Expression of Multidrug Resistance Proteins and Accumulation of Cisplatin in Human Non-small Cell Lung Cancer Cells

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In order to understand and overcome multidrug resistance (MDR) of human non-small cell lung cancer (NSCLC), mRNA and protein expression levels of P-glycoprotein (MDR1), multidrug resistance-associated protein 1 (MRP1), and lung resistance-related protein (LRP) were investigated and compared with the chemosensitivity and the intracellular/intranuclear cisplatin accumulation of three NSCLC cell lines (Ma-10, Ma-31, and Ma-46). Ma-31 was more resistant than Ma-10 and Ma-46 to cisplatin, carboplatin, etoposide, and paclitaxel. The mRNA level of MDR1 was extremely low, and MDR1 protein was not detected in all cell lines. MRP1 mRNA expression was highest in Ma-31 and lowest in Ma-10, but there was no notable difference between the MRP1 protein expression in three cell lines. LRP mRNA/protein was equally expressed in Ma-10 and Ma-31, but was nominal in Ma-46. The intracellular/intranuclear cisplatin accumulation of the cells was determined to be Ma-31 > Ma-46 > Ma-10. Thus, MDR1, MRP1, and LRP mRNA and protein expression levels were not correlated with the chemosensitivity or the intracellular/intranuclear cisplatin accumulation of each cell line. The present results indicate that MDR proteins (MDR1, MRP1, and LRP) may not play an important role in the chemoresistance and drug efflux of NSCLC cells.

Key words multidrug resistance protein; cisplatin accumulation; non-small cell lung cancer; P-glycoprotein (MDR1); multidrug resistance-associated protein 1 (MRP1); lung resistance-related protein (LRP)

Lung cancer is currently the leading cause of cancer deaths worldwide. Primary lung cancers are classified into two main histological groups: small cell lung cancer (SCLC) and non-small cell lung cancer (NSCLC). NSCLC constitutes approximately 85% of all lung cancers and often shows intrinsic multidrug resistance (MDR), whereas SCLC almost always responds well to various anticancer agents. Cisplatin is an established antitumor agent for the treatment of advanced human NSCLC and is employed in cisplatin-based adjuvant chemotherapy. Although the development of resistance to cisplatin is one of the major obstacles in the successful treatment of NSCLC, the molecular mechanisms involved remain poorly understood. In order to overcome the problems related to drug resistance and to improve the clinical outcome of patients with NSCLC, the mechanisms of cancer chemoresistance must be more clearly elucidated.

Our previous in vitro study using three NSCLC cell lines (Ma-10, Ma-31, and Ma-46) has demonstrated that cisplatin has triggered apoptosis more easily in the chemosensitive human NSCLC cell line than the chemoresistant cell line, based on biochemical and morphological findings. We have also reported that an increased transcriptional level of constitutive signal transducer and activator of transcription (STAT) 3 may be related to the suppressive regulation of the apoptotic pathway in intrinsically chemoresistant NSCLC cells. However, multiple factors are involved in the chemoresistance of cancer cells, suggesting that other mechanisms or factors may also contribute to the resistance of the NSCLC cell lines. Therefore, it is needed to further study other cellular mechanisms of chemoresistance, such as ATP-binding cassette transporters and lung resistance-related protein (LRP), important in drug disposition and in the development of MDR, together with measuring the concentration of anticancer agent in cancer cells.

During the past decade, there have been many studies linking various transporters to MDR both in cell culture and in the clinical setting. Of these proteins, P-glycoprotein (MDR1), multidrug resistance-associated protein 1 (MRP1), and LRP have attracted considerable attention in studies of cancer chemotherapy. In tumor cell lines, MDR is often associated with overexpression of ATP-dependent drug efflux proteins belonging to the superfamily of ATP-binding cassette transporters: the 170-kDa MDR1 and the 190-kDa MRP1. These proteins bind to and transport structurally unrelated compounds to maintain their intracellular concentrations below cytotoxic levels. In addition to an overall decrease in intracellular drug concentration, redistribution of the drug from the nucleus to the cytoplasm has also been implicated in MDR of cancer cells. Recently, another drug resistance-related protein, referred to as LRP, has been identified. LRP has been found to be identical to the human major vault protein, which is the major component of vaults. Vaults are mainly located in the cytoplasm, and approximately 5% of cellular vaults are located in the nuclear pore complexes that allow the bidirectional nucleocytoplasmic exchange of materials. Therefore, LRP is considered to mediate drug redistribution by regulating both cytoplasmic and nucleocytoplasmic transport. Furthermore, it has been reported that LRP expression is correlated with in vitro resistance of various cancer cells to platinum drugs, cisplatin and carboplatin.

These findings suggest that MDR proteins such as MDR1, MRP1, and LRP may mediate the drug resistance of our NSCLC cell lines by reducing the anticancer drug concentra-
tion at intracellular targets. However, to our knowledge, there have been no reports concerning a possible linkage between intranuclear cisplatin accumulation and LRP in NSCLC cells.

In the present study, we therefore investigated the mRNA and protein expression of MDR1, MRP1, and LRP and compared these expression data with the chemosensitivity and intracellular/intranuclear cisplatin accumulation of NSCLC cells in order to clarify the contribution of these proteins to the cellular chemoresistance.

MATERIALS AND METHODS

**Materials** Cisplatin, carboplatin, etoposide, and paclitaxel were obtained from Bristol-Myers Squibb Co. (Tokyo, Japan). 3-(4,5-Dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) was purchased from Sigma Aldrich Japan (Tokyo, Japan). TaqMan One-Step RT-PCR Master Mix Reagents, TaqMan β-actin Control Reagents, MicroAmp Optical 96-well Reaction Plates, Optical Adhesive Covers, and Optical Cover Compression Pads were purchased from Applied Biosystems (Foster City, CA, U.S.A.).

**Cell Lines** Three NSCLC cell lines were used (the Ma-10 and Ma-31 adenocarcinoma cell lines and the Ma-46 squamous cell carcinoma cell line). These cell lines, which were kindly provided by Dr. E. Shimizu, were obtained from Japanese patients and established at the Prefectural Habikino Hospital using a culture technique described by Masuda et al.\(^\text{15}\) The characteristics of these cell lines are shown in Table 1. Ma-10 was derived from a patient who showed a partial response to chemotherapy, Ma-31 was derived from a patient who showed no response to chemotherapy or surgery, and Ma-46 was derived from a patient who showed a complete response after surgery without chemotherapy. The cell lines were grown at 37 °C under a humidified atmosphere containing 10% heat-inactivated fetal calf serum (PAA laboratories, Linz, Austria) and 60 μg/ml gentamicin (Schering-Plough, Osaka, Japan).

**MTT Cell Viability Assay** The cells were seeded in 96-well plates (Costar; Corning Inc., Corning, NY) for 24 h, and then exposed to various concentrations of cisplatin, carboplatin, etoposide, and paclitaxel for 72 h. The cells were incubated with 10 μl of 2-mg/ml MTT in phosphate-buffered saline (PBS) for 3 h, and the formazan crystals were lysed with 100 μl of 20% sodium dodecyl sulfate (SDS) for 3 h at 37 °C. Absorbance at 550 nm relative to a reference wavelength of 630 nm was then measured with a microplate reader (Dainipponseiyaku, Osaka, Japan). Absorbance values were expressed as percentages relative to untreated controls, and the concentrations resulting in 50% inhibition of cell growth (IC\(_{50}\) values) were calculated.

**Quantitative Real-Time Reverse Transcriptase-Polymerase Chain Reaction** Cultured cells were seeded into 12-well plates (Costar; Corning) at a concentration of 3 × 10\(^5\) cells/well and incubated with medium alone for 24 h. Then, total RNA was extracted from the cells using the RNasy Mini Kit and QIAshredder (Qiagen, Hilden, Germany) following the manufacturer’s protocol. The mRNA levels of MDR1, MRP1, LRP, and pregnane X receptor (PXR) were measured by quantitative real-time reverse transcriptase-polymerase chain reaction (RT-PCR) using an ABI PRISM 7700 Sequence Detection System (Applied Biosystems). The primers and probes for MDR1, MRP1, and PXR used in the RT-PCR have been described previously.\(^\text{15}\) The primers and probe for LRP were newly designed as follows.

**Preparation for Fractionation** Cultured cells were seeded into 100-mm tissue culture plates (Costar; Corning) and incubated with medium alone for 24 h. Then, cell fractionation was prepared as follows.

To analyze the subcellular localization of MRP1, subcellular fractions (nuclear, mitochondrial, microsomal, and soluble cytosolic fractions) were prepared by the method of Rior-\(^\text{17}\) dan and Ling.\(^\text{17}\) The microsomal fraction was also used for Western blotting of MDR1.

For MDR1 and MRP1 Western blotting assays, cell membrane was purified from the cells according to the procedure described by Lin et al.\(^\text{18}\) The cell membrane pellet was resuspended in the appropriate PBS.

For LRP Western blotting assays, the cytoplasm was extracted from the cells by a modification of the method described by Takahashi et al.\(^\text{19}\) Cytoplasmic extracts were prepared by five cycles of freezing, thawing, and vortexing of the cells in PBS, followed by centrifugation at 9500 g for 10 min to obtain the supernatant.

For Western blotting of LRP and intranuclear cisplatin

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**Table 1. Characteristics of NSCLC Cell Lines**

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Age</th>
<th>Sex</th>
<th>Subtype</th>
<th>Stage</th>
<th>Origin</th>
<th>Prior Therapy</th>
<th>Response</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ma-10</td>
<td>56</td>
<td>Male</td>
<td>Ad</td>
<td>T3N3M1</td>
<td>PLE</td>
<td>CVM</td>
<td>PR</td>
</tr>
<tr>
<td>Ma-31</td>
<td>59</td>
<td>Male</td>
<td>Ad</td>
<td>T2N0M0</td>
<td>Lung</td>
<td>CVM, OP</td>
<td>NC</td>
</tr>
<tr>
<td>Ma-46</td>
<td>74</td>
<td>Male</td>
<td>Sq</td>
<td>T2N0M0</td>
<td>Lung</td>
<td>OP</td>
<td>CR</td>
</tr>
</tbody>
</table>

Ad, adenocarcinoma; Sq, squamous cell carcinoma; PLE, pleural effusion; CVM, cisplatin plus vindesine plus mitomycin C; OP, operation; PR, partial response; NC, no change; CR, complete response.
measurement, the nuclear fraction was prepared as described in the literature with minor modifications. The cells were suspended in buffer A (10 mM Tris–HCl at pH 7.4, 150 mM NaCl, 5 mM MgCl₂, and 0.5 mM phenylmethylsulfonyl fluoride [PMSF]). The cell suspension was lysed with buffer B (buffer A plus 5 mM 2-mercaptoethanol and 0.2% Triton X-100). The lysate was placed on buffer C (10 mM Tris–HCl at pH 7.4, 5 mM MgCl₂, 0.5 mM PMSF, 0.2 mM 2-mercaptoethanol, and 25% glycerol) and then centrifuged at 220 g for 10 min. After one-half of the volume of supernatant was removed, the nuclei were obtained by centrifugation at 9500 g for 10 min.

**Antibody Preparation** Twenty-aminoc-acid peptides were synthesized (MDR1: 775-QGFTFKAGEILTKRLRYMYM- 9500, MRP1: 156-TTLATFIQLERRKGVQSPSFYM-399) using a peptide synthesizer (PSSM-8; Shimadzu, Kyoto, Japan). Anti-MDR1 and anti-MRP1 antisera were obtained by immunizing rabbits according to the method of Liu et al. 21) The IgGs were isolated from the serum using HiTrap γ Protein A Columns (Amersham Pharmacia Biotech, Tokyo, Japan) according to the method specified by the manufacturer. To assess the cross-reactivity of the antibodies generated, dot blot analysis of the synthetic peptides of MDR1 and MRP1 immunoprobed with the antibodies were performed. Western blot assays were further performed to test the specificity of the antibody for MDR1 or MRP1. Mouse kidney microsomal fraction, a positive control for MDR1, and HepG2 cell lysate (BD Transduction Laboratories, Lexington, KY), a positive control for MRP1, were used. The specific band corresponding to MDR1 or MRP1 was detected, and the band was eliminated by competition with the synthetic peptides as antigen (data not shown).

**Western Blotting** Protein concentrations were measured using the Micro BCA Protein Assay Reagent Kit (Pierce, Rockford, IL) with bovine serum albumin as the standard. Proteins were separated by 10% SDS-polyacrylamide gel electrophoresis and transferred to a polyvinylidene difluoride membrane (ATTO, Tokyo, Japan). Membranes were incubated with Blocking Solution in Tris-buffered saline (TBS) for Immunoassay (Nacalai Tesque, Kyoto, Japan) according to the method specified by the manufacturer. To assess the cross-reactivity of the antibodies generated, dot blot analysis of the synthetic peptides of MDR1 and MRP1 immunoprobed with the antibodies were performed. Western blot assays were further performed to test the specificity of the antibody for MDR1 or MRP1. Mouse kidney microsomal fraction, a positive control for MDR1, and HepG2 cell lysate (BD Transduction Laboratories, Lexington, KY), a positive control for MRP1, were used. The specific band corresponding to MDR1 or MRP1 was detected, and the band was eliminated by competition with the synthetic peptides as antigen (data not shown).

**Chemosensitivity of NSCLC Cell Lines to Anticancer Drugs** The effects of cisplatin, carboplatin, etoposide, and paclitaxel on the growth of lung cancer cells were evaluated by measuring cell viability using the MTT assay. The IC₅₀ values of anticancer drugs in the three NSCLC cell lines (Ma-10, Ma-31, and Ma-46) are shown in Table 2. Ma-31 showed a 3-fold higher IC₅₀ for cisplatin, a 6-fold higher IC₅₀ for carboplatin, a 128-fold higher IC₅₀ for etoposide, and an 886-fold higher IC₅₀ for paclitaxel, compared with Ma-10. Ma-46 showed an almost 15-fold higher IC₅₀ for etoposide and a 6-fold higher IC₅₀ for paclitaxel, compared with Ma-10. Therefore, Ma-31 was the most resistant to these drugs. Ma-10 was the most sensitive to etoposide and paclitaxel. Ma-46, which was derived from a patient who had not undergone chemotherapy, showed intrinsic resistance to the anticancer agents.

In a previous study, we determined DNA fragmentation, the translocation of phosphatidylserine at the cell surface, and nuclear condensation in the three cell lines treated with cisplatin accumulation in the NSCLC cell lines was analyzed according to the method of Nakajima et al. 22) with minor modifications. Briefly, 5×10⁶ cells were seeded into 100-mm tissue culture plates and incubated for 24 h. The cells were incubated with 150 µM cisplatin for 2 h at 37°C and were then washed three times with PBS. After whole cell extracts and nuclear fractions were prepared, the mass of intracellular or intranuclear platinum was determined using a polarized Zeeman atomic absorption spectrophotometer (model Z-8200 or Z-5710; Hitachi, Ltd., Tokyo, Japan). The absolute mass of platinum in the cellular and nuclear samples was determined from a calibration curve prepared using a platinum standard solution (Kanto Chemical Co., Inc., Tokyo, Japan). The intracellular/intraneuclear platinum content was normalized to the DNA content, which was estimated by the method of Labarca and Paigen 23) with calf thymus DNA (Nacalai Tesque) as the standard.

**Statistical Analysis** Statistical analysis was performed using the Student’s t test. A value of p<0.05 was considered to be statistically significant.

### RESULTS AND DISCUSSION

The IC₅₀ values were calculated from the dose-response curves. The IC₅₀ values were calculated from the dose-response curves. The IC₅₀ values were calculated from the dose-response curves.
cisplatin. DNA fragmentation in Ma-46 increased after treatment with cisplatin, whereas no significant DNA fragmentation was observed in Ma-31. The translocation of phosphatidylserine at the cell surface occurred in Ma-46, but not observed in Ma-31. Cisplatin induced nuclear condensation in Ma-46. On the other hand, no significant nuclear condensation was observed in Ma-31. The findings of these biochemical and morphological studies showed that cisplatin apparently induced apoptotic cell death in Ma-46. On the contrary, cisplatin did not easily induce apoptosis in Ma-31, suggesting that apoptosis inducibility is responsible for the anti-cancer drug susceptibility of NSCLC cells.

**mRNA Expression of MDR1, MRP1, and LRP in NSCLC Cells**

Cisplatin is an important base drug for the treatment of NSCLC, but intrinsic or acquired resistance to anticancer agents is a significant clinical problem, leading to treatment failure in patients with this type of cancer. The mechanism of cisplatin resistance is known to be multifactorial: elevation of cellular glutathione, increased DNA repair capability, and reduced intracellular accumulation of the drug. Of these, the reduced intracellular accumulation of cisplatin is considered to be a consistent finding in a variety of cisplatin-resistant cancer cell lines. Previous studies have reported MDR1 or MRP1 expression in cisplatin-resistant SCLC and ovarian cancer. Therefore, a decrease in the cellular cisplatin concentration through elevated drug efflux pumps such as MDR1 and MRP1 would be responsible for the cancer chemoresistance of NSCLC as well as other cancers. So, we determined the expression of MDR1 and MRP1, and compared the results with the chemosensitivity data using heterogeneously chemosensitive NSCLC cell lines. We also investigated the expression of LRP, which has been considered to be involved in the regulation of the nucleocytoplasmic transport of drugs and resistance to anticancer drugs, including cisplatin. The relationship between LRP expression and intranuclear cisplatin accumulation in NSCLC cells had previously not been investigated and was therefore a subject of the present study.

mRNA expression levels of MDR1, MRP1, and LRP were examined by quantitative real-time RT-PCR. The TaqMan method is exquisitively sensitive and permits the quantitative evaluation of the very small amounts of mRNA, as reported previously. Table 3 shows the ratio of target mRNA expression to β-actin mRNA expression in the three cell lines. β-actin was used as the endogenous control in the measurement of target mRNA. A recent study has demonstrated that the nuclear receptor PXR may be involved in the induction of MDR1 expression, and we therefore investigated the expression of PXR mRNA. The PXR mRNA level was extremely low in all NSCLC cell lines. The mRNA level of MDR1 was extremely low in Ma-31 and Ma-46, and was undetectable in Ma-10. With regard to MRP1 mRNA expression, the chemoresistant Ma-31 cell line showed the highest level and the chemosensitive Ma-10 cell line showed the lowest level. LRP mRNA was equally expressed in Ma-10 and Ma-31, but was nominal in Ma-46.

Based on these findings, the chemosensitivity of these NSCLC cell lines cannot be explained by the expression levels of MDR1 and LRP mRNA. On the other hand, the expression of MRP1 mRNA is likely to be associated with the chemoresistance of the cells, but the protein expression of MRP1 and the intracellular cisplatin accumulation need to be considered.

**Protein Expression of MDR1, MRP1, and LRP in NSCLC Cells**

To confirm the findings for mRNA expression, protein expression of MDR1, MRP1, and LRP were determined by Western blotting. After microsomal protein was subjected to immunoblotting studies with purified rabbit IgG against human MDR1, a band at 170 kDa, corresponding to MDR1, was detected in the microsomal fraction from mouse kidney (positive control), but was not detected in the fraction from NSCLC cells (Fig. 1A, top). Although the cell membrane fraction was further purified, no protein band reacting with anti-MDR1 antibody was detected in any of the cell lines (Fig. 1A, middle). This finding indicates that MDR1 is not expressed in these NSCLC cell lines, which is in agreement with the results for MDR1 mRNA expression in the cells (Table 3).

When the subcellular localization of MRP1 was investigated in Ma-10, an immunoreactive protein band corresponding to MRP1 was detected in the microsomal fraction (Fig. 1B, top). An MRP1 band was detected in the purified cell membrane fraction, but there was no notable difference between the bands from the three cell lines (Fig. 1B, middle), although there was notable difference in MRP1 mRNA expression (Table 3). The difference in expression observed between MRP1 mRNA and protein suggests that there is strong post-transcriptional regulation of MRP1 expression in these NSCLC cell lines and that the detection of MRP1 mRNA does not always predict the presence of the corresponding protein.

When the chemosensitivity data in Table 2 were assessed in relation to the protein expression of MDR1 and MRP1, no significant correlations were found between them, suggesting that neither MDR1 nor MRP1 plays a major role in determining the differences in chemosensitivity between these cell lines.

The expression of LRP protein in the cytoplasm and the nuclear fraction of NSCLC cells was determined by Western blot analysis with anti-LRP antibody. An immunoreactive protein band at 110 kDa, corresponding to LRP, was detected in both fractions of Ma-10 and Ma-31. On the other hand,
LRP was not detected in Ma-46 (Fig. 1C), indicating that LRP is not expressed in Ma-46. These data are in accord with the expression of LRP mRNA as shown in Table 3. In agreement with the finding of Dingemans et al., there was no significant correlation between LRP expression and chemosensitivity in the three NSCLC cell lines, suggesting that LRP is not involved in the chemoresistance of these NSCLC cells.

**Intracellular and Intranuclear Cisplatin Accumulation in NSCLC Cells**

The nucleus is a critical action site for cisplatin. We therefore investigated intranuclear cisplatin accumulation in the three NSCLC cell lines. In order to investigate intranuclear cisplatin accumulation, the nuclear fraction was isolated from cells exposed to 150 μM cisplatin for 2 h, and the platinum concentration was measured. As shown in Fig. 2B, the intranuclear cisplatin accumulation in Ma-10 was one-ninth that in Ma-31 (0.24, 2.23, and 0.78 pmol/μg DNA in Ma-10, Ma-31, and Ma-46, respectively). More than 69% of the intracellular cisplatin reached the nuclei of the cells (80%, 69%, 93% in Ma-10, Ma-31, Ma-46, respectively). As was the case for intracellular cisplatin accumulation analysis (Fig. 2A), no significant correlation was found between intranuclear cisplatin accumulation and chemosensitivity in these cancer cells. Thus, the higher intranuclear concentration of cisplatin accumulated in NSCLC cells did not necessarily induce higher chemosensitivity of these cells.

Since there has been no previously reported link between LRP and intranuclear cisplatin accumulation, the intranuclear cisplatin accumulation data were assessed in relation to the mRNA/protein expression of LRP. No significant correlations were found between them, suggesting that LRP is not necessarily involved in the regulation of the nucleocytoplasmic transport of cisplatin in these NSCLC cell lines.
In the present study, we demonstrated that there was no correlation between MDR1, MRP1, and LRP mRNA/protein expression and chemosensitivity or intracellular/intranuclear cisplatin accumulation in these NSCLC cells. The results indicate that these MDR proteins and the increased drug efflux may not play an important role in the chemoresistance of NSCLC cells, which can be significant information for clinical oncologists to select the proper chemotherapy. Intrinsic or acquired defects in the apoptotic machinery, which can profoundly alter the therapeutic outcome, may be responsible for the cisplatin susceptibility of these NSCLC cells. Obtaining a clearer understanding of the details of such regulation should permit the development of more effective treatments for multidrug resistant NSCLC.

Acknowledgments This work was supported in part by a Sasakawa Scientific Research Grant from The Japan Science Society. We acknowledge David G. Spear, M.D. for reviewing the manuscript.

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