Multifactorial Drug Resistance in an Adriamycin-resistant Human Small Cell Lung Carcinoma Cell Line

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ABSTRACT

In a human small cell lung carcinoma cell line, GLC, Adriamycin (ADR) resistance was induced. In the resistant cell line, GLC/ADR, a 45% decreased intracellular ADR level was found compared to GLC, but this could not fully explain the resistance. Evaluation of DNA damage in both cell lines after incubation with ADR by alkaline and neutral elution revealed single-strand breaks, DNA-protein cross-links, and double-strand breaks (DSB). At all incubation concentrations there was a decreased amount of all types of DNA damage in GLC/ADR. The number of DSB was decreased also when corrected for the decreased intracellular concentration. This can at least partly be explained by the decreased stability of ADR induced DSB. After removal of ADR, 80% of DSB was repaired in 1 h in GLC/ADR against no repair in GLC. X-ray induced DSBs were also repaired faster: in GLC/ADR \( t_{1/2} = 10 \) min and in GLC, \( t_{1/2} = 23 \) min.

Ratios for single strand breaks/DSBs and single strand breaks/DNA-protein cross-links between GLC and GLC/ADR after exposure to ADR differed; these differences were compatible with differences in the distribution of the various types of DNA damage induced in the cell lines due to an altered ADR-topoisomerase II interaction. In this human small cell lung carcinoma cell line the resistance is multifactorial with decreased intracellular ADR levels, increased DNA repair, and altered ADR-topoisomerase interaction.

INTRODUCTION

In small cell lung cancer patients, a first remission can be induced with ADR \(^3\) containing regimens in 70–90% of the cases \(^1\) but relapse is the rule. Subsequently during reinduction treatments remission rates decline and remission durations shorten \(^1\). This decrease in drug effectiveness is probably due to the acquisition of drug resistance \(^1–3\) by the tumor. Elucidation of the mechanisms involved in drug resistance could indicate ways to circumvent such a development and could become clinically important.

In order to study acquired ADR resistance in human tumor cells, an hSCLC cell line with resistance to ADR was developed \(^4\). As far as resistance mechanisms in this cell line are concerned we focussed on the presence of the drug in the cell and its effects on DNA.

Changes in intracellular drug levels are to be expected in the presence of PDR \(^2–3\). This form of resistance is based on a decreased intracellular drug level caused by an active outward transport of the drug \(^5, 6\). The drugs involved are “natural products” and usually include ADR. It is not known whether this form of resistance for ADR is prevalent in human tumors.

(3) or whether it can explain the occurrence of resistance to the drug in the clinical situation.

As far as DNA damage is concerned we especially studied the occurrence of DSBs in relation to intracellular ADR concentrations. Furthermore the stability of these DSBs after drug removal was evaluated. DNA damage brought about by ADR is mediated by topoisomerase II \(^7–9\). Resistance against this drug could be the result of changes in this enzyme. Indications for such changes might be found by studies investigating the ratio between the different types of DNA damage as measured by alkaline elution assay. We therefore measured SSBS, DSBs, and DPCs in both the resistant and sensitive cell line.

MATERIALS AND METHODS

Cell Line. The GLC cell line was derived in our laboratory from a pleural effusion of a patient with small cell lung cancer. GLC was cultured in RPMI 1640 (Gibco, Paisley, Scotland)-10% FCS (Flow Laboratories, Irvine, Scotland) at 37°C and 5% CO2 in a humidified atmosphere. It grows partly as loose floating aggregates and partly attached, with a doubling time of 26 h. It has the characteristics of a variant type hSCLC \(^4, 10\).

Induction of Drug Resistance. GLC was cloned after 100 passages by culturing a single cell suspension in 0.3% agarose in DME/F12, 40% conditioned RPMI 1640, and 20% FCS for 21 days. Individual colonies were isolated and cultured in RPMI 1640-10% FCS. In addition a cloning experiment was performed in 18 nM ADR. A well-growing colony was selected and cultured to be used for further drug resistance induction. Culturing this subclone in RPMI 1640, 10% FCS, and 18 nM ADR resulted initially in about 90% cell death. After 3 passages with this drug concentration, however, less than 50% cell kill was observed and the ADR concentration was then doubled. This procedure was repeated every third passage until an ADR concentration of 1152 nM was reached. After 20 passages at 1152 nM there was no apparent cell death, and the cell line could be passed twice a week in growth medium with 1152 nM ADR. The thus established ADR resistant cell line was called GLC/ADR. The doubling time remained at 26 h.

Clonogenic Assay. Underlayers consisting of 0.5% agar (Difco, Detroit, MI) in DME (Flow Laboratories)/F12 nutrient mixture (Flow Laboratories)-20% FCS in a 35-mm Petri dish were used in all experiments. For clonogenic assay with continuous drug incubation a single cell suspension in 0.3% agarose (FMG, Rockland, ME) in DME/F12, 20% FCS with the appropriate drug concentration was used as upper layer. In other cases, when only a 1-h drug incubation period was used the cells were incubated with the drug at various concentrations in RPMI 1640-10% FCS at 37°C. After washing 3 times with PBS a suspension was prepared in 0.3% agarose in DME/F12-20% FCS without drug and plated as upper layer. For GLC, 3000 viable cells (triply blue exclusion) and for the resistant subline 5000 viable cells were cloned per dish. Colonies containing more than 40 cells were counted at about day 8. Drug resistance was measured by plotting the percentage of the colonies grown in the drug treated compared to the untreated control against the log of the drug concentration.

Resistance was evaluated for ADR (Farmitalia Carlo Erba, Milano, Italy), and vincristine (Eli Lilly, Indianapolis, IN) after both 1 h and continuous incubation in a clonogenic assay. cis-Diaminedichloroplatinum, VP 16-213 (both Bristol-Myers, Madrid, Spain), vindesine (Eli-Lilly), actinomycin D (MSD, Rahway, NJ), melphalan (Wellcome, London, England), and colchicine (Roussel, Paris, France) were tested.

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3 The abbreviations used are: ADR, Adriamycin; PDR, pleiotropic drug resistance; SSBS, single strand break; DSB, double strand break; DPC, DNA-protein cross-link; hSCLC, human small cell lung carcinoma; DME, Dulbecco’s modified Eagle’s (medium); FCS, fetal calf serum; PBS, phosphate buffered saline (136 mM NaCl-2.5 mM KCl-6.5 mM Na2HPO4-1.5 mM KH2PO4, pH 7.3); SDS, sodium dodecyl sulfate; VP 16-213, etoposide.
in the assay with 1 h drug incubation. For the testing of radiosensitivity, cells were irradiated for various times using a Philips X-ray source at 200 kV, 15 mA, and 0.5 mm Cu/Al filter giving 200 rads/min at 21 cm and clamped as described above.

Labeling, Incubation, and Irradiation Procedures. Prior to all procedures GLC4/ADR was cultured 10 days in drug-free culture medium. For DNA studies, log phase cells were labeled for 48 h with [3H]-thymidine (New England Nuclear, Boston, MA), 0.1 μCi/ml (50 mCi/mmol). Before ADR incubation there was a 2-h chase period. For drug incubation 1 ml cell suspension containing 6–8 × 10^6 cells for SSBs and DPCs or 2–3 × 10^6 cells for DSBs was added to 1 ml culture medium containing twice the desired drug concentration. ADR solutions were freshly prepared. For irradiation, cells in culture medium were placed on ice and irradiated using an X-ray source (15 mA; 200 kV; 0.5 mm Cu/Al filter; 900 rads/min; Philips, Eindhoven, The Netherlands).

Intracellular ADR Concentration. Cells were incubated with ADR for 1 h under the same conditions as for DSB assay. After incubation the cell suspension was cooled on ice and washed 3 times with 5 ml ice-cold PBS. The pellet was extracted overnight at -20°C in the dark with 3 ml 0.3 N HCl/50% ethanol. Total fluorescence of the supernatant was measured with a Kontron spectrofluorometer at an excitation of 474 nm and an emission of 549 nm. A calibration curve was prepared by dissolving a stock solution of ADR in 0.3 N HCl/50% ethanol (11).

SSBs. Alkaline elution according to the method of Kohn et al. (12) was used with some minor changes as described earlier (13). Briefly, 6–8 × 10^6 cells were layered on mixed cellulose ester filters (Millipore S.A., Molsheim, France), pore size 3.0 μm, after drug incubation and washed with 30 ml ice-cold PBS. Cells were lysed with 2 ml 0.2% sodium lauryl sarcosine (Sigma, St. Louis, MO) in 0.04 M EDTA-2 M NaCl, pH 9.6; after 15 min, 20 ml ice-cold 0.02 M EDTA, pH 10 were added and allowed to flow by gravity.

Proteins were removed with a second lysing solution, 2% SDS (Sigma)-0.02 M EDTA, pH 9.6 with 0.5 mg/ml proteinase K (catalogue no. 24568; Merck, Darmstadt, West Germany) for 1 h. After removal of the lysing solution, 27 ml elution buffer-0.02 M EDTA-0.1% SDS, pH 12.4 were added and run with an elution speed of 0.05 ml/min. Nine fractions of 3 ml each were collected. The fractions and pumped-dried filters were dissolved in 5 ml Picofluor (Packard, Downers Grove, IL), and radioactivity was measured by liquid scintillation counting. Each run contained untreated and X-irradiated control samples. The fraction remaining on the filter after 21 ml was used for calculation of the SSBs in radequivalents (that dose of X-ray inducing an equivalent fraction remaining on the filter after 21 ml was used for calculation of IL), and radioactivity was measured by liquid scintillation counting.

RESULTS

Clonogenic Assay. In Fig. 1 the results are shown of a clonogenic assay using a 1-h ADR incubation of GLC4 and GLC4/ADR. There is a 44-fold increase in drug concentration inhibiting colony formation by 50%.

The concentration of drug inhibiting colony formation by 50% and resistance factor of GLC4 and GLC4/ADR for several other drugs in the clonogenic assay using both 1 h and continuous incubation are shown in Table 1. Cross-resistance was found for vincristine, vindesine, VP 16-213, cis-diaminedichloroplatinum, and X-irradiation, while there was no cross-resistance for melphanal, colchicine, and actinomycin D.

Intracellular ADR Concentrations. The intracellular ADR concentrations in both cell lines after 1 h incubation with ADR are shown in Fig. 2. The intracellular ADR concentration is reduced in GLC4/ADR at all incubation concentrations tested with a mean decrease of 45% (P < 0.02; t test).

SSBs. Results are shown in Table 2. The number of SSBs in GLC4/ADR was consistently lower than in GLC4 (P < 0.01; t test) for concentrations below 20 μM.

Table 1. Cross-resistance: IC^50 and resistance factor in clonogenic assay after continuous and 1 h incubation for GLC4 and GLC4/ADR

<table>
<thead>
<tr>
<th>Drug</th>
<th>IC^50 of GLC4 (μm)</th>
<th>IC^50 of GLC4/ADR (μm)</th>
<th>Resistance factor</th>
</tr>
</thead>
<tbody>
<tr>
<td>Continuous incubation</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ADR</td>
<td>0.04</td>
<td>1.265</td>
<td>32</td>
</tr>
<tr>
<td>VCR</td>
<td>0.0017</td>
<td>0.0055</td>
<td>3.2</td>
</tr>
<tr>
<td>1 h incubation</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ADR</td>
<td>0.285</td>
<td>12.5</td>
<td>44</td>
</tr>
<tr>
<td>Vincristine</td>
<td>0.060</td>
<td>0.347</td>
<td>5.7</td>
</tr>
<tr>
<td>Vindesine</td>
<td>0.15</td>
<td>4.5</td>
<td>30</td>
</tr>
<tr>
<td>VP 16-213</td>
<td>3.4</td>
<td>127</td>
<td>37.5</td>
</tr>
<tr>
<td>cis-Diaminedichloroplatinum</td>
<td>5</td>
<td>10.7</td>
<td>2.1</td>
</tr>
<tr>
<td>Melphanal</td>
<td>1.8</td>
<td>0.75</td>
<td>0.42</td>
</tr>
<tr>
<td>Actinomycin D</td>
<td>0.24</td>
<td>0.22</td>
<td>0.92</td>
</tr>
<tr>
<td>Colchicine</td>
<td>1.67</td>
<td>0.8</td>
<td>0.48</td>
</tr>
<tr>
<td>X-ray (rads)</td>
<td>91</td>
<td>296</td>
<td>3.2</td>
</tr>
</tbody>
</table>

^IC^50, concentration of drug inhibiting colony formation by 50%.

Stability of ADR Induced DSBs. The elution procedure was identical to DSB detection. Both cell lines were incubated with 10 μM ADR as described above. ADR was removed after 1 h by dilution and centrifugation. The cells were incubated at 37°C in drug free medium for the appropriate time. Repair was stopped by rapid cooling on ice and immediate assay of DSBs. The number of DSBs remaining after the incubation period was expressed as a percentage of the initial number of DSBs at r = 0.

Repair of X-Ray Induced DSBs. A cell suspension with 2–3 × 10^6 cells in 0.5 ml was placed on ice and irradiated with 3600 rads. Repair was initiated by placing the cells at 37°C. Repair was stopped by dilution with ice-cold culture medium and rapid cooling on ice. DSBs were assayed immediately.
Fig. 2. Intracellular ADR concentration in GLC4 (•) and GLC4/ADR (D) after 1 h incubation. Ordinate, concentration of ADR in incubation medium in μM; abscissa, intracellular ADR concentration in pmol/10^6 cells; bars, SE (n = 6). P < 0.02 by t test for each incubation concentration.

Fig. 3. DSBs detected by neutral elution in GLC4 (•) and GLC4/ADR (D) in radequivalents (Radeq) plotted against the intracellular ADR concentration in pmol ADR/10^6 cells. Bars, SD.

Fig. 4. Stability of DSBs induced by 1 h incubation with 10 μM ADR in GLC4 (•) and GLC4/ADR (D). DSBs detected by neutral elution are expressed as a percentage of the initial amount of DSBs and plotted against the repair time in h. Bars, SE; n = 4.

**DISCUSSION**

It was possible to induce ADR resistance *in vitro* in this established hSCLC cell line. The drug resistance observed was found to be limited not only to ADR. In the spectrum of cross-resistance there were definite differences with the so-called PDR. In contrast to the pattern described in PDR (2, 3), the GLC4/ADR cell line showed no cross-resistance against at least 2 natural products, colchicine and actinomycin D. In PDR the mechanism supposedly responsible for drug resistance was an active outward drug transport, resulting in lowered intracellular
The amount of DSBs is related to the intracellular ADR concentration; there are less DSBs per amount of ADR in GLC4/ADR (Fig. 3). The DSBs after 1 h are the net effect of newly induced damage and already ressealed breaks. Therefore an explanation for the lower amount of DSBs in GLC4/ADR can be a decreased DSB stability. We have indeed detected this increased DSB repair after ADR removal (Fig. 4). Whether this increased disappearance rate of DSBs in the resistant cell line is the result of an improved repair capacity is not certain. The fact, however, that there is also an increased rate of rescaling of X-ray induced DSBs (Fig. 5) in GLC4/ADR indeed suggests an improved DNA repair mechanism.

A different interaction of ADR with topoisomerase II resulting in a diminished amount of DSBs or in less stable DSBs might also interfere with resistance (17, 21, 22). Indications for this altered ADR-topoisomerase interaction can be deduced from the ratios between the different types of DNA damage. The SSB/DSB and SSB/DPC ratios are different in both cell lines, after exposure to comparable ADR concentrations. This altered ratio might be the effect of a modified topoisomerase. Modification of topoisomerase II can be related to drug resistance as recently has been shown in resistance to VP 16-213 (22, 23).

The results discussed in this paper indicate that acquired ADR resistance in this cell line is only partly explained by a decreased intracellular drug concentration. The DNA damage is diminished in the resistant cell line and the most lethal type of damage is less stable possibly due to an increased DNA repair capacity or an altered ADR-topoisomerase II interaction. The clinical implication can be that circumvention of drug resistance has to be based on multiple principles instead of only increasing the intracellular drug uptake.

REFERENCES


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