Differential cytotoxicity of anticancer agents in hMutSα-deficient and -proficient human colorectal cancer cells

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Abstract: Mismatch repair (MMR)-deficient cells exhibit drug resistance to several anticancer agents including N-methyl-N'-nitro-N-nitrosoguanidine (MNNG), cisplatin, and adriamycin. Since these agents are potent mutagens, it is possible to select resistant clones of tumor cells during chemotherapy. Prior to determining whether drug cytotoxicity was altered by MMR-deficiency, mutation in the (A)₈ repeat region of the hMSH3 gene of the MMR-deficient human colorectal cancer cell line HCT116 and the MMR-proficient human chromosome 3-transferred HCT116 (HCT116+ch3) was confirmed. A screening method was then determined using MNNG cytotoxicity in both cell lines and 20 additional anticancer agents were examined. Clonogenic cytotoxic assay revealed in 8 anticancer agents (streptozotocin, 5-fluorouracil, tegafur, bleomycin, mitomycin C, vinblastine, vincristine, and nidoran) maintaining the desired level of cytotoxicity required a higher concentration in HCT116 than in HCT116+ch3. Cytosine-13-arabinofuranoside, chlorambucil, and epirubicin were more cytotoxic to HCT116. Dacarbazine, nitrogen mustard, 3'-azido-3'-deoxythymidine, aclarubicin, neocarzinostatin, actinomycin D, and peplomycin possessed similar cytotoxicity. These results suggest that drugs with higher or uncompromised sensitivity can circumvent drug resistance due to MMR-deficiency in tumor cells.

Key words: mismatch repair deficiency, drug resistant, human colorectal cancer cells, circumvention

Introduction

The mismatch repair (MMR) system plays an important role in an increase in DNA replication and transcription fidelity¹. MMR is a highly conserved cellular process found in organisms from Escherichia coli to humans. It results in the recognition, excision, and re-synthesis of a newly synthesized DNA strand containing mismatched bases². Six human MMR genes such as hMSH2(³,⁴), hMSH3(⁵), hMSH6 (GTBP or p160)⁶, hMLH1(⁷,⁸), hPMS2, and hPMS1(⁹) have been defined by cytogenetic, biochemical, and molecular biological studies. hMSH2, dimerized with either hMSH6(⁵) or hMSH3(¹¹), recognizes mismatched bases and subsequently recruits the hMLH1 and hPMS2 complex, hMutLa(¹²), proliferative cell nuclear antigen(¹³), and other MMR-related proteins. The hMSH2 and hMSH6 complex, hMutSα, recognizes one base mismatch and one unpaired base³. The hMSH2 and hMSH3 complex, hMutSβ, binds to two or more unpaired bases¹¹. A defective MMR system is associated with hereditary nonpolyposis colorectal cancer (HNPPC)³,⁴,⁶,⁸,¹⁴,¹⁵ and certain sporadic colon cancers¹,¹⁶-¹⁸. MMR deficient cells have a higher frequency of both spontaneous mutation and alterations in microsatellite repeat sequences (the
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Microsatellite instability (MSI) phenotype and are resistant to some antitumor agents such as N-methyl-N'-nitro-N-nitrosoguanidine (MNNG), 6-thioguanine, cisplatin, adriamycin, and etoposide.

One pair of the human colorectal cancer cell lines, HCT116 and normal human chromosome 3-transferred HCT116 (HCT116+ch3), provides an excellent experimental system to understand the biological roles of MMR gene products because of their similar genetic and cytological composition. HCT116 has the homozygously mutated hMLH1 gene located in 3p21-23 and exhibits microsatellite instability, lack of MMR and transcription-coupled excision repair (TCR) activities, and resistance to some of the anticancer drugs mentioned above. Conversely, HCT116+ch3 restores these MMR-deficient phenotypes because of adequate expression of the wild-type hMLH1 gene on the transferred chromosome. Recently, another mutation in a microsatellite sequence, (A)8, in exon 7 of the hMSH3 gene located in 5q11-13 of HCT116 has been identified. Thus, hMutSa, but not hMutSβ, is assumed to be active in HCT116, while the MMR pathway is blocked at the hMutLα step.

Drug resistance due to MMR deficiency is a serious problem in cancer chemotherapy. Since anticancer agents are potent mutagens, treatment with these agents may lead to the regeneration or proliferation of drug-resistant MMR-deficient tumor cells. A mere two-fold difference in cisplatin sensitivity has been demonstrated to be sufficient to regenerate a resistant tumor in a mouse model. Knowledge concerning the sensitivity of anticancer agents in MMR-deficient cells is limited. Thus, it is important to determine the sensitivity of cancer chemotherapeutic agents frequently used in treating MMR-deficient malignant tumor cells. In this study, MNNG cytotoxicity in a pair of cell lines, HCT116 and HCT116+ch3, was used to determine the best of 3 assay methods. The sensitivity of 20 anticancer agents was then screened by clonogenic cytotoxic assay using the pair of cell lines. The agents were classified into three groups: those less effective on hMutSa-deficient than proficient cells, those more effective on hMutSa-deficient cells; and those with no difference in effectiveness. The potential utility of drugs for treatment of MMR-deficient tumors is then discussed.

Materials and Methods

Materials

MNNG, streptozotocin, dacarbazine, chlorambucil, 3'-azido-3'-deoxythymidine (AZT), 5-fluorouracil (5-FU), tegafur, cytosine β-D-arabinofuranoside (Ara-C), aclacinomycin, mitomycin C, actinomycin D, vincristine, vinblastine, bleomycin, and 3-(4,5-dimethyl-thiazole-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Neocarzinostatin was kindly donated by Dr. Shigeo Matsumoto of the Kayaku Co. Ltd., Tokyo. Nitrogen mustard, nidoran, peplomycin, epirubicin, ifosfamide, and ranimustine were generous gifts of Yoshitomi Pharmaceutical Industries, Ltd., Osaka, Sankyo Co., Ltd., Tokyo, Nippon Kayaku Co., Ltd., Tokyo, Kyowa Hakko Kogyo Co., Ltd., Tokyo, Shionogi & Co., Ltd., Osaka, and Tokyo Tanabe Co., Ltd., Tokyo, respectively.

Cells and cell culture

HCT116 was maintained in minimum essential medium (Earle's salt) supplemented with 10% fetal bovine serum. HCT116+ch3 was cultured in the same medium with 400 μg geneticin (Gibco-BRL)/ml. Cells were grown in a 5% CO2 in air in a humidified atmosphere.

Mutation analysis

DNA was isolated from cultured cells with a DNAzol Reagent (GIBCO BRL Life Technologies, Inc., Grand Island, NY). A set of primers (sense 5'-GAGATAATGACTGATACTTCTACC-3' ; antisense 5'-CATTGTTTCCTACCTGCAAAG-3') for polymerase chain reaction (PCR) and sequencing was specific to a part of the hMSH3 gene (Gene Bank accession J104810). Reaction mixture of PCR contained 0.1 μg template DNA, primers, 0.2 mM each dNTP mix, and 2.5 U Taq DNA polymerase, and amplification buffer consisting of 10 mM Tris-Cl, 50 mM KCl, and 1.5 mM MgCl2, pH 8.8, in a total volume of 100 μl. Thermal condition was 30 cycles of denaturing at 96°C, anneal-
ing at 58°C, extension at 72°C for 1 min, and final extension for 8 min in a PCR Thermal Cycler (model PJ2000, Perkin-Elmer Co., Foster City, CA). The PCR product was used for single strand conformational polymorphism (SSCP) as described with small modifications. Aliquot of the PCR product was mixed with denaturing solution containing 95% formamide and the mixture was heated at 95°C for 3 min, immediately placed on an ice-bath for 5 min, and electrophoresed with a 12% polyacrylamide gel (20 x 40 x 0.1 cm) at 20°C for 20 hrs with 3 W constant power. The abnormal bands were detected by comparing those of normal DNA. DNA sequencing was performed using a Taq DyeDeoxy Terminator Cycle Sequence kit (Perkin-Elmer) as recommended by the supplier. The reaction mixture was analyzed on an automated DNA sequencer (Perkin-Elmer, Applied Biosystems Division, model 373S).

Preparation of single cell suspension

Exponentially growing cells in a 25 cm² flask were harvested by trypsinization, washed with medium supplemented with 10% fetal bovine serum by centrifugation, and resuspended with 10 ml of medium in a 15 ml tube. To make a single cell suspension, the tube was left to stand for 15–20 min at room temperature and 6 ml of top supernatant was removed to another tube. The tube was centrifuged and 100 μl of medium was added. After the cell suspension was up and down several times with a 200 μl tip, fresh medium was added for further dilutions. This step was repeated until cells became a single cell suspension. Confirmation of a single cell suspension and determination of the cell number were done with a hemocytometer.

Cytotoxic assays

MNNG was dissolved with dimethylsulfoxide at a concentration of 20 mM and further dilutions were made with sterile phosphate-buffered saline (PBS) consisting of 136.9 mM NaCl, 2.7 mM KCl, 8.1 mM Na₂HPO₄, and 1.5 mM KH₂PO₄, pH 7.3. Other cytotoxic agents were dissolved in Milli-Q grade water and filtered with a 0.22 μm pore-sized membrane. Further dilutions were made with sterile PBS.

For clonogenic assay, 200 single cells suspended in 2 ml medium in a well of a 6-well plate were cultured in a CO₂ incubator. After 24 hrs, cells were exposed to various concentrations of an agent for 1 hr, 24 hrs, or continuously (168 hrs). Culture was continued another 7 days. Cells were fixed and stained with a mixture consisting of 0.05% crystal violet, 4.2% neutralized formalin, and 10% ethanol. Colonies consisting of more than 50 cells were counted. The number of colonies in the culture without agents was defined as 100% and 50% inhibitory concentration (IC₅₀) was estimated in triplicate. Colony efficiencies of HCT116 and HCT116+ch₃ were 0.7–0.75 and 0.6–0.65, respectively.

MTT assay was carried out as described with small modifications. Cells were plated into a well of a 96-well plate at a cell density of 10⁴/well in a total culture volume of 100 μl. After 24 hrs, cells were treated with various concentrations of MNNG in quadruplicate for 1 hr and washed with medium. After 3 day cultivation, 10 μl of MTT solution was added and incubated for 3 hrs. After removal of the supernatant by suction, 50 μl of fresh medium and 100 μl of 0.04 N HCl in isopropanol were added and thoroughly mixed with a microplate mixer. The absorbance at 545 nm was measured by a microplate reader (model 550, Bio-Rad Laboratories, Hercules, CA, USA).

Dye-staining was performed as described with small modifications. Cells (10⁴) in 200 μl of culture medium were added to each well of a 96-well microplate. After incubation for 24 hrs at 37°C, cells were treated with MNNG for 1 hr in quadruplicate and culture was continued for another 3 days. After cultivation, cells were washed, fixed, and stained with crystal violet. The absorbance at 610 nm was measured by a microplate reader.

Statistical analysis

The values in triplicate or quadruplicate samples are expressed as means and SD. Paired Student’s t-test 1-tail p values were calculated from 3 to 5 independent experiments. p value less than 0.05 was determined as significant difference.
Results

Mutation in the \((A)_8\) repeat region of the \(hMSH3\) gene

We studied the mutation in the \((A)_8\) repeat region of the \(hMSH3\) gene of the HCT116+ch3 cells with PCR-SSCP analysis. As expectedly, the band pattern of DNA of HCT116+ch3 was identical to the parent cell line, HCT116 (Fig. 1). Sequence analysis showed homozygous one A deletion in both cell lines (data not shown). These results indicate that the \(hMSH3\) gene of HCT116+ch3 has the same mutation as that of HCT116 and the hMutSβ heterodimer consisting of hMSH2 and hMSH3 in both cell lines is dysfunction. Therefore, the MMR pathway mediated by hMutSa in HCT116+ch3, but not HCT116, is assumed to be proficient.

Comparison of cytotoxic assays

To determine an appropriate assay method for the drug sensitivity in the hMutSa-deficient HCT116 cell line and the hMutSa-proficient HCT116+ch3 cell line, the clonogenic, MTT, and dye-staining cytotoxic assays were evaluated, using MNNG, which is known to be approximately 5-fold resistant to MMR-deficient cell lines\(^{23}\). Though HCT116 was shown to be resistant to MNNG by all the assay methods, the clonogenic cytotoxic assay was the most sensitive and reliable (Fig. 2). Thus, the clonogenic assay was selected to determine the cytotoxicity of the other antitumor agents.

Cytotoxicity of anticancer agents

The cytotoxic effects of 5 alkylating agents on HCT116 and HCT116+ch3 were studied to determine the resistance of hMutSa-deficient cells. HCT116 was resistant to streptozotocin, a potent DNA-methylation agent, at all concentrations tested. The survival curves of both cell lines treated with various concentrations of streptozotocin are shown in Fig. 3A. Four independent experiments with streptozotocin revealed an approximately 2.7-fold resistance in hMutSa-deficient cells (\(p = 0.002\)), when comparing the IC\(_{50}\) values for both cell lines (Table 1). Although more HCT116 cells than HCT116+ch3 cells survived when treated with nidoran and dacarbazine (Fig. 3B and C), as well as nitrogen mustard, no significant differences in IC\(_{50}\) values of these agents were observed (Table 1). In contrast to the DNA methylating agents, such as MNNG and streptozotocin, HCT116 was more sensitive to chlorambucil than HCT116+ch3 (Fig. 3D). Similar results were obtained in 3 independent experiments: an approximately 1.3-fold resistance was noted in HCT116+ch3 cells (Table 1). Ifomide and ranimustine displayed no cytotoxicity at any concentration examined.
Fig. 2. Comparison of sensitivity of clonogenic (A), MTT (B), and dye-staining (C) assays in determining MNNG cytotoxicity to the human colorectal cancer cell lines, HCT116 and HCT116+ch3. Single cell suspensions of HCT116 (open symbols) and HCT116+ch3 (closed symbols) were inoculated into wells of a 6 well-culture plate, and the cytotoxicity of various concentrations of MNNG indicated in figure was determined as described under Materials and Methods. Bars show SD.

Fig. 3. Survival curves of HCT116 and HCT116+ch3 treated with alkylating agents. Percent survival of HCT116 (open symbols) and HCT116+ch3 (closed symbols) treated with various indicated concentrations of streptozotocin (A) for 1 hr, nidoran (B) for 24 hrs, dacarbazine (C) for 7 days, and chlorambucil (D) for 1 hr, determined by the clonogenic assay in triplicate. Bars show SD.
The IC50 values were determined from the survival curves and are expressed as means ± SD of the number of independent experiments in triplicate.

Index was calculated by following formula: mean of the 1050 value for HCT116/mean of the IC50 value for the HCT116+ch3.

p values were calculated using paired t-test with 1-tail p.

The concentration of neocarzinostatin is expressed as μg per ml.

Since 6-thioguanine, a purine derivative, is known to be less effective in HCT11625), the drug sensitivity of 5 nuclear base-related analogs was then examined. HCT116 was 2.5- and 1.7-fold more resistant to 5-FU and tegafur, respectively (Fig. 4A and B, Table 1). Unexpectedly, Ara-C was more cytotoxic to HCT116 than HCT116+ch3 (Fig. 40, Table 1). Aclarubicin and AZT had similar cytotoxicity to HCT116 and HCT116+ch3 (Fig. 4C, Table 1). Vinca alkaloids were more cytotoxic to HCT116+ch3 than HCT116 (Fig. 6, Table 1).

Bleomycin and mitomycin C were less cytotoxic to HCT116 than HCT116+ch3 (Fig. 5A and B). These antibiotics were approximately half as effective with HCT116, as determined by IC50 values (Table 1). Like chlorambucil and Ara-C, HCT116 had a 1.2-fold sensitivity to epirubicin (Fig. 5D, Table 1). Actinomycin D, neocarzinostatin, and peplomycin were almost equally cytotoxic to both cell lines (Fig. 5C, Table 1). Vinca alkaloids were more cytotoxic to HCT116+ch3 than HCT116 (Fig. 6, Table 1).
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Fig. 4. Survival curves of HCT116 and HCT116+ch3 treated with nucleoside analogs. Percent survival of HCT116 (open symbols) and HCT116+ch3 (closed symbols) treated with various indicated concentrations of 5-FU (A) for 1 hr, tegafur (B) for 7 days, aclarubicin (C) for 1 hr, and Ara-C (D) for 1 hr, determined by clonogenic assay in triplicate. Bars show SD.

Discussion

The present study aimed to determine the sensitivity of cancer chemotherapeutic agents in MMR-deficient cells. Twenty-one cytotoxic agents, including MNNG, were screened using a clonogenic cytotoxic assay system with a pair of human colon cancer cell lines: HCT116, an hMLH1 deficient cell line, and HCT116+ch3, which is proficient in MMR due to microcell fusion of a wild type copy of the hMLH1 gene. The cytotoxic agents were either more, less or equally cytotoxic to MMR-deficient cells. The efficacies of 5-FU, bleomycin, and vinblastine in HCT116 cells, as determined by IC_{50} values, were approximately 2- to 3-fold higher than those in HCT116+ch3 cells. The cytotoxic effects of nitodar, tegafur, mitomycin C, and vincristine on HCT116 were less than those with HCT116+ch3. Fink et al. have demonstrated that an approximately 2-fold difference in cisplatin cytotoxicity between MMR-deficient and -proficient cells in vitro is sufficient to enable regrowth of MMR-deficient tumor cells in mice treated with cisplatin. Furthermore, proliferation of MMR-deficient cells cocultured with proficient cells and an increased mutation rate in MMR-deficient...
Fig. 5. Survival curves of HCT116 and HCT116+ch3 treated with antitumor antibiotics. Percent survival of HCT116 (open symbols) and HCT116+ch3 (closed symbols) treated with various indicated concentrations of bleomycin (A), mitomycin C (B), actinomycin D (C), and epirubicin (D), for 1 hr, determined by the clonogenic assay in triplicate. Bars show SD.

Fig. 6. Survival curves of HCT116 and HCT116+ch3 treated with vinca alkaloids. Percent survival of HCT116 (open symbols) and HCT116+ch3 (closed symbols) treated with various indicated concentrations of vinblastine (A) and vincristine (B), for 1 hr, determined by clonogenic assay in triplicate. Bars show SD.
cells\textsuperscript{38} have been demonstrated when the cells were drugs to which they possessed only limited resistance such as platinum compounds, adriamycin, and etoposide. Thus, it is likely that the 8 additional chemotherapeutic agents with lower cytotoxicity to HCT116 cells in this study may produce similar results to other drugs with compromised cytotoxicity such as MNNG, platinum compounds, adriamycin, 6-thioguanine, and etoposide, namely, proliferation of MMR-deficient tumor cells and an increase in the mutation rate in tumor cells.

The mechanism of MMR-deficient cell resistance to alkylating agents and platinum compounds has been extensively studied. hMutSa, a heterodimer consisting of hMSH2 and hMSH6, binds to methylated DNA and platinum adducts, and MMR-proficient cells cease to proliferate because they are unable to pass the G2 checkpoint\textsuperscript{23,24,39\textendash}41. In MMR-deficient cells, because of the lack of cell cycling signalling, most cells pass through the checkpoints, leading to resistance for certain drugs. Streptozotocin, an antibiotic derivative of MNNG\textsuperscript{42}, which was least cytotoxic to HCT116 among the drugs tested here, may act in a similar manner to MNNG. Major DNA damage induced by antitumor antibiotics such as bleomycin, mitomycin C, neocarzinostatin, and actinomycin D includes cross-linking and/or cleavage of single or double strand DNA. Besides cleavage of DNA strands, mitomycin C also generates DNA adducts\textsuperscript{43}, which may bind to hMutSa. Though there is no report describing bleomycin-related DNA adducts, it is possible to generate bleomycin-specific adducts or cleavage sites in DNA, which may be recognized by hMutSa. Neocarzinostatin is reported to generate DNA adducts\textsuperscript{44}, though no difference in cytotoxicity was observed. Perhaps cleavage action to DNA by the neocarzinostatin-chromophore is more rapid than other antibiotics\textsuperscript{45}. Actinomycin D, a DNA intercalator, which generates no DNA adduct showed no difference in cytotoxicity. The cytotoxicity of 5-FU and its metabolic analog, tegafur, were lower in HCT116 than in HCT116+ch3, indicating that 5-FU incorporated into the thymidine residue of a DNA strand may be recognized by hMutSa. Vinka alkaloids cause both depolymerization of mitotic interpolar microtubules and cell metaphase block\textsuperscript{46,47} and do not appear to result in direct DNA damage. Therefore, the mechanism responsible for MMR-deficient cell resistance to vinka alkaloids may differ from the DNA damaging agents mentioned above. Possibly, resistance is triggered by events downstream of the MMR signaling pathway, events which lead to arrest the cell cycle.

Unexpectedly, Ara C, chlorambucil, and epirubicin were significantly more cytotoxic to MMR-deficient cells. To our knowledge, anticancer drugs belonging in this category have not been reported previously. These DNA damaging agents inhibit DNA replication. Ara-C inhibits the action of DNA polymerase $\alpha$\textsuperscript{48}. Chlorambucil inhibits DNA replication by alkylation and induces $G_2$ phase arrest\textsuperscript{49,50}. Epirubicin acts as a DNA intercalator and its maximal cell kill occurs during the S phase of the cell cycle\textsuperscript{51}. Because no common target molecule or pathway is known, further detailed investigation is required. During the preparation of this manuscript, the modulating activity of aphidicolin, a DNA polymerase inhibitor, was observed in MMR-deficient cells\textsuperscript{52}. These agents appear to have utility in treating drug resistant tumor due to MMR deficiency either alone or in combination with less cytotoxic drugs such as cisplatin, 5-FU, and tegafur, which have been widely utilized in solid tumor chemotherapy.

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