Biochemical Characterization of Resistance to Mitoxantrone and Adriamycin in Caco-2 Human Colon Adenocarcinoma Cells: A Possible Role for Glutathione S-Transferases

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ABSTRACT

Cytotoxicity of Adriamycin on human colon adenocarcinoma cell lines was investigated. Concentrations of Adriamycin producing 50% inhibition were very similar in HT29, Sw480, Sw620, and Sw1116 cells, whereas Caco-2 cells were relatively insensitive. As compared to the Sw1116 cell line, Caco-2 cells were also insensitive to mitoxantrone. Sensitivity to cisplatin, 5-fluorouracil, or ethacrynic acid was comparable in both cell lines. To find the mechanism for this mitoxantrone and Adriamycin resistance, several potential Adriamycin-detoxifying systems were characterized and quantified in both Sw1116 and Caco-2 cells. No dramatic differences in glutathione content and expression of both selenium-dependent- and independent glutathione peroxidase, UDP-glucurononyltransferase, and cytochrome P-450 were found. However, highly significant differences in glutathione S-transferase activity were present, the expression of both class ε and class α glutathione S-transferases being much higher in the Caco-2 cell line. In addition, a slightly higher content of P-170 glycoprotein was present in the Caco-2 cells. These findings suggest that glutathione S-transferases, and to a lesser extent the P-170 glycoprotein, may be involved in mitoxantrone and Adriamycin resistance of Caco-2 colon carcinoma cells.

INTRODUCTION

In the treatment of gastrointestinal malignancies with anticancer drugs, the frequent occurrence of resistance to these drugs represents a significant problem. One can distinguish two forms of drug resistance: primary, or intrinsic resistance, and secondary, or acquired resistance. In the latter case, a tumor, after an initial response, becomes insensitive to a given treatment. A better understanding of the mechanism leading to drug resistance, whether intrinsic or acquired, could indicate ways to overcome the anticancer drug resistance.

In this respect, it would be elucidating to study malignant cells or tissue obtained before and after treatment with chemotherapeutics from patients in whom the tumor has become resistant. However, this kind of material is scarce, and the data available are restricted (1–5). From these few studies, there are indications for a role of glutathione and glutathione S-transferases, a group of detoxifying enzymes (6), in the phenomenon of acquired resistance.

In several in vitro studies of cell lines resistant to a broad range of chemotherapeutics, further evidence was obtained that increased levels of glutathione and glutathione S-transferases may be related to acquired resistance (6–25). Resistance to anticancer drugs was generated by expression of class α and π glutathione S-transferases via transfection of their copy DNAs in various types of cells (26–28).

In addition, it was shown that sensitivity to anticancer drugs increased by lowering levels and activity of glutathione and glutathione S-transferases, respectively (13, 23, 29–31). Investigation of paired specimens from both normal and malignant tissue from the same patients indicates that glutathione S-transferase enzyme activity generally is higher in refractory tumors (32–35). Glutathione S-transferases are involved in the metabolism of anticancer drugs (36, 37) such as melphalan (38), chlorambucil (39), phenylalanine mustard (40), cyclophosphamide (41), and mitoxantrone (42). As a result, increased glutathione S-transferase enzyme activity may lead to a decreased cytotoxic effect of anticancer drugs with respect to tumors or tumor-derived cell lines.

There is a large body of evidence, however, that other mechanisms for drug resistance may be present. Of special importance seems to be the plasma membrane-located P-170 glycoprotein or multidrug resistance gene product which requires ATP for functional activity, thereby pumping the cytotoxic drugs out of the cell (see ref. 43 and references therein).

In the search for the mechanism of anticancer drug resistance in colon tumors, we compared the biochemical characteristics of the colon adenocarcinoma-derived cell lines Sw1116 and Caco-2, which are sensitive and relatively insensitive to Adriamycin, respectively.

MATERIALS AND METHODS

Reagents. The following reagents were used: Adriamycin (Farmitalia, Rotterdam, The Netherlands); methotrexate (Multipharma, Weesp, The Netherlands); cis-diammine dichloroplatinum (cisplatinum; Pharmacemie BV, Haarlem, The Netherlands); mitoxantrone (Lederle Nederland BV, Etten-Leur, the Netherlands); ethacrynic acid, MTT,2 UDP-glucurononyltransferase, and cytochrome P-450 were found. However, highly significant differences in glutathione S-transferase activity were present, the expression of both class ε and class α glutathione S-transferases being much higher in the Caco-2 cell line. In addition, a slightly higher content of P-170 glycoprotein was present in the Caco-2 cells. These findings suggest that glutathione S-transferases, and to a lesser extent the P-170 glycoprotein, may be involved in mitoxantrone and Adriamycin resistance of Caco-2 colon carcinoma cells.

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glucuronyltransferase (50), and glutathione S-transferase class μ (51) and θ (34) were given in earlier papers. Monoclonal antibodies to class α glutathione S-transferases were developed recently. These antibodies give a strong reaction with the B, B1, B2, and B2, B3 isomers and do not cross-react with class μ or θ isoenzymes.3

Immunoblots were quantified by densitometric analysis, using a laser densitometer (Ultrascan XL; LKB, Bromma, Sweden).

Quantification of P-170 Glycoprotein. Plasma membrane-enriched fractions from both Sw116 and Caco-2 cells were prepared as follows. After homogenization, cells were centrifuged at 400 × g for 10 min, and subsequently, the supernatant was centrifuged at 10,000 × g for 15 min. Pellets were resuspended in 20 mM Tris/HCl buffer (pH 7.4), containing 1% dithiothreitol and 0.25 mM succrose, and subjected to SDS polyacrylamide gel electrophoresis, Western blotting, and subsequent incubation with a monoclonal antibody against the P-170 glycoprotein (C219; Centocor, Malvern, PA). Detection was performed using the enhanced chemiluminescence method, according to the instructions of the manufacturer (Amersham, Buckinghamshire, United Kingdom). Staining intensity on the film (X-OMAT; Kodak, Rochester NY) of the immunoblot by enhanced chemiluminescence (see Fig. 3), using the monoclonal antibody “219, and is expressed in arbitrary absorbance units per mg protein.

RESULTS

IC50 values for Adriamycin were determined in the human colon adenocarcinoma cell lines Caco-2, HT29, Sw480, Sw620, and Sw116. Under the specific MTT assay conditions used here, values were very close to 20 μM, except for the Caco-2 cells for which a concentration of 525 ± 209 μM (mean ± SEM) Adriamycin was needed to kill 50% of the cells (Table 1). Glutathione S-transferase enzyme activity was also assayed in these cell lines, and the activity was highest in the Caco-2 cells (Table 1).

In addition, cytotoxicity of several other drugs was tested in the Caco-2 cells and in a cell line much more sensitive to Adriamycin, the Sw116 cell line. Results are given in Table 2. Caco-2 cells are also insensitive to mitoxantrone, whereas cisplatin, 5-fluorouracil, and ethacrynic acid have more or less similar effects on both Caco-2 and Sw116 cells; the cytotoxic effect of 5-fluorouracil is very low.

To elucidate the mechanism responsible for the insensitivity of Caco-2 cells with regard to Adriamycin and mitoxantrone, several systems potentially involved in removal or metabolism of these compounds were quantified (Table 3). Content and enzyme activity of glutathione, selenium dependent- and independent glutathione peroxidase, cytochrome P-450, UDP-glucuronyltransferase, and P-170 glycoprotein did not differ dramatically between the Caco-2 and Sw116 cells. However, content and activity of glutathione S-transferases, and more specifically glutathione S-transferases α and θ, were much higher in the Caco-2 cells. This is visualized in the Figs. 1 and 2. Fig. 1 shows the cytosolic protein profiles from both cell lines, indicating the high expression in the Caco-2 cells of a protein comigrating with glutathione S-transferase α. Densitometric analysis of the Sw116 and Caco-2 cytosolic protein fractions from both Sw116 and Caco-2 cells were prepared as follows. After homogenization, cells were centrifuged at 400 x g for 10 min, and subsequently, the supernatant was centrifuged at 10,000 x g for 15 min. Pellets were resuspended in 20 mM Tris/HCl buffer (pH 7.4), containing 1% dithiothreitol and 0.25 mM succrose, and subjected to SDS polyacrylamide gel electrophoresis, Western blotting, and subsequent incubation with a monoclonal antibody against the P-170 glycoprotein (C219; Centocor, Malvern, PA). Detection was performed using the enhanced chemiluminescence method, according to the instructions of the manufacturer (Amersham, Buckinghamshire, United Kingdom). Staining intensity on the film (X-OMAT; Kodak, Rochester NY) of the immunoblot (Fig. 2, panel 1, shows the purified glutathione S-transferases from Sw116 and Caco-2 cells and indicates that glutathione S-transferase α is the most prominent isoenzyme in both cell lines. However, some higher molecular mass class α transferases.

Table 1 Adriamycin cytotoxicity and glutathione S-transferase enzyme activities in several human colon adenocarcinoma cell lines

<table>
<thead>
<tr>
<th>Cell line</th>
<th>IC50 value for Adriamycin (μM)</th>
<th>Glutathione S-transferase activity (nmol/mg protein/min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Caco-2</td>
<td>525 ± 209</td>
<td>3680 ± 236</td>
</tr>
<tr>
<td>HT29</td>
<td>19 ± 4</td>
<td>206 ± 54</td>
</tr>
<tr>
<td>Sw480</td>
<td>17 ± 2</td>
<td>248 ± 40</td>
</tr>
<tr>
<td>Sw620</td>
<td>18 ± 7</td>
<td>249 ± 26</td>
</tr>
<tr>
<td>Sw116</td>
<td>48 ± 20</td>
<td>285 ± 52</td>
</tr>
</tbody>
</table>

*IC50 values were determined by using the MTT assay (see "Materials and Methods") as follows: Sw116 and Caco-2 cells were seeded (10⁶ cells and 5 × 10⁶ cells/well, respectively) in 96-well tissue culture plates. After 24 h, the cells were exposed to Adriamycin for 2 h and subsequently cultured without drug for 48 h. Thereafter, cells were exposed to MTT for 4 h, and the amount of formazan was quantified.

Table 2 IC50 values for (antineoplastic) drugs in Caco-2 and Sw116 colon carcinoma cells

<table>
<thead>
<tr>
<th>Drug</th>
<th>IC50 (μM) Caco-2</th>
<th>IC50 (μM) Sw116</th>
<th>Ratio Caco/Sw</th>
</tr>
</thead>
<tbody>
<tr>
<td>Adriamycin</td>
<td>525 ± 10</td>
<td>48 ± 20</td>
<td>11</td>
</tr>
<tr>
<td>Mitoxantrone</td>
<td>1600 ± 30</td>
<td>8 ± 1</td>
<td>207</td>
</tr>
<tr>
<td>Cisplatin</td>
<td>230 ± 120</td>
<td>430 ± 8</td>
<td>0.53</td>
</tr>
<tr>
<td>5-Fluorouracil</td>
<td>&gt;19,230</td>
<td>9100 ± 1800</td>
<td>±2.1</td>
</tr>
<tr>
<td>Ethacrynic acid</td>
<td>54 ± 11</td>
<td>35 ± 7</td>
<td>1.5</td>
</tr>
</tbody>
</table>

*IC50 values were determined using the MTT assay as described for Adriamycin in Table 1. IC50 value for 5-fluorouracil in Caco-2 cells is only slightly higher than the indicated value (19,230 μM) since here 47% of the cells have died. It was practically impossible, however, to use higher concentrations of 5-fluorouracil in the MTT assay.

Table 3 Biochemical characteristics of Sw116 and Caco-2 colon carcinoma cells

<table>
<thead>
<tr>
<th>Drug</th>
<th>Sw116</th>
<th>Caco-2</th>
<th>Ratio Caco/Sw</th>
</tr>
</thead>
<tbody>
<tr>
<td>Doubling time (day)</td>
<td>0.35 ± 0.04*</td>
<td>0.81 ± 0.18</td>
<td>2.3</td>
</tr>
<tr>
<td>Glutathione (nmol/mg protein)</td>
<td>17.4 ± 1.7</td>
<td>13.1 ± 0.7</td>
<td>0.8</td>
</tr>
<tr>
<td>Selenium dependent glutathione peroxidase (nmol/mg protein/min)</td>
<td>89 ± 6</td>
<td>42 ± 1</td>
<td>0.5</td>
</tr>
<tr>
<td>Selenium independent glutathione peroxidase (nmol/mg protein/min)</td>
<td>17.0 ± 0.9</td>
<td>5.6 ± 0.4</td>
<td>0.33</td>
</tr>
<tr>
<td>Glutathione S-transferase (nmol/mg protein)</td>
<td>285 ± 52</td>
<td>3680 ± 236</td>
<td>12.9</td>
</tr>
<tr>
<td>Glutathione S-transferase α (μg/mg protein/min)</td>
<td>6.0 ± 0.6</td>
<td>37.6 ± 3.0</td>
<td>6.3</td>
</tr>
<tr>
<td>Glutathione S-transferase α (μg/mg protein/min)</td>
<td>60.0 ± 1.5</td>
<td>75.0 ± 2.0</td>
<td>1.25</td>
</tr>
<tr>
<td>Cytochrome P-450 (units/mg protein)</td>
<td>2.08</td>
<td>1.52</td>
<td>0.7</td>
</tr>
<tr>
<td>UDP-glucuronyltransferase (units/mg protein)</td>
<td>1.84</td>
<td>0.98</td>
<td>0.5</td>
</tr>
<tr>
<td>P-170 glycoprotein (units/mg protein)</td>
<td>9.7 ± 0.1</td>
<td>15.6 ± 3.8</td>
<td>1.6</td>
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*Mean ± SEM. All experiments were performed at least in triplicate.

Table 4 Biochemical characteristics of Sw116 and Caco-2 colon carcinoma cells

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ADRIAMYCIN RESISTANCE IN CACO-2 HUMAN COLON CARCINOMA CELLS

Increased glutathione S-transferase or glutathione peroxidase enzyme activity (9, 12, 22, 60). However, in other studies of the mechanism of Adriamycin resistance in mammary (1) or colon adenocarcinoma (61) cell lines, there was no evidence for a role of the P-170 glycoprotein.

The human colon adenocarcinoma cell lines we investigated here were not made resistant in vitro. The Caco-2 cells may have had primary resistance or the insensitivity toward Adriamycin is the result of acquired resistance due to the treatment with chemotherapeutics given to the patient from whom the cell line was derived (62).

As compared to the SW1116 cell line, the Caco-2 cells only have a high degree of resistance toward Adriamycin and mitoxantrone. These two compounds are structurally related and share in common the quinone groups, which are capable of reduction and autoxidation to produce oxygen radicals (61). However, mitoxantrone may be metabolized by cytochrome P-450-catalyzed oxidation, resulting in highly electrophilic intermediates (42, 63). As a consequence, alkylation may be another mode of action of mitoxantrone. Subsequently, these intermediates may be conjugated with glutathione and glucuronic acid and catalyzed by glutathione-S-transferases and UDP-glucuronyltransferases, respectively (42, 63).

The Adriamycin-/mitoxantrone-insensitive Caco-2 cells, as are also expressed in the Caco-2 cells. These observations are confirmed in the immunoblots of Fig. 2, where cytosolic fractions from both cell lines were treated with monoclonal antibodies against class π (panel 2) and class α (panel 3) glutathione S-transferases. Class glutathione S-transferases were undetectable on immunoblots of both cell lines.

Fig. 3 shows the P-170 glycoprotein in plasma membrane-enriched fractions of both cell lines. Staining intensity of the P-170 glycoprotein was quantified by densitometry. In Caco-2 cells, the P-170 glycoprotein content is 1.6-fold higher than in the SW1116 cells (Table 3).

DISCUSSION

Several cell lines that were made resistant in vitro to one drug often exhibit a cross-resistance to a wide variety of other drugs, which are structurally or mechanistically unrelated (9, 57). This multidrug resistance was often associated with the overexpression of the P-170 glycoprotein or MDR-1 gene product (57, 58).

Recently this mechanism was shown to be present in the SW620 human colon adenocarcinoma cell line made resistant to Adriamycin (59). In addition to overexpression of the P-170 glycoprotein, other mechanisms may contribute to the multidrug resistant phenotype, as shown in an Adriamycin-resistant P388 leukemia (12) and a rat mammary adenocarcinoma (60) cell line. These are an earlier onset of DNA repair (12) and an
that similar experiments in MCF7 breast tumor cells do not support this hypothesis (65-67).

Despite the fact that other mechanisms not investigated here, such as increased DNA repair or decreased topoisomerase II activity (68), may be responsible for, or contribute to, the drug resistance, the observed insensitivity to Adriamycin/mitoxantrone with concomitant high expression of class \( \pi \) and \( \alpha \) glutathione S-transferases suggests that these enzymes may contribute to the resistance in Caco-2 cells.

REFERENCES


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