DT-Diaphorase as a Critical Determinant of Sensitivity to Mitomycin C in Human Colon and Gastric Carcinoma Cell Lines

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ABSTRACT

Mitomycin C (MMC), a known cytotoxic agent, requires cellular enzyme-mediated activation for effective antitumor activity. To study the bioreductive enzymes responsible for MMC activation in tumor cells, we examined the enzyme activities of DT-diaphorase (DTD) and NADPH:cytochrome P-450 reductase in 13 colon and gastric carcinoma cell lines and then compared these activities to the respective cellular MMC sensitivity. We found that cell lines with nonexistent or marginal DTD activity, such as St-4 and MKN7, showed resistance to MMC, in comparison to cell lines with DTD activity ranging from 210 to 1420 nmol/min/mg protein. No correlation was found between NADPH:cytochrome P-450 reductase activity and MMC sensitivity in these cell lines. To confirm the role of DTD in cellular MMC sensitivity, we constructed an expression vector containing NQO1, a gene that codes for DTD, and transfected the vector into St-4 cells expressing no DTD activity. Several transfectants clones with DTD activity from 144 to 2085 nmol/min/mg protein were obtained. All of the transfectants showed 5-10-fold higher sensitivity to MMC compared to the parental St-4 cells. Consistent with the MMC sensitivity, we also found that MMC-DNA adduct was formed more extensively in the NQO1 transfectants than in the St-4 cells. These results indicate that DTD activity is required for effective cytotoxicity of MMC in colon and gastric carcinoma cells.

INTRODUCTION

MMC is a clinically useful antitumor drug that is effective in the treatment of solid tumors. To exert its antitumor activities, MMC must be activated through bioreductive pathways that are catalyzed by cellular enzymes. Two pathways are involved in this reductive bioactivation. The first pathway, catalyzed by P-450 reductase (1, 2), xanthine oxidase (2), cytochrome b5 reductase (3), and xanthine dehydrogenase (4), is the one-electron reduction of MMC to its semiquinone radical. The MMC semiquinone radical is a highly reactive molecule. The molecule rearranges to generate two electrophilic centers that interact with the two-amino function of guanine residues in DNA (5, 6). The second pathway is the two-electron reduction of MMC to form MMC hydroquinone, without the production of the semiquinone. The MMC hydroquinone also possesses potent DNA-alkylating activity (6, 7). A major two-electron reductase is NAD(P):quinone oxidoreductase, which is known as DTD (EC 1.6.99.2).

Several reports strongly suggest an important role for DTD in the reductive bioactivation of MMC. For example, a number of MMC-resistant cell lines show reduced or nonexistent DTD activity (7-14), and MMC can actually be activated in vitro to MMC hydroquinone by purified DTD in a pH-dependent manner (7, 15). However, in some reports, DTD plays a questionable role in cellular MMC activation (16, 17), e.g., no correlation was found between DTD activity and MMC sensitivity in 15 cell lines, including lung, breast, and colon cancer cells. One method to demonstrate directly the role of DTD in MMC activation not yet appearing in a published study is to modulate MMC sensitivity through transfer of NQO1, the gene that codes for DTD (18). Because of the ambiguity of these published results, the role of DTD in MMC cytotoxicity has not yet been accepted widely.

To elucidate the role of DTD in cellular MMC sensitivity, we have examined the enzyme activities of DTD and P-450 reductase and the IC50 of MMC in 13 colon and gastric carcinoma cell lines. We have found that cell lines with nonexistent or marginal DTD activity show resistance to MMC, and cell lines with DTD activity from 210 to 1420 nmol/min/mg protein are comparatively sensitive to MMC. Furthermore, we have demonstrated that St-4 cells, which have no DTD activity and are MMC resistant, become sensitive to MMC after cellular transfection of the NQO1 gene. The MMC-DNA adduct is also formed more extensively in the NQO1 transfectants than in St-4 cells. This is the first report to demonstrate directly the role of DTD in cellular MMC sensitivity.

MATERIALS AND METHODS

Chemicals. MMC, KW2149, and ADM were generous gifts from Kyowa Hakko Kogyo Co., Ltd. (Tokyo, Japan). cDDP was provided kindly by Bristol-Myers Squibb Co. Ltd. (Tokyo, Japan). NADH and NADPH were obtained from Nacalai Tesque Co. Ltd. (Tokyo, Japan). 2,6-Dichlorophenol indophenol and phenylmethylsulfonyl fluoride were from Sigma Chemical Company. Dicoumarol and TEMPO were from Wako Pure Chemicals, Inc. (Osaka, Japan). All other reagents were of analytical grade.

Cell Culture and Growth Inhibition Assay. All cell lines were cultured in RPMI 1640 (Nissui, Japan) containing 10% fetal bovine serum (Life Technologies, Inc., Grand Island, NY) and 100 μg/ml of kanamycin (Meiji Seika, Tokyo, Japan) and maintained in a humidified atmosphere of 95% air and 5% CO2 at 37°C.

The growth inhibition assay was performed as described previously (19). In brief, exponentially growing cells were plated into six-well plates in 2 ml growth medium. The colon cell lines were plated at a density of 2 × 104 cells/ml, and stomach cell lines and transfectants were plated at a density of 4 × 105 cells/ml. After the overnight culture at 37°C, graded concentrations of drugs were added to the medium. After 2 days, cells were trypsinized and counted with a Coulter counter. The drug concentrations needed for inhibition of cell growth by 50% (IC50 values) were determined as described previously (20).

Cytosol Preparation and Enzyme Assays. All cells were grown to subconfluence under standard conditions. The cell layer was washed with PBS (136.9 mM NaCl, 2.7 mM KCl, 8.1 mM Na2HPO4, 1.5 mM KH2PO4), and then scraped and collected by centrifugation. The pelleted cells were then suspended in ice-cold reticulocyte standard buffer [10 mM TRIS-HCl (pH 7.4), 10 mM NaCl, and 1.5 mM MgCl2] containing 0.1 mM phenylmethylsulfonyl fluoride and were allowed to stand for 10 min at 4°C. After this incubation, the cells were lysed by sonication for 10 s. The homogenate was demulsified by centrifugation at 1,500 g for 10 min at 4°C, and used for P-450 reductase enzyme assay. The demulsified homogenate was further centrifuged at 105,000 × g for 30 min at 4°C. This cytosol was used as DTD enzyme source. Protein concentrations were determined with the bicinchoninic acid protein assay kit (Pierce Chemical Co., Rockford, IL).
DTD activity was assayed according primarily to the method of Ernster (21) as modified by Benson et al. (22). P-450 reductase activity was measured according to the method of Vermillion and Coon (23).

Cloning and Transfection of the NQO1 Gene. DNA probes to screen full-length cDNA of the NQO1 gene (18) were obtained by RT-PCR of an mRNA fraction from MKN45 cells. Two sets of primers were used: 5'-AGG CTG GGT TGA GCC AGT GTT C-3' (392-413; sense-NQO1) and 5'-ATT TGT AAG CAA ACT CTC C-3' (435-417; antisense-NQO1). A cDNA library of human colon carcinoma HT29 cells was constructed using the ZAP-cDNA™ Synthesis Kit (Stratagene cloning system, San Diego, CA), and 5×10⁵ plaques were screened sequentially by plaque hybridization using the first and the second DNA probes described above. Six clones containing full-length NQO1 cDNA were obtained. An expression construct containing the NQO1 coding region was obtained by PCR amplification of a full-length NQO1 cDNA using the primers sense-NQO1 36–53 and 5'-CCA GGC TAA GGA ATC TCA-3' (890-873; antisense-NQO1), and this construct was inserted directly into the eukaryotic cell expression vector pCR III (Invitrogen, San Diego). The cloned NQO1 coding region was sequenced to confirm the absence of mutations that could result in a substitution of amino acids. Transfection was performed by electroporation (960 μF, 270 mV) (Gene Pulser™; Bio-Rad, Richmond, CA). After 2 days of incubation, the cells were lysed and DNA was prepared, as described previously (12). The radioactivity of [³H]MMC in the cell lines was measured, and four clones with different DTD activity were obtained. The success ful transfection of the NQO1 gene into the transfectants was confirmed by Southern blot analysis, as described previously (24).

Immunoblot Analysis. The immunoblot analysis of DTD was performed using a mouse anti-human DTD monoclonal antibody (12), and the DTD protein bands were detected by enhanced chemiluminescence with the Western blotting protocol (Amersham Japan. Tokyo, Japan).

Measurement of DNA Alkylation. Cells (3×10⁵) were incubated with 18.5 kBq/ml of MMC (Amersham Japan, Tokyo, Japan) for 2 h at 37°C. To inhibit, respectively, DTD or P-450 reductase activity, 200 μM dicoumarol was added to the culture 30 min before MMC treatment, or 10 mM TEMPOl was added just before MMC treatment. After the incubation period, the cells were lysed and DNA was prepared, as described previously (12). The radioactivity of [³H]MMC in the genomic DNA was determined by liquid scintillation counting.

RESULTS

Comparison of Enzyme Activities and MMC Sensitivity. Enzyme activities of DTD and P-450 reductase were examined in eight colon and five gastric cancer cell lines (Table 1). DTD activity in colon cell lines ranged from 223 to 1000 nmol/min/mg protein, except in HT29/MMC cells, which are deficient in DTD and thus show MMC resistance (12). In gastric cell lines, DTD activity was not detected in St-4 cells, was detected slightly in MKN7 cells, and was detected abundantly in the other three cell lines. P-450 reductase activity ranged from 0.67 to 1.22 nmol/min/mg protein in colon cancer cell lines, whereas the activity was below the detectable level in three out of five gastric cell lines, yet it was rich in St-4 cells. No correlation was found between the two enzyme activities in the cell lines.

We next determined the IC₅₀ of MMC in these cell lines and compared these values with the enzyme activities (Table 1 and Fig. 1). As shown in Fig. 1A, cell lines with no (St-4 and HT29/MMC) or marginal (MKN7) DTD activity showed higher IC₅₀ values compared to the other cell lines expressing DTD activity from 210 to 1420 nmol/min/mg protein. This result is consistent with previous reports, which demonstrate reduced or defective DTD activity in MMC-resistant cells (7, 9–12, 14). However, it is notable that cellular MMC sensitivity did not correlate with a DTD activity above 210 nmol/min/mg protein. In the case of P-450 reductase activity, we could not find any correlation with MMC sensitivity (Fig. 1B).

Transfection of NQO1 Gene into St-4 Cells. To directly elucidate the role of DTD in MMC sensitivity, we introduced NQO1, the gene encoding DTD, into St-4 cells, which have no intrinsic DTD activity. Four clones were obtained and designated SDT1, SDT2, SDT3, and SDT4. The growth rate of the transfectants did not vary more than 15% from that of the parental St-4 cells (data not shown). The cloned transfectants expressed DTD activity from 144 to 2083 nmol/min/mg protein (Fig. 2). SDT-AS, a transfectant with the NQO1 gene in an antisense orientation, showed no DTD activity, as did parental St-4 cells. Consistent with the observed DTD enzyme activity, the transfectant cells expressed DTD protein, detectable with an anti-DTD antibody.

Table 1 Enzyme activities and IC₅₀ values of MMC in colon and gastric carcinoma cell lines

<table>
<thead>
<tr>
<th>Cell lines</th>
<th>Enzyme activity</th>
<th>IC₅₀ * of MMC</th>
</tr>
</thead>
<tbody>
<tr>
<td>Colon</td>
<td></td>
<td></td>
</tr>
<tr>
<td>HT29</td>
<td>837 ± 84</td>
<td>1.22 ± 0.28</td>
</tr>
<tr>
<td>HCC2998</td>
<td>1000 ± 96</td>
<td>0.68 ± 0.13</td>
</tr>
<tr>
<td>KM12</td>
<td>395 ± 32</td>
<td>1.03 ± 0.16</td>
</tr>
<tr>
<td>KM20L2</td>
<td>544 ± 118</td>
<td>0.67 ± 0.01</td>
</tr>
<tr>
<td>WiDr</td>
<td>672 ± 49</td>
<td>0.77 ± 0.03</td>
</tr>
<tr>
<td>HCT15</td>
<td>570 ± 31</td>
<td>0.86 ± 0.01</td>
</tr>
<tr>
<td>HCT116</td>
<td>223 ± 35</td>
<td>0.75 ± 0.05</td>
</tr>
<tr>
<td>HT29/MMC</td>
<td>ND</td>
<td>1.20 ± 0.10</td>
</tr>
<tr>
<td>Stomach</td>
<td></td>
<td></td>
</tr>
<tr>
<td>ST-4</td>
<td>ND</td>
<td>1.82 ± 0.15</td>
</tr>
<tr>
<td>MKN7</td>
<td>37 ± 7.4</td>
<td>ND</td>
</tr>
<tr>
<td>MKN28</td>
<td>242 ± 13</td>
<td>ND</td>
</tr>
<tr>
<td>MKN45</td>
<td>1420 ± 63</td>
<td>ND</td>
</tr>
<tr>
<td>MKN74</td>
<td>210 ± 21</td>
<td>0.48 ± 0.04</td>
</tr>
</tbody>
</table>

* nmol/min/mg protein.

* ng/ml.

< 5 nmol/min/mg protein.

< 0.05 nmol/min/mg protein.
DT-DIAPHORASE AS A DETERMINANT OF MMC SENSIVITY

Fig. 2. Immunoblot and activity of DT-diaphorase in St-4 and NQOl gene transfectants. Cell lysates (20 μg) from St-4, SDT-AS, SDT1, SDT2, SDT3, and SDT4 were subjected to immunoblot analysis. DT activity was measured as described in "Materials and Methods." N.D. <5 nmol/min/mg protein.

Table 2. Sensitivity of NQOl transfectants to various antitumor agents

<table>
<thead>
<tr>
<th>Cells</th>
<th>MMCb</th>
<th>KW2149b</th>
<th>ADMb</th>
<th>cDDPc</th>
</tr>
</thead>
<tbody>
<tr>
<td>Parental cell</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>St-4</td>
<td>102 ± 2.2</td>
<td>5.9 ± 1.2</td>
<td>15 ± 0.5</td>
<td>0.4 ± 0.1</td>
</tr>
<tr>
<td>Antisense</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>SDT-AS</td>
<td>91 ± 2.2 (0.89)d</td>
<td>NT*</td>
<td>NT</td>
<td>NT</td>
</tr>
<tr>
<td>SDS</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>S1TD1</td>
<td>10 ± 1.9 (0.10)</td>
<td>3.1 ± 0.8 (0.53)</td>
<td>14 ± 0.5 (0.93)</td>
<td>0.2 ± 0.0 (0.50)</td>
</tr>
<tr>
<td>S1TD2</td>
<td>20 ± 5.6 (0.20)</td>
<td>7.4 ± 1.6 (1.25)</td>
<td>12 ± 1.0 (0.80)</td>
<td>0.5 ± 0.1 (1.25)</td>
</tr>
<tr>
<td>S1TD3</td>
<td>10 ± 1.5 (0.10)</td>
<td>5.3 ± 0.0 (0.90)</td>
<td>15 ± 0.3 (1.00)</td>
<td>0.5 ± 0.1 (1.25)</td>
</tr>
<tr>
<td>S1TD4</td>
<td>13 ± 2.2 (0.13)</td>
<td>6.3 ± 2.0 (1.07)</td>
<td>14 ± 0.5 (0.93)</td>
<td>0.5 ± 0.1 (1.25)</td>
</tr>
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</table>

* IC50 values are the means ± SD of triplicate determinations.
* μg/ml.
* μmol.
* Numbers in parentheses show resistance indices calculated from IC50 in transfectants divided by the IC50 in parental St-4.
* NT, not tested.

monoclonal antibody. The P-450 reductase activity was also measured, but it did not differ significantly in the transfectants (data not shown).

We next examined the sensitivity of the transfectants to antitumor agents such as MMC, KW2149, ADM, and cDDP (Table 2). The IC50 of MMC in the parental St-4 and SDT-AS cells were 102 and 91 ng/ml, respectively, whereas those in the NQOl transfectants ranged from 10 to 20 ng/ml. This result indicates clearly that expression of DTD in St-4 cells sensitized the cells to MMC 5-10-fold. The expression level of DTD in the transfectants, however, did not correlate with MMC sensitivity, an observation in good agreement with the results from the 13 colon and gastric cell lines described above (Table 1; Fig. 1A).

The sensitivity to KW2149, a MMC analogue that does not require reductive bioactivation (25), was not changed in the NQOl transfectants, except for S1TD1, which showed approximately 2-fold higher sensitivity. The SDT1 cells also exhibited 2-fold higher sensitivity against cDDP compared to the other NQOl transfectants and the parental St-4 cells (the reason for this phenomenon has not been clarified at this moment). The IC50 values of ADM in the transfectants were almost comparable to those in the parental St-4 cells, indicating that DTD is not involved in ADM cytotoxicity.

MMCC-DNA Adduct in NQOl Transfectants. MMC exhibits its cytotoxic activity by binding to cellular DNA (6, 7). Therefore, DNA-bound MMC was measured in the NQOl transfectants and the parental St-4 cells (Table 3). Without inhibitors of reductive bioactivation, the amount of DNA-bound MMC in the NQOl transfectants was approximately 2.8-fold higher than that in the St-4 cells. Although the DTD activities in S1TD1 and S1TD4 cells differed more than 14-fold, the amount of DNA-bound MMC was almost comparable in these transfectants, suggesting that MMC activation in the cells becomes saturated at a DTD activity greater than 144 nmol/min/mg protein. This saturation effect may account for the lack of further sensitization of transfectants expressing DTD levels higher than S1TD1 cells.

In the presence of dicoumarol, an inhibitor of DTD, the amount of DNA-bound MMC was only marginally reduced in St-4 cells, while markedly diminished in DTD proficient S1TD1 and S1TD4 cells. TEMPOL, which inhibits one-electron reductive activation of MMC, significantly reduced the DNA-bound MMC in S1TD, S1TD1, and S1TD4 cells, suggesting that a considerable amount of MMC was activated by the one-electron reductive pathway in all of these cells, irrespective of DTD activity.

DISCUSSION

MMC, one of the most potent antitumor drugs against solid tumors, requires reductive activation by cellular enzymes to initiate its antitumor capabilities. Several lines of evidence suggest that DTD, which catalyses the two-electron reductive activation of MMC, plays an important role in cellular MMC activation (1, 7-14). However, until now, no data had been presented that showed a direct correlation between DTD activity and MMC sensitivity. Furthermore, the role of DTD in cellular sensitivity to MMC had not yet been demonstrated directly. In this study, we intended to clarify whether DTD activity correlates with MMC sensitivity in colon and gastric carcinoma cell lines. We also aimed to demonstrate directly by gene transfer experiments the role of DTD in the bioactivation of MMC.

DTD activity in 13 colon and gastric carcinoma cell lines did not correlate well with cellular MMC sensitivity. We found, however, that the cell lines with nonexistent or marginal DTD activity demonstrated resistance to MMC, as compared with other similarly MMC-sensitive cell lines having DTD activity levels above 210 nmol/min/mg protein. This result suggests that substantial DTD activity is required for effective MMC activation in colon and gastric carcinoma cell lines. To demonstrate further the requirement of DTD in MMC sensitivity, we introduced the NQOl gene into St-4 cells, which do not normally demonstrate cellular DTD activity. As compared with the parental St-4 cells, the NQOl transfectants expressed DTD activity at 144-2085 nmol/min/mg protein and showed 5-10-fold higher sensitivity to MMC. The actual IC50 of MMC in these transfectants were at similar levels as those in DTD-proficient colon and gastric cell lines, suggesting that MMC resistance in St-4 cells is primarily due to the...
deficiency in DTD activity in this cell line. The sensitivities of the transfectants to other antitumor drugs were not changed significantly. These results indicate that DTD is required for effective activation of MMC in colon and gastric cell lines.

During the course of these experiments, we noted two interesting observations. The sensitivity to MMC was comparable in colon and gastric cell lines having DTD activity above 210 nmol/min/mg protein. Additionally, NQO1-transfected St-4 cells expressing DTD at activity levels of 144-2085 nmol/min/mg protein also exhibited comparable MMC sensitivity. These results suggest that the activation of MMC by DTD is saturated in these cells. At levels above the saturating activity of DTD, MMC may be fully activated to carry out its cytotoxic activity in the cells. The possibility of saturation of MMC activation is supported by the results of the DNA alkylation experiment, in which DNA from S1T1 (DTD activity, 144 nmol/min/mg protein) and S1T4 (DTD activity, 2085 nmol/min/mg protein) were alkylated equally by MMC. This result suggests that in the cells, a DTD activity of 144 nmol/min/mg protein can activate MMC as well as a DTD of 2085 nmol/min/mg protein can. As of now, we were not able to determine the DTD activity needed for saturated activation of MMC in the cells. However, because MKN7 cells, with a DTD activity of 37 nmol/min/mg protein, are moderately resistant to MMC, saturation of MMC activation may possibly be achieved in cells having a higher DTD activity than that in the MKN7 cells.

The requirement for DTD activity in the MMC sensitivity of colon and gastric carcinoma cell lines implies that this enzyme may have a use in clinical diagnosis as a predictor of tumor MMC sensitivity. Further studies are needed to elucidate the clinical implications of DTD activity in cancer chemotherapy involving MMC.

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