Detection of a mouse OGG1 fragment during caspase-dependent apoptosis: Oxidative DNA damage and apoptosis

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We investigated the expression of mouse 8-oxoguanine DNA glycosylase 1 (mOGG1) in mouse non-parenchymal hepatocytes (NCTC) during etoposide- or mitomycin C (MMC)-induced apoptosis. We observed mOGG1 fragmentation in apoptotic cells. The apoptosis accompanying the fragmentation of mOGG1 was caspase-dependent. The mOGG1 fragment existed in both the cytoplasm and nucleus of the etoposide-treated NCTC, indicating that the mOGG1 fragment could be transferred into the nucleus. In addition, 8-hydroxyguanine (8-OH-Gua, 7,8-dihydro-8-oxoguanine) accumulated in the DNA of NCTC treated with etoposide, suggesting that the mOGG1 fragment might not function as a normal repair enzyme in etoposide-treated NCTC. Although we have not clarified in detail the mechanism and the significance of the mOGG1 fragmentation, further study of the fragmentation of DNA repair enzymes might provide insights into the relationship between oxidative DNA damage and apoptosis. (Cancer Sci 2004; 95: 634–638)

Cells are damaged by a number of environmental stresses, such as ultraviolet radiation, X-rays, heat shock, and chemical agents. Some of the stresses generate reactive oxygen species (ROS), which are highly toxic to cell maintenance processes and survival. ROS are known to induce several types of oxidative DNA damage, including premutagenic lesions, such as thymine glycol,[1)] 5-hydroxymethyluracil,[2)] 2-hydroxyadenine,[3)] and 8-hydroxyguanine (8-OH-Gua).[4)]

8-OH-Gua is thought to be responsible for carcinogenesis, because it induces GC to TA transversion-type point mutations in DNA *in vitro*.[5,6)] Experiments in both cultured cells and animals have supported this premise.[7–11)] Therefore, analyses of oxidative DNA damage should be useful to understand in detail the mechanisms of carcinogenesis. Moreover, the genes for the human and mouse glycosylase-type repair enzymes for 8-OH-Gua (*hOGG1* and *mOGG1*) have been cloned.[12–14)] The corresponding proteins have also been analyzed to study the mechanisms of carcinogenesis and to assess the cancer risks of chemical substances or environmental factors.[7–11)]

Several recent reports have suggested a relationship between oxidative DNA damage and the apoptotic process,[15–17)] although, to our knowledge, only a few studies have investigated the relationship between 8-OH-Gua and apoptosis. Apoptosis is a physiological process of programmed cell death, which is characterized by distinct morphological changes and internucleosomal DNA fragmentation. The DNA damage induced by various stresses, including ROS, activates p53, which is an important molecule for the induction of cell cycle arrest or apoptosis in order to prevent oncogenesis.[18–21)] The anticancer activities of some cancer chemotherapeutic agents, such as etoposide, mitomycin C (MMC), and cisplatin, are due to the induction of apoptosis.[22–24)] Among these agents, etoposide has a topoisomerase II inhibitory action that is responsible for inducing apoptosis, and it recently was shown to be a pro-oxidant.[24)]

In our previous report, we detected a 32-kDa mOGG1 fragment in the livers of mice fed with 0.06% 3′-methyl-4-dimethylaminoazobenzene.[25)] Although we could not confirm the mechanism of the mOGG1 fragmentation, it may be related to the apoptotic process.

In this study, we analyzed the expression of mOGG1 in non-parenchymal hepatocytes (NCTC) exposed to etoposide or MMC by means of immunoblotting assays, and we studied the relationship between oxidative DNA damage and apoptosis.

Materials and Methods

Cell culture and anticancer agent treatment. The mouse non-parenchymal cell lines, NCTC and NMuLi, were obtained from the Health Science Research Resources Bank (HSRRB, Osaka, Japan) and were cultured in NCTC135 medium (ICN Biomedicals, Inc., OH) with 10% horse serum. The mouse fibroblast cell line, 3T3-Swiss albino (HSRRB), was cultured in Dulbecco's modified Eagle's medium with 10% fetal bovine serum. When the cells were ~70% confluent, etoposide (Sigma, MO) or MMC (Sigma) was added to the medium to a final concentration of 100 μ*M* or 30 μ*M*, respectively. As a control, an equivalent volume of dimethylsulfoxide (DMSO, the vehicle of the reagents) was added to the medium. After 6, 12, 24, and 48 h of cultivation, the cells were harvested, washed with sterile PBS, and subjected to analyses. The cell viability was monitored by means of the trypan blue exclusion test.

Apoptosis assay. Nuclear morphology was assessed using Hoechst 33342 fluorescence staining. The percentage of apoptotic cells was determined by counting the number of nuclei showing the chromatin condensation and fragmentation characteristic of apoptosis, after observing a total of at least 300 cells. Low-molecular-weight DNA fragments were determined by gel electrophoresis. DNA extraction from the cells was performed with a MEBCYTO Apoptosis ladder detection kit from MBL Medical & Biological Laboratories Co. (Nagoya, Japan), according to the manufacturer's instructions. DNA samples were electrophoresed in 1.5% agarose gels (50 V) for approximately 1 h. Gels were stained with ethidium bromide (0.5 μg/ml) and evaluated under UV illumination.

Preparation of anti-mouse OGG1 polyclonal antibody. To perform the immunoblot analysis, we prepared a polyclonal antiserum raised against the mOGG1 protein as described elsewhere.[25)] Briefly, the amino acid sequence of the antigenic peptide corresponded to aa 43–52: QSFRWKEQSP. After binding the peptide to BSA (peptide/BSA=0.3 mg/1 mg), the peptide solution was conjugated with GERBU ADJUVANT 100 (GERBU Biotechnik GmbH, Gaiberg, Germany). One milliliter of peptide-

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adjuvant solution was injected s.c. into female Japanese white rabbits (Seac Yoshitomi, Ltd., Fukuoka, Japan) three times, at intervals of 2 weeks. After immunization, the blood was collected directly from the heart under anesthesia with sodium pentobarbital.

SDS-PAGE and western blotting. Western blot analyses were performed to examine the expression of mOGG1 in anticancer agent-treated NCTC. The cells were homogenized with a Potter-type homogenizer in cold buffer (50 m*M* Tris-HCl (pH 7.5), 50 m*M* KCl, 3 m*M* EDTA, 5 m*M* magnesium acetate, and 3 m*M* β-mercaptoethanol) containing protease inhibitors (5 ng/ml each of leupeptin, antipain, pepstatin, and chymostatin). The homogenates were centrifuged (12,000*g*, 30 min) and the supernatants were collected as whole-cell extracts. The protein concentrations of the extract were determined with the Bio-Rad Protein Assay (Bio-Rad, Richmond, CA). An aliqot containing 50 mg of protein extract was loaded on each lane of a 4–12% SDS-PAGE gel. The proteins were fractionated for 2.5 h at 100 V, and were transferred to a PVDF membrane (Millipore, Bedford, MA). The membranes were blocked and incubated with the anti-mOGG1 antiserum overnight at 37°C, washed twice, and incubated with an alkaline phosphatase-coupled secondary antibody (EY Laboratories, Inc., CA) at 37°C for 3 h. The antigen-antibody complexes were visualized with a BCIP-NBT solution kit (Nacalai Tesque, Inc., Kyoto, Japan).

Collection of cytoplasmic and nuclear fractions. To determine whether the mOGG1 fragment could be transferred into the nucleus, the cytoplasmic and nuclear fractions were separated and subjected to an immunoblot analysis. These fractions were obtained according to the method of Schreiber *et al.*,[26] with some modifications. Briefly, $0.5–1\times10^6$ cells were collected, washed with 1 ml of PBS, and pelleted by centrifugation at 5000 rpm for 1 min. The pellet was resuspended in 400 ml of cold buffer A (10 m*M* Hepes-KOH (pH 7.8), 10 m*M* KCl, 0.1 m*M* EDTA (pH 8.0), 0.1% NP-40, 1 m*M* DTT) containing protease inhibitors (0.5 m*M* PMSF and 2 mg/ml each of pepstatin, leupeptin, chymostatin, and antipain) by pipetting. The cells were disrupted completely by vortexing and were centrifuged. The supernatant was collected as the cytoplasmic fraction. The pellet was resuspended in buffer C (50 m*M* Hepes-KOH (pH 7.8), 420 m*M* KCl, 0.1 m*M* EDTA (pH 8.0), 5 m*M* $MgCl_2$, 20% glycerol, 1 m*M* DTT) containing protease inhibitors (2 mg/ml each of pepstatin, leupeptin, chymostatin, and antipain) and was incubated with rotation at 4°C for 30 min. After the incubation, the samples were centrifuged at 15,000 rpm at 4°C for 15 min. The supernatant was collected as the nuclear fraction.

Caspase activity assay. Total caspase activity was measured by using the "CaspACE" Assay System (Promega, Madison, WI) according to the manufacturer's protocol. Briefly, 24 h after the cells were seeded, etoposide was added to the medium to a final concentration of 100 μ*M* with or without the Z-VAD-FMK inhibitor (final concentration was 50 μ*M*). After 24 h of cultivation, the cells were harvested, lysed by freezing and thawing, and centrifuged to obtain the supernatant fraction. The caspase activity in the supernatant fraction was determined by measuring the absorbance at 450 nm.

Analysis of 8-OH-Gua in cellular DNA. The assay for measuring 8-OH-Gua was described elsewhere.[27, 28] Briefly, the cellular genomic DNA was isolated by the sodium iodide method, using a DNA Extraction WB Kit (Wako Pure Chemical Industries, Ltd., Osaka, Japan). For cell homogenization, a lysis solution containing 1 m*M* desferal (deferoxamine mesylate, Sigma Chemical Co., MO) was used. The isolated DNA was digested with nuclease P1 (Yamasa Corp., Choshi, Japan) to obtain a deoxynucleoside mixture. The solution was filtered with an Ultrafree-Probind filter (Millipore) and was injected into a high-

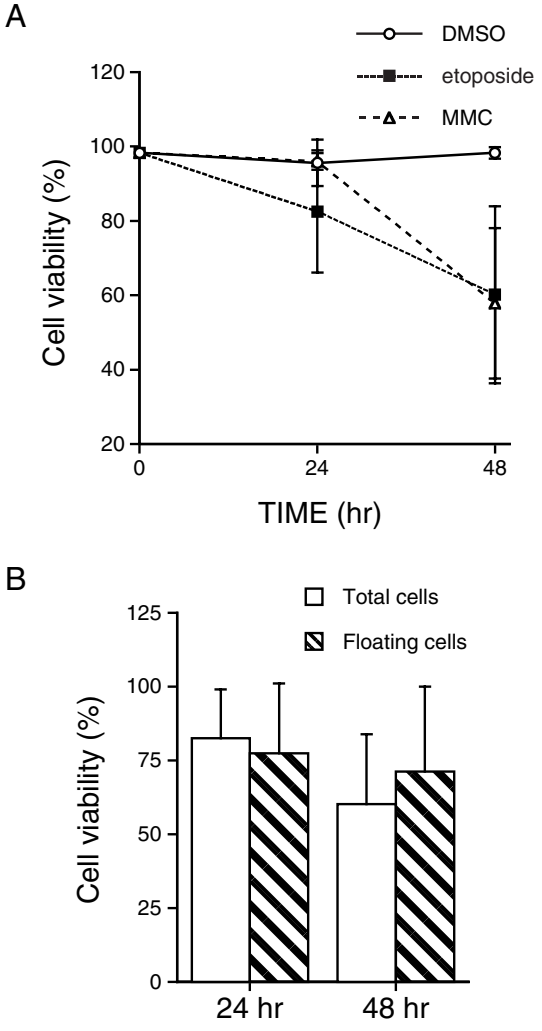

Fig. 1. (A) Time course (0, 24, and 48 h) of viability of NCTC treated with etoposide (100 μ*M*), MMC (30 μ*M*), and DMSO. Cell viability was assayed by means of the trypan blue exclusion test. The volume of DMSO was equal to that of the etoposide or MMC solution. Mean±SD, *n*=4. (B) Viability of total cells and floating cells of etoposide (100 μ*M*)-treated NCTC (24 and 48 h). Mean±SD, *n*=4.

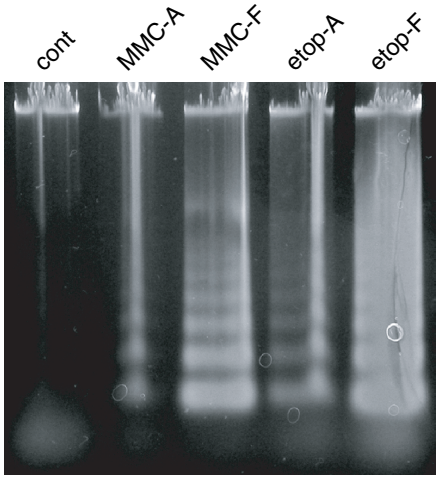

Fig. 2. The formation of DNA cleavage products corresponding to 180–200 bp ladders was clearly observed 24 h after MMC or etoposide treatment.

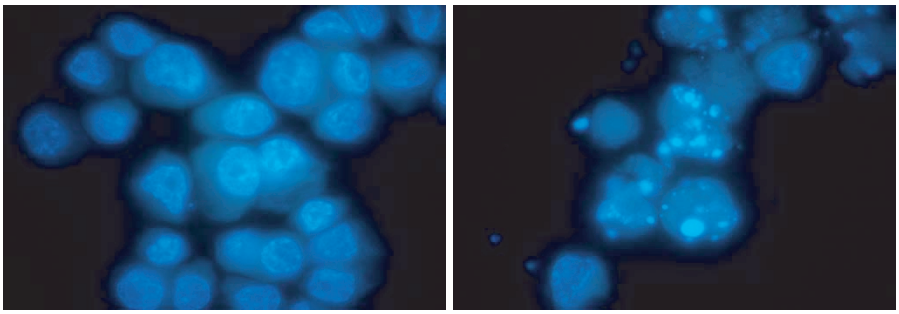

Fig. 3. A typical example of the formation of apoptotic nuclei in NCTC 24 h after etoposide treatment.

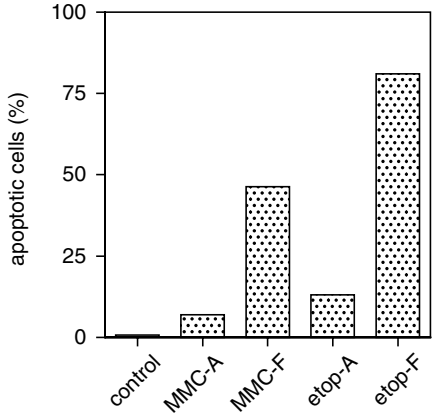

Fig. 4. Increase in apoptotic cells after MMC or etoposide treatment.

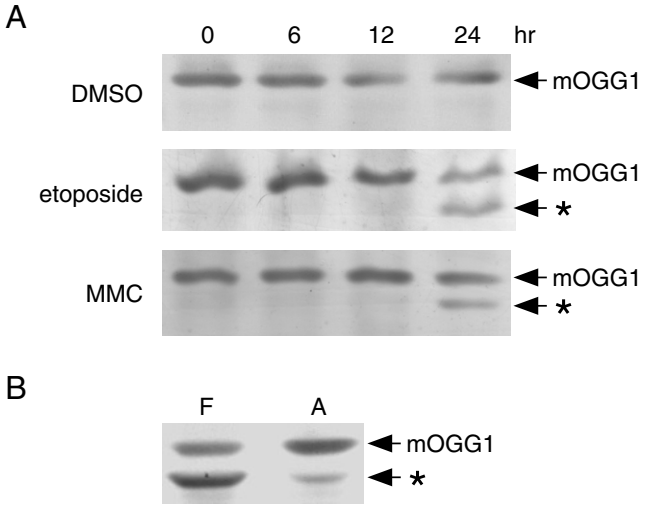

Fig. 5. Immunoblot analyses of mOGG1 expression. The asterisk (*) indicates the mOGG1 fragment. (A) mOGG1 expression in the cells treated with DMSO (top), 100 μ*M* etoposide (middle), and 30 μ*M* MMC (bottom) at 0, 6, 12, 24 h of cultivation. The volume of DMSO was equal to that of the etoposide or MMC solution. (B) mOGG1 expression in the floating cells (F) and the adherent cells (A) was also analyzed in NCTC treated with etoposide.

performance liquid chromatography (HPLC) column (Shiseido Capcell Pak C18 MG) equipped with an electrochemical detector (ECD)(Coulochem II, ESA, USA). The 8-OH-Gua value in the DNA was calculated as the number of 8-OH-Gua per 10^6 guanine (Gua).

Statistical analysis. Values in the text represent the means±SD. The statistical significance was calculated using Student's *t* test. *P* values less than 0.05 were considered to indicate significant differences.

Results

Cell viability and apoptosis detection. The cell viability is shown in Fig. 1. More than 80% of the cells survived among the etoposide- or MMC-treated NCTC at 24 h, whereas only about 60% of the cells survived at 48 h (Fig. 1A). Since some of the reagent-treated NCTC became detached from the dishes, we also checked the viability of the floating cells. Almost the same proportion of floating cells survived in comparison to the total cells treated with etoposide (Fig. 1B). Based on these findings, the treatment time with the reagents in the following experiments was set at 24 h. At 24 h, these cells exhibited signs of apoptosis, such as DNA ladder formation (Fig. 2) and changes in nuclear morphology (Fig. 3). The number of apoptotic cells increased with the MMC and etoposide treatments, and was higher in the floating cells than in the adherent cells (Fig. 4).

Mouse OGG1 expression in apoptotic cells. A band was clearly observed at the position of 38 kDa (the molecular weight of full-length mOGG1) by immunoblotting. Besides the 38-kDa band, another discrete band was also visible at a position of 35 kDa, only in the etoposide- or MMC-treated NCTC (24 h) (Fig. 5A). Furthermore, to examine whether this fragment of mOGG1 was related to cell damage, we analyzed the mOGG1 expression levels in the floating cells and the adherent cells independently and compared them. The intensity of the lower molecular band from the floating cells was clearly stronger than that from the adherent cells (Fig. 5B).

Inhibition of caspase activity. To determine if the appearance of the mOGG1 fragment was related to caspase-dependent apoptosis, we inhibited the caspase activity of etoposide-treated NCTC by the addition of Z-VAD-FMK, which is a potent, irreversible, and cell-permeable pan-caspase inhibitor (Fig. 6B). When the caspase activity was inhibited, no mOGG1 fragments were detected, even in etoposide-treated NCTC, by western blot analysis (Fig. 6A).

Mouse OGG1 expression in the cytoplasm and nucleus of apoptotic cells. To confirm whether the mOGG1 fragment could transfer into the nucleus, we separately examined the mOGG1 expression in the cytoplasmic and nuclear fractions prepared from etoposide-treated NCTC by the method of Schreiber *et al.*[26] The mOGG1 fragment was detected in the nucleus, and the ratio of the mOGG1 fragment to the full-length mOGG1 was not significantly different between the cytoplasm and the nucleus (Fig. 7).

8-OH-Gua levels in the cellular DNA. Analyses of the 8-OH-Gua levels in the cellular DNA revealed that the 8-OH-Gua level

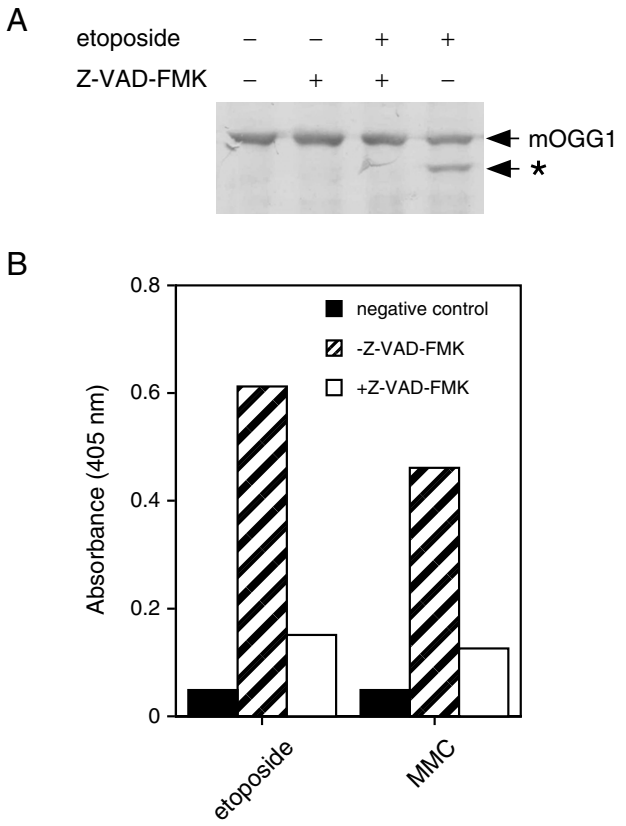

Fig. 6. Effect of caspase inhibitor on mOGG1 expression. (A) Immunoblot analyses failed to detect the mOGG1 fragment in the etoposide-treated NCTC grown in the presence of Z-VAD-FMK. The asterisk (*) indicates the mOGG1 fragment. (B) The caspase activity induced by etoposide was inhibited by the addition of Z-VAD-FMK.

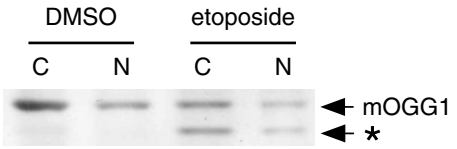

Fig. 7. mOGG1 expression in the cytoplasm and the nucleus of NCTC-treated with DMSO or 100 μ*M* etoposide. The volume of DMSO was equal to that of the etoposide solution. The asterisk (*) indicates the mOGG1 fragment. C, cytoplasm; N, nucleus.

was higher in the NCTC treated with etoposide, in comparison with the DMSO-treated NCTC. Notably, the level of 8-OH-Gua was even higher in the floating cells than in the adherent cells (Fig. 8). However, when cells were treated with etoposide in the presence of Z-VAD-FMK, no increase of 8-OH-Gua was observed.

Discussion

OGG1 is believed to be the main enzyme that removes 8-OH-Gua from nuclear DNA, leading to fewer ROS-derived mutations and reduced cancer risk in mammalian tissues. Therefore, for cancer risk assessments of substances or environmental factors, the expression level and/or the activity level of mOGG1, as well as the accumulation level of 8-OH-Gua in the cellular DNA, should be analyzed as a biomarker of cancer risk. On the other hand, some types of DNA damage are known to be involved in cell death, and recent studies have suggested that the

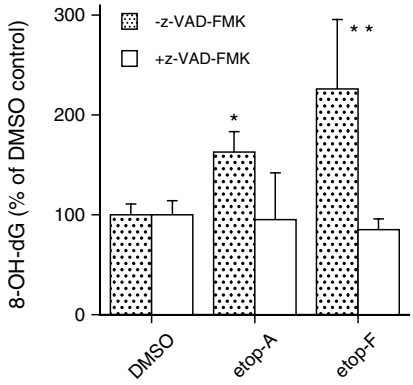

Fig. 8. 8-OH-Gua accumulates in the DNA of NCTC cells treated with etoposide. The etoposide-treated NCTC were separated into adherent cells (etop-A) and floating cells (etop-F). The ratios (%) of 8-OH-Gua levels in the etoposide-treated cells to those in the control cells are shown. Actual values (8-OH-Gua/10^6 Gua) of controls are 0.83 (–Z-VAD-FMK) and 1.78 (+Z-VAD-FMK).Mean±SD, n=3–5. * P=0.0035 vs. DMSO, ** P=0.0089 vs. DMSO.

overproduction of 8-OH-Gua is responsible for apoptosis, rather than carcinogenesis. Chung and his collaborators reported that OGG1-deficient human leukemia cells (KG-1) could be forced toward apoptosis by the addition of 8-hydroxydeoxyguanosine to the culture medium.[29] Thus, recent interest has also focused on the relationship between DNA damage and apoptosis, since this could be used to design therapeutics against tumors by inducing apoptosis in the target tissues. However, with regard to apoptosis, DNA repair mechanisms have not been considered as thoroughly as 8-OH-Gua accumulation. DNA damage and repair capacity are both important, and should be monitored closely to understand the exact role of DNA damage in apoptosis.

We previously found that dietary administration of the hepatocarcinogen, 3′-methyl-4-dimethylaminoazobenzene, induced the production of a 32-kDa fragment of mOGG1, which is smaller than the full-length mOGG1 (38 kDa), in mouse liver,[25] although we could not confirm the relationship between the fragmentation of mOGG1 and apoptosis by immunohistochemistry. Therefore, we wished to determine whether the fragmentation of mOGG1 might be related to the apoptotic process induced by the carcinogen. Based on our previous findings, we designed the present study to examine mOGG1 expression in apoptotic cells. We observed a similar phenomenon to that seen in our previous experiment[25]; that is, two clear bands, the full-length mOGG1 (38 kDa) and a lower-molecular-weight protein (35 kDa), were observed in apoptotic cells by immunoblot analyses. Interestingly, the mOGG1 fragment disappeared in caspase-inhibited NCTC, suggesting that this phenomenon is related to a caspase-dependent apoptotic pathway. Moreover, this mOGG1 fragment was more prevalent in the floating cells than in the adherent cells during apoptosis. This result also supports the idea that the mOGG1 fragment is related to cell death. However, more direct evidence for this hypothesis should be obtained from biochemical experiments.

Caspases are proteases that cleave proteins at specific amino acid sequences. According to our present findings, it seems reasonable to expect that the fragmentation of mOGG1 is due to caspase activity. To our knowledge, several amino acid sequences specific for caspase activity have been determined.[30] However, the mOGG1 amino acid sequence lacks these sequences, suggesting that either an unknown type of caspase acted on mOGG1 or mOGG1 carries an unidentified caspase cleavage site. As a next step, the caspase and the cleaved amino acid sequence should be identified.

In addition, we examined whether the mOGG1 fragment could transfer into the nucleus, because the C-end terminal region of OGG1 has a nuclear localization signal (NLS)[31] and its translocation into the nucleus should be an important step for its DNA repair function. Our results indicated that this fragment of mOGG1 was present in the nucleus, suggesting that this molecule carried the NLS and could transfer into the nucleus (Fig. 7). This result agrees with our previous immunoblot analysis, which indicated that the mOGG1 fragment carried the NLS.[25] We found that the 8-OH-Gua levels were increased in the apoptotic cells in comparison to the control cells (Fig. 8). Etoposide is known as an inhibitor of topoisomerase II, and is effective for cancer therapy. Notably, recent research indicated that etoposide[24] and MMC[32] acted as pro-oxidants. Therefore, we can speculate that 8-OH-Gua accumulation in the cellular DNA reflects an imbalance between DNA damage and repair. In short, the disturbance of DNA repair enhances the accumulation of 8-OH-Gua. In addition, more 8-OH-Gua existed in the DNA of the floating cells than in that of the adherent cells, suggesting that the increase in 8-OH-Gua accumulation, as well as the fragmentation of mOGG1, was related to cell death.

Finally, we performed similar experiments and western blot analyses to study the mOGG1 expression in other mouse cell lines (3T3-Swiss albino and NMuLi) treated with etoposide. No fragmentation of mOGG1 was seen in the experiment using 3T3-Swiss albino cells (mouse fibroblast cells), while the same fragmentation was seen in the experiment using NMuLi (mouse non-parenchymal epithelial liver cells) (data not shown). Therefore, the mOGG1 fragmentation observed in this study might be a phenomenon occurring in specific tissues, including mouse non-parenchymal hepatocytes.

In summary, the mOGG1 fragment was detected during caspase-dependent apoptosis and could be transferred into the nucleus. However, it presumably could not repair 8-OH-Gua, and therefore 8-OH-Gua accumulated. The significance of the mOGG1 fragmentation is not yet clear, and even its mechanism remains obscure. Further experiments will be required to address these phenomena.

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