Depletion of O^6-methylguanine-DNA methyltransferase by O^6-benzylguanine enhances 5-FU cytotoxicity in colon and oral cancer cell lines

JUN MURAKAMI[1], YOU-JIN LEE[2], SUSUMU KOKEGUCHI[3], HIDETSUGU TSUJIGIWA[4], JUN-ICHI ASAUMI[1], HITOSHI NAGATSUKA[4], KAZUHIRO FUKUI[3], MASAHIRO KURODA[5], NORIAKI TANAKA[6] and NAGAHIDE MATSUBARA[6]

[1]Department of Oral and Maxillofacial Radiology, Okayama University Graduate Schools of Medicine, Dentistry and Pharmaceutical Sciences, Okayama, Japan; [2]Department of Oral Histology-BK21, College of Dentistry, Chosun University, 375 Seosuk-dong, Dong-gu, Gwangju, Korea; [3]Departments of Oral Microbiology, [4]Oral Pathology, [5]Radiology, [6]Gastroenterological Surgery and Surgical Oncology, Okayama University Graduate Schools of Medicine, Dentistry and Pharmaceutical Sciences, Okayama, Japan

Received February 20, 2007; Accepted March 26, 2007

Abstract. O^6-methylguanine-DNA methyltransferase (MGMT) is a DNA repair enzyme whose expression is controlled by its promoter methylation. A cell that expresses a low amount of MGMT is known to be more sensitive to the antiproliferative effects of alkylating agents. We have previously shown that the colorectal cancer patients treated with 5-fluorouracil (5-FU) as adjuvant chemotherapy had a better prognosis when the tumor revealed hypermethylation in its *MGMT* promoter. Therefore, we sought to investigate the relationship between the expression levels of MGMT and the antitumor effect of 5-FU *in vitro* by using two colon adenocarcinoma and four oral cancer cell lines with a variety of MGMT expression. We also investigated the effects of MGMT depletion by O^6-benzylguanine (O^6-BG), a potent inhibitor of MGMT. The 5-FU treatment uniformly depleted protein and mRNA expression of MGMT in all cell lines examined. Cell lines expressing low levels of MGMT were sensitive to 5-FU. On the other hand, cells expressing high levels of MGMT were less sensitive to 5-FU. The 5-FU treatment exhibited a better antiproliferative effect on the cells expressing high levels of MGMT by the pretreatment of O^6-BG. Depletion of MGMT by O^6-BG enhanced the antitumor effect of 5-FU. Assessment of the levels of MGMT expression in cancer cells and the control of its expression could contribute to the effective chemotherapy by 5-FU especially in patients who previously were considered as low-responsive individuals whose tumors have high levels of MGMT.

Correspondence to: Dr Jun Murakami and Dr Jun-ichi Asaumi, Department of Oral and Maxillofacial Radiology, Field of Tumor Biology, Okayama University Graduate School of Medicine, Dentistry and Pharmaceutical Sciences, 2-5-1, Shikata-cho, Okayama 700-8558, Japan
E-mail: jun-m@md.okayama-u.ac.jp, asaumi@md.okayama-u.ac.jp

Key words: 5-fluorouracil, O^6-methylguanine-DNA methyltransferase, O^6-benzylguanine

Introduction

5-Fluorouracil (5-FU) is among the most commonly used anti-neoplastic agents applied to a variety of malignancies, including colon and oral cancers. However, not all cancer cells respond in similar manner to 5-FU treatment. The catabolic enzymes of 5-FU in tumor tissue, including thymidylate synthase (TS) and dihydropyrimidine dehydrogenase (DPD), have been assessed for their effectiveness in clinical practice, however, this remains controversial (1,2).

O^6-methylguanine-DNA methyltransferase (MGMT) is a DNA repair enzyme that repairs the adduct at the O^6-position of guanine (3-10). Since alkylating agents generate a complex spectrum of adducts at the O^6-position of guanine, the MGMT levels in a cell are a good marker to assess the effects of these agents. Several human tumor cell lines with little or no methyltransferase activity have been shown to be hypersensitive to the alkylating agents (11-14). Attempts at reducing MGMT activity in tumors theoretically sensitizes cells to the O^6-alkylating agent (15), and several attempts, such as the pre-administration of a methylating drug before administration of the alkylating agent (16) or administration of MGMT-specific inhibitors (17), have been made to inactivate MGMT. O^6-benzylguanine (O^6-BG) is a specific MGMT inhibitor and is used to sensitize chloroethylators or methylators (17-19). O^6-BG treatment has been approved as a phase I study (20) and is currently being administered in combination with BCNU, an O^6-alkylating agent, for the treatment of glioblastoma.

We have shown that colorectal cancer (CRC) patients who receive 5-FU as adjuvant chemotherapy have a better prognosis when the tumor reveals hypermethylation in its *MGMT* promoter (21). These results indicate that CRC cells

with low MGMT expression could be sensitive to 5-FU treatment. However, the relationship between MGMT expression and responsiveness to 5-FU treatment in cancer cell lines *in vitro* has not been elucidated. Other than alkylating agents, a favorable response to the cisplatin treatment has also been reported among cancer cells with promoter methylation of *MGMT* (22). This response could be explained by the possibility that cisplatin abrogate MGMT activity (23,24). The relationship, however, between MGMT expression and responsiveness to 5-FU or other cancer therapeutic agents in cancer cell lines *in vitro* is unknown.

In the present study, we examined the relationship between MGMT expression and responsiveness to 5-FU *in vitro* using CRC cell lines (generally expressing high levels of MGMT) and oral cancer cells (showing a variety of MGMT expression).

Materials and methods

Cell lines and culture. Human oral cancer cell lines (HSC4, HSC3, SAS, and Hep2) were obtained from the Cell Resource Center for Biomedical Research Institute of Development, Aging, and Cancer, Tohoku University. Human colon cancer cell lines (LoVo and RPMI-4788) were obtained from ATCC. All cell lines were maintained in Dulbecco's modified Eagle's medium (MDEM) (Nissui Pharmaceutical Co. Ltd., Tokyo, Japan) supplemented with 10% fetal bovine serum (FBS) (Hyclone Laboratories Inc., UT), 100 units/ml penicillin (Meiji Seika Ltd., Tokyo, Japan) and 100 μg/ml streptomycin (Meiji Seika Ltd.) in a CO_2 incubator (Sanyo Electric Co., Ltd., Osaka, Japan) with 95% air plus 5% CO_2 at 37˚C.

Chemicals. *N*-methyl-*N*-nitrosourea (MNU) (Nacalai Tosque, Inc., Kyoto, Japan), *N*-methyl-*N*'-nitro-*N*-nitrosoguanidine (MNNG) (Nacalai Tosque, Inc.), 5-fluorouracil (5-FU) (Wako Pure Chemical Industries, Ltd. Osaka, Japan), and O^6-Benzylguanine (O^6-BG) (Sigma Chemical Co. St. Louis, MO) diluted in water were added to the MDEM to the final concentration indicated in each treatment.

RNA isolation and RT-PCR. Extraction of total cellular RNA was carried out using Trizol reagent (Invitrogen Corp., Carlsbad, CA) according to the manufacturer's instructions. RNA was reverse-transcribed with Superscript II reverse transcriptase and oligo dT primers (Invitrogen Corp., Carlsbad, CA). Amplification of cDNAs was performed under the following PCR conditions: 7 min at 94˚C for 1 cycle; then 26 cycles at 94˚C for 30 sec, 59˚C for 30 sec, 72˚C for 30 sec; and a final elongation step at 72˚C for 10 min. The primers used for the amplification were: *MGMT*; sense: 5'-GCCGGCTC TTCACCATCCCG-3', antisense: 5'-GCTGCAGACCACTC TGTGGCACG-3', GAPDH; sense: 5'-GAAGGTGAAGG TCGGAGTC-3', antisense: 5'-CAAAGTTGTCATGGAT GACC-3' (25). *MGMT* primers were used to amplify a 211-bp product spanning the sequence 339-527 from the GenBank accession number: M29971. The amplified GAPDH fragment was used as a positive control. The RT-PCR products were separated by electrophoresis on a 2% agarose gel, stained with ethidium bromide, and viewed by UV. The intensities of the bands were quantified using Image J 1.33u (National Institutes of Health, USA).

Western blotting. Proteins in cell-free extracts were separated by electrophoresis in 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS PAGE), and the individual proteins were transferred to polyvinylidene difluoride membranes (Bio-Rad Laboratories, Hercules, CA) using a semi-dry electrophoretic transfer apparatus (LKB-Produkter AB, Bromma, Sweden) at room temperature. The blotted membranes were blocked for 1 h in TBS-T (containing 0.1% Tween-20) plus 5% powdered skin milk and then probed for 2 h with mouse anti-MGMT monoclonal antibody MT 3.1 Ab-1 (Neomarkers, Fremont, CA) diluted 1:800 in TBS-T. The membranes were then washed three times in TBS buffer and incubated for 1 h with the appropriate secondary antibody horseradish peroxidase-conjugated AffiniPure goat anti-mouse IgG (H+L) (ImmunoResearch Laboratories Inc., West Grove, PA) in TBS-T. Bound antibody was detected using an ECL plus kit (Amersham Pharmacia Biotech Inc., Little Chalfont, UK) according to the manufacturer's instructions. The mouse monoclonal antibody for ß-actin, ß-actin AC-15-ab6276, purchased from Abcam Limited (Cambridge, UK) was diluted 1:5000 in TBS-T and was utilized in the same manner. The intensities of the bands were quantified using Image J 1.33u (National Institutes of Health).

MNU and 5-FU treatment. Cells ($5x10^5$) were seeded in 5 ml MDEM in a flask (Nalge Nunc International, Roslilde, Denmark). Then, 24 h after seeding, the medium was exchanged for one containing an appropriate drug (MNU or 5-FU), and the flask was immersed in a 37˚C water bath (Taitec, Co., Ltd., Saitama, Japan). Following drug treatment for 1 h, the cells were rinsed three times with drug-free medium and their survival rates were determined as described below.

Cell survival assay for 5-FU and MNU. Cell survival was assayed by measuring the colony-forming ability of the cells in triplicate. Only colonies containing >50 cells were counted. After treatment with drugs, the cells were dispersed with trypsin, seeded at adequate concentrations, and incubated at 37˚C in a CO_2 incubator. Surviving cells were fixed in 10% formaldehyde and stained with 10% Giemsa stain solution. Cell-survival rates were corrected for the seeding efficacy of untreated controls.

O^6-BG treatment and an alteration of 5-FU sensitivity. Alteration of the chemosensitivity for each condition was evaluated using the MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrasodium bromide] assay. The MTT assay was carried out using an MTT cell growth kit (Chemicon International, Inc. Temecula, CA) according to the manufacturer's instructions. For O^6-BG-treated groups, $1x10^5$ cells incubated in the medium containing 75 μM of O^6-BG for 4 days were rinsed three times with fresh medium, and the cells were then seeded into 96-well plates. Eight replicate wells/per assay condition were seeded at a density of $1.5x10^4$ cells in 0.1 ml of medium containing the appropriate amount of O^6-BG (37.5 or 75 μM). As O^6-BG-untreated control groups, cells were also seeded into 96-well plates at the same density in medium without O^6-BG. Cells were incubated for 24 h at 37˚C. Stock solutions of 5-FU were prepared by dissolving the drug

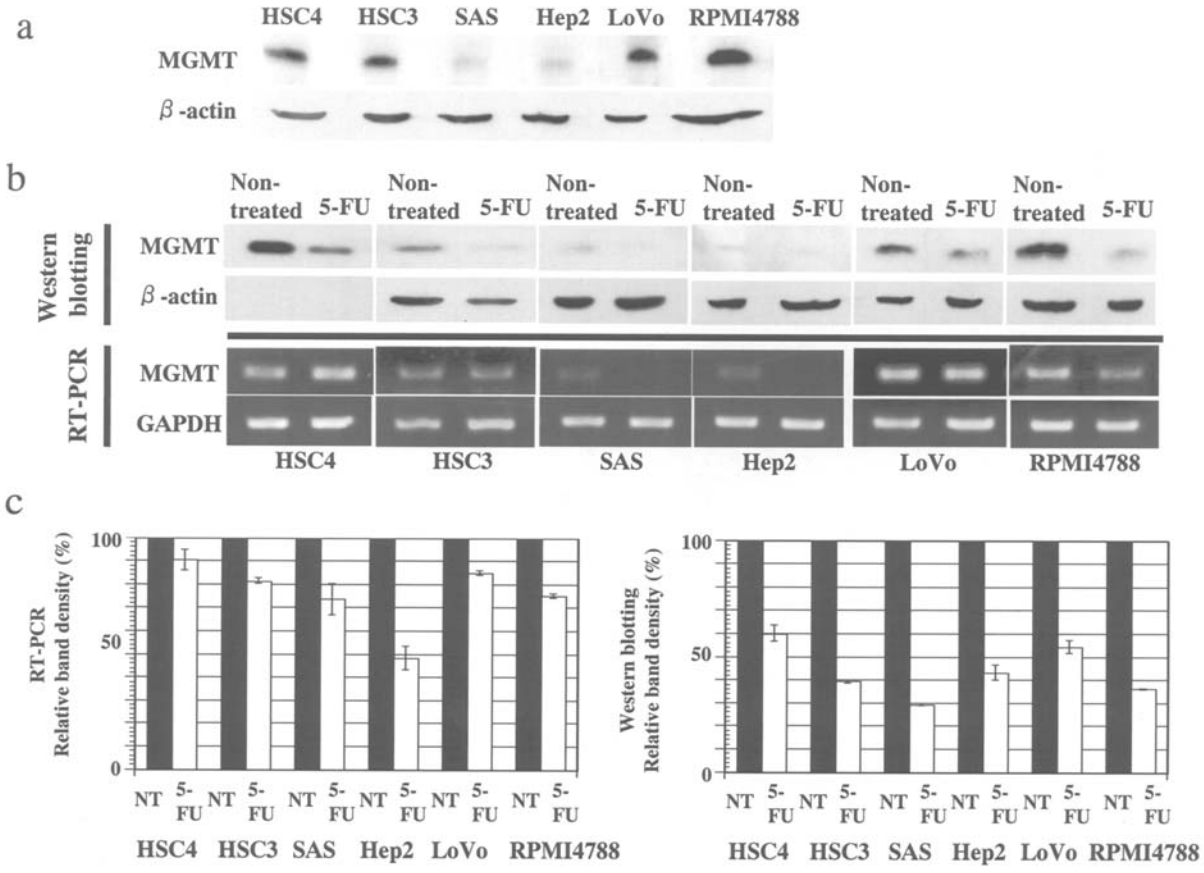

Figure 1. Effects of 5-FU treatment on the expression of MGMT in colon and oral cell lines. (a) MGMT protein expression was assessed in colon and oral cancer cell lines by Western blot analysis. Equal amounts of extracted protein from the cell lines were loaded onto a 10% SDS-PAGE gel and electrophoresed. Proteins were electroblotted onto a PVDF membrane that was probed with monoclonal antibody MT 3.1 specific for human MGMT. (b) Effects of 5-FU treatment on the expression of *MGMT* mRNA and protein in the cell lines. For the 5-FU-treated groups, $1x10^5$ cells were incubated in the medium containing 200 μM of 5-FU for 38 h. The protein and mRNA expressions of *MGMT* were measured by Western blotting or RT-PCR. (c) Intensities of the bands were quantified by the proportion of MGMT versus ß-actin or GAPDH with Image J 1.33u (National Institutes of Health). The relative band intensity represents the intensity of 5-FU treated sample/5-FU non-treated control sample. Black columns represent cells with no 5-FU treatment. White columns represent cells treated with 5-FU. The significance of the differences was tested by the Student's t-test: $p<0.05$. Each column is the average of three measurements; bars, SD. The band intensities in groups treated with 5-FU were significantly different from those without 5-FU.

at a concentration of 10 mM in distilled water no more than 2 h prior to use in an experiment; final concentrations were obtained by diluting the stock solution directly into the tissue-culture medium. Cells were incubated with several concentrations of 5-FU ranging from 100-800 μM for an additional 38 h. At the end of the 5-FU exposure, 10 μl of MTT (5 mg/ml) was added to each well for 4 h at 37˚C to allow the MTT to form formazan crystals by reacting with metabolically active cells. Next, 100 μl of color development solution (isopropanol with 0.04 N HCl) was added to each well. Within an hour, the absorbance of each well was measured in a microplate reader (Corona microplate leader MTP-120, Corona Electric Co., Ltd., Japan) with a test wavelength of 570 nm. The percentage of cell-growth inhibition was calculated by comparison of the absorbance reading from treated versus untreated control cells. Each experimental condition consisted of >5 wells.

Statistical analysis. Statistical analysis was conducted with JMP 5.0.1 J software (SAS Institute Inc. Cary, NC). We used the Student's t-test for comparisons between the values. A significant difference was defined as $p<0.05$.

Results

5-FU treatment depletes the level of MGMT in colon and oral cancer cell lines. MGMT activity is closely correlated with the spontaneous MGMT protein expression detected by the immuno-reactive method, including Western blot analysis or immunohistochemical study (26,27). We studied the MGMT protein expression status of two CRC and four oral cancer cell lines by Western blotting. A high level of MGMT expression was observed in 2 of the CRC cell lines (LoVo and RPMI-4788) and 2 of the oral cancer cell lines (HSC4 and HSC3). On the other hand, low expression levels were observed in 2 of the oral cancer cell lines (SAS and Hep2) (Fig. 1a). To examine the alteration of MGMT expression by the 5-FU treatment, we assessed the protein and mRNA expression levels by Western blotting and RT-PCR after treating the cell lines with 5-FU *in vitro*. 5-FU treatment uniformly reduced MGMT protein content in all the cell lines examined (Fig. 1b). The RT-PCR results, however, revealed that the mRNA content was slightly degraded by the 5-FU treatment in all cell lines. These results suggested that the MGMT depletion was primarily a post-translational consequence of 5-FU treatment.

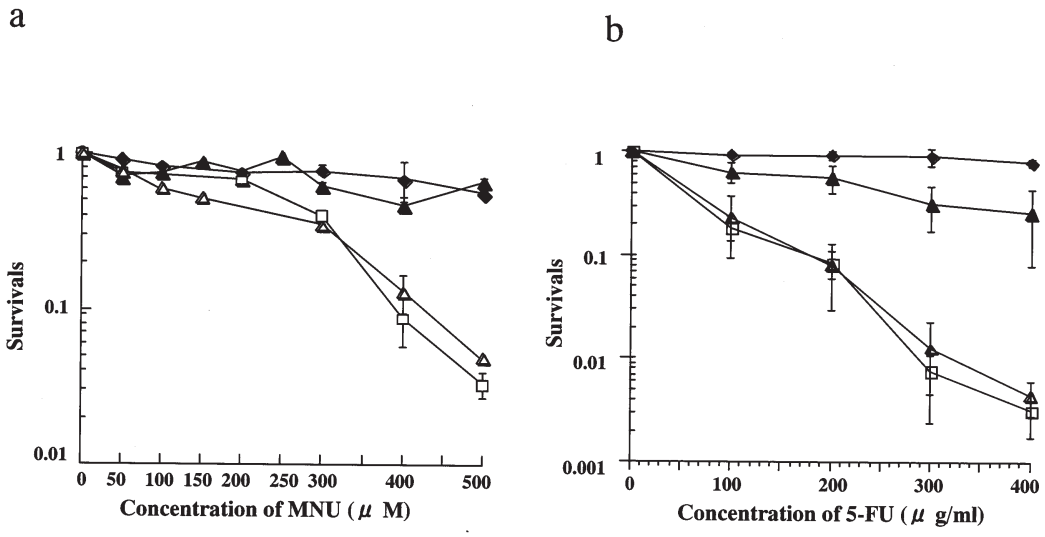

Figure 2. Sensitivity to anticancer agents in oral cancer cell lines. (a) Cellular sensitivity to alkylating agent MNU. (b) Cellular sensitivity to 5-FU. The significance of the differences was tested by the Student's t-test. Each point is the average of three independent experiments, each performed in triplicate; bars, SD. HSC4, ◆; HSC3, ▲; SAS, □; Hep2, △.

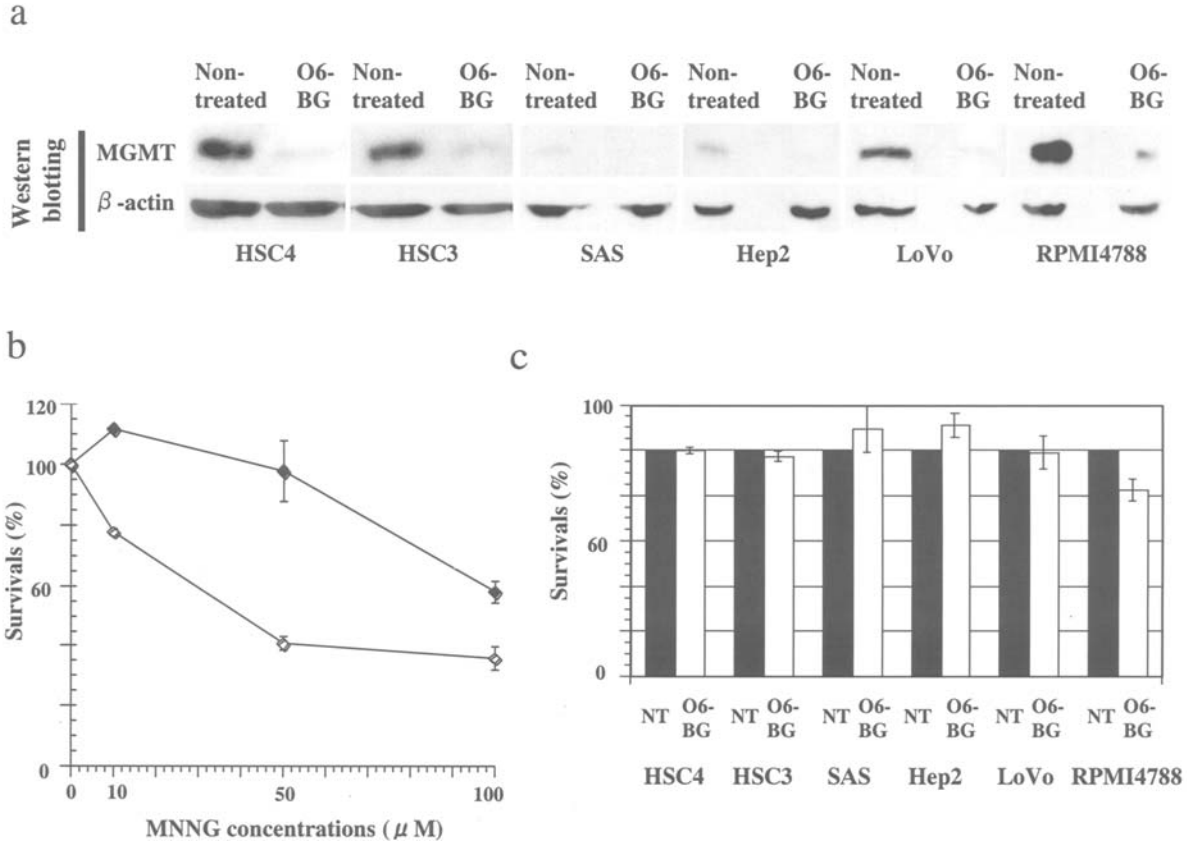

Figure 3. Effects of O^6-BG in cancer cell lines. To examine whether MGMT depletion enhances the anti-tumor effect of 5-FU, we evaluated the combined effects of 5-FU and MGMT inhibitor O^6-BG on cell growth. For the O^6-BG-treated groups, cells were exposed to 75 μM O^6-BG for 4 days. First, we confirmed whether our O^6-BG protocol was effective in depleting MGMT protein and in restoring the cellular sensitivity to the alkylating agent. (a) Western blot analysis of MGMT protein before and after O^6-BG treatment. (b) The restoration of MNNG sensitivity by O^6-BG in HSC4 cells. The significance of the differences was tested by the Student's t-test: $p<0.05$. Each point is the average of more than five wells; bars, SD. (c) The cytotoxicity for O^6-BG in cell lines. Each column is the average of more than five wells; bars, SD. Black columns represent cells with no O^6-BG treatment. White columns represent cells treated with O^6-BG.

Antiproliferative effects of treatment with the alkylating agent MNU and 5-FU. To observe the cellular response to the anti-tumor agents, four oral cancer cells with a variety of MGMT expression were subsequently treated with anti-tumor agents. Fig. 2 shows the results obtained following treatment of the cell lines with varying concentrations of the alkylating agent

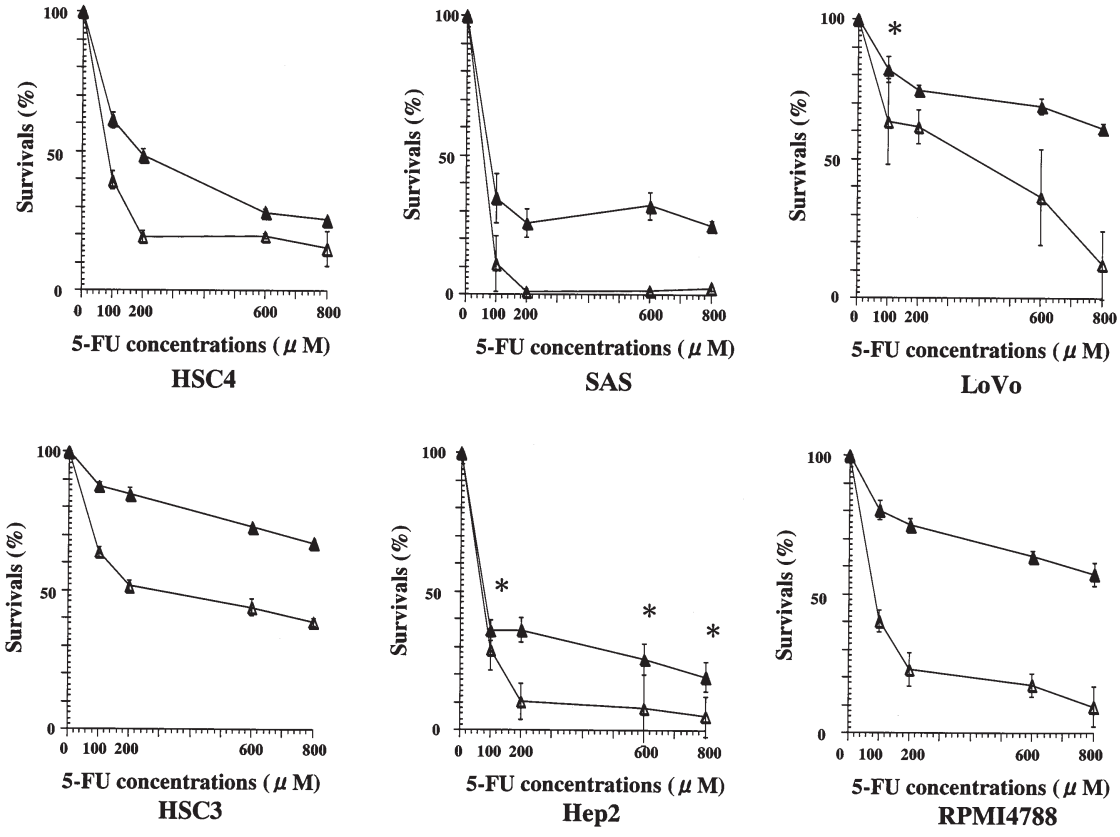

Figure 4. Effects of O^6-BG on cellular sensitivities with 5-FU treatment. To determine whether MGMT depletion may enhance the anti-tumor effect of 5-FU, we evaluated the combined effects of 5-FU and MGMT inhibitor O^6-BG on cell growth. The closed symbols represent the survival of 5-FU single-treated groups, while the open symbols represent the survival of O^6-BG/5-FU combined-treated groups. Each point is the average of more than five wells; bars, SD. The cell-survival rates in groups treated with 5-FU together with O^6-BG were significantly different ($p<0.05$) from those without O^6-BG by the Student's t-test, except for the asterisk-added groups.

MNU or the non-alkylating agent 5-FU. Two of the four cell lines expressing relatively large amounts of MGMT protein (HSC4 and HSC3) appeared to be resistant to the lethal effects of MNU, depending on the concentrations used (Fig. 2a). In contrast, growth of the cell lines expressing low levels of MGMT protein (SAS and Hep2) was inhibited in a dose-dependent manner by MNU ($p<0.05$, Fig. 2a). Although, the different response to the alkylating agent was thought to depend on the MGMT expression status, it was surprising to find that the responsiveness of the four cell lines to the 5-FU was exactly the same as that to MNU (Fig. 2b). MGMT low-expressing cell lines (Hep2 and SAS) were sensitive to 5-FU, just as to MNU, in a dose-dependent manner. In contrast, the cell lines expressing a large amount of MGMT (HSC4 and HSC3) were not inhibited in their proliferation by the treatment with 5-FU ($p<0.05$, Fig. 2b).

Cellular sensitivities in response to 5-FU treatment in combination with O^6-BG. To examine whether the depletion of MGMT expression may enhance the chemotherapeutic effect of 5-FU in the treatment of cancer patients, we evaluated the combined effect of 5-FU and an MGMT inhibitor, O^6-benzylguanine (O^6-BG), on cell growth. For the O^6-BG-treated groups, cells were treated with 75 μM O^6-BG for 4 days. The levels of MGMT protein were then examined. Depletion of MGMT proteins were observed in all cell lines treated with O^6-BG (Fig. 3a). Next, the cells were washed and seeded into 96-well plates with a medium containing the appropriate amount of O^6-BG (37.5 or 75 μM). After incubation for 24 h, the cells were exposed to 5-FU in several concentrations for an additional 38 h. To confirm that our O^6-BG combined protocol was an effective means of restoring cellular sensitivity to the alkylating agent, the HSC4 cell line showing resistance to the alkylating agent was treated with our combined regimen with an alkylating agent MNNG (Fig. 3b). Significant enhancement of cellular sensitivity to the MNNG was observed. To exclude the cytotoxicity of the solitary administration of O^6-BG, cell survival from a single treatment of O^6-BG was evaluated (Fig. 3c). Given that the cell survival in the O^6-BG-treated groups was always >80% compared to the untreated groups, the cytotoxicity of O^6-BG was confirmed as minimum. Growth inhibition was observed in every cell line examined in a dose-dependent manner after 5-FU treatment for 38 h within a concentration range from 100-800 μM. Treatment with 5-FU in combination with O^6-BG revealed enhanced anti-tumor effects compared with the results obtained with 5-FU treatment alone (Fig. 4). The survival rates of the cells treated with O^6-BG and 5-FU significantly decreased compared with those treated with 5-FU alone, except for the points indicated by asterisks ($p<0.05$). Among the four MGMT-expressed cell lines (HSC4, HSC3, LoVo, and RPMI-4788), O^6-BG especially enhanced the cytotoxicities of HSC3 and

RPMI-4788 to 5-FU. Two 5-FU-sensitive cell lines with low expression of MGMT (SAS and Hep2) also increased their sensitivity to the cytocidal effects of 5-FU under the O^6-BG/ 5-FU combined regimen.

Discussion

To our knowledge, this is the first study to examine the relationship between the levels of MGMT expression in cancer cells and the anticancer effects of 5-FU *in vitro*. Several studies have indicated that catabolic enzymes of 5-FU in tumor cells are critical factors in the 5-FU effect against cancer cells (1,2). Among the enzymes involved in 5-FU catabolism, TS and DPD are considered to be most important for the 5-FU effect. Since several attempts have been made to predict the effectiveness of the 5-FU by assessing these enzyme activities, the correlation between the effectiveness of 5-FU treatment and these enzyme activities remains controversial (28,29). On the other hand, the mismatch repair (MMR) system is involved in the effectiveness of 5-FU. Carethers *et al* have demonstrated that colon cancer cell lines deficient in MMR activity (loss of *hMLH1*) are significantly more tolerant of the 5-FU effect than the MMR-proficient HCT116+chr3 cells established by chromosomal transfer (30). Accordingly, 5-FU responsiveness might be more complex than has been thought.

MGMT is one of the DNA repair enzymes that rapidly repair adducts at the O^6-position of guanine and that act with the MMR system. Although its expression is known to modulate the effectiveness of the alkylating agents or cisplatin (11-14,22), almost no study has focused on the relationship between MGMT activity and the 5-FU effect on cells. We have previously shown that CRC patients who are treated with 5-FU as adjuvant chemotherapy have a better prognosis when the tumor reveals hypermethylation in its *MGMT* promoter (21). Therefore, we sought to investigate whether a cell expressing low levels of MGMT is more sensitive to 5-FU *in vitro* by using colon adenocarcinoma cell lines, which commonly express high levels of MGMT, and human oral cancer cell lines, which show a variety of MGMT expression.

In this study, we found that after 5-FU treatment, the protein content of MGMT was markedly reduced, while the *MGMT* mRNA content was preserved in all cell lines examined. To our knowledge, this is the first study demonstrating MGMT protein depletion in response to treatment with 5-FU in cancer cell lines, depletion that is thought to be caused by a post-translational modification. Bibby *et al* have reported that molecular combination of 5-FU and alkylating agent 2-chloroethyl-1-nitrosourea (CNU) results in a significant growth retardation of BCNU-resistant murine colon cancer and human breast cancer xenografts in mice *in vivo* (15). These anti-tumor effects are potentiated by the addition of O^6-benzylguanine (BG), a pseudo-substrate of MGMT. We also took into account in this study that the up-regulation of the CNU effect by 5-FU might have been achieved by the depletion of MGMT caused by 5-FU.

The mechanism involved in the depletion of MGMT protein by 5-FU is unknown. Since metabolites of 5-FU cause both RNA- and DNA-directed cytotoxicities (31), 5-FU may also generate DNA adducts (such as O^6-alkylating DNA adducts generated by alkylating agents), and MGMT is consumed in the process. It remains to be determined in future attempts whether or not 5-FU generates such adducts. We examined whether the depletion of MGMT causes a cytocidal effect on cancer cells. The cancer cells that showed reduced or no expression of MGMT and were sensitive to alkylating agent (i.e. SAS and Hep2) were also sensitive to 5-FU treatment. Cells with high levels of MGMT (i.e. HSC4 and HSC3) were not sensitive to 5-FU. MGMT depletion by O^6-BG, pseudosubstrate of MGMT (17-19), potentiated the anti-tumor effect of 5-FU. Since MGMT activity has been recognized as an important marker of sensitivity to alkylating agents, many attempts have been made to deplete MGMT by using the specific inhibitor O^6-BG to enhance the anti-tumor effects of alkylating agents (17). In our study, O^6-BG depleted the MGMT protein content and restored sensitivity to 5-FU and to the alkylating agent, MNNG. Single administration of O^6-BG to the cells has been reported as non-toxic (32). Although pretreatment of cancer cells by O^6-BG showed minimal cytocidal effect in our study, our co-incubation time of 5-FU and O^6-BG in our protocol was slightly longer compared to other studies. Nevertheless, we found that the O^6-BG/5-FU combined regimen produced supra-additive cytocidal effects in all cells examined. An enhancement of 5-FU sensitivity was achieved even in cells with quite a low MGMT expression (SAS and Hep2). Our results demonstrate that MGMT activity is one of the causes of 5-FU resistance, which provides the first evidence for an essential involvement of MGMT in 5-FU-induced cellular toxicity. As such, depletion of MGMT in cancer cells could become an important therapeutic target for sensitizing cells to 5-FU treatment.

In summary, MGMT depletion uniformly occurs in response to 5-FU in colon and oral cancer cell lines, irrespective of the original expression levels of MGMT. The levels of MGMT expression were related to the sensitivity to 5-FU, and an enhancement of the anti-tumor effect of 5-FU was observed in response to MGMT depletion. Our current *in vitro* findings in addition to our previous clinical studies propose important clinical applications related to the enhancement of 5-FU anti-tumor effect for colorectal cancer and other malignancies with promoter hypermethylation of *MGMT* by controlling the levels of MGMT in the tumor.

Acknowledgements

We are most grateful to Professor Shoji Kawasaki for his generous support for making this collaboration possible. This study was supported by Grant-in-Aid (15591277, 16791240, 17791458) for Scientific Research from the Ministry of Education, Science, Sports and Culture of Japan.

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