ERK-signal pathway inhibition in colon cancer cell lines

P-gp expression levels were depressed significantly in SW480/VCR and CoLo205/VCR Cells after 48 h of treatment with VCR (30 ng/ml) + PD098059 (15 nmol/ml), compared to treatment with only VCR (30 ng/ml) (Fig.2). With PD098059, P-gp expression was at a similar level or slightly lower level compared to the onset of the assay. In contrast, MRP, LRP, GST-π and TOPOII expression showed no significant difference

within 48 h, after treatment with either VCR (30 ng/ml) + PD098059 (15 nmol/ml)or with VCR (30 ng/ml) alone. These results suggested that mdr1 gene expression could be inhibited by modulating MAPK pathway in colon cancer.

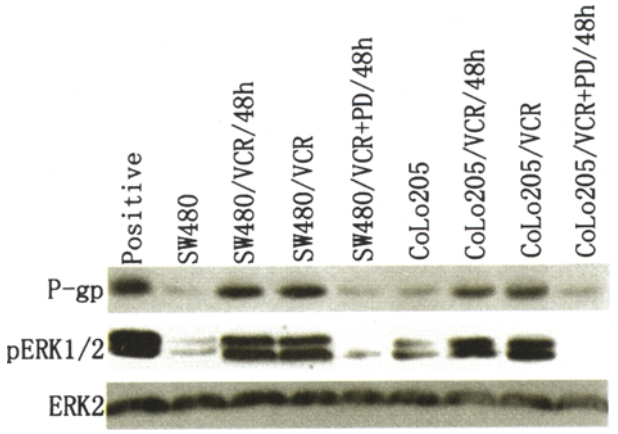

Fig.2. Down-regulated mdr1 gene expression by specific ERK signal pathway inhibition, PD098059, in SW480/VCR and CoLo205/VCR cells. SW480/VCR/48h: SW480/VCR cells exposed to vincristine for 48 h; SW480/VCR: SW480/VCR cells before exposure to vincristine; SW480/VCR+PD/48 h: SW480/VCR cells exposed to vincristine and PD098059 for 48 h; CoLo205/VCR/48h: CoLo205/VCR cells exposed to vincristine for 48 h; CoLo205/VCR: CoLo205/VCR cells before exposure to vincristine; CoLo205/VCR+PD/48h: CoLo205/VCR cells exposed to vincristine and PD098059 for 48 h.

MTT assay to drug sensitivity

The IC_{50} (248 ± 19.6 and 215 ± 10.7 ng/ml) to VCR of SW480/VCR and CoLo205/VCR cells exhibited a 2.16 and 2.03-fold higher resistance than the negative control group (115 ± 15.6 and 106 ± 11.9 ng/ml) and a 1.35 and 1.21-fold higher resistance than the group (184 ± 21.8 and 177 ± 19.4 ng/ml) treated with VCR (30 ng/ml) + PD098059 (Table 1). These results also indicate that PD098059, a specific ERK signal pathway inhibitor, can partially reverse drug resistance induced by VCR.

DISCUSSION

Different tumors display different sensitivity to chemotherapeutic drugs, and drug resistance can be induced by anti-tumor chemical substances. The failure of different regimens of combination chemotherapy for different malignant tumors is mainly due to the overexpression of MDR associated genes. Various cancer cell lines show a MDR phenotype after long-term exposure to gradient concentrations of chemical drugs. Also, a MDR phenotype can be induced by transient exposure to chemotherapeutic drugs. Chaudhary, et al. found that the expression of mdr1 mRNA in the drug-sensitive leukemia cell line K562 had been upregulated by short-term exposure to different chemotherapeutic drugs. [6] Tomida, et al. Reported mdr1 gene overexpression in drug-sensitive human colon carcinoma cell line HT-29 after transient exposure to adriamycin. [13] P-gp expression also was upregulated in gastric cancer cell after transient exposure them to VCR. [14] Our results showed that a comparatively higher level of P-gp expression was found after exposuring colon cancer cells SW480 or CoLo205 to VCR for 72 h, but the expression levels of MRP, LRP, GST-π and TOPOII were not increased.

Mitogen-activated protein kinases (MAPKs), found in all eukaryotes, are common participants in signal-transduction pathways from the membrane to the nucleus, and play an important role in cell proliferation, differentiation and apoptosis. [15] Signal-transduction pathways play critical roles in pathogenesis and progression of tumors. Good correlations among H-Ras expression levels and those of the upstream and downstream signaling proteins of EGFR, MEK and ERK were found in cases of malignancy with distant

Table 1. Drug sensitivity to VCR under the treatment of VCR/48 h or VCR+PD/48 h in SW 480/VCR and CoLo 205/VCR cells

Group	SW480	SW 480/VCR/48h	SW 480/VCR+PD/48h	CoLo 205	CoLo 205/VCR/48h	CoLo 205/VCR+PD/48h
IC_{50} (ng/ml)	115 ± 15.6	248 ± 19.6	184 ± 21.8	106 ± 11.9	215 ± 10.7	177 ± 19.4
Fold	1	2.16	1.60	1	2.03	1.67

metastasis in colorectal cancers, suggesting that Ras/Raf/MEK/ERK signaling pathways may play significant roles in carcinogenesis and progression of colorectal cancer.[16]

The activation of MAPK and expression of MDR-associated genes can be induced by anti-cancer drugs,[17] but the mechanism of their relationship is not understood. Kisucka et al. [10] found that PD098059 significantly reduced the survival of murine vincristine-resistant L1210/VCR cells with a decrease of LC_{50} to vincristine from 2.65 mmol/L to 0.67 mmol/L. Those results demonstrate that a MEK1/2 inhibitor can function as a reversal agent of VCR resistance in L1210/VCR cells, but the precise mechanism by which PD098059 modulates MDR has not as yet been resolved. In this regard, the role of ERK-mediated phosphorylation cascade should be considered. S Ding, et al. reported that PD098059 re-sensitised the Taxol- resistant human ovarian cancer cell line A1847/TX at least 20-fold. But when mdr1 cDNA was inserted into the wild-type cell line to generate a highly Taxol-resistant sub-line 1847/MDR5, MAPK kinases again became activated, which demonstrated that the increased activity of the signaling pathway in taxol-resistance lines was directly attributable to mdr1 overexpression and was not due to the effects of taxol itself. MAPK may regulate mdr1 expression, but the mechanism of action between MAPK and mdr1 needs further study. [18] Our studies show that PD098059 can reduce drug resistance and enhance the cytotoxic action of VCR in colon cancer cells.

Our study also shows that the specific MEK inhibitor, PD098059, can partially reverse drug resistance by reducing the IC_{50} of SW480/VCR and CoLo205/VCR cells, while at the same time P-gp expression was down-regulated. Chen, et al. (in press) showed, that when the gastric cancer cell line MGC803 was treated with VCR, the induction might be transduced from activation of the MAPK signaling pathway to the mdr1 gene, and the expression of mdr1. The results were directly associated with the fact that VCR was pumped out of the cells and multidrug resistance developed.

In our research, we showed that the inhibitor of MEK1/2, PD098059, could reduce mdr1 expression in the SW480 and CoLo205 cells exposed to VCR by blocking the ERK1/2 signal-transduction pathway in colon cancer, but the precise mechanism between mdr1 and the MAPK signal transduction pathway needs further study.

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