Manganese Superoxide Dismutase Expression Correlates with Chemosensitivity in Human Gastric Cancer Cell Lines

Gwong-Cheung Hur,1 Sung Jin Cho,1 Chan-Hyung Kim,2 Min Kyu Kim,1 Soo In Bae,3,4 Seon Young Nam,3,4 Jong-Wan Park,2 Woo Ho Kim,3,4 and Byung Lan Lee1
1Departments of Anatomy, 2Pharmacology, and 3Pathology and 4Cancer Research Institute, College of Medicine, Seoul National University, Seoul, Korea

ABSTRACT

Purpose: The purpose is to investigate the potential correlation between antioxidant enzyme (AOE) levels and resistance to anticancer drugs in human gastric carcinoma cell lines.

Experimental Design: Protein contents of AOE such as manganese superoxide dismutase (MnSOD), copper/zinc superoxide dismutase, catalase, glutathione S-transferase–p-glycoprotein, and multidrug resistance-associated protein were observed by Western blot analysis, and MnSOD activity was measured in six Korean gastric cancer cell lines. The direct correlation between AOE and the chemosensitivity to doxorubicin (DOX), mitomycin C, 5-fluorouracil, and vinblastine was analyzed by cytotoxicity test. MnSOD was overexpressed by transient transfection of human MnSOD cDNA.

Results: Expressions of AOE in gastric cancer cell lines were variable. MnSOD expression was related with the resistance to DOX and mitomycin C but not with that to 5-fluorouracil and vinblastine. In comparison, expressions of other AOE, p-glycoprotein and multidrug resistance-associated protein, were not correlated with tumor sensitivity to any of the drugs used. Cell lines with a high MnSOD protein content showed higher MnSOD activity than those with a low MnSOD protein content. In addition, MnSOD overexpression increased the resistance of gastric carcinoma cells to DOX.

Conclusions: MnSOD is an important factor of drug response to reactive oxygen species-generating anticancer drugs in the gastric cancer cells. Thus, measurement of MnSOD levels in clinical samples may provide an indication of subsequent treatment response of gastric cancer patients.

INTRODUCTION

Gastric cancer is one of the most common malignancies worldwide and the major cause of cancer death in Asian countries (1). Chemotherapy for gastric cancer patients, however, is not effective. Thus, evaluation of the chemosensitivity of gastric cancer cells to anticancer agents based on the phenotype differences of individual cell lines will provide more useful information for choosing correct drugs for gastric cancer patients.

AOEs, including SOD,1 CAT, glutathione peroxidase, and GST are thought to be necessary for life processes in all oxygen-metabolizing cells by removing ROS, but biological significance of AOE in the transformed cells is not clear. Previously, the correlation between AOE expression and the clinical outcome of cancer patients has been investigated using biopsy specimens. In malignant gliomas, high immunoreactivity of GST–p, but not that of Cu/ZnSOD, was related to the short survival time after recurrence (2). In malignant mesothelioma, high CAT and/or coordinated high expression of MnSOD and CAT decreased tumor progression (3). In gastric cancers, immunoreactivity for MnSOD, but not that for Cu/ZnSOD, correlated with a poor overall survival rate (4, 5).

The development of drug resistance of tumors is multifactorial and still poorly understood. There have been studies on the relation between the AOE expressions and the chemosensitivity to ROS-generating anticancer drugs (6–8) in several cancer cells. A Chinese hamster ovary cell clone with MnSOD overexpression showed a higher resistance to DOX and MMC than the control clones (9). In murine leukemic cells, the DOX-resistant cells showed lower activities of GST–p (10). In human mesothelioma cells with a higher level of MnSOD compared with the normal mesothelial cells, enhanced capacity of CAT, but not that of MnSOD, showed protective effect against epirubicin (3). Glutathione peroxidase overexpression partially inhibits apoptosis in DOX-treated human breast carcinoma cells (7). From these studies, not only scavenging of superoxide (O2·−) by MnSOD, but also subsequent decomposition of hydrogen peroxide (H2O2), appears to be critical in the resistance of several cancer cells to various ROS-generating agents. Thus, the relation between AOE expression and the cell resistance to the chemotherapy may differ according to the species and the cell type investigated.

Although there was speculation regarding the correlation between the patient survival and the drug resistance to ROS-generating anticancer drugs caused by MnSOD (11), the direct relationship between endogenous AOE expressions and re-
sponse to chemotherapeutic agents in the gastric carcinoma cells has not been well studied. Thus, the present study assessed the expression levels of the endogenous AOs such as MnSOD, Cu/ZnSOD, CAT, and GST-π in gastric carcinoma cells and measured the sensitivity to DOX and MMC as drugs known to kill by generating ROS and 5-FU and Vin, which theoretically kill by nonredox mechanism. In addition, we measured the expression levels of PGP and MRP that have been suggested as important factors in the failure of chemotherapy (12).

MATERIALS AND METHODS

Reagents. DOX, MMC, 5-FU, Vin, H2O2, DHR, xanthine oxidase, xanthine, and lucigenin (10,10'-dimethyl bis-9,9' biaxridium nitrate) were purchased from Sigma Chemical Co. (St. Louis, MO). The antibodies used were polyclonal antibodies against human MnSOD or Cu/ZnSOD (StressGen Biotechnologies Co., Victoria, British Columbia, Canada), GST-π (Novo-
castra Laboratories Ltd., Newcastle, United Kingdom), CAT (Athens Research & Technology, Inc., Athens, GA), PGP (Santa Cruz Biotechnology, Inc., Santa Cruz, CA), and MRP (MRPr1; Signet Lab., Inc., Bedham, MA). The Lipofectamine reagents were purchased from Life Technologies, Inc. (Grand Island, NY). All other chemicals were reagent grade and were purchased from Sigma Chemical Co.

Cell Culture. Six Korean gastric cancer cell lines (SNU-216, SNU-484, SNU-601, SNU-638, SNU-668, and SNU-719), which have already been well characterized (13–16), were purchased from the Korean Cell Line Bank (Seoul, Korea). They were cultured in RPMI 1640 (Life Technologies, Inc.) supplemented with 10% heat-inactivated fetal bovine serum, 2 mg/ml sodium bicarbonate, 100 units/ml penicillin, and 100 μg/ml streptomycin (Life Technologies, Inc.) at 37°C in 5% CO2.

Western Blot Analysis. For immunoblot analysis of AOs, equal amounts of the proteins of the cell lysates in 1× SDS lysis buffer [125 mM Tris-HCl (pH 6.8), 4% SDS, 0.004% bromophenol blue, and 20% glycerol] were loaded onto 10% SDS/polyacrylamide gels and electrophoretically transferred to nitrocellulose membranes. The membranes were incubated with either rabbit antihuman MnSOD (1:1000), Cu/ZnSOD (1:1000), CAT (1:1000), GST-π (1:1000), or β-actin (1:1000) for 1 h. For PGP and MRP, 6.5% SDS/polyacrylamide gels and incubation with rabbit anti-PGP (1:2000) or rat anti-MRP (1:50) for 16 h were used. Horseradish peroxidase-conjugated antirabbit IgG (Zymed Laboratories, Inc., San Francisco, CA) or antirat IgG (Santa Cruz Biotechnology) diluted 1:5000 was used as the secondary antibody. Enhanced chemiluminescence (Amersham International plc., Buckinghamshire, United Kingdom) was used to visualize the complexes. Signals were measured by Scion Image (Scion Co., Frederick, MD) and expressed as a percentage to the density of the blot from SNU-216. An equal loading of the protein was confirmed by β-actin.

Chemosensitivity Assay. Plating cell densities of individual cell lines that allow logarithmic growth throughout the experiment were determined after cells were cultured without any treatment for 60 h. Various cell numbers (3.62 × 105 cells of SNU-216, 3.3 × 105 cells of SNU-638, 2.75 × 105 cells of SNU-484, 3.15 × 105 cells of SNU-668, 3.71 × 105 cells of SNU-601, and 4.75 × 105 cells of SNU-719) were seeded into each well of 24-well plates. After 24 h, cells were exposed to varying concentrations of MMC, DOX (range, 1 ng/ml to 3 μg/ml), 5-FU (range, 1 ng/ml to 100 μg/ml), or Vin (range, 1 ng/ml to 50 μg/ml). Control columns contained cells without drug and blank columns contained medium alone. After 36 h, cell numbers were measured indirectly using the method reported by Kim et al. (17). Briefly, cells were stained with 0.2% crystal violet aqueous solution in 20% methanol for 10 min, dissolved in 1% SDS, transferred into 96-well plates, and the absorbance was measured at 570 nm using an ELISA reader. Absorbance in the treated cells was expressed as a percentage of control, and IC50's were estimated using a Sigma Plot. Three parallel samples were treated in each concentration point. Each treatment was performed more than three times separately.

Detection of MnSOD Activity. MnSOD activities in various SNU cell lines were measured by a highly sensitive method using lucigenin-based chemiluminescence as described previously (18). After reaching confluence, cells were harvested from the dish with the trypsin-EDTA solution. Cells were spun down at 300 g at 4°C for 5 min, and the pellet was homogenized in a cold buffer solution containing 130 mM NaCl, 2 mM KCl, 2% (v/v) NP40, 2 mM EDTA, 5 mM KCN, 0.5 mM phenylmethylsulfonyl fluoride, 20 mM Tris/HCl, and pH 7.4. The concentrations of proteins in the lysates were adjusted to 10 μg/100 μl by the addition of the homogenation buffer. To measure MnSOD activity, 400 μl of the lysate was incubated in a cuvette with 4 μM of xanthine oxidase and 25 μM xanthine at 27°C for 5 min. One hundred μl of a lucigenin solution containing 100 μM lucigenin in the homogenation buffer was injected into the cuvette before reading and chemiluminescence was monitored for 30 s using a Multi-Biolumat LB9505C photodetector (Berthold). One unit of MnSOD is defined as the activity required for 50% inhibition under the control reaction omitting the cell lysates for the reaction mixture.

Detection of H2O2 Using a Fluorogenic Probe. To compare the MnSOD activity in response to ROS-generating anticancer drugs, conversion of H2O2 from O2 after DOX treatment was detected by a method reported by Negre-Salvayre et al. (19) with slight modifications. Generation of cellular H2O2 was observed using a fluorogenic probe DHR that reacts with H2O2, not with O2, produced in the mitochondria. Twenty-four h after plating, cells were exposed either to IC50 of DOX for 36 h or to 100 μM of H2O2 for 1 h. Control cells were not treated with DOX or H2O2. DOX-treated cells were loaded with DHR (5 μM) for 30 min. In cases of H2O2 treatment, cells were loaded with DHR for 30 min just before H2O2 treatment. Fluorescent intensity of treated cells and control cells was observed under fluorescent microscope.

Construction and Transfection of the MnSOD Expression Vector. Human MnSOD cDNA was transfected into SNU-484, and SNU-601 to examine the effect of increased MnSOD on the chemoresistance. An expression vector, pcDNA3 with a human MnSOD cDNA insert (780 bp), was kindly provided by Dr. Bernardetta Palazzotti at the Catholic University of Italy. This construct (0.4 μg) or empty pcDNA3 vector (0.4 μg) was transfected into 107 cells/well in 24-well plates using Lipofectamine Reagent according to the manufacturer’s recommendations, and the transfected cells were treated with 78 ng/ml DOX for 36 h to determine the drug resistance.
**Statistical Analysis.** The results were analyzed by a non-parametric Mann-Whitney U test to compare the correlation between the IC\(_{50}\) for anticancer drugs and the antioxidant content in each of the cancer cell lines. After transfection, a significant difference was determined by a one-way ANOVA test followed by a Newman-Keuls test. \( P < 0.05 \) was considered to be statistically significant.

**RESULTS**

**Expression Levels of Endogenous AOE s.** Fig. 1 shows varying protein contents of MnSOD, Cu/ZnSOD, CAT, and GST-\(\pi\) in all of the tested cell lines. The MnSOD expression level was significantly higher in SNU-216, SNU-638, and SNU-668 in comparison with SNU-484, SNU-601, and SNU-719 \(( P < 0.001; \text{Fig. } 1A)\). The expression level of Cu/ZnSOD was lower in SNU-216 and that of CAT was lower in SNU-216 and SNU-638 than in the other cell lines \(( P < 0.05; \text{Fig. } 1, B \text{ and } C)\). The GST-\(\pi\) expression level was significantly higher in SNU-601 than in the other cell lines \(( P < 0.05; \text{Fig. } 1D)\).

**Correlation between Antioxidant Expression and Chemosensitivity.** Six human gastric cancer cell lines were treated with various concentrations of DOX, MMC, 5-FU, and Vin. Table 1 and Fig. 2 show the in vitro chemosensitivity responses of individual cell lines to the drugs. Cell lines expressing a high level of MnSOD showed statistically significant higher resistance to the cytotoxicity of ROS-generating drugs.

![Fig. 1 Western blot analysis for AOE s.](image)

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**Table 1**  IC\(_{50}\) to anticancer drugs in each cell line

<table>
<thead>
<tr>
<th>Cell lines</th>
<th>MMC(^a) (ng/ml)</th>
<th>DOX(^a) (ng/ml)</th>
<th>5-FU ((\mu g/ml))</th>
<th>Vin (ng/ml)</th>
<th>MnSOD expression</th>
</tr>
</thead>
<tbody>
<tr>
<td>SNU-216</td>
<td>919.0 ± 11.0(^c)</td>
<td>872.0 ± 125.5(^d)</td>
<td>16.5 ± 0.5</td>
<td>11788.3 ± 1043.5(^d)</td>
<td>High</td>
</tr>
<tr>
<td>SNU-484</td>
<td>22.1 ± 3.1</td>
<td>58.2 ± 18.0</td>
<td>18.1 ± 2.3</td>
<td>2308.3 ± 619.1(^e)</td>
<td>Low</td>
</tr>
<tr>
<td>SNU-601</td>
<td>82.6 ± 17.0</td>
<td>27.6 ± 7.8</td>
<td>0.2 ± 0.2</td>
<td>9.6 ± 0.5</td>
<td>Low</td>
</tr>
<tr>
<td>SNU-638</td>
<td>203.1 ± 28.3(^d)</td>
<td>475.5 ± 67.8(^e)</td>
<td>0.8 ± 0.2</td>
<td>3.3 ± 0.6</td>
<td>High</td>
</tr>
<tr>
<td>SNU-668</td>
<td>267.8 ± 21.5(^d)</td>
<td>891.0 ± 246.1(^e)</td>
<td>49.7 ± 2.0(^d)</td>
<td>21732.5 ± 6071.0(^d)</td>
<td>High</td>
</tr>
<tr>
<td>SNU-719</td>
<td>14.9 ± 4.1</td>
<td>33.0 ± 13.0</td>
<td>6.2 ± 2.8</td>
<td>52.0 ± 6.0</td>
<td>Low</td>
</tr>
</tbody>
</table>

\(^a\) IC\(_{50}\)s were evaluated by the Sigma plot, and results are presented as mean ± SD of three independent experiments.

\(^b\) DOX, MMC: ROS-generating anticancer drugs.

\(^c\) \( P < 0.001 \) compared with SNU-484, -601, and -719.

\(^d\) \( P < 0.001 \) compared with SNU-484, -601, -638, and -719.

\(^e\) \( P < 0.001 \) compared with SNU-601, -638, and -719.

\(^f\) \( P < 0.01 \) compared with SNU-216 and -484.
such as DOX and MMC compared with cell lines expressing a low level of MnSOD ($P < 0.001$). Although two of the three gastric cancer cells expressing a high level of MnSOD such as SNU-216 and SNU-668 appeared resistant to Vin, the correlations were not statistically significant. In comparison, expression of Cu/ZnSOD, CAT, and GST-π did not show any correlation with chemosensitivity to any of the anticancer drugs. Thus, among the AOE examined, only MnSOD expression appears to contribute to the resistance to the ROS-generating anticancer drugs in gastric cancer cells.

**MnSOD Activity.** To compare the MnSOD expression level to the activity of MnSOD, the activity of MnSOD in each cell line was monitored. As shown in Fig. 3, MnSOD activities of SNU-216, SNU-638, and SNU-668 expressing a high level of MnSOD protein were significantly higher than those of the other cell lines expressing a low level of MnSOD protein ($P < 0.05$). Although MnSOD activity of SNU-484 with a low MnSOD protein content was higher than those of SNU-484 and SNU-719, this data were not statistically significant ($P > 0.05$).

**Expression of PGP and MRP.** Fig. 4 shows that protein contents of PGP in SNU cell lines were not detectable, whereas those of MRP are variable regardless the chemosensitivity to the DOX, MMC, or Vin. Hep3B, a hepatocellular carcinoma cell line, was used as positive control for PGP and MRP (20).

**Effects of DOX on $O_2^-$ Generation.** Our data confirmed that inside living cells, DOX treatment causes the generation of $O_2^-$ that is converted to $H_2O_2$ by mitochondrial MnSOD (21). Therefore, after DOX treatment, fluorescent intensity to $H_2O_2$ was increased in SNU-216, SNU-638, and SNU-668 expressing a high level of MnSOD but not in SNU-484, SNU-601, and SNU-719 with a low level of MnSOD (Fig. 5A). In cases of H$_2$O$_2$ treatment, fluorescent intensity was increased in all of these six cell lines (Fig. 5B).

**MnSOD Overexpression and Chemosensitivity to DOX.** Transiently transfected SNU-484 and SNU-601 with MnSOD cDNA were used to confirm the effects of MnSOD overexpression on chemosensitivity to a ROS-generating drug. DOX. Fig. 6, A and B, show that MnSOD transfection increases MnSOD expression (Fig. 6A) and, subsequently, enhances cell viability after DOX treatment (Fig. 6B).

**DOX-Induced Changes in MnSOD Expression.** To evaluate the effect of DOX-induced MnSOD expression on the resistance to DOX cytotoxicity, Western blot analysis was performed using cell lines exposed to increasing concentrations of DOX for 36 h. Treatment of DOX increased the expression level of MnSOD with the same pattern in both of DOX-resistant cell lines (Fig. 7A) and nonresistant cell lines (Fig. 7B). Thus,
DOX-induced MnSOD expression does not appear to cause the difference in the chemosensitivity to DOX between the cell lines.

DISCUSSION

SOD, a major AOE, catalyzes the dismutation of $O_2^-$ to $H_2O_2$. There are two main forms of SOD in eukaryotic cells: type I-Cu/ZnSOD, located primarily in the cytosol but also in the nucleus, and type II-MnSOD, sited in the mitochondrial matrix (22, 23). MnSOD expression was originally reported to be lower or absent in various cancers (24). Recent studies have, however, documented high levels of MnSOD in malignant tumors of the mesothelium, stomach, ovary, brain, and cervix (3, 5, 25–27).

There have been several studies in the literature linking high MnSOD expression in a variety of tumor types with protection against ROS-generating drugs (10, 28). On the other hand, several studies (29–32) using transfection of the SOD gene, transgenic animals, or cells treated with tumor necrosis factor $\alpha$ to cause MnSOD induction have indicated that high levels of SOD convey minimal protection or no protection at all or even increase susceptibility to oxidant effects. Thus, the role of MnSOD in the host defense against exogenous oxidants remains inconclusive.

MnSOD levels have previously been reported in gastric cancer in relation to prognosis (4, 5) but not to drug resistance. Our results showed that AOE expressions varied in individual gastric cancer cell lines. Among them, the cell lines expressing a higher level of MnSOD were more resistant to DOX and MMC ($P < 0.001$), but not to 5-FU and Vin, when compared with the other cell lines. This result suggests that high MnSOD expression in the gastric cancer is positively correlated with the resistance to DOX and MMC. In comparison, endogenous expressions of Cu/ZnSOD, CAT, and GST-$\pi$ did not affect the resistance to any of the anticancer drugs. Thus, removal of $O_2^-$, but not that of $H_2O_2$, may be important in the chemoresistance of gastric cancer cells to ROS-generating anticancer drugs but not in the multidrug resistance.

In the present study, the results of the MnSOD activity assay were in agreement with those of the Western blotting. The activities of cell lines with a high MnSOD content were significantly higher than those of the other cell lines with a low MnSOD content ($P < 0.05$). In addition, detection of $H_2O_2$ produced in the mitochondria using a fluorogenic probe indirectly demonstrated the MnSOD activity (19). Our results indicate that $O_2^-$ is generated by DOX and converted to $H_2O_2$ by mitochondrial MnSOD, which is more effectively performed in cell lines expressing a high level of MnSOD.

Two or more mechanisms often coexist to confer drug-resistant phenotype. The resistance to DOX by some tumor cells

![Fig. 5](https://example.com/figure5.png) Fluorescent microscopy after loaded with DHR. Detection of ROS ($H_2O_2$) in cancer cell lines treated with DOX for 36 h (A) or with $H_2O_2$ for 1 h (B). Compared with the control of each cell line, fluorescent intensity was increased in SNU-216, SNU-638, and SNU-668 with a high MnSOD content but not in SNU-484, SNU-601, and SNU-719 with a low MnSOD content. On the other hand, $H_2O_2$ treatment increased fluorescent intensity in all of these six cell lines.
is mainly caused by the effect of PGP and MRP (33). In addition, in some cancer cells, expression of PGP confers resistance to Vin as well as DOX (34, 35). In the gastric cancer, it is still controversial whether PGP or MGP confers resistance to DOX (36–38). In the present study, the expression of PGP in SNU cell lines was undetectable, and MRP was highly expressed in two of the three cell lines that are chemosensitive to DOX and MMC. Furthermore, SNU-601, SNU-638, and SNU-719, Vin-sensitive cell lines, showed high to moderate MRP expression. These results suggest that PGP or MRP is not significantly involved in the chemoresistance of SNU cell lines to DOX, MMC, and Vin.

DOX interferes with the topoisomerase II-DNA complex, which in turn inhibits DNA duplication and transcription to mRNA (39). A previous study, however, reported that the association between the cellular resistance to the DOX treatment and topoisomerase II activity and/or protein in the gastric cancer cells was not clear (38).

To further test the effect of the MnSOD expression on the resistance to the ROS-generating DOX in the gastric cancer, SNU-484 and SNU-601 cells were transiently transfected. Our data demonstrated that an augmentation of intracellular MnSOD by ~1.4- and 5.4-fold leads to an approximate 2.17- and 3.26-fold increase, respectively, in cell viability after DOX treatment. Thus, it was further substantiated that MnSOD in the gastric cancer cells plays a protective role against DOX cytotoxicity. Taken together, our results suggest that in the gastric cancer cells, removal of superoxide is critical in the resistance to ROS-generating drugs.

Because MnSOD is induced by cytotoxic drugs (40), we examined the DOX-induced change in MnSOD expression levels in individual cell lines. After treatment with DOX for 36 h, same pattern of increment was shown in all of the cell lines. This result indicates that the difference in the drug resistance between the gastric cancer cell lines depends upon that in the constitutive MnSOD expression but not that in the DOX-induced MnSOD expression. We speculate that the decrease in the MnSOD expression at relatively higher concentrations of DOX may come from the cell death by DOX cytotoxicity.

The present study cannot explain why cytotoxicity of ROS-generating drugs was related with MnSOD expression, but not with that of Cu/ZnSOD, although both SOD enzymes are known to catalyze the O$_2^-$ to H$_2$O$_2$. We speculated that damage of mitochondria which is particularly susceptible to damage induced by ROS (41) is important in the cell death of gastric cancers treated by ROS-generating drugs. Furthermore, it is reported that elevated levels of Cu/ZnSOD in cells and animals creates an oxidative stress (42).

In conclusion, our results strongly support the belief that MnSOD plays a central role in protecting gastric cancer cells against ROS-generating anticancer drugs by decreasing the mitochondrial ROS. Thus, measuring the MnSOD contents in individual gastric cancers may predict the efficacy of chemotherapy for gastric cancer patients.
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