2004년 8월
박사학위논문

전립선암 세포주에서 방사선에 의한 8-oxoG 축적에 대한 Metallothionein-Ⅲ의 억제효과

조선대학교 대학원
의 학 과
정 충 식

전립선암 세포주에서 방사선에 의한 **8-oxoG** 축적에 대한 **Metallothionein-Ⅲ**의 억제효과

Metallothionein-Ⅲ prevents γ-ray-induced 8-oxoG accumulation in prostatic cell line

2004년 8월

조선대학교 대학원

의 학 과

정 충 식

전립선암 세포주에서 방사선에 의한 **8-oxoG** 축적에 대한 **Metallothionein-Ⅲ**의 억제효과

지도교수 김 철 성

이 논문을 의학박사학위신청 논문으로 제출함.

2004 년 6 월

조선대학교 대학원

의 학 과

정 충 식

정충식의 박사학위논문을 인준함

위원장	대학교 교수	인
위 원	대학교 교수	인
위 원	대학교 교수	인
위 원	대학교 교수	인
위 원	대학교 교수	인

2004년 6월 일

조선대학교 대학원

CONTENTS

Abstract----------4

Ⅰ. Introduction----------6

Ⅱ. Experimental procedures----------9

Ⅲ. Results----------15

Ⅳ. Discussion----------26

Reference----------30

CONTENTS OF TABLES

Table 1. Activities of the antioxidant enzymes ----------18

Table 2. Mutation frequencies at the hypoxanthine-guanine phosphoribosyl transferase (*HPRT*) locus in pcDNA3 transfected cells, MT-III transfected clone-7 cells---------- 25

CONTENTS OF FIGURES

Fig. 1. Expresssion of MT in DU145 cells ---------- 15

Fig. 2. MT-Ⅲ inhibits γ - radiation-induced 8-oxoG accumulation ---- 17

Fig. 3. The effect of MT-Ⅲ on the hOGG1 expression ---------- 17

Fig. 4. Effect of γ - radiation on the hOGG1 protein level and 8-oxoG glycosylase activity ---------- 19

Fig. 5. siRNA-mediated down-regulation of the hOGG1 mRNA and protein in the DU145 cells ---------- 21

Fig. 6. siRNA-mediated down-regulation of the hOGG1 leads to increase in γ - ray 8-oxoG accumulation ---------- 22

Fig. 7. Effect of MT-Ⅲ on the γ - ray-induced 8-oxoG accumulation in hOGG1 depleted cells ---------- 23

Fig. 8. siRNA-mediated down-regulation of the hOGG1 leads to increase in γ - radiation-induced mutant frequency ---------- 24

ABSTRACT

Metallothionein-III prevents γ-ray-induced 8-oxoG accumulation in prostatic cell line

Choong-Sik Jeong
Advisor : Kim chul-sung,MD Ph.D
Department of Medicine,
Graduate School of Chosun University

Metallothioneins (MT) play an important biological role in preventing oxidative damage to cells. We have previously demonstrated that the efficiency of the protective effect of MT-III against the DNA degradation from oxidative damage was much higher than that of MT-I/II. As an extension of the latter investigation, this study aimed to assess the ability of MT-III to suppress 8-oxoguanine (8-oxoG), which is one of the major base lesions formed after an oxidative attack to DNA, and the mutant frequency of the hypoxanthine-guanine phosphoribosyl transferase (*HPRT*) gene in human prostate cancer cells, DU145 cells upon exposure to γ-rays. We found that human MT-III expression decreased the level of 8-oxoG and mutation frequency in the γ-irradiated human prostate cancer cells, DU145. Using a 8-oxoguanosine DNA-glycosylase (OGG1)-specific siRNAs, we also found that MT-III expression resulted in the suppression of the γ-radiation-induced 8-oxoG accumulation and

mutation in the OGG1 depleted cells, which is the major repair enzyme of 8-oxoG. Moreover, the down-regulation of MT in cells induced by MT specific siRNA led to a significant increase in the 8-oxoG level, after exposure to γ-irradiation. These results suggest that under the conditions of γ-ray oxidative stress, MT-III prevents the γ-radiation-induced 8-oxoG accumulation and mutation in normal and hOGG1 depleted prostate cancer cells, DU145 and this suppression might, at least in part, contribute to the anticarcinogenic role of MT-III.

INTRODUCTION

The metallothioneins (MT) are a group of intracellular metal binding proteins of a low molecular mass (6-7 kDa) that are widely distributed in a broad range of eukaryotic species from yeast to mammals (1,2). In both mice and humans, there are four classes of quite similar MT proteins, MT-I to MT-IV. MT-I and MT-II are widely expressed in all tissues, whereas MT-III and MT-IV are expressed mainly in the central nervous system and the squamous epithelia, respectively (3-7). While much is known about the chemical properties and genetic regulation of MT, the actual physiological role of MT is largely unknown. The first recognized function of MT was the detoxification of heavy metals such as cadmium and mercury (8, 9). Subsequently, a number of cellular functions have been proposed for MT, including regulating the essential metal homeostasis (10, 11), contributing to the control of cellular proliferation and apoptosis (12, 13), and protecting against radiation and oxidative damage (14, 15). The role of MT in oxidative damage has been aggressively investigated, and the vast majority of studies show that MT are a potent antioxidant that protects against various oxidative damage from reactive oxygen species (ROS) *in vitro* and *in vivo* because of their multiple cysteines. *In vitro*, up-regulation of MT has been correlated with the resistance to cytotoxicity induced by various hydroxyl radical generators, and the rate constant of MT for a reaction with hydroxyl radicals is more than 100 times higher than that of glutathione (16-21). In addition, MT is 50 times more effective in protecting DNA from hydroxyl radicals than glutathione on a molar basis (22). *In vivo*, the induction of MT expression by a variety of stresses associated with oxidative injury is consistent with MT functioning as an antioxidant (23-25).

MT-III, the brain-specific member of MT family, was discovered as an inhibitory neuronal growth factor that appeared at lower levels in Alzheimer's disease brains (4, 26). However, the mechanism of this inhibition and the physiological significance of MT-III are not clear. MT-III regulation has been studied in a number of animal models of brain damage (27, 28), suggesting that this MT isoform is involved in reparative and/or protective processes in the

brain. Moreover, it has been recently shown that mice deficient in MT-III are more susceptible to seizures induced by kainic acid and exhibit greater neuronal injury than normal mice, and transgenic mice containing elevated levels of MT-III were more resistant to neuronal injury (29). These results suggest that MT-III could play a neuroprotective role; however, the mechanisms underlying such a protective role have not been fully known. In view of MT's proposed protective role as a free radical scavenger, it may also have relevance in brain neurological diseases. Therefore, MT-III, which is a unique protein, requires further study to further define its antioxidant nature and its involvement in neuroprotection.

ROS-induced damage to the cellular macromolecules has been implicated in the etiology of cancer and ageing, as well as in other human diseases (30). Among the oxidative lesions, 8-oxoguanine (8-oxoG) is one of the major base lesions formed after an oxidative attack to DNA (31). Relative large quantities of 8-oxoG are produced in mammalian cells, either as a byproduct of the normal oxidative metabolism, or as a result of the exogenous sources of ROS, such as ionizing radiation (32). 8-oxoG preferentially mispairs with adenosine during replication and thereby gives rise to G:C to T:A transversion mutations (33). Because of the relative abundance and potent mutagenicity, 8-oxoG is believed to represent a major source of ROS-induced mutagenesis in all aerobic cells. The cellular damage caused by radiation is mainly oxidative damage as a result of the formation of several types of ROS including hydroxyl radicals and superoxide radicals by the radiolysis of water in cells (34, 35). DNA is the presumed target of attack of either the primary radiolysis products of ionizing radiation such as hydroxyl radicals or secondary radicals, which are derived from the reactions of hydroxyl radicals with species in close proximity to the DNA (36). A number of reports have demonstrated that MT can protect against radiation-induced DNA damage (14). Therefore, MT may be one of the most important defense mechanisms against ROS-induced mutagenesis, particularly those produced by irradiation. Supporting this hypothesis, several groups have found a correlation between the elevated MT levels and the decreased number of mutations after exposure to oxidative stress (37, 38). We have previously demonstrated that the MT-III expressing cells are highly protected from the ROS-induced DNA damage (39), and that purified MT-III has the most efficient protective effect against hydroxyl radical-induced DNA single-strand breaks compared to that of MT-I/II (40).

Based on these considerations, this study evaluated the protection provided by MT-III in γ-irradiated human prostate cancer DU145 cells. Attempts were made to determine whether or not MT-III expression could modulate the γ-irradiation-induced 8-oxoG accumulation and mutagenesis. The amount of 8-oxoG accumulation was determined using hig performance liquid chromatography (HPLC) combined with an electrochemical detector, and the mutant frequency was determined by measuring the mutation frequency of the hypoxanthine-guanine phosphoribosyl transferase (*HPRT*) gene locus. This study demonstrates that MT-III expression can effectively inhibit the accumulation of 8-oxoG as well as suppress the mutant frequency of the *HPRT* gene after exposure to γ-irradiation in DU145cells. Most notably, this study demonstrates that MT-III expression leads to a reduction in the level of γ-ray-induced 8-oxoG formation and mutation in 8-oxoguanosine DNA-glycosylase (OGG1) depleted cells, which is the major repair enzyme of 8-oxoG. These findings suggest that a MT-III plays a critical role in the protection of γ-irradiation-induced 8-oxoG accumulation as well as in γ-irradiation-induced mutation in normal and hOGG1 depleted cells.

EXPERIMENTAL PROCEDURES

Maintenance of Cell Line-The human prostate cancer DU145 cells (Coriell Institute for Medical Research) were maintained in Earle's minimum essential medium (EMEM) supplemented with 10% fetal bovine serum (FBS), 100 units of penicillin/ml and 100 μg of streptomycin/ml (Life Technologies, Inc.). The cells were maintained in 5% CO_2/95% air at 37 °C in a humidified incubator.

Preparation of Constructs and Clones-The construct of the human MT-III, MT-I and MT-II is described elsewhere (39, 40). Human OGG1 (hOGG1) cDNA, was amplified by RT-PCR using the hOGG1 oligo primer (5`-ATGCCTGCCCGCGCGCTTCTGCC-3`, 5`-CTAGCCTTCCGGCCCTTTGGAAC-3`) from human prostate cancer DU145 cells. After confirming the DNA sequence, the hOGG1 cDNA was cloned into a pcDNA3 mammalian expression vector, which was driven by the CMV promoter (Invitrogen). The transfections were performed using the LipofectAMINE method (Promega) according to the manufacturer's instructions. After transfection, the cells were incubated with complete medium containing 400 μg/ml G418 for 5 weeks. The cell clones resistant to G418 were isolated and analyzed.

Irradiation of Cells with γ-Rays-The cells were seeded into 100-mm diameter tissue culture dishes and allowed to attach for a period of 16 ~ 24 hr at 37°C. The cells were then irradiated on ice with various γ-ray doses (0- 100 Gy) using a gamma cell irradiator (Clonac 600C; Linac, USA) at a dose rate of 0.96 Gy/min. The control cells were subjected to a similar treatment, but without the irradiation. After irradiation, the cells were washed with ice-cold phosphate-buffered saline and harvested for the various measurements.

LDH activity measurement- After cells were irradiated on ice with 50 and 100 Gy of γ-ray, culture medium was collected. Total LDH activity was determined by spectrophotometry using LDH kit (Sigma Co, USA). A total of 100 μl of supernatant were used to estimate LDH activity by measuring the oxidation of NADH at 340 nm. Extinction was recorded at 340 nm for 2 min. The results were expressed as international units of enzyme activity per liter of medium (U/l).

Production and Purification of the human Metallothionein-III Antibody-Polyclonal antibodies are produced by immunizing New Zealand White rabbits conjugated with bovine serum albumin and a synthetic peptide (CKGEEGAKAEAE) corresponding to residues 51-62 of human MT-III. A cysteine residue was attached to the N-terminus of the peptide to introduce an SH residue for coupling. Approximately 500 μg of the conjugated peptide in Freund's complete adjuvant (Sigma) was intradermally injected into two New Zealand White rabbits, followed by boosting the conjugate in Freund's incomplete adjuvant (Sigma) four times at 2 weeks intervals. Two weeks after the final boost, the serum was harvested and the immunoglobulin was purified using Protein A agarose (Oncogene) and serum collected. Specific antibodies were prepared from the antiserum by affinity column chromatography using the peptide antigen (CKGEEGAKAEAE) coupled to SulfoLink Coupling Gel (PIERCE Biotechnology).

Western Blotting-The cells were washed with PBS, and lysed at 0 °C for 30 min in a lysis buffer (20 mM Hepes, PH 7.4, 2 mM EGTA, 50 mM-glycerol phosphate, 1% triton X-100, 10% glycerol, 1 mM dithiothreitol (DTT), 1 mM phenylmethylsulfonyl fluoride (PMSF), 10 μg/ml leupeptin, 10 μ/ml aprotinin, 1 mM Na_3VO_4, and 5 mM NaF). The protein content was determined using a Bio-Rad dye-binding microassay (Bio-Rad, Hercules, CA, USA); and 20 μg protein per lane was electrophoresed on the 10% SDS polyacrylamide gels after boiling for 5 min in a Laemmli sample buffer. The proteins were blotted onto Hybon ECL membranes (Amersham-Phamacia, Biotech). The markers (MBI) were used as the size standards. After electroblotting, the membranes were blocked with Tris-buffered saline with Tween-20 (10 mM Tris-HCl, PH 7.4, 150 mM NaCl, 0.1% Tween-20) containing 5% milk, and were incubated with the anti-hOGG1, α-tubulin (Santa Cruz Biotech, CA, USA) and MT-I/II (BD Biosciences, San Diego, CA) and MT-III antibodies diluted in a blocking buffer for 4 h. The primary antibody dilutions were those recommended by the manufacture. The membranes were then washed, incubated with the appropriate secondary antibodies (1:4,000) in a blocking buffer for 2 h, and repeatedly washed again. The biotted proteins were detected using an enhanced chemiluminescence detect system (iNtRON, Biotech., Seoul, Korea).

Semiquantitative Reverse Transcriptase Polymerase Chain Reaction-RNA extraction was conducted using the RNA-STAT-60 according to the manufacturer's instructions (TEL-

TEST, Inc., Friendswood, Texas). Briefly, after homogenizing the cells in the RNA STAT-60. the homogenate was mixed with chloroform (5:1;v:v), shaken vigorously for 15 sec, and then centrifuged at 13,000 rpm for 15 min at 4 °C. The RNA present in the upper colorless aqueous phase was precipitated by adding isopropanol, which was washed twice with 70% ethanol and then air dried for 10 min. The RNA was then resuspended in DEPC. 10 µl RNA aliquots were prepared and stored at -70 °C until needed. 2 µg of the total RNA was reverse transcribed using a M-MLV cDNA synthesis system (Promega) and the reverse transcribed DNA was subjected to a polymerase chain reaction (PCR). The profile of the replication cycles was denaturation at 94 °C for 50 sec, annealing at 58 °C for 50 sec, and polymerization at 72 °C, for 1 min. In each reaction, the same amount of *glyceraldehydes-3-phosphate dehydrogenase* (*GAPDH*) was used as the internal control. The primers used for the PCR are as follows: *hOGG1* forward, 5'-CTG CCT TCT GGA CAA TCT TT-3'; *hOGG1* reverse, 5'- TAG CCC GCC CTG TTC TTC-3' designed to amplify a 551 bp region; *hMT-III* forward, 5`- TCA CTG GCA GCA GCT GCA CTT CTC -3`; *hMT-III* reverse, 5`- ATG GAC CCT GAG ACC TGC CCC TGC-3` designed to amplify a 207 bp region; *hMT-I* forward, 5`- ATG GAT CCC AAC TGC TCC TG-3`; *hMT-I* reverse, 5`- ACT TCT CCG ATG CCC CTT -3` designed to amplify a 227 bp region; *hMT-II* forward, 5`- ATG GAT CCC AAC TGC TCC TG-3`; *hMT-II* reverse, 5`-AGC TGC ACT TGT CCG ACG-3` designed to amplify a 175 bp region; *GAPDH* forward, 5'- CCA TGG AGA AGG CTG GGG-3'; and *GAPDH* reverse, 5'- CAA AGT TGT CAT GGA TGA CC-3' designed to amplify a 194 bp region (total number of cycles:26). The PCR products were resolved on 1% agarose gels, stained with ethidium bromide, and then photographed.

SiRNA-Sequence information regarding the human OGG1 and MT-III mRNA was extracted from the NCBI Entrez nucleotide database. Three target sites within the human OGG1 and MT-III gene were chosen from the human OGG1 and MT-III mRNA sequences (GenBankTM accession AF003595 and NM_005954, respectively). Following selection, each target site was searched with NCBI Blast to confirm the specificity only to the human OGG1 and MT-III. The sequences of the 21-nucleotide (nt) sense and antisense RNA are as follows: hOGG1-siRNA1, 5`-GUAUGGACACUGACUCAGUAUU-3` (sense) and 5`-UCUGAGUCAGUGUCCAUACUU-3` (antisense) for the *hOGG1* gene (nt 241-261);

hOGG1-siRNA2, 5`-GUACUUCCAGCUAGAUGUUUU-3` (sense) and 5`-AACAUCUAGCUGGAAGUACUU-3` (antisense) for the *hOGG1* gene (nt 348-368); human *MT*-siRNA, 5`-AUGCACCCUCCUGCAAGAAGUU-3` (sense) and 5`-CUUCUUGCAGGAGGGUGCAUUU-3` (antisense) for the human *MT* gene (nt 148-168); LacZ siRNA, 5`-CGUACGCGGAAUACUUCGAUU-3` (sense), 5`-AAUC GAAGUAUUCCGCGUACGUU-3` (antisense) for the *LacZ* gene. These siRNAs were prepared by a transcription-based method using a Silencer siRNA construction kit (Ambion, Austin, TX) according to manufacturer's instructions. LacZ siRNA was used as a negative control. The cells were transfected with the siRNA duplexes by using Oligofectamine (Invitrogen).

8-oxoG Glycosylase Activity Assay-The cells at the exponential phase were centrifuged at 800 × *g* for 5 min. The cell pellets (10^6 cells per each assay) were then suspended in 2 volumes of a homogenization buffer (50 mM Tris-HCl, 50 mM KCl, 1 mM EDTA, 5% glycerol and 0.05% 2-mercaptoethanol, pH 7.5) and homogenized. The homogenates were mixed with streptomycin (final concentration 1.5%) to remove the nucleic acids. The supernatants obtained by centrifugation were dialyzed extensively against the homogenization buffer and used as cell extracts for the endonuclease nicking assay. 8-oxoguanine (8-oxoG)-containing the 21mer with the sequence 5'-CAGCCAATCAGT**X**CACCATTC-3' (**X** = 8-oxoG) and its complementary strand were chemically synthesized (The Midland Certified Reagent Co., Midland, TX, USA). The oligonucleotides were 3`-end-labeled using terminal transferase and [α-^{32}P]ddATP (Amersham, 3000 Ci/mmol). The end-labeled oligomer was annealed with its complementary oligonucleotide and the resulting duplex DNA was used as the assay substrate. The duplex substrate DNA (20 pmol) was incubated with the cell extracts (10 μg protein) at 37 °C for 1 h in 1 ml of the reaction mixture (50 mM Tris-HCl, 50 mM KCl, and 1 mM EDTA, pH 7.5). The reaction was terminated by heating at 90 °C for 3 min. The reaction products were electrophoresed on 20% denaturing (7M urea) polyacrylamide gels (DNA sequencing gel). The gels were wrapped in a saran wrap and exposed to the film (Kodak) for visualization.

Analysis of 8-oxoG in the Cellular DNA-The 8-oxoG levels in the DNA of the cells were measured using a slight modification of a method described elsewhere (41). Briefly, the

cellular DNA was isolated using a DNA extractor WB kit (Wako, Osaka, Japan). 50 μg of the isolated DNA was digested with 2 units of nuclease P1 (Boehringer Mannheim Co., Mannheim, Germany) in a 100 μl solution containing 1 mM EDTA and 10 mM sodium acetate (pH 4.5). The nucleotide solution was filtered with an Ultrafree-Probind filter (Millipore, Bedford, MA), and a 20 μl aliquot of the sample was injected into an HPLC column (YMCPack ODS-AM, 5 m, 4.6 300 mm) equipped with an electrochemical detector (ECD) (Coulochem II; ESA, Chelmsford, MA) at a flow rate of 1 ml/min. The mobile phase consisted of 12.5 mM citric acid, 25 mM sodium acetate, 30 mM NaOH, and 10 mM acetic acid containing 3% methanol. dG (Sigma Chemical Co., St. Louis) and 8-oxoG (Cayman Chemical Co., Ann Arbor, MI) were used as the standards. The 8-oxoG level in the DNA was expressed as 8-oxoG/ 10^7 dG.

Antioxidant Determinations-The cell lysates were prepared by repetitive freezing and thawing in 10 mM Tris HCl, pH 7.5, 0.1% Triton X-100, and the proteins were extracted by successive freezing and thawing. The enzymatic measurements were performed on the 17,000×g supernatant. The spectrophotometric catalase assay was performed using a slight modification of a procedure described elsewhere (42). Monitoring at a wavelength of 240 nm indicated a consumption of 20 mM H_2O_2 (25°C). One unit is defined as 1 μmol H_2O_2 consumed/min. The activities were normalized to the cell protein content. The data is reported as a mean ± s.d. of triplicates. The total superoxide dismutase (SOD) (Mn SOD and Zn/Cu SOD) activity was measured by the ferricytochrome *c* assay according to a procedure derived from that described by Mccord *et al.* (43). Briefly, the reduction rate of 20 μM ferricytochrome *c* was monitored at a wavelength of 550 nm in the presence of 100 μM hypoxanthine, 0.35 unit catalase and xanthine oxidase whose activity was adjusted to yield an absorbance change of <0.015/min in the absence of SOD (25 °C). One SOD unit is defined as the amount that reduces the ferricytochrome *c* reduction rate by 50%. The data, which was normalized to the cell protein content, represent the mean ± s.d. of triplicates. The glutathione peroxidase activity was assayed according to a slight modification of the procedure described elsewhere (44). The consumption rate of 0.15 mM NADPH at 37°C was monitored at 340 nm in the presence of 0.07 units of glutathione reductase, 4 mM GSH and 0.57 mM *tert*-BuOOH. One unit is defined as 1 μmol NADPH consumed/min. The activities were normalized to the

cell protein content.

γ-ray-induced HPRT Mutation Assay-The method used to determine the frequency of the γ-ray-induced *HPRT* mutants has been described (45). Briefly, the cells were cultured in HAT medium (EMEM + 10% FBS, 100 μM hypoxanthine, 0.4 μM aminopterin, 16 μM thymidine) for at least 14 days to remove existing the *HPRT* mutants and plated at 5×10^4 per 100 mm-diameter dish. After γ-irradiation, the cells were allowed a further 12–14 days to express the *HPRT* mutants before plating in selective medium containing 30 μM 6-thioguanine (6-TG, Sigma, St. Louis, MO). A separate set of cells was plated at a cloning density in a medium lacking 6-TG that was used to determine the cloning efficiency of the cells at the time of selection. The mutant colonies were stained with 0.5% crystal violet in 50% methanol and scored after 12–14 days. For each dose and for the untreated control, the frequency of the mutants was calculated from the number of 6-TG-resistant colonies formed divided by the number of cells selected. This frequency was corrected for the cloning efficiency of the cells at the time of selection. The induced frequencies were calculated by subtracting the background frequencies in the control population.

RESULTS

MT-III Suppresses the Level of γ-ray-Induced 8-oxoG -In order to identify the potential role of MT-III in the accumulation of 8-oxoG, human MT-III was subcloned into the pcDNA3 vector, to form pcDNA3-MT-III. This construct was transfected into the human prostate cancer DU145 cells. Twelve stable transfected cell lines were established after selection using G418 for five weeks. Semi-quantitative RT-PCR and Western blot analysis revealed that the MT-III expression levels in the three individual MT-III transfected cells were significantly higher than that of the empty vector transfected cells (Fig. 1, A and B). We also made MT-I and MT-II expressing cells to compare the inhibitory effect of MT-III on the γ-radiation-induced 8-oxoG accumulation with that of MT-I and MT-II (Fig. 1, C and D).

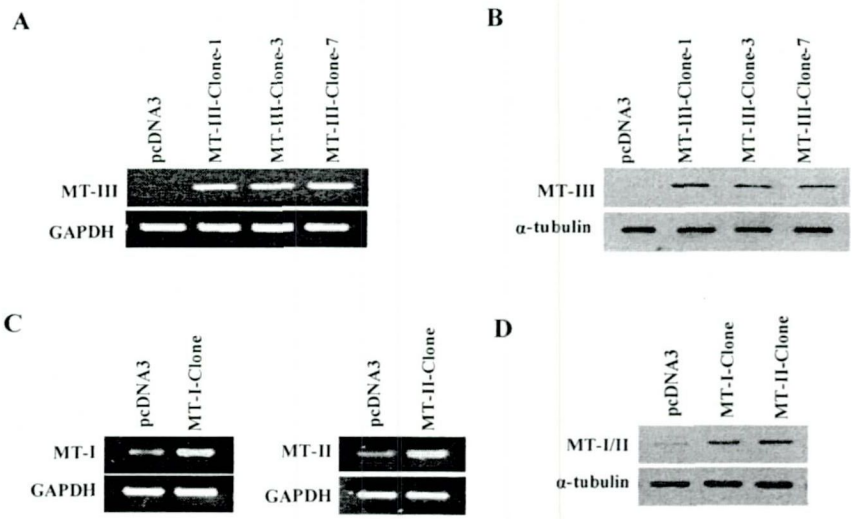

Fig. 1. Expression of MT in DU145 cells.

The effect of MT-III on γ-radiation-induced cellular toxicity was initially evaluated. Cells were exposed to 50 and 100 Gy of γ-ray. 12 h after treatment, the medium from cultures were collected and the LDH activity was measured. As shown in Fig. 2A, when the cells were

irradiated at 50 and 100 Gy, MT-III expressing clones had 23 ± 3.4 ~ 26 ± 4.1 and 30 ± 4.3 ~ 33 ± 4.9 % greater decrease in LDH release than did empty vector expressing cells, respectively. In order to investigate the effect of MT-III on the γ-radiation-induced oxidative DNA lesion in the DU145 cells, the amount of 8-oxoG in genomic DNA was measured. Parental cells, one empty vector expressing the clone, and three individual MT-III expressing clones were exposed to 20, 40, 60, 80 and 100 Gy of γ-ray, and the 8-oxoG measurement in the DNA of cells exposed to γ-rays was performed using HPLC in combination with an electrochemical detector. The 8-oxoG levels in the parental and empty vector expressing cells exposed to 20, 40, 60, 80 and 100 Gy of γ-radiation were 11.4 ± 1.5~13.6 ± 2.4, 19.3 ± 2.9 ~22.1 ± 3.2, 28.7 ± 3.3~31.5 ± 3.8, 33.4 ± 4.7~36.5 ± 4.9 and 40.7 ± 5.2~43.3 ± 5.4 per 10^7 dG, respectively. However, all three MT-III expressing clones contained 2~5 times less genomic 8-oxoG than the parent and empty vector transfected cells ($P<0.01$). The 8-oxoG level in the MT-III expressing clones (clone-1, clone-3 and clone-7) exposed to 20, 40, 60, 80 and 100 Gy of γ-radiation were 5.1 ± 1.4~7.3 ± 1.6, 6.2 ± 1.5~8.1 ± 1.9, 8.6 ± 2.3 ~13.3 ± 2.9, 12.1 ± 2.5~ 16.3 ± 3.1 and 15.4 ± 2.9~19.4 ± 3.3 per 10^7 dG, respectively (Fig. 2B). In contrast to the MT-III expressing cells, MT-I and MT-II expressing cells had only a 20 ~25 % decrease in γ-ray-induced 8-oxoG accumulation as compared to empty vector transfected cells (Fig. 2C). Therefore, MT-III, MT-I and MT-II expression prevent the γ-ray-mediated increase in the accumulation of 8-oxoG in human prostate cancer DU145 cells, and the effect of MT-III is greater than that of MT-I and MT-II..

The Effect of MT-III on the Expression of hOGG1-8-oxoG is excised by the DNA glycosylase/lyase activity of hOGG1, which is a representative member of the DNA glycosylase/lyase family of repair enzymes (46-48). Therefore, low amounts of 8-oxoG in genomic DNA from the MT-III expressing clones might reflect the increased repair of 8-oxoG. In order to test this hypothesis, the hOGG1 mRNA and protein in the empty vector transfected clone and the MT-III expressing clones was compared. Using semiquantitative RT-PCR using the specific primers for hOGG1 and western blotting with a specific antibody against hOGG1 to evaluate the level of the hOGG1 mRNA and protein, it was found that the levels of the hOGG1 mRNA and protein in the MT-III expressing and control cells were similar (Fig. 3, A and B). These suggested that MT-III expression has no effect on the

hOGG1 expression level.

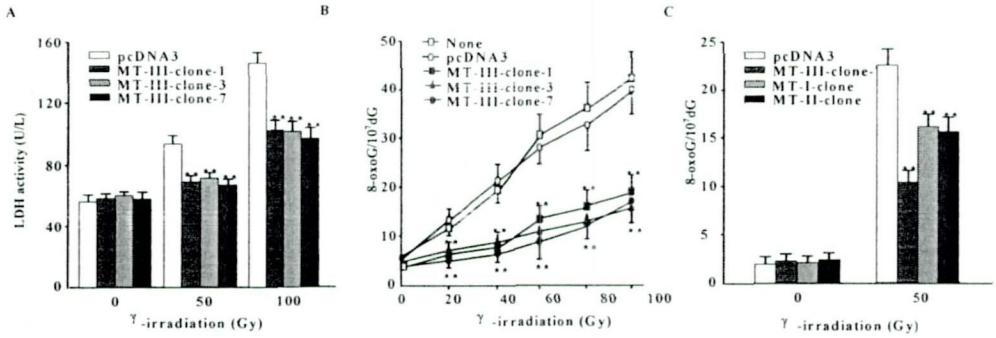

Fig. 2. MT-III inhibits γ-radiation-induced 8-oxoG accumulation.

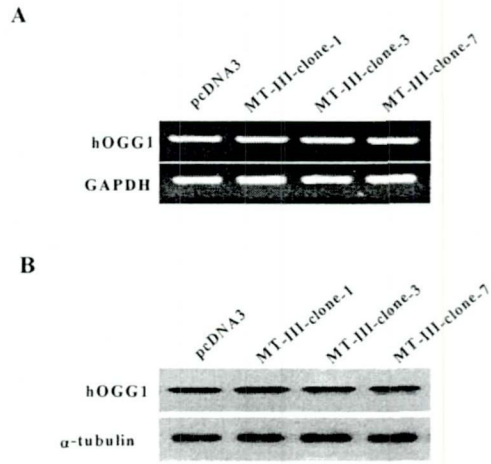

Fig. 3. The effect of MT-III on the hOGG1 expression.

The Effect of MT-III on the Antioxidant Enzyme Activity-Because the direct consequence of the ROS interaction with DNA is the generation of oxidative DNA damage, such as 8-oxoG, this study next examined whether or not MT expression leads to the modulation of the antioxidant enzyme activity. The activities of catalase, superoxide dismutase and glutathione peroxidase were determined in the control and MT-III expressing clones. As shown in Table 1, the levels of these antioxidant enzyme activities in the MT-III expressing and control cells were similar, suggesting that MT-III expression has no effect on the antioxidant enzyme activity.

Table 1. Activities of antioxidant enzymes. Each value is the mean±s.d. from the four to five independent determinations. All activities were determined in cell homogenates as described under "Experimental Procedures"

	SOD U/mg protein	Catalase U/mg protein	GSH-Px U/mg protein
DU145	2.11 ± 0.07	0.65 ± 0.16	19.4 ± 2.75
DU145 + pcDNA3	1.96 ± 0.06	0.71 ± 0.14	21.3 ± 2.69
DU145/MT-III-clone-1	2.14 ± 0.08	0.74 ± 0.18	20.9 ± 3.14
DU145/MT-III-clone-3	2.09 ± 0.05	0.63 ± 0.15	19.9 ± 2.80
DU145/MT-III-clone-7	2.12 ± 0.06	0.69 ± 0.16	21.1 ± 2.71

8-oxoG Glycosylase Activities in γ-irradiated Cells-A previous study revealed that the 8-oxoG repair activity was reduced in the γ-irradiated cells (49). Therefore, we investigated whether γ-radiation can affect hOGG1 expression and its activity in MT-III expressing cells. The MT-III expressing clone-7 cells were treated with 20, 40, and 60 Gy of γ-radiation and the hOGG1 protein levels were determined in the attached cells. Western blotting with a specific antibody against hOGG1 showed that the hOGG1 was reduced by 21 % and 52 % in

the 40 and 60 Gy of γ-irradiated cells as compared with those of control cells, respectively (Fig. 4A). In order to confirm the γ-radiation-mediated decrease in hOGG1 expression, nuclear extracts from the γ-irradiated cells were prepared and examined for their ability to cleave 8-oxoG using a 21-mer oligonucleotide containing a single 8-oxoG at nucleotide 13. As shown in Fig.4B, the γ-irradiated cells resulted in a concentration-dependent decrease in the ability of the nuclear extracts to cleave the 8-oxoG:C substrate. These results suggest that the γ-irradiated MT-III expressing cells have a lower 8-oxoG repair activity.

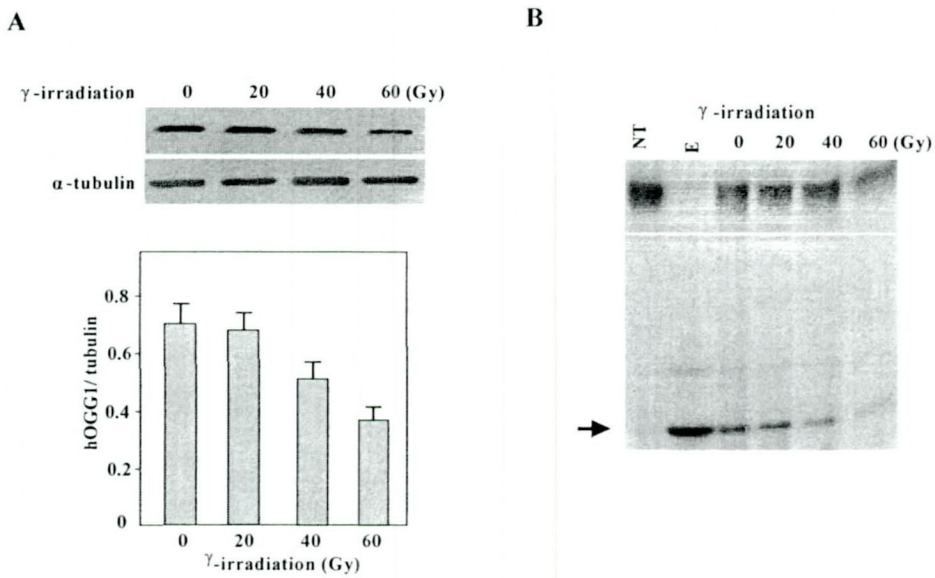

Fig. 4. Effect of γ-radiation on the hOGG1 protein level and 8-oxoG glycosylase activity.

siRNA-Mediated Down-Regulation of hOGG1-The above results suggest that γ-irradiation decreased the 8-oxoG repair activity. Therefore, the potential role of MT-III in the γ-ray-induced 8-oxoG formation in hOGG1 depleted cells was next analyzed using the hOGG1 specific siRNAs. Two target regions of the human hOGG1 mRNA (*i.e.* 241-261 and 348-368 sequences) were selected by scanning the length of the hOGG1 gene for the AA-

dinucleotide sequences and downstream 19 nucleotides without a significant homology to the other genes using an appropriate genome database. The antisense strands of the synthesized hOGG1-siRNAs are the reverse complement of the target sequences. The sense strands of the hOGG1-siRNAs have the same sequences as the target mRNA sequences with the exception that they lack the 5`-AA sequence. A uridine dimer was incorporated at the 3`-end of the sense strand siRNAs. Therefore, the end products are three double-stranded 21-mer siRNAs (*i.e.* hOGG1-siRNA1 and hOGG1-siRNA2), which should theoretically reduce the hOGG1 mRNA and protein expression levels, and the control siRNA (lacZ siRNA), which should not be effective in hOGG1 gene silencing. Two different 21-base pair siRNA constructs, hOGG1-siRNA1, hOGG1-siRNA-2, the mock, and the control siRNA, were transfected into the DU145 cells to test their ability to suppress hOGG1 expression. In order to obtain the quantitative hOGG1 mRNA expression values, semiquantitative RT-PCR experiments were performed 24 h after treating the cells with either the hOGG1 siRNAs, the mock, or the control siRNA. Treatment with the hOGG1 siRNAs resulted in a decrease in the hOGG1 mRNA level to $85 \pm 6 \sim 90 \pm 5$ %, compared to the mock-and control siRNA transfected cells (Fig. 5A). The protein extracts were obtained 24-96-h after transfection, and Western blot analyses were performed for the hOGG1 protein and the normalized to the α-tubulin expression level. It was found that both two siRNAs to the different sequences within the hOGG1 gene effectively inhibited hOGG1 protein expression 48 h after transfection (Fig. 5B). By 96-h post-transfection, the hOGG1 protein levels had increased to the levels comparable to the control siRNA-transfected cells (data not shown). The level of 8-oxoG repair activity was also tested, and it was confirmed that the hOGG1 siRNAs transfected cells had almost no 8-oxoG repair activity (Fig. 5C). These results demonstrate that all hOGG1 siRNAs were specific to hOGG1. Because there is a strong correlation between the hOGG1 mRNA and protein suppression by siRNA, these results suggest that hOGG1 silencing in the DU145 cells result from a reduction in the amount of hOGG1 mRNA available for translation. This suggests that the hOGG1-siRNAs are highly specific and efficient in hOGG1 gene silencing in the DU145 cells.

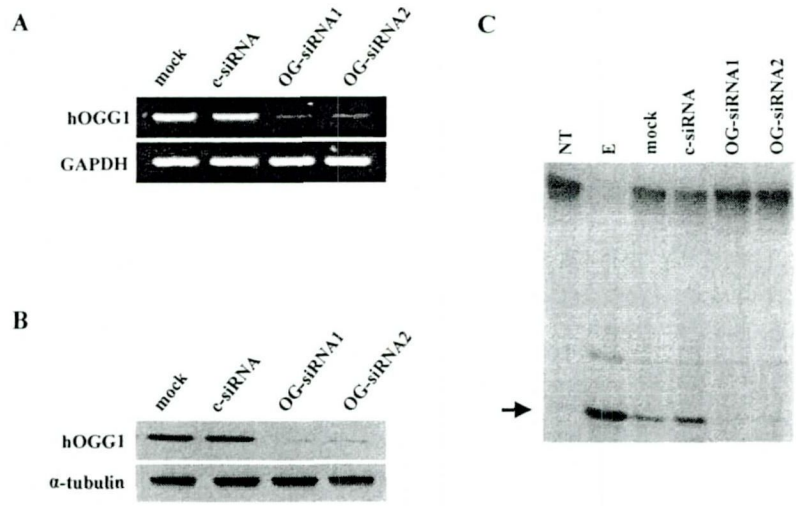

Fig. 5. siRNA-mediated down-regulation of the hOGG1 mRNA and protein in the DU145 cells.

Down-Regulation of hOGG1 via siRNA Leads to an Increase in the Level of γ-radiation-Induced 8-oxoG-Because hOGG1 is involved in the BER pathway and is known to play a crucial role in the repair of the 8-oxoG (46-48), the 8-oxoG levels was measured in the genomic DNA from hOGG1 depleted DU145 cells. The DU145 cells were transfected with either the mock, control-siRNA or hOGG1-siRNAs. Forty-eight hours after transfection, the cells were then exposed to 10, 20, 30, 40 and 50 Gy of γ rays and the amount of 8-oxoG in the genomic DNA was then measured by HPLC with an electrochemical detector. As shown in Fig. 6, in the mock and control-siRNA transfected cells, a 8-oxoG levels for the cells exposed to 10, 20, 30, 40 and 50 Gy of γ-radiation were 6.2 ± 2.5~9.1 ± 3.4, 10.6 ± 3.7~14.8 ± 4.1, 13.8 ± 3.9 ~17.2 ± 4.1, 19.3 ± 6.2~21.3 ± 7.5, and 26.5 ± 7.1~29.6 ± 7.4 per 10^7 dG, respectively. However, in the cells transfected with the hOGG1-siRNA, the γ-ray-induced 8-oxoG levels were significantly higher compared with those of the mock and control-siRNA transfected cells. The hOGG1 siRNA-transfected cells demonstrated that an 8-oxoG level for

the cells exposed to 10, 20, 30, 40 and 50 Gy of γ-radiation were 29.5 ± 3.9~35.3 ± 4.5, 42.4 ± 4.1~48.3 ± 4.9, 60.1 ± 6.7~64.3 ± 7.2, 83.4 ± 11.4~84.1 ± 12.3, and 101,5 ± 12.3~105.6 ± 14.5 per 10^7 dG, respectively.

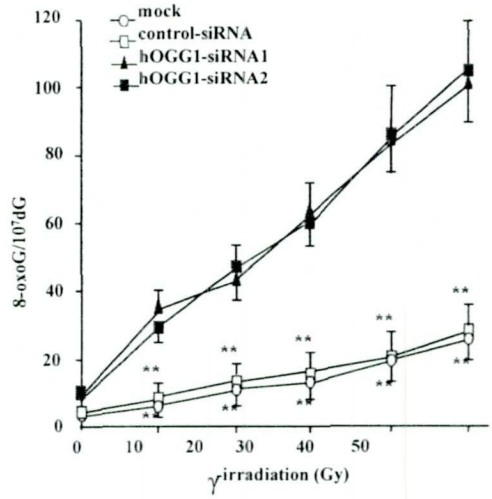

Fig. 6. siRNA-mediated down-regulation of the hOGG1 leads to increase in γ- ray 8-oxoG accumulation.

Expression of MT-III Suppresses the Level of γ ray-Induced 8-oxoG Accumulation in the hOGG1 Depleted Cells-In order to investigate the effect of MT-III on the accumulation of the 8-oxoG in hOGG1 depleted cells, the empty vector pcDNA3 transfected cells and the MT-III clone-7 cells were treated with hOGG1-siRNAs. Forty-eight hours after treatment, the cells were then exposed to 50 Gy of γ-irradiation and the 8-oxoG level in the genomic DNA was measured. We found that the amount of 8-oxoG in genomic DNA from the hOGG1-siRNA treated pcDNA3 clone cells exposed to 50 Gy of γ-radiation were 95.3 ± 6.4 ~106.1 ± 12.5 per 10^7 dG. However, the genomic DNA of the hOGG1-siRNA treated MT-III clone-7 contains a 57 ~ 61 % decrease in γ-ray-induced 8-oxoG accumulation as compared to hOGG1-siRNA treated empty vector transfected cells (Fig 7).

The amount of 8-oxoG in the genomic DNA from the hOGG1-siRNA treated MT-III clone-7 cells exposed to 50 Gy of γ-radiation were 39.7 ± 5.6~43.4 ± 9.5 per 10^7 dG. In contrast to the effect of MT-III, the hOGG1-siRNA treated MT-I and MT-II clones has only a 25~29 % decrease in γ-ray-induced 8-oxoG accumulation as compared to hOGG1-siRNA treated empty vector transfected cells. These results suggest that MT-III can prevent the γ ray-induced 8-oxoG accumulation in hOGG1 depleted cells, and the inhibitory effect of MT-III on the 8-oxoG accumulation is higher than that of MT-I and MT-II.

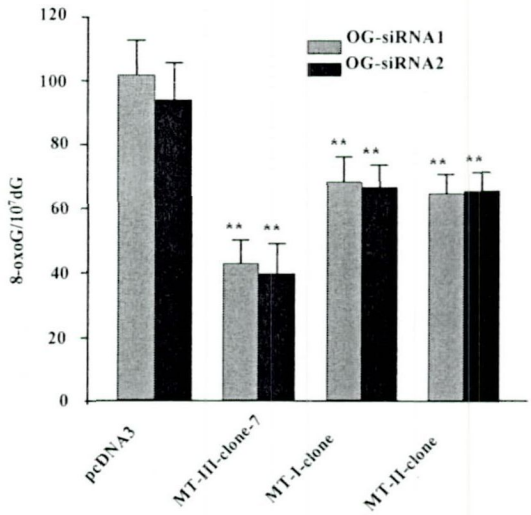

Fig. 7. Effect of MT-III on the γ- ray-induced 8-oxoG accumulation in hOGG1 depleted cells.

Expression of MT-III Prevents γ ray-Induced Mutant Frequency of the HPRT Gene in

hOGG1 Depleted Cells-In order to investigate whether or not MT-III expression may inhibit the γ-irradiation-induced mutation in the hOGG1 depleted cells, γ-radiation-induced mutation in the hOGG1 depleted human prostate cancer DU145 cells were initially evaluated. We investigated the γ-irradiation-induced mutagenesis of the *HPRT* gene, and found that a mutation of this gene leads to 6-thioguanine (6-TG) resistance of mutant cells. The DU145 cells were transfected with hOGG1-siRNAs. Forty-eight hours after transfection, the cells were exposed to 2, 4, 6 and 8 Gy γ rays, and the γ ray-induced mutant frequencies of the *HPRT* gene were measured. As shown in Fig. 8, the hOGG1 siRNAs transfected cells showed a significant higher mutant frequency than the mock- and control siRNA-transfected cells. The mutant frequencies of the *HPRT* were 49 ± 9~51 ± 11 x 10^{-6}, 93 ±21~105 ± 24 x 10^{-6}, 142 ± 28~154 ± 31 x 10^{-6}, and 206 ± 31~ 216 ± 34 x 10^{-6} in the hOGG1 siRNA-transfected cells exposed to 2, 4, 6 and 8 Gy γ rays, respectively. These values were 2~3-fold higher than the *HPRT* mutant frequency of the γ-irradiated mock and control siRNA-transfected cells.

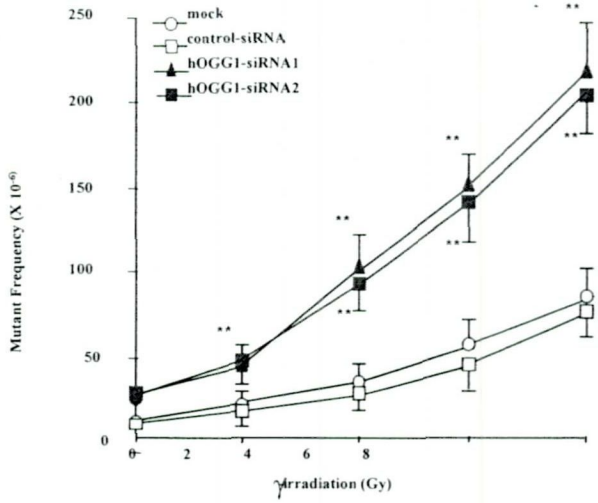

Fig. 8. siRNA-mediated down-regulation of the hOGG1 leads to increase in γ- radiation-induced mutant frequency.

The role of MT-III in the inhibition of γ ray-induced mutation in hOGG1 depleted cells was next investigated. The pcDNA3 clone and MT-III clone-7 cells were treated with hOGG1-siRNAs. Forty-eight hours after treatment, the cells were then exposed to either 5 Gy or 10 Gy of γ-radiation and the *HPRT* mutant frequencies were then determined. As shown in Table 2, the hOGG1-siRNA treated MT-III clone-7 cells have a decreased mutation frequency at the *HPRT* locus, compared to that of the hOGG1-siRNA treated control cells. The *HPRT* mutant frequencies were 61 ± 29~64 ± 35 x 10^{-6} and 124 ± 45~138 ± 57 x 10^{-6} in hOGG1-siRNA treated MT-III clone-7 cells exposed to 5 and 10 Gy of γ rays, respectively. However, the γ-ray-induced mutation was significantly increased in hOGG1 siRNA transfected pcDNA3 clone cells. The *HPRT* mutant frequencies were 139 ± 45~143 ± 49 x 10^{-6} and 269 ± 64~272 ± 69 x 10^{-6} in the hOGG1-siRNA treated pcDNA3 clone cells exposed to 5 and 10 Gy γ rays, respectively. These results suggest that MT-III is important for suppressing the γ-radiation-induced mutation, when the hOGG1 is down-regulated.

Table 2. Mutation frequencies at the HPRT locus in pcDNA3 transfected cells, MT-III transfected clone-7 cells

Dose of γ-irradiation	Cell line	Mutant Frequency [a] (x 10^{-7})	Relative mutant frequency [b]
5 Gy	pcDNA3	39 ± 12	1
	pcDNA3 + OG-siRNA1	139 ± 45	3.6
	pcDNA3 + OG-siRNA2	143 ± 49	3.7
	MTIII-clone-7 + OG-siRNA1	61 ± 29	1.6
	MTIII-clone-7 + OG-siRNA2	64 ± 35	1.7
10 Gy	pcDNA3	94 ± 35	2.4
	pcDNA3 + OG-siRNA1	269 ± 64	6.9
	pcDNA3 + OG-siRNA2	272 ± 69	7.0
	MTIII-clone-7 + OG-siRNA1	124 ± 45	3.2
	MTIII-clone-7 + OG-siRNA2	138 ± 57	3.5

a Mutations /cell/generation.

b Frequencies are relative to that observed in 5 Gy irradiated pcDNA3 cells

DISCUSSION

The results of this study demonstrate the essential role of MT-III in inhibiting 8-oxoG formation, the major mutagenic base lesion in DNA caused by exposure to ROS. Ionizing irradiation damages the DNA for the most part by ionizing water to produce ROS. These results suggest that the formation of 8-oxoG in human prostate cancer cells is increased as a result of exposure to γ-irradiation, resulting in the dose-dependent accumulation of 8-oxoG, and that the increased 8-oxoG level are suppressed significantly by MT-III expression. In addition, elevated MT-III cells caused a further decrease in the γ-irradiation-induced 8-oxoG formation and γ-irradiation-induced mutation frequency in the hOGG1 depleted cells, compared to that of the hOGG1 depleted control cells. Therefore, it is likely that MT-III is important for suppressing the γ-irradiation-mediated increase in 8-oxoG accumulation and mutation.

Recently, several lines of evidence have suggested that MT has a protective effect against ROS-induced DNA damage, including γ-irradiation. For example, increased MT expression levels led to a significant decrease in the radiation-induced chromosome and DNA damage (51, 52). Although the detailed mechanism of MT in protection against radiation needs to be elucidated, many studies support the hypothesis that MT, acting as ROS scavengers, leads to protection against radiation-induced cellular toxicity in normal and tumor cells (53-55). MT has a high cysteine content, they can freely exchange metals with electrophiles, and thereby prevent Fenton reactions, or react directly with H_2O_2 or hydroxyl radicals (16, 56). It has also been demonstrated that the *in vitro* oxidation of the MT cysteines induces the release of metals, and that ROS can react directly with MT sulfhydryl groups (16-21). Therefore, MT scavenges ROS before they can react with the DNA. The *in vivo* and *in vitro* studies show that the efficiency of the protective effect of MT against DNA degradation from oxidative damage was much higher than that of the reduced glutathione (17, 18, 22). Ionizing radiation generates hydroxyl radicals either directly by the hydrolysis of water, or indirectly by forming other ROS, including the superoxide anion, hydrogen peroxide and peroxyl radicals. These ROS may subsequently be converted to hydroxyl radicals by further reactions during the cellular metabolic processes (34-36). Approximately 90 % of the cellular

DNA damage produced by ionizing radiation is caused by hydroxyl radicals, which generates more than 30 different base adducts as well as various amino acids, proteins and lipid addition products, strand breaks and crosslinks (57). Of the various types of DNA modifications induced by ionizing radiation, 8-oxoguanine (8-oxoG) is one of the most prevalent DNA damage products. 8-oxoG preferentially mispairs with adenosine during replication and thereby gives rise to G:C to T:A transversion mutations (31). Consequently, the formation of 8-oxoG that occurs as a result of a reaction of hydroxyl radicals from ionizing radiation with DNA is likely to play a role in DNA mutagenesis and carcinogenesis (33). MT overexpression was found to protect the Chinese hamster G12 cells against mutagenesis by oxidative mutagens, as well as against the oxidative stress induced by the tumor promoter, 121-O-tetradecanoylphorbol-13-acetate (TPA) (37, 58). Similarly, increased levels of MT in the pancreatic β-cells can prevent the streptozotocin-induced DNA damage, which is probably caused by ROS (59). Moreover, the under expression of MT with the antisense RNA led to an increase in spontaneous mutagenesis, which was accompanied by increased levels of oxidative stress (38). Using the DNA strand break assay and comet assay following the hydrogen peroxide treatment, we have previously demonstrated that MT-III could prevent ROS-induced DNA damage, and the efficiency of the protective effect of MT-III against DNA degradation from oxidative damage was much higher than that of MT-I/II (39). Therefore, MT-III may play an important role in suppressing mutagenesis through a decrease in the 8-oxoG accumulation in response to ROS-induced DNA damage, including ionizing radiation. However, there is no biochemical study reporting the precise role of MT-III in 8-oxoG accumulation. In order to evaluate the potential role of MT-III in the accumulation of 8-oxoG after ionizing radiation, the coding region of human MT-III was cloned and permanently transfected into the DU145 cells. As shown in Fig. 2, the 8-oxoG levels in the MT-III expressing clones following exposure to γ-irradiation, expressed as the ratio of 8-oxoG to dG, were significantly decreased when compared to that in the parental and empty vector expressing cells. The inhibitory effect of MT-III to γ-ray-induced 8-oxoG accumulation was higher than MT-I and MT-II. It was also demonstrated that MT-III expression did not affect the regulation of the other antioxidant defense system, such as SOD, catalase, glutathione peroxidase and glutathione (Table 1), and that MT-III expression also

did not affect the hOGG1 expression level along with its activity (Fig. 3). These results suggest that MT-III itself effectively suppresses γ-irradiation-induced 8-oxoG formation.

Escherichia coli contains a GO system that prevents the mutagenic effect of 8-oxoG. The bacterial GO system consists of three proteins: MutM, a DNA glycosylase/lyase that recognizes 8-oxoG:C and catalyzes the excision of 8-oxoG; MutY, which is a DNA glycosylase that recognizes 8-oxoG:A and catalyzes the excision of A; and MutT, a specific phosphatase to cleave 8-oxo-dGTP (60). In mammalian cells, the main defense enzyme against the mutagenic effects of 8-oxoG in cellular DNA is 8-oxoguanine-DNA glycosylase (OGG1), which is the structurally unrelated but functionally similar to Mut M. The inactivation of the *OGG1* gene in yeast and mice leads to an increase in the spontaneous mutation frequency in the cells. The human *OGG1* (*hOGG1*) gene is found on chromosome 3p26.2, and allelic deletions of this region frequently occur in a variety of human cancers (46-48). In addition, the *hOGG1* gene is somatically mutated in some cancer cells and is highly polymorphic among the human populations (61, 62). Recently, Mei *et al.*, reported that the 8-oxoG repair activity was significantly reduced in the γ-irradiated cells compared to that in the control cells (49). In this study, it was confirmed that the γ-irradiation appeared to decrease the hOGG1 protein expression and its repair activity (Fig. 4). Therefore, the exposure of the cells to γ-irradiation may lead to a significant increase in 8-oxoG accumulation through both ROS generation and inhibition of the 8-oxoG repair activity. These observations indicate that MT-III plays an important role in the suppression of 8-oxoG formation when the hOGG1 level is decreased. In order to explore this hypothesis, small interfering RNAs (siRNAs) in the form of two independent, non-overlapping, 21-base pair RNA duplexes, which target human OGG1 (hOGG1), was used so as to inhibit its expression. The transfection of the parent DU145 and pcDNA3 transfected cells with the hOGG1-siRNA resulted in a significant increase in the 8-oxoG accumulation response to γ-irradiation in a dose-dependent manner compared to the mock- and control siRNA-transfected cells. However, the amount of 8-oxoG formation from the hOGG1 depleted MT-III expressing cells exposed to γ-irradiation was markedly suppressed compared to that of the hOGG1 depleted control cells (Fig. 7). Furthermore, the γ-ray-induced mutation was significantly increased in the hOGG1 depleted cells, and this increase was also suppressed by MT-III expression (Table 2). These results

strongly suggest that MT-III can prevent the γ-irradiation-induced accumulation of 8-oxoG as well as the γ-irradiation-induced mutation frequency in the normal and hOGG1 depleted cells.

In summary, oxidative DNA damage is the result of an imbalance between the production of ROS, which are believed to be responsible for most of the initial DNA damage caused by oxidative stress, and the ability of the cellular antioxidant defense systems. Cells have developed elaborate networks to deal with potentially damaging radicals, which include enzymatic and non-enzymatic antioxidative systems. Although antioxidant enzymes including SOD, catalase and glutathione peroxidase are important for the cellular antioxidant defense systems, they do not contribute to the protection of oxidative DNA damage because the nucleus lacks these antioxidant enzymes (99). In contrast to the antioxidant enzymes, MT are present in both the nucleus and cytoplasm in the normal circumstances. Moreover, DNA damaging agents such as UV-irradiation lead to an increase in the MT expression level as well as an increase in the stimulation of the nuclear translocation of MT (100-102). This nuclear accumulation of MT is an important factor that determines the role of MT in the protection from ROS-induced DNA damage, because DNA is quite sensitive to oxidative stress, whereas the nucleus does not contain antioxidant enzymes. MT-null mice have high incidence to tumor formation caused by chemical carcinogens such as 7, 12-dimethylbenz[a]anthracene (DMBA), 121-O-tetradecanoylphorbol-13-acetate (TPA) and N-butyl-N-(4-hydroxybutyl)nitrosamine (62, 103). In addition, the carcinogenicity caused by X-rays and anticancer drugs such as cisplatin and melphalan have been prevented by a pretreatment with bismuth and zinc, both of which are well known MT inducers (104, 105). We demonstrate that MT-III is important for suppressing the level of γ-ray-induced 8-oxoG and mutations in the normal and hOGG1 depleted cells. These results strongly suggest that MT-III protect against oxidative DNA damage, and this protection may, at least in part, contribute to the anticarcinogenic role of MT-III.

REFERENCES

1. Miles, A.T., Hawksworth, G.M., Beattie, J.H., and Rodilla, V. (2000) *Crit. Rev. Biochem. Mol. Biol.* **35**, 35-70
2. Hidalgo, J., Aschner, M., Zatta, P., and Vasak, M. (2001) *Brain Res. Bull.* **15**, 133-145
3. Waalkes, M.P., and Klaassen, C.D. (1985) *Fundam Appl. Toxicol.* **5**, 473-477
4. Uchida, Y., Takio, K., Titani, K., Ihara, Y., and Tomonaga, M. (1991) *Neuron* 7, 337-347
5. Palmiter, R.D., Findley, S.D., Whitmore, T.E., and Durnam, D.M. (1992) *Proc. Natl. Acad. Sci. USA.* **89**, 6333-6337
6. Quaife, C.J., Findley, S.D., Erickson, J.C., Froelick, G.J., Kelly, E.J., Zambrowicz, B.P., and Palmiter, R,D. (1994) *Biochemistry* **33**, 7250-7259
7. Masters, B.A., Quaife, C.J., Erickson, J.C., Kelly, E.J., Froelick, G.J., Zambrowicz, B.P., Brinster, R.L., and Palmiter, R.D. (1994) *J. Neurosci.* **14**, 5844-5857
8. Kagi, J.H.R., and Vallee, B.L. (1960) *J. Biol. Chem.* **235**, 3460–3465
9. Durnam, D.M., and Palmiter, R.D.(1987) *Experientia Suppl.* **52**, 457–463
10. Jacob, C., Maret, W., and Vallee, B.L. (1998) *Proc. Natl. Acad. Sci. USA.* **95**, 3489–3494
11. Klaassen, C.D., Liu, J., and Choudhuri, S. (1999) *Annu. Rev. Pharmacol. Toxicol.* **39**, 267-294
12. Cherian, M.G., and Apostolova, M.D. (2000) *Cell. Mol. Biol.* **46**, 347-356
13. Jin, R., Chow, V.T., Tan, P.H., Dheen, S.T., Duan, W., and Bay, B.H. (2002) *Carcinogenesis* **23**, 81-86
14. Cai, L., Satoh, M., Tohyama, C., and Cherian, M.G. (1999) *Toxicology* **132**, 85-98
15. Lazo, J.S., Kuo, S.M., Woo, E.S., and Pitt, B.R. (1998) *Chem. Biol. Interact.* **111-112**, 255-262
16. Chubatsu, L.S., and Meneghini, R. (1993) *Biochem. J.* **291**, 193–198
17. Mello-Filho, A.C., Chubatsu, L.S., and Meneghini, R. (1988) *Biochem.J.* **256**, 475–479
18. Thornalley, P.J., and Vasak, M. (1985) *Biochim. Biophys. Acta.* **827**, 36–44
19. Abel, J., and N. de Ruiter (1989) *Toxicol. Lett.* **47**, 191–196
20. Greenstock, C.L., Jinot, C.P., R.P. Whitehouse,R.P., and Sargent, M. D. (1987) *Free Radic. Res. Commun.* **2**, 233–239
21. Cai, L., Tsiapalis, G., and Cherian, M.G. (1998) *Chem. Biol. Interac.* **115**, 141–151
22. Miura, T., Muraoka, S., and Ogiso, T. (1997) *Life Sci.* **60**, 301-309
23. Shiraishi, N., Yamamoto, H., Takeda, Y., Kondoh, S., Hayashi, H., Hashimoto, K., and Aono, K. (1986) *Toxicol. Appl. Pharmacol.* **85**, 128–134
24. Koropatnick, J., Leibbrand, M., and Cherian, M.G. (1989) *Radiat. Res.* **119**, 356–365
25. Bauman, J.W., Liu, J., Liu, Y.P., and Klaassen, C.D. (1991) *Toxicol. Appl. Pharmacol.* **110**, 347–354
26. Yu, W.H., Lukiw, W.J., Bergeron, C., Niznik, H.B., and Fraser, P.E. (2001) *Brain Res.* **894**, 37–45
27. Hozumi, I., Inuzuka, T., Hiraiwa, M., Uchida, Y., Anezaki, T., Ishiguro, H., Kobayashi, H., Uda, Y., Miyatake, T., and Tsuji, S. (1995) *Brain Res.* **688**, 143–148
28. Yamada, M., Hayashi, S., Hozumi, I., Inuzuka, T., Tsuji, S., and Takahashi, H. (1996) *Brain Res.* **735**, *257–264*
29. Erickson, J.C., Hollopeter, G., Thomas, S.A., Froelick, G.J., and Palmiter, R.D. (1997) *J.*

Neurosci. **17,** 1271–1281
30. Hogg, N. (1998) *Semin. Reprod. Endocrinol.* **16,** 241-248
31. Cadet, J., Berger, M., Douki, T., and Ravanat, J.L. (1997) *Rev. Physiol. Biochem. Pharmacol.* **131,** 1-87
32. Wood, M.L., Dizdaroglu, M., Gajewski, E., and Essigmann, J.M. (1990) *Biochemistry* **29,** 7024-7032
33. Cheng, K.C., Cahill, D.S., Kasai, H., Nishimura, S., and Loeb, L.A. (1992) *J. Biol. Chem.* **267,** 166-172
34. Chapman, J.D., Reuvers, A.P., Borsa, J., and Greenstock, C.L. (1973) *Radiat. Res.* **64,** 291–306
35. Roots, R., and Okada, S. (1975) *Radiat. Res.* 64, 306–320
36. Burkitt, M. J., and Gilbert, B.C. (1990) *Free Radic. Res. Commun.* **10,** 265–280
37. Rossman, T.G., and Goncharova, E.I. (1998) *Mutat. Res.* **402,** 103-110
38. Rossman, T.G., Goncharova, E.I., Nadas, A., and Dolzhanskaya, N. (1997) *Mutat. Res.* **373,** 75-85
39. You, H.J., Lee, K.J., and Jeong, H.G. (2002) *FEBS Lett.* **521,** 175-179
40. You, H.J., Oh, D.H., Choi, C.Y., Lee, D.G., Hahm, K.S., Moon, A.R., and Jeong, H.G. (2002) *Biochim. Biophys. Acta.* **1573,** 33-38
41. Lee, M.R., Kim, S.H., Cho, H.J., Lee, K.Y., Moon, A.R., Jeong, H.G., Lee, J.S., Hyun, J.W., Chung, M.H., and You, H.J. (2004) *J. Biol. Chem.* **279,** 9857-9866
42. Shigenaga, M.K., Park, J.W., Cundy, K.C., Gimeno, C.J., and Ames, B.N. (1990) *Methods Enzymol.* **186,** 521–530
43. Beers, R.F., and Sizer, I.W. A (1952) *J. Biol. Chem.* **195,** 133-140
44. Mccord, J.M., and Fridovich, I. (1969) *J. Biol. Chem.* **224,** 6049-6055
45. Flohe, L., and Gunzler, W.A. (1984) *Methods Enzymol.* **105,** 114-121
46. Glaab, W.E., Tindall, K.R., and Skopek, T.R. (1999) *Mutat. Res.* **427,** 67-78
47. Radicella, J.P., Dherin, C., Desmaze, C., Fox, M.S., and Boiteux, S. (1997) *Proc. Natl Acad. Sci. USA.* **94,** 8010–8015
48. Roldan-Arjona, T., Wei, Y.F., Carter, K.C., Klungland, A., Anselmino, C., Wang, R.P., Augustus, M., and Lindahl, T. (1997) *Proc. Natl. Acad. Sci. USA.* **94,** 8016–8020
49. Rosenquist, T.A., Zharkov, D.O., and Grollman, A.P. (1997) *Proc. Natl. Acad. Sci. USA.* **94,** 7429–7434
50. Mei, N., Tamae, K., Kunugita, N., Hirano, T., and Kasai, H. (2003) *Environ Mol. Mutagen.* **41,** 332-338
51. Aschner, M. (1998) *Neurotoxicology* **19,** 653-660
52. Cai, L., and Cherian, M.G. (1996) *Mutat. Res.* **369,** 233-241
53. Vukovic, V., Pheng, S.R., Stewart, A., Vjk, C.H., and Hedley, D.W. (2000) *Int. J. Radiat. Biol.* **76,** 757-762
54. Renan, M.J., and Dowman, P.I. (1989) *Radiat. Res.* **120,** 442-455
55. Sato, M., and Bremner, I. (1993) *Free Radic. Biol. Med.* **14,** 325-337
56. Eichholtz-Wirth, H., Reidel, G., and Hietel, B. (1993) *Br. J. Cancer* **67,** 1001-1006
57. Cai, L., and Cherian, M.G. (2003) *Toxicol. Lett.* **136,** 193-198
58. Riley, P.A. (1994) *Int. J. Radiat. Biol.* **65,** 27-33
59. Suzuki, J.S., Nishimura, N., Zhang, B., Nakatsuru, Y., Kobayashi, S., Satoh, M., and Tohyama, C. (2003) *Carcinogenesis* **24,** 1123-1132

60. Chen, H., Carlson, E.C., Pellet, L., Moritz, J.T., and Epstein, P.N. (2001) *Diabetes* **50,** 2040-2046
61. Tajiri, T., Maki, H., and Sekiguchi, M. (1995) *Mutat. Res.* **336,** 257-267
62. Chevillard, S., Radicella, J.P., Levalois,C., Lebeau, J., Poupon, M.F., Oudard, S., Dutrillaux, B., and Boiteux, S. (1998) *Oncogene* **16,** 3083–3086
63. Penkowa, M., Espejo, C., Martinez-Caceres, E.M., Poulsen, C.B., Montalban, X., and Hidalgo, J. (2001) *J. Neuroimmunol.* **119,** 248-260
64. Penkowa, M., Espejo, C., Martinez-Caceres, E.M., Montalban, X., and Hidalgo, (2003) *J. Cell Mol Life Sci.* **60,** 185-197
65. Penkowa, M., and Hidalgo, J. (2003) *J. Neurosci. Res.* **72,** 574-586
66. Carrasco, J., Penkowa, M., Hadberg, H., Molinero, A., and Hidalgo, J. (2000) *Eur. J. Neurosci.* **12,** 2311-2322
67. Penkowa, M., Carrasco, J., Giralt, M., Moos, T., and Hidalgo, J. (1999) *J. Neurosci.* **19,** 2535-2545
68. Giralt, M., Penkowa, M., Lago, N., Molinero, A., and Hidalgo, J. (2002) *Exp. Neurol.* **173,** 114-128
69. Puttaparthi, K., Gitomer, W.L., Krishnan, U., Son, M., Rajendran, B., and Elliott, J.L. (2002) *J. Neurosci.* **22,** 8790-8796
70. Carrasco, J., Penkowa, M., Giralt, M., Camats, J., Molinero, A., Campbell, I.L., Palmiter, R.D., and Hidalgo, (2003) *J. Neurobiol. Dis.* **13,** 22-36
71. Irie, Y., and Keung, W.M. (2001) *Biochem. Biophys. Res. Commun.* **282,** 416-420
72. Irie, Y., and Keung, W.M. (2003) *Brain Res.* **960,** 228-234
73. Uchida, Y., Ihara, Y., and Tomonaga, M. (1988) *Biochem. Biophys. Res. Commun.* **150,** 1263-1267
74. Uchida, Y., and Tomonaga, M. (1989) *Brain Res.* **481,** 190-193
75. Chung, R.S., Vickers, J.C., Chuah, M.I., Eckhardt, B.L., and West, A.K. (2002) *Exp. Neurol.* **178,** 1-12
76. Ceballos, D., Lago, N., Verdu, E., Penkowa, M., Carrasco, J., Navarro, X., Palmiter, R.D., and Hidalgo, (2003) *J. Cell Mol. Life Sci.* **60,** 1209-1216
77. Montoliu, C., Monfort, P., Carrasco, J., Palacios, O., Capdevila, M., Hidalgo, J., and Felipo, V. (2000) *J. Neurochem.* **75,** 266-273
78. Uchida, Y., Gomi, F., Masumizu, T., and Miura, Y. (2002) *J. Biol. Chem.* **277,** 32353-32359
79. Kohno, T., Shinmura, K., Tosaka, M., Tani, M., Kim, S.R., Sugimura, H., Nohmi, T., Kasai, H., and Yokota, J. (1998) *Oncogene* **16,** 3219–3225
80. Chen, Y., Irie, Y., Keung, W.M., and Maret, W. (2002) *Biochemistry* **41,** 8360-8367
81. Lee, J.Y., Kim, J.H., Palmiter, R.D., and Koh, J.Y. (2003) Exp. Neurol. 184, 337-347
82. Sakamoto, T., Kawazoe, Y., Uchida, Y., Hozumi, I., Inuzuka, T., and Watabe, K. (2003) *Neuroreport* **14,** 2147-2151
83. Perry, G., Nunomura, A., Hirai, K., Zhu, X., Perez, M., Avila, J., Castellani, R.J., Atwood, C.S., Aliev, G., Sayre, L.M., Takeda, A., and Smith, M.A. (2002) *Free Radic. Biol. Med.* **33,** 1475-1479
84. Liu, P.K. (2001) *Curr. Top. Med. Chem.* **1,** 483-495
85. Brooks, P.J. (2000) *Neurochem. Int.* **37,** 403-412
86. Gabbita, S.P., Lovell, M.A., and Markesbery, W.R. (1998) *J. Neurochem.* **71,** 2034–2040

87. Lyras, L., Cairns, N.J., Jenner, A., Jenner, P., and Halliwell, B. (1997) *J. Neurochem.* **68,** 2061–2069
88. Bogdanov, M.B., Andreassen, O.A., Dedeoglu, A., Ferrante, R.J., and Beal, M.F. (2001) *J. Neurochem.* **79,** 1246-1249
89. Sanchez-Ramos, J., Overvik, E., and Ames, B.A. (1994) *Neurodegeneration* **3,** 197–204
90. Nunomura, A., Perry, G., Aliev, G., Hirai, K., Takeda, A., Balraj, E.K., Jones, P.K., Ghanbari, H., Wataya, T., Shimohama, S., Chiba, S., Atwood, C.S., Petersen, R.B., and Smith, M.A. (2001) *J. Neuropathol. Exp. Neurol.* **60,** 759-767
91. Browne, S.E., Bowling, A.C., MacGarvey, U., Baik, M.J., Berger, S.C., Muqit, M.M., Bird, E.D., and Beal, M.F. (1997) *Ann. Neurol.* **41,** 646-653
92. Iida, T., Furuta, A., Nishioka, K., Nakabeppu, Y., and Iwaki, T. (2002) *Acta. Neuropathol (Berl).* **103,** 20-25
93. Lovell, M.A., Xie, C., and Markesbery, W.R. (2000) *Brain Res.* **855,** 116-123
94. Floyd, R.A. (1999) *Proc. Soc. Exp. Biol. Med.* **222,** 236-245
95. Uchida, Y. (1994) *Biol. Signals* **3,** 211-215
96. Sogawa, C.A., Miyazaki, I., Sogawa, N., Asanuma, M., Ogawa, N., and Furuta, H. (2000) *Brain Res.* **853,** 310-316
97. Kramer, K.K., Zoelle, J.T., and Klaassen, C.D. (1996) *Toxicol. Appl. Pharmacol.* **141,** 1-7
98. Kramer, K.K., Liu, J., Choudhuri, S., and Klaassen, C.D. (1996) *Toxicol. Appl. Pharmacol.* **136,** 94-100
99. Meneghini, R. (1997) *Free Radic. Biol. Med.* **23,** 783-792
100. Nagano, T., Itoh, N., Ebisutani, C., Takatani, T., Miyoshi, T., Nakanishi, T., and Tanaka, K. (2000) *J. Cell. Physiol.* **185,** 440-446
101. Levadoux, M., Mahon, C., Beattie, J.H., Wallace, H.M., and Hesketh, J.E. (1999) *J. Biol. Chem.* **274,** 34961-34966
102. Jourdan, E., Emonet-Piccardi, N., Didier, C., Beani, J.C., Favier, A., and Richard, M.J. (2002) *Arch. Biochem. Biophys.* **405,** 170-177
103. Kondo, Y., Himeno, S., Endo, W., Mita, M., Suzuki, Y., Nemoto, K., Akimoto, M., Lazo, J.S., and Imura, N. (1999) *Carcinogenesis* **20,** 1625-1627
104. Kagimoto, O., Naganuma, A., Imura, N., Toge, T., Niwa, O., and Yokoro, K. (1991) *J. Radiat. Res(Tokyo).* **32,** 417-428
105. Satoh, M., Kondo, Y., Mita, M., Nakagawa, I., Naganuma, A., and Imura, N. (1993) *Cancer Res.* **53,** 4767-4768

FIGURE LEGENDS.

Fig.1. Expression of MT in DU145 cells. The total RNA and the total protein were extracted from the empty vector-transfected cells (pcDNA3), the MT-III-transfected cells (MT clone-1, MT clone-3 and MT clone-7) (A and B), and the MT-I-(MT-I-clone) and MT-II-(MT-II-clone) expressing clones (C and D). MT-III, MT-I and MT-II mRNA levels were evaluated by semiquantitative RT-PCR using the *MT-III-*, *MT-I*, and *MT-II*-specific primers on 24 cycles. Fifty μg of the total protein was loaded on SDS polyacrylamide gel for western blot analysis. The antibodies against MT-III and MT-I/II were used. The detection of α-tubulin was used as the loading control.

Fig.2. MT-III inhibits γ-radiation-induced 8-oxoG accumulation. A. The parental cells (None), empty vector-transfected cells (pcDNA3) and MT-III transfected cells (MT-III-clone-1, MT-III-clone-3 and MT-III-clone-7) were exposed either to 50 Gy γ rays. At 12 hr after γ-radiation, medium were collected and LDH activity was measured, as described in "Experimental Procedures." Results were presented as LDH U/l for the mean± s.d. from four separate experiments. ** denotes $p<0.01$. B. The parental cells (None), empty vector-transfected cells (pcDNA3) and MT-III transfected cells (MT-III-clone-1, MT-III-clone-3 and MT-III-clone-7) were exposed either to 20-100 Gy γ rays. The amount of 8-oxoG in the genomic DNA was measured by HPCL in combination with electrochemical detector as described in "Experimental Procedures." The amounts of 8-oxoG in the DNA from the MT-III expressing clones treated with the γ rays were significantly lower than those of the empty vector expressing cells. The values are reported as a mean± s.d. from six separate experiments. ** denotes $p<0.01$. C. The empty vector-transfected cells (pcDNA3), MT-I (MT-I-clone), MT-II (MT-II-clone) and MT-III (MT-III-clone-7) transfected cells were exposed either to 50 Gy γ rays. The amount of 8-oxoG in the genomic DNA was measured by HPCL in combination with electrochemical detector as described in "Experimental Procedures." The values are reported as a mean± s.d. from six separate experiments. ** denotes $p<0.01$.

Fig. 3. The effect of MT-III on the hOGG1 expression. A. The mRNA was extracted from three individual MT-III expressing DU145 clones (MT-III-clone-1, MT-III-clone-3 and MT-III-clone-7), one MT-I expressing clone (MT-I-clone), one MT-II expressing clone (MT-II-clone), and one empty vector expressing clone (pcDNA3), and the *hOGG1* mRNA levels were evaluated by semiquantitative RT-PCR using the *hOGG1*-specific primers on 26 cycles. B. The total protein was extracted from three individual MT-III expressing DU145 clones (MT-III-clone-1, MT-III-clone-3 and MT-III-clone-7) and one empty vector expressing clone (pcDNA3) and quantified, as described in "Experimental Procedures." Fifty μg of the total protein was loaded on SDS polyacrylamide gel for western blot analysis. The antibodies against hOGG1 were used. The detection of α-tubulin was used as the loading control.

Fig. 4. Effect of γ-radiation on the hOGG1 protein level and 8-oxoG glycosylase activity. A, western blot analysis of the hOGG1 protein levels in the γ-irradiated DU145 cells. The DU145 cells were treated with various γ ray doses (20- 60 Gy). The cells lysates were then prepared from the γ-irradiated cells, and equal amounts (50 μg proteins) of the cell lysates were separated by 12 % SAS-PAGE, and then transferred onto a nitrocellulose membrane. The membrane was immunoblotted with either anti-hOGG1 or anti-α-tubulin antibodies. The hOGG1 and α-tubulin were detected using the enzyme-linked chemiluminescence. The protein expression levels were quantified using a Bio-Rad Versa-Doc imager and Quantity One analysis software. The results are representative of three similar experiments. B. a 21-mer containing an 8-oxoG lesion was incubated with the cell extracts from the γ-irradiated (0-60 Gy) cells and oligonucleotide cleavage products (lane 3-6) were analyzed on the DNA sequencing gels and subjected to autoradiography as described in the "Experimental Procedures." Human OGG1 (E, lane 2) and buffer alone (NT, lane1) serve as positive and negative controls, respectively. The arrow indicates the DNA cleavage products (13mer).

Fig. 5. siRNA-mediated down-regulation of the hOGG1 mRNA and protein in the DU145 cells. A, the cells were transfected with either the mock, control siRNA (c-siRNA) or hOGG1 siRNAs (OG-siRNA1 and OG-siRNA2). Twenty-four hours after transfection, the total RNA was extracted from the cells and analyzed using semiquantitative RT-PCR, as

described in "Experimental Procedures." B, the cells were treated with the mock, control siRNA or hOGG1 siRNAs. Forty-eight hours later, the cell lysates were prepared from the mock-, control siRNA- or hOGG1 siRNAs-treated cells. Equal amounts (50 μg proteins) of the cell lysates were separated by 12 % SAS-PAGE, and transferred onto a nitrocellulose membrane. The membrane was immunoblotted with either anti-hOGG1 or anti-α-tubulin antibodies. The hOGG1 and α-tubulin were detected using the enzyme-linked chemiluminescence. C, a 21-mer containing an 8-oxoG lesion was incubated with the cell extracts from the mock-, the control siRNA (c-siRNA)- or hOGG1 siRNAs (OG-siRNA1, OG-siRNA2)-treated cells for 48 h and oligonucleotide cleavage products (lane 3-6) were analyzed on DNA sequencing gels and subjected to autoradiography as described in the "Experimental Procedures." Human OGG1 (E, lane 2) and buffer alone (NT, lane1) serve as positive and negative controls, respectively. The arrow indicates the DNA cleavage products (13mer).

Fig. 6. siRNA-mediated down-regulation of the hOGG1 leads to increase in γ ray-induced 8-oxoG accumulation. The cells were transfected with the mock, control siRNA or hOGG1 siRNAs (hOGG1-siRNA1 and hOGG1-siRNA2). Forty-eight hours after transfection, the cells were exposed with various doses of γ ray, and the amount of 8-oxoG in the DNA was measured as described in "Experimental Procedures." The values are presented as a mean ± s.d. from four separated experiments. ** denotes $p<0.01$.

Fig. 7. Effect of MT-III on the γ ray-induced 8-oxoG accumulation in hOGG1 depleted cells. The empty vector-transfected cells (pcDNA3), MT-III expressing the clone-7 cells (MT-III-clone-7), MT-I expressing the clone cells (MT-I-clone), and MT-II expressing the clone cells (MT-II-clone) were treated with hOGG1 siRNAs (OG-siRNA1 and OG-siRNA2). Forty-eight hours later, the cells were exposed with 50 Gy of γ ray, and the amount of 8-oxoG in the DNA were measured as described in "Experimental Procedures." The values are presented as a mean ± s.d. from four separated experiments. ** denotes $p<0.01$.

Fig. 8. siRNA-mediated down-regulation of the hOGG1 leads to increase in γ-radiation-

induced mutant frequency. The mock-, the control siRNA- and the hOGG1 siRNAs (hOGG1-siRNA1 and hOGG1-siRNA2)-transfected cells were exposed either to 2-8 Gy of γ-radiation, and the induction of mutations at the *hprt* locus were measured, as described in Materials and Methods. The induced mutant frequencies in the hOGG1 siRNA-transfected cells treated with γ-irradiation were significantly higher than those of the mock- and control siRNA-transfected cells. The values are presented as a mean ± s.d. from four separated experiments. ** denotes $p<0.01$.