Modes of actions of two type of anti-neoplastic drugs, dacarbazine and ACNU, to induce apoptosis

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O6-Methylguanine and O6-chloroethylguanine, which are the primary cytotoxic DNA lesions produced by 5-(3,3-dimethyl-1-triazeno)imidazole-4-carboxamide (dacarbazine) and 1-(4-amino-2-methyl-5-pyrimidyl)methyl-3-(2-chloroethyl)-3-nitrosourea (ACNU), respectively, can be repaired by O6-methylguanine-DNA methyltransferase (MGMT), coded by the MGMT gene. However, the two types of drugs exhibit different effects on cells defective in both MGMT and MLH1 functions, the latter being related to the cellular activity to recognize mismatched bases of DNA for inducing apoptosis. Human cells deficient in both MGMT and MLH1 are resistant to the killing effect of dacarbazine and exhibit an increased mutant frequency after treatment with dacarbazine. On the other hand, the double deficient cells are sensitive to the killing action of ACNU and there is no significant increase in ACNU-induced mutant frequency. A mismatch recognition complex, composed of MSH2, MSH6, MLH1, PMS2 and PCNA, is formed after exposing MGMT-deficient cells to dacarbazine, but not in cells treated with ACNU. In contrast, the phosphorylation of Chk1 efficiently occurs in cells treated with dacarbazine as well as with ACNU, the former being in MLH1-dependent manner, whereas the latter in MLH1-independent manner. Therefore, the signals delivered from different sources would merge at the step of Chk1 activation or at an earlier step, and the subsequent process leading to apoptosis appears to be common.

Introduction

Among the various types of drugs designed for use in cancer chemotherapy, many have the potential for alklylation. These chemicals alkylate DNA bases, thereby preventing the multiplication of rapidly growing tumor cells. These alkylating agents can be divided into at least three groups, according to their preferred sites of action and their ability to form interstrand cross-links in DNA (1–7). The first and the second type of drugs can be represented by 5-(3,3-dimethyl-1-triazeno)imidazole-4-carboxamide (dacarbazine) and 1-(4-amino-2-methyl-5-pyrimidyl)methyl-3-(2-chloroethyl)-3-nitrosourea (ACNU), respectively. There is also a third type of drug, which acts on DNA in a different way. The last type of drugs, represented by cyclophosphamide, yields 7-substituted guanine and other alkylated bases, which also make cross-links in the DNA. Reflecting the fact that the primary killing lesions induced by the first two classes of drugs are O6-substituted guanine residues, mice defective in O6-methylguanine methyltransferase are considerably more sensitive to the killing actions of these drugs than wild-type mice (8). The LD50 values for dacarbazine of Mgmt−/− and Mgmt−/− mice were 450 and 20 mg/kg body wt, while the values for ACNU were 70 and 14 mg/kg body wt, respectively. On the other hand, no significant difference was observed in the susceptibility of the two types of mice to cyclophosphamide.

Dacarbazine is a methylating drug used for chemotherapy in subjects with malignant melanoma, soft-tissue sarcoma, osteogenic sarcoma, neuroblastomas and Hodkgin’s disease (1). Following administration to the cell, this drug is converted into active forms, which subsequently methylate the DNA bases. The primary metabolic pathway of dacarbazine involves N-hydroxylation at one of its N-methyl residues and conversion to 5-(3-hydroxymethyl-3-methylimidazole-4-carboxamide (5). This metabolite is chemically unstable and will lose formaldehyde to generate the corresponding monomethyltriazenine, which can release the methylidyazionium ion. Consistent with this proposed pathway, the administration of dacarbazine to animals results in the formation of O6-methylguanine in the DNA of various tissues and in the proportions that would be expected if a methylidyazionium ion was acting as the ultimate methylating agent (9,10). ACNU, on the other hand, yields bulky lesions, which interfere the DNA replication. This drug has been used either alone or in combination with other agents for the treatment of brain tumors (2,4). Following cellular exposure, its chloroethyl group is transferred to the O6 position of guanine residues in the DNA. The O6-chloroethylguanine further reacts with the cytosine residue in the opposite strand, yielding an interstrand cross-link (1-(N6-deoxyctydyl)-2-(N6-deoxyguanosinyl)-ethane in the DNA (11).

Both dacarbazine and ACNU cause cell death, which is related to apoptosis (10,12). However, the processes leading to apoptosis appear to be different, reflecting the fact that the DNA lesions produced by the two types of drugs cause different effects on the cellular DNA synthesis. O6-Methylguanine, produced by dacarbazine, would allow progression of the DNA replication forks and, therefore, retard DNA replication, yields an O6-methylguanine–thymine mispair. It has been shown that such a mispair, synthesized in vitro, can be recognized by the mismatch recognition protein to activate ATR kinase (13). In contrast, O6-chloroethylguanine, a bulky lesion produced by ACNU, and its resulting cross-link would prevent DNA replication and transcription, which itself induces apoptosis (14). Important roles of O6-methylguanine-DNA methyltransferase (MGMT) and mismatch repair proteins on apoptosis induced by O6-methylguanine and O6-chloroethylguanine have been reported by several groups (15–22). In the present study, we have performed systematic analyses of cell survival, mutation induction and formation of mismatch recognition complex after treatment with dacarbazine and ACNU to reveal the initial steps of the apoptotic processes induced by the actions of the two types of anti-neoplastic drugs in human cells. The results herein obtained may provide useful information for determining the applicability of these drugs to patients with malignant tumors.

Materials and methods

Chemicals

Dacarbazine and ACNU were purchased from Kyowa Hakko Co. (Tokyo, Japan) and Sankyo Co., Ltd (Tokyo, Japan), respectively. These drugs were dissolved in Dulbecco’s modified Eagle’s medium (D-MEM) supplemented with NaHCO3, amino acids, vitamins and antibiotics (100 U penicillin/ml and 100 μg streptomycin/ml), neutralized with NaOH and sterilized by filtration.
through 0.45 μm filter unit (Millipore, Billerica, MA, USA) just before use. S9–cofactor set was obtained from Oriental Yeast Co., Ltd (Tokyo, Japan). The S9 mixture was prepared by adding one volume of S9 (microsomal fraction prepared from rat liver) to nine volumes of cofactor solution (MgCl2, KCl, glucose-6-phosphate, NADPH and NADH in sodium phosphate buffer, pH 7.4) as described previously (10). D-MEM and fetal calf serum (FCS) were obtained from ICN Biochemicals, Inc. (Solon, OH, USA) and HyClone (Logan, UT, USA), respectively. Penicillin and streptomycin were obtained from Invitrogen Corp. (Carlsbad, CA, USA). Amino acid- and vitamin-enriched media were obtained from Nissin Pharmaceutical Co., Ltd (Tokyo, Japan). 6-Thioguanine (6-TG) was obtained from Sigma (St.Louis, MO, USA).

**Cell lines**

D-MEM supplemented with 10% FCS was used for the cell culture, and the culture was usually maintained at 37°C in 5% CO2. Human cell lines, HeLa S3 (wild-type) and its methyltransferase-deficient derivative MR, were obtained from H.Yayakawa. SW48, a human tumor-derived cell line deficient in both MGMT and MLH1 expression, was purchased from American Type Culture Collection (Manassas, VA, USA).

**Survival of cells after drug treatment**

Approximately 300 cells were placed in D-MEM–10% FCS in a well, incubated at 37°C for 1 day and washed with phosphate-buffered saline. The cells were then treated with 2 mg/ml of D-MEM containing 10 mM S9 mixture and various concentrations of dacarbazine or ACNU for 4 h. After the medium was replaced by D-MEM–10% FCS, cells were incubated for 7 days. Cells were then fixed with 10% (v/v) formaldehyde and stained with 0.1% (v/v) crystal violet. Cell survival was expressed as a proportion of the colony number of drug-treated cells to that of non-treated cells. Each determination was performed at least three times and the data are presented as the mean ± SD.

**Small interference RNA transfection**

Small interference RNAs (siRNAs) corresponding to two regions of the MLH1 sequence, 5'-AAAGCTCTACACGCGTAAAT-3' (for M1) and 5'-AAAGTG-CATCTTTACCTCTTC-3' (for M2) and control RNA, 5'-AATTCCTCG-AAACGTTACGT-3' (Qiagen, Hilden, Germany). After culturing HeLa MR cells (1.5 × 10^5 cells per well) in 24-well plate for 1 day, the cells were transfected with 1 μg of each RNA, using the RNAiFect transfection reagent according to the manufacturer’s protocol (Qiagen Hilden, Germany). To assess the effects of these siRNAs, whole cell extracts were prepared as described (12), and 1.3 μg protein of cell extracts were used for immunodetection. The primary antibodies used for western blotting were anti-MLH1 (BD PharMingen, SanJose, CA, USA), anti-PMS2 (BD PharMingen, SanJose, CA, USA) and anti-glyceraldehyde-3-phosphate dehydrogenase (from Ambion, Austin, TX, USA) antibodies. Antigens transferred were detected by a D-MEM–10% FCS, cells were incubated for 7 days. Cells were then exposed to the two types of drugs, and their survivals were determined (Figure 1A and B). HeLa MR, which is deficient in MGMT activity, is considerably more sensitive to both dacarbazine and ACNU than the wild-type strain, HeLa S3. It is evident, therefore, that the human methyltransferase is capable of repairing O6-methylguanine and O6-chloroethylguanine, which are produced by dacarbazine and ACNU, respectively.

**SW48 cells, on the other hand, exhibit different responses to the two types of drugs. This cell line is as resistant to dacarbazine as it is wild-type HeLa S3, but it exhibits an increased sensitivity to ACNU. SW48 was originally isolated from human colorectal adenocarcinoma and is known to have defects in various gene functions. It has been shown that SW48 cells lack methyltransferase activity and are devoid of MLH1 as well as MSH6 proteins, due to the transcriptional silencing of the genes (24,25). In addition, this cell line carries mutations in other genes, including those for DNA polymerase δ and transforming growth factor-β type receptor (26,27). To resolve the complexity associated with the human tumor-derived cell line, we performed an RNA interference experiment. When two types of siRNAs (M1 and M2), which could bind to different regions of MLH1 mRNA, were applied to HeLa MR cells, the expression levels of the MLH1 protein in M1- and M2-treated cells were reduced to ~50% of the level of the control RNA-treated cells (Figure 2A). Almost the same degree of reduction was observed with PMS2, which forms a complex with MLH1, consistent with the previous observation that these proteins are unstable unless they form a complex (28,29). In addition, these reduced levels of MLH1 as well as PMS2 protein were retained for at least 4 days, while the survival
assays were conducted by exposing the cells to the two types of drugs at 2 days after siRNA treatment. Figure 2B and C show the survival of siRNA-treated HeLa MR cells following exposure to the two types of drugs. The methyltransferase-deficient HeLa MR cells demonstrated high levels of resistance to dacarbazine following application of siRNAs (M1 and M2). However, there is essentially no difference in sensitivities of M1/M2- and control RNA-treated cells to ACNU. This indicates that the MLH1 function is indeed required for cell death caused by dacarbazine, but not by ACNU. It is remarkable that the HeLa MR cells have acquired resistance to dacarbazine, even though significant amounts of MLH1 protein are still present after siRNA administration. This haploinsufficient nature of the MLH1 function will be addressed in the Discussion.

Mutagenic actions of dacarbazine and ACNU
To see if the MLH1 function is required for O6-alkylguanine-induced mutagenesis, we determined the frequencies of 6-TG-resistant mutants in MLH1-proficient HeLa MR and -deficient SW48 cells, after treatment with dacarbazine and ACNU. For each combination of cell types and drugs, three determinations were performed and the results are summarized in Figure 3. Even under normal conditions, SW48 cells showed a higher mutant frequency in comparison with HeLa MR.
cells, thus implying that SW48 cells would accumulate mutations, due to their inability for correcting errors associated with DNA replication. After treatment with dacarbazine, the mutant frequency of SW48 cells increases considerably, and the value reaches six times that of the same cells without drug treatment. In contrast, no significant increase in the mutant frequency was observed with SW48 cells treated with ACNU than with non-treated cells. These results suggest that dacarbazine-induced lesions can be processed through a pathway in which mismatch repair-related proteins are involved, whereas ACNU-induced cell death occurs without the involvement of these proteins.

Formation of a mismatch protein complex on damaged DNA
A protein complex composed of PCNA, MutSα (a heterodimer of MSH2 and MSH6) and MutLα (a heterodimer of MLH1 and PMS2) is formed on the chromosomal DNA, when human cells deficient in MGMT activity are exposed to relatively low doses of MNU (N-methyl-N-nitrosourea) (13,23). To determine if such a complex is formed in the cells treated with these two types of drugs, the following experiment was performed. HeLa MR cells stably expressing FLAG epitope-tagged PMS2 [HeLa MR (ePMS2)] were treated with dacarbazine or ACNU for 4 h and further incubated in a drug-free growth medium. At certain times, the cells were permeabilized with digitonin and then treated with 3,3′-dithiobis-sulfosuccinimidylpropionate to stabilize protein complexes. The chromatin fractions were prepared from these cells, and the mismatch recognition protein complex was immunoprecipitated with an anti-FLAG antibody. The immunoprecipitated materials were treated with a reducing agent to cleave the cross-linking and then subjected to SDS–PAGE, followed by the detection of each component by specific antibodies.

To compare the effects of dacarbazine and ACNU, the concentrations of the two types of drugs were adjusted to comparable lethal levels on methyltransferase-deficient HeLa MR cells. These values were estimated from the survival curves shown in Figure 1, where the LD_{50} of MR cells for dacarbazine is 0.33 mM, whereas the value for ACNU is 8.5 μM. The cells were therefore treated with 0.66 and 2 mM dacarbazine or 17 and 51 μM ACNU, which give two and six lethal hits, respectively.

Figure 4 shows the result of analyses of the PMS2-bound protein components associated with the chromatin DNA of HeLa MR cells treated with the two types of drugs. An association of MSH2, MLH1 and PCNA to FLAG-tagged PMS2 was observed in the cells exposed to dacarbazine. The association was more evident with samples prepared at 16 h after treatment, compared with those at 8 h, and larger amounts of proteins were bound with increasing concentrations of the drug. No comparable association of the protein components with PMS2 was observed when the cells were treated with ACNU, even at doses that yield the same lethal effects to the cells as those exerted by dacarbazine.
Phosphorylation of Chk1

The activation of ATR kinase causes the initiation of the signaling cascades, which thus leads to cell-cycle checkpoint and cell death in response to DNA replication fork block and DNA damage by alkylating agents (30–32). We therefore have examined whether this activation occurs in response to dacarbazine and ACNU by monitoring Chk1 phosphorylation. For this, HeLa MR cells deficient in the methyltransferase activity were exposed to 2 mM dacarbazine or 51 μM ACNU, doses sufficient to induce apoptosis. Measurement of the caspase-3 activity confirmed that apoptosis was indeed induced under these conditions. Three days after exposure to dacarbazine and ACNU, the levels of caspase-3 activity increased 2.4 and 2.2 times than that of untreated cells, respectively. The levels of phosphorylation of Chk1 protein was then measured, and the results are shown in Figure 5A. The amounts of phosphorylated Chk1 increase after treatment of cells with either type of drugs.

Furthermore, the dacarbazine-induced Chk1 phosphorylation occurs in HeLa MR cells, but not in SW48 cells, which lack both MGMT and MLH1 functions, whereas the ACNU-induced phosphorylation proceeds equally well in both HeLa MR and SW48 cells (Figure 5B). Therefore, the signals initiated from the two different types of DNA lesions, which are delivered in mismatch protein-dependent and -independent manners, appear to merge at the point of Chk1 phosphorylation or earlier (Figure 6).

Discussion

O6-Methylguanine and O6-chloroethylguanine, which are produced by dacarbazine and ACNU, respectively, can be repaired by the MGMT, coded by the MGMT gene (8,33,34). Indeed, human cell lines deficient in this methyltransferase activity are hypersensitive to lethal effects of both of these drugs. However, the two types of drugs exhibit different effects on cells defective in both MGMT and MLH1 functions, the latter being related to the cellular activity to recognize mismatched bases of DNA for their processing. It is remarkable that HeLa MR cells that received siRNA for MLH1 acquired high levels of resistance to dacarbazine, even though these siRNA-treated cells still contain considerable amounts of MLH1 protein (a half of the normal level). Such a haploinsufficient character of the MLH1 gene is also observed in gene-targeted mice as well as cells derived from such mice. Mgmt−/− Mlh1+/+ mice, which carry almost half the amount of MLH1 protein in comparison with Mlh1+/+ mice, are significantly more resistant to the killing action of MNU than Mgmt−/− Mlh1+/+ mice (35). A similar result was obtained with the cell lines derived from these mice (12). This haploinsufficient nature may be related to the fact that MLH1 protein forms complexes with one of its related molecules, such as PMS1, PMS2 and MLH3 (36,37). In conjugation with MutSb (MSH2/MSH6 heterodimer), MutLx (MLH1/PMS2 heterodimer) participates in the recognition of mismatched bases, whereas other forms of dimers, such as MLH1/MLH3 heterodimer, are presumably involved in repair of small insertion/deletion loops. It is possible that during the competition between MLH1 homologs for heterodimer formation, any changes in their concentrations could affect the activity of the heterodimer itself. The level of MLH1 expression may be important for both maintaining genomic stability and for controlling the induction of apoptosis after DNA damage.

When HeLa MR cells, which are deficient in the methyltransferase activity, are exposed to an alkylating agent MNU, a protein complex composed of PCNA, MutSα and MutLα is formed on the chromosomal DNA carrying O6-methylguanine (23). The in vivo formation of this complex occurs with the progression of DNA replication forks and it is inhibited by aphidicolin, a DNA polymerase α inhibitor, thus implying that an O6-methylguanine–thymine pair, which is formed from the O6-methylguanine–cytosine pair after one round of DNA replication, may be a target for making such a complex. This possibility is consistent with a recent in vitro study, which indicated that MutSα-containing complex binds to O6-methylguanine–thymine mispair but not to O6-methylguanine–cytosine (13,38). The present study also demonstrated that formation of the mismatch protein complex occurs in HeLa MR cells treated with dacarbazine, which yields O6-methylguanine as the primary cytotoxic lesion. No such complex was formed in the cells which had been exposed to comparatively lethal doses of ACNU.

O6-Chloroethylguanine and its cross-linking products, produced by ACNU, are bulky DNA lesions that would prevent the progression of DNA replication forks. The blockage of DNA replication by such

![Fig. 5. The phosphorylation of Chk1 protein. (A) Treatment of HeLa MR (ePMS2) cells with dacarbazine and ACNU. HeLa MR (ePMS2) cells were treated with or without 2 mM dacarbazine or 51 μM ACNU for 4 h. The cells were incubated in 10% FCS-containing medium at 37°C and collected at the times indicated. (B) Chk1 phosphorylation in HeLa MR and SW48 cells. The two types of cells were treated with or without 2 mM dacarbazine or 51 μM ACNU for 4 h and collected at 24 h after treatment. In both cases, cells were lysed with SDS–PAGE sample buffer containing phosphatase inhibitors, sonicated and then the cell extracts were prepared by centrifugation. Immunoblotting was performed with the use of antibodies that specifically recognize Chk1 and serine 317-phosphorylated Chk1 proteins, respectively. MR: HeLa MR and SW: SW48. + and − denote samples with and without dacarbazine and ACNU treatment, respectively.](https://academic.oup.com/carcin/article-abstract/28/12/2657/2476354)
lesions may be sensed by the ATM/ATR system (32), which thus activates the signaling cascades leading to the cell-cycle checkpoint and apoptotic pathways for cell death. The signaling cascades initiated by the replication-permitting DNA lesions, such as O6-methylguanine–thymine mispairs, are transmitted through the mismatch recognition complex and then are further delivered to specific receptors where the signal would merge with that initiated by DNA replication blockage. It was recently shown that after the treatment of cells with S1-type methylating agents, ATR kinase is activated, which cation blockage. It was recently shown that after the treatment of cells

Fig. 6. A model for the initial steps of apoptosis induced by ACNU and dacarbazine. MMR complex: mismatch recognition protein complex.

The involvement of mismatch repair-related proteins for dacarbazine-induced apoptosis raises the question as to the applicability of this type of drugs to patients, particularly those with tumors, which are deficient of these proteins. A considerably high proportion of tumors are deficient in MGMT or MLH1 protein and, in some cases, both gene products are absent due to transcriptional silencing (41). Tumors deficient in both of these proteins would exhibit a higher resistance to the monofunctional triazenes, such as dacarbazine, and patients with such tumors may bear an increased risk for secondary tumors after the administration of these drugs. Precautions, based on the level of expression of these genes, are particularly advisable when considering the clinical application of these drugs. In this regard, ACNU and related compounds may therefore be more dependable since the sensitivity of cells to this drug is not affected by the mismatch repair status. However, there remains a possibility that some other gene functions may affect the responsiveness of the cells to these drugs. In addition to O6-methylated DNA lesions, N-methylated DNA adducts, such as N3-methylguanine and N7-methyladenine, are also produced in DNA when treated with such chemotherapeutic agents. These lesions do not exhibit cytotoxicity because they are removed by base excision repair. Methoxyamine specifically inhibits the base excision repair process by stabilizing the abasic sites and preventing their processing by apurinic/apyrimidinic endonucleases, resulting in cell death (42). A recent study reported that methoxyamine enhances the therapeutic efficacy of some alkylating agents and that this drug also potentiates cytotoxicity regardless of the MGMT or the mismatch repair status of the cells (43). Further studies are clearly necessary in order to provide a sound basis of application for the chemotherapeutic agents to patients having tumors with different genetic and epigenetic status.

Funding

Ministry of Education, Culture, Sports, Science and Technology of Japan (including Frontier Research Grant).

References


Received April 25, 2007; revised July 20, 2007; accepted August 13, 2007

Apoptosis induced by dacarbazine and ACNU