Occurrence of Cytosolic Protein and Phosphoprotein Changes in Human Colon Tumor Cells with the Development of Resistance to Mitomycin C

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ABSTRACT

The cytosolic proteins and phosphoproteins of a mitomycin C-sensitive cell line were examined as progressively greater doses of mitomycin C were administered over a period of 44 weeks. Resistance of the human colon carcinoma cell line increased from a 50% inhibitory concentration of 1 to 6 μM over this time period. Changes in cytosolic protein patterns included increases in the amounts of three proteins with molecular weight \( \times 10^{-3} \) apparent isoelectric point (Mr/pl) values of 56/6.2, 37/7.3, and 27/6.1. Analysis of in vitro \( ^{32} \)P-labeled phosphoprotein patterns revealed reductions in the amounts of four proteins with Mr/pl values of 42/6.3, 40/7.7, 31/6.3, and 25/6.1. One increase was detected in a phosphoprotein with a Mr/pl value of 33/6.1. These changes in cytosolic components paralleled the development of resistance to mitomycin C and may reflect changes in the clonal composition of the cell line as it becomes progressively more resistant to mitomycin C or changes in critical proteins or enzymes involved in the activation or biotransformation of the drug.

INTRODUCTION

Drug resistance remains as one of the major obstacles in the chemotherapy of cancer (1). Defects in known mechanisms by which drug resistance can develop include altered membrane transport, biotransformation, failure to activate, or enhanced excision-repair capability (2-4).

MMC is thought to be taken up rapidly into cells and to be bioactivated through reduction at the quinone ring. This mechanism requires NADPH and results in the loss of the tertiary methoxy group and rearrangement of the aziridine ring and takes place under anaerobic conditions (5). Resistance toward this drug is of particular mechanistic interest since it displays cross-resistance with other alkylating agents in some tumor cell lines, while it does not in others (6). Consequently, Hoshino et al. (6) have suggested that MMC may have two modes of action with the potential to lose one or both of its activities through resistance mechanisms. Although the precise mechanisms by which MMC resistance is acquired are unknown, it is thought that preexistent, resistant variants within the tumor cell population or intracellular changes that occur during drug exposure are responsible for the development of larger resistant cell populations.

A model cell system for the study of MMC resistance has been developed which is based upon repeated exposure of a sensitive human colon carcinoma line (HCT 116) to MMC in vitro (7). An increase in a Mr 148,000 cell surface glycoprotein was reported to correlate positively with the development of MMC resistance. To study this model further and to determine whether cytosolic proteins and enzymes might also reflect changes in resistance to MMC, the proteins themselves and their phosphoprotein counterparts were examined by high-resolution, two-dimensional polyacrylamide gel electrophoresis and autoradiography.

Changes in the synthesis of a cytosol protein, V19, have already been shown to correlate with increased resistance to vincristine in DC-3F/VCRD-5 cells and in vincristine-resistant mouse cells (8). We have shown in an earlier report (9) that cytosol proteins and antigens of human colon tumor cell lines can serve as indicators that are useful in the grouping of cell lines of similar phenotype, rates of growth, and degrees of differentiation (9). If altered cellular regulatory mechanisms are involved in the development of drug resistance, then changes in protein phosphorylation may also be observed. Protein phosphorylation is known to be involved in many cellular regulatory mechanisms (10, 11).

The changes in protein and phosphoprotein pattern that were observed are described and discussed in view of known mechanisms for the development of drug resistance and in terms of their potential value as “markers” for the clonal variants of HCT 116 cells selected by pressure with MMC.

MATERIALS AND METHODS

Cell Culture and Development of Resistant Variants. Human tumor cell line HCT 116 (12-14) was maintained as a monolayer in McCoy’s Medium 5A supplemented with 10% fetal bovine serum (12). Resistant cell populations were generated by weekly 1-h treatments with MMC (3-5 μg/ml). Following drug treatment cells were passed in the McCoy’s culture medium without drug to minimize the influence of acute drug effects. Resistant cell lines corresponding to 11, 26, and 44 treatments with MMC were carried through 107, 92, and 74 passages in the absence of drug, respectively, and were compared with untreated HCT 116 cells. Variants had 50% inhibitory concentration values which ranged from 1 to 6 μM and which have remained stable for more than 1 year.

In Vivo \( ^{32} \)P Labeling and Preparation of Cytosol Fractions. Cells were labeled in vivo in phosphate-free medium containing carrier free \( ^{32} \)P, (1.5 mCi/ml) for 16 h at 37°C (15). Cells were isolated and washed in 0.15 mM NaCl/0.01 mM sodium phosphate, pH 7.2 (phosphate-buffered saline) containing 1.0 mM phenylmethylsulfonyl fluoride/0.1 mM leupeptin. Cytosols were prepared by homogenizing phosphate-buffered saline-washed cells with a SDT Series Tissumizer fitted with the SDT-080EN shaft and generator (Tekmar Co., Cincinnati, OH) in 0.05 mM Tris/0.025 mM KCl/0.005 mM MgCl₂ buffer, pH 7.4, containing 1.0 mM phenylmethylsulfonyl fluoride/0.1 mM leupeptin and 10 mM sodium fluoride. A 50% homogenization supernatant was prepared as described previously (9). Unlabeled cytosol fractions were prepared from confluent cell cultures as described previously without sodium fluoride in the homogenizing buffer.

Two-Dimensional Gel Electrophoresis, Silver Staining, and Autoradiography. Samples were concentrated by lyophilization and redis-
solved in water to a final concentration of 2 mg/ml. Samples were precipitated with 4 volumes of ethanol at −20°C for 16 h. Samples were redissolved at a concentration of 1.25 mg/ml in 2% ampholine, pH 3.5–10 (LKB Instruments, Inc., Gaithersburg, MD), 9 M urea, and 2 mM dithiothreitol. A 40-μl aliquot was centrifuged in a Beckman Airfuge at 98,000 × g for 5 min (Beckman Instruments, Palo Alto, CA) and 20 μl were loaded on an isoelectric focusing gel. Phosphorylated protein samples were adjusted to 3 × 10^6 cpm, 50 μg of carrier protein were added, and samples were precipitated with ethanol. Two-dimensional polyacrylamide gel electrophoresis was performed as described by Takami and Busch (16) with isoelectric focusing (pH 3.5–10 ampholines) in the first dimension and SDS/polyacrylamide gel electrophoresis in a 10% acrylamide gel in the second dimension (17). Unlabeled specimens were detected by staining with 0.2% Coomassie Brilliant Blue R and by silver staining as described by Wray et al. (18). Phosphoprotein patterns were obtained by exposing Kodak X-Omat AR film (XAR-5; Eastman Kodak, Rochester, NY) to dried gels. The pH gradients established in isoelectric focusing gels were determined by mixing multiple gel slices in 1.0 ml of deionized water and measuring the pH after diffusion of ampholines for 16 h. Bio-Rad low-molecular-weight standards (phosphorylase b, 92,500; bovine serum albumin, 66,200; ovalbumin, 45,000; carbonic anhydrase, 31,000; soybean trypsin inhibitor, 21,500; and lysozyme, 14,000) were used to estimate molecular weight in the SDS-containing second-dimension gel (Bio-Rad, Richmond, CA).

Enzyme Assays. Glutathione S-transferase activity was measured by the procedure of Habig and Jakoby (19) utilizing recrystallized 1-chloro-2,4-dinitrobenzene (Eastman Kodak) and glutathione (Sigma Chemical Co., St. Louis, MO) as substrates. Specific activities were based upon protein concentrations as determined by the protein-dye binding assay of Bradford (20).

RESULTS

Development of Resistant Variants. The HCT 116 cell line, established from an adenocarcinoma of the human colon, has been determined to have a variety of biological criteria to be poorly differentiated in terms of its biological characteristics (9, 21). To establish a series of MMC-resistant variants, this line and cultures previously treated in the series were exposed to multiple, weekly 1-h treatments with 3- to 5-μg/ml doses of MMC and allowed to undergo passage in drug-free medium. Treated lines were exposed to their 50% inhibitory concentration and suitable numbers and amounts of selected cultures, corresponding to 11, 26, and 44 treatments, were frozen. A tabulation of those biological characteristics that define the HCT 116 and its MMC-resistant variants are shown in Table 1.

Two-Dimensional Polyacrylamide Gel Electrophoretic Analysis of HCT 116 Cells and Their Mitomycin C-Resistant Variants. A silver-stained isoelectric focusing/SDS two-dimensional polyacrylamide gel is shown in Fig. 1A. Over 350 polypeptide components were detected on this electrophoretogram. Subsequent gels shown in Figure 1, B to D, correspond to the silver-stained two-dimensional patterns obtained for cytosol fractions prepared from variant cultures treated 11, 26, and 44 times with MMC, respectively.

A detailed analysis of the patterns obtained from six experiments, derived from separate cell culture preparations, revealed that there were six three protein spot changes that were observed six times. These spots showed increases in their stain density with the development of resistance and were identified by Mr/pi values of 56/6.2, 37/7.3, and 27/6.1. Although several other changes were observed on one or more of the patterns and many additional differences could be found in a careful examination of the gels shown in Fig. 1, none of these additional changes were uniformly observed throughout this study. The increases in the amounts of these three proteins as resistance developed suggest that their expression may be related to (a) the phenotype of subclones within the HCT 116 cell line that begin to dominate the resistant variant culture lines, (b) increases in the levels of detoxification enzymes important in the metabolism of MMC, or (c) genes with low activity that have been activated.

Autoradiographic Analysis of Two-Dimensional Polyacrylamide Gels of In Vivo 32P-labeled Proteins from HCT 116 Cells and Their Mitomycin C-resistant Variants. Cultures corresponding to the specimens analyzed in Fig. 1 were labeled for 16 h with 32P (1.5 μCi/ml) and cytosol fractions were prepared as described in “Materials and Methods.” The autoradiograms resulting from exposure of X-ray film to dried two-dimensional gels of these samples are shown in Fig. 2. A set of three separate preparations of labeled cells were examined to detect consistent increases or decreases in relative phosphorylation.

Only one increase in relative phosphorylation was observed (Fig. 2, upward pointing arrowhead) and correspond to a Mr/pi of 33/6.1. Reduced phosphorylation was observed for four phosphoproteins. These were marked with downward pointing arrowheads and correspond to Mr/pi values of 42/6.3, 40/6.7, 31/6.3, and 25/6.1. Several other changes were observed within a single experiment; however, only those changes in relative spot density that were consistently observed have been reported. The phosphoprotein spot which increased in autoradiographic density (33/6.1) did not correspond in molecular weight but did correspond in pl to the silver-stained protein spot identified by Mr/pi as 27/6.1 that increased in stain density with the development of drug resistance. These two proteins are sufficiently similar in pl but different in molecular weight that they are thought to be different proteins.

Comparison of Glutathione S-Transferase Levels in HCT 116 Cells and Their Mitomycin C-resistant Variants. A similarity was noted between the Mr/pi value of the 27/6.1 peptide shown to increase in stain density in Fig. 1 and the value reported for the ψ subunit of the anionic form of GST isolated from human liver, lung, and placenta (22, 23). It was decided, therefore, to measure the GST enzymatic activity in each of the cytosol fractions being studied to determine whether it had increased. Enzymatic assays utilizing 1-chloro-2,4-dinitrobenzene and reduced glutathione as substrates were performed on each cytosol fraction. The results of these assays are summarized in Table 2. GST levels were shown to increase throughout the development of resistance (53%) but to increase most substantially (47%) after 26 treatments with MMC. The increases in

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Description</th>
<th>50% inhibitory concentration</th>
<th>Growth rate (h)</th>
</tr>
</thead>
<tbody>
<tr>
<td>HCT 116</td>
<td>Parental</td>
<td>0.95 ± 0.05</td>
<td>14–18</td>
</tr>
<tr>
<td>HCT 116R11</td>
<td>11 × MMC</td>
<td>2.30 ± 0.20</td>
<td>20–24</td>
</tr>
<tr>
<td>HCT 116R26</td>
<td>26 × MMC</td>
<td>3.90 ± 0.30</td>
<td>20–24</td>
</tr>
<tr>
<td>HCT 116R44</td>
<td>44 × MMC</td>
<td>6.40 ± 1.30</td>
<td>20–24</td>
</tr>
</tbody>
</table>

* Mean ± SD determined from three dose-response curves in a cell proliferation assay (7). 
* Previously reported (14). 
* Treatments 11, 26, and 44 times for 1 h each at 37°C as described in "Materials and Methods."
enzymatic activity appear to parallel the increases in stain density observed for the protein with a Mr/pl value of 27/6.1.

**DISCUSSION**

Examination of cytosolic protein and phosphoprotein changes that accompanied the development of resistance to the drug MMC revealed a limited number of reproducible changes. Increases were observed in the amounts of three cytosolic proteins with Mr/pl values of 56/6.2, 37/7.3, and 27/6.1, while reductions were detected in either the amount or the degree of phosphorylation of four phosphoproteins with Mr/pl values of 42/6.3, 40/6.7, 31/6.3, and 25/6.1. Only one phosphoprotein was observed to undergo an increase in amount or in its degree of phosphorylation. The phosphoprotein spot which increased in autoradiographic density corresponded to a protein with a Mr/pl value of 33/6.1. In each case the phosphoprotein spots that underwent changes in phosphate content corresponded to minor spots on the silver-stained gels and required several observations before they could be accepted as reproducible changes.

Some changes that appeared between adjacent sampling points in the treatment program did not persist throughout the series. These changes in pattern spot density may reflect transient fluxes in the clones within the culture as autonomy is quantitatively and qualitatively changed. This type of competition between preexistent variants is consistent with one of the mechanisms proposed for the development of resistance to MMC (24), as well as current evidence for the clonal diversity of human colon cancer (25).

The changes that occur in the amounts of enzymes that play key roles in the NADPH-requiring anaerobic activation of MMC would probably represent very minor protein constituents of the cytosol and thus would not necessarily be seen on silver-stained gels. Since the microsomal fraction was removed by centrifugation in the preparation of the cytosols used in this study, cytochrome P-450 and other components of the oxidative system should have been largely if not completely removed.

An effort was made to bind cytosolic proteins to MMC immobilized on Sepharose 4B and to elute those proteins having an affinity for MMC. No binding was observed for any of the components detected on two-dimensional gels. It appeared from these negative results that if interactions exist between MMC and the proteins reported to increase in amount in this study, they did not involve strong or long-term bonds of the type reported for ligandin (26). They may, however, be involved in weak or transitory interactions that were not detected by this approach.

These data are consistent with the following interpretations. Treatment of HCT 116 cells with MMC probably unseats a preexisting autonomy among clonal variants of the cell line. As a result more resistant variants are selected and give rise to a new clonal dominance. The resistance of the ultimate clonal variants may be (a) the result of increases in the amounts of detoxification enzymes involved in the inactivation and ultimate clearance of MMC or (b) decreases in the content of key enzymes involved in the anaerobic, NADPH-requiring, activation of the drug. One or more of the cytosolic phosphoproteins observed to decrease in amount with the corresponding development of resistance may be involved in the activation of MMC either directly or through a regulatory route.

It is interesting to point out that one of the cytosol proteins which increases in relative amount (Mr/pl of 27/6.1) is similar in Mr/pl to the value reported for the ψ subunit of the anionic form of human glutathione S-transferase that has been isolated from human liver, lung, and placenta (22, 23). Recently Siegers et al. (27) have reported the detection of both GSH S-aryltransferase and GSH peroxidase activity in human intestinal mucosa as well as in the mucosa of the colon (27). Their data showed higher GSH-S-aryltransferase levels in tumors derived from the sigmoid and the rectum than could be measured in nontumorous specimens from the corresponding regions of the colon or the rectum. Our results show a 53% increase in GST levels in HCT 116R44 cells when compared to levels measured in extracts of the parental HCT 116 cells. Further studies are required, however, to establish whether the Mr/pl 27/6.1 protein spot is a subunit of the anionic GST and to determine if GST is involved in the development of resistance to MMC by HCT 116 cells.

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Fig. 1. Isoelectric focusing/SDS two-dimensional polyacrylamide gels of cytosolic proteins from parental and MMC resistant HCT 116 cells. The patterns correspond to HCT 116 (A), HCT 116R11 (B), HCT 116R26 (C), and HCT 116R44 (D). Upward pointing arrowheads, protein spots undergoing increased stain density; A, actin. The nonequilibrium pH gradient is indicated on the scale over each gel; the molecular weight (MW) markers are designated on the left side of each gel. Gels were silver stained by the method of Wray et al. (18).
Fig. 2. Autoradiographs of isoelectric focusing/SDS two-dimensional gels of [32P]-labeled cytosolic phosphoproteins from parental and MMC-resistant HCT 116 cells. The patterns correspond to HCT 116 (A), HCT 116R11 (B), HCT 116R26 (C), and HCT 116R44 (D). Upward pointing arrowhead, protein spot undergoing increased phosphorylation; downward pointing arrowheads, protein spots undergoing decreased phosphorylation. The nonequilibrium pH gradient is indicated below each autoradiogram; the migration of molecular weight (mw) markers is designated on the right side of each autoradiogram.
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