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ORIGINAL ARTICLE

Toxicity against gastric cancer cells by combined treatment with 5-fluorouracil and mitomycin *c*: implication in oxidative stress

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Abstract
Purpose Combined chemotherapy of 5-fluorouracil (5FU) and mitomycin *c* (MMC) is clinically used for gastric cancer, but the precise conditions and molecular mechanism of these agents when used together remain unclear. We examined the administration sequence of combining 5FU with MMC to maximize toxicity against a human gastric cancer cell line, and then investigated the possible molecular mechanisms underlying the observed toxic effects.
Methods Human gastric cancer MKN45 cells were treated with a combination of 5FU and MMC, and the changes in cell viability and apoptosis-related proteins were determined by a tetrazolium dye-based cytotoxicity assay and Western blot analysis, respectively. The intracellular levels of reactive oxygen species (ROS) were monitored using a fluorescent probe or by a cytochrome *c* reduction assay.
Results Pretreatment for 24 h with 5FU augmented the toxic effect of MMC in MKN45 cells. The synergic effect was mediated mainly via ROS formation and the p53-dependent apoptotic pathway, leading to mitochondrial dysfunction and caspase activation. In vitro experiments using extracts of the treated cells showed superoxide anion generation in a redox cycle of MMC, involving alterations in superoxide dismutase.
Conclusions Pretreatment with 5FU enhanced the MMC-induced toxicity against gastric cancer cells *via* alterations in antioxidant enzymes with resulting ROS generation. This observation will need confirmation in the clinical setting.

Keywords Gastric cancer · 5-Fluorouracil · Mitomycin *c* · Reactive oxygen species · Superoxide dismutase

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Introduction

5-Fluorouracil (5FU) has been used for decades as a treatment for patients with advanced and metastatic cancers of the stomach and colon. Because response to 5FU was not always satisfactory, trials to improve response rates led to the use of combination therapy with two or three different drugs. One combination, 5FU and MMC, has been used widely for the therapy of advanced gastric cancer [1, 2]. However, there are limited data regarding the precise protocol that might offer the best therapeutic effect. One study used MMC on day 1 with subsequent addition of 5FU [3], while another pretreated with 5FU [4]. However, neither combination was compared to each other nor to other drug schedules. The mode of action of both drugs differs, suggesting that better understanding of the combination might lead to a more correct use of the drugs.

The basis of the therapeutic effect of 5FU depends upon metabolic conversion into three active metabolites. One of the metabolites, fluorodeoxyuridine monophosphate,

blocks DNA synthesis by inactivating thymidylate synthase, leading to a reduced concentration of thymidylate and DNA degradation [5, 6]. The other two metabolites, fluorouridine triphosphate and fluorodeoxyuridine triphosphate, result in an inhibition of RNA maturation and an induction of single-strand breaks in DNA, respectively [7, 8]. MMC has two major modes of action. First, it inhibits DNA topoisomerase-II. Second, this 1,4-quinone forms a semiquinone radical that can transfer an electron to oxygen with generation of a superoxide radical. This reaction is catalyzed by flavoenzymes such as NADPH-cytochrome P-450 reductase [9, 10]. Both the semiquinone or superoxide radicals can generate hydroxyl radicals that can covalently bind to DNA and cause strand breaks, which inhibits DNA replication and repair [11, 12]. In addition to the crucial toxic effect of DNA intercalation, under aerobic conditions reactive oxygen species (ROS) generated in the redox cycle of MMC are also thought to participate in MMC-induced cellular toxicity [9]. This effect has been demonstrated in cultured cells exposed to the anthracycline antibiotics, adriamycin [13] and idarubicin [14]. In experiments using tumor cells, it has been suggested that ROS-dependent apoptotic signaling including mitochondrial dysfunction and caspase activation is involved in toxic events induced by MMC [15, 16].

Addition of 5FU to gastric cancer cells treated with a platinum-based drug, cisplatin, was effective in controlling cell growth [17]. Moreover, apoptosis induced by 5FU were associated with p53 gene status in gastric cancer cell lines [18]. Although the potency of a variety of combinations has so far been examined by numerous investigators both clinically and in cell cultures, the molecular mechanism(s) of the combined effects of these drugs on toxicity against gastric cancer cells still remains unclear. In this paper, we have therefore focused on the combined use of 5FU and MMC against gastric cancer MKN45 cells, and investigated the addition sequence of the two drugs, and the mechanism of the toxic effect with special attention to alterations in apoptotic-related factors, changes in antioxidant enzymes, and ROS production as factors of potential importance.

Materials and methods

Materials

5FU and MMC were kindly supplied by Kyowa Hakko Chemical. 2-(4-Iodophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium mono sodium salt (WST-1) was purchased from Wako Pure Chemical Industries; 6-carboxy-2′, 7′-dichlorodihydrofluorescein diacetate di(acetoxymethyl-ester) (DCFH) was from molecular probes; Cu, Zn-superoxide dismutase (SOD) was from UBE industrials; and acetyl Asp-Glu-Val-Asp *p*-nitroanilide and acetyl Leu-Glu-His-Asp *p*-nitroanilide were from Sigma-Aldrich. All other chemicals were of the highest grade that could be obtained commercially.

Cell culture and treatment

Human gastric cancer cell line MKN45 and MKN28 (Health Science Research Resources Bank) were cultured in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and antibiotics in a 5% CO_2 incubator. The cells were seeded at densities of 2×10^5 cells/dish and 1×10^4 cells/well into a 60-mm dish and a 96-well microplate, respectively. After reaching 70–80% confluence of the cells, the medium was replaced with DMEM containing 2% FBS 24 h before treatment of drugs. The cells were treated concomitantly with 5FU and MMC (FM treatment), or with MMC after 24-h pretreatment with 5FU ($F_{24}M$ treatment).

Cell viability was measured by the cytotoxicity assay, based on the activity of mitochondrial dehydrogenase reducing 3-(4,5-dimethyl-2-thizalyl)-2,5-diphenyl-2H tetrazolium bromide (MTT) to a purple insoluble formazan compound, but using WST-1 as the chromogen [19].

Western blot analysis

For the analysis of cytochrome *c* release from the mitochondria, the cells were washed twice with Dulbecco's phosphate-buffered saline (DPBS) supplemented with 40 μg/ml saponin, and homogenized by passing the suspension through a 26 gauge needle (20 strokes). The cytosolic fraction of the homogenate was obtained by centrifugation at 12,000×*g* for 15 min. In the analyses of other proteins, the cells washed with DPBS were suspended before sonication in DPBS containing 0.5% Triton X-100 and 0.3 mM phenylmethanesulfonyl fluoride. The cell extracts were isolated by centrifuging at 12,000×*g* for 15 min, and protein concentration in the extracts was determined with a bicinchoninic acid Protein Assay System (Pierce). The proteins in the cytosolic fractions and extracts of the cells were electrophoretically separated on a 7.5 or 12% SDS-polyacrylamide gel under reducing conditions, and then transferred to a PVDF membrane (Millipore) by electroblotting. After blocking with 5% skim milk, the membrane was allowed to react with primary monoclonal antibodies against human Bcl-2 (BD Transduction Laboratories), Bcl-XL (BD Transduction Laboratories), Bax (Santa Cruz Biotechnology), cytochrome *c* (Trevigen), CuZnSOD (Binding Site) and MnSOD (Binding Site). The immunoreactive proteins were detected by being reacted with peroxidase-conjugated secondary antibody (Bio-rad), in which the peroxidase activity was visualized by means of

an enhanced chemiluminescence substrate system (Amersham Biosciences).

Assay of enzyme activity

Cells were washed twice with DPBS, suspended in 10 mM Tris–HCl, pH 7.4, containing 5 mM 2-mercaptoethanol and 20% glycerol, and then homogenized by passing the suspension through a 26 gauge needle. The homogenate was centrifuged at 12,000×*g* for 15 min, and MMC reductase activity in the supernatant was determined by monitoring oxidation rate of NADPH at 340 nm. The assay mixture consisted of 0.1 M potassium phosphate, pH 7.4, 0.1 mM NADPH, 9 μM MMC and the cell extract, in a total volume of 2.0 ml. The rate was corrected for the nonenzymatic NADPH oxidation by MMC in the mixture without the extract. One unit (U) of enzyme activity was defined as the amount of enzyme that catalyzes the formation or oxidation of 1 μmol NADPH per minute at 25°C.

For assay of caspase activity, the cell extracts were prepared as described above, except that 50 mM HEPES–NaOH, pH 7.4, containing 5 mM 3-[(3-cholamidopropyl) dimethylammonio]-propanesulfonic acid and 5 mM dithiothreitol was used as the homogenized solution. The activities of caspase-3 and caspase-9 were spectrophotometrically measured at 415 nm using acetyl Asp-Glu-Val-Asp *p*-nitroanilide and acetyl Leu-Glu-His-Asp *p*-nitroanilide, respectively, as the substrates [20].

Superoxide dismutase activity in the cell extract was determined using a SOD Activity Detection Kit (Wako Pure Chemical) according to the manufacturer's instruction.

Measurement of ROS

Level of intracellular hydrogen peroxide was estimated by DCFH oxidation as previously reported [21]. Following washes with DPBS, the cells were incubated for 20 min in fresh medium containing 10 μM DCFH, and again sufficiently washed with DPBS. DCF fluorescence-positive cells were counted using a FACScan equipped with a fluorescein isothiocyanate filter and Cell Quest Pro software (Becton-Dickinson).

Superoxide anions generated by reduction of MMC in the cell extract were determined by the method of McCord and Fridovich [22]. The assay mixture consisted of 0.1 M potassium phosphate buffer, pH 7.4, 0.5 mM NADPH, 0.1 mM EDTA, 50 μM ferricytochrome *c*, 9 μM MMC and the cell extract, in a total volume of 2.0 ml. The formation rate of ferrocytochrome *c* at 37°C was spectrophotometrically monitored at 550 nm.

Measurement of 8-hydroxy-deoxyguanine

8-Hydroxy-deoxyguanine (8-OH-dG) concentration in the cell extracts was determined using a 8-OH-dG enzyme-linked immunosorbent assay kit according to the manufacturer's instructions (Japan Institute for the Control and Aging).

Measurement of intracellular reduced glutathione

Glutathione (GSH) concentration was measured by HPLC as its *o*-phthalaldehyde adduct [23]. Cells were washed twice with DPBS and lysed by sonication for 1 min.

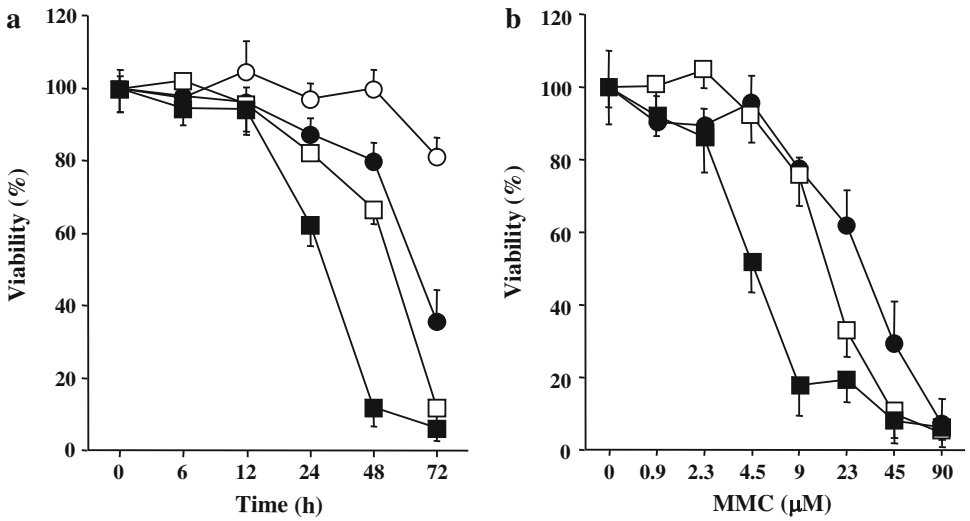

Fig. 1 Viability of MKN45 cells in response to drug treatment. **a** Time-course experiment. Cells were treated for the indicated times with 5FU (23 μM) alone (*open circle*), MMC (9 μM) alone (*filled circle*) or FM (*open square*). In the $F_{24}M$ treatment, the cells were pretreated for 24 h with 5FU prior to treating with MMC (*filled square*). The values are normalized to those in cells treated with dimethylsulfoxide alone. **b** Dose–response. The cells were treated with MMC alone (*filled circle*), FM (*open square*) and $F_{24}M$ (*filled square*), and the treatments were terminated 48 h after adding the indicated concentrations of MMC to the medium

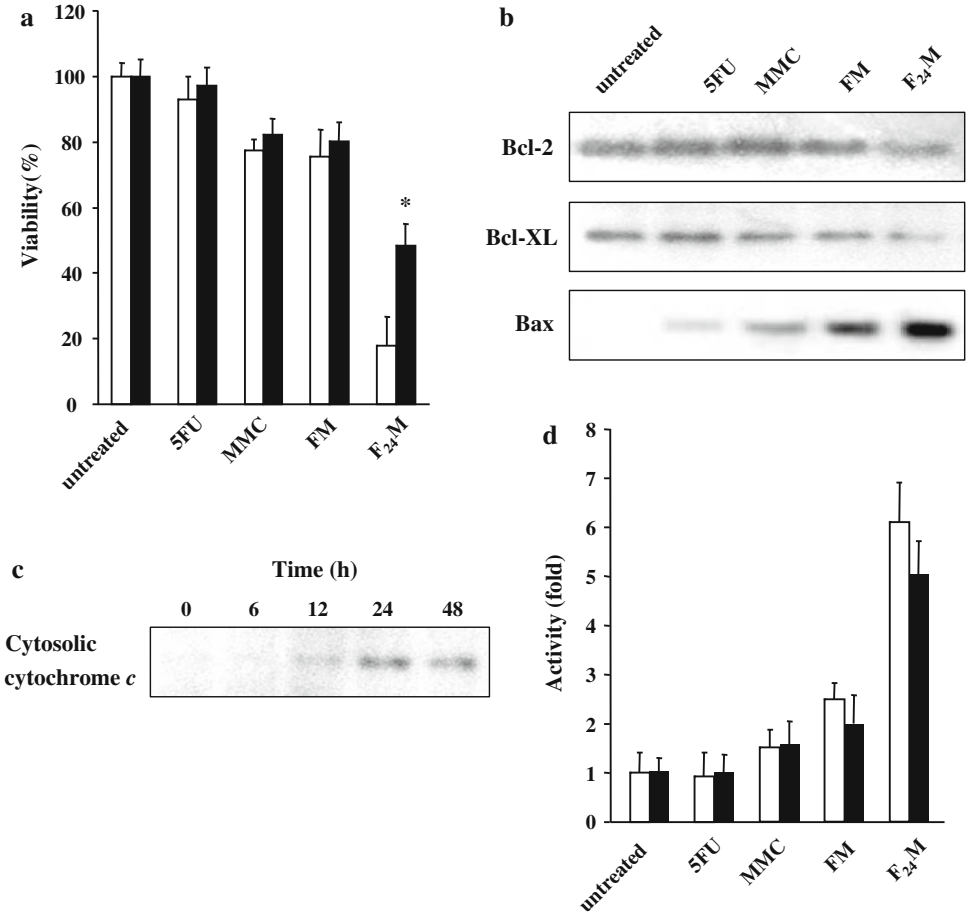

Fig. 2 Apoptotic signaling in MKN45 cells induced by $F_{24}M$ treatment. **a** Comparison of toxicities of 5FU and MMC between MKN45 (*open bar*) and MKN28 cells (*filled bar*). The cells were treated with 5FU, MMC alone, FM, or $F_{24}M$, and the treatments were terminated 48 h after initiation of MMC (9 μM). The values are normalized to that in cells treated with dimethylsulfoxide. *Difference from the $F_{24}M$-treated MKN45 cells, $p < 0.05$. **b** Alterations in Bcl-2, Bcl-XL and Bax. The cells were treated as described in **a**, except that the incubation with MMC was terminated at 24 h. Cell extracts (20 μg of protein) were analyzed by Western blots probed with antibodies against Bcl-2, Bcl-XL and Bax. **c** Release of cytochrome *c* into cytosol after $F_{24}M$ treatment of MKN45 cells. The cells were pretreated for 24 h with 5FU and then treated for 0, 6, 12, 24 and 48 h with MMC (9 μM). The cytosolic fraction (40 μg of protein) was analyzed by Western blotting. **d** Activation of caspases. Cells were treated as noted in **a**, and caspase-3 (*open bar*) and caspase-9 (*filled bar*) activities were measured. Each activity is expressed as the fold increase in the ratio of the activity in the treated group compared with that in the untreated group

Following centrifugation at 12,000×*g* for 15 min, GSH in the supernatant was derivatized by incubating for 30 min with *o*-phthalaldehyde, and an aliquote of the derivatized preparation was subjected to HPLC using a C_{18} column, which was eluted isocratically with a mobile phase consisting of 0.15 M sodium acetate/methanol (91.5:8.5) at a flow rate of 0.5 ml/min. The *o*-phthalaldehyde-GSH adduct was monitored using a fluorescence detector operating at excitation and emission wavelengths of 250 and 410 nm, respectively.

Statistical analysis

Data are expressed as the means ± SD of at least three independent experiments. Statistical evaluation of the data was performed by using the unpaired Student's *t* test and ANOVA followed by Fisher's test. A p value <0.05 was considered statistically significant.

Results

Pretreatment with 5FU potentiates MMC-induced toxicity against gastric cancer MKN45 cells

Treatment with 9 μM MMC decreased viability of MKN45 cells, reaching 34% by 72 h, although the viability was less affected by treating with 23 μM 5FU (Fig. 1a). Concomitant treatment with 5FU (*FM treatment*) slightly strengthened the MMC-induced cytotoxicity. In contrast, pretreatment of the cells for 24 h with the same concentration of 5FU (*$F_{24}M$ treatment*) drastically increased the

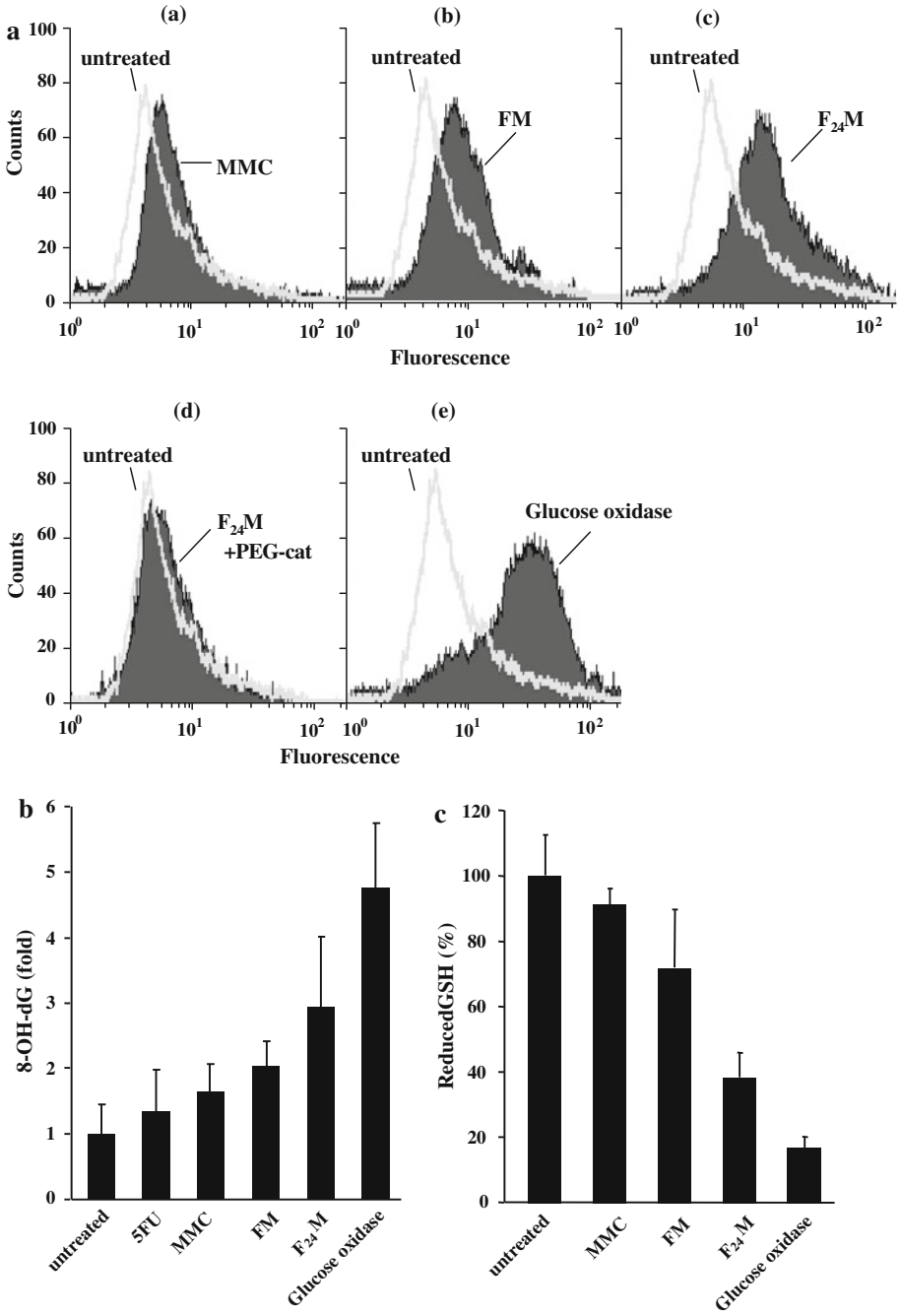

Fig. 3 Intracellular ROS generation enhanced by $F_{24}M$ treatment. **a** DCFH oxidation. MKN45 cells were treated with MMC (*a*), FM (*b*), or $F_{24}M$ (*c*), and the treatments were terminated 24 h after initiation of MMC (9 μM). PEG-cat (200 U/ml) was added to the culture media of (*c*) 2 h before treating with MMC (*d*). The cells were also treated for 4 h with glucose oxidase (20 mU/ml) in the presence of glucose (20 mM) (*e*). **b** 8-OH-dG formation. The cells were treated as noted in **a**, and in the 5FU group the cells were treated for 72 h with 5FU (23 μM). The values are expressed as the fold increase in the ratio of 8-OH-dG concentration in the treated cells compared with that in the untreated cells. **c** Intracellular GSH level. The cells were treated as noted in **a**, and GSH level was measured by HPLC analysis. The values are normalized to that in untreated cells and expressed as the percentage of reduced GSH level in the treated cells to that in the untreated cells

susceptibility to MMC. As a dose–response experiment of MMC in the $F_{24}M$ treatment is shown in Fig. 1b, the marked reduction in the viability was observed when the cells were treated for 48 h with higher concentrations (≥9 μM) of MMC. The potentiating effect of the 5FU pretreatment for 24 h on the MMC-induced cytotoxicity was almost the same as that of the longer time (≥48 h) pretreatment, and was stronger than that of the MMC pretreatment on the 5FU-induced toxicity (data not shown). Therefore, the 24-h pretreatment with 5FU was adopted in all subsequent experiments.

Activation of p53-dependent apoptotic mechanisms in the $F_{24}M$-treated cells

To examine the involvement of p53 in the cytotoxicity induced by MMC and 5FU, we compared difference in susceptibility to the two drugs and their combined treatments (FM and $F_{24}M$) between poorly differentiated MKN45 and highly differentiated MKN28 cells that bear wild-type p53 and its mutated form, respectively (Fig. 2a). No significant difference in the viability between the two cells was observed in the treatments of single drug (5FU or MMC)

and FM, whereas in the $F_{24}M$ treatment the viability of MKN45 cells was significantly low compared to that of MKN28 cells. Levels of Bcl-2 and Bcl-XL were low and that of Bax was greatly increased in the $F_{24}M$-treated cells, compared to the cells treated with the single drug and FM (Fig. 2b). In addition, the release of cytochrome *c* into the cytosol of the $F_{24}M$ treated cells was observed at 12 h after the incubation with MMC (Fig. 2c). Marginal activations in caspase-3 and caspase-9 were furthermore induced only by the $F_{24}M$ treatment (Fig. 2d). It should be noted that release of cytochrome *c* and activation of caspases observed during treatment of MMC alone or FM were much less than those during the $F_{24}M$ treatment.

Pretreatment with 5FU increases ROS production and GSH depletion by MMC

The DCFH oxidation assay was used to estimate amounts of intracellular H_2O_2-derived oxidants. The DCF-derived fluorescence was slightly enhanced in the cells treated for 24 h either with MMC alone or FM (Fig. 3a, *panels a and b*). In contrast, 5FU pretreatment greatly augmented the ratio of DCF-positive/total cells following addition of MMC (*panel c*). The ratio of DCF-positive/total cells was normalized by addition of polyethyleneglycol-conjugated catalase (PEG-cat) (*panel d*), confirming that the DCF oxidation reflected H_2O_2 production under the experimental conditions. The ratio was not so strong as that observed in cells treated with glucose oxidase, which causes continuous H_2O_2 production during the oxidation of glucose (*panel e*). In addition, $F_{24}M$ treatment of MKN45 elicited an increase in 8-OH-dG level (Fig. 3b) and a decrease in reduced GSH (Fig. 3c) in the cells compared to treatment of MMC alone. These results demonstrated that ROS-dependent apoptotic signaling is highly activated by 5FU pretreatment and participates in the MMC-induced toxicity against MKN45 cells. Pretreatment with ROS inhibitors, cell-membrane permeable GSH ethyl ester (GSH ester) and PEG-cat, almost completely suppressed endogenous cytochrome *c* release in the $F_{24}M$-treated cells (Fig. 4a), and improved cell viability resulted from the 5FU pretreatment (Fig. 4b).

Mechanism of superoxide anion generation by $F_{24}M$ treatment

The NADPH-linked reductase activity of MMC was detected in the extracts of MKN45 cells, and was not significantly changed by MMC and $F_{24}M$ treatments (Fig. 5a). When the reductive rates of cytochrome *c* coupled with the in vitro reduction of MMC by the cell extracts were compared, the $F_{24}M$-treated cells showed higher reduction rate than the cells treated with MMC alone (Fig. 5b, *curve a*) and without drugs (data not shown). The $F_{24}M$ treatment

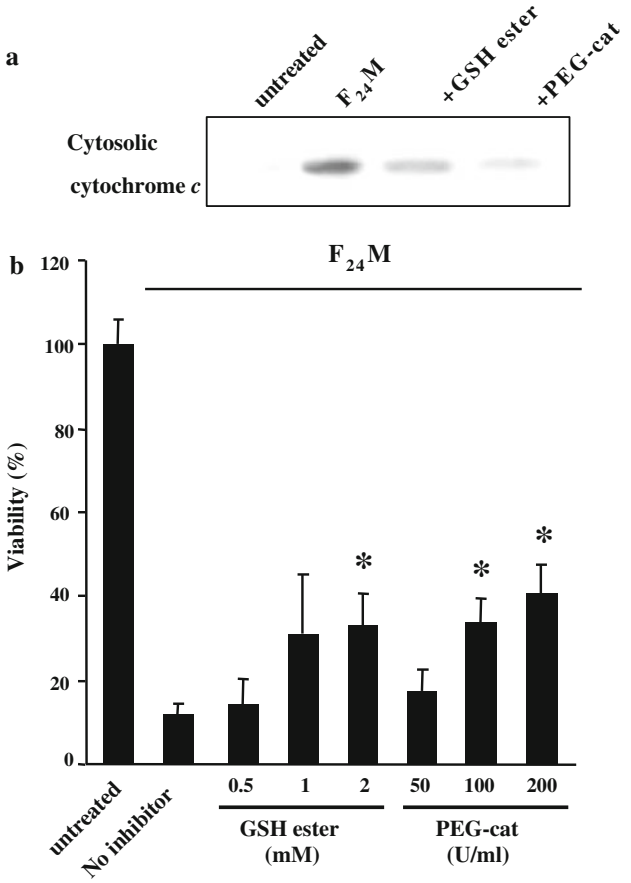

Fig. 4 Effect of ROS inhibitors on cytochrome *c* release and cytotoxicity induced by $F_{24}M$ treatment. **a** Cytochrome *c* release. Cells were pretreated for 24 h with 5FU and then treated for 24 h with MMC (9 μM). GSH ester (2 mM) or PEG-cat (200 U/ml) was added to the culture medium 2 h before initiation of MMC treatment. Cytosolic cytochrome c was assayed as in Fig. 2. **b** Cell viability. The cells were pretreated for 24 h with 5FU and then treated for 48 h with MMC (9 μM). The indicated concentrations of GSH ester or PEG-cat were added to the culture medium 2 h before initiation of MMC treatment. *Difference from the $F_{24}M$ group (*no inhibitor*), $p < 0.05$

resulted in a noticeable decrease in SOD activity, whereas such decrease was not detected in the cells treated with 5FU or MMC alone (Fig. 6a). In the $F_{24}M$-treated cells, the protein levels of CuZnSOD and MnSOD were decreased by 36 and 67%, respectively, of the basal level at the time point of 48 h after the addition of MMC, as estimated on densitometric analysis of the Western blotting results (Fig. 6b, c). The loss of SOD activity was partially reversed by ROS inhibitors (Fig. 6d).

Discussion

In this study, we address the optimal conditions to maximize toxicity against gastric cancer in combination of 5FU with MMC, and have found that the pretreatment for 24 h

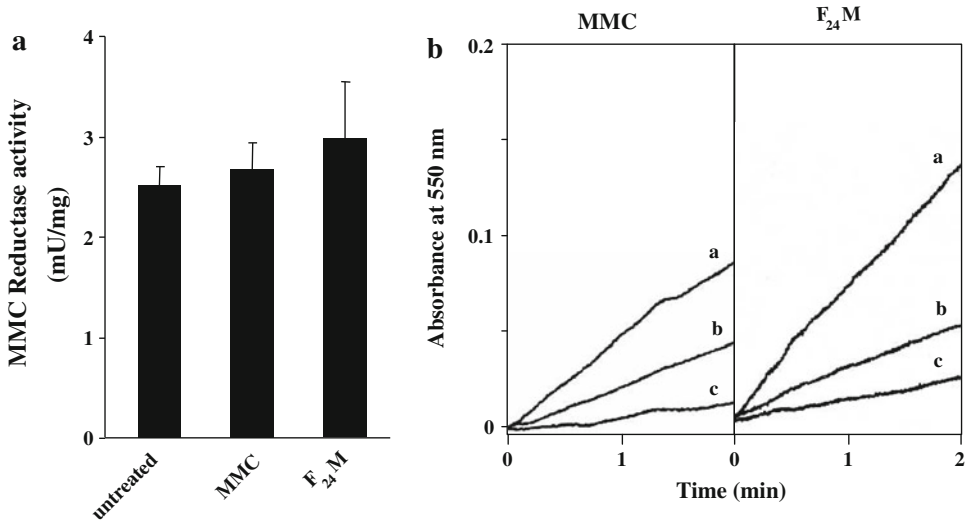

Fig. 5 MMC reduction in MKN45 cells. **a** MMC reductase activity. The cells were pretreated for 24 h without (*MMC*) or with 5FU ($F_{24}M$), and followed by treating for 24 h with MMC (9 μM). MMC reductase activity in cell extracts (100 μg) was spectrophotometrically determined by monitoring oxidation rate of NADPH. **b** Cytochrome *c* reduction coupled with MMC reduction. The extracts (100 μg) prepared in **a** were incubated with cytochrome *c* and 9 μM MMC in the absence (*curve a*) or presence (*curve b*) of 200 U CuZnSOD. The reduction rate of cytochrome *c* was spectrophotometrically monitored at 550 nm. No significant reduction of cytochrome *c* was observed without MMC (*curve c*)

with 5FU results in a great enhancement of the susceptibility of MKN45 cells to a range of MMC commonly used in clinical treatment (Fig. 1). Previous literature showed that MMC is known to upregulate thymidine phosphorylase, a tumoral enzyme that converts 5FU into its active form, fluorouridinemonophosphate [24]. It is therefore speculated that the MMC-induced metabolic activation of 5FU also plays an important role in the combined chemotherapy of 5FU plus MMC. The results using the MKN45 and MKN28 cells (Fig. 2a) suggest that the additive effect of 5FU and MMC is mainly mediated via p53-dependent apoptotic signaling. In the experiment, the demonstration that mutation of p53 is involved in chemoresistance of gastric cancer toward the combined treatment with 5FU and MMC is consistent with the report that combined treatment with 5FU and cisplatin induces apoptosis in MKN45 cell but not in MKN28 cells [17]. The up-regulation of Bax protein, which is transcriptionally regulated by p53 [25], increases mitochondrial permeability via an alteration of balance between Bax and Bcl-2 (or Bcl-XL), and releases mitochondria-derived proteins, such as cytochrome *c*, leading to caspase activation [26]. As expected, the $F_{24}M$ treatment can alter these proteins and caspases involved in the p53-dependent signaling pathway (Fig. 2b–d). A variety of apoptotic stimuli, such as ceramide [21], hydrogen peroxide [27] and other chemotherapeutic agents [28] produce similar changes in cultured cell lines. During 5FU treatment, p53 activates mitochondrial ferredoxin reductase [29], which sensitizes cells to oxidative stress-induced apoptosis [30]. Therefore, the activation of ferredoxin reductase might be responsible for the synergic effect of 5FU on the MMC-induced cell death.

Malignant cells are sensitive to oxidative stress injury by exogenous ROS inducers, although cancer cells also have counter-regulatory mechanisms to quench intrinsic oxidative stress [31, 32]. In this report, pretreatment with 5FU augmented the MMC-induced ROS production and the enhanced ROS triggered further oxidative changes in gastric cancer cells (Fig. 3a, b). Since p53 is believed to be up-regulated and activated by 8-OH-dG formation, an indicator of oxidative damage for DNA [33], ROS generated during the $F_{24}M$ treatment might contribute to the increased to the p53-dependent apoptosis. GSH plays an essential role in many detoxification processes, and accumulation of oxidants causes its depletion through excessive GSSG formation or efflux from cells, linking to apoptotic initiation [34]. In the $F_{24}M$-treated cells the intracellular level of GSH decreased proportionately with an increase in intracellular hydrogen peroxide (Fig. 3c). In addition, pretreatment with exogenous GSH ester and PEG-cat partially reversed the $F_{24}M$ treatment-induced cytochrome *c* release into the cytosol, the reduced cell viability, and the impaired SOD activities (Figs. 4, 6d). These results demonstrate that ROS-dependent pathways participate in the $F_{24}M$ treatment-induced cytotoxicity. However, the lack of complete reversal by anti-oxidant compounds could suggest that ROS-independent pathways also contribute to the toxic effects of the combined drugs.

Reactive oxygen radicals are chemically reactive and potentially toxic to cells when high levels accumulate, and

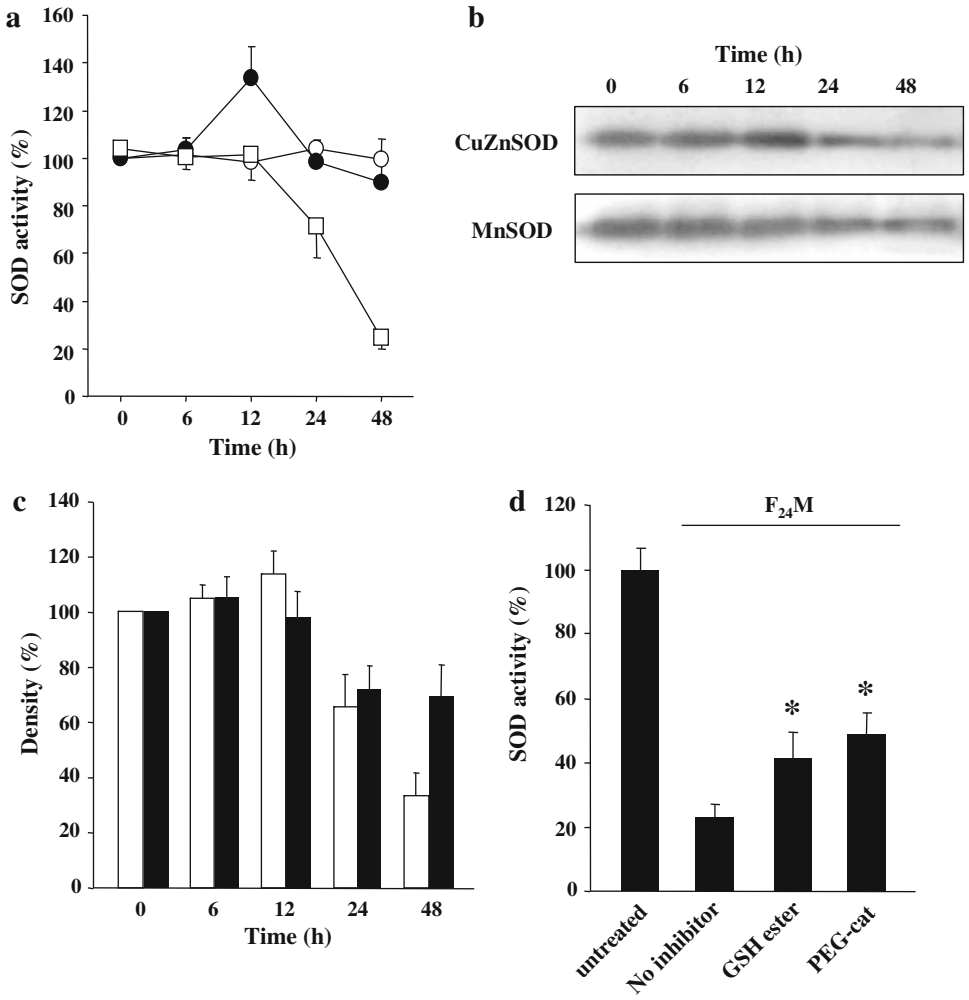

Fig. 6 $F_{24}M$ treatment attenuates SOD activity in MKN45 cells. **a** Time-course changes in SOD activity. The cells were treated with MMC (*filled circle*) or $F_{24}M$ (*open square*), and the treatments were terminated 24 h after initiation of MMC (9 μM). The cells were also treated for 72 h with 5FU (23 μM) alone (*open circle*). The values are normalized to that in cells before treatment and expressed as the percentage of the activity in the treated group to that in the untreated group. **b** Alterations in CuZnSOD and MnSOD. The proteins (20 μg) in the $F_{24}M$-treated cell cytosol prepared in **a** were analyzed by Western blotting. **c** Densitometric data of the bands of CuZnSOD (*open bar*) and MnSOD (*filled bar*) shown in **b**. The values are expressed as the percentage of the band density in the treated group to that in the untreated group. **d** Effect of ROS inhibitors on SOD activity. The cells were pretreated for 24 h with 5FU and then treated for 24 h with MMC (9 μM). GSH ester (2 mM) or PEG-cat (200 U/ml) was added to the culture medium 2 h before initiation of MMC treatment. The values are expressed as the percentage of the activity in the treated group to that in the untreated group. *Difference from the $F_{24}M$ group (*no inhibitor*), $p < 0.05$

need to be promptly eliminated from cells by a highly regulated antioxidant system that includes enzymes such as SOD, catalase, and GSH peroxidase. Our current study showed that the $F_{24}M$ treatment does not elicit any significant change in the MMC reductive activity in the cells (Fig. 5a). This result is not consistent with the hypothesis that accelerated turnover of free electrons in the MMC-redox cycle was driving the accumulation of ROS. Another explanation for the accumulation of ROS is that increased formation of intracellular ROS can reduce the levels of antioxidant proteins, such as SOD and GSH peroxidase [35]. In addition, inhibition of RNA synthesis by 5FU is theoretically thought to quench de novo syntheses of cellular proteins. It is therefore suggested that the marked reduction of SOD by the $F_{24}M$ treatment is mediated by both oxidant-induced degradation and inhibition of the de novo synthesis of the enzyme. The 5FU-induced inhibition of the de novo synthesis of SOD is supported by the data in Fig. 6a, in which the expression of SOD was transiently enhanced, possibly due to a cytoprotective effect, in the cells treated for 12 h with MMC, whereas such enhancement was not detected in the $F_{24}M$ treatment group. Thus, the data in the current study favor a decrease in anti-oxidant function (levels of GSH and CuZnSOD) as the major cause

of increased ROS accumulation, rather than an increase in ROS production per se.

Collectively, our study shows that in combined chemotherapy of 5FU and MMC, 24-h pretreatment with 5FU maximizes the MMC-induced toxicity against gastric cancer, and the mechanism includes ROS accumulation and the resultant reduction in anti-oxidant enzymes, especially CuZnSOD. By comparison to previous data [3, 36], it is needed to evaluate efficacy of our observations in the clinical setting.

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References

1. Rudi J, Werle S, Bergtholdt D et al (2005) Infusional 5-fluorouracil and mitomycin C: an effective regimen in the treatment of advanced gastric cancer. Onkologie 28:128–132
2. Hartmann JT, Pintoffl JP, Al-Batran SE et al (2007) Mitomycin C plus infusional 5-fluorouracil in platinum-refractory gastric adenocarcinoma: an extended multicenter phase II study. Onkologie 30:235–240
3. Kretzschmar A, Reichardt P, Thuss-Patience PC et al (2000) Weekly 24 hour infusion of high-dose 5-fluorouracil plus folinic acid in combination with mitomycin C for the treatment of advanced gastric cancer. Oncology 59:14–17
4. Hofheinz R, HartungG SamelS et al (2002) High-dose 5-fluorouracil/folinic acid in combination with three-weekly mitomycin C in the treatment of advanced gastric cancer. A phase-II study. Onkologie 25:255–260
5. Ren Q, Van Groeningen CJ, Hardcastle A et al (1997) Determinants of cytotoxicity with prolonged exposure to fluorouracil in human colon cancer cells. Oncol Res 9:77–88
6. Wadler S, Horowitz R, Mao X et al (1996) Effect of interferon on 5-fluorouracil-induced perturbations in pools of deoxynucleotide triphosphates and DNA strand breaks. Cancer Chemother Pharmacol 38:529–535
7. Ghoshal K, Jacob ST (1994) Specific inhibition of pre-ribosomal RNA processing in extracts from the lymphosarcoma cells treated with 5-fluorouracil. Cancer Res 54:632–636
8. Ciccolini J, Cuq P, Evrard A et al (2001) Combination of thymidine phosphorylase gene transfer and deoxyinosine treatment greatly enhances 5-fluorouracil antitumor activity in vitro and in vivo. Mol Cancer Ther 1:133–139
9. Keyes SR, Fracasso PM, Heimbrook DC et al (1984) Role of NADPH:cytochrome *c* reductase and DT-diaphorase in the biotransformation of mitomycin C1. Cancer Res 44:5638–5643
10. Verma RP (2006) Anti-cancer activities of 1, 4 naphthoquinones-a QSAR study. Anticancer Agents Med Chem 6:489–499
11. Iyer VN, Szybalski W (1963) A molecular mechanism of mitomycin action: linking of complementary and DNA strands. Proc Natl Acad Sci USA 50:355–362
12. Lee YJ, Park SJ, Ciccone SL et al (2006) An in vivo analysis of MMC-induced DNA damage and its repair. Carcinogenesis 27:446–453
13. Vásquez-Vivar J, Martasek P, Hogg N et al (1997) Endothelial nitric oxide synthase-dependent superoxide generation from adriamycin. Biochemistry 36:11293–11297
14. Celik H, Arinç E (2008) Bioreduction of idarubicin and formation of ROS responsible for DNA cleavage by NADPH-cytochrome P450 reductase and its potential role in the antitumor effect. J Pharm Pharm Sci 11:68–82
15. Kim R, Emi M, Matsuura K et al (2005) Therapeutic potential of antisense (AS) Bcl-2 as a chemosensitizer for patients with gastric and breast carcinoma. Gan To Kagaku Ryoho 32:1540–1545
16. Park IC, Park MJ, Hwang CS et al (2000) Mitomycin C induces apoptosis in a caspases-dependent and Fas/CD95-independent manner in human gastric adenocarcinoma cells. Cancer Lett 158:125–132
17. Matsuhashi N, Saio M, Matsuo A et al (2005) The evaluation of gastric cancer sensitivity to 5-FU/CDDP in terms of induction of apoptosis: time- and p53 expression-dependency of anti-cancer drugs. Oncol Rep 14:609–615
18. Osaki M, Tatebe S, Goto A et al (1997) 5-Fluorouracil (5-FU) induced apoptosis in gastric cancer cell lines: role of the p53 gene. Apoptosis 2:221–226
19. Usui S, Matsunaga T, Ukai S et al (1997) Growth suppressing activity for endothelial cells induced from macrophages by carboxymethylated curdlan. Biosci Biotechnol Biochem 61:1924–1925
20. Kotamraju S, Konorev EA, Joseph J et al (2000) Doxorubicin-induced apoptosis in endothelial cells and cardiomyocytes is ameliorated by nitrone spin traps and ebselen. Role of reactive oxygen and nitrogen species. J Biol Chem 275:33585–33592
21. Matsunaga T, Kotamraju S, Kalivendi SV et al (2004) Ceramide-induced intracellular oxidant formation, iron signaling, and apoptosis in endothelial cells: protective role of endogenous nitric oxide. J Biol Chem 279:28614–28624
22. McCord JM, Fridovich I (1969) Superoxide dismutase. An enzymic function for erythrocuprein (hemocuprein). J Biol Chem 244:6049–6055
23. Tietze F (1969) Enzymic method for quantitative determination of nanogram amounts of total and oxidized glutathione: applications to mammalian blood and other tissues. Anal Biochem 27:502–522
24. Ogata Y, Matono K, Sasatomi T et al (2006) Upregulation of thymidine phosphorylase in rectal cancer tissues by mitomycin C. J Surg Oncol 93:47–55
25. Miyashita T, Reed JC (1995) Tumor suppressor p53 is a direct transcriptional activator of the human bax gene. Cell 80:293–299
26. Narita M, Shimizu S, Ito T et al (1998) Bax interacts with the permeability transition pore to induce permeability transition and cytochrome *c* release in isolated mitochondria. Proc Natl Acad Sci USA 95:14681–14686
27. Dhanasekaran A, Kotamraju S, Kalivendi SV et al (2004) Supplementation of endothelial cells with mitochondria-targeted antioxidants inhibit peroxide-induced mitochondrial iron uptake, oxidative damage, and apoptosis. J Biol Chem 279:37575–37587
28. Kalivendi SV, Konorev EA, Cunningham S et al (2005) Doxorubicin activates nuclear factor of activated T-lymphocytes and Fas ligand transcription: role of mitochondrial reactive oxygen species and calcium. Biochem J 389:527–539
29. Hwang PM, Bunz F, Yu J et al (2001) Ferredoxin reductase affects p53-dependent, 5-fluorouracil-induced apoptosis in colorectal cancer cells. Nat Med 7:1111–1117
30. Liu G, Chen X (2002) The ferredoxin reductase gene is regulated by the p53 family and sensitizes cells to oxidative stress-induced apoptosis. Oncogene 21:7195–7204
31. Szatrowski TP, Nathan CF (1991) Production of large amounts of hydrogen peroxide by human tumor cells. Cancer Res 51:794–798
32. Toyokuni S (1998) Oxidative stress and cancer: the role of redox regulation. Biotherapy 11:147–154
33. Ernst P (1999) The role of inflammation in the pathogenesis of gastric cancer. Aliment Pharmacol Ther 13(Suppl 1):13–18
34. Tampo Y, Kotamraju S, Chitambar CR et al (2003) Oxidative stress-induced iron signaling is responsible for peroxide-dependent oxidation of dichlorodihydrofluorescein in endothelial

cells: role of transferrin receptor-dependent iron uptake in apoptosis. Circ Res 92:56–63
35. Fujii J, Taniguchi N (1999) Down regulation of superoxide dismutases and glutathione peroxidase by reactive oxygen and nitrogen species. Free Radic Res 31:301–308
36. Trumper M, Ross PJ, Cunningham D et al (2006) Efficacy and tolerability of chemotherapy in elderly patients with advanced oesophago-gastric cancer: a pooled analysis of three clinical trials. Eur J Cancer 42:827–834