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유방암 내성 단백질 BCRP 발현 저하와 P-glycoprotein 과발현의 관계에 관한 연구

조선대학교 대학원

의 학 과

강 길

유방암 내성 단백질 BCRP 발현 저하와

P-glycoprotein 과발현의 관계에 관한 연구

Relationship between down-regulated expression of
breast cancer resistance protein and overexpression
of P-glycoprotein

2006年 2月 24日

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P-glycoprotein 과발현의 관계에 관한 연구

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이 논문을 의학박사학위 청구논문으로 제출함.

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조선대학교 대학원 의학과

P-glycoprotein (Pgp), multidrug resistance-associated protein (MRP) 및 breast cancer resistance protein (BCRP) 등의 세포막 수송체의 과발현은 암세포에 다약물 내성을 일으키는 주된 원인이 되고 있다. 본 연구는 이 수송체들의 발현이 서로 연관되어 있는지 확인하기 위하여 수행되었다. 유전자 발현은 RT-PCR방법, Western blot 및 면역화학염색법으로 분석하였다.

약물 감수성 폐암 세포주인 SK-MES-1/WT세포는 낮은 수준의 Pgp, 중등도 수준의 MRP 및 높은 수준의 BCRP를 발현하였으나, doxorubicin내성 세포주인 SK-MES-1/DX1000세포는 높은 수준의 Pgp, 중등도 수준의 MRP 및 낮은 수준의 BCRP을 발현 하였다. 본 실험에서 관찰된 SK-MES-1/DX1000세포에서 Pgp발현이 증가에 의해 BCRP 발현이 감소하는 현상이 Pgp 억제제(PSC833 및 verapamil)와 MDR1 siRNA를 이용해 Pgp발현을 knock-down시킨 방법으로 부분적으로 회복되었다. 이 결과는 오염된 클론에 의해 얻어진 것이 아니라는 것을 이중 면역화학염색법을 이용하여 공초점 현미경으로 확인하였다. 이 같은 결과는 같은 세포내에서 Pgp가 특이적으로 BCRP발현을 기능적으로 저하한다는 것을 시사한다. PSC833은 SK-MES-1/DX1000세포에서 BCRP발현 및 c-Jun의 인산화를 증가시켰다. Dominant-negative c-Jun (DN-c-Jun)의 발현은 PSC833에 의한 BCRP발현 증가를 부분적으로 차단하였다.

초록

이상의 결과는 폐암 세포주 SK-MES-1/DX1000세포에서 Pgp가 BCRP의 발현을 기능적으로 감소시키며, 이 같은 현상에 c-Jun이 적어도 부분적으로 매개한다는 최초의 증거를 제시하고 있다.

I. INTRODUCTION

The development of multidrug resistance (MDR) by tumor cells is a major obstacle to successful cancer chemotherapy. P-glycoprotein (ABCB1/MDR1, Pgp), multidrug resistance-associated protein 1 (ABCC1/mrp1, MRP1), and breast cancer resistance protein (ABCG2/bcrp, BCRP) are members of the ATP-binding-cassette (ABC) superfamily of membrane transporters (Cole et al., 1992; Maliepaard et al., 1999; Riordan and Ling, 1979). These proteins are thought to function as energy-dependent efflux pumps of a variety of structurally diverse chemotherapeutic agents, thereby decreasing their intracellular drug accumulation. Other than the fact that these resistant proteins belong to the ABC superfamily, they are quite different in terms of gene loci, amino acid sequences, structures and substrates. Although the physiologic functions of ABC transporters are not well known, they are expressed constitutively in not only tumor cells but also normal cells in the digestive system including the small intestine, large intestine, liver, and pancreas; epithelial cells in the kidneys, adrenals, brain, and testes; and endothelial cells (Bodo et al., 2003). From the aspect of the tissue distribution, ABC transporters are thought to participate in the absorption and secretion of endogenous and exogenous substances. When cancer originates not only from cells normally expressing efflux pump but also cells having genes but not expressing, gene expression is initiated due to the exposure to anticancer drugs, resulting in resistance to anticancer drugs, eventually interfering with chemotherapy.

It has been proposed that down-regulation of one ABC transporter may up-regulate others to compensate for the decreased transport activity. It was reported that Mrp1 and Mdr1b are up-regulated but Mrp2 and the sister of Pgp down-regulated in endotoxemic rat liver (Vos et al., 1998). But it is not well known about the exact mechanisms of differential expression and regulation of transporters.

In this study, I have investigated how ABC transporters such as Pgp and BCRP are related in terms of gene expression in a lung cancer cell SK-MES-1/WT and its doxorubicin-resistant cell subline SK-MES-1/DX1000.

II.   MATERIALS AND METHODS

Cell culture

The doxorubicin-resistant lung cancer cell subline SK-MES-1/DX1000 was selected from the drug-sensitive parental cell line SK-MES-1/WT after a chronic exposure to doxorubicin on an intermittent dosage schedule at sufficient time intervals to permit the expression of the resistance phenotypes. Selecting drugs were started from 1 x IC_{50} and the concentration was increased escalated at an increasing rate of 50%, and then finally the cells were cultured in fixed concentrations, 1000 ng/ml of doxorubicin.

Cytotoxicity assay

The in vitro cytotoxicity of the drugs was determined by using the MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide, Sigma, ST. Louis, MO, USA] assay (Pieters et al., 1988). 90 μl aliquots of the cells suspensions, at 2 x 10^5 cells/ml in D-MEM medium containing 10% fetal bovine serum, were seeded into a 96-well microplate which already contained 10 μl of a drug. The wells containing no drugs were used as a control of cell viability and the wells containing no cells were used for calibrating and zeroing the spectrophotometer. A stock solution of 5 mg/ml of MTT was prepared in saline and then stored at -20℃. After the cells were incubated at 37℃ or for 3 days, an aliquot of 10 μl of MTT solution was added to each well, shaken for 1 min, and the microplates were incubated for 5 hr. Formazan crystals were dissolved with dimethylsulfoxide (DMSO). The optical density of the wells was measured with a microplate reader at set at a wavelength of 540 nm. The 50% inhibitory concentration (IC_{50}) of a particular agent was defined as that drug concentration that causes a 50% reduction in the cell number versus the untreated

control. The IC_{50} values were directly determinedd directly from the semilogarithmic dose-response curves. All experiments were carried out at least in triplicate.

Functional drug accumulation assay and effects of chemosensitizers

Cell suspensions($1x10^6$cells/ml) in phosphate-buffered saline were exposed to daunorubicin (5 g/ml, Cerubidine[R], Rhone-Poulenc Rorer, Canada) with or without PSC833 (5 μM, generously given from Novartis. phama, Basel, Switzerland) at 37℃ for 30 min. Cells were subsequently analyzed for their cellular drug fluorescence by a flow cytometer (Becton Dickinson, San Jose, CA, USA), in which a focused argon laser beam (488 nm) excited cells in a laminar sheath flow and their fluorescence emissions (575 nm) were collected to generate a histogram.

Western blot analysis for BCRP and Pgp

Proteins were solubilized and then fractionated by SDS-PAGE. Western blotting was performed by a slight modification of the method first described by Towbin et al. (Towbin et al., 1992). Proteins were transferred onto a nitrocellulose membrane by electroblotting at a current of 60 V overnight. The membrane was incubated in blocking solution (5% skim milk) for 1 hr at room temperature, washed, and then incubated with primary antibodies for Pgp, MRP1 and BCRP (diluted 1:1000, Santa Cruz Biotech, CA, USA).The membrane was washed, and incubated with horseradish peroxidase-conjugated rabbit-antimouse IgG (diluted 1:2000, Amersham, NJ, USA) for 1 hr. The membrane was then stained using the detection reagent of the ECL detection kit (Amersham, NJ, USA). Protein concentration was determined with a Bio-Rad protein assay kit and standardized with bovine serum albumin.

RNA extraction and reverse transcription-polymerase chain reaction (RT-PCR) assay

The total RNA was prepared from the cells by using a RNeasy midi kit (Quiagen, Hilden, Germany). *MDR1, mrp1, bcrp* and *β-actin* mRNA transcripts were detected using the RT-PCR assay. The *MDR1* mRNA expression was detected with 3' and 5'primers corresponding to nucleotides 1179-1201 and 907-930, respectively, of the published cDNA sequence (Chen et al., 1986), yielding a 295-bp PCR product. The *MRP1* mRNA expression was detected with 3' and 5' primers corresponding to nucleotides 4551-4568 and 4180-4197, respectively, of the published cDNA sequence (Cole et al., 1992), yielding a 355-bp PCR product. The *bcrp* mRNA expression was detected with 3' and 5' primers corresponding to nucleotides 2136-2151 and 2590-2610, respectively, of the published cDNA sequence (Doyle et al., 1998), yielding a 475-bp PCR product. The β-actin expression as a control of the RNA amount was detected with 3' and 5' primers corresponding to nucleotides 2392-2412 and 1912-1932, respectively, of the published cDNA sequence (Nakajima-Iijima et al., 1985), yielding a 501-bp PCR product.

RNAs from each sample were reverse transcribed using units of Moloney murine leukemia virus reverse transcriptase (Invitrogen, CA, USA) and oligo (dT) primer for 1 hr at 37°C. The resulting cDNA were diluted 1:5 with water and then were amplified with 2.5 units of Taq polymerase (Promega, WI, USA) and 10 pmole of each primers in a GeneAmp PCR9600 (Perkin-Elmer-Cetus, CT, USA) for 21 cycles (but 15 cycles for -actin) of sequential denaturation (at 95°C for 30 s), annealing (at 53°C except at 65°C in *MDR1*), and extension (at 72°C for 30 s). After the last cycle, all PCR products were subjected to a final extension for 5 min at 72°C. For quantitation, 5 μ Ci of [α-^{32}P] dCTP were added to each reaction mixture. After PCR, PCR products were combined and then electrophoresed on 7.5% nondenaturing polyacrylamide gels. The bands were scanned with a densitometer. The amounts of each mRNA transcripts were normalized with that of *β-actin* mRNA.

Double immunochemical staining

The cells were incubated for 3 days with 10 μM of PSC833 at a chamber slide (Nunc, Roskilde, Denmark) and washed 3 times with phosphate-buffered saline (PBS), fixed and permeabilized in cold methanol-acetone solution (1:1) for 30 min and washed more than 3 times with PBS. The cells were incubated for 50 min with 1 % normal goat serum in PBS and incubated at 37℃ for 1 hour with polyclonal rabbit anti-human Pgp antibody (diluted 1:25) or, polyclonal mouse anti-human BCRP antibody (diluted 1:25) containing 0.1% Triton X-100 in a humid chamber at room temperature. After the cells were washed 3 times with PBS, the cells were incubated with fluorescence isothiocyanate (FITC) conjugated goat anti-rabbit or alexa fluor 488 goat anti-mouse IgG at 37℃ for 1 hr, respectively. The cells were washed 2 times with PBS, once with water and then observed on an Olympus IX70 FV300 confocal microscope (Ina, Japan) equipped with the appropriate optics.

Down-regulation of Pgp expression using siRNA

MDR1 siRNA commercial obtained from Sant Cruz (CA, USA) is a target-specific 20-25nt siRNA. The SK-MES-1/DX1000 cells were washed three times with PBS and transfected with the siRNA at the final concentration of 10 μM by using Lipofectamine reagent (Invitrogen, CA, USA) according to the manufacturer's protocol. After 30 hr incubation, cells were rinsed with PBS, treated with PSC833, and incubated for the indicated time.

Construction of plasmids and transfection of dominant negative c-jun (DN-c-jun)

Construction of a mammalian expression vector for the DN-c-jun was previously described (Ioroi et al., 2003). The mammalian expression vector containing DN-c-Jun-EGFP was prepared by PCR amplification using the NH_2-terminal primer 5'-CTTCGAATTCTGATGACTAGCCAGAACACGCTG-3' containing Eco*RI* site and the

COOH-terminal primer 5'-GACCGGTGGATCCCGAAATGTTTGCAACTGCTGCGTTAG -3' containing a Bam*H*/ site using the pEF-BOS-DN-c-Jun as template. The amplification product was gel purified, digested with the restriction endonucleases, Eco*R*/ and Bam*H*/, and ligated into a similarly digested pEGFP-N1 (Clontech Laboratories, Inc, CA, USA) to produce the pDN-c-Jun-EGFP-N1 expression plasmid (Fig. 1). Cells were transfected with the vector with or without insert, using Lipofectamine reagent (Invitrogen, CA, USA) according to the manufacturer's protocol. After 24 h of incubation, cells were rinsed with PBS, treated with PSC833.

Statistical analysis

   Statistical significance of the data was determined by the Student's t-test. P-values less than 0.05 were taken as statistically significant.

III. RESULTS

Selection of doxorubicin-resistant SK-MES-1 subline

  Doxorubicin-resistant lung cancer cells were selected from the drug-sensitive lung cancer cells (SK-MES-1/WT) by chronic exposure to gradually increasing concentrations of doxorubicin from 100 ng/ml (IC_{50} value) to 1000 ng/ml on an intermittent schedule. The doxorubicin-resistant SK-MES-1 subline was clonally selected and designated SK-MES-1/DX1000. The SK-MES-1/DX1000 cells appeared to be apparently similar to the drug-sensitive parental cell in cell size. But they showed slightly increased doubling times, as compared to 4 days of the SK-MES-1/WT cells. The SK-MES-1/DX1000 cells showed resistance not only to a selecting drug doxorubicin (66.6-fold) but also to paclitaxel (172.1-fold), vincristine (20.3-fold) and mitoxantrone (5.3-fold) as compared with the SK-MES-1/WT cells (Fig. 2, Table 1). Considering the MDR phenotype and the substrate specificity, the SK-MES-1/DX1000 cells has been shown to overexpress Pgp.

Expression and functional analyses of two transporters in the SK-MES-1/DX1000 cells

  Western blot analysis was carried out to confirm which resistance mechanism is involved in the SK-MES-1/DX1000 cells. The RT-PCR assay and Western blot analysis showed that the gene expression levels of ABC transporters in the SK-MES-1/WT are Pgp (+/-), MRP (++), and BCRP (+++) whereas those in the SK-MES-1/DX1000 are Pgp (+++), MRP (++), and BCRP(+) (Fig 3). MRP data were not shown in this study. The increased amount of proteins related to drug resistance was often but not always due to increased mRNA resulting from gene amplification. In this study, Southern blot analysis was not performed to examine the gene copy number of each gene. Instead, to test whether the altered levels of the transporter

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proteins were correlated with the amounts of each mRNA, RT-PCR assay was performed. The steady-state level of *MDR1* or *bcrp* mRNA was correlated with that of each protein (Fig. 3). These results suggest that altered expression of Pgp and BCRP takes place at the transcriptional level. Gene amplification of *MDR1* in the SK-MES-1/DX1000 cells remains to be determined. It is of interest that the BCRP expression is down-regulated by overexpression of Pgp in the SK-MES-1/DX1000 cells, which may be responsible for the low level of resistance to metoxantrone, a well-known good substrate for BCRP.

The effects of PSC833, a potent Pgp inhibitor, on the growth of the SK-MES-1/DX1000 cells in the presence of 1000 ng/ml doxorubicin, and the daunorubicin accumulation of the SK-MES-1/WT and SK-MES-1/DX1000 cells were investigated. PSC833 reversed the resistance of the SK-MES-1/DX1000 cells to doxorubicin (Fig 4A). In addition, intracellular accumulation of daunorubicin in SK-MES-1/DX1000 was increased by 5 μM PSC833 (Fig. 4B). The effectiveness of Pgp inhibitors on both chemosensitivity and drug accumulation in the SK-MES-1/DX1000 cells suggests the functional involvement of Pgp as an MDR mechanism.

Effects of Pgp inhibition on the activity and expression of BCRP in the SK-MES-1/DX1000 cells

To determine whether Pgp is involved in the down-regulated expression of BCRP in the SK-MES-1/DX1000 cells, Pgp inhibitors (PSC833 and verapamil) and *MDR1* siRNA were used to inhibit the activity and expression of Pgp. PSC833 not only inhibit the function of Pgp but also decreased the levels of *MDR1* mRNA and Pgp (Fig. 5), which may account for its strong Pgp inhibition. It also concomitantly increased the steady-state level of *bcrp* mRNA and BCRP in the SK-MES-1/DX1000 cells (Fig. 5). This result suggests that the down-regulated expression of BCRP in

the SK-MES-1/DX1000 cells was partly restored by treatment with PSC833. Verapamil, another Pgp inhibitor, showed the similarresults as showed in PSC833 treatment although it increased steady-state level of *MDR1* mRNA (Fig. 5). This result suggests that Pgp plays a functionally important role in BCRP expression.

To rule out a possibility that the down-regulated expression of BCRP with increased expression of Pgp in SK-MES-1/DX1000 was due to contaminated clones, double immunohistochemical staining was carried out. The confocal microscopy showed the same result to Western blot and RT-PCR analyses (Fig. 6). This result demonstrates that the down-regulated BCRP expression by overexpression of Pgp occur not in the different clones but in the same clone.

To exclude a possibility of unknown effects of Pgp-inhibiting drugs, an *MDR1* siRNA experiment was carried out. *MDR1* siRNA was transfected into the SK-MES-1/DX1000 cells and its expression was determined on 1 and 3 days using RT-PCR and Western blot analyses. *MDR1* siRNA decreased mRNA and protein levels of Pgp in the SK-MES-1/DX1000 cells by the expression level of the SK-MES-1/WT cells, but increased those of *bcrp* mRNA and BCRP on 3 days after transfection (Fig. 7). This result suggests strongly that Pgp induces the down-regulated expression of BCRP in a Pgp-specific manner.

Involvement of c-Jun in PSC833-induced expression of BCRP in the SK-MES-1/DX1000 cells

One of the most relevant signaling pathways involved in stress, inflammation and apoptosis is the mitogen-activated protein kinase (MAPK), a family of serine/threonine kinases. Three major MAPKs, c-Jun-N-terminal kinase (JNK), p38 and extracellular signal-regulated kinase (ERK), have been shown to regulate apoptosis (Chuang et al., 2000; Kyriakis and Avruch, 2001). In this study, there were no changes in the expression and phosphorylation of c-Jun between the

SK-MES-1/WT and the SK-MES-1/DX1000 cells (Fig. 8). But, PSC833 increased the levels of phosphorylated c-Jun (p-c-Jun) as well as c-Jun in the both cells. Since PSC833 increased BCRP expression, it was tested whether significant increase of p-c-Jun by PSC833 was involved in BCRP expression. To inhibit activity of endogenous c-Jun, dominant-negative c-Jun (DN-c-Jun) was transfected. The DN-c-Jun was generated by deleting the transactivational domain of amino acids 3 -122 of the wild-type c-Jun by PCR (Ioroi et al., 2003). The DN-c-Jun gene was ligated into pEGFP-NI plasmid, producing the pDN-c-Jun-EGFP-N1. Transfection of pDN-c-Jun-EGFP-N1 partly inhibited PSC833-induced expression of BCRP, suggesting the involvement of c-Jun in this event (Fig. 9).
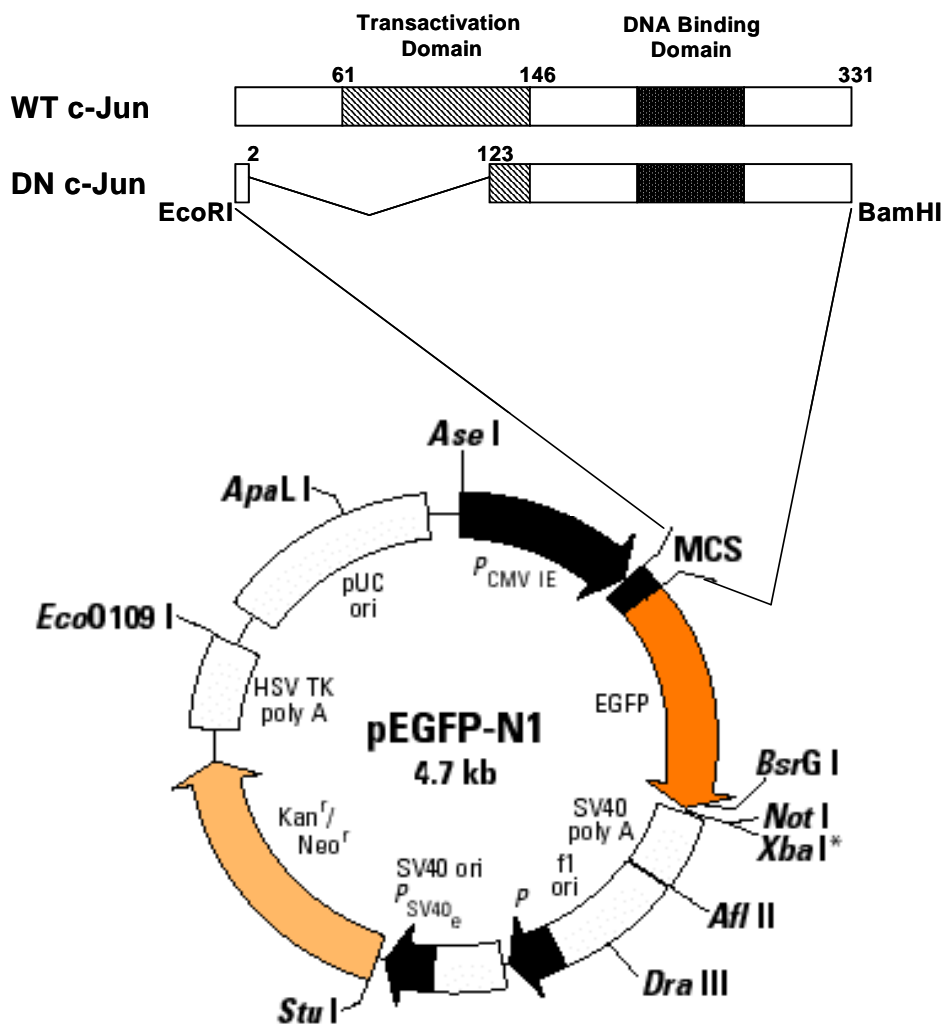
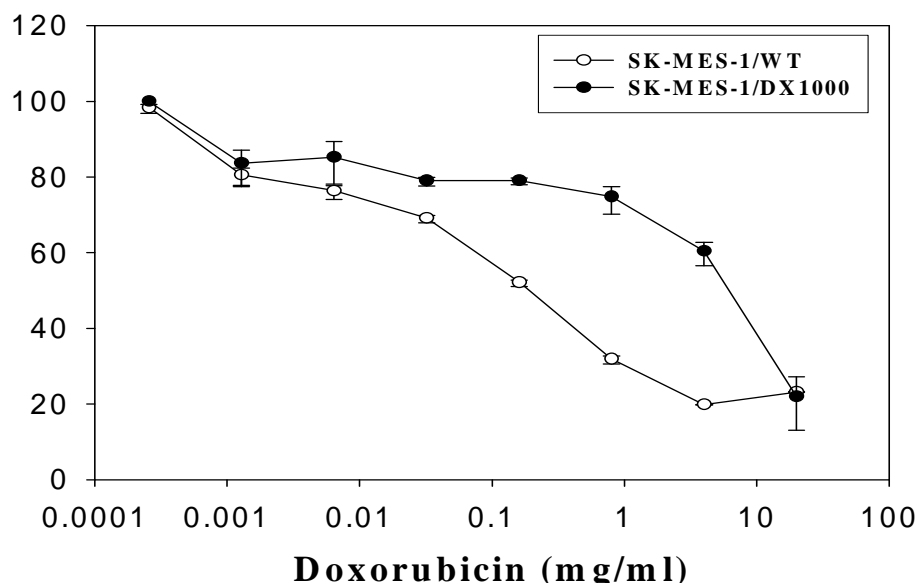**FIG. 1.** Scheme of pDN-c-Jun-EGFP-N1 expression plasmid.

FIG. 2. Cytotoxicity of doxorubicin in the parental drug-sensitive cell line SK-MES-1/WT and its doxorubicin-resistant subline SK-MES-1/DX1000. WT, wild-type.
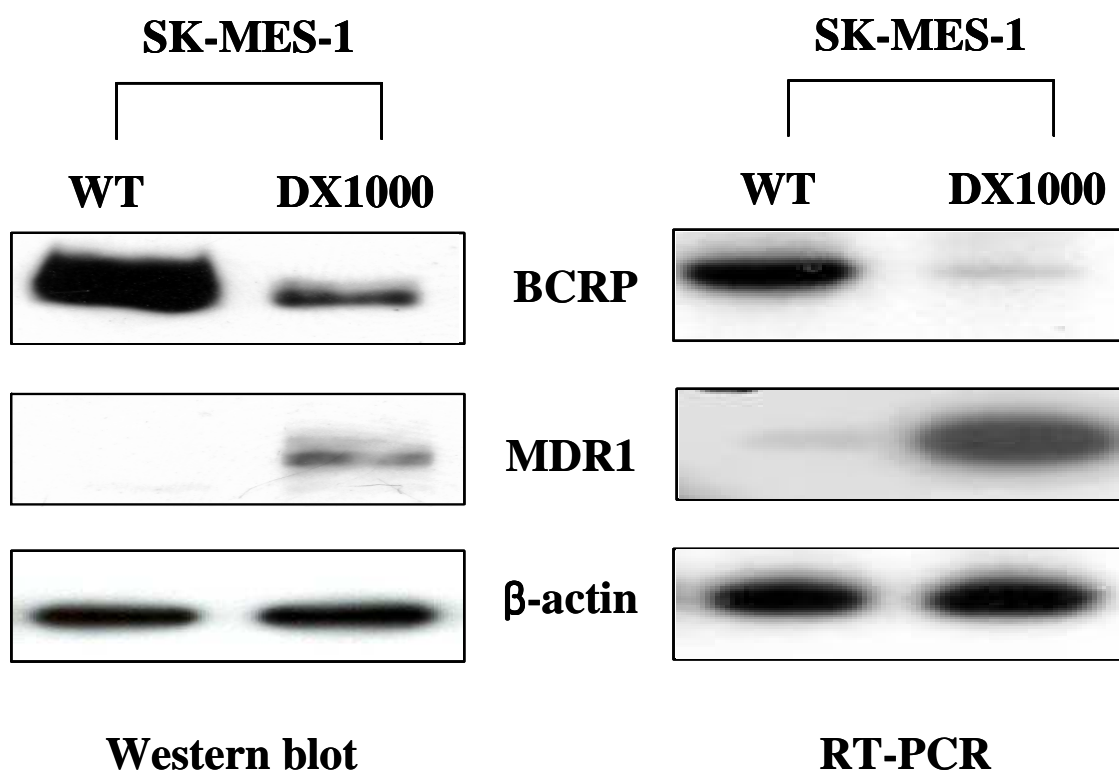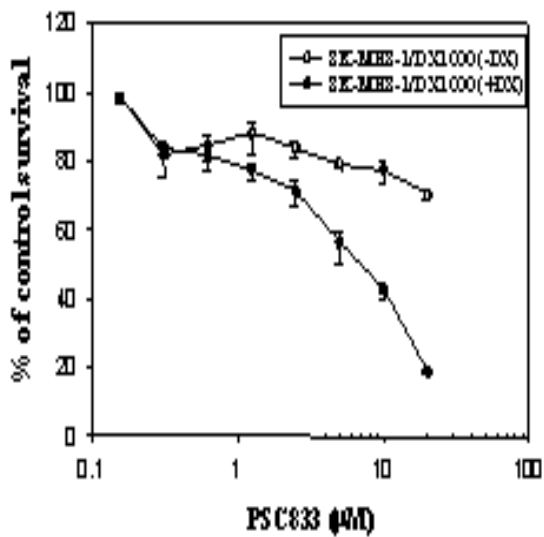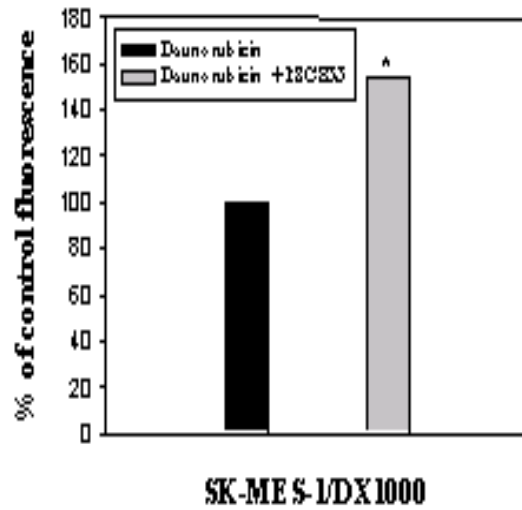
FIG. 3. Expression profiles of MDR1 and BCRP of the SK-MES-1/WT and SK-MES-1/DX1000 cells.

(A)

(B)

FIG. 4. The effects of PSC833 on the growth and daunorubicin accumulation of the SK-MES-1/WT and SK-MES-1/DX1000 cells. (A) PSC833 was incubated with SK-MES-1/DX1000 cells in a microplate containing 1000 ng/ml doxorubicin for 3 days and then MTT assay was performed. (B) Cell suspensions (1×10^6/ml) in PBS were exposed to daunorubicin (3 μg/ml) with or without PSC833 (5 μM) at 37℃ for 30 min and then fluorescence was determined by a flow cytometer. Control represents mean fluorescence in the absence of PSC833. DX, doxorubicin.
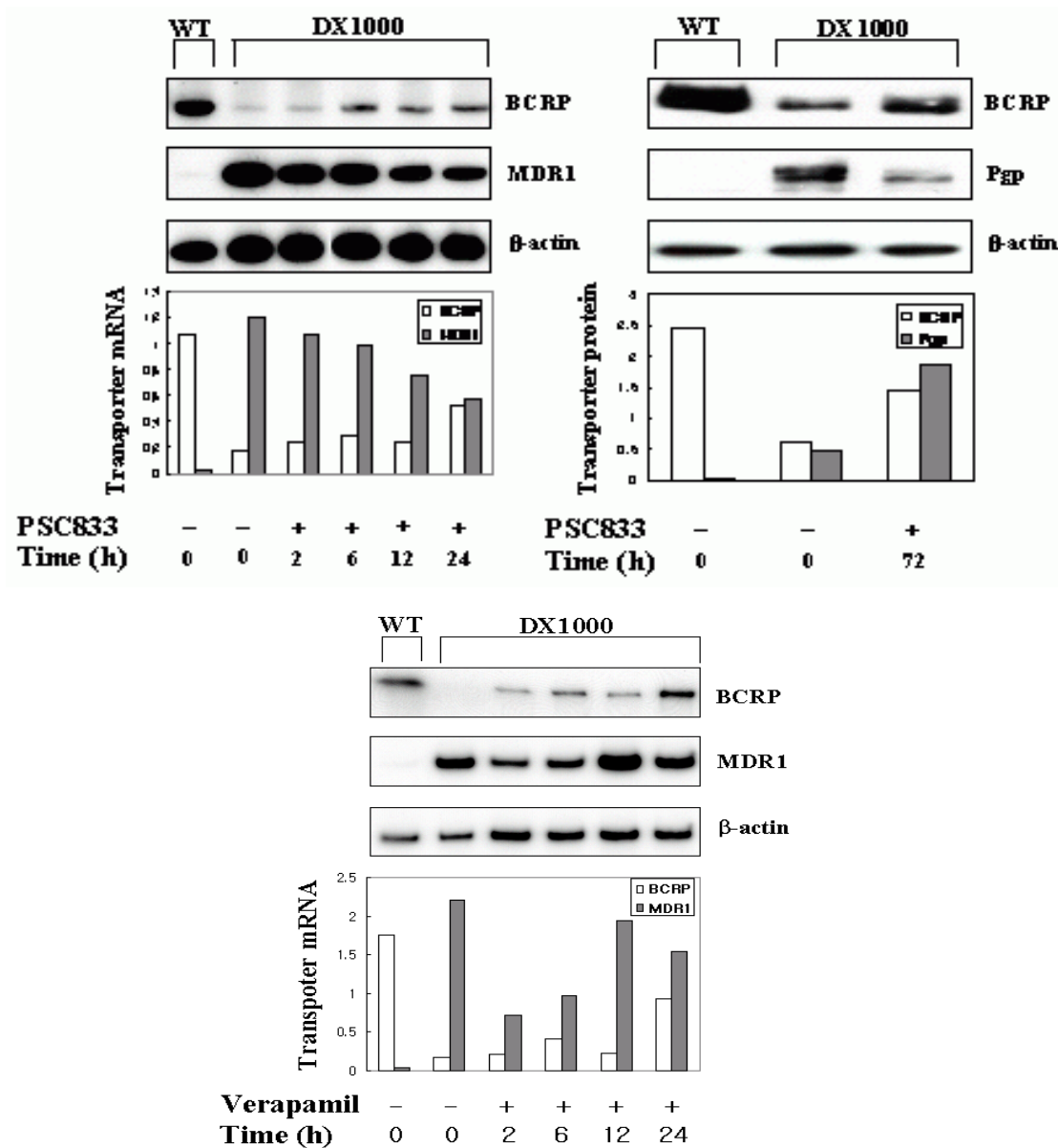
FIG. 5. The effects of Pgp inhibitors in expression of BCRP. Cells treated with PSC833 or verapamil were analyzed by RT-PCR and Western blotting assays. Steady-state mRNA and protein levels were normalized by each β-actin level and then expressed as ratio values on Y axis.
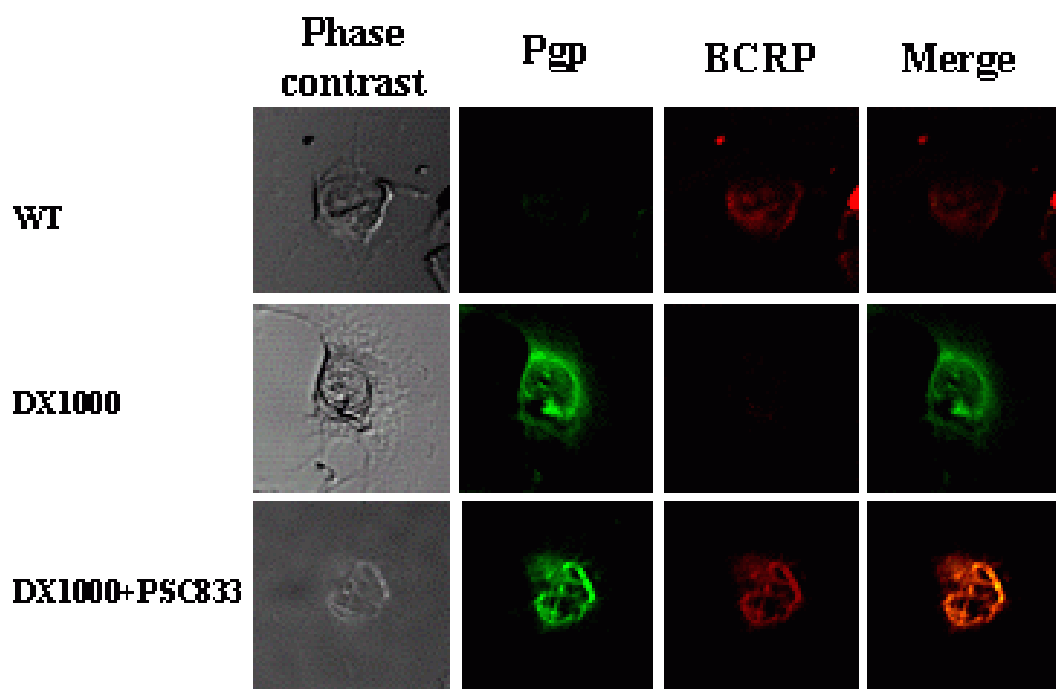
FIG. 6. Double immunochemical staining for Pgp and BCRP in SK-MES-1/WT and SK-MES-1/DX1000 cells.
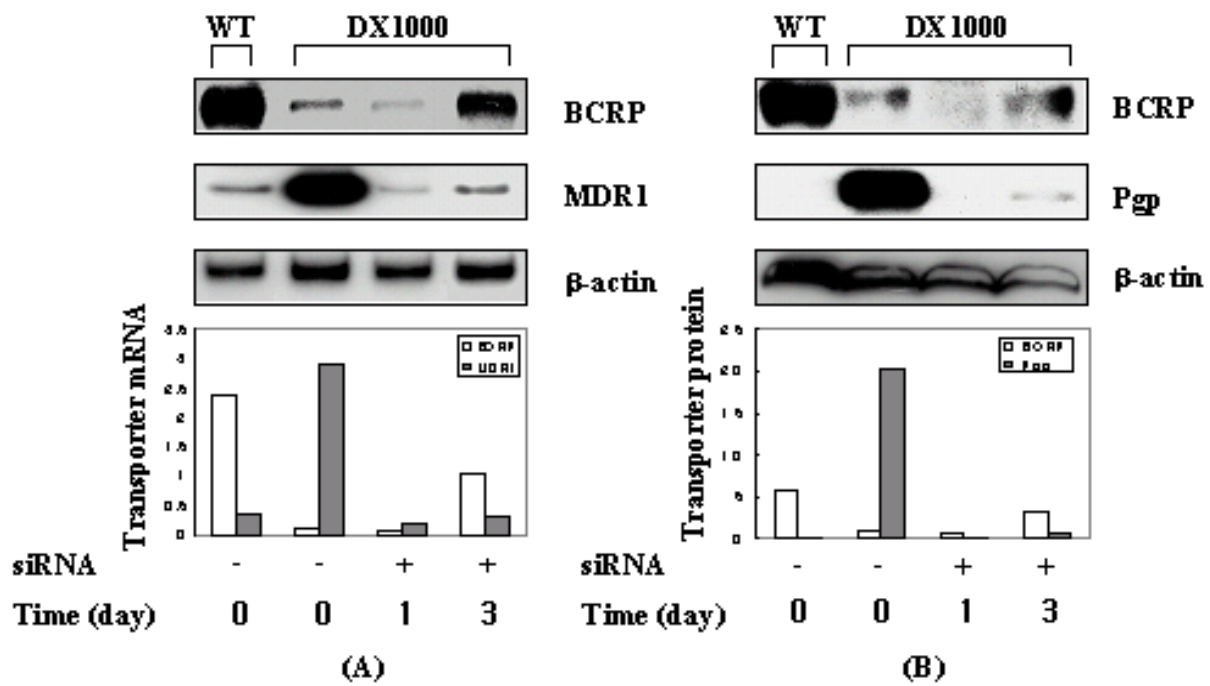
FIG. 7. The effects of *MDR1* siRNA on BCRP expression in the SK-MES-1/DX1000 cells. Steady state mRNA (A) and Protein (B) levels were normalized by β-actin and then expressed as ratio values on Y axis.
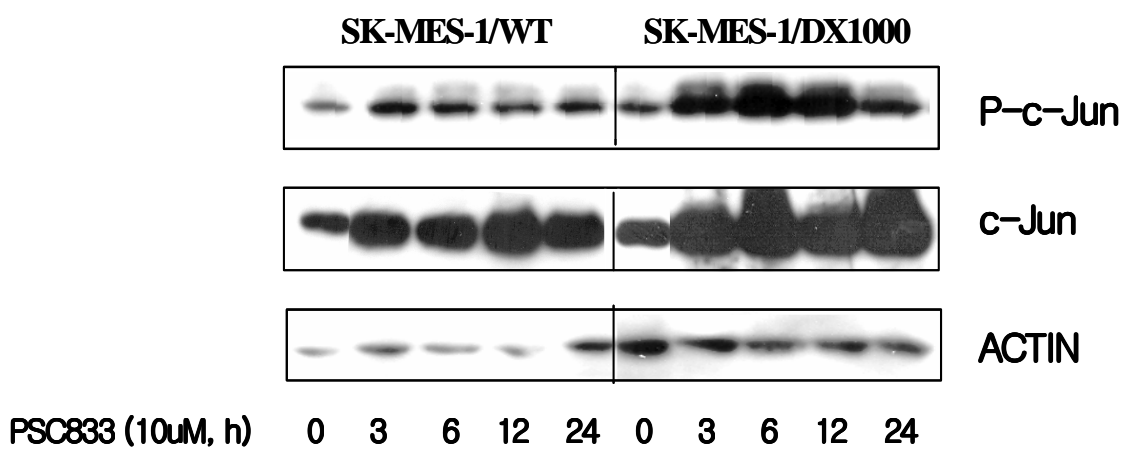
FIG. 8. Phosphorylation and expression of c-Jun in the SK-MES-1/WT and SK-MES-1/DX1000 cells.
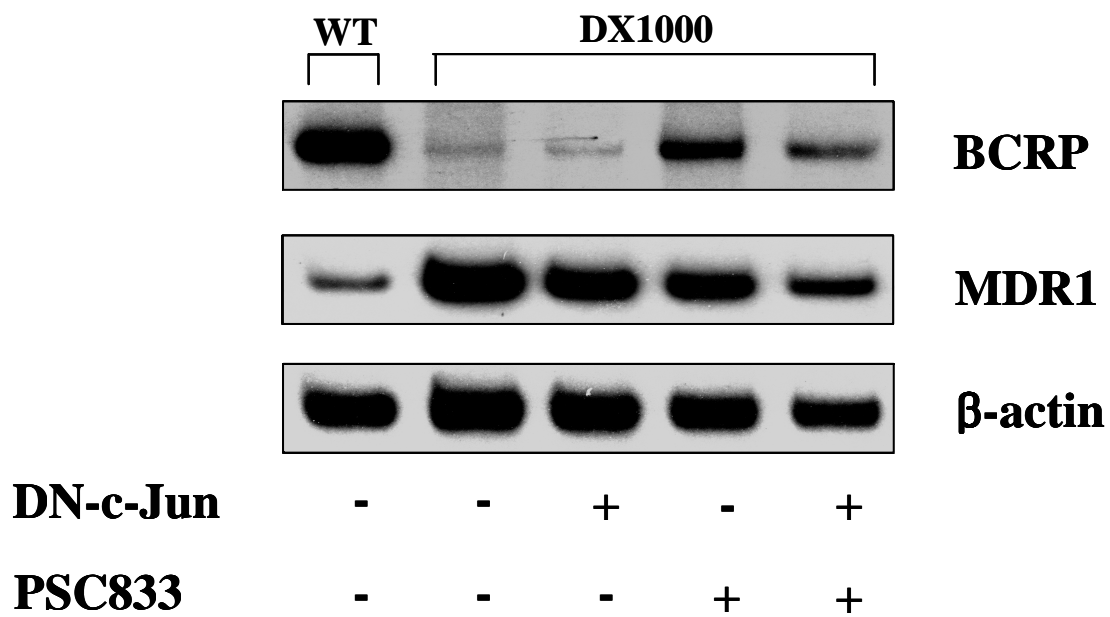
Fig. 9. Involvement of c-Jun in expression of BCRP by PSC833 in the SK-MES-1/DX1000 cells. The effect of dominant negative c-Jun (DN-c-Jun) on PSC833-induced BCRP expression. The SK-MES-1/DX1000 cells were treated with 10 μM of PSC833 following transient transfection of DN-c-Jun vector. Levels of *bcrp* and *MDR1* mRNAs were determined by the RT-PCR assay.

Table 1. Cytotoxicity of doxorubicin in the parental drug-sensitive cell line SK-MES-1/WT and its doxorubicin-resistant subline SK-MES-1/DX1000

	IC_{50} (μg/ml)		Relative resistance (fold)
	SK-MES-1/WT	**SK-MES-1/DX1000**	
Doxorubicin	0.095	6.33	66.6
Paclitaxel	6.06	1040	172.1
Vincristine	0.41	8.22	20.0
Mitoxantrone	0.08	0.42	5.3

IV. DISCUSSION

MDR is one of the major causes in the failure of clinical chemotherapy of cancer patients. There is no exception in lung cancer which shows still poor prognosis, at least in part, due to MDR. Overexpression of efflux pumps such as Pgp, MRP and BCRP located in the plasma membrane is involved in MDR. In order to determine how the expression of these ABC transporters can be linked each other, the expression of three transporters were determined in the doxorubicin-resistant lung cancer cell subline SK-MES-1/DX1000 selected from its drug-sensitive cell line SK-MES-1/WT. The SK-MES-1/WT cells expressed the low level of Pgp, the intermediate level of MRP and the high level of BCRP whereas the SK-MES-1/DX1000 cells showed overexpression of Pgp and down-regulated expression of BCRP but no change in the level of MRP. These results mean overexpression of Pgp and down-regulated expression of BCRP, but no change in the level of MRP in the SK-MES-1/DX1000 cells, suggesting an inversely related possibility between Pgp and BCRP expression. The confocal microscopy using double immunohistochemical staining of Pgp and BCRP also showed the inversely relation between Pgp and BCRP, indicating that this finding was not due to the contamination of clones differently selected. It is a first report in cancer cells that overexpression of Pgp can down-reglate BCRP. This result is strongly supported by a recent report that the defective Pgp in the mutant *mdr1a* (-/-) mice is associated with increased *bcrp* mRNA at the mouse blood-brain barrier (Cisternino et al., 2004). In this study, a potent Pgp inhibitor PSC833 in the SK-MES-1/DX1000 cells not only decreased expression of Pgp but also concomitantly increased BCRP expression. Another Pgp inhibitor verapamil showed the same result obtained by PSC833 as well. The cotreatment of PSC833 with epirubicin prevented the increase in Pgp expression induced by epirubicin alone in a dose-dependent manner although PSC833 alone had any significant effect on the Pgp expression (Hu et al., 1996). The Pgp antagonists such as verapamil, nifedipine, and cyclosporin A increased Pgp

expression in the human colon carcinoma cell line and its drug-resistant sublines (Herzog et al., 1993). Although PSC833 and verapamil have opposite effects on the expression of Pgp, both inhibitors increased the BCRP expression down-regulated by overexpression of Pgp in the SK-MES-1/DX1000 cells,suggesting that Pgp could play a functionally important role in BCRP expression. In addition, the knock-down expression of Pgp by transfection of *MDR1* siRNA also increased BCRP expression. This result supports that the increased level of BCRP by Pgp inhibitors in the SK-MES-1/DX1000 cells is not due to unknown other effects of Pgp-inhibiting drugs but a Pgp-specific mechanism. So far, a couple of reports have suggested that the ABC transporters could be linked in term of gene expression. The change in the MRP level was inversely correlated with Pgp in childhood neuroblastoma that had been treated with retinoic acid (Bordow et al., 1994). Examination of early passages of human small cell lung cancer H69/VP cells showed that overexpression of MRP occurred prior to Pgp although both protein was coexpressed at any time (Brock et al., 1995). In human colon carcinoma cells, tumor necrosis factor-alpha influences MRP and LRP gene expression in opposite ways (Stein et al., 1997). Overexpression of MRP was inversely related to that of Pgp in the doxorubicin-resistant AML sublines in a concentration-dependent manner (Choi et al., 1999). Expression of MRP and Pgp did not correlate positively in primary oral squamous cell carcinoma (Friedrich et al., 2004). In addition, BCRP and MRP2 transcripts were more abundant in jejunum than MDR1 transcripts. However, BCRP exhibited a 100-fold lower transcript level in Caco-2 cells compared with jejunum (Taipalensuu et al., 2001). On the other hand, there have been reports about positive correlation between the expression of transporters. There was a significant correlation between *bcrp* and *MDR1* mRNA expression in 51 AML patients (Galimberti et al., 2004). The expressions of *MDR1* and *mrp1* genes were correlated with each other in human breast carcinoma (Kanzaki et al., 2001). Expression of Pgp and BCRP moderately positively correlated in primary oral squamous cell carcinoma.(Friedrich et al., 2004). Intergrating these reports, it has been shown that the expression of ABC membrane transporters and their relations could vary according to the type of cells as well as

drug concentrations.

How does Pgp down-regulate BCRP expression? It could be hypothesized that Pgp might act as an efflux pump of any inducer(s) of ABC transporters. Endogenous substrates for Pgp include corticosterone (Wolf and Horwitz, 1992), beta-estradiol 17beta-D-glucuronide, an endogenous cholestatic metabolite of estradiol (Liu et al., 1996), glutamate (Liu and Liu, 2001) and endorphin (King et al., 2001). It was also recently reported that Pgp has the function of removing beta-amyloid, which was reported as the causal substance of Alzheimer's disease (Lam et al., 2001). It remains to be determined what molecules as Pgp substates are involved in the BCRP expression.

In this study, the involvement of the MAPK pathways in BCRP and Pgp expression was tested. The MAPK members include JNK, p38 and ERK pathways. Signaling through the JNK and P38 pathways is associated with induction of apoptosis whereas the ERK pathway promote cell growth and survival (Papa et al., 2004). A Pgp inhibitor PSC833 not only inhibited the function of Pgp but also decreased Pgp expression in the SK-MES-1/DX1000 cells. It is of interest that PSC833 increased the levels of p-c-Jun as well as expression of c-Jun in the SK-MES-1/WT and /DX1000 cells. Since p-c-Jun itself can induce c-Jin expression (Dunn et al., 2002), it appeared that increased level of c-Jun by PSC833 may be due to increased level of p-c-Jun. In addition, since it has demonstrated that c-Jun activation down-regulates Pgp expression in human MDR cells (Miao and Ding, 2003), PSC833 has been shown to down-regulate Pgp expression through increased level of p-c-Jun in the SK-MES-1/1000 cells. PSC833 also increased BCRP expression in the SK-MES-1/DX1000 cells. It was therefore tested whether significant increase of p-c-Jun by PSC833 induced BCRP expression. Expression of DN-c-Jun inhibited partly PSC833-induced expression of BCRP, suggesting the involvement of c-Jun in this event in the SK-MES-1/DX1000 cells.

Overall, these results provide the first evidence that Pgp can functionally down-regulate BCRP expression in the SK-MES-1/DX1000 cells, which is at least in part mediated by c-Jun activation.

Ⅴ. SUMMARY & CONCLUSION

Overexpression of membrane transporters including P-glycoprotein (Pgp), multidrug resistance-associated protein (MRP), and breast cancer resistance protein (BCRP) confer multidrug resistance to tumor cells. This study was attempted to examine if the expression of these transporters would be linked each other. The gene expression was determined by RT-PCR and Western blot analyses. The SK-MES-1/WT cells expressed the low level of Pgp, the intermediate level of MRP and the high level of BCRP whereas the SK-MES-1/DX1000 cells showed overexpression of Pgp and down-regulated expression of BCRP but no change in the level of MRP. The down-regulated expression of BCRP by overexpression of Pgp in the SK-MES-1/DX1000 cells was partially restored by treatment with Pgp inhibitors (PSC833 and verapamil) or the knock-down of Pgp expression using siRNA. This result suggests strongly that Pgp can functionally down-regulate expression of BCRP in a Pgp-specific manner in the same cell, which is confirmed by confocal microscopy using double immunohistochemical staining. PSC833 increased BCRP expression as well as c-Jun phosphorylation in the SK-MES-1/DX1000 cells. Expression of dominant-negative c-Jun (DN-c-Jun) inhibited partly PSC833-induced expression of BCRP, suggesting the involvement of c-Jun in this event.

Overall, these results provide the first evidence that Pgp can functionally down-regulate BCRP expression in the SK-MES-1/DX1000 cells in Pgp-specific manner, which is at least in part mediated by c-Jun activation.

VI. ACKNOWLEDGEMENTS

VII REFERENCES

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