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Different initial steps of apoptosis induced by two types of antineoplastic drugs

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ABSTRACT

O^6-Methylguanine and O^6-chloroethylguanine are primary DNA lesions produced by two types of antineoplastic drugs, 8-carbamoyl-3-methylimidazo[5,1-*d*]-1,2,3,5-tetrazin-4(3H)-one (temozolomide, TMZ) and 1-(4-amino-2-methyl-5-pyrimidinyl) methyl-3-(2-chloroethyl)-3-nitrosourea (ACNU), respectively. They can be repaired by O^6-methylguanine-DNA methyltransferase, coded by the *Mgmt* gene. Otherwise, these two types of lesions induce apoptosis in different ways. O^6-Chloroethylguanine blocks DNA replication thereby inducing apoptosis. On the other hand, O^6-methylguanine does not block DNA replication and the resulting O^6-methylguanine-thymine mispair is recognized by mismatch repair-related proteins, including MLH1, thereby inducing apoptosis. Reflecting this, mouse cells lacking both MGMT and MLH1 are resistant to TMZ, but not to ACNU. The translocation of phosphatidylserine in cell membrane as well as a change of mitochondrial transmembrane potentials occurred in an MLH1-dependent manner after treatment with TMZ, but no such MLH1 dependency was observed in the case of ACNU treatment. By using cell lines defective in both APAF-1 and MGMT, it was revealed that the APAF-1 function is required for execution of apoptosis induced by either TMZ or ACNU. There is almost 12 h delay in occurrence of apoptosis-related mitochondrial depolarization in TMZ-treated cells in comparison to those of ACNU-treated cells, reflecting the fact that at least one cycle of DNA replication is required to trigger apoptosis in the former case, but not in the latter.

1. Introduction

Various chemicals with alkylation capacity have been developed to treat subjects with malignant tumors. These chemicals exert profound cytotoxic effects on actively growing cells and this is the basic rationale for the use of these agents in cancer chemotherapy. Those antineoplastic drugs with alkylating capacity can be divided into several groups, according to their preferred sites of action and their ability to form interstrand cross-links in DNA [1]. Among them, the monofunctional triazenes and the bifunctional chloroethylnitrosoureas are most notable, because they produce O^6-substituted guanine residues as the primary killing lesions in DNA. The monofunctional triazenes can be represented by dacarbazine (5-(3,3-dimethyl-1-triazeno)imidazole-4-carboximide) and temozolomide (TMZ: 8-carbamoyl-3-methylimidazo[5,1-*d*]-1,2,3,5-tetrazin-4(3H)-one) which produce O^6-methylguanine with and without metabolic activation,

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respectively [2–6]. The chloroethylnitrosoureas, on the other hand, yield O^6-chloroethylguanine, which can form an interstrand cross-link in DNA. ACNU (1-(4-amino-2-methyl-5-pyrimidinyl) methyl-3-(2-chloroethyl)-3-nitrosourea) and BCNU (1,3-bis-(2-chloroethl)-1-nitrosourea) are examples of this type of drugs [7,8].

TMZ and ACNU were chosen to investigate the modes of action of these two types of drugs. Neither of which do require metabolic activation for their actions. TMZ is a newly introduced anti-tumor drug, which can be used for clinical treatment of brain tumors and metastatic melanomas [2,4,9,10]. TMZ is rapidly and completely absorbed after oral administration and undergoes spontaneous hydrolysis in aqueous solution at physiological pH to form its active metabolite, 5-(3-methyltriazene-1-yl)imidazole-4-carboxamine, which acts on DNA to produce O^6-methylguanine. In addition, this drug has advantages in clinical use, since the intact TMZ molecule easily crosses the blood brain barrier and is then activated in the brain compartment. ACNU, on the other hand, yields bulky lesions, which interfere with DNA replication. Following cellular exposure, its chloroethyl group is transferred to the O^6-position of guanine residues in DNA and this O^6-chloroethylguanine further reacts with the cytosine residue in the opposite strand to form an interstrand DNA cross-link. This drug has been used either alone or in combination with other agents for the treatment of brain tumors [7,8].

Both TMZ and ACNU cause apoptotic cell death. However, the processes leading to apoptosis appear different, reflecting the fact that the DNA lesions produced by the two types of drugs cause different effects on cellular DNA synthesis. O^6-Methylguanine, produced by TMZ, would allow progression of DNA replication fork and produce an O^6-methylguanine-thymine mispair. By using an *in vitro* reconstituted system, it was shown that MutSα, composed of MSH2 and MSH6 proteins, binds to such a mispair in DNA [11]. In cells exposed to this type of simple alkylating agents, a complex consisting of MutSα, MutLα (MLH1 and PMS2) and PCNA is formed, which is prerequisite for execution of apoptosis [12]. On the other hand, ACNU-induced O^6-chloroethylguanine, which prevents progression of DNA replication fork, induces apoptosis in a different way. The mismatch recognition complex is not formed in the cells treated with ACNU, and ACNU-induced apoptosis proceeds in the absence of MLH1, an essential component of the complex [3]. Therefore, the initial steps for apoptosis induced by the two types of DNA lesions are apparently different. This raises a question how signals initiated from base mispairing and those from DNA replication blockage activate downstream events for apoptosis. At least one cycle of DNA replication is required to form the O^6-methylguanine-thymine mispair, while apoptotic signal can be initiated once replication forks stumble over DNA lesions by ACNU. It is therefore conceivable that the occurrence of TMZ-induced apoptotic events would be delayed, in comparison to that for ACNU-induced event, which are triggered by blockage of DNA replication itself.

With these in mind, apoptosis-related events were investigated in the cells treated with TMZ and ACNU. To better comprehend the results, genetically defined mouse cell lines were used in these studies. For this, mouse lines defective in either one of the genes whose products may be involved in the prospective apoptotic pathway were constructed, and then the cell lines were established from such mice. One of the key factors in the lethal action of TMZ and ACNU is O^6-methylguanine-DNA methyltransferase, the product of *Mgmt* gene, since this enzyme eliminates O^6-methylguanine as well as O^6-chloroethylguanine [13–15]. Therefore, to provide the maximum efficacy for the cytotoxic actions of alkylating drugs, an MGMT-deficient character was further introduced into those mouse lines. These cells lines, were used to investigate mitochondrial permeabilization and activation of APAF-1 (apoptotic protease activating factor-1) in TMZ- and ACNU- induced apoptosis.

2. Materials and methods

2.1. Chemicals

TMZ, obtained from LKT Laboratories, Inc. (St. Paul, MN), was dissolved in DMSO and stored at −30 °C. ACNU was purchased from Kyowa Hakko Co. (Tokyo, Japan), dissolved in water and stored at −30 °C. The stock solution was added to serum-free Dulbecco's modified essential medium (D-MEM), immediately before use.

2.2. Cell lines

D-MEM supplemented with 10% fetal calf serum (FCS) was used for cell culture, which was maintained at 37 °C in 5% CO_2. Cell lines defective in the *Mgmt* and/or *Mlh1* gene were established from lung fibroblasts of corresponding gene-targeted mice [16]. These include YT100 ($Mgmt^{+/+}Mlh1^{+/+}$), YT100M ($Mgmt^{+/+}Mlh1^{+/+}$ cells overexpressing human MGMT protein), YT102 ($Mgmt^{-/-}Mlh1^{+/+}$) and YT103 ($Mgmt^{-/-}Mlh1^{-/-}$).

The generation of *Apaf-1* null-mutant mice has been described by Yoshida et al. [17]. By mating $Mgmt^{-/-}Apaf1^{+/+}$ mice with $Mgmt^{+/+}Apaf1^{+/-}$ mice, $Mgmt^{+/-}Apaf1^{+/-}$ mice were obtained. Finally, $Mgmt^{-/-}Apaf1^{+/-}$ mice were obtained by mating $Mgmt^{+/-}Apaf1^{+/-}$ mice. To prepare embryonic fibroblast cells carrying genotypes of $Mgmt^{-/-}Apaf1^{+/+}$ and $Mgmt^{-/-}Apaf1^{-/-}$, $Mgmt^{-/-}Apaf1^{+/-}$ mice were mated and an E14.5 embryo was removed. Small pieces of sliced embryo were placed in the medium, and cultured for several days. After several passages, pSVtsA58 ort(-), a transforming vector carrying cDNA for SV40 large T antigen (obtained from Riken DNA Bank, RDB#1126, http://www.rtc.riken.go.jp) was introduced into the primary cells by the $CaPO_4$ precipitation method (Mammalian Transfection Kit; Stratagene, LaJolla, CA). Cells that survived the 15th passage were considered immortal and used for these experiments. Genotypes of these cells were determined by PCR analysis, using appropriate primers described previously [17,18]. The established cell lines were designated YT105 ($Mgmt^{-/-}Apaf1^{+/+}$) and YT106 ($Mgmt^{-/-}Apaf1^{-/-}$).

2.3. Cell survival after drug treatment

Approximately 500 cells were seeded in a 100-mm dish and incubated at 37 °C for 1 day in 5% CO_2. The cells were washed

three times with phosphate-buffered saline (PBS), then incubated in serum-free D-MEM containing various concentrations of TMZ or ACNU for 1 h. After the medium was replaced with D-MEM with 10% FCS, the cells were further cultured for 5 days. The cells were then fixed with 10% (v/v) formaldehyde and stained with 0.1% (v/v) crystal violet. The number of colonies was counted and the survival rate was obtained by dividing the number of colonies of treated cells by that for untreated cells. Each determination was performed at least three times and the data are presented as means ± S.D.

2.4. Phosphatidylserine translocation

The cells were washed three times with PBS, then were incubated in serum-free D-MEM containing various concentrations of TMZ or ACNU for 1 h. After the medium was replaced with D-MEM containing 10% FCS, cells were harvested by trypsinization, as described above. After one wash with PBS, cells were stained with propidium iodide (PI) and annexin V, by using the Vybrant Apoptosis Kit#2 (InVitrogen Corp., Carlsbad, CA), according to the manufacturer's directions. The samples were analyzed using FACScan with 10,000 events per determination. Each determination was performed at least three times and the data are presented as means ± S.D.

2.5. Mitochondrial depolarization

About 2×10^5 cells were seeded in a 100-mm dish for flow cytometry analysis. The cells were washed three times with PBS, then incubated in serum-free D-MEM containing various concentrations of TMZ or ACNU for 1 h. The medium was replaced by D-MEM with 10% FCS, and the culture was further incubated at 37 °C. For mitochondrial membrane potential assay, fluorescence dye $DiOC_2(3)$ was used, based on the instruction for the MitoProbeTM $DiOC_2(3)$ assay kit (InVitrogen Corp., Carlsbad, CA) with some modifications. At various time points, the cells were stained with this dye (50 nM) for 30 min. After trypsinization (0.5 mg/ml trypsin, 0.53 mM EDTA), followed by the addition of trypsin inhibitor to stop the trypsinization, cells were washed once with PBS. The cells were then applied to flow cytometric assay, by using FACScan (Becton Dickinson, Franklin Lakes, NJ,), with 10,000 events per determination. Each determination was performed at least three times and the data are presented as means ± S.D.

3. Results

3.1. Requirement of the MLH1 function for TMZ-, but not for ACNU-induced cell death

Three types of cells were used, which were established from gene-targeted mice [16]. Since strain YT100, derived from wild-type mice, possesses a relatively low level of MGMT activity, strain YT100M, which carries cDNA for human MGMT, was used as *Mgmt*$^{+/+}$ cell. Strain YT102 is defective in the *Mgmt* gene, while strain YT103 is devoid of both *Mgmt* and *Mlh1* genes. Fig. 1 shows the survival of the three types of cells after treatment with TMZ and ACNU. *Mgmt*$^{-/-}$ cells exhibited increased levels of sensitivity to the two types of drugs in comparison to MGMT-proficient wild-type cells. This indicates that MGMT is capable of repairing O^6-chloroethylguanine produced by ACNU as efficiently as O^6-methylguanine formed by TMZ.

An interesting observation is the fact that the two drugs act on *Mgmt*$^{-/-}$*Mlh1*$^{-/-}$ cells in different ways. These cells are as resistant to TMZ as are wild-type cells, but exhibit the same high degree of sensitivity to ACNU as do *Mgmt*$^{-/-}$ cells. It seems that MLH1, a component of mismatch recognition complex, is required for apoptosis triggered by O^6-methylguanine whereas this protein is not concerned with the apoptotic process caused by O^6-chloroethylguanine. This implies that O^6-methylguanine and O^6-chloroethylguanine cause cell killing through different mechanisms.

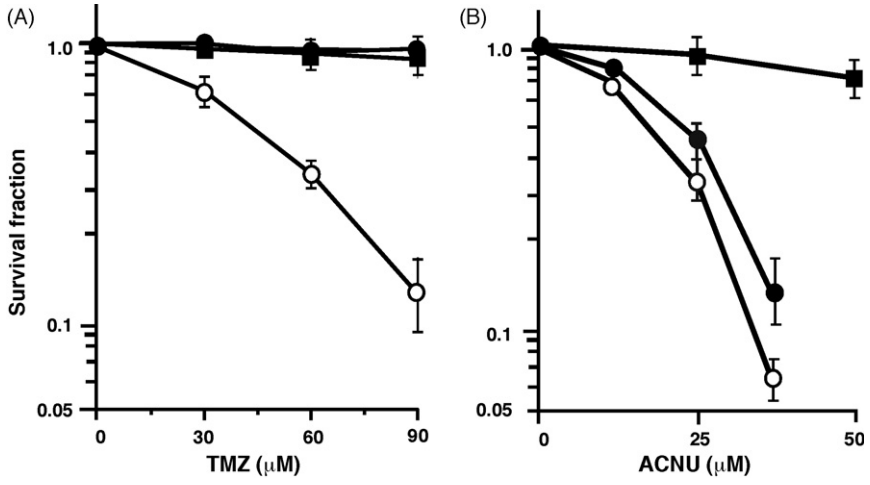

Fig. 1 – Sensitivity of *Mgmt*- and/or *Mlh1*-deficient cells to TMZ and ACNU. The cells with different genetic backgrounds were incubated in serum-free D-MEM containing various concentrations of TMZ or ACNU at 37 °C for 1 h, followed by continuous culture in drug-free D-MEM containing 10% FCS. The survival rates were determined after 5 days of culture. (A) Treated with TMZ. (B) Treated with ACNU. (■) YT100M (*Mgmt*$^{+/+}$*Mlh1*$^{+/+}$ cells overexpressing human MGMT); (○) YT102 (*Mgmt*$^{-/-}$*Mlh1*$^{+/+}$); (●) YT103 (*Mgmt*$^{-/-}$*Mlh1*$^{-/-}$).

The translocation of phosphatidylserine from the inner to the outer leaflet of the plasma membrane is a well-defined hallmark of apoptosis [19]. An annexin V binding assay was used to examine if this event occurs in cells carrying mismatched base pairs. In this analysis, annexin V and PI double negative cells are regarded as viable, and changes in this cell population after drug treatment were monitored. To compare the effects of TMZ and ACNU, the concentrations of the two types of drugs were adjusted to give comparable lethal hits to methyltransferase-deficient cells. Those concentrations were estimated from the survival curves shown in Fig. 1, where the LD_{37} of *Mgmt*$^{-/-}$ cells for TMZ is 58 μM while the value for ACNU is 23.7 μM. Cells were therefore treated with 348 μM TMZ or 142 μM ACNU, to give six lethal hits per cell.

As shown in Fig. 2A, an elevated level of phosphatidylserine translocation in response to TMZ was observed with *Mgmt*$^{-/-}$*Mlh1*$^{+/+}$ cells, but only a minor response was observed with *Mgmt*$^{-/-}$*Mlh1*$^{-/-}$ cells. On the other hand, ACNU-induced translocation occurred in both types of cells (Fig. 2B). These situations are more clearly shown in Fig. 2C and D, where the relative number of viable cells is plotted against the time after treatment. The apoptosis-related phosphatidylserine translocation induced by TMZ requires the MLH1 function and its induction becomes evident 24 h after treatment. In the case of ACNU, the event occurs as early as 12 h after treatment in an MLH1-independent manner.

3.2. *Loss of mitochondrial membrane potentials*

Based on significant role of mitochondria in the transduction of apoptotic signals [20–22], the involvement of mitochondria was further investigated in TMZ- and ACNU-induced apoptosis. The assay we adopted was to monitor the loss of its membrane potential using a fluorescence dye [23]. The loss of membrane potential is preceded by several apoptotic events such as the release of cytochrome *c* or the translocation of phosphatidylserine [20–22].

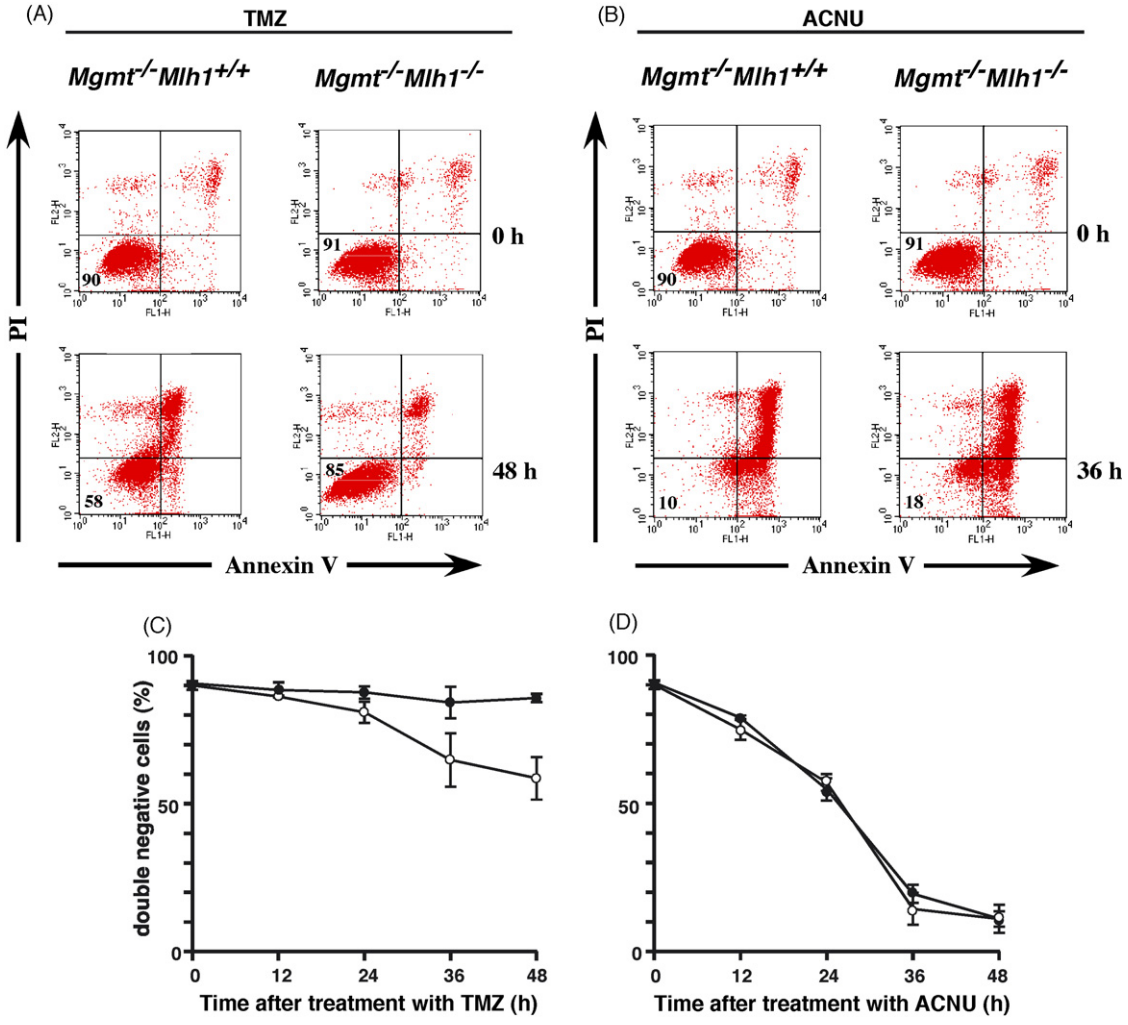

Fig. 2 – Phosphatidylserine translocation after drug treatment. The cells were incubated in serum-free D-MEM containing 348 μM TMZ or 142 μM ACNU at 37 °C for 1 h, followed by continuous culture in drug-free D-MEM containing 10% FCS. (A and B) Flow cytometric analyses were performed at various time points after treatment with TMZ (for A) and ACNU (for B). The horizontal axis shows annexin V staining and the vertical axis shows PI staining. The numbers in the left lower panels show percentages of annexin V and PI double negative cells. (C and D). Time courses of the changes of double negative cell population after treatment with TMZ (for C) and ACNU (for D). (○), YT102 (*Mgmt*$^{-/-}$*Mlh1*$^{+/+}$); (●), YT103 (*Mgmt*$^{-/-}$*Mlh1*$^{-/-}$).

Mlh1-proficient and deficient cells with a *Mgmt*$^{-/-}$ genotype were treated with 348 μM TMZ or 142 μM ACNU, as used for the annexin V binding assay (Fig. 2), and changes in mitochondrial membrane potentials were measured. Comparison of the results shown in Fig. 3A and B reveals that potential changes are induced after TMZ treatment in an MLH1-dependent manner while more rapid and extensive changes are caused by ACNU treatment in both *Mlh1*$^{+/+}$ and *Mlh1*$^{-/-}$ cells. When the number of depolarized cells was plotted against the time after drug treatment, the curves shown in Fig. 3C and D were obtained. It is evident that the MLH1-dependent process induced by TMZ, occurs 24 h after treatment, while the MLH1-independent process induced by ACNU, starts as early as 12 h after treatment.

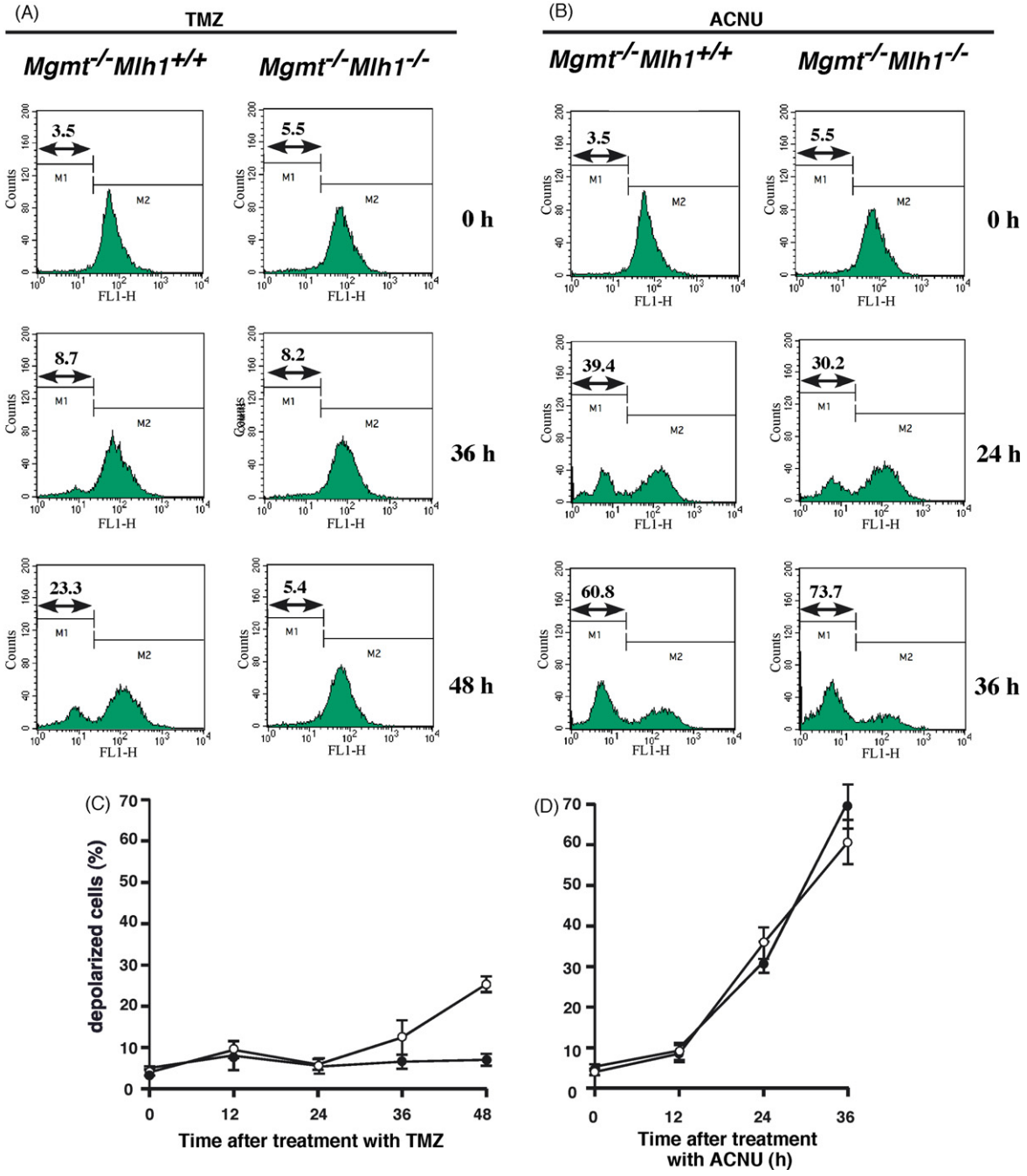

Fig. 3 – Changes of mitochondrial transmembrane potentials after drug treatment. The cells were incubated in serum-free D-MEM containing 348 μM TMZ or 142 μM ACNU at 37 °C for 1 h, followed by continuous culture in drug-free D-MEM containing 10% FCS. (A and B) The loss of membrane potential was evaluated by $DiOC_2(3)$ staining followed by flow cytometric analysis. The number in each panel shows the percentage of the cell population exhibiting a lower transmembrane potential (M1), defined as depolarized cells. (C and D). The time course of the changes of depolarized cells after treatment with TMZ (for C) or ACNU (for D). (○), YT102 (*Mgmt*$^{-/-}$*Mlh1*$^{+/+}$); (●), YT103 (*Mgmt*$^{-/-}$*Mlh1*$^{-/-}$).

3.3. *Involvement of APAF-1 in cell death caused by TMZ and ACNU*

APAF-1 is a scaffold protein which causes the activation of the caspase cascade. It binds to cytochrome *c* released from depolarized mitochondria, and forms a multi-protein complex, called an apoptosome, which initiates cleavage of procaspases [17,20]. To see if APAF-1 is required for TMZ- and ACNU- induced cell death, APAF-1-defective cells with an *Mgmt*$^{-/-}$ genotype were constructed. For this, *Mgmt*$^{-/-}$*Apaf-1*$^{+/-}$ mice were mated and E14.5 embryo was used to prepare embryonic fibroblast cells with the genotypes of *Mgmt*$^{-/-}$*Apaf-1*$^{+/+}$ and *Mgmt*$^{-/-}$*Apaf-1*$^{-/-}$. The use of early embryos was necessary since most of *Apaf-1*$^{-/-}$ mice (more than 90%) die prior to birth [17]. After several passages, SV40-derived transforming vectors were introduced into these primary cells. Cells which survived the 15th passage were considered immortal, and they were used as established cell lines, YT105 (*Mgmt*$^{-/-}$*Apaf1*$^{+/+}$) and YT106 (*Mgmt*$^{-/-}$*Apaf1*$^{-/-}$).

The sensitivity of these cell lines to TMZ and ACNU was determined by counting colony-forming units after exposure to various concentrations of the drugs. Fig. 4 shows that *Mgmt*$^{-/-}$*Apaf-1*$^{-/-}$ cells are more resistant to both types of drugs in comparison to *Mgmt*$^{-/-}$*Apaf-1*$^{+/+}$ cells, suggesting that the APAF-1 function is required for cell death induced by TMZ as well as by ACNU.

The requirement of APAF-1 function in apoptosis in these cells was further examined using the annexin V binding assay. Based on the survival curves, 288 μM TMZ and 102 μM ACNU, which correspond to six times of the LD_{37} values of *Mgmt*$^{-/-}$ cells, were applied to the two types of cells. As shown in Fig. 5, numbers of *Apaf-1*-proficient viable cells decrease in a fashion significantly faster than do those of *Apaf-1*-deficient viable cells, when treated by either TMZ or ACNU. Therefore, it is evident that the normal function of APAF-1 is required for execution of apoptosis triggered by the two types of alkylating drugs. It is noted that apoptosis induced by ACNU in *Mgmt*$^{-/-}$ cells, proceeds more rapidly than does the process triggered by TMZ, consistent with the results of mitochondrial potential change.

4. Discussion

Tumor-derived cell lines with a related gene mutation are often used to establish the role of a specific gene function(s) in a certain biological process. However, these cells usually carry multiple mutations and transcriptional alterations in various classes of genes. For instance, SW48, a cell line isolated from human colorectal adenocarcinoma, lacks O^6-methylguanine-DNA methyltransferase activity and is devoid of MLH1 as well as MSH6 proteins, due to transcriptional silencing of the genes [24,25]. This cell line also carries mutations in other genes, including those for DNA polymerase δ and transforming growth factor-β type receptor [26,27]. To avoid such complexity, cell lines isolated from gene-targeted mice carrying defined genetic characteristics have been used. These include YT102 (*Mgmt*$^{-/-}$*Mlh1*$^{+/+}$), YT103(*Mgmt*$^{-/-}$*Mlh1*$^{-/-}$) and YT100M (MGMT-overproducing wild-type cells), all of which were established in a previous study [16]. In the present study, two cell lines, YT105 (*Mgmt*$^{-/-}$*Apaf1*$^{+/+}$) and YT106 (*Mgmt*$^{-/-}$*Apaf1*$^{-/-}$), were further isolated from the embryonic tissues of mice with the corresponding characteristics.

Both O^6-methylguanine, which are produced by dacarbazine and TMZ, and O^6-chloroethylguanine formed by the action of ACNU and BCNU, can be repaired by the methyltransferase enzyme, coded by the *Mgmt* gene [13–15]. *Mgmt*$^{-/-}$ cells established from the gene-targeted mice are indeed hypersensitive to lethal effects of both types of drugs (shown in Figs. 1 and 2). However, the two types of drugs exhibit different effects on cells defective in both MGMT and MLH1 functions. The latter effects are related to the cellular ability to recognize mismatched DNA bases to induce apoptosis. *Mgmt*$^{-/-}$*Mlh1*$^{-/-}$ cells were as resistant to TMZ as are wild-type cells, but exhibit an increased sensitivity to ACNU. Similar results have been obtained with human cell lines with defects in related genes [12,28,29]. Of interest is our finding that a protein complex, composed of PCNA, MutSα, and MutLα, is formed on the chromosomal DNA carrying O^6-methylguanine [12]. The formation of this complex occurs with the progression of the DNA replication forks and it is inhibited by aphidicolin, a DNA polymerase α inhibitor. This suggests that an O^6-methylgua-

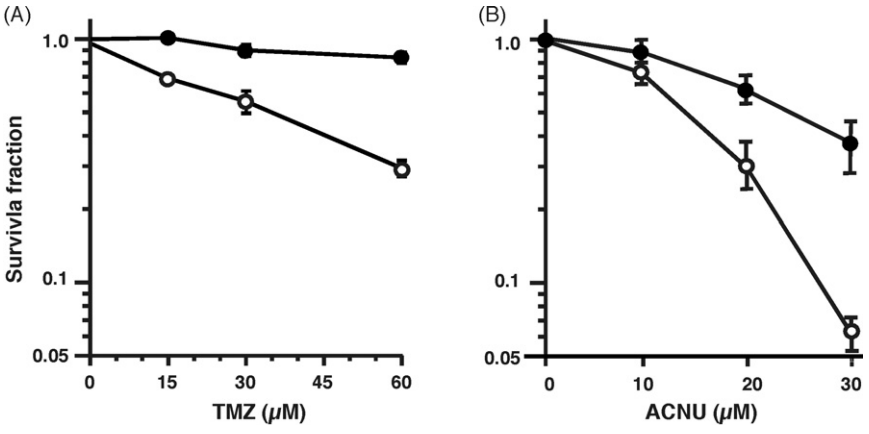

Fig. 4 – Sensitivity of *Apaf1*-deficient cells to TMZ and ACNU. The cells were incubated in serum-free D-MEM containing various concentrations of TMZ (for A) and ACNU (for B) at 37 °C for 1 h, followed by continuous culture in drug-free D-MEM containing 10% FCS. Thereafter, the survival rates were determined after 5 days of culture. (○) YT105 (*Mgmt*$^{-/-}$*Apaf1*$^{+/+}$); (●) YT106 (*Mgmt*$^{-/-}$*Apaf1*$^{-/-}$).

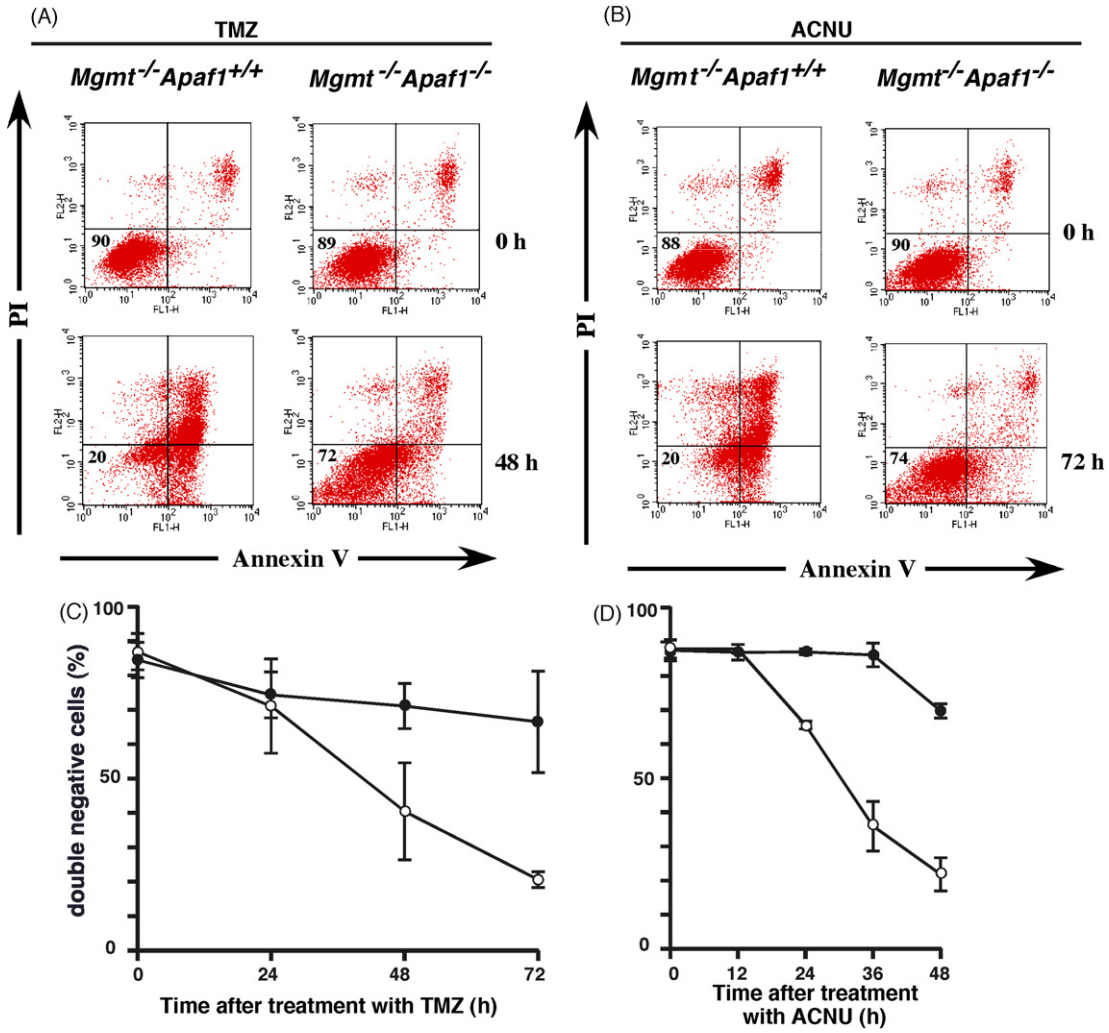

Fig. 5 – Phosphatidylserine translocation in *Mgmt*$^{-/-}$ and *Mgmt*$^{-/-}$*Apaf1*$^{-/-}$ cells after drug treatment. The cells were incubated in serum-free D-MEM containing 288 μM TMZ or 102 μM ACNU at 37 °C for 1 h, followed by continuous culture in drug-free D-MEM supplemented with 10% FCS. (A and B). Flow cytometric analyses were performed at various time points after treatment with TMZ (for A) and ACNU (for B). The horizontal axis shows annexin V staining and the vertical axis shows PI staining. The number in each panel shows the percentage of annexin V and PI double negative cells. (C and D). The time course of the changes in the viable cells treated by TMZ (for C) and by ACNU (for D). (○), YT105 (*Mgmt*$^{-/-}$*Apaf1*$^{+/+}$); (●), YT106 (*Mgmt*$^{-/-}$*Apaf1*$^{-/-}$).

nine-thymine pair, which is formed from the O^6-methylguanine-cytosine pair after one round of DNA replication, may be a target for the formation of such a complex. This is consistent with the results of *in vitro* study, which indicated that a MutSα-containing complex binds to an O^6-methylguanine-thymine mispair but not to an O^6-methylguanine-cytosine pair [11]. Furthermore, a mismatch protein complex is formed in cells treated with dacarbazine, which yields O^6-methylguanine, but not in cells exposed to ACNU [3].

O^6-Chloroethylguanine and its cross-linking products, produced by ACNU and BCNU, are bulky DNA lesions that prevent the progression of DNA replication forks. The blockage of DNA replication by such lesions may be sensed by the ATM/ATR system [30], which then activates the signaling cascades leading to the cell-cycle checkpoint and apoptotic pathway for cell death. On the other hand, the signaling cascades initiated by replication-permitting DNA lesions, such as O^6-methylguanine-thymine mispair, may be transmitted through the mismatch recognition complex and further delivered to specific receptors where the signal would merge with the process initiated by DNA replication block. It has recently been shown that after treatment of cells with Sn1-type methylating agents, ATR kinase is activated, which then leads to the phosphorylation of Chk1 protein [31]. Since phosphorylation of Chk1 occurs in HeLa MR cells after treatment with either dacarbazine or ACNU [3], it is suggested that the signals delivered from the two sources would merge at this or an earlier step. The present study, using well defined mouse cells lines, revealed that translocation of phosphatidylserine from the inner to the outer leaflet of the plasma membrane as well as depolarization of mitochondria are induced after treatment with TMZ and ACNU. In addition, the

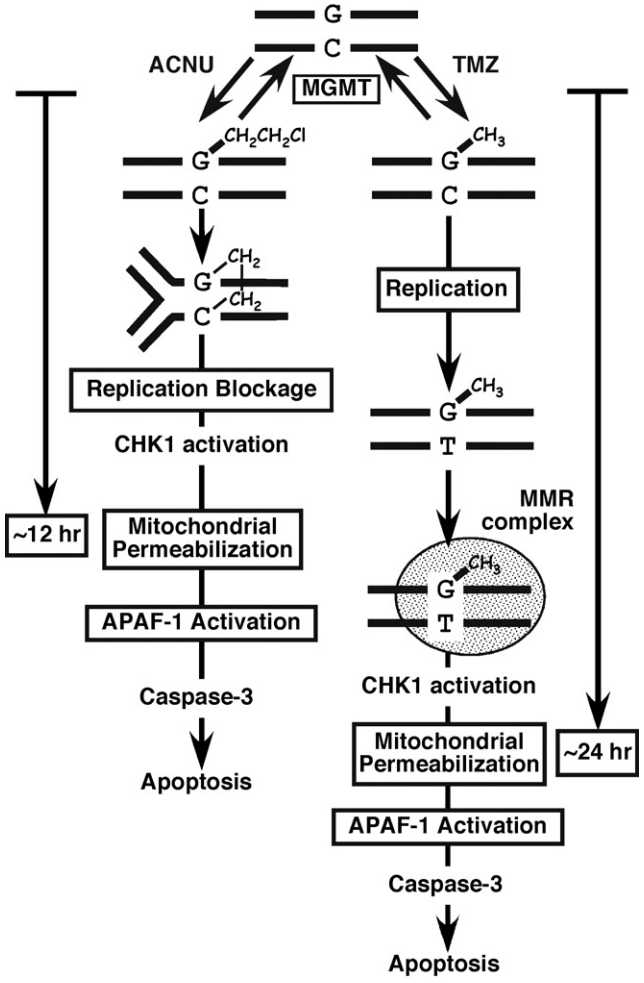

Fig. 6 – A model for the initial steps of apoptosis induced by ACNU and TMZ. MMR complex: mismatch recognition protein complex.

normal function of APAF-1 is required for excursion of apoptosis triggered by the two types of alkylating drugs. It has been shown that activation of caspase-3, which is hallmark of apoptosis induced by blocking DNA synthesis, also occurs in the O^6-methylguanine mediated apoptosis created by alkylating carcinogen [16,32,33].

Tomic et al. [34] reported that *Apaf-1* knockout mouse fibroblasts are more resistant to *N*-methyl-*N*-nitro-*N*-nitosoguanidine in comparison to wild-type fibroblasts under conditions where the methyltransferase activity is inhibited by O^6-benzylguanine. To avoid the complexity caused by the use of an inhibitor, we constructed a cell line defective in both *Mgmt* and *Apaf-1* genes and used them to determine if the APAF-1 function is indispensable for O^6-methylguanine-mediated apoptosis. These results showed that $Mgmt^{-/-}Apaf1^{-/-}$ cells are considerably more resistant to TMZ as well as ACNU than are $Mgmt^{-/-}Apaf1^{+/+}$ cells. The requirement of APAF-1 function in apoptosis caused by the two types of agents was further demonstrated using the annexin V binding assay.

Changes in mitochondrial transmembrane potential as well as APAF-1 activation induced by ACNU became evident around 12 h after treatment while those induced by TMZ required at least 24 h for their appearance. This time difference may be related to the mechanisms of initiation of apoptotic reactions induced by the two types of agents. It is thought that at least one round of DNA replication is required for formation of O^6-methylguanine-thymine mispair, the target of signaling molecules for TMZ-induced apoptosis, whereas blockage of DNA replication occurs as soon as the replication fork encounters a bulky DNA lesion.

These findings can be summarized in a model as shown in Fig. 6. The precise details of this mechanism remain obscure, particularly those for damage recognition and the following initiation steps for the two different apoptotic pathways. It is necessary to identify proteins involved in these steps, and studies with this in mind are presently in progress in this laboratory.

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