PCNA–MutSα-mediated binding of MutLα to replicative DNA with mismatched bases to induce apoptosis in human cells

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ABSTRACT

Modified bases, such as O6-methylguanines, are produced in cells exposed to alkylating agents and cause apoptosis. In human cells treated with N-methyl-N-nitrosourea, we detected a protein complex composed of MutSα, MutLα and PCNA on damaged DNA by immunoprecipitation method using chromatin extracts, in which protein–protein interactions were stabilized by chemical crosslinking. Time course experiments revealed that MutSα, consisting of MSH2 and MSH6 proteins, and PCNA bind to DNA to form an initial complex, and MutLα, composed of MLH1 and PMS2, binds to the complex when the DNA is damaged. This sequential mode of binding was further confirmed by the findings that the association of PCNA–MutSα complex on chromatin was observed even in the cells that lack MLH1, whereas in the absence of MSH2 no association of MutLα with the chromatin was achieved. Moreover, reduction in the PCNA content by small-interfering RNA or inhibition of DNA replication by aphidicolin, an inhibitor of DNA polymerase, significantly reduced the levels of the PCNA–MutSα–MutLα complex and also suppressed an increase in the caspase-3 activity, a hallmark for the induction of apoptosis. These observations imply that the induction of apoptosis is coupled with the progression of DNA replication through the action of PCNA.

INTRODUCTION

Base mismatches are constantly produced at low levels during the normal process of DNA replication and cause base substitution mutations if they are not repaired. The occurrence of such events is accelerated when the cells are exposed to certain agents that modify DNA bases. O6-methylguanine, produced by the action of alkylating agents, is one such lesion; it can pair with thymine as well as cytosine during DNA replication, leading to a G·C to A·T transition (1,2). To prevent such outcomes, organisms from bacteria to human are equipped with a specific DNA repair enzyme, O6-methylguanine-DNA methyltransferase, which transfers a methyl group from the O6-methylguanine moiety of the alkylated DNA to its own molecule, thereby repairing the DNA lesion in a single-step reaction (3,4). MGMT−/− mice, which are defective in the methyltransferase gene, are hypersensitive to the killing effect of alkylating agents and, when administered sublethal doses of the agents, produce a large number of tumors in their organs (5,6). In these mice, the damage by the alkylating agents is confined to tissues with rapidly growing cells, such as those in the bone marrow and intestinal mucosa, indicating that cell proliferation is required for killing the cells containing O6-methylguanine in the DNA. Studies with MGMT−/− cells further revealed that the O6-methylguanine-induced cell death occurs by apoptosis, which requires at least one round of DNA replication for its induction (7–9).

Another notable feature of O6-methylguanine-induced apoptosis is the involvement of mismatch repair proteins in the process. Mismatch repair has been defined as a mechanism to correct replication errors, including mismatched bases and small insertions/deletions, by excising the error-containing region of the newly replicated strand, followed by repair synthesis and strand joining. The molecular mechanism of mismatch repair has been well characterized in Escherichia coli, in which at least 11 enzymes or protein components are involved (10). Among them, MutS and MutL play important roles in recognizing mismatched regions and initiating the repair reactions. Many mammalian counterparts of MutS and MutL have been found, among which MutSα, a heterodimer composed of the MutS homologs MSH2 and MSH6, and MutLα, a dimer composed of the MutL homologs MLH1

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and PMS2, are regarded as being essential for the recognition of base mismatches (11–14). Early studies with cell lines derived from human tumors revealed that the lack of some of these gene functions rendered O6-methylguanine-DNA methyltransferase-deficient cells resistant to alkylating agents (15,16). Although tumor-derived cells would possess mutations and exhibit transcriptional silencing of some other genes, recent studies with a more defined system, namely MGMT−/− MLH1−/− cells derived from the gene-targeted mice, have clearly shown that the mismatch repair-related function is indeed required for the execution of apoptosis triggered by O6-methylguanine (17,18).

In eukaryotic cells, the mismatch repair proteins appear to be involved in at least two different processes, one to repair replication-associated errors in a strand-specific manner and the other to signal for inducing apoptosis in cells with mismatched DNA bases. This was clearly shown by recent findings that certain MSH2 or MSH6 missense mutations can cause a deficiency in mismatch repair, whereas retaining the signaling functions that confer sensitivity to chemotherapeutic agents (19,20). The molecular mechanism of the former process, mismatch repair, has been elucidated by analyzing the interactions and reconstructing the protein complex in vitro (21,22). However, studies on the mechanism of the latter process, modified base-induced apoptosis, have been hampered mainly by the lack of appropriate systems for investigating the process occurring in cells. In this regard, human cell lines defective in the MGMT gene would be noteworthy, since the exposure of the cells to simple alkylating agents would induce a large number of O6-methylguanine lesions in the chromosomal DNA, which would trigger apoptosis. The O6-methylguanine thus formed would persist in the DNA through the progression of the cell cycle and, hence, the conditions and timing required for the formation of an apoptosis-related protein complex could be followed in the cells. Moreover, by comparing the results obtained with O6-methylguanine-DNA methyltransferase-proficient and -deficient cells, it is possible to ascertain if the molecular events observed are related to O6-methylguanine (23,24).

Taking advantage of this system, we have investigated an early step of apoptosis triggered by O6-methylguanine. Since the protein complex formed on O6-methylguanine-containing DNA is unstable, to stabilize the multi-protein complex formed on the chromatin, we treated cells undergoing the early process of apoptosis with a protein crosslinking agent. After the isolation of the complex, the crosslinks were cleaved and its components were analyzed. In this way, we were able to determine the nature of the complex and resolve the conditions and timing required for its formation.

MATERIALS AND METHODS
Cell lines and cell culture
Human cell lines, HeLa S3 (wild-type), and its O6-methylguanine-DNA methyltransferase-deficient derivative, HeLa MR (25), were obtained from H. Hayakawa (Kyushu University, Fukuoka, Japan). SW48, a human tumor-derived cell line deficient in both Mgmt and Mlh1 expression, was purchased from American Type Culture Collection. LoVo, a human colorectal adenocarcinoma cell line deficient in Msh2 (26), was obtained from S. Oda (National Kyushu Cancer Center, Fukuoka, Japan). The HeLa MR line stably expressing FLAG epitope-tagged PMS2 was constructed as follows. A DNA fragment encoding the hPMS2 protein was amplified by PCR using pcDNA3.1/V5-His-hPMS2 plasmid DNA (27), obtained from S. Fukushige (Tohoku University School of Medicine, Miyagi, Japan), as a template and was inserted into the HindIII–BamHI site of the p3xFLAG-CMV-10 expression vector (Sigma). The resulting plasmid, named p3xFLAG-hPMS2, was digested with Scal, and the linearized DNA was introduced into HeLa MR cells by electroporation using a Gene pulser (Bio-Rad). Stable transfectants were selected as G418 (700 µg/ml) resistant colonies, and a line expressing the FLAG-tagged hPMS2 protein was isolated and designated as HeLa MR (ePMS2). These cells were cultured in DMEM supplemented with 10% fetal bovine serum (FBS) and antibiotics at 37°C in a 5% CO2 atmosphere.

Chemicals
N-Methyl-N-nitrosourea (MNU) and 3,3′-dithiobis-sulfosuccinimidylpropionate (DTSSP) were obtained from Nacalai Tesque Inc. (Kyoto, Japan) and Pierce, respectively. O6-benzylguanine and aphidicolin were purchased from Sigma.

Preparation of whole cell extracts
The cells were washed twice with phosphate-buffered saline (PBS) and scraped from the dish. The collected cells by centrifugation at 3000 g for 5 min at 4°C were suspended with 2× SDS–PAGE sample buffer [120 mM Tris–HCl (pH 6.8), 4% SDS, 20% glycerol, 200 mM DTT and 0.002% bromophenol blue], sonicated and boiled. The material was centrifuged at 20,000 g for 10 min, and the supernatant fraction was taken as the whole cell extract.

Preparation of chromatin extracts
Approximately 3 × 10^6 cells were seeded in a 10 cm dish and incubated for 1 day. The cells were washed twice with PBS and then treated with 1 mM MNU in serum-free DMEM for 1 h. After the medium was replaced with DMEM containing 10% FBS, the cells were incubated for the appropriate duration. The cells were permeabilized and washed extensively on the dish with ice-cold buffer A [20 mM HEPES–KOH (pH 7.9), 5 mM KCl, 1.5 mM MgCl2, 0.25 M sucrose and 0.1 mM EGTA] containing 50 µg/ml of digitonin (Wako) and protease inhibitors (Roche), and then were treated with 1 mM DTSSP (Pierce) for 2 h at 4°C. The crosslinking reaction was stopped by the addition of 50 mM Tris–HCl (pH 7.5). The cells were scraped from the dish and collected by centrifugation at 3000 g for 10 min at 4°C. The cell pellet was suspended in ice-cold buffer B [20 mM Tris–HCl (pH 8.0), 0.1 M NaCl, 1.5 mM MgCl2, 10% glycerol and 0.1% Triton X-100] containing protease inhibitors and then was sonicated. The material was centrifuged at 20,000 g for 15 min at 4°C, and the supernatant fraction was taken as the chromatin extract.

Immunoprecipitation and western blotting
For the immunoprecipitation of the FLAG-tagged PMS2-bound proteins, 10 µl of anti-FLAG M2-agarose (Sigma) were added to 1 ml of chromatin extract, prepared as described
above, and incubated for 4 h at 4°C. After extensive washings of the beads with buffer B, the proteins bound to the beads were eluted in 40 μl of buffer B containing 200 μg/ml of 3x FLAG peptide (Sigma). To immunoprecipitate the proteins bound to MSH2 or PMS2, 10 μl of anti-MSH2 (Zymed) or anti-PMS2 antibody conjugated with Protein G–Sepharose (Amersham Biosciences) were used as the secondary antibody. Rabbit IgG conjugated to horseradish peroxidase (Amersham (BD Biosciences), anti-PCNA (Santa Cruz Biotechnology) antibodies used were as follows: anti-FLAG M2 (Sigma), anti-MLH1 (BD Biosciences), anti-MSH2 (Zymed), anti-MSH6 (BD Biosciences), anti-PCNA (Santa Cruz Biotechnology) and anti-Histone H3 (abcam). Anti-mouse IgG and anti-rabbit IgG conjugated to horseradish peroxidase (Amersham Biosciences) were used as the secondary antibody.

siRNA transfection

siRNA for the PCNA sequence (siPCNA), 5’-AAGCACCACAAACCAAGGAGAAAG-3’, was purchased from Qiagen. After culturing 1 × 10⁶ cells in a 10 cm dish for 1 day, the cells were transfected with 40 nM of siRNA, using the Lipofectamine2000 reagent (Invitrogen) according to the manufacturer’s protocol. For the control transfection, the negative control siRNA (Qiagen) was used.

Assay of caspase-3 activity

One day after the siRNA transfection, the cells (1–5 × 10⁵) were seeded in a 6 cm dish and incubated at 37°C for 1 day. The cells were treated with 1 mM MNU for 1 h and incubated for another 3 days. The cell lysate preparation and the caspase activity assay were performed, according to the instructions in the EnzChek caspase-3 assay kit #2 (Molecular Probes). To assay the caspase-3 activity, the rate of hydrolysis of the synthetic substrate Z-DEVD-R110 was measured in the presence or absence of the inhibitor, Ac-DEVD-CHO. The hydrolysis products were quantified using a spectrofluorometer, Fusion α (PerkinElmer), with excitation at 496 nm and emission at 520 nm. The values obtained with the inhibitor were subtracted from those obtained without the inhibitor to express the specific activity of caspase-3.

Flow cytometric analysis

Cells (1 × 10⁶) on a 10 cm dish were harvested by a 0.25% trypsin-0.02% EDTA treatment, washed with PBS and suspended in 400 μl of PBS containing 0.1% Triton X-100, 25 μg/ml of propidium iodide and 0.1 mg/ml of RNase A. Samples were analyzed using a FACS Calibur flow cytometer (Becton Dickinson), with 10,000 events per determination.

RESULTS

Formation of a protein complex on O⁶-methylguanine-containing DNA

Accumulating evidence suggests that an initial step in the apoptosis induced by O⁶-methylguanine would be the binding of mismatch recognition proteins to the DNA with the lesion. However, it was difficult to isolate such a protein–DNA complex from the cells, since it is unstable and decomposed during ordinary extraction procedures. To overcome this difficulty, we treated the cells with a crosslinking agent, before the materials were isolated. The cells were permeabilized with digitonin and then treated with DTSSP, to stabilize the complex. After sonication of the cells, the chromatin was extracted and then the crosslinks were cleaved by a treatment with a reducing agent. In this way, we were able to resolve the components of the complex and follow the conditions needed for its formation.

O⁶-methylguanine, which is produced in DNA by treating cells with simple alkylating agents, can be repaired by a specific DNA repair enzyme, O⁶-methylguanine-DNA methyltransferase. HeLa MR cells are defective in this enzyme and readily undergo apoptosis after exposure to relatively low doses of MNU. We used this cell line and its parental line, HeLa S3, which is proficient in the methyltransferase activity, to determine whether a protein–DNA complex is formed in an O⁶-methylguanine-dependent manner. The two types of cells were treated with 1 mM MNU for 1 h and incubated in MNU-free growth medium for another 11 h, and then the cells were collected for examination of the protein complexes. From the same amounts of chromatin fractions prepared from each sample, DTSSP-linked protein complexes were immunoprecipitated with the use of an anti-MSH2 monoclonal antibody, and then the protein components were dissociated by the treatment with the reducing agent. The materials thus obtained were subjected to SDS–PAGE, followed by the detection of each component by specific antibodies.

Figure 1 shows analyses of the MSH2-bound protein components on the chromatin DNA of the two types of cells, with or without MNU treatment. In HeLa MR cells without MNU treatment, certain amounts of MSH6 and PCNA were co-immunoprecipitated with MSH2, indicating that low, but significant amounts of Msh6 and PCNA are associated with the chromatin, even under normal conditions. When the cells were exposed to MNU, the association of these components with the chromatin increased, and moreover, new bands, corresponding to MLH1 and PMS2, appeared. Since only very low signals for these proteins were detected in untreated cells, it is likely that the association of MutLα with the chromatin is strictly dependent on the formation of O⁶-methylguanine in the DNA. This notion is supported by the results obtained with HeLa S3 cells, in which the O⁶-methylguanine is repaired efficiently by its intrinsic repair enzyme activity. With and without MNU treatment, there were no significant changes in the patterns of these protein bands.

Association of the protein components with chromatin after MNU treatment

To address this hypothesis for the complex formation on the damaged chromosome in detail, we constructed HeLa MR
and FLAG-tagged PMS2, the components of MutL (Figure 2C and D), dramatic increases in the levels of MLH1 and PMS2, the latter of which was FLAG-tagged, in the complex increased rapidly and concomitantly up to 12 h after the MNU treatment (Figure 3A and B). It seems that certain amounts of MutSα and PCNA are associated with the normal form of the chromatin, and when the DNA is damaged, MutLα, composed of MLH1 and PMS2, is further bound to the complex.

To monitor the time course of formation of the complex, the chromatin-associated PMS2 was immunoprecipitated with the use of its FLAG-tag, and the components of the mismatch protein complex were examined. As shown in Figure 3C and D, relatively small amounts of PMS2 were present on the chromatin without the MNU treatment, and the associations of the other components with PMS2 were hardly detected. After the MNU treatment, the amounts of the PMS2-associated forms of the protein components increased dramatically and reached a maximum level at the 12 h of treatment, consistent with the observation made in the preceding experiment (Figure 3B). Thus, the sequential binding of MutSα and MutLα was indicated by the reciprocal immunoprecipitation experiments using the chromatin extracts.

Mutations affecting the complex formation

To confirm this notion, we examined the complex formation in various cell lines with defects in some of the mismatch repair proteins. The cell line SW48, derived from a human colorectal adenocarcinoma, is deficient in $O^6$-methylguanine-DNA methyltransferase activity and lacks the MLH1 protein, owing to transcriptional silencing of the genes (29,30). In immunoprecipitation assays using the same amount of chromatin extracts from cells treated with or without MNU, the MNU-induced interactions of MSH2 with the components of MutSα, MLH1 and PMS2, were detected in the HeLa MR, but not in the SW48 (Figure 4A). Moreover, the interaction between MSH2 and PCNA was observed, regardless of the MNU treatment, in SW48 as well as in HeLa MR. These findings indicate that the MLH1 function is dispensable for the interaction between MutSα and PCNA on chromatin.

Suppression of the complex formation was observed with the cell line LoVo, which is defective in the MSH2 function. Since this cell line has functional $O^6$-methylguanine-DNA methyltransferase, the experiment was performed with the use of $O^6$-benzylguanine, a specific inhibitor of the methyltransferase (31,32). In the presence of $O^6$-benzylguanine, methyltransferase-proficient HeLa S3 cells, which are otherwise defective in MNU-induced complex formation (Figure 1), were able to produce the complex in response to the MNU treatment. Under the same conditions, LoVo cells showed no response to the MNU treatment (Figure 4B). These results indicate that the complex formation is indeed triggered by the $O^6$-methylguanine lesions produced on the chromosomal DNA and also that the association of MutLα with the chromatin is MutSα-dependent.

Role of PCNA in $O^6$-methylguanine-induced apoptosis

The results of these experiments indicated that PCNA is concomitantly associated with MutSα on normal chromatin
and further binds to MutLα to form a PCNA–MutSα–MutLα complex upon MNU treatment of the cells. To determine whether PCNA is prerequisite for the complex formation and if such a complex is involved in the progression of apoptosis, we employed the RNA interference method (33). For this, siPCNA, a siRNA specific for a part of the PCNA mRNA sequence, and siCont., a negative control RNA, were synthesized and introduced into HeLa MR (ePMS2) cells. At 48 h after the application of siPCNA, the cellular level of PCNA decreased to 39% of the level attained with cells that received the siCont., whereas the expression levels of other mismatch-related proteins were not affected (Figure 5A). Then, the siPCNA-treated cells and control cells were exposed to 1 mM MNU, and the chromatin extracts were prepared after 12 h for immunoprecipitation. Under PCNA knockdown conditions, the loading of the mismatch repair proteins to chromatin was severely inhibited (Figure 5B). A significantly lower amount of FLAG-PMS2 was immunoprecipitated with an anti-FLAG antibody, as compared with that of the control cells (Figure 5C). Simultaneously, the amounts of the PMS2-associated forms of MSH2 and MLH1 decreased to 60% (for MSH2) and 47% (for MLH1) of levels for the control, respectively. Thus, PCNA is actually required for the recruitment of the mismatch repair proteins to chromatin and for the formation of the mismatch recognition complex on the damaged chromosome. Similar observation was reported in a cell-free reconstituted system, by using purified PCNA, MutSα (MSH2/MSH6) complex and mismatch-containing heteroduplex DNA (34).

Caspase-3, a member of the cysteine protease family, is induced during the course of apoptosis, induced by TNF, gamma rays and many other means (35). This is also the hallmark of the progression of apoptosis triggered by O6-methylguanine: caspase-3 activation occurred in Mgmt-deficient cells, but not in cells defective in both Mgmt and Mlh1, when these cells were treated with MNU (18). Therefore, we next examined whether the caspase-3 induction in HeLa MR (ePMS2) cells exposed to MNU is affected by the PCNA knockdown. The result shown in Figure 5D indicates that the treatment of the cells with siPCNA significantly

Figure 2. Association of the protein components with chromatin after MNU treatment. (A) HeLa MR (ePMS2) cells were treated with or without 1 mM MNU for 1 h and collected at the indicated times and the whole cell extracts were prepared as described in Materials and Methods. Immunoblotting was performed by the use of antibodies that specifically recognize MSH2, MSH6, MLH1, FLAG-PMS2, PCNA and β-actin. β-Actin is a loading control. (B) The intensities of the bands for each protein, shown in (A), were quantified, and the relative intensities as compared with that at 0 h are plotted. Open squares, MSH2; open circles, MSH6; closed squares, MLH1; closed circles, FLAG-PMS2; and open triangles, PCNA. (C) HeLa MR (ePMS2) cells treated in the same way in (A) were collected and the chromatin extracts were prepared as described in Figure 1. The amounts of proteins bound to chromatin were analyzed by immunoblotting using specific antibodies against mismatch repair proteins, PCNA and histone H3. Histone H3 is a loading control. (D) The relative intensities of the bands for each protein, shown in (C), are calculated in the same way as in (B), and are plotted. Open squares, MSH2; open circles, MSH6; closed squares, MLH1; closed circles, FLAG-PMS2; and open triangles, PCNA.
reduced the induction of caspase-3. This implies that PCNA is a necessary component of the mismatch recognition complex, which is required for the execution of MNU-induced apoptosis.

**Requirement of DNA replication for the recognition of O\textsuperscript{6}-methylguanine base pairs**

Previous studies with Mgmt-knockout mice (5) revealed that bone marrow, spleen, thymus and intestinal mucosa, in which cells are actively growing, are most severely damaged by MNU administration, implying that DNA replication is required for O\textsuperscript{6}-methylguanine-triggered apoptosis. To determine whether DNA replication is a prerequisite for the formation of the mismatched base-associated protein complex, we examined the effects of aphidicolin, an inhibitor of DNA polymerase, on the formation of the complex. HeLa MR (ePMS2) cells were incubated for 16 h in a medium containing aphidicolin, to inhibit DNA replication, and then were treated with MNU for 1 h. The cell culture was divided into two portions; one incubated in the absence of aphidicolin and the other in the presence of the drug. The cells were collected at the indicated times after MNU treatment and were subjected to flow cytometry and immunoprecipitation analyses. As shown in Figure 6A, most of the cells treated with aphidicolin are arrested at the G1/S boundary and remained at this stage as long as the drug was present in the medium. When the aphidicolin was removed, the treated cells progressed into the S phase of the cell cycles. Immunoblotting analyses of these samples revealed that total amounts of each mismatch repair protein and PCNA were almost constant (data not shown); however, the level of PCNA bound to chromatin remarkably increased when the cells progressed into S phase (Figure 6B). In accordance with this increase, immunoprecipitation analyses showed that in the absence of aphidicolin, the association of MSH2, MLH1 and PCNA with FLAG-tagged PMS2 occurs gradually when the cells progress into the S phase, whereas the formation of the complex is severely inhibited when the drug is present in the medium (Figure 6C). Thus, it seems that the formation of the mismatch recognition complex induced by MNU treatment is dependent on the progression of DNA replication.
Figure 4. Inability of MLH1- and MSH2-defective cells to form the protein complex. (A) Effect of MLH1 deficiency. HeLa MR (methyltransferase-deficient) and SW48 (deficient in both methyltransferase and MLH1) cells were treated with or without 1 mM MNU for 1 h and then were incubated for another 11 h. The chromatin extracts were prepared as described in Figure 1 and the same amount of extracts were subjected to immunoprecipitation with an anti-MSH2 antibody, followed by immunoblotting. (B) MSH2-dependent interaction of MutLα with PCNA. HeLa S3 and LoVo (MSH2-deficient) cells were pre-incubated in a medium containing 25 mM O6-benzylguanine for 2 h and then exposed to MNU for 1 h. After incubation for another 11 h in the presence of O6-benzylguanine, the chromatin extracts were prepared, immunoprecipitated with the anti-PMS2 antibody, and immunoblotted.

Figure 5. Effects of a PCNA knockdown on the formation of the mismatch recognition complex and the apoptotic induction. (A) PCNA knockdown by siRNA. HeLa MR (ePMS2) cells were transfected with siRNAs for PCNA and negative control sequences, and then were cultivated for 48 h. The whole cell extracts prepared were used for immunoblotting to analyze the total amounts of mismatch-related proteins. β-Actin is a loading control. (B) Chromatin-bound proteins under PCNA knockdown condition. siRNA-transfected cells in (A) were treated with 1 mM MNU. After 12 h incubation, chromatin extracts were prepared and the amounts of mismatch-related proteins and histone H3 were analyzed by immunoblotting. Histone H3 is a loading control. (C) Complex formation determined by immunoprecipitation analysis. The chromatin extracts were used for immunoprecipitation with the anti-FLAG antibody and then subjected to immunoblotting with the anti-FLAG, MSH2, MLH1 and PCNA antibodies. (D) Induction of apoptosis as measured by caspase-3 activity. Caspase-3 activity was determined at 72 h after treatment with or without 1 mM MNU. Values obtained with the MNU-treated cells were divided by those of the untreated cells, and the relative caspase-3 activities are shown. Experiments were performed three times and the standard deviations are shown in bars.
DISCUSSION

There are two types of DNA lesions that cause apoptosis, one that blocks DNA replication and another that allows progression of the DNA replication forks. Unless otherwise repaired, both types of lesions can induce cell death, but in different ways. Blockage of DNA replication by bulky DNA lesions is sensed by the ATM/ATR system, which activates the mitochondria-dependent apoptotic pathway for cell death (36). However, modified bases, which allow DNA replication, are recognized by a protein complex, composed of the bacterial mismatch repair protein homologs MutSα and MutLα, to induce apoptosis (18,24). The latter process is of utmost biological and medical significance, since the survival of cells carrying such mismatches would cause base substitution mutations, leading to the induction of tumors and the disposition of hereditary diseases.

Studies on the mechanism of apoptosis caused by replication-permitting base mispairs have been hampered by the lack of an appropriate in vivo system, since such mispairs are rarely formed during the normal DNA replication. In vitro reconstitution systems with synthetic DNA strands carrying such mismatches have been devised, and protein complexes interacting with the DNA fragments were isolated (37,38). However, it was sometimes difficult to analyze the relevant roles of these complexes, found in vitro, in the induction of apoptosis, since base mismatches would cause entirely different outcomes, including DNA repair, apoptosis and some others, according to the conditions in vivo. In this regard, O6-methylguanine-induced mispairs are unique, since most of the cells with these lesions undergo apoptosis (18). O6-methylguanine can be produced in the chromosomal DNA in situ by exposing cells or organisms to simple alkylating agents and, moreover, the amount of the mismatched bases in the DNA can be controlled by regulating the doses of the agents. In the present study, we have taken advantage of this system to identify a protein complex that interacts with mismatched DNA under physiological conditions, in which the protein interactions on the intact chromatin are fixed by a chemical crosslinker introduced into permeabilized cells, and have related it to the induction of apoptosis.

When human cells deficient in O6-methylguanine-DNA methyltransferase activity were exposed to appropriate doses of MNU, a protein complex composed of PCNA, MutSα and MutLα was formed on the chromosomal DNA. Without exposure to the alkylating agent, no such complex was formed. In normal cells, PCNA and MutSα exist as forms associated with the chromatin, and this association was observed even in cells that lack MLH1, a component of MutLα. In the absence of MutSα, owing to the lack of its component MSH2, no association of MutLα with the chromatin was achieved. Time course experiments revealed that the formation of the PCNA–MutSα–MutLα complex occurs gradually after MNU treatment and reaches a maximum of ~12 h after the treatment, implying that at least one cycle of DNA replication is required for initiating the complex.

Figure 6. Effects of aphidicolin on the formation of the mismatch recognition complex. (A) Arrest of cell cycle progression by aphidicolin. HeLa MR (ePMS2) cells were cultivated in a medium containing 5 μM aphidicolin for 16 h and then treated with 1 mM MNU for 1 h. The culture was divided into two portions, one incubated in the presence of aphidicolin and the other in the absence of the drug. Cells were collected at the indicated time points and subjected to flow cytometric analyses to monitor cell cycle progression. (B) Chromatin-bound PCNA during DNA replication. Chromatin extracts were prepared from cells at 0, 4 and 8 h after MNU treatment, in the presence or absence of aphidicolin, and the amounts of histone H3 and PCNA were analyzed by immunoblotting. The relative ratios of PCNA are also shown. (C) Formation of protein complex during DNA replication. Chromatin extracts in (B) were used for immunoprecipitation with an anti-FLAG antibody. Immunoblotting was performed with the anti-FLAG, MSH2, MLH1 and PCNA antibodies.
formation. This notion was supported by our finding that the prevention of DNA replication by a treatment with aphidicolin, a DNA polymerase inhibitor, caused significant decrease in the levels of complex formation. After one round of DNA replication, the O6-methylguanine–cytosine pair, produced by the action of alkylating agents, can be converted to an O6-methylguanine–thymine pair, which may be the target for the MutSα–PCNA complex. Notably, the pancytopenia observed in MNU-treated MGMT−/− mice is associated with the death of actively growing cells in bone marrow and intestinal mucosa, and this impairment is completely suppressed by the introduction of the MihI mutation into the mice (5,17).

PCNA is a component of the DNA replication machinery, and its role in mismatch repair has also been implicated. In in vitro reconstitution experiments, physical interactions of MutSα with PCNA were detected, and co-localization of MutSα and PCNA in the nucleus was also shown (37,39–41). In the present study, we found that PCNA persists on the chromatin in a form associated with MutSα and, on the exposure of cells to an alkylating agent, it further makes a complex with MutLα. When siRNA for PCNA was introduced, the amount of PCNA as well as those of the components of MutSα and MutLα, forming the complex on the chromatin, decreased considerably. Consistent with this reduction, the induction of caspase-3, an event associated with apoptosis, was significantly suppressed. These results support the view that PCNA is required for the progression of O6-methylguanine-induced apoptosis, in addition to its fundamental roles in DNA replication and repair.

How is the O6-methylguanine-triggered apoptosis executed? The PCNA–MutSα complex may act as a genome surveillant and bind to the O6-methylguanine–thymine pair, which is produced after one round of DNA replication. This would then facilitate further binding of MutLα to the complex, and the PCNA–MutSα–MutLα complex thus formed might exert its action on certain molecules, which would transmit the apoptotic signal to downstream members. Since the activation of caspase-3 occurs during the course of O6-methylguanine-induced apoptosis (18), as observed in the processes triggered by TNF and blockage of DNA replication forks (42,43), it seems that the signals delivered from different sources converge at the step of caspase-3 induction, which then activates the subsequent common steps necessary for the execution of apoptosis, including DNA fragmentation and the formation of apoptotic bodies. To understand the entire scheme of the O6-methylguanine-induced apoptotic pathway, the components functioning downstream of mismatch repair proteins must be identified. Studies aimed at elucidating this process by both biochemical and genetic means are in progress in the laboratory.

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REFERENCES

1. Coulondre.C. and Miller,J.H. (1977) Genetic studies of the lac repressor. IV. Mutagenic specificity in the lacI gene of Escherichia coli. J. Mol. Biol., 117, 577–606.
2. Ito,T., Nakamura,T., Maki,H. and Sekiguchi,M. (1994) Roles of transcription and repair in alkylation mutagenesis. Mutat. Res., 314, 273–285.
3. Sekiguchi,M., Nakabeppu,Y., Sakumi,K. and Tsuzuki,T. (1996) DNA repair methyltransferase as a molecular device for preventing mutation and cancer. J. Cancer Res. Clin. Oncol., 122, 199–206.
4. Olsson,M. and Lindahl,T. (1980) Repair of alkylated DNA in Escherichia coli. Methyl group transfer from O6-methylguanine to a protein cysteine residue. J. Biol. Chem., 255, 10569–10571.
5. Tsuzuki,T., Sakumi,K., Shiraishi,A., Kawate,H., Igarashi,H., Ikawama,T., Tominaga,Y., Zhang,S., Shimizu,S., Ishikawa,T. et al. (1996) Targeted disruption of the DNA repair methyltransferase gene renders mice hypersensitive to alkylating agent. Carcinogenesis, 17, 1215–1220.
6. Sakumi,K., Shiraishi,A., Shimizu,S., Tsuzuki,T., Ishikawa,T. and Sekiguchi,M. (1997) Methylnitrosourea-induced tumorigenesis in MGMT gene knockout mice. Cancer Res., 57, 2415–2418.
7. Tominaga,Y., Tsuzuki,T., Shiraishi,A., Kawate,H. and Sekiguchi,M. (1997) Alkylation-induced apoptosis of embryonic stem cells in which the gene for DNA-repair, methyltransferase, had been disrupted by gene targeting. Carcinogenesis, 18, 889–896.
8. Kaina,B., Ziouta,A., Ochs,K. and Coquerelle,T. (1997) Chromosomal instability, reproductive cell death and apoptosis induced by O6-methylguanine in Mex−.Mex− and methylation-tolerant mismatch repair compromised cells: facts and models. Mutat. Res., 381, 227–241.
9. Meikrantz,W., Bergom,M.A., Memisoglu,A. and Samson,L. (1998) O6-alkylguanine DNA lesions trigger apoptosis. Carcinogenesis, 19, 369–372.
10. Burdett,V., Baitinger,C., Viswanathan,M., Lovett,S.T. and Modrich,P. (2001) In vivo requirement for RecJ, ExoVII, Exol and ExoX in methyl-directed mismatch repair. Proc. Natl Acad. Sci. USA, 98, 6765–6770.
11. Drummond,J.T., Li,G.M., Longley,M.J. and Modrich,P. (1995) Isolation of an hMSH2-p160 heterodimer that restores DNA mismatch repair to tumor cells. Science, 268, 1909–1912.
12. Palombo,F., Gallinari,P., Iaccarino,I., Lettieri,T., Hughes,M., D’Arrigo,A., Truong,O., Hsuan,J.J. and Jiricny,J. (1995) GTBP, a 160-kilodalton protein essential for mismatch-binding activity in human cells. Science, 268, 1912–1914.
13. Li,G. and Modrich,P. (1995) Restoration of mismatch repair to nuclear extracts of H6 colorectal tumor cells by a heterodimer of human MutL homologs. Proc. Natl Acad. Sci. USA, 92, 1950–1954.
14. Kolodner,R. and Marsischky,G. (1999) Eukaryotic DNA mismatch repair. Curt. Opin. Genet. Dev., 9, 89–96.
15. Branch,P., Aquilina,G., Bignami,M. and Karran,P. (1993) Defective mismatch binding and a mutator phenotype in cells tolerant to O6-methylguanine DNA lesions. Nature, 362, 652–654.
16. Kat,A., Thilly,W.G., Fang,W.H., Longley,M.J., Li,G.M. and Modrich,P. (1993) An alkylation-tolerant, mutator human cell line is deficient in strand-specific mismatch repair. Proc. Natl Acad. Sci. USA, 90, 6424–6428.
17. Kawate,H., Sakumi,K., Tsuzuki,T., Nakatsu,Y., Ishikawa,T., Takahashi,S., Takano,H., Noda,T. and Sekiguchi,M. (1998) Separation of killing and tumorigenic effects of an alkylating agent in mice defective in two of the DNA repair genes. Proc. Natl Acad. Sci. USA, 95, 5116–5120.
18. Takagi,Y., Takahashi,M., Sanada,M., Ito,R., Yamaizumi,M. and Sekiguchi,M. (2003) Roles of MGMT and MLH1 proteins in alkylation-induced apoptosis and mutagenesis. DNA Repair (Amst), 2, 1135–1146.
19. Yang,G., Scherer,S., Shell,S., Yang,K., Kim,M., Lipkin,M., Kucherlapati,R., Kolodner,R. and Edelmann,W. (2004) Dominant effects.
of an Msh6 missense mutation on DNA repair and cancer susceptibility. Cancer Cell, 6, 139–150.
20. Lin,D.P., Wang,Y., Scherer,S.J., Clark,A.B., Yang,K., Avdievich,E., Jin,B., Werling,U., Parris,T., Kurihara,N. et al. (2004) An Msh2 point mutation uncouples DNA mismatch repair and apoptosis. Cancer Res., 64, 517–522.
21. Genschel,J. and Modrich,P. (2003) Mechanism of 5’-directed excision in human mismatch repair. Mol. Cell, 12, 1077–1086.
22. Dzantiev,L., Constantin,N., Genschel,J., Iyer,R.R., Burgers,P.M. and Modrich,P. (2004) A defined human system that supports bidirectional mismatch provoked excision. Mol. Cell, 15, 31–41.
23. Ochs,K. and Kaina,B. (2000) Apoptosis induced by DNA damage. O6-methylguanine is Bcl-2 and Caspase-9/S regulated and Fas/Caspase-8 independent. Cancer Res., 60, 5815–5824.
24. Hickman,M.J. and Samson,L.D. (2004) Apoptotic signaling in response to a single type of DNA lesion, O6-methylguanine. Mol. Cell, 14, 105–116.
25. Sanada,M., Takagi,Y., Ito,R. and Sekiguchi,M. (2004) Killing and mutagenic actions of dacarbazine, a chemotherapeutic alkylating agent, on human and mouse cells: effects of Mgmt and Mlh1 mutations. DNA Repair (Amst.), 3, 413–420.
26. Oki,E., Oda,S., Maehara,Y. and Sugimachi,K. (1999) Mutated gene-expression in human and mouse cells: effects of Mgmt and Mlh1 mutations. Nucleic Acids Res., 27, 2143–2147.
27. Kondo,E., Horii,A. and Fukushige,S. (2001) The interacting domains of three MutL heterodimers in man: hMLH1 interacts with 36 homologous amino acid residues within hMLH3, hPMS1 and hPMS2. Nucleic Acids Res., 29, 1695–1702.
28. Harlow,E. and Lane,D. (1988) Antibodies: A Laboratory Manual. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
29. Kane,M.F., Loda,M., Gaida,G.M., Lipman,J., Mishra,R., Goldman,H., Jessup,J.M. and Kolodner,R. (1997) Methylation of the hMLH1 promoter correlates with lack of expression of hMLH1 in sporadic colon tumors and mismatch repair-defective human tumor cell lines. Cancer Res., 57, 808–811.
30. Baranovskaya,S., Soto,J.L., Perucho,M. and Malkhosyan,S.R. (2001) Functional significance of concomitant inactivation of hMLH1 and hMSH6 in tumor cells of the microsatellite mutator phenotype. Proc. Natl Acad. Sci. USA, 98, 15107–15112.
31. Friedman,H.S., Koir,S., Pegg,A.E., Houghton,P.J., Colvin,O.M., Moschel,R.C., Bigner,D.D. and Dolan,M.E. (2002) O6-benzylguanine-mediated enhancement of chemotherapy. Mol. Cancer Ther., 1, 943–948.
32. Pegg,A. (2000) Repair of O6-alkylguanine by alkyltransferases. Mutat. Res., 462, 83–100.
33. Elbashir,S.M., Harborth,J., Lendeckel,W., Yalcin,A., Weber,K. and Tuschl,T. (2001) Duplexes of 21-nucleotide RNAs mediate RNA interference in cultured mammalian cells. Nature, 411, 494–498.
34. Lau,P.J. and Kolodner,R.D. (2003) Transfer of the MSH2–MSH6 complex from proliferating cell nuclear antigen to mispaired bases in DNA. J. Biol. Chem., 278, 14–17.
35. Shi,Y. (2002) Mechanisms of caspase activation and inhibition during apoptosis. Mol. Cell, 9, 459–470.
36. Zhou,B.B. and Caspase-9/3. (2004) Apoptotic signaling in response to a single type of DNA lesion, O6-methylguanine. Mol. Cell, 14, 105–116.
37. Lau,P.J. and Kolodner,R.D. (2003) Transfer of the MSH2–MSH6 complex from proliferating cell nuclear antigen to mispaired bases in DNA. J. Biol. Chem., 278, 14–17.
38. Shi,Y. (2002) Mechanisms of caspase activation and inhibition during apoptosis. Mol. Cell, 9, 459–470.
39. Zhou,B.B. and Caspase-9/3. (2004) Apoptotic signaling in response to a single type of DNA lesion, O6-methylguanine. Mol. Cell, 14, 105–116.