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The Association of Increased Lung Resistance Protein Expression with Acquired Etoposide Resistance in Human H460 Lung Cancer Cell Lines

Eunmyong Lee and Soo-Jeong Lim

Department of Bioscience and Biotechnology, Sejong University, Seoul, Korea

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Chemoresistance remains the major obstacle to successful therapy of cancer. In order to understand the mechanism of multidrug resistance (MDR) that is frequently observed in lung cancer patients, here we studied the contribution of MDR-related proteins by establishing lung cancer cell lines with acquired resistance against etoposide. We found that human H460 lung cancer cells responded to etoposide more sensitively than A549 cells. Among MDR-related proteins, the expression of p-glycoprotein (Pgp) and lung resistance protein (LRP) were much higher in A549 cells compared with that in H460 cells. When we established H460-R1 and -R2 cell lines by progressive exposure of H460 cells to increasing doses of etoposide, the response against etoposide as well as doxorubicin was greatly reduced in R1 and R2 cells, suggesting MDR induction. Induction of MDR was not accompanied by a decrease in the intracellular accumulation of etoposide and the expression of MDR-related proteins that function as drug efflux pumps such as Pgp and MRP1 was not changed. We found that the acquired resistance paralleled an increased expression of LRP in H460 cells. Taken together, our data suggest the implicative role of LRP in mediating MDR in lung cancer.

Key words: Multidrug resistance, Lung resistance protein, Etoposide, Lung cancer

INTRODUCTION

Intrinsic or acquired resistance to chemotherapeutic drugs is one of the major obstacles to effective cancer treatment. Almost all non-small cell lung cancer (NSCLC) patients display intrinsic chemoresistance, generally limiting the chance of successful chemotherapy (Ihde and Minna, 1991). The most frequent form of resistance observed in lung cancer patients is multidrug resistance (MDR), which is characterized by cross-resistance to a wide variety of structurally unrelated drugs, including the anthracyclines, some vinca alkaloids, and the epipodophyllotoxins (Borst *et al.*, 2000). Several mechanisms of MDR in lung cancer have been identified, including the overexpression of the ATP-binding cassette (ABC) superfamily of transporters, which function as energy-dependent pumps to extrude anticancer drugs from cancer cells (Borst *et al.*, 2000). P-glycoprotein (Pgp) and the multidrug resistance-associated protein 1 (MRP1) are two of the most extensively studied ABC transporters and have been shown to actively transport a wide variety of cytotoxic agents. In NSCLC patients, overexpression of MRP1 is frequently observed and has been correlated inversely with chemosensitivity against diverse anticancer drugs (Young *et al.*, 2001). Breast cancer resistance protein (BCRP), another member of ABC family, was recently found to be frequently overexpressed in many types of cancer patients (Sugimoto *et al.*, 2005). BCRP confers high levels of resistance to mitoxantrone as well as to the anthracyclines, the camptothecins and topotecan (Zhang *et al.*, 2005).

In 1993, Scheper *et al* first described lung resistance protein (LRP) in an lung cancer cell line selected for resistance against doxorubicin (Scheper *et al.*, 1993). LRP expression was found to and correlates with resistance to cisplatin in lung cancer (Berger *et al.*, 2000). Evidence that LRP is involved in exhibiting MDR phenotype was found in a variety types of cancers including NSCLC, astrocytic brain tumor, acute myeloid leukaemia and B-cell lymphoma (Berger *et al.*, 2001; Filipits *et al.*, 1998;

Correspondence to: Soo-Jeong Lim, Department of Bioscience and Biotechnology, Sejong University, 98 Kunja-dong, Kwangjin-gu, Seoul 143-747, Korea
Tel: 82-2-3408-3767, Fax: 82-2-3408-3334
E-mail: sjlim@sejong.ac.kr

Ohsawa *et al.*, 2005; Rybarova *et al.*, 2004). Since its original description, LRP has been identified as the major vault protein (Mossink *et al.*, 2003). Vaults are complex ribonucleoprotein particles containing at least two high molecular weight proteins and a small RNA molecule, in addition to the 110 kd major vault protein. LRP does not belong to the ABC-transporter family and the precise role of LRP in MDR-phenotype is unclear (Mossink *et al.*, 2003). The entrapment of drugs into vesicular compartments, resulting in decreased nuclear/cytoplasmic ratios, have been observed in some MDR cell lines overexpressing LRP (Izquierdo *et al.*, 1996), suggesting that LRP may block the nuclear transport of substrate drugs.

In the present study, we aimed to investigate the contribution of MDR-related proteins in lung cancer by establishing lung cancer cell lines with acquired resistance against etoposide, one of widely used chemotherapeutic drugs.

MATERIALS AND METHODS

Materials

Etoposide and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) were obtained from Sigma (St. Louis, MO, USA). Doxorubicin was obtained from LKT Laboratories (Minneapolis, MN). Antibodies to human LRP were purchased from Chemicon International (Temecula, CA, U.S.A.). Anti-human b-actin antibodies were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, U.S.A.). All other chemicals were of reagent grade and used without further purification.

Cell lines and cultures

The human H460 and A549 NSCLC cell lines were purchased from the American Type Culture Collection (ATCC, Manassas, VA, U.S.A.). The cell lines were cultured in RPMI 1640 medium, each supplemented with 10% heat-inactivated fetal bovine serum (FBS) (Hyclone, Logan, UT, U.S.A.) and 100 units/mL each of penicillin and streptomycin. To develop chemoresistance to etoposide, H460 cells were exposed to progressively increasing concentrations of etoposide for 6 months. Two etoposide-resistant cell lines were established: R1 was cultured in presence of etoposide up to 2 μM and R2 was cultured up to 16 mM. Resistant cell lines were then maintained in the indicated concentration of etoposide.

Cytotoxicity assay

Cells were seeded into 96-well plates. The next day varying doses of etoposide or doxorubicin was added, and the cells were incubated for 72 h, after which cell growth and viability were measured using MTT. The ability of cells to form formazan crystals by active mitochondrial respiration was determined using a microplate spectrophotometer (Molecular Devices) after dissolving the crystals in DMSO (Lee *et al.*, 2003).

MRP1, Pgp, BCRP and LRP expression

Total cellular RNA was isolated from cells using a Qiagen RNeasy mini kit (Qiagen, Santa Clarita, CA). Single-stranded oligo(dT)-primed cDNA was generated from 2 μg RNA in a 25 μL reaction using Moloney murine leukemia virus reverse transcriptase (Promega, Madison, WI, U.S.A.). Primer sequences used for analysis of human MRP1, Pgp, BCRP and LRP are summarized in Table I. The amplification reaction was carried out with 2 μL cDNA product for 27 cycles, and each cycle consisted of 94°C for 45 s, 57°C for 50 s, and 72°C for 50 sec, followed by a final 1 min elongation at 72°C. The final PCR products were electrophoresed in 1% agarose gel.

Cellular accumulation of etoposide

Cells plated in 100 cm^2 dishes were treated with media containing 20 μM of etoposide. At 1-h post-incubation, cells were harvested, washed three times to remove extracellular VP-16. With cell lysates obtained, concentrations of VP-16 were analyzed by the reported HPLC method (Aita *et al.*, 1999; Kang *et al.*, 2005) with a slight modification. Diazepam was used as an internal standard and samples were extracted using a liquid-liquid extraction process. The HPLC system consisted of a variable wavelength UV detector (UV/VIS 151, Gilson, Middleton, WI, U.S.A.), HPLC pump (Model 305/306, Gilson), sampling injector (Model 231 XL, Gilson), syringe pump (Model 402, Gilson), dynamic mixer (Model 811D, Gilson), column heating control system (CH-500, Eppendorf, Westbury, NY, USA) and operation software (UniPoint™ 3.0 for Window, Gilson). The separation was achieved using a reversed phase (C_{18}) column (Nucleosil 100-5; 25 cm, *I* × 4.6 mm, i.d.; particle size, 5 μm, Macherey-Nagel, MN, U.S.A.). The mobile phase, acetonitrile : water : glacial acetic acid (35:61:1, v/v/v) were run at a flow rate of 1 mL/

Table I. RT-PCR primer sequences for MRP1, PGP, BCRP and LRP mRNA amplication

	Primer sequences (5'→ 3')	PCR product sizes
MRP1	Sense: CGTGTACTCCAACGCTGAC Antisense: CTGGACCGCTGACGCCCGTGAC	325 bp
PGP	Sense: GGAAGCCAATGCCTATGACTTTA Antisense: GAACCACTGCTTCGCTTTCTG	193 bp
BCRP	Sense: TGGCTGTCATGGCTTCAGTA Antisense: GCCACGTGATTCTTCCACAA	205 bp
LRP	Sense: TGGCTTTGAGACCTCGGAAG Antisense: TCCAGTCTCTGAGCCTCATGC	230 bp

min and the column effluent was monitored by UV detector set at 254 nm. The retention times for VP-16 and diazepam were approximately 5 and 20 min, respectively. Quantitation was based on the internal standard method using ratios of peak areas of VP-16 and diazepam and a calibration curve for concentrations ranging between 0.1 and 2 μg/mL, with the limit of sensitivity being 0.1 μg/mL. All results were normalized to cellular protein content.

Immunoblotting

Treated cells were scraped from the culture, washed twice with PBS and incubated for 15-30 min on ice in lysis buffer containing 150 mM NaCl, 10 mM Tris, 0.2% Triton X-100, 0.3% NP-40, 0.2 mM Na_3VO_4 and protease inhibitors (pH 7.4) (Roche). After centrifugation at 16,000 g for 15 min at 4°C, supernatants were collected and the protein concentration in each was measured by the Bradford method. Aliquots of supernatants containing equal amounts of protein were boiled in SDS-reducing buffer for 5 min, electrophoresed on SDS-polyacrylamide gels and transferred to nitrocellulose membranes. Membranes were blocked with 5% nonfat dry milk and probed with specific primary antibodies, followed by incubation with appropriate peroxidase-conjugated secondary antibodies. Blots were developed with ECL Plus reagent (Amercham, Arlington Heights, IL) according to the manufacturer's protocol.

RESULTS

Etoposide sensitivity and MDR-related protein expression in H460 and A549 cells

Cytotoxic effects of etoposide on lung cancer cells were evaluated by measuring cell viability using the MTT assay. When H460 and A549 cells were treated with increasing dose of etoposide, we found that A549 cells were more resistant than H460 cells (Fig. 1A). To investigate whether the etoposide resistance in A549 cells is associated with increased expression of MDR-related proteins, we semiquantitatively assayed the mRNA level of MRP1, Pgp, BCRP and LRP. Both cell lines had similar expression of MRP1 and BCRP. In contrast, the Pgp and LRP mRNA was detectable in A549 cells, but not in H460 cells (Fig 1B). High expression of LRP in A549 cells has been consistently shown in earlier studies (Meschini *et al.*, 2002). It suggests that the resistance of A549 cells to etoposide may be at least partly related to increased expression of PGP or LRP.

Chemoresistance of etoposide-selected H460 cells

After sequential exposure of H460 cells to increasing doses of etoposide, the response of H460-R1 and H460-R2 cells against chemotherapeutic drugs was investigated in comparison with parental H460 cells. As shown in Fig.

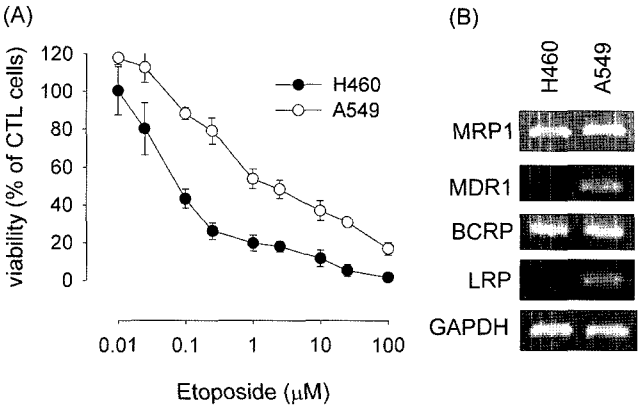

Fig. 1. Etoposide sensitivity and MDR-related proteins expression in human H460 and A549 NSCLC cells. (A) Cells were seeded at a density of 5×10^3/well in 96-well plates and, starting 24 h later, were incubated with varying doses of etoposide. At 48 h post-incubation, cell growth and viability of cells were determined by MTT assay. Results are expressed as percentage growth (mean ± S.D. of triplicate wells) relative to untreated cells (B) Expression of MRP1, PGP, BCRP and LRP mRNA in H460 and A549 cells was determined by semi-quantitative RT-PCR, with GAPDH mRNA serving as a loading control.

2A, H460-R1 and -R2 cells were significantly more resistant to the etoposide treatment. The etoposide concentration inhibiting the cell growth by 50% (IC_{50}) was higher than 20 μM in both R1 and R1 cell lines, whereas it was less than 0.1 μM in parental H460 cells. No significant difference in IC_{50} was found between R1 and R2 cells despite different cell culture conditions.

To investigate whether etoposide exposure caused cross-resistance to other MDR-related chemotherapeutic drugs in H460 cells, we also compared the sensitivity of R1 and R2 cells against doxorubicin, another well-known MDR-related drug. R1 and R2 cells were approximately 4.1- and 8.3-fold more resistant to doxorubicin, compared with parental H460 cells (Fig. 2B). These data suggest that progressive exposure of H460 cells with increasing dose of etoposide induced MDR in these cells.

Etoposide accumulation in etoposide-selected H460 cells

Earlier studies have shown that cancer cells with acquired resistance against MDR-related drugs generally have increased expression of MDR-related proteins. To collectively evaluate the involvement of MDR-related proteins that function as ATP-dependent drug efflux pumps, we examined whether the intracellular accumulation of etoposide was decreased in R1 and R2 cells. We found that the intracellular accumulation of etoposide in R1 and R2 cells were not significantly different from that in parental H460 cells (Fig. 3). Our data suggest that the acquired MDR in R1 and R2 cells was not caused by a decrease in the intracellular etoposide accumulation.

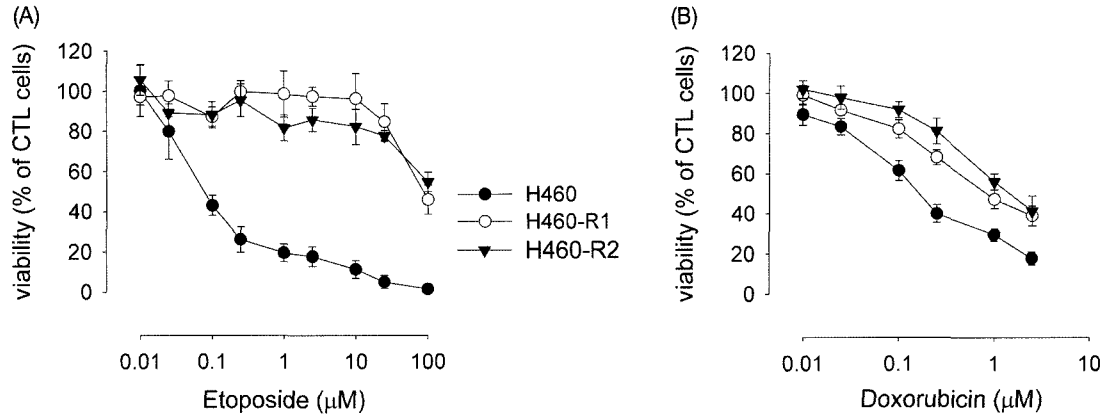

Fig. 2. Acquired resistance against etoposide and doxorubicin in etoposide-selected H460 cells. H460, H460-R1 and H460-R2 cells were incubated with varying doses of (A) etoposide or (B) doxorubicin, and then subjected to MTT assay. Results are expressed as percentage growth (mean ± S.D. of triplicate wells) relative to untreated cells.

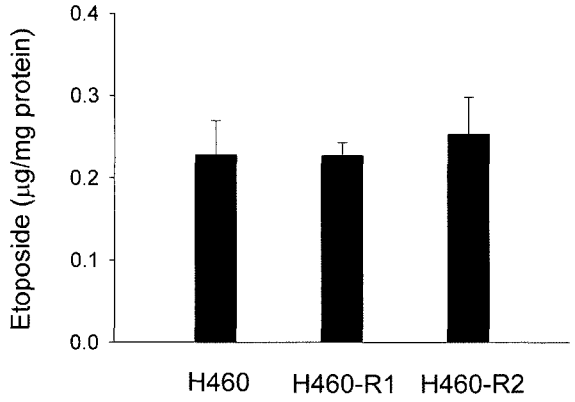

Fig. 3. Intracellular accumulation of etoposide in etoposide-selected H460 cells. Cells plated in 100 cm^2 dishes were incubated with 20 mM etoposide for 1 h. Cells were harvested, washed three times with PBS, and lysed with lysis buffer, and each cell lysate was directly subjected to HPLC analysis for the quantitative determination of intracellular etoposide concentration (n=3).

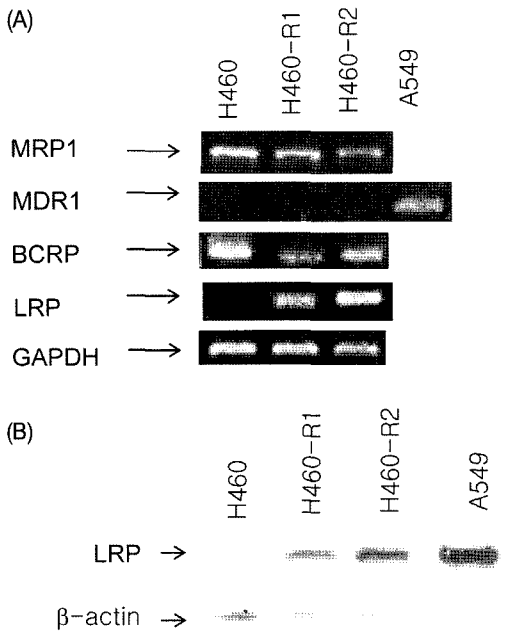

Fig. 4. Expression of MDR-related proteins in etoposide-selected H460 cells. (A) Expression of MRP1, PGP, BCRP and LRP mRNA in H460, H460-R1 and H460-R2 cells was determined by semi-quantitative RT-PCR, with GAPDH mRNA serving as a loading control. (B) Immunoblot analysis of LRP protein expression in cells. Immunoblotting with an antibody to b-actin was used to ensure equal loading of proteins in each lane.

Expression of MDR-related proteins in etoposide-selected H460 cells

To further investigate the mechanism inducing MDR in etoposide-selected cells, we assayed the expression of Pgp, MRP1, BCRP and LRP by RT-PCR analysis. The expression of MRP1 and BCRP mRNA was similar among H460, H460-R1 and H460-R2 cells (Fig. 4A). Expression of Pgp mRNA was not detected in any of these cell lines. In contrast, expression of LRP mRNA was detected in both R1 and R2 cells, whereas it was not in parental H460 cells. In accordance with the RT-PCR analysis data, increased expression of LRP protein was found in R1 and R2 cells, compared with parental cells (Fig. 4B). The expression of MRP1 and Pgp protein remained unchanged at barely detectable level regardless of cell lines (data not shown).

DISCUSSION

We have shown here that etoposide-selected H460 lung cancer cells acquired cross-resistance to MDR-related drugs, etoposide and doxorubicin. Acquired MDR seems likely to be associated with LRP, rather than other MDR-related proteins that function as ATP-dependent drug efflux pumps. This is supported by data showing that the intracellular accumulation of etoposide was not

decreased in R1 and R2 cells, and that the expression of LRP, but not those of members of ABC transporter family (Pgp, MRP1 and BCRP), was increased in these cells. In accordance with our data, the association of etoposide resistance with increased LRP expression was found in several earlier studies (Berger *et al.*, 2000; Hu *et al.*, 2002; Kitazono *et al.*, 2001). Since expression of LRP was also found in clinical samples obtained from NSCLC patients (Dingemans *et al.*, 1996), LRP may be a critical component of a pathway inducing multidrug resistance in NSCLC. Therefore, strategies developing inhibitors of LRP may help to improve the chemotherapeutic response of lung cancer patients.

Although LRP has been implied in the development of non-P-glycoprotein-mediated drug resistance in cancer, it remains unclear how LRP is involved in mediating the chemoresistance in a variety of cancer. Since LRP may play a role in blocking nuclear accumulation of chemotherapeutic drugs (Kitazono *et al.*, 1999), it will be of interest to further investigate whether the nuclear accumulation of etoposide was decreased in R1 and R2 cells.

It is well-known that drug resistance in tumor cells is not caused by a single factor, but rather by the coordinated effects of multiple factors involved in the drug response. In our study, we observed that the etoposide sensitivity of H460-R1 and –R2 cells was lower than that of A549 cells, despite lower LRP expression. Therefore, there is a possibility that factors other than LRP may also be induced, causing MDR in etoposide-selected H460 cells. For example, acquired etoposide resistance in H460 cells may also be caused by an increased expression of enzymes responsible for resisting oxidative damage, since the cytotoxic effects of etoposide have been shown to be mediated by the induction of oxidative stress inside cells *via* ROS generation (Sawada *et al.*, 2001; Troyano *et al.*, 2001). We are currently investigating whether other factors play a role in inducing MDR by alone or in coordination with LRP in cells with acquired resistance to etoposide.

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