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Effect of Depletion and Oxidation of Cellular GSH on Cytotoxicity of Mitomycin c in Small Cell Lung Cancer Cells

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Abstract – Effect of the depletion or oxidation of GSH on mitomycin c (MMC)-induced mitochondrial damage and cell death was assessed in small cell lung cancer (SCLC) cells. MMC induced cell death and the decrease in the GSH contents in SCLC cells, which were inhibited by z-LEHD.fmk (a cell permeable inhibitor of caspase-9), z-DQMD.fmk (a cell permeable inhibitor of caspase-3) and thiol compound, N-acetylcysteine. MMC caused nuclear damage, release of cytochrome c and activation of caspase-3, which were reduced by N-acetylcysteine. The depletion of GSH due to L-butionine-sulfoximine enhanced the MMC-induced cell death and formation of reactive oxygen species in SCLC cells, whereas the oxidation of GSH due to diamide or NH_2Cl did not affect cytotoxicity of MMC. The results show that MMC may cause cell death in SCLC cells by inducing mitochondrial dysfunction, leading to activation of caspase-9 and -3. The MMC-induced change in the mitochondrial membrane permeability, followed by cell death, in SCLC cells may be significantly enhanced by the depletion of GSH. In contrast, the oxidation of GSH may not affect cytotoxicity of MMC.

Keywords □ mitomycin c, small cell lung cancer cells, mitochondrial membrane permeability, cell death, depletion and oxidation of GSH

INTRODUCTION

The membrane permeability transition of mitochondria has been shown to be involved in a variety of toxic and oxidative forms of cell injury as well as apoptosis. Opening of the mitochondrial permeability transition pore causes a depolarization of the transmembrane potential, releases of Ca^{2+} and cytochrome c and loss of oxidative phosphorylation, which results in loss of cell viability (Bernardi, 1996; Mignotte and Vayssière, 1998). In addition to DNA, mitochondria are postulated as a cellular target for anticancer drugs (Pritsos *et al.*, 1997; Hong *et al.*, 2003).

Mitomycin c (MMC) is commonly used in combination with other drugs for the treatment of breast, lung and prostate cancers. This drug is activated by the enzymatic reduction of its quinone moiety and then binds covalently to DNA, which shows a cytotoxic effect on tumor or normal cells (Sartorelli *et al.*, 1994; Cummings *et al.*, 1998). The enzymatic bioactivation produces reactive oxygen species (ROS), which are involved in oxidative damage of cell components (Nakano *et al.*, 1984; Pritsos and Sartorelli, 1986). MMC damages the DNA and membrane integrity in mitochondria in EMT6 mouse mammary carcinoma cells (Pritsos *et al.*, 1997). In contrast, in human pulmonary adenocarcinoma A549 cells the morphological changes of mitochondria due to MMC are not demonstrated (Simamura *et al.*, 2001). It is also uncertain whether MMC-induced cell death is mediated by caspase-3 activation (Kobayashi *et al.*, 2000; Pirnia *et al.*, 2002). Therefore, it is necessary to clarify whether opening of the mitochondrial membrane permeability pore in cells exposed to MMC induces the activation of caspases through the release of cytochrome c.

The drops in GSH levels and oxidation of GSH have been shown to increase the sensitivity of cells to the damaging effect of toxic substances (Reed, 1990) and are associated with mitochondrial dysfunction (Jurma *et al.*, 1997). The toxicity of anticancer drugs may largely depend on the intracellular level of reduced GSH. The depletion of GSH due to L-buthionine-(S,R)-sulfoximine increases the toxicity of etoposide on K562 human erythroleukemia cells (Gantchev and Hunting, 1997). In contrast, the GSH depletion does not affect the toxicity of etoposide and doxorubicin on U-937 human promonocytic leukemia cells (Troyano *et al.*, 2001). The mitochondrial membrane

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permeability is postulated to be affected by the redox state of dithiols (Constantini *et al.*, 1996). Therefore, it is necessary to explore whether the MMC-induced change in the mitochondrial membrane permeability and cell death is modulated by depletion and oxidation of GSH.

Although mitochondria are suggested as a cellular target for MMC, effect of the depletion and oxidation of GSH on MMC-induced mitochondrial damage has not been clarified. The purpose of the present study was to explore the influence of the depletion or oxidation of GSH against cytotoxicity of MMC. We examined the toxic effect of MMC on SCLC cells by measuring the effect on the cytochrome c release, caspase-3 activity, GSH contents and ROS formation.

MATERIALS AND METHODS

Materials

Quantikine® M human cytochrome c assay kit was purchased from R&D systems (Minneapolis, MN, USA), and ApoAlert™ CPP32/caspase-3 assay kit was from CLONTECH Laboratories Inc. (Palo Alto, CA, USA). Mitomycin c (MMC), diamide, N-acetylcysteine, z-Leu-Glu-(O-ME)-His-Asp(O-Me) fluoromethyl ketone (z-LEHD.fmk), z-Asp-(OMe)-Gln-Met-Asp(OMe) fluoromethyl ketone (z-DQMD.fmk), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), Hoechst 33258, 2',7'-dichlorofluorescin diacetate ($DCFH_2$-DA), diamide, glutathione (GSH, reduced form), 5,5'-dithio-bis-(2-nitrobenzoic acid) (DTNB), NADPH, glutathione reductase, phenylmethylsulfonylfluoride (PMSF) and RPMI 1640 medium were obtained from Sigma-Aldrich Inc. (St. Louis, MO, USA).

Preparation of monochloramine

NH_2Cl was prepared freshly on the day of use by the NaOCl-induced oxidation of ammonium chloride (Tamai *et al.*, 1991). One volume of NaOCl was mixed with 4 volume of 20 mM NH_4Cl in 10 mM K_2HPO_4 buffer, pH 8.0 at 4°C. The concentration of NH_2Cl was determined using a molar extinction coefficient of 42.9 at 242 nm.

Culture of small cell lung cancer cells

The human small cell lung cancer (SCLC) cells (NCI-H889) were obtained from the Korean cell line bank (Seoul, Korea). Cells were maintained in RPMI supplemented with 10% heat-inactivated fetal bovine serum (FBS), 100 U/mL penicillin and 100 μg/mL streptomycin in 5% CO_2 atmosphere at 37°C. The culture medium was changed every 3 days, and the cells were subcultured about once a week. Cells (1×10^7) were plated on polystyrene 60×15 mm cell culture dishes (Corning Incorporated, Corning, NY, USA) 48-72 h before experiments. Cells were washed with RPMI containing 1% FBS and replated onto 96 well plates at a density of 4×10^4 cells per well in a volume of 200 μL (or various numbers of cells/mL in 24 well plates). Cells were treated with MMC in RPMI containing 1% FBS for 24 h at 37°C.

Cell viability assay

Cell viability was measured by using the MTT assay, which is based on the conversion of MTT to formazan crystals by mitochondrial dehydrogenases (Mosmann, 1983). SCLC cells (4×10^4) were treated with MMC for 24 h at 37°C. The medium (200 μL) was incubated with 10 μL of 10 mg/mL MTT solution for 2 h at 37°C. Culture medium was removed, and 100 μL of dimethyl sulfoxide was added to each well to dissolve the formazan. Absorbance was measured at 570 nm using a microplate reader (Molecular Devices Co., Spectra MAX 340, Sunnyvale, CA, USA). Cell viability was expressed as a percentage of the value in control cultures.

Morphological observation of nuclear change

SCLC cells (1×10^6 cells/mL) were treated with MMC for 24 h at 37°C, and the nuclear morphological change was assessed using Hoechst dye 33258 (Oberhammer *et al.*, 1992). After treatment, the medium was centrifuged at 412 *g* for 10 min in a microplate centrifuge, and medium was removed. The pellets were washed twice with phosphate buffered saline (PBS). SCLC cells were incubated with 1 μg/mL Hoechst 33258 for 3 min at room temperature, and nuclei were visualized using an Olympus Microscope with a WU excitation filter (Tokyo Japan).

Measurement of DNA fragmentation

Apoptosis was assessed by measuring the DNA fragmentation, which occurs following the activation of endonucleases. SCLC cells (1×10^5 cells/mL) were treated with MMC for 24 h at 37°C, washed with PBS and fixed with formaldehyde solution. Nucleotide (dNTP) was incorporated at the 3' ends of DNA fragments using terminal deoxynucleotidyl transferase (TdT). This nucleotide was detected using a horseradish-peroxidase and TACS-Sapphire according to TiterTACS protocol. Data were expressed as absorbance at 450 nm.

Measurement of cytochrome c release

The release of cytochrome c from mitochondria into the cytosol was assessed by using a solid phase ELISA kit for the detection of human cytochrome c. SCLC cells (5×10^5 cells/mL) harvested by centrifugation at 412 *g* for 10 min were washed twice with PBS and resuspended in 250 mM sucrose, 20 mM HEPES-KOH (pH 7.5), 10 mM KCl, 1.5 mM $MgCl_2$, 1 mM EDTA, 1 mM EGTA, 0.5 mM dithiothreitol and 0.1 mM PMSF. Cells were further homogenized by successive passages through a 26-gauge needle. The homogenates were centrifuged at 100,000 *g* for 30 min, and the supernatant obtained was used for analysis of cytochrome c. The supernatants were added into the 96-well microplates coated with monoclonal antibody specific for human cytochrome c that contains cytochrome c conjugate. The procedure was performed as described in the assay kit. Absorbance of samples was measured at 450 nm in a microplate reader. A standard curve was constructed by adding the diluted solutions of cytochrome c standard, handled like samples, to the microplates coated with monoclonal antibody. The amount was expressed as nanograms/mL by reference to the standard curve.

Measurement of caspase-3 activity

The activation of caspase-3 occurred during the apoptotic process in cells was assessed (Mignotte and Vayssière, 1998). SCLC cells (2×10^6 cells/mL) were treated with MMC for 24 h at 37°C, and caspase-3 activity was determined as described in user's manual of ApoAlert™ CPP32/Caspase-3 assay kit. The supernatant obtained by a centrifugation of cells dissolved was added to the reaction mixture containing dithiothreitol and caspase-3 substrate (*N*-acetyl-Asp-Glu-Val-Asp-*p*-nitroanilide) and was incubated for 1 h at 37°C. Absorbance of the chromophore *p*-nitroanilide produced was measured at 405 nm. The standard curves were obtained from absorbances in the *p*-nitroanilide standard reagent diluted with cell lysis buffer (up to 20 nM). One unit of the enzyme was defined as 1 nmol of chromophore *p*-nitroanilide produced.

Measurement of Intracellular ROS Formation

The dye $DCFH_2$-DA, which is oxidized to fluorescent DCF by hydroperoxides, was used to measure relative levels of cellular peroxides (Fu *et al.*, 1998). After exposure to MMC, SCLC cells (4×10^4) were incubated with 50 μM dye for 30 min at 37°C and then were washed with PBS. The cell suspensions were centrifuged at 412 *g* for 10 min, and medium was removed. Cells were dissolved with 1% Triton X-100, and fluorescence was measured at an excitation wavelength of 485 nm and an emission wavelength of 530 nm using a fluorescence microplate reader (SPECTRAFLUOR, TECAN, Salzburg, Austria).

Measurement of total glutathione

The total glutathione (reduced form GSH+oxidized form GSSG) was determined using glutathione reductase (van Klaveren *et al.*, 1997). SCLC cells (4×10^4) were treated with MMC for 24 h at 37°C, centrifuged at 412 *g* for 10 min in a microplate centrifuge, and medium was removed. The pellets were washed twice with PBS. Cells were dissolved with 2% 5-sulfosalicylic acid (100 μL) and then incubated in 100 μL of the reaction mixture containing 22 mM sodium EDTA, 600 μM NADPH, 12 mM DTNB and 105 mM NaH_2PO_4, pH 7.5 at 37°C. Glutathione reductase (20 μL, 100 U/mL) was added and the mixture incubated for a further 10 min. Absorbance was measured at 412 nm using a microplate reader. The standard curve was obtained from absorbance of the diluted commercial GSH that was incubated in the mixture as in samples.

Measurement of oxidized glutathione

SCLC cells (4×10^4) were treated with MMC for 24 h at 37°C, 5-sulfosalicylic acid added to dissolve cells and centrifuged at 412 *g* for 10 min. The supernatants were used for analysis of GSSG (Hissin and Hilf, 1976). Supernatants (100 μL) were reacted with 40 μL of 40 mM N-ethylmaleimide for 30 min at room temperature, and to this mixture 0.86 mL of 0.1 N NaOH was added. This mixture (100 μL) was mixed with 1.8 mL of 0.1 N NaOH solution and then were reacted with 100 μL of *o*-phthalaldehyde (final concentration, 500 μg) for 15 min at room temperature. The amounts of GSSG were measured at an excitation wavelength of 350 nm and an emission wavelength of 420 nm using a fluorescence microplate reader. The standard curve was obtained from absorbance of the diluted commercial GSSG incubated in the mixture as in samples.

Statistical analysis

Data are expressed as the means ± SEM. Statistical analysis was performed by one-way analysis of variance. When significance was detected, post hoc comparisons between the different groups were made using the Duncan's test for multiple comparisons. A probability less than 0.05 was considered to be statistically significant.

RESULTS

Cell death and decrease in GSH contents due to MMC

MMC caused cell death in human SCLC cells, which was approximately correlated with decrease in cellular GSH contents. The mechanism by which MMC shows toxicity against SCLC cells was investigated. Cell death due to 15 μg/mL MMC was inhibited by 40 μM z-LEHD.fmk (a cell permeable inhibitor of caspase-9), 40 μM z-DQMD.fmk (a cell permeable inhibitor of caspase-3) and 1 mM N-acetylcysteine (Fig. 1A). Like the effect on cell death, caspase inhibitors (z-LEHD.fmk and z-DQMD.fmk) interfered with decrease in the GSH contents due to MMC (Fig. 1B). The production of ROS within cells was determined by monitoring conversion of $DCFH_2$-DA to DCF. Treatment with 15 μg/mL MMC showed an increase in the formation of ROS, which was depressed by 1 mM N-acetylcysteine (Fig. 1C).

To assess apoptotic cell death due to MMC and clarify the inhibitory effect of thiol compound on cytotoxicity of MMC, we investigated the nuclear damage in MMC-treated cells. During the process of apoptosis, DNA fragmentation is caused by activation of endonucleases. Nuclear staining with Hoechst 33258 demonstrated that control SCLC cells had regular and round-shaped nuclei. In contrast, the condensation and fragmentation of nuclei, characteristic of apoptotic cells, were evident in SCLC cells treated with 15 μg/mL MMC for 24 h at 37°C (Fig. 2A). One mM N-acetylcysteine depressed the MMC-induced nuclear damage, while the nuclear morphology in cells treated with thiol compound alone was similar to that in the control cells.

Fragmented DNA was also assessed by measuring the binding of dNTP to the 3 ends of DNA fragments, which was detected by quantitative colorimetric assay. SCLC cells (1×10^5 cells/mL) were treated with 15 μg/mL MMC in the presence of N-acetylcysteine for 24 h at 37°C. The control cells show 0.224 ± 0.011 of absorbance (n=6). Treatment with 15 μg/mL MMC for 24 h caused about 2.5-fold increase in absorbance (Fig. 2B). N-acetylcysteine (1 mM) depressed the MMC-induced increase in absorbance, while absorbance in cells treated with N-acetylcysteine alone was not significantly different from that in the control cells.

Cytochrome c release and caspase-3 activation due to MMC

By investigating the effect on the mitochondrial membrane permeability we assessed the cytotoxic effect of MMC. Opening of the mitochondrial membrane permeability transition pore as an early phenomenon in apoptotic cell death causes a

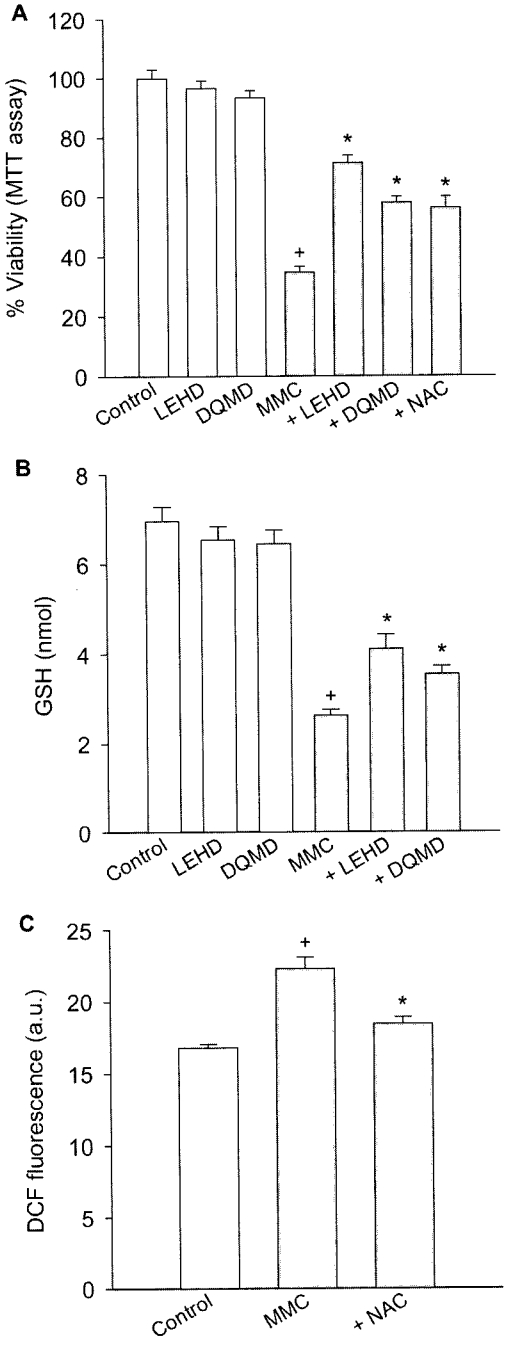

Fig. 1. Cell death and GSH depletion due to MMC. SCLC cells (4×10^4) were treated with 15 μg/mL MMC in the presence of caspase inhibitors [40 μM of z-LEHD.fmk (LEHD) and z-DQMD.fmk (DQMD)] or 1 mM N-acetylcysteine (NAC) for 24 h at 37°C. Data are expressed as the percentage of cell viability in MTT assay (A), nmol in GSH contents (B) and arbitrary units (a.u.) of fluorescence in ROS formation (C). The values represent the means ± SEM (n=6). $^+P < 0.05$, significantly different from control (percentage of control); $^*P < 0.05$, significantly different from MMC alone.

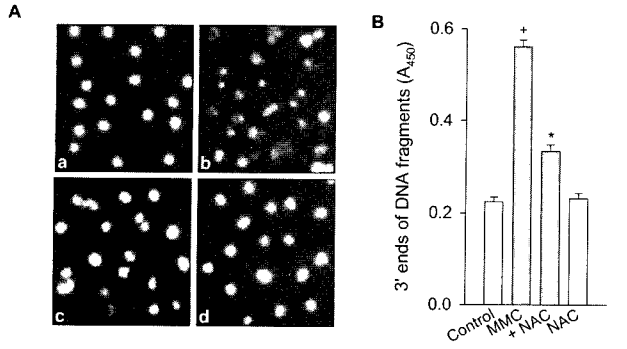

Fig. 2. MMC-induced nuclear damage. In the experiment A, SCLC cells (1×10^6) were treated with 15 μg/mL MMC in the presence of 1 mM N-acetylcysteine for 24 h at 37°C. Cells were observed by fluorescence microscopy after nuclei staining with Hoechst 33258. Figure represents microscopic morphology of the control cells (a), cells treated with MMC alone (b), cells treated with MMC and N-acetylcysteine (c) and cells treated with N-acetylcysteine alone (d). All the subparts are representative of four different experiments. In the experiment B, SCLC cells (1×10^5) were treated with 15 μg/mL MMC and 1 mM N-acetylcysteine (NAC) for 24 h at 37°C. The 3 ends of DNA fragments were detected as described in Materials and methods. Data are expressed as absorbance and represent means ± SEM (n=6). $^+P < 0.05$, significantly different from control; $^*P < 0.05$, significantly different from MMC alone.

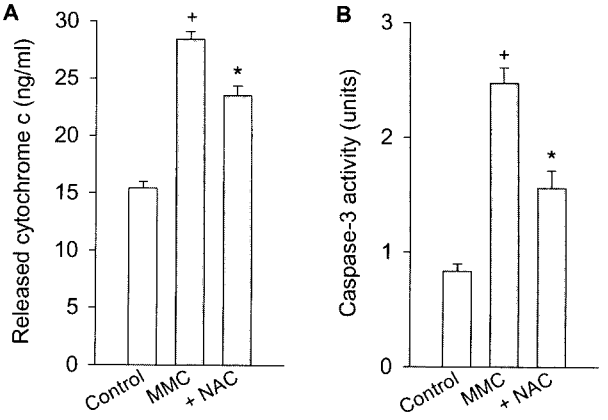

Fig. 3. MMC-induced release of cytochrome c and activation of caspase-3. SCLC cells were treated with 15 μg/mL MMC in the presence of 1 mM N-acetylcysteine (NAC) for 24 h at 37°C. In the assay of cytochrome c (A), SCLC cells (5×10^5) were treated with MMC, and in the assay of caspase-3 activity (B), 2×10^6 cells were treated with MMC. Data represent the means ± SEM (n=6). $^+P < 0.05$, significantly different from control; $^*P < 0.05$, significantly different from MMC alone.

release of cytochrome c from mitochondria into the cytosol, leading to the activation of caspases (Mignotte and Vayssière, 1998). The MMC-induced cell death was assessed by measuring a release of cytochrome c into the cytosol and subsequent activation of caspase-3. Treatment of SCLC cells with 15 μg/mL MMC for 24 h showed a significant increase in cytochrome c release (Fig. 3A). One mM N-acetylcysteine significantly attenuated the MMC-induced release of cytochrome c.

Control SCLC cells had caspase-3 activity of 0.834 ± 0.064 U in 2×10^6 cells. The activity of caspase-3 in SCLC cells treated with 15 μg/mL MMC for 24 h increased to 2.473 U/2×10^6 cells. Treatment with 1 mM N-acetylcysteine significantly inhibited the caspase-3 activation due to MMC (Fig. 3B).

Effect of depletion or oxidation of GSH on cytotoxicity of MMC

We investigated the effect of the GSH depletion on the cytotoxicity of MMC. SCLC cells were treated with 500 μM L-buthionine sulfoximine, a specific inhibitor of γ-glutamylcysteine synthetase (the rate-limiting step in glutathione biosynthesis), for 24 h, and then cells were treated with 15 μg/mL MMC for 6 h. L-buthionine sulfoximine for a 30 h-treatment decreased the GSH contents in SCLC cells by 57% (Fig. 4A). However, this treatment did not significantly induce formation of ROS and cell death (Fig. 4B, C). In this experimental condition, the formation of ROS due to MMC in the presence of L-buthionine sulfoximine was slightly greater than that of MMC alone, but which had not statistical difference. Treatment with L-buthionine sulfoximine significantly enhanced the MMC-induced cell death (Fig. 4C). The results suggest that the depletion of GSH enhances the toxic effect of MMC against SCLC cells.

The present study examined the effect of the GSH oxidation on the cytotoxicity of MMC. SCLC cells were treated with MMC in the presence of diamide (50-200 μM), a thiol oxidant. Diamide increased the formation of GSSG for a 6 h-treatment in a dose-dependent manner, and at 200 μM it caused a significant decrease in the GSH contents (Fig. 5A, B). However, the oxidation and depletion of GSH due to MMC in the presence of diamide was less than the sum of the effect of MMC and diamide. In this experimental condition, diamide at 100-200 μM caused the casapse-3 activation and cell death in SCLC cells, and cell death due to MMC in the presence of diamide was greater than the effect of MMC alone (Fig. 5C, D). However, the cytotoxic effect of MMC in the presence of diamide was less than the sum of the each effect of MMC and diamide. The thiol oxidant NH_2Cl caused the formation of GSSG in SCLC cells in a dose-dependent manner, but the oxidation of GSH due to MMC in the presence of NH_2Cl was less than the sum of the effect of MMC and NH_2Cl (Fig. 6A). Unlike diamide, cell death due to MMC plus NH_2Cl was not significantly different

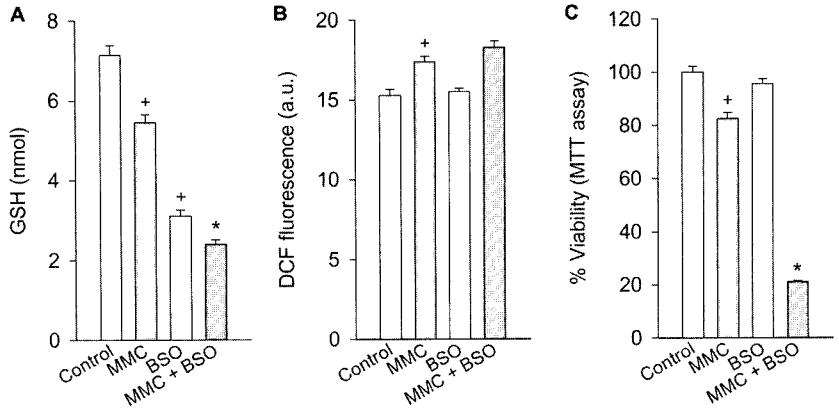

Fig. 4. Increase in cytotoxicity of MMC by GSH depletion. SCLC cells (4×10^4) were treated with 500 μM L-buthionine sulfoximine (BSO) for 24 h at 37°C, and then in this mixture, cells treated with 15 μg/mL MMC for 6 h at 37°C. The values are expressed as the nmol in total GSH contents (A), arbitrary units of fluorescence in ROS formation (B) and percentage of cell viability (C). Data represent means ± SEM (n=6). ^+P <0.05, significantly different from control; $^*P$ < 0.05, significantly different from MMC alone.

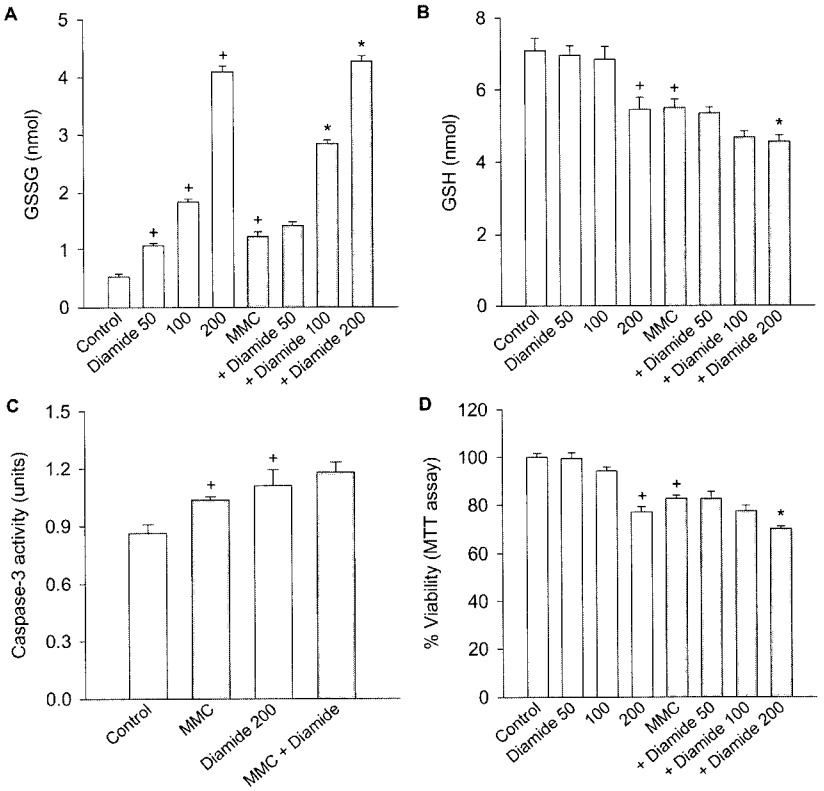

Fig. 5. Effect of thiol oxidant diamide on cytotoxicity of MMC. SCLC cells (4×10^4) were treated with 15 μg/mL MMC in the presence of diamide (50-200 μM) for 6 h at 37°C. The values are expressed as the nmol in GSSG (A), total GSH contents (B) and units in caspase-3 activity (C) and percentage of cell viability (D). Data represent the means ± SEM (n=6). ^+P <0.05, significantly different from control; $^*P$ < 0.05, significantly different from MMC alone.

from the effect of MMC alone (Fig. 6B). The results suggest that the oxidation of GSH may not affect the toxic effect of MMC against SCLC cells.

DISCUSSION

Apoptotic cell death is suggested to be mediated by interaction of ligand with cell surface CD95 receptor, leading to the

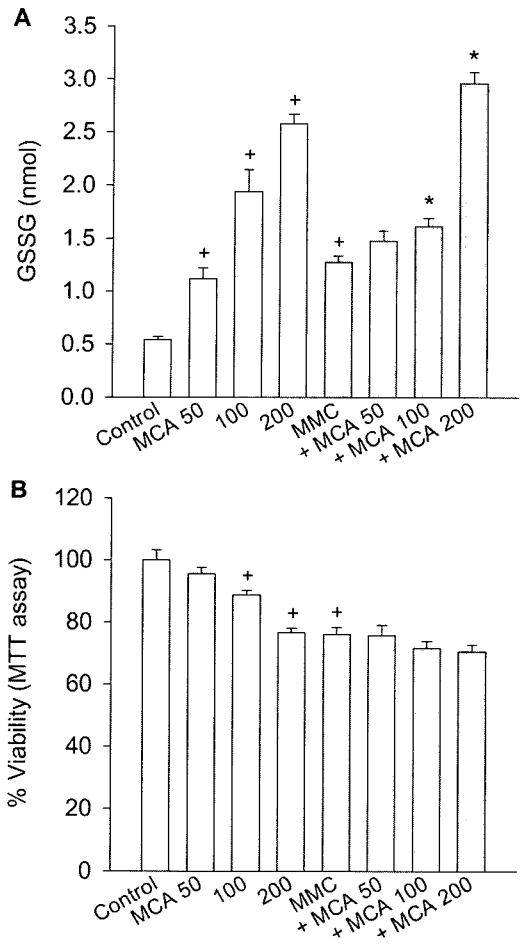

Fig. 6. Effect of thiol oxidant NH_2Cl on cytotoxicity of MMC. SCLC cells (4×10^4) were treated with NH_2Cl (MCA, 50-200 μM) for 6 h at 37°C. The values are expressed as nmol in GSSG (A) and percentage of cell viability (B). Data represent the means ± SEM (n=6). ^+P <0.05, significantly different from control; $^*P$ < 0.05, significantly different from MMC alone.

activation of caspase-8, and by mitochondrial dysfunction, results in the release of cytochrome c and subsequent activation of caspase-9 and -3 (Mignotte and Vayssière, 1998; Chandra *et al.*, 2000). MMC causes disruption of the mitochondrial transmembrane potential in normal human lymphocytes AHH-1, leading to activation of caspase-3 (Guilouf *et al.*, 1999). In contrast to this report, the cytotoxic effect of MMC on human breast cancer MCF-7 cell line is not mediated by the activation of caspase-3 (Pirnia *et al.*, 2002). MMC can induce caspase-8 activation and apoptosis in the absence of CD95 receptor interaction (Wesselborg *et al.*, 1999). The mechanism by which MMC causes cell death is therefore uncertain. The present study investigated the cytotoxicity of MMC in relation to mitochondrial membrane permeability. A significant cytotoxic effect of MMC on SCLC cells was demonstrated by monitoring cell viability and by measuring DNA fragments as nuclear damage. In the present study, the inhibitory effect of by specific caspase inhibitors (z-LEHD.fmk and z-DQMD.fmk) suggests that MMC-induced apoptotic cell death and depletion of GSH in SCLC cells is mediated by the activation of caspase-9 and -3.

Loss of the mitochondrial membrane potential causes the release of cytochrome c from mitochondria to the cytosol, followed by the activation of caspase-3 that is involved in apoptotic cell death (Mignotte and Vayssière, 1998). The condensation and fragmentation of nuclei (Fig. 2) and a significant increase in caspase-3 activity (Fig. 3) were evidence for apoptotic death following exposure to MMC in SCLC cells. It has been suggested that anticancer drugs cause cell injury by altering the mitochondrial membrane permeability (Amarante-Mendes *et al.*, 1998; Guilouf *et al.*, 1999). However, because some anticancer drugs cause apoptosis without the cytosolic accumulation of cytochrome c, the role of cytochrome c in anticancer drug-induced cell death has not been clearly elucidated (Tang *et al.*, 1998). One of the aims of this study was therefore to clarify whether the cytotoxic effect of MMC is mediated by the mitochondrial membrane permeability change and subsequent release of cytochrome c. The present results suggest that mitochondrial damage due to MMC causes the release of cytochrome c into the cytosol, leading to activation of caspase-9 and -3. This process may elicit apoptotic cell death in SCLC cells. The ROS formation and GSH depletion due to anticancer drugs may cause mitochondrial dysfunction and subsequent cytochrome c release, which leads to cell viability loss (Troyano *et al.*, 2001; Xu and Ashraf, 2002; Hong *et al.*, 2003). The inhibitory effect of N-acetylcysteine on the toxicity of MMC and the formation of ROS suggest that the MMC-induced cytochrome c release and caspase-3 activation, which lead to cell death in SCLC cells, may be mediated by the formation of free radicals and the GSH depletion.

The oxidation of both GSH and NAD(P)H of mitochondria due to oxidative stress has been suggested to induce the mitochondrial membrane permeability transition (Constantini *et al.*, 1996). Drops in GSH levels and concomitant increase in ROS are found during the glutamate-induced apoptotic process in the immortalized hippocampal cell line, HT22 cell (Tan *et al.*, 1998). Anti-cancer drugs have been suggested to reveal the cytotoxic effect through reduction of the intracellular GSH levels (Troyano *et al.*, 2001). Nevertheless, the depletion of GSH due to L-buthionine sulfoximine increases or does not affect the toxicity of anticancer drugs, such as etoposide and doxorubicin

(Gantchev and Hunting, 1997; Troyano *et al.*, 2001). Preincubation with L-buthionine sulfoximine does not induces significantly production of ROS and cell death in intact cells (Chandra *et al.*, 2000; Troyano *et al.*, 2001). MMC caused a formation of ROS and the decrease in the GSH contents in SCLC cells. Because GSH is the main antioxidant system in cells, the drops in GSH levels is suggested to increase the sensitivity of cells to damaging effect of toxic substances (Reed, 1990). N-acetylcysteine significantly inhibited MMC-induced mitochondrial damage and cell death. The GSH depletion due to L-buthionine sulfoximine markedly enhanced MMC-induced cell death. These results suggest that the toxicity of MMC on SCLC cells is significantly enhanced by the depletion of intracellular GSH level due to formation of ROS. However, in contrast to the effect of the GSH depletion, the thiol oxidation due to diamide or NH_2Cl did not affect the cytotoxicity of MMC (ROS formation, caspase-3 activation and cell death) on SCLC cells. The results suggest that the oxidation of intracellular GSH does not affect the toxicity of MMC on SCLC cells.

In conclusion, the results show that MMC may cause cell death in SCLC cells by inducing the mitochondrial damage, leading to caspase-3 activation. The toxicity of MMC on SCLC cells appears to be prevented by the recovery of GSH contents and be enhanced by the depletion of intracellular GSH. In contrast, the oxidation of cellular GSH not appears to affect the toxicity of MMC.

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