Mitomycin C Resistance in a Human Colon Carcinoma Cell Line Associated with Cell Surface Protein Alterations

James K. V. Willson, Byron H. Long, Michael E. Marks, Diane E. Brattain, John E. Wiley, and Michael G. Brattain

ABSTRACT

A human colon carcinoma cell line resistant to mitomycin C (MMC) was obtained by repeated exposure of a previously described sensitive parental line, HCT 116, to MMC in vitro. Xenografts grown from the MMC-resistant phenotype were not inhibited in MMC-treated animals, while MMC treatment produced growth inhibition in parental cell xenografts. The MMC-resistant phenotype exhibited a greater amount of a Mr 148,000 cell surface protein than did the parental line. The increase in this Mr 148,000 cell surface protein correlated positively with the degree of MMC resistance. Alkaline elution of filter-bound DNA from resistant cells exposed to MMC in vitro showed a decrease in DNA cross-link formation such that a 10-fold higher MMC concentration was required to produce similar cross-link formation in the resistant cell as compared to the parental cell. The development of MMC resistance was not associated with in vitro cross-resistance to other natural product cytotoxic drugs. This model for resistance to MMC will be useful in future studies to define the mechanisms for MMC action and resistance in human colon carcinoma cells.

INTRODUCTION

There has been little improvement in chemotherapy of colon carcinoma over the past 2 decades (6, 8, 21). MMC is one of the few drugs which has been shown to have even marginal activity against this disease (6). The marginal activity of this drug suggests that an improved understanding of the mechanisms of action and resistance to MMC might lead to the development of better methodology for the utilization of the drug in chemotherapy and/or analogues of MMC with increased antitumor activity (13).

A number of investigators have selected subpopulations of murine and human cancer cells which are resistant to natural products with anticancer activity by repeated exposure of the target cells to a sublethal concentration of drug (2, 3, 10–12, 16). We have utilized a similar approach to develop a model for MMC resistance in human colon carcinoma. MMC-resistant cells were selected from the previously described HCT 116 human colon carcinoma cell line (4, 17). We have developed a number of human colon carcinoma cell lines (4, 19), but the HCT 116 cell line was of particular interest because the growth of xenografts in athymic mice from cultured HCT 116 cells is inhibited by treatment of tumor-bearing animals with MMC (17). The characterization of a MMC-resistant variant of HCT 116 cells is described in this report.

MATERIALS AND METHODS

Cell Culture. Cells were maintained in McCoy's 5A tissue culture medium supplemented with 10% heat-inactivated fetal bovine serum in 25-cm² flasks (Coming, Corning, NY) and subcultured weekly as described previously (4). Cells are free of Mycoplasma contamination as documented by Hoechst stain (Flow Laboratories, McLean, VA) and aerobic and anaerobic culture (Wisconsin State Laboratory of Hygiene, Madison, WI).

Resistant Phenotype. A MMC-resistant cell was selected from the parental line HCT 116 by repeated exposure to MMC under the following conditions. HCT 116 cells in log-phase growth were exposed for 1 hr to 3 μM MMC. Treated cells were cultured in fresh medium until survivors resumed log-phase growth, at which time cells were passaged and the MMC treatment cycle was repeated. The interval between MMC treatments ranged from 3 to 4 weeks in the initial 4 exposure cycles to a stable weekly interval following Cycle 11. In this report, the MMC-resistant cells are identified by the number of MMC cycles prior to passage of the resistant cells without MMC exposure; e.g., HCT 116R11 identifies resistant cells following 11 MMC treatment cycles.

Cytotoxicity Assays. Cells were plated into 25-cm² flasks containing 5 ml of growth medium on Day 1 at concentrations of 1.4 x 10⁶ cells/ml (HCT 116 and HCT 116R). On Day 2, growth medium was removed and replaced with growth medium with drug concentrations (5 ml) ranging from 0.1 to 10 μM MMC, VP-16, VM26, bleomycin (all from Bristol Laboratories, Syracuse, NY), and Adriamycin (Adria Laboratories, Columbus, OH); 0.01 to 1.0 μM vincristine (EI Lilly, Indianapolis, IN); and 0.001 to 1.0 μM actinomycin D (Merck, Sharpe and Dohm, West Point, PA). After incubation for 1 hr at 37°C in medium containing drug, cultures received a complete change of medium without drug. Growth inhibition was determined by hemocytometer counts on Day 7 after suspension of the cells by treatment with 0.2% trypsin (Grand Island Biological Co., Grand Island, NY). 0.01 mg EDTA for 5 min at 37°C. Control cultures were treated in an identical manner, except that they received a complete change on Day 2 without drug. On Day 7, control cultures contained 1.8 x 10⁶ cells/ml for both HCT 116 and HCT 116R. Growth inhibition of drug-treated cells was expressed as the fraction of surviving cells on Day 7 relative to untreated control cultures x 100%. Control cultures were treated with 0.2% trypsin (Grand Island Biological Co., Grand Island, NY), 0.01 mg EDTA for 5 min at 37°C. Control cultures were treated in an identical manner, except that they received a complete change on Day 2 without drug. On Day 7, control cultures contained 1.8 x 10⁶ cells/ml for both HCT 116 and HCT 116R. Growth inhibition of drug-treated cells was expressed as the fraction of surviving cells on Day 7 relative to untreated control cultures x 100%. Cell survival studies after a 1-h MMC exposure at concentrations 0.03 and 10 μM MMC were carried out in a clonogenic assay using conditions described previously (22).

Treatment of Xenografts with MMC. Female (8-week-old) athymic mice of BALB/c parentage were obtained from Charles River Breeding Laboratories (Stone Ridge, NY). Experiments were performed with...
groups of 5 drug-treated animals bearing xenografts of each subpopulation and 5 control animals bearing untreated xenografts of each subpopulation. Each subpopulation was suspended from 25- to 200-μm flasks by treatment with calcium-free tissue culture medium for 1 hr, and 1 x 10^6 cells suspended in tissue culture medium and 8% fetal bovine serum were injected s.c. All mice given injections of cells suspended in this manner developed progressively growing tumors 7 to 10 days following inoculation. Mice were treated i.p. with 1.6 mg/kg body weight of MMC on Days 1, 8, and 15. Control mice were treated with 0.9% NaCl solution on Days 1, 8, and 15. This dosage was selected on the basis of optimal doses of MMC determined in normal BALB/c mice. Tumor size was measured serially in individual animals with calipers, and volumes were estimated by the formula

\[ \frac{a \times b^2}{2} \]

where \( a \) is the maximum length of the tumor and \( b \) is the perpendicular to \( a \). No experimental deaths were encountered and, at the end of 3 weeks, animals were sacrificed.

Alkaline Elution Assay for DNA Cross-Links. Cultures were generally started in 75-cm² flasks 4 days before harvesting so that cells were in exponential growth for the experiment. Two separate populations of cells were incubated with [2-3H]thymidine (0.01 μCi/ml) or [methy1-3H] thymidine (0.01 μCi/ml) for 48 hr prior to harvesting with trypsin-EDTA. Sedimented cells were suspended at 5 x 10^6 cells/ml in fresh medium. Various concentrations of drugs were added to 1-ml aliquots of cells containing [3H]DNA for incubation at 37°C. Following a 1-hr incubation with drug, the cells were placed on ice and irradiated with 600 rads of γ-radiation from a MCo source in a Gammacell 220 irradiator to introduce random single-strand breaks in order to observe cross-link formation (20). Irradiated, drug-treated cells were then layered over polycarbonate filters in 25-mm Swinnex filter holders affixed to 50-ml syringe barrels as described by Kohn et al. (15) and washed twice by filtration at 4°C with 20 ml of cold PBS (0.15 M NaCl, 0.01 M NaH₂PO₄, pH 7.5). Cells containing [3H]DNA were irradiated with 300 rads on ice and distributed among the filters in aliquots of 5 x 10^6 cells to provide an internal elution standard as described previously (7, 23). The cells were once again washed with PBS using gravity filtration. Cells were lysed on the filters at room temperature with 3 ml of SDS-EDTA lysis solution, followed by 2 ml of SDS-EDTA lysis solution containing proteinase K (0.5 mg/ml) that had been heated at 56°C for 1 hr (14). Elution of radioactive DNA was conducted at pH 12.1 with 10 ml of tetrapropylammonium hydroxide-glucose, and 1.0 mCi of Na₁²⁵I. After the 10- to 12-min incubation period, the reaction mixture was removed, and the cells were washed 3 times with PBS, mechanically harvested in PBS, and centrifuged. The cell pellet was solubilized in 2.5% SDS (w/v)/50 mM β-mercaptoethanol (v/v) and 3 M urea using SDS-PAGE loading buffer containing 0.1% SDS (w/v)/50 μM EDTA (v/v). The eluted fractions were processed for radioactive counting as described previously (15). Resulting 125I and 3H cpm of the eluted fractions were expressed as a percentage of the sum of cpm remaining on the filter and tubing, present in the lysis flow through, and collected in the eluted fractions.

Characterization of Cell Surface Proteins. Lactoperoxidase and glucose oxidase were purchased from Sigma (St. Louis, MO). Carrier-free Na¹²⁵I was from Amersham (Arlington Heights, IL). All reagents required for SDS-polyacrylamide gel electrophoresis were obtained from Bio-Rad (Richmond, CA).

Cell surface proteins were labeled using the lactoperoxidase iodination procedure. Confluent cell cultures were washed 3 times in situ with PBS (0.01 M sodium phosphate, 0.154 M sodium chloride), pH 7.4 and then incubated at room temperature for 10 to 12 min in 3 ml of PBS containing 1.5 units of lactoperoxidase, 1.5 units of glucose oxidase, 30 μM of β-o-glucose, and 1.0 mM of Na¹²⁵I. After the 10- to 12-min incubation period, the reaction mixture was removed, and the cells were washed 3 times with PBS, mechanically harvested in PBS, and centrifuged. The cell pellet was solubilized in 2.5% SDS (w/v)/50 mM β-mercaptoethanol (v/v) and 3 M urea using SDS-PAGE loading buffer containing 0.1% SDS (w/v)/50 μM EDTA (v/v). The eluted fractions were processed for radioactive counting as described previously (15). Resulting 125I and 3H cpm of the eluted fractions were expressed as a percentage of the sum of cpm remaining on the filter and tubing, present in the lysis flow through, and collected in the eluted fractions.

Karyotype Determination. Cells from log-phase cultures were exposed to Colcemid (0.5 μg/ml) (Grand Island Biological Co.) for 1 hr. Cells were disaggregated by incubation for 1 hr in a calcium-free medium and incubated in 0.075 M KCl for 30 min at 37°C fixed with 25% glacial acetic acid in anhydrous methanol. Trypsin-Giemsa banding was performed, and multiple karyotypes were determined. The modal karyotype is presented.

RESULTS

Response of the Parental (HCT 116) and MMC-resistant Variants (HCT 116R) to MMC in Vitro. Growth inhibition curves as a function of concentration of MMC treatment for HCT 116 and the resistant cell lines HCT 116R11 and HCT 116R22 are shown in Chart 1. A total of 3 survival curves were generated for each cell line, and the resulting IC₅₀ values are presented in Table 1. Resistance to MMC increased with continued exposures to MMC. HCT 116R22 has an IC₅₀ which is 4.8-fold higher than that of the parent cell line (HCT 116) and 2.2-fold higher than that of the earliest resistant cells, HCT 116R11. The difference in MMC sensitivity could not be explained by changes in growth radioactivity were fractionated by electrophoresis on 5 to 10% acrylamide gradient slab gels in 0.1% SDS (w/v) using 4% acrylamide stacking gels. Preparation of the 5 and 10% acrylamide solutions and electrophoresis was performed as described in "Materials and Methods."

<table>
<thead>
<tr>
<th>MMC Concentration (μM)</th>
<th>HCT 116</th>
<th>HCT 116R11</th>
<th>HCT 116R22</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>90 ± 0.1°C²</td>
<td>2.0 ± 0.2°C²</td>
<td>4.2 ± 0.1°C²</td>
</tr>
<tr>
<td>0.75 ± 0.1°C²</td>
<td>3.0 ± 0.6°C²</td>
<td>4.3 ± 0.6°C²</td>
<td>6.2 ± 0.8°C²</td>
</tr>
</tbody>
</table>

² Mean ± S.D. determined from 3 to 6 dose-response curves in a cell proliferation assay as described in "Materials and Methods."
ATHYMIC MICE. Points, mean of 5 animals. A, saline-treated; O, MMC-treated. A, growing xenografts in untreated mice, with an average tumor volume of 684 ± 50 (S.E.) cu mm at 21 days postinjection. As shown in Chart 2, the parent cell line, HCT 116, formed rapidly growing xenografts from MMC-treated mice, with an average tumor volume of 684 ± 50 (S.E.) cu mm at 21 days postinjection. As reported previously (17), the volume of HCT 116 xenografts from MMC-treated mice was approximately 50% of those of untreated animals. There was no difference between the size of tumors from treated and untreated mice bearing HCT 116R11 xenografts.

DNA Cross-Link Formation Induced by MMC in HCT 116 and HCT 116R11 Cells. DNA-DNA cross-link formation is illustrated readily by alkaline elution techniques (6, 15) and has been used to study MMC-induced cross-link formation and repair in the HCT 116 system of cell lines (17). MMC-induced cross-link formation was evaluated in the HCT 116 cells, shown to be sensitive to MMC in vivo, and the HCT 116R11 cells, shown to be resistant to MMC in vivo. Both nontreated controls and MMC-treated cell populations were irradiated following MMC treatment to expose DNA-DNA cross-links, which are indicated by decreased elution of DNA from drug-treated, irradiated cells relative to the elution of irradiated control cells (Chart 3). A dose-dependent relationship between MMC concentrations and DNA-DNA cross-links is demonstrated in HCT 116 cells by the slower elution of DNA from drug-treated cells relative to the elution of DNA from control cells (Chart 3A). However, no significant cross-link formation was detected in HCT 116R11 cells within the limits of sensitivity for the assay at MMC doses of 1 to 4 µM but cross-links were demonstrated at 10 µM MMC (Chart 3B). These studies reveal that drug resistance is reflected by decreased levels of DNA-DNA cross-links in MMC-resistant cells such that 10 µM MMC resulted in similar cross-link formation in HCT 116R22 cells as that produced by approximately 1 µM MMC in HCT 116 cells. We have reported previously (17) that the kinetics of cross-link removal from the resistant cells and the parent line were similar. Therefore, an increase in cross-link repair does not appear to account for differences in MMC sensitivity in this model.

Characterization of Cell Surface Proteins in HCT 116 and MMC-resistant Variants. The cell surface labeling patterns of the MMC-sensitive parental cell line HCT 116 and the MMC-resistant variants HCT 116R11 and HCT 116R22 were compared. MMC-resistant cells exhibited a quantitative increase of a Mr, 148,000 cell surface protein which was not a prominent cell surface protein in the MMC-sensitive cells. The amount of the Mr, 148,000 cell surface protein was greater in the HCT 116R22 cells than in the HCT 116R11 cells and, therefore, the amount of this protein correlated positively with the degree of MMC resistance. Autoradiograms of 125I-labeled cell surface proteins from HCT 116R22 (Fig. 1A) and HCT 116 cells (Fig. 1B) showed the increased Mr, 148,000 cell surface protein in the HCT 116R22 cells. Several other differences between the cell surface labeling pattern of the HCT 116R22 and HCT 116 cells are apparent in Fig. 1; however, only the Mr, 148,000 cell surface protein alteration was found to be consistently in greater amount on the MMC-resistant variants relative to the parental cells.

In Vitro Sensitivity of HCT 116 and HCT 116R26 Cells to Other Cytotoxic Agents. The IC50 values for 6 additional agents were determined in the MMC-resistant cell HCT 116R26 and in the parent cell line HCT 116. The MMC resistance of HCT 116R26 was identical to that of the HCT 116R22 line. The HCT 116R26 cells were selected for these studies because they had received the greatest number of MMC exposures, and therefore, they were thought to be the most likely among the MMC-resistant variants to exhibit cross-resistance. These studies revealed that the development of MMC resistance in this model is not associated with in vitro cross-resistance to these additional natural product cytotoxic drugs (Table 2). The HCT 116R26 and, by implication, HCT116R11 and HCT116R22 do not manifest the pleiotropic resistance described in many previously reported laboratory models (2, 3, 10, 11). It should be pointed out that those models showing pleiotropic resistance have much higher levels of resistance (102- to 104-fold) to the drug utilized for selection than the levels of MMC resistance developed for the HCT 116 system. Higher levels of MMC resistance in HCT 116 cells might also be associated with a pleiotropic resistance phenotype.

Karyotype. The chromosome analysis of the cell line exhibiting the greatest degree of MMC resistance, HCT 116R22, was compared with parental line, HCT 116. The HCT 116 cell line has a 46,XY,10q+,18p+,t(11;14)(p15;q13) (Fig. 2). The resistant

<table>
<thead>
<tr>
<th>Table 2</th>
<th>IC50 values for cytotoxic drugs against HCT 116R26 and HCT 116 cells in vitro</th>
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<tbody>
<tr>
<td>Drug</td>
<td>HCT 116</td>
</tr>
<tr>
<td>MMC</td>
<td>0.9</td>
</tr>
<tr>
<td>Actinomycin D</td>
<td>0.008</td>
</tr>
<tr>
<td>Adriamycin</td>
<td>0.34</td>
</tr>
<tr>
<td>Bleomycin</td>
<td>0.5</td>
</tr>
<tr>
<td>Vinblastine</td>
<td>0.12</td>
</tr>
<tr>
<td>VP-16</td>
<td>10.0</td>
</tr>
<tr>
<td>VM-26</td>
<td>0.7</td>
</tr>
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</table>

IC50 values determined from dose-response curves over a range of drug concentrations as described in "Materials and Methods.”
cell line HCT 116R22 shares the 10q+ and 18p+ with the parent line. The preservation of these markers supports the conclusion that the MMC-resistant variants are derived from HCT 116 cell line. The HCT 116R22 line differs from the parental line by the loss of one each of chromosomes 16 and 17 and in being trisomic for chromosomes 19 and 20. The absence in the resistant cell of the 11/14 rearrangement present in the parent cells suggests that a variant present in low frequency in the parental population may have been selected by repeated exposure to MMC. It is interesting that both lines have retained a modal number of 46 chromosomes, even though they are markedly different in their chromosome constitution. Neither the resistant nor the parent line contain any double minutes or homogeneously staining regions.

**DISCUSSION**

We have isolated a MMC-resistant human colon carcinoma cell line from a sensitive parental line following repeated exposure of the sensitive line to MMC. In this model, the resistance appears to be specific for MMC, as the relative in vitro sensitivities of the resistant and parental phenotypes to several other anticancer drugs did not differ.

An important feature of this model for MMC resistance, as well as a previously described model for MMC cytotoxicity (17), is the fact that in vitro differences in MMC sensitivity were reflected by the in vivo response of xenografts grown from the lines in MMC-treated athymic mice. Moreover, the biological characteristics of the resistant lines are related closely to the parental line with respect to tumorigenicity in nude mice and clonogenicity in semisolid medium. The lack of response by HCT 116R11 in vivo supports the use of this line in future studies to define mechanisms for resistance to MMC and to investigate means of circumventing MMC resistance.

In the HCT 116 model, we have identified a potential marker for resistance to MMC. A major cell surface protein at M, 148,000 was found in SDS electrophoretic profiles of the resistant phenotype, which was not a major protein in the profiles of sensitive parental cells. The quantitative difference in this protein between resistant and sensitive parental cells increased as MMC resistance of the cells increased. The increased expression of this cell surface glycoprotein alteration in drug-resistant phenotypes may be exploitable as a marker for MMC resistance in clinical cancer.

We are currently attempting to develop antibodies to the M, 148,000 protein isolated by slab gel electrophoresis in order to evaluate it as a marker for MMC resistance.

The mechanism of action of MMC is thought to result from its intracellular activation to a reactive species that alkylates and cross-links DNA (18). The mechanism(s) of human cancer cell resistance to MMC is yet to be defined; however, a number of possible mechanisms have been suggested (13), including (a) decreased intracellular drug concentration, (b) reduced intracellular activation, and (c) increased repair of DNA strand breaks. In the HCT 116R cell and the described previously model for...
native resistance in the HCT 116 system (17), the number of DNA cross-links following in vitro treatment correlated closely with sensitivity of the line to MMC. In addition, repair was not increased in either the selected resistant line, HCT 116R11, or the native resistant line, HCT 116b (17). Therefore, it is likely that either reduced intracellular drug concentrations or decreased drug activation may account for resistance in this model. The quantitative changes in cell surface protein that distinguish the HCT 116-resistant and sensitive cells might be associated with alterations in membrane transport of MMC and thus alter intracellular drug concentrations. The HCT 116R model will be useful in further studies to determine the mechanism(s) of resistance of MMC and for the evaluation of new strategies to circumvent this resistance.

ACKNOWLEDGMENTS

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REFERENCES


MITOMYCIN C RESISTANCE
Fig. 2. A, karyotype HCT 116, 46,XY,10q+,18p+,t(11;14)p15;q13). B, HCT 116R22, 46,XY,10q+,18p+,-16,-17,+19,+20.

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