Resistance Mechanisms in Three Human Small Cell Lung Cancer Cell Lines Established from One Patient during Clinical Follow-up


ABSTRACT

Mechanisms for resistance were studied in three classic type, human small cell lung cancer cell lines, GLC14, GLC16, and GLC19, that were established from one patient during clinical follow-up. Clinically the tumor changed from sensitive (GLC14) to completely resistant to (chemo)therapy (GLC19) during this period. The stain with JSB-1 antibody, detecting the M, 170,000 multidrug resistance associated glycoprotein, was most pronounced in GLC14 and absent in GLC19. Intracellular Adriamycin (Adr) concentrations were decreased in GLC14 and GLC19 versus GLC16. Glutathione levels were 12.9, 15.5, and 16.6 μg/ml; total sulphydryl groups were 36.5, 45.7, and 48.8 μg/ml; and glutathione S-transferase activity was 13, 29, and 43 nmol l-chloro-2,4-dinitrobenzene/min/mg protein for GLC14, GLC16, and GLC19, respectively. Incubation with DL-buthionine-S,R-sulfoximine increased Adr and cisplatin induced cytotoxicity, whereas X-ray induced cytotoxicity remained the same. Catalase activity increased from 0.88 to 1.73 to 3.83 μmol H2O2/min/mg protein in, respectively, GLC14, GLC16, and GLC19. Compared to GLC14 and GLC16, Adr induced a higher amount of DNA strand breaks in GLC19. In none of the three cell lines could Adr induced DNA strand breaks be repaired. X-ray induced a comparable amount of DNA strand breaks in all three cell lines but all cell lines were capable of repairing the X-ray induced DNA strand breaks within 90 min.

It is concluded that a number of different mechanisms are operative and that some but not all of the observed changes in mechanisms for drug resistance in these lines correlate with the clinical data.

INTRODUCTION

The development of acquired resistance to antineoplastic drugs represents a significant problem in the treatment of patients with cancer. Elucidation of the mechanisms involved in drug resistance could indicate ways to circumvent such a development. Several studies concerning in vitro developed resistance in human tumor cell lines have been reported (1–6). These studies dealt especially with in vitro acquired resistance for Adr, in which the resistance factor is often very high. Little is known, however, about the correlation between the mechanisms found for in vitro acquired resistance and the mechanisms responsible in the clinic. Therefore the study of cell lines derived from tumors with acquired resistance in vivo might broaden our understanding of the mechanisms involved. Thus far only few cell lines derived from one patient before as well as after therapy have been characterized (7–9), but no studies on mechanisms of in vivo acquired resistance have been published. Mechanistic studies in lines obtained from tumors with in vivo acquired resistance most probably will better reflect the in vivo situation in the patient.

In the present study, a number of possible mechanisms for in vivo acquired resistance were evaluated in three cell lines (GLC14, GLC16, and GLC19). The lines were derived from one patient with small cell lung cancer during clinical follow-up and treatment. During this period the tumor changed from sensitive to completely resistant to (chemo)therapy.

MATERIALS AND METHODS

Chemicals. Adr was provided by Farmitalia Carlo Erba, Milan, Italy. CDDP was obtained from Bristol Myers S.A.E., Madrid, Spain. RPMI was obtained from Gibco, Paisley, Great Britain. Fetal calf serum was obtained from Flow Laboratories, Irvine, Great Britain. BSO was obtained from Sigma, St. Louis, MO. H2O2 and dimethyl sulfoxide were from Merck, Darmstadt, West Germany. BSO was obtained from Chemalog, South Plainfield, NJ. Ethidium bromide was from Serva, Heidelberg, West Germany.

Patients and Cell Lines. Three classic-type, human small cell lung cancer cell lines (GLC14, GLC16, and GLC19) have been established from one patient during longitudinal follow-up. During this period the tumor changed from sensitive to completely resistant to (chemo)-therapy. The characterization of these lines is described by Berendsen et al. (7). In short, GLC14 was derived before therapy. Treatment consisted of cyclophosphamide 1 g/m2 every 3 weeks, Adr 45 mg/m2 every 3 weeks, and etoposide 100 mg/m2/days 1, 3, and 5 every 3 weeks (cyclophosphamide/Adr/etoposide). After a complete response lasting 7 months, because of tumor recurrence induction with cyclophosphamide/Adr/etoposide was started. After 4 cycles a partial response was seen and therapy was discontinued. At that moment GLC16 was derived. Radiotherapy (10 × 300 rad) was given to the left hilar region and the mediastinum. After recurrence of disease GLC19 was established from a biopsy of the left upper lobe, the area which had been irradiated. Two additional cycles of cyclophosphamide/Adr/etoposide were given, but the tumor progressed.

GLC14, GLC16, and GLC19 were kept in continuous culture in RPMI 1640 with 10% heat inactivated fetal calf serum. GLC14 grows almost completely attached to the culture flask, whereas GLC16 and GLC19 grow floating in aggregates. The culture doubling time was 26, 27, and 44 h, respectively, GLC14, GLC16, and GLC19. For all experiments, cells were incubated 1:1 with 0.02% EDTA solution (0.35 g NaHCO3, 5,5-dithiobis(2-nitrobenzoic acid), and MTT was obtained from Gibco, Paisley, Great Britain. Fetal calf serum was obtained from Flow Laboratories, Irvine, Great Britain. GSH, l-chloro-2,4-dinitrobenzene, catalase, 5,5-dithiobis(2-nitrobenzoic acid), and MTT were obtained from Sigma, St. Louis, MO. H2O2 and dimethyl sulfoxide were from Merck, Darmstadt, West Germany. BSO was obtained from Chemalog, South Plainfield, NJ. Ethidium bromide was from Serva, Heidelberg, West Germany.

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Sensitivity Assay. The microculture tetrazolium assay is based on the cellular reduction of MTT by the mitochondrial dehydrogenase of viable cells to a blue formazan product which can be measured spectrophotometrically (10, 11). Before the assays were performed the linear relationship of cell number to MTT formazan crystal formation was checked and cell growth studies were performed. Cell survival studies were performed on day 4 in all three cell lines. Thus, all lines were in an exponential phase of growth and had proceeded two to three cell divisions.

Equal numbers of cells (35,000/well for these cell lines) were incubated in a total volume of 0.1 ml of culture medium with one of the following agents, Adr, cDDP, and H2O2, for 1 h, in 96-well culture plates (Nunc; Gibco). For X-ray treatment (Philips X-ray source at 200 kV, 15 mA, 0.5 mm Cu/Al filter), cells were irradiated in tubes in a volume of 0.5 ml and then transferred to 96-well culture plates. For experiments after BSO incubation under conditions as described below, cells in medium without BSO were incubated with Adr or cDDP for 4 h or irradiated. After the incubation, cells, in wells, were washed three times with medium and then MTT solution was added. The plates were incubated for 4 h before the purple formazan product was aspirated and the color was measured at 550 nm. The absorbance was calculated as the percentage of the control value.

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2 To whom requests for reprints should be addressed.

The abbreviations used are: Adr, Adriamycin; cDDP, cisplatin [cis-diammine-dichloroplatinum(II)]; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazoliumbromide; BSO, dl-buthionine-S,R-sulfoximine; PBS, phosphate buffered saline (0.14 M NaCl, 2.7 mM KCl, 6.4 mM NaHCO3, 2-H2O2, and 1.5 mM KH2PO4; pH 7.4); MDR, multidrug resistance; GSH, glutathione; GST, glutathione S-transferase.

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times by removing the medium after centrifugation of the microtiter plates (10 min, 180 \( \times \) g) followed by addition of fresh medium. After a culture period of 4 days, 20 \( \mu \)M of MTT solution (5 mg MTT/ml PBS) were added to each well for 3.5 h. Thereafter the plates were centrifuged (30 min, 180 \( \times \) g) and the supernatant was carefully aspirated, in order not to disturb the formazan crystals. Dimethyl sulfoxide (100%; 200 \( \mu \)l) was added to solubilize the formazan crystals and the plate was read immediately at 520 nm using a scanning microtiter well spectrophotometer (Titertek Multiskan, Flow Laboratories). The percentage of cell survival was calculated as the mean of three test samples/mean of three untreated samples. Controls consisted of media without cells (background extinction), and cells were incubated in wells with medium instead of the drug.

P-Glycoprotein Expression. The monoclonal antibody JSB-1 which is directed against the M, 170,000 MDR associated glycoprotein was tested on cytospins using an indirect immunoperoxidase staining protocol (12).

Intracellular Adr Level. Cellular Adr levels were determined after 1 h incubation with Adr at 0°C and at 37°C. After the incubation cells were washed three times with ice-cold PBS, extracted with 0.3 N HCl-50% ethanol, and centrifuged. Adr fluorescence was measured in the supernatant at excitation and emission wavelengths of 474 and 549 nm, respectively, in a Kontron spectrofluorometer (13). Intracellular Adr levels were calculated by subtracting the vales measured at 0°C (extracellular bound Adr) from the corresponding values measured at 37°C (14).

Sulfhydryl Compounds and Enzyme Assays. Cells in the logarithmic phase of growth were harvested 4 days after passage. Cells were washed with ice-cold PBS and resuspended in a relevant concentration in PBS or another solution mentioned in the assay procedure. All measurements were performed under Vmax conditions for GSH determination. For GSH determination cells were resuspended in ice-cold 5% trichloroacetic acid, vortexed, and centrifuged at 4°C (15 min, 10,000 \( \times \) g). The supernatant was assayed for total GSH by the enzyme recycling method under conditions similar to those described by Tietze (detection limit, 0.1 \( \mu \)g/mg protein) (15). Total sulfhydryl groups were assayed as described by Siedlik and Lindsay (16). Cells were resuspended in Tris-EDTA buffer, pH 8.2, and sonicated. Cell homogenate was incubated with 5,5-dithiobis(2-nitrobenzoic acid), Ellman's reagent, for 15 min followed by centrifugation (15 min, 3000 \( \times \) g). Absorbance of the supernatant was measured at 412 nm. For measurement of the GST activity by the method of Habig et al. (17), 1-chloro-2,4-dinitrobenzene was used as substrate and the enzyme reaction was measured by the absorbance change at 340 nm. The catalase activity was measured following the decomposition of H2O2 mediated by catalase in a cell suspension of 3 \( \times \) 10^7 cells/ml at 240 nm according to the method of Beers and Sizer (18). For protein determination the assay of Lowry et al. was used (19). All measurements were performed on a Zeiss PMQ spectrophotometer.

BSO Treatment. Cells were cultured for 65 h in the presence of 50 \( \mu \)M BSO. At that moment GSH was no longer detectable in the three cell lines. GSH levels of GLC6 and GLC9 were significantly decreased in GLC6 and GLC9 as compared to GLC4.

BSO increased both Adr and cDDP induced cytotoxicity (Figs. 4 and 5) whereas X-ray induced cytotoxicity remained the same (not shown).

Fig. 6 shows the Adr induced strand breaks for the three lines. After 1 h incubation with Adr, an increased amount of DNA strand breaks was induced in GLC9 compared to GLC4.
MECHANISMS INVOLVED IN IN VIVO ACQUIRED DRUG RESISTANCE

Table 1  Sulfhydryl compounds, the activity of the enzymes GST and catalase, and the presence of the M, 170,000 glycoprotein, as assessed with JSB-1, in the three different cell lines

<table>
<thead>
<tr>
<th>No. of experiments</th>
<th>GLC\textsubscript{14}</th>
<th>GLC\textsubscript{16}</th>
<th>GLC\textsubscript{19}</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total GSH (µg/mg protein)</td>
<td>6</td>
<td>12.9 ± 1.9\textsuperscript{a}</td>
<td>15.5 ± 1.4 (P &lt; 0.05)\textsuperscript{f}</td>
</tr>
<tr>
<td>Total sulfhydryl groups (µg/mg protein)</td>
<td>8</td>
<td>36.5 ± 9.4</td>
<td>45.7 ± 11.7 (NS)\textsuperscript{f}</td>
</tr>
<tr>
<td>GST (nmol CDNB/min/mg protein)</td>
<td>4</td>
<td>13 ± 7</td>
<td>29 ± 4 (P &lt; 0.0025)\textsuperscript{f}</td>
</tr>
<tr>
<td>Catalase (µmol H\textsubscript{2}O\textsubscript{2}/min/mg protein)</td>
<td>3</td>
<td>0.88 ± 0.19</td>
<td>1.73 ± 0.65 (P &lt; 0.05)\textsuperscript{f}</td>
</tr>
<tr>
<td>JSB-1</td>
<td>3</td>
<td>Positive, 0-1%</td>
<td>Positive, 5%</td>
</tr>
</tbody>
</table>

\textsuperscript{a} Mean ± SD.
\textsuperscript{f} GLC\textsubscript{14} versus GLC\textsubscript{16}.
\textsuperscript{g} GLC\textsubscript{16} versus GLC\textsubscript{19}.
\textsuperscript{f} NS, not significant; CDNB, 1-chloro-2,4-dinitrobenzene.

GLC\textsubscript{14} and GLC\textsubscript{16}. In none of the three cell lines, Adr induced damage (10 µM, 1 h) could be repaired within 90 min (Fig. 6). For X-ray an identical amount of DNA strand breaks was induced in the three cell lines (Fig. 7). All three cell lines were capable to repair the 900-rad X-ray induced DNA damage completely within 90 min.

DISCUSSION

The three cell lines GLC\textsubscript{14}, GLC\textsubscript{16}, and GLC\textsubscript{19} which were established from one patient during clinical follow-up (7) may permit the study of in vivo acquired resistance to cytotoxic drugs and irradiation, provided that the mechanisms concerned remain present during in vitro culturing and that in vivo conditions do not select out for other clones of cells. The sensitivity of the patient's tumor at the time GLC\textsubscript{14} was developed is not reflected in a pronounced sensitivity of the cell line for the drugs tested, neither in comparison with the cell lines estab-
lished later nor with other cell lines with a known clinical pedigree (1). However, clinically relevant concentrations of Adr may be limited to the lower range of the concentrations tested in Fig. 1, where the sequence of sensitivity correlates with the clinical findings. Mechanisms that could be relevant to the development of resistance that were tested in this study included decreased intracellular drug levels, which are usually a result of an altered membrane transport (2, 22, 23); a changed intracellular enzyme capacity to detoxify electrophilic agents and drug induced free radicals (24, 25); and/or changes in DNA damage or repair capacity (1, 6, 26).

Intracellular Adr levels decrease in the cell lines concomitantly with the emergence of clinical resistance. This is a characteristic of MDR, and the patient was treated with so-called natural products. The cell line GLC10 turned out to be more resistant for Adr and X-ray in comparison with GLC14 and GLC19. Analysis of the proteins present in multidrug resistant cell lines has revealed one consistent change, overproduction of a 170,000, protein, or P-glycoprotein (27). The stain with JSB-1 antibody detecting the M, 170,000 glycoprotein (12) was most pronounced in GLC10. Surprisingly no reactivity was seen in GLC19. This last finding does not exclude that MDR plays a role in all three cell lines, but it does show that using only a stain with monoclonal antibody against M, 170,000 antigen does not predict clinical tumor drug resistance, although we are aware of the fact that, e.g., GLC19 could be just another clonal line which has been selected for which handles Adr differently.

Detoxifying systems often increase in capacity during exposure to antitumor therapy (4, 5). The elevated amount of GSH and the increased activity of GST can explain resistance to alkylating agents and agents that act by releasing free radicals. Accordingly, sensitivity increased after exposure of the cell lines to BSO (28, 29). The observed elevation of the catalase activity in the sequence GLC14, GLC16, and GLC19 shows that there is in this sequence also an increased capacity to detoxify free radicals, especially hydrogen peroxide.

The amount of X-ray induced DNA strand breaks is not different in the three cell lines, and all lines have an efficient repair of this damage. In contrast, Adr induced DNA strand breaks are not repaired in any of the lines within the observation period of 90 min. The high amount of DNA damage in GLC19 did not correlate with the low intracellular drug levels in that line. Probably also the compartmentalization and differences in efflux or metabolism of drugs in the cell can influence their effect.

It is concluded that the exposure of this patient’s tumor to combination chemotherapy and radiation coincided with the emergence of tumor cell clones with a number of adaptation mechanisms. From these mechanisms, the intracellular drug restriction and the capacity to detoxify electrophilic agents and drug induced free radicals correlate with the clinical development of resistance of the tumor.

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