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-glucuronyltransferase, and cytochrome P-450 were found. However, highly significant differences in glutathione S-transferase activity were present, the expression of both class π 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 Sw1116 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 serious 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). Investigations 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 melphanal (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; Pharmachemie BV, Haarlem, the Netherlands); mitoxantrone (Lederle Nederland BV, Etten-Leur, the Netherlands); ethacrynic acid, MTT, glutathione, glutathione agaroese, 1-chloro 2,4-dinitrobenzene, and 5,5'-dithio dihydroxybenzoic acid (Sigma Chemical Co., St Louis, MO).

Cell Culture. Sw1116 and Caco-2 colon carcinoma cells were obtained from the American Type Culture Collection (Rockville, MD) and were grown as monolayers in Dulbecco’s modified Eagle’s medium supplied with 10% (v/v) fetal bovine serum, 1% nonessential amino acids, 1 mM L-glutamine, 20 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid, and 50 μg/ml gentamycin in a 5% CO₂ atmosphere at 90-100% humidity. Cells were passaged after a prior incubation for 3-5 min with a trypsin (0.05%, w/v)/EDTA (0.02%, w/v) solution. All culture media products were obtained from Flow Laboratories (Irvine, Scotland). Cells were harvested and homogenized as described before (44). Microsomal (150,000 g pellet) and cytosolic (150,000 g superna tant) fractions were made essentially by using recently published methods (45).

Drug Sensitivity Assay. Cytotoxicity was determined and IC₅₀ values were calculated by using the microculture tetrazolium assay as described by Alley et al. (46) and Carmichael et al. (47).

SDS Polycrylamide Gel Electrophoresis and Immunoblotting. SDS polycrylamide gel electrophoresis was done according to the method of Laemmli (48). Western blotting and subsequent incubation with antibodies were performed as described before (49). A semidry blotting system (Novablot II; Pharmacia, Uppsala, Sweden) was used. Details of the monoclonal antibodies against cytochrome P-450 (45), UDP

1 The abbreviations used are: MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-dipheny lltetrazolium bromide; IC₅₀, concentration producing 50% inhibition; SDS, sodium dodecyl sulfate.
glucuronyltransferase (50), and glutathione S-transferase class μ (51) and π (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 Bb, B2, and B2B2 isoforms and do not cross-react with class n or π transferases.1

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

Quantification of P-170 Glycoprotein. Plasma membrane-enriched fractions from both Sw1116 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 mM 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 band comigrating with the P-170 marker protein was quantified by densitometry.

Miscellaneous. Reduced glutathione was assayed by the method of Anderson (52), protein by the method of Lowry et al. (53), glutathione S-transferase activity by the method of Habig et al. (54), and glutathione peroxidase by the method of Wendel (55). Glutathione S-transferases were purified from 150,000-g supernatants by affinity chromatography, as described before (56).

RESULTS

IC50 values for Adriamycin were determined in the human colon adenocarcinoma cell lines Caco-2, HT29, Sw480, Sw620, and Sw1116. 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 Sw1116 cell line. Results are given in Table 2. Caco-2 cells are also insensitive to mitoxantrone, whereas cisplatin, 5-fluorouracil, and ethylic acid have more or less similar effects on both Caco-2 and Sw1116 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 Sw1116 cells. However, content and activity of glutathione S-transferases, and more specifically glutathione S-transferases π and α, 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 π. Densitometric analysis of the Sw1116 and Caco-2 cytosolic protein patterns revealed a glutathione S-transferase π protein content of 0.5 ± 0.1 and 4.2 ± 0.5% of total protein, respectively. These values are in accordance with the data obtained after quantification of glutathione S-transferase π protein content from the immunoblot (Fig. 2, Table 3).

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>HT-29</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 ± 96</td>
</tr>
<tr>
<td>Sw1116</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: Sw1116 and Caco-2 cells were seeded (104 cells and 5 × 105 cells/well, respectively) in 96-wells 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 Sw1116 colon carcinoma cells

<table>
<thead>
<tr>
<th>Drug</th>
<th>IC50 value (μM)</th>
<th>Ratio Caco/Sw</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Sw1116</td>
<td>Caco-2</td>
</tr>
<tr>
<td>Adriamycin</td>
<td>525 ± 10</td>
<td>11</td>
</tr>
<tr>
<td>Mitoxantrone</td>
<td>1660 ± 30</td>
<td>207</td>
</tr>
<tr>
<td>Cisplatin</td>
<td>230 ± 120</td>
<td>0.53</td>
</tr>
<tr>
<td>5-Fluorouracil</td>
<td>&gt;19,230</td>
<td>2.08 1.52</td>
</tr>
<tr>
<td>Ethyl oracil</td>
<td>54 ± 11</td>
<td>0.53</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>3.05 ± 0.4</td>
</tr>
<tr>
<td></td>
<td></td>
<td>17.4 ± 1.7</td>
</tr>
<tr>
<td></td>
<td></td>
<td>93.0 ± 1.6</td>
</tr>
<tr>
<td></td>
<td></td>
<td>17.0 ± 0.9</td>
</tr>
<tr>
<td></td>
<td></td>
<td>5.6 ± 0.4</td>
</tr>
<tr>
<td></td>
<td></td>
<td>285 ± 52</td>
</tr>
<tr>
<td></td>
<td></td>
<td>3680 ± 236</td>
</tr>
<tr>
<td></td>
<td></td>
<td>6.0 ± 0.6</td>
</tr>
<tr>
<td></td>
<td></td>
<td>376 ± 3.0</td>
</tr>
<tr>
<td></td>
<td></td>
<td>6.0 ± 0.6</td>
</tr>
<tr>
<td></td>
<td></td>
<td>2.9 ± 0.9</td>
</tr>
<tr>
<td></td>
<td></td>
<td>3.08 ± 1.52</td>
</tr>
<tr>
<td></td>
<td></td>
<td>1.84 ± 0.98</td>
</tr>
<tr>
<td></td>
<td></td>
<td>9.7 ± 0.1</td>
</tr>
</tbody>
</table>

* Mean ± SEM for at least three determinations, except for cytochrome P-450 and UDP-glucuronyltransferase which were assayed only once.


4 Mean ± SEM. All experiments were performed at least in triplicate.

Table 3 | Biochemical characteristics of Sw1116 and Caco-2 colon carcinoma cells

<table>
<thead>
<tr>
<th>Drug</th>
<th>Caco-2</th>
<th>Sw1116</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>3.03</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 (μg/mg protein)</td>
<td>6.0 ± 0.6</td>
<td>376 ± 3.0</td>
<td>6.3</td>
</tr>
<tr>
<td>Glutathione S-transferase protein (μg)</td>
<td>2.9 ± 0.9</td>
<td>High</td>
<td></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>
</tr>
</tbody>
</table>

* Mean ± SEM for at least three determinations, except for cytochrome P-450 and UDP-glucuronyltransferase which were assayed only once.

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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 \( \pi \) (panel 2) and class \( \alpha \) (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

Fig. 1. Cytosolic protein profile of Sw1116 and Caco-2 colon carcinoma cells. Cytosolic protein (68 μg) from Sw1116 (Sw) and Caco-2 (Ca) colon carcinoma cells was subjected to SDS polyacrylamide gel electrophoresis (10%, w/v, acrylamide). In lane M, 0.2 μg purified glutathione S-transferase \( \pi \) from human placenta was applied. The proteins were stained with Coomassie brilliant blue. Arrow, high expression in the Caco-2 cells of a protein with a molecular mass identical with glutathione S-transferase \( \pi \).

Fig. 2. Characterization of glutathione S-transferases from Sw1116 and Caco-2 colon carcinoma cells. In 1, glutathione-agarose-purified glutathione S-transferases from Sw1116 (Sw; 0.4 μg protein) and Caco-2 (Ca; 1.0 μg protein) colon carcinoma cells were separated by SDS polyacrylamide gel electrophoresis (10%, w/v, acrylamide) and stained with Coomassie brilliant blue. Lane M, 0.5 μg of purified glutathione S-transferase \( \pi \) from human placenta; arrow, glutathione S-transferase subunits with higher molecular mass as compared to the class \( \alpha \) transferase. This band represents class \( \alpha \) glutathione S-transferase (see also panel 3). In 2, cytosols (30 μg protein) from Sw1116 and Caco-2 cells were subjected to SDS polyacrylamide gel electrophoresis (10%, w/v, acrylamide) and subsequent Western blotting. The blot was incubated with a monoclonal antibody against glutathione S-transferase \( \pi \). Lane M, 0.5 μg purified glutathione S-transferase \( \pi \) from human placenta. Staining intensity was quantified by densitometry and results are given in Table 3. In 3, detection of glutathione S-transferase \( \alpha \) in Sw1116 and Caco-2 colon carcinoma cells is shown. Cytosols (60 μg protein) from Sw1116 and Caco-2 cells were treated as described for 2. Glutathione S-transferase \( \alpha \) was immunodetected with a monoclonal antibody. Lane M, 0.13 μg of purified glutathione S-transferase \( \alpha \) from human liver. Staining intensity was quantified by densitometry and results are given in Table 3. The apparent difference in mobility of the \( \alpha \) subunits from liver and Caco-2 cells may be due to disturbances in the SDS slab gel matrix, since in other experiments no difference was noted.

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compared to the Sw1116 cells, have a significantly higher glutathione S-transferase activity, whereas the P-170 glycoprotein level is only slightly higher. The other potential detoxifying systems investigated are somewhat lower in the Caco-2 cells and, therefore, may not contribute to the Adriamycin/mitoxantrone resistance. If we take into account the previously published data concerning Adriamycin-resistant human colon adenocarcinoma cell lines (59, 61), it may be concluded that the contribution of the P-170 glycoprotein to drug resistance in colon carcinoma cell lines is at variance and most probably is not the only mechanism of importance.

Both \( \pi \) and \( \alpha \) class transferases are responsible for the high glutathione S-transferase enzyme activity in the Caco-2 cells. Class \( \alpha \) glutathione S-transferases are absent in the sensitive Sw1116 cell line. A similar observation was made recently by Schisselbauer et al. (64) in erythroleukemia cells, in which a class \( \alpha \) glutathione S-transferase was expressed only in the Adriamycin-resistant cells. Since evidence has now been gained for a role of glutathione conjugation in the metabolism of mitoxantrone (42, 63), the search for a possible involvement of cystolic glutathione \( S \)-transferases to catalyze such reactions should be encouraged, especially since transfection studies with copy DNAs for glutathione \( S \)-transferase \( \pi \) (26–28) and \( \alpha \) (26, 28) have shown increased resistance to Adriamycin in several types of cells, also suggesting a direct role for these transferases in the metabolism of Adriamycin. However, it should be noted 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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