Jpn. J. Cancer Res. **93**, 247–252, March 2002

Lack of Direct Involvement of 8-Hydroxy-2′-deoxyguanosine in Hypoxanthine-guanine Phosphoribosyltransferase Mutagenesis in V79 Cells Treated with N,N′-Bis(2-hydroxyperoxy-2-methoxyethyl)-1,4,5,8-naphthalenetetracarboxylic-diimide (NP-III) or Riboflavin

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The object of this study is to investigate the relationship between a typical product of oxidative DNA damage, 8-hydroxy-2′-deoxyguanosine (8OHdG), and mutagenesis in V79 cells through a molecular analysis of hypoxanthine-guanine phosphoribosyltransferase (*hprt*) gene mutants. We performed a direct sequencing analysis of the cDNA of mutants obtained after treatment with N,N′-bis(2-hydroxyperoxy-2-methoxyethyl)-1,4,5,8-naphthalenetetracarboxylic-diimide (NP-III) or riboflavin, each of which induces the formation of 8OHdG in cellular DNA upon UVA irradiation. The frequency of mutation after both treatments was no more than 2 to 5 times the control value. A considerable number of the mutants could not be amplified by RT-PCR, and this was also the case for the control mutants. Among the mutants analyzed, deletions and a TA→AT transversion occurred predominantly. The reasons for the weak association of induction of 8OHdG with frequency of mutation and the possible mechanism of oxidative-stress-derived mutagenesis are discussed.

Key words: 8-Hydroxy-2′-deoxyguanosine — hprt — Mutation — Deletion

8-Hydroxy-2′-deoxyguanosine (8OHdG), a typical oxidative marker compound in DNA, is widely used as an indicator of carcinogenesis.[1–6] However, in a previous study,[7] only a weak correlation was observed between 8OHdG and the frequency of mutation when N,N′-bis(2-hydroxyperoxy-2-methoxyethyl)-1,4,5,8-naphthalenetetracarboxylic-diimide (NP-III) was used as a source of hydroxyl radical (OH·). NP-III is a photo-Fenton reagent known to generate OH· upon UVA irradiation.[8,9] It was suggested that the damaged guanine would not directly contribute to errors in DNA that cause mutation and that the evaluation of 8OHdG would not necessarily reflect mutagenicity in a cellular system. In addition, it was possible that the mutation in our system was NP-III-dependent and would occur regardless of the generation of OH· or formation of 8OHdG. In this study, we investigated the relationship between 8OHdG and mutagenicity in V79 cells by sequence analysis of hypoxanthine-guanine phosphoribosyltransferase (*hprt*) gene mutants obtained through treatment with NP-III. Further, we also analyzed the effect of riboflavin (Rf), a photosensitizer known to induce the formation of 8OHdG.[10–12]

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MATERIALS AND METHODS

Cells and reagents Chinese hamster lung fibroblasts, V79 (V79 379A, IFO#50082), were grown in Eagle's minimum essential medium supplemented with 10% heat-inactivated fetal calf serum, 100 U/ml penicillin and 100 μg/ml streptomycin. NP-III was kindly provided by Dr. Seiichi Matsugo at Yamanashi University. Rf was purchased from Sigma Chemical (St. Louis, MO).

Cell treatments and 8OHdG determination The cells were treated as described in a previous study.[7] Briefly, V79 cells were plated at 5×10^5/60-mm dish and incubated for 24 h at 37°C in 5% CO_2. The cells were washed and incubated in Dulbecco's phosphate-buffered saline for 15 min with either 20 μM NP-III or 20 μg/ml Rf under UV irradiation at 366 nm. The dose of UVA in this condition was ~10 kJ/m^2.[7] After the treatment, the cells were washed and some were stored as a cell pellet at −80°C until the determination of 8OHdG. The determination was made under anaerobic conditions as described[13] to minimize experimental artifacts and by HPLC with an electrochemical detection system.

Determination of mutagenicity and sequence analysis The mutagenicity of the cells was evaluated by hypoxantine-guanine phosphoribosyltransferase (HPRT) mutation

Table I. Induction of 8OHdG and Frequency of Mutation in V79 Cells

Treatment	8OHdG induction		Mutation frequency	
	8OHdG/10^5 dG (Mean±SD)	Induction ratio (fold)	$6TG^r/10^6$ cells (Mean or mean±SD)	Induction ratio (fold)
NP-III/UV	n=3		n=6	
−/−	0.688±0.184	1.0	7.4±4.2	1.0
+/+	4.760±0.582[a)]	6.9	15.5±16.3	2.1
Rf/UV	n=7		n=2	
−/−	0.433±0.174	1.0	8.5	1.0
+/+	1.586±0.838[a)]	3.7	32.0	3.8

a) Increase was significant (P<0.001) compared with controls (−/−) in each group.

assay as described previously.[7)] 6-Thioguanine-resistant mutants ($6TG^r$) from the mutation assay were isolated and total cellular RNA was extracted. A reverse transcription (RT) reaction was carried out using a first-strand cDNA synthesis kit (Pharmacia Biotech, Piscataway, NJ) with pd(T)12-18 as a primer. Amplification and purification of *hprt* cDNA were performed as described by Yadollahi-Farsani *et al*.[14)] The sequence of the purified full-length cDNA (657 bp) was determined with the primers used in ref. 14 using a Prism Dye Terminator Cycle Sequencing Ready Reaction FS kit (ABI, Foster City, CA) and analyzed automatically by a 310 Genetic Analyzer (ABI). Ethyl methanesulfonate (EMS), a typical mutagen, was used as a positive control in the mutation assay and the sequence analysis.

Detection of large deletions with a part of the samples was carried out by means of a multiplex PCR method described by Xu *et al*.[15)]

Statistical analysis The significance of the differences in the data for 8OHdG and frequency of mutation was analyzed by using the *t* test.

RESULTS

The results on 8OHdG are shown in Table I. Both treatments produced a significant increase in cellular 8OHdG, but there was a difference in the induction ratio. The cells treated with NP-III and UV (NPUV) showed about a 7-fold increase in 8OHdG compared to the control cells treated with PBS only, while Rf and UV treatment (RfUV) increased the level of 8OHdG in cells by about 4-fold. Treatment with either NP-III or Rf alone did not result in any increase in 8OHdG (data not shown).

The results of the mutation assay are also shown in Table I. The data for each assay and the results of sequence analysis are shown in Table II. EMS (0.5 mg/ml), known to induce a GC→AT transition, was used as a positive mutagenic agent. The frequency of mutation caused by NPUV or RfUV was not as high as that in the case of EMS treatment and the induction (compared to the control cells in each assay) for each treatment was no more than 2- to 5-fold, while with EMS it was 40-fold.

As shown by the results in Table II, a considerable percentage of the NPUV or RfUV mutants could not be amplified by RT-PCR (NPUV 47%, RfUV 73%) for sequence analysis. The same was true for the control mutants (75%), possibly for the reasons described in "Discussion."

In NPUV mutants, mutations occurred predominantly at the TA base pair (bp) (21 of 26 mutants). The major type was a TA→AT transversion (10 of 26) and deletions (8 of 26). A base exchange at GC bp was found in only 4 of 26 mutants analyzed. In RfUV mutants, large deletions within an exon were predominant (4 of 6) and one-base substitution occurred at TA bp (2 of 6), though only 6 mutants could be analyzed. The results for the samples treated with NP-III or Rf alone are also shown in Table II, though the number of mutants available for the sequence analysis is small. Treatment without UV irradiation produced similar types of mutations to treatment with UV. As previously described,[16, 17)] most of the EMS-treated mutants (12 of 14) in our assay had a GC→AT transition. Even in the case of EMS treatment, 22% of the samples could not be amplified by RT-PCR.

DISCUSSION

8OHdG is considered a useful marker of oxidative stress-related carcinogenesis, yet the mechanism leading to mutagenesis and to cancer *in vivo* is still ambiguous. 8OHdG has been shown to be highly mutagenic (with a mutation frequency of 0.5–4.0%) by studies using transfected DNA containing synthetic 8OHdG[18–20)] and to cause transversion at a modified guanine base as well as the adjacent position. In a previous study,[7)] however, we presented evidence that cells in which 8OHdG was highly

Table II. Mutation Assay and Sequence Analysis of V79 Cells Treated with NP-III, Rf or EMS

Mutation assay			RT-PCR			Sequence analysis			
Assay	Colonies/ assay	$6TG^r/10^6$ cells (Mean±SD)	Total	Not amplified (%)	Amplified	No.	Site	Type of mutation	Target sequence
	Control								
A	7/5 dish	10.3	3	3[a] (100)	0	3[a]	exon 2–9	deletion (by multiplex PCR)	
B	2/5 dish	3.1	2	0	2	2	151	GC→TA	5′ GCC CGA GAT 3′
C	26/5 dish	27.4	10	6 (60)	4	4	197–233	deletion	CTC TGT·····CTG CTG
D	6/5 dish	11.2	5	5 (100)	0				
E	4/5 dish	5.7	4	4 (100)	0				
	Total	(11.5±9.5)	24	18 (75)	6				
	NP								
B	1/5 dish	1.4	1	0	1	1	196	TA→AT	CTC TGT GTG
C	4/10 dish	4.3	4	2 (50)	2	2	595	TA→AT	TAC TTC AGG
	Total	(1.9)	5	2 (40)	3				
	NPUV								
A	13/5 dish	22.6	10	0	10	5	564	deletion	TTT GT**T** **G**[c]TT
						2	491 & 514	TA→CG & GC→TA	TTG C**TG** GTG
						1	409	TA→AT	AGT GTT GGA
						1	616–626	deletion	ATA ATT GAC
						1	exon 8	deletion	AAT CAT·····AGT GAA
B	15/5 dish	22.9	15	3 (20)	12	6	395 & 396	TA→AT & TA→CG	TTG AT**T** **G**TT
						3	398	TA→AT	ATT **GT**T GAG
						2	130	GC→CG	ATG GAC AGG
						1	211	deletion	GGG GGC TAT
C	32/10 dish	33.3	24	20 (83)	4	4	136	TA→GC	AGG ACT GAA
	Total	(19.7±14.0)	49	23 (47)	26				
	Rf								
D	17/5 dish	29.6	14	14 (100)	0				
E	6/5 dish	9.8	5	3 (60)	2	2	exon 6	deletion	
	Total	(19.7)	19	17 (89)	2				
	RfUV								
D	17/5 dish	53.1	15	15 (100)	0				
E	8/5 dish	10.9	7	1 (14)	6	2	473	TA→GC	ATG GTT AAG
						1	exon 5	deletion	
						1	exon 6	deletion	
						1	exon 8	deletion	
						1	exon 2–4	deletion	
	Total	(32.0)	22	16 (73)	6				
	EMS								
B	139/4 dish	347.5	8	1 (13)	7	2	400	GC→AT	GTT GAG GAC
						1	208	GC→AT	AAG GGG GGC
						1	419	GC→AT	ACT GGT AAA
						1	539	GC→AT	GTT GGA TTT
						1	580	GC→AT	CTT GAC TAT
						1	460	insertion	TAC gAA[b] CCT
C	83/2 dish	466.3	4	2 (50)	2	1	74	GC→AT	ATT CCT AAT
						1	exon 8	deletion	
D	129/2 dish	542.0	4	0	4	1	151	GC→AT	GCC CGA GAT
						1	508	GC→AT	TCT CGA AGT
						1	544	GC→AT	TTT GAA ATT
						1	589	GC→AT	AAT GAG TAC
E	76/2 dish	342.3	2	1 (50)	1	1	400	GC→AT	GTT GAG GAC
	Total	(424.5±97.0)	18	4 (22)	14				

a) DNA samples of the three Control mutants in assay A were analyzed by a multiplex PCR method to detect large deletions from exon 2 to exon 9.
b) The guanine inserted at position 460 is expressed in lower case.
c) Mutations that occurred adjacent to guanine are expressed in bold.

induced by NP-III have only a low rate of mutation. In the present study, to examine the direct involvement of 8OHdG in the mutagenic process of the cell, we performed a sequence analysis of the *hprt* gene in $6TG^r$ mutants obtained by using two kinds of photosensitizer, NP-III or Rf, for the induction of 8OHdG.

In the molecular analysis of *hprt*, a multiplex PCR method is commonly used to examine deletions of exons in genomic DNA.[15, 21–25] The method, however, is not suitable to detect small base changes such as the GC→TA transversion that would be expected from the induction of 8OHdG. In this study, we have chosen a direct sequencing method to screen for mutations within full-length cDNA. However, as shown in Table II, some of the samples could not be amplified by RT-PCR even among the PBS control mutants. The reason for this might be large deletions, which have been shown to occur at high frequency in the *hprt* gene regardless of the experimental treatment.[22–29] A study by Schmidt and Kiefer[22] using V79 cells as a target found that 13–19% of spontaneous mutants contained complete gene deletions, and this figure rose to 55–76% when partial deletions (≥2 exons) were included. Studies using human cells[24–28] have also shown spontaneous large deletions, including the complete loss of genes. The extent of deletions leading to failure of amplification should be examined with various kinds of methods. RT-PCRs were carried out with a part of the unamplified samples from NPUV assay using different sets of primers introduced by Yadollahi-Farsani *et al.*[14] for sequence analysis, with the result that neither NPUV nor control mutants could be amplified with any set of primers (data not shown). Using a multiplex PCR method by Xu *et al.*,[15] we found the DNA of three of the control mutants in assay A to have large deletions from exon 2 to exon 9 (data not shown). Further detection, such as deletion analysis involving flanking regions of the *hprt* locus or sequencing of the breakpoint junction, as used in a recent study,[21] would also be required.

Treatment-specific mutation patterns could be detected in NPUV mutants and most mutations occurred at TA bp, predominantly as a TA→AT transversion. In a previous study,[30] the TA→AT transversion was proposed as a reactive oxygen species (ROS)-related mutation following either O_2 or H_2O_2 treatment. NP-III, when irradiated with UVA, is considered to produce $OH^{\cdot}$ near DNA and has been shown to cause DNA cleavage at -GG- sites *in vitro*.[8, 9] The same process may be involved in our NPUV assay to induce the formation of 8OHdG in the cell. At the same time, considering that $OH^{\cdot}$ is the major radical species produced from H_2O_2 in the presence of Fe^{2+}, the NP-III-derived $OH^{\cdot}$ could also be involved in the base exchanges at bp TA.

However, there remains the question of whether 8OHdG is required for the mutagenic process. It has been shown that mammalian cells have an effective system of repairing 8OHdG[31–33] and that the damage can be rapidly removed.[7, 34, 35] In our system, the amount of 8OHdG declined to half in 6 h after the treatment and to the control level in 24 h.[7] If most of the 8OHdG could be repaired in a period shorter than the doubling time of the cells, the damage would not directly contribute to mutation. This could partly explain the weak correlation between the ability to induce 8OHdG and the low frequency of mutation in the NPUV assay. Furthermore, it was notable that, instead of the -GG- DNA cleavage site shown *in vitro* in the former study, most of the mutations at bp TA were adjacent to a guanine base (16 of 26, expressed in bold in Table II). If these guanine bases are damaged, it is possible that the formation of 8OHdG would prescribe the sites of the mutation and that the repair process involving adjacent bases might cause mutation in a dynamic way. Further study is expected to clarify the mechanism of the ROS-related mutagenesis leading to the base exchange at TA and to identify the kind of DNA modifications which may be associated with the process.

Riboflavin is a naturally distributed photosensitizer which induces 8OHdG in cellular DNA,[11, 12] but this process is considered to occur without the generation of ROS.[36] It has also been shown *in vitro* that 8OHdG was preferentially formed at the 5′ site of 5′-GG-3′ sequences.[10] From our results, neither induction of 8OHdG nor mutation of RfUV occurred at high frequency. The major types of mutation detected were large deletions in exons, which were also observed in the control mutants. Yamamoto *et al.*[12] have shown that Rf could be mutagenic when illuminated by visible light, which was also present in our experimental system as a background factor. These results suggest that the addition of Rf to the cellular system with or without UV irradiation would merely cause an additional effect on the mutagenic process that might spontaneously occur with the agent endogenously present in the cells. In other words, it is possible that the spontaneous mutation which occurred at high frequency in our assay was caused by an endogenous photosensitizer, such as Rf within cells and illumination during the experiments.

In this study, since the frequency of mutation induced by the treatments was low, the number of samples obtained for the sequence analysis was limited. Moreover, among the mutation spectra, some mutation patterns were identical among several mutants. These mutants were supposed to have originated from one mutant clone and this might be caused by a drift during the expression period of the experiment. As described in the recent article,[21] which dealt with a small number of mutant cells, propagation during the expression should be maintained more strictly and on a larger scale to avoid drift and to increase the number of mutants from independent clones. In conclu-

sion, the patterns of mutation caused by NPUV or RfUV treatment were more similar to spontaneous mutations than to the guanine-specific modifications which have been reported to be correlated with the formation of 8OHdG. This suggests that 8OHdG which is acutely induced in the cellular system may not be directly involved in the mutagenic events. A comprehensive study of the process, including the repair system and detection of multiple DNA damage, is needed to clarify the mechanism leading from DNA damage to cancer *in vivo*.

ACKNOWLEDGMENTS

We thank Dr. Seiichi Matsugo (Yamanashi University) for providing NP-III synthesized at his laboratory and giving instructions for its use. We would also like to thank Dr. X.-Q. Mao (Kyoto University Graduate School of Medicine) for her great assistance and advice in setting up the RT-PCR and the sequence analysis system.

(Received September 21, 2001/Revised December 5, 2001/ Accepted December 20, 2001)

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