

Regulatory Network of Mitomycin C Action in Human Colon Cancer Cells

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A network composed of activation and inactivation pathways to regulate mitomycin C (MMC) action is suggested to exist in human cancer cells. COLO201 colon cancer cells were stably transfected with human *NQO1* cDNA that encodes NAD(P)H:quinone oxidoreductase (DT-diaphorase, DTD), and a clonal cell line with about 57-fold elevated DTD activity was obtained. Northern analysis revealed that expression of the NADPH:cytochrome P450 reductase (P450 reductase) gene was decreased in the transfectant, COLO201/NQO1, associated with the increase of *NQO1* expression. Biochemical characterization of the cells showed a significant increase of the glutathione (GSH) content concomitantly with the decrease of the P450 reductase activity. As a result of these coordinated modulations, sensitivity of COLO201/NQO1 to MMC was not increased as compared to the parent cells. Analyses of inhibition by specific inhibitors of DTD, P450 reductase and glutathione *S*-transferase (GST) in 5 human colon cancer cell lines including the transfectant showed that DTD and P450 reductase play significant roles in MMC activation in cells with sufficiently high DTD activity and with marginal DTD activity, respectively. In contrast, GST appeared to participate in MMC inactivation in cells with a high level of GST activity. These results indicated that DTD, P450 reductase, GSH and GST may act together compensatively or competitively, depending on their levels in cells, to determine the cellular sensitivity to MMC.

Key words: Colon cancer — Mitomycin C — *NQO1* — NADPH:cytochrome P450 reductase — Drug resistance

Mitomycin C (MMC) is one of the most active agents against gastro-intestinal cancers, but nevertheless has a response rate of only about 20%.¹⁾ This shortcoming has stimulated research aimed at *in vitro* prediction of the sensitivity of individual tumors to MMC and at biochemical modulation of its activity. The mechanism(s) of MMC-induced cytotoxicity remains unclear, although reductive metabolism resulting in the generation of electrophilic alkylating species capable of interacting with cellular macromolecules, especially DNA, is generally assumed to be a prerequisite for the cytotoxic effect of MMC. Efforts, therefore, have been directed toward identifying the enzyme(s) responsible for the MMC activation. At least 5 different enzymes have been shown to be capable of reductively activating MMC and most studies on enzymatic activation of MMC have focused on NADPH:cytochrome P450 reductase (P450 reductase) and NAD(P)H:quinone oxidoreductase (DT-diaphorase, DTD).²⁾ However, there may be cell-specific differences in metabolic pathways of MMC and/or MMC activity may not be determined only by the activation metabolism.

We previously reported that the activity of MMC was determined by the interplay between activation by DTD and inactivation by glutathione *S*-transferase (GST) in cells with sufficiently high DTD activity.³⁾ MMC was most active against cells that have only DTD activity, active against DTD-predominant cells, and less active against GST-predominant cells. In contrast, P450 reductase may play an important role in the MMC activation in cells with marginal DTD activity, such as COLO201 and COLO320DM. These human colon cancer cells had extremely low DTD activity, but were nonetheless moderately sensitive to MMC. Modulation of the DTD activity did not vary their sensitivity to MMC at all.³⁾

Recently, expression of the *DTD* gene has been shown to be coordinated with that of γ -glutamylcysteine synthetase (γ -GCS), the rate-limiting step enzyme for *de novo* glutathione (GSH) synthesis in human colon mucosa.⁴⁾ It has also been reported that the expressions of γ -GCS and *MRP* (multi-drug resistance associated protein) are coordinated in human colorectal cancers.⁵⁾ The ability to develop drug resistance is a cellular defense mechanism. There may exist an intricate network, which may be composed of activation and inactivation pathways, to determine MMC action in cells.

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In this study, we transfected *NQO1* cDNA encoding DTD into COLO201 cells and investigated the resulting genetic and biochemical alterations related to intracellular MMC activation and inactivation. Through comparisons with the parent COLO201 cells and 3 other human colon cancer cells, we attempted to clarify the role of DTD in MMC activation, to identify the enzyme(s) responsible for the MMC activation in each cell line, and to elucidate whether there exists a coordinated pattern of alteration of the activation and inactivation pathways.

MATERIALS AND METHODS

Chemicals MMC was kindly provided by Kyowa Hakko Kogyo Co., Ltd. (Tokyo). Dicoumarol (DIC), metyrapone (MPN), rotenone, GSH, 2,6-dichlorophenol indophenol (DCPIP), horse heart cytochrome *c*, and 1-chloro-2,4-dinitrobenzene (CDNB) were obtained from Sigma (St. Louis, MO). All other chemicals were of analytical grade and were purchased from Wako Pure Chemicals (Osaka), unless otherwise indicated.

Cell lines and cell culture A multi-drug-resistant variant of a human myelogenous leukemia cell line, K562/DOX, was kindly provided by Dr. T. Tsuruo (Tokyo). Human colon adenocarcinoma lines, COLO201 and COLO320DM, were obtained from Japanese Cancer Research Resources Bank (Tokyo). HCC-48 and HCC-50 cell lines established by Dr. K. Yanagihara (Tokyo) from a xenotransplantable human colon cancer cell line CH-4, and a colon cancer cell line CH-5, respectively,⁶ were kindly provided by their originator. All cell lines were cultured in RPMI 1640 medium containing 10% heat-inactivated fetal bovine serum (FBS) at 37°C in a humidified atmosphere of 5% CO₂, and maintained in continuous exponential growth by passage every 3 days.

Evaluation of MMC efficacy Exponentially growing cultured cells were concentrated to 5×10⁶/ml and exposed to the indicated drug concentrations for 30 min. After two washes in drug-free medium, cells were resuspended at a density of 2.5×10⁴/ml in RPMI 1640 with 10% FBS and seeded in 24-well plates. After 72 h incubation at 37°C, cells were trypsinized and counted with a Coulter counter (Hialeah, FL) and by use of the Trypan blue exclusion test.

Assays of enzyme activity Activities of DTD, P450 reductase, and GST were measured as we described previously.³ DTD activity was measured as the DIC-sensitive reduction of DCPIP. The velocity of the reduction of DCPIP was measured by spectrophotometry at 600 nm. P450 reductase activity was measured using cytochrome *c* as the electron acceptor. Increase in absorbance at 550 nm due to the NADPH-dependent reduction of cytochrome *c* was taken as the index of P450 reductase activity. GST activity was measured with CDNB as a substrate by mon-

itoring the enzyme-dependent change in absorbance at 340 nm.

Protein content was measured with the Bio-Rad protein assay (Bio-Rad Laboratory, Hercules, CA) using bovine serum albumin as a standard.

Cellular GSH content Total cellular GSH content was measured as previously described.³ GSH is conventionally assayed by an enzyme recycling procedure in which it is sequentially oxidized by 5,5'-dithiobis-(2-nitrobenzoic acid) and reduced by NADPH in the presence of GSH reductase. GSH content was calculated from the rate of change in absorption at 412 nm, on the basis of a standard curve for each experiment.

RNA preparation and northern analysis Total RNA isolation and northern analysis were performed as described previously.³ The probes used for hybridization were: (1) a 1.1-kb fragment of the human *NQO1* cDNA derived from K562 cells⁷; (2) a 0.75-kb *EcoRI* cDNA insert of the human *GSTπ* clone pGPi2⁸; (3) a 0.7-kb fragment of human *MDR1* cDNA derived from K562 cells⁹; (4) a 0.9-kb *EcoRI* cDNA fragment of the human *MRP10*; (5) a 0.8-kb fragment of human NADPH:cytochrome *c* (P450) reductase cDNA derived from HCC-48 cells¹¹; (6) a 0.85-kb fragment (containing exons 4, 5, 6, 7 and 8) of human glyceraldehyde-3-phosphate dehydrogenase gene (*GAPDH*).¹² The sizes of mRNA were determined from the mobilities of 18S and 28S ribosomal RNA.

Construction of an expression plasmid for human *NQO1* The total RNA from K562 cells was reverse-transcribed to cDNA using Ready-To-Go You-Prime First-Strand Beads and random hexamer (Pharmacia Biotech, Piscataway, NJ) according to the manufacturer's instructions. Human *NQO1* cDNA was obtained by polymerase chain reaction (PCR) from the total cDNA. The pair of primers used was 5'-AACCACGAGCCCAGCCAATC-3' (nucleotides 7-26) and 5'-TTGAGCCCAGTCGGATT-TTG-3' (nucleotides 1090-1071).⁷ The amplified cDNA fragment (1084 bp) containing the *NQO1* coding region (825 bp) and the 5'- (44 bp) and 3'-noncoding regions (215 bp) was cloned into the plasmid pGEM-T (Promega, Madison, WI). After selecting a plasmid that has the insert in an appropriate direction, whole nucleotides in the insert were sequenced to confirm the absence of mutations. Utilizing restriction enzyme sites in the vector flanking the inserted cDNA, the *NQO1* cDNA was excised with *NotI* (5' flanking) and *ApaI* (3' flanking) and subcloned into the *NotI* and *ApaI* sites of the eukaryotic expression vector pRc/CMV (Invitrogen, Carlsbad, CA). The resulting plasmid was designated as pRc/CMV-NQO1.

Transfection of *NQO1* COLO201 cells were transfected with pRc/CMV-NQO1 using liposomes of cationic lipids (Tfx-50; Promega) according to the manufacturer's proto-

cols. Cells were selected by growing them for 2 weeks in RPMI 1640 medium with 10% FBS and 300 $\mu\text{g}/\text{ml}$ of Geneticin (G418 sulfate, Life Technologies, Inc., Grand Island, NY). Transfected colonies resistant to Geneticin were screened for the expression of *NQO1*. Another transfection with the vector pRc/CMV was also performed as a control experiment.

Data analysis Student's *t* test and linear regression analysis were used as appropriate.

RESULTS

To clarify the role of DTD in MMC activation, *NQO1* cDNA was transfected into COLO201 cells, which have extremely low DTD activity. One COLO201 clonal cell line was obtained and designated as COLO201/NQO1, which was stably transfected with *NQO1* cDNA. The growth rate of COLO201/NQO1 cells was similar to those of the parental and vector-transfected COLO201 cells that

were designated as COLO201/vector (data not shown). Northern analysis with *NQO1* cDNA as a probe demonstrated that COLO201/NQO1 cells abundantly expressed a 1.5-kb mRNA in addition to intrinsic mRNAs of 1.1 kb and 2.6 kb (Fig. 1). The additional mRNA was thought to have been derived from the *NQO1* cDNA in the plasmid (pRc/CMV-NQO1) used for the transfection, since it was not detected from the COLO201/vector cells (Fig. 1). The size of the additional mRNA matched that of a transcript which starts from the CMV promoter region and terminates at the transcription termination sequences of the bovine growth hormone genes in the plasmid. With normalization by using *GAPDH* mRNA as a standard, total radioactivity of the *NQO1* mRNA bands from COLO201/NQO1 cells was about 25 times that of COLO201 cells.

The transfectant COLO201/NQO1, however, was less sensitive to MMC than COLO201 (Fig. 2). The MMC concentration causing 50% cell growth inhibition (IC_{50}) was 3.6 and 2.2 $\mu\text{g}/\text{ml}$ for COLO201/NQO1 and for COLO201 cells, respectively. The increased *NQO1* mRNAs may not be utilized for production of active DTD and/or may cause some functional modulations of other properties linked to MMC action. Therefore, the enzymatic activities of DTD, P450 reductase, and GST, and the total amount of GSH were measured (Table I). DTD activities (nmol/min/mg protein) were 689 in COLO201/

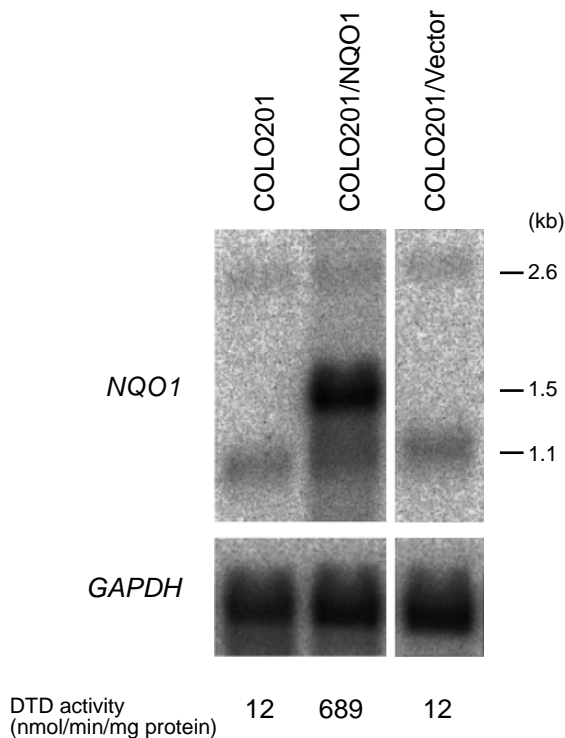


Fig. 1. Northern analysis of the expression of *NQO1* mRNA and DTD activities in COLO201, COLO201/NQO1 and COLO201/vector cell lines. *NQO1* expression was remarkably increased after transfection with *NQO1* cDNA into COLO201 cells. Total RNA (10 $\mu\text{g}/\text{lane}$) from each cell line was separated in 1% agarose gel, blotted, and hybridized with *NQO1* probe. The membrane was subsequently re-hybridized with *GAPDH* probe after the *NQO1* probe had been stripped off. DTD activity was measured as described in "Materials and Methods."

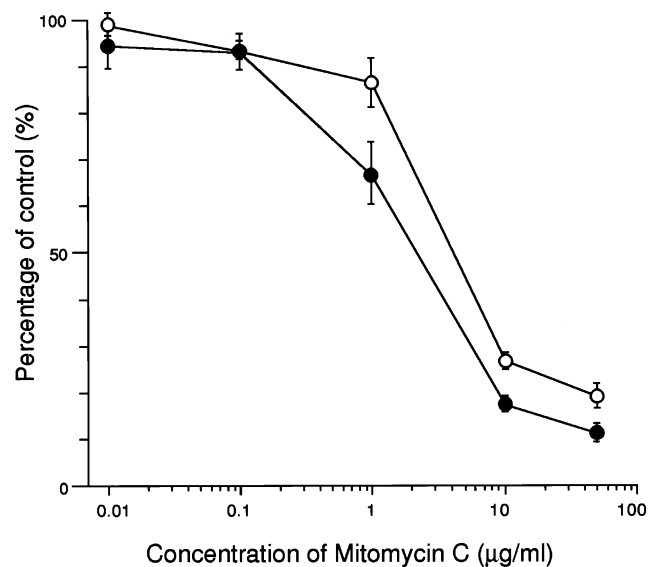


Fig. 2. Cell growth inhibition induced by MMC in COLO201 (closed circles) and COLO201/NQO1 (open circles) cells. The cellular sensitivity of COLO201/NQO1 to MMC was not changed as compared to the parent COLO201 cells. Experimental conditions are described in "Materials and Methods." Each data point represents the mean \pm SD of triplicate samples.

Table I. Properties Putatively Linked to Mitomycin C Activity in COLO201 and COLO201/NQO1 Cells

	COLO201	COLO201/NQO1	P-value
Enzymatic activity (nmol/min/mg protein)			
DTD	12±2 ^{a)}	689±39	0.0011
P450 reductase	4.6±0.2	2.9±0.1	0.0103
GST	118±14	113±12	NS ^{b)}
GSH content (nmol/mg protein)	13.8±2.9	19.3±2.0	0.0098

a) Each value represents the mean±SD of 9 samples in 3 separate experiments.

b) Not significant (*P*-value <0.05 was considered to be significant). DTD, NAD(P)H:quinone oxidoreductase; P450 reductase, NADPH:cytochrome P450 reductase; GST, glutathione *S*-transferase; GSH, glutathione.

NQO1 and 12 in COLO201. The DTD activity of the transfectant was significantly higher than that of COLO201 (about 57-fold, *P*=0.0011), so the transfection of *NQO1* cDNA into COLO201/NQO1 cells had led to an increase of the DTD activity. Interestingly, the transfection caused a decrease of P450 reductase activity (*P*=0.0103) and an increase of GSH amount (*P*=0.0098) in the cells. Since GSH is generally assumed to act in MMC detoxification, the *NQO1* transfection may also have activated the GSH-mediated detoxification of MMC.

The coordinated modulation of DTD and P450 reductase was also seen in the gene expression analysis. A decreased level of expression of the P450 reductase gene was observed in the *NQO1*-transfected cells, while no significant change was detected in the COLO201/vector cells (Fig. 3). To characterize the COLO201/NQO1 cells in more detail, expressions of other genes that may be related to MMC sensitivity were also examined by northern analysis. However, no significant changes in gene expressions of *GSTπ*, *MDR1* and *MRP* were detected as compared to the parent COLO201 cells (Fig. 3). These results suggest that DTD may not be involved in MMC activation and P450 reductase may be of key importance in MMC activation in COLO201 and COLO201/NQO1 cells.

To elucidate the roles of DTD and P450 reductase in MMC activity, inhibition assays were performed in 5 human colon cancer cells including COLO201 and COLO201/NQO1 (Fig. 4). The influence of GST inhibition on MMC efficacy was also investigated to clarify the participation of GST in MMC inactivation. Inhibition of DTD by 50 μM DIC caused a significant decrease in MMC efficacy in HCC-48 and COLO201/NQO1. This indicates that DTD participates in MMC activation in HCC-48, and exogenously increased DTD in COLO201/

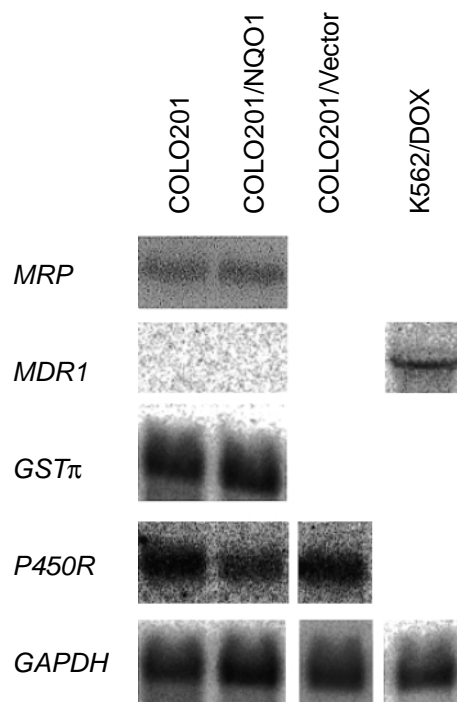


Fig. 3. Northern analysis of the expression of P450 reductase gene (*P450R*), *GSTπ*, *MDR1*, and *MRP* in COLO201 and COLO201/NQO1 cell lines. Expression of *P450R* in COLO201/NQO1 cells was lower than those in COLO201 and COLO201/vector cells. Total RNA (10 μg/lane) from each cell line was separated in 1% agarose gel, blotted, and hybridized with the indicated probes. Total RNA from K562/DOX was used as a positive control of *MDR1* expression.

NQO1 cells also works for MMC activation. On the other hand, GST inhibition by 3.3 μM ethacrynic acid (EA) caused an increase of the MMC effect on HCC-48 and HCC-50 cells. This indicates that GST plays an important role in MMC inactivation in HCC-48 and HCC-50. However, MMC activity decreased significantly in COLO320DM, COLO201 and COLO201/NQO1 when the cells were treated with MMC in the presence of MPN, an inhibitor of P450 reductase, at 5 mM. In contrast to the case of HCC-48, P450 reductase appeared to play an important role in MMC activation in COLO320DM and COLO201. In COLO201/NQO1 cells, P450 reductase plays a more important role than DTD in the activation.

Among the 5 cell lines, DTD activity [in nmol/min/mg protein] was highest in COLO201/NQO1 [689], high in HCC-48 [90], low in HCC-50 [76], and lower in COLO320DM [11] and COLO201 cells [12]. GST activity [in nmol/min/mg protein] was also highest in HCC-48 [202], high in HCC-50 cells [180], and low in COLO201 [118], COLO320DM [113] and COLO201/NQO1 [113]

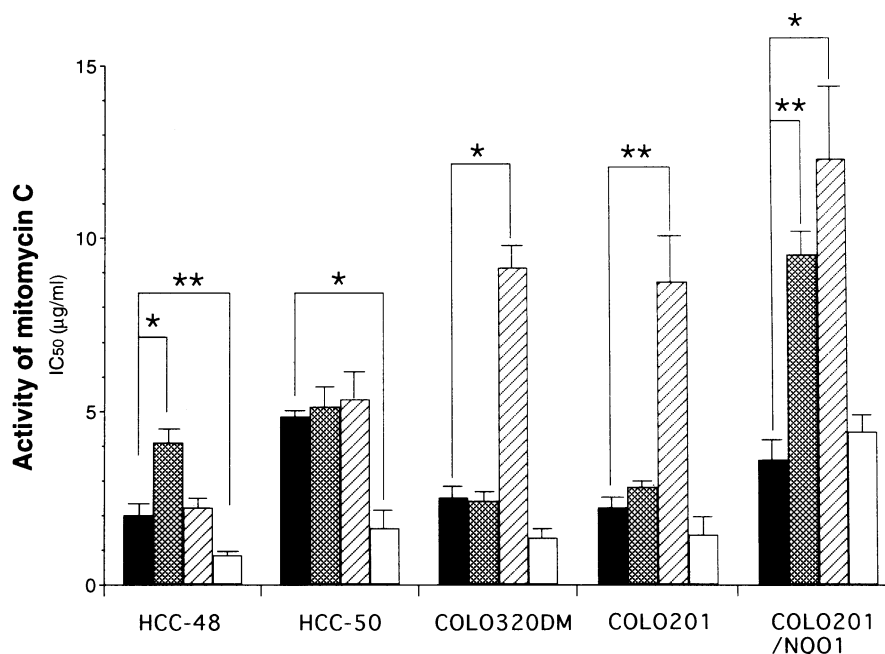


Fig. 4. Effect of inhibition of DTD, P450 reductase and GST activities on MMC efficacy. Exponentially growing cells (5×10^6 /ml) were exposed to MMC for 30 min in the absence (solid bar) or presence of a nontoxic inhibitor of DTD ($50 \mu\text{M}$ DIC, cross-hatched bar), P450 reductase (5 mM MPN, hatched bar), or GST ($3.3 \mu\text{M}$ EA, open bar), after which cytotoxicity was assessed by means of the trypan blue dye exclusion test. MMC-induced cytotoxicity was decreased by the inhibition of DTD activity in HCC-48, while it was decreased by the inhibition of P450 reductase in COLO320DM and COLO201 cells. Cellular sensitivity of COLO201/NQO1 to MMC was decreased by inhibition of the activities of both DTD and P450 reductase. In contrast, MMC activity in HCC-48 and HCC-50 was increased by inhibition of GST activity; * $P < 0.01$, ** $P < 0.05$. Results are the means \pm SDs of 9 samples in 3 separate experiments.

(Table I). These results suggest that MMC activity may be determined by an interplay between activation by DTD and inactivation by GST in cells that have sufficiently high activities of DTD and GST, such as HCC-48 and HCC-50. In these cells, P450 reductase was relatively uninvolved, as we previously suggested.³⁾ In contrast, P450 reductase may activate MMC in cells that have a marginal DTD activity, a low GST activity and a low GSH content, such as COLO201 and COLO320DM. In COLO201/NQO1 cells, although their DTD activity became very high after transfection of *NQO1*, P450 reductase still plays a more important role than DTD in the activation.

DISCUSSION

Both DTD and P450 reductases have been proven to be involved in MMC activation in human colon cancer cells. The predominant activating enzyme of MMC is likely to be determined by the balance of the activities of these enzymes in cells, as we previously suggested.³⁾ When cells have sufficiently high DTD activity, DTD plays a significant role in the reductive activation of MMC,

while P450 reductase is relatively uninvolved. On the other hand, in cells with marginal DTD levels, such as COLO320DM and COLO201, P450 reductase mainly participates in the activation metabolism.

Despite intensive studies, the functional significance of DTD and P450 reductase in reductive activation of MMC remains controversial. Although several reports have suggested an important role for DTD or P450 reductase in the reductive activation of MMC, contrary results have also been reported.³⁾ As shown here, those contradictory results can be attributed to the relative activity levels in cells. From studies using cells transfected with P450 reductase gene, P450 reductase has been demonstrated to bioreductively activate MMC in living cells.¹³⁾ The parent CHO cells and the transfectants, however, have extremely low DTD activity (approximately 10 nmol/min/mg protein). DTD was also proven to be a critical determinant of sensitivity to MMC in human colon and gastric carcinoma cells through comparisons with *NQO1* transfectants and the parent ST-4 cells.¹⁴⁾ The P450 reductase activity in ST-4 cells is only 1.82 nmol/min/mg protein. These variations of DTD and P450 reductase levels in cells may complicate their functional significance in MMC activa-

tion. This is also supported by the results of Joseph *et al.* (1996), who concluded that the enzymatic activation of MMC is catalyzed by P450 reductase, but that the activation also involves DTD when it is present at extremely high levels.¹⁵⁾

Interestingly, overexpression of *NQO1*, the gene which codes for DTD, in COLO201 cells that originally have only marginal DTD activity was also found to cause a decrease of the P450 reductase activity and an increase of the GSH amount in the cells. Although the increased activity of DTD in the transfectant favored MMC activation, the associated reduction in P450 reductase activity and the increase of GSH content prevented sensitization of the cells to MMC. In conjunction with our previous finding that MMC activity was determined by the competition between DTD-mediated activation and GST-mediated inactivation in cells with sufficiently high DTD activity,³⁾ a network composed of activation and inactivation pathways might regulate the MMC-induced cytotoxicity to protect cells from the drug stress. Thus, DTD and/or P450 reductase play significant roles in the activation of MMC, while GSH and/or GST are of key importance in the inactivation.¹⁶⁻¹⁸⁾ Those data also suggest that transfection of *NQO1* into malignant cells without investigating the details of the coordinated modulation may not always be effective to establish the role of DTD in MMC activation directly and may not be a prototype for gene therapy-mediated tumor sensitization.

Our results are consistent with the findings of O'Dwyer *et al.* (1996), who showed that the expressions of the γ -GCS and *DTD* genes were coordinated.⁴⁾ Although we did not investigate γ -GCS expression and activity, the total amount of GSH in COLO201/*NQO1* increased significantly (Table I). It is noteworthy that the increase in DTD activity induced by transfection with *NQO1* might repress the expression of P450 reductase gene in COLO201/

NQO1. Since the increased expression of *NQO1* did not affect expressions of *GST π* , *MRP* and *MDR1*, there might be a different signal from the one that was suggested to be involved in the coordinate expression of several xenobiotic detoxifying enzymes.^{4,5)} Recently, multiple *cis*-acting DNA sequences that regulate expression of the rat P450 reductase gene have been identified. There was an indication that the gene is under complex control, involving both positive and negative regulators.¹⁹⁾ Unfortunately, the promoter region of human P450 reductase gene has not yet been isolated and it is not known whether the human gene also contains similar regulatory elements. Further analyses of the promoter of human P450 reductase gene and factors that interact within the promoter should provide the molecular basis for understanding the transcriptional-regulatory mechanisms of genes for redox cycle-related enzymes.

In summary, this study presents the first demonstration of the existence of an intricate regulation pattern among gene expressions for DTD and P450 reductase in human colon cancer cells. Biochemical data on GSH in cells transfected with *NQO1* (COLO201/*NQO1*) also suggest that the expressions of γ -GCS and *NQO1* may be coordinated. DTD and P450 reductases play significant roles in MMC activation in cells with sufficiently high DTD activity and with marginal DTD activity, respectively. In contrast, GSH and GST appear to participate in MMC inactivation. These results indicate that DTD, P450 reductase, GSH and GST may act together compensatively or competitively, depending on their levels in cells, to determine the cellular sensitivity to MMC. Further studies are required to find the factor(s) that control the regulatory network.

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