Determinants of Cisplatin and Irinotecan Activities in Human Lung Adenocarcinoma Cells: Evidence of Cisplatin Accumulation and Topoisomerase I Activity

TADASHI MATSUMURA¹, NAGIO TAKIGAWA³, KATSUYUKI KIURA¹, TAKUO SHIBAYAMA³, MASAKAZU CHIKAMORI¹, MASAHIRO TABATA¹, HIROSHI UEOKA² and MITSUNE TANIMOTO²

¹Department of Respiratory Medicine (Thoracic Oncology), Okayama University Hospital, 2-5-1 Shikata-cho, Okayama 700-8558;
²Department of Hematology, Oncology, and Respiratory Medicine, Okayama University Graduate School of Medicine and Dentistry, 2-5-1 Shikata-cho, Okayama 700-8558;
³Department of Internal Medicine, National Minami-Okayama Hospital, 4066 Hayashima, Tsukubo-gun, Okayama 701-0304, Japan

Abstract. To elucidate the sensitivity of adenocarcinoma of the lung to cisplatin and irinotecan, intracellular glutathione (GSH) and glutathione S-transferase (GST)-π concentrations and topoisomerase (topo) I activity were investigated using six adenocarcinoma cell lines. The antiproliferative activity was determined by MTT assay in terms of inhibition concentration (IC50) values. The IC50 values to cisplatin were not correlated with the amounts of intracellular GSH or GST-π, but with intracellular accumulation of platinum (r=−0.91, p=0.013). IC50 values to SN-38 were correlated with topo I activity determined by relaxation assay of pBR322 (r=−0.83, p=0.040). These results suggest that platinum accumulation and topo I activity have definite impacts on the sensitivity of lung adenocarcinoma to cisplatin and irinotecan, respectively.

Resistance to chemotherapy is frequently observed in small cell lung cancer (SCLC) and non-small cell lung cancer (NSCLC). NSCLC is considered to be intrinsically resistant to anticancer agents, whereas SCLC is thought to acquire resistance after several cycles of chemotherapy (1). We have investigated acquired resistance using ex vivo-selected drug-resistant SCLC cell lines (2-4). In this study, we examined intrinsic resistance using NSCLC cell lines, which were not exposed to any drugs ex vivo. Fewer than half of patients with NSCLC experience a partial response and, even if there is a survival benefit, it is trivial. The poor response and the poor treatment outcome are clinically critical problems. The mechanism responsible for intrinsic drug resistance in NSCLC is still obscure. The factors of resistance include the intracellular concentration of a drug, intracellular glutathione (GSH) and glutathione S-transferase (GST)-π concentrations, and topoisomerase (topo) I activity (1). Adenocarcinoma is the most common type of NSCLC in Japan, the U.S. and some other countries and a gradual increase in its incidence has been recognized (5). We investigated factors influencing the drug sensitivity of human lung adenocarcinoma cells.

Materials and Methods

Chemicals and reagents. The drugs were obtained from the following sources; cisplatin, etoposide and cis-1, 1-cyclobutanedicarboxylato (2R)-2-ethyl-1, 4-butanediamine platinum (II) (NK121) from Nippon Kayaku Co., Ltd. Tokyo, Japan; vindesine, vincristine, nedaplatin from Shionogi & Co., Ltd., Osaka, Japan; 7-ethyl-10-hydroxy-camptothecin (SN-38: an active metabolite of irinotecan) from Yakult Honsha Co., Ltd., Tokyo, Japan; carboplatin from Bristol-Myers Squibb K.K., Tokyo, Japan; 1,1-cyclobutanedicarboxylato(2-aminomethylpyrrolidione) platinum (II) (DWA2114R) from Chugai Pharmaceutical Co., Ltd., Tokyo, Japan; Adriamycin from Kyowa Hakko Kogyo Co., Ltd., Tokyo, Japan. MTT (3-[4,5]dimethyl-thiazol-2-yl]-2,5-diphenyl tetrazolium bromide) and DL-Buthionine-S,R-sulfoximine (BSO) were purchased from Sigma Chemical Co., St. Louis, MO, USA.

Cell lines. Six human lung adenocarcinoma cell lines were used, which included ABC-1 (JCRB0815), ABC-3 and ABC-5 established in our department (6, 7), and A549 (JCRB0076), PC-3 (JCRB0077) and RERF-LC-MS (JCRB0081) supplied by the Japanese Cancer Research Resource Bank (JCRB, Tokyo, Japan). All the cell lines were maintained in tissue culture flasks at 37°C, under a humidified
atmosphere supplemented with 5% CO₂ in air. The culture medium used in this study was RPMI-1640 (Life Technologies, Inc., Grand Island, NY, USA), supplemented with 10% fetal bovine serum (ICN Biomedicals Japan Co., Ltd., Tokyo, Japan), penicillin-G (100 U/ml) and streptomycin (100 µg/ml) (RPMI-FBS).

**Drug sensitivity tests.** Drug sensitivity was determined by MTT assay, which was modified from the original method reported by Mossman (8), as described previously (2, 3). Briefly, 1,000 to 5,000 cells/well were seeded to a 96-well microplate, which contained each drug prepared in advance, and were continuously exposed to each drug for 96 hours in a 5% CO₂ incubator. Four hours after adding MTT, MTT formazan was dissolved in fresh isopropanol. The absorbance of the wells at 560 nm was measured using a Model 3550 microplate reader (Bio-Rad Laboratories, Richmond, CA, USA). The concentration of each drug required to inhibit the growth of tumor cells by 50% (IC₅₀) was determined by plotting the surviving cell fraction to a drug concentration with a custom-made program in Excel (Microsoft), based on linear interpolation between data points. Determinations were carried out in quadruplicate in each experiment and the results were confirmed by two or more separate experiments.

**Cell growth rate and cell cycle.** The growth rate of cells was also determined by MTT assay (2, 3). Cells growing in the exponential phase were seeded in 96-well microplates. The doubling-time of each cell line was estimated from the time-course of cell increments determined by measuring the mean absorbance of eight wells for seven successive days. The ratio of the S period was determined by flow cytometry using a one color method stained with Cell Cycle Test kit (Becton Dickinson Co., Ltd.).

**Intracellular GSH / GST-α.** Intracellular GSH and GST-α were measured as described previously (2, 3). Briefly, GSH was assayed by the method reported by Tietze (9) and GST-α was assayed using a GST-α EIA kit (one-step sandwich EIA; Dainihon Seiyaku, Osaka, Japan). The GSH and GST-α concentrations were expressed as the ratio to mg protein as determined by the method of Bradford (10).

**DNA topo I activity.** Crude nuclear extracts were prepared according to the method of Tsutsui et al. (11), as described previously (2). DNA topo I activity was assayed by relaxation of pUC utilizing plasmid DNA pBR322 (11). The protein assay was performed by the method reported by Tietze (9) and GST-α was assayed using a GST-α EIA kit (one-step sandwich EIA; Dainihon Seiyaku, Osaka, Japan). The GSH and GST-α concentrations were expressed as the ratio to mg protein as determined by the method of Bradford (10).

**Intracellular accumulation study of cisplatin and SN-38.** The cells were incubated in RPMI-FBS containing 10 µM of cisplatin or 1 µM SN-38 at a cell density of 5 x 10⁶/ml in a 37°C / 5% CO₂ incubator for incubated in RPMI-FBS containing 10 µM of cisplatin or 1 µM SN-38. The cells were collected and washed three times with cold phosphate-buffered saline (PBS). The cells were resuspended in 500 µl of distilled water and sonicated with a 30-sec burst using a Bioruptor (model UC100D; Olympus, Tokyo, Japan). The cell extracts for cisplatin were analyzed for platinum by atomic absorption spectrophotometry using a Hitachi polarized Zeeman atomic spectrophotometer, Model z-7000 (Hitachi, Tokyo, Japan) (4). The cell extracts for SN-38 were analyzed by spectrophotometry (12). SN-38 was detected at wavelength 542 nm when it was exited at wavelength 300 nm.

**Accumulation study of vincristine, adriamycin and etoposide.** Cells were incubated at a cell density of 1 x 10⁶/ml in RPMI-FBS containing 0.1 µM [³H]-vincristine (Moravek Biochemicals, Brea, CA, USA), 0.5 µM [³H]-Adriamycin (Amersham Pharmacia Biotech Ltd., Tokyo, Japan) and 100 µM [³H]-etoposide (Moravek Biochemicals) at 37°C. After 30, 60 and 120 min, 0.2 ml of the samples were removed from the culture and filtered with a semiautomatic cell harvester (Labo Mash, Labo Science Co., Ltd, Tokyo, Japan). The cells on the filter were solubilized by Clear-sol I (Nakarai Tesque, Inc., Kyoto, Japan). The radioactivity in the cells was determined by a liquid scintillation counter (Aloka LSC-700, Aloka, Tokyo, Japan) (2, 4).

**Results**

**Drug sensitivity.** Table I shows the mean IC₅₀ values of the drugs tested to cell lines. PC-3 cells were generally resistant to the drugs tested, while the ABC-1 and RERF-LC-MS cells were sensitive to them. IC₅₀ values to cisplatin were well correlated with those to platinum analogs: CBDCA (r=0.99); 254-S (r=0.99); NK121 (r=0.89); DWA2114R (r=0.94).

**Doubling-time and rate of S-phase.** The doubling-time and rate of S-phase are shown in Table II. They did not have significant correlation with the IC₅₀ values for any drugs.

**DNA topo I activity.** The appearance of relaxed DNA bands and the disappearance of supercoiled forms are regarded as evidence of an adequate topo I activity in the nuclear extracts (Figure 1). Topo I activity was significantly correlated with IC₅₀ values to SN-38 (r=-0.83, p=0.040) (Figure 2).
Intracellular GSH / GST-κ. The amounts of intracellular GSH and GST-κ, shown in Table II, were not correlated with the IC50 values to cisplatin and its analogs. Treatment with BSO decreased the intracellular GSH content in all five cell lines tested: 0.40 μg/mg protein in ABC-1; 0.18 μg/mg protein in ABC-3; 0.11 μg/mg protein in A549; 2.0 μg/mg protein in PC-3; 1.3 μg/mg protein in RERF-LC-MS. The ratio of the GSH content in the untreated cells to that in the treated cells ranged from 0.004 to 0.572. Table I shows the IC50 values to cisplatin in the cells treated with BSO. When they were treated with BSO, sensitivity to cisplatin was increased 1.30 to 1.85 times in terms of IC50 values.

Intracellular accumulation of drugs. Figure 3 illustrates the intracellular concentration of drugs at 30 min and 120 min after drug exposure. The concentration of Adriamycin, etoposide and SN-38 in most of the cell lines did not change between 30 min and 120 min. However, the intracellular accumulation of cisplatin and vincristine in all the cell lines increased at 120 min compared with that at 30 min. The intracellular accumulation of cisplatin at 120 min was correlated with the IC50 values of the drug (r = −0.91, p = 0.013) (Figure 4); that of other drugs showed no correlation with sensitivity in terms of IC50 values.

Discussion

Drug resistance is a major problem in chemotherapy for NSCLC including adenocarcinoma. The mechanisms of resistance are multifactorial (1). We studied the accumulation of intracellular drugs, GSH, GST-κ and topo I activity and compared them with drug sensitivity. In this study, the cisplatin and SN-38 activities were mainly determined by the platinum accumulation and topo I activity of the cells, respectively.

The mechanisms of cisplatin resistance include: (i) decreased accumulation of cisplatin, (ii) increased detoxification systems, such as GSH, GST-κ and metallothionein, and (iii) decreased DNA damage or increased repair (1). Morikage et al. reported an inverse correlation between the IC50 values to cisplatin and the cellular accumulation of cisplatin in NSCLC lines (13). We confirmed these results. A strategy to overcome resistance to cisplatin includes the development of cisplatin analogs, which are more potent than cisplatin, and modulators to enhance cisplatin activity (13). We examined cisplatin analogs such as carboplatin, nedaplatin and DWA2114R (14) and a modulator such as BSO, which decreases the intracellular GSH levels (15). The IC50 values to cisplatin analogs were not less than those to cisplatin in all the cell lines tested. In addition, although BSO dramatically decreased the GSH levels, the increment of sensitivity to cisplatin was small. Attempts should focus on increasing the accumulation of cisplatin by simultaneous use of drugs such as amphotericin B (13).

Kanzawa et al. (16) and Kubota et al. (17) reported that the mechanisms of irinotecan resistance were decreased accumulations of SN-38 and decreased amount and activity of topo I in irinotecan-resistant NSCLC cell lines. A half-molecule ABC transporter BCRP/MXR/ABCP, glucuronidation of SN-38, and deleted TOP1 mRNA have recently

Table II. Characteristics of lung adenocarcinoma cell lines.

<table>
<thead>
<tr>
<th></th>
<th>ABC-1</th>
<th>ABC-3</th>
<th>ABC-5</th>
<th>A549</th>
<th>PC-3</th>
<th>RERF-LC-MS</th>
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</thead>
<tbody>
<tr>
<td>Doubling-time (h)</td>
<td>23.8</td>
<td>46.8</td>
<td>37.9</td>
<td>27.0</td>
<td>39.9</td>
<td>41.7</td>
</tr>
<tr>
<td>% S-phase</td>
<td>37.0</td>
<td>39.8</td>
<td>15.0</td>
<td>15.7</td>
<td>12.2</td>
<td>27.6</td>
</tr>
<tr>
<td>DNA topo I activity (a.u.)</td>
<td>1.52</td>
<td>1.28</td>
<td>0.52</td>
<td>0.56</td>
<td>0.97</td>
<td>0.49</td>
</tr>
<tr>
<td>GST-κ (μg/mg protein)</td>
<td>1.47</td>
<td>6.53</td>
<td>9.83</td>
<td>5.10</td>
<td>0.05</td>
<td>1.75</td>
</tr>
<tr>
<td>GSH (μg/mg protein)</td>
<td>9.40</td>
<td>38.9</td>
<td>5.47</td>
<td>0.19</td>
<td>16.4</td>
<td>16.3</td>
</tr>
</tbody>
</table>

Figure 1. DNA topo I activity determined by relaxation assay of pBR322. The amount of nuclear extracts are 0.05 μg for lanes 1 (ABC-1), 2 (ABC-3), 3 (ABC-5), 4 (PC-3), 5 (RERF-LC-MS) and 6 (A549).

Figure 2. Scatterplots of IC50 values to SN-38 versus topo I activity (r = −0.83, p = 0.040).
been reported to be involved in irinotecan resistance (18-20). The sensitivity of tumor cells to irinotecan was associated with the intracellular topo I level. Itomachi et al. also reported a significant correlation between topo I activity response to SN-38 in clear cell carcinoma of the ovary (21). To our knowledge, however, no one has reported a significant relationship between topo I activity and response to SN-38 in lung cancer cell lines. We observed that topo I activity was significantly correlated with IC50 values to SN-38. However, methods to increase topo I activity remain unknown and further study will be needed to increase topo I activity and, therefore, sensitivity of irinotecan.

In Japan, combination chemotherapy of cisplatin and irinotecan is becoming one of the standard chemotherapies for NSCLC and extensive-stage SCLC (22, 23). However, steps must be taken to overcome resistance to cisplatin and irinotecan.

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References


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Figure 4. Scatterplots of IC50 values to cisplatin versus intracellular cisplatin at 120 min (r=-0.91, p=0.013).