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DNA BASE DAMAGE IN CHROMATIN OF γ-IRRADIATED CULTURED HUMAN CELLS

ZEENA NACKERDIEN,[1,2] RYSZARD OLINSKI[1,3] and MIRAL DIZDAROGLU[1]†

[1]*Chemical Science and Technology Laboratory, National Institute of Standards and Technology, Gaithersburg, Maryland 20899, USA*
[2]*Department of Radiotherapy, University of Stellenbosch, Tygerberg, South Africa*
[3]*Department of Clinical Biochemistry, Medical School, 85-094 Bydgoszcz, Poland*

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We report on the chemical characterization of DNA base damage in chromatin of γ-irradiated cultured human cells. Chromatin was isolated from unirradiated and irradiated cells and analyzed by gas chromatography/mass spectrometry with selected-ion monitoring after acidic hydrolysis of chromatin and trimethylsilylation of hydrolysates. Prior to analysis of chromatin samples, experimental conditions for acidic hydrolysis were optimized by determining the relative molar response factors of modified bases under non-acidic and acidic conditions, and their release from DNA under various acidic conditions. A number of modified bases in chromatin isolated from irradiated cells were identified and quantitated. These were 5-hydroxy-5-methylhydantoin, 5-hydroxyhydantoin, 5-(hydroxymethyl)uracil, cytosine glycol, thymine glycol, 5,6-dihydroxycytosine, 4,6-diamino-5-formamidopyrimidine, 8-hydroxyadenine, 2-hydroxyadenine, 2,6-diamino-4-hydroxy-5-formamidopyrimidine, and 8-hydroxyguanine. Radiation doses ranging from 42 to 420 Gy ($J \cdot kg^{-1}$) were used. Background levels of all modified bases were observed in chromatin isolated from unirradiated cells. The radiation yields of a number of modified bases were increased significantly over their background levels at a dose as low as 42 Gy. In most cases, linear dose-yield relationships were obtained up to ≈200 Gy. At radiation doses higher than 420 Gy, no additional increase in the yields of modified bases was observed. The yields of guanine-derived bases amounted to ≈45% of the total net yield of modified bases measured, followed by almost equal yields of adenine-, cytosine- and thymine-derived bases. Modified bases identified were typical products of hydroxyl radical attack on DNA bases, indicating the involvement of hydroxyl radical, although their induction in part by the direct effect of ionizing radiation through ionization of DNA bases cannot be excluded. The yields of modified bases were lower than those previously measured after γ-irradiation of fully expanded chromatin in aqueous buffer solutions.

KEY WORDS: ionizing radiation, hydroxyl radical, mass spectrometry, formamidopyrimidines, indirect effect of radiation, chromatin damage.

ABBREVIATIONS: ·OH, hydroxyl radical; e^-_{aq}, hydrated electron; 5,6-diHThy, 5,6-dihydrothymine; 5-OH-5-Me-Hyd, 5-hydroxy-5-methylhydantoin; 5-OH-Hyd, 5-hydroxyhydantoin; 5-OH-Ura, 5-hydroxyuracil; 5-OH-Cyt, 5-hydroxycytosine; 5-OH-6-HThy, 5-hydroxy-6-hydrothymine; 5-OH-6-HUra, 5-hydroxy-6-hydrouracil; 5-OH-6-HCyt, 5-hydroxy-6-hydrocytosine; 5-OHMe-Ura, 5-(hydroxymethyl)uracil; Cyt glycol, cytosine glycol; Thy glycol, thymine glycol; 5,6-diOH-Ura, 5,6-dihydroxyuracil; 5,6-diOH-Cyt, 5,6-dihydroxycytosine; FapyAde, 4,6-diamino-5-formamidopyrimidine; 8-OH-Ade, 8-hydroxyadenine; 2-OH-Ade, 2-hydroxyadenine; FapyGua, 2,6-diamino-4-hydroxy-5-formamidopyrimidine; 8-OH-Gua, 8-hydroxyguanine; BSTFA, bis(trimethylsilyl)trifluoroacetamide; GC/MS-SIM, gas chromatography/mass spectrometry with selected-ion monitoring; Gy, Gray ($J \cdot kg^{-1}$); RMRF, relative molar response factor; Me_3Si, trimethylsilyl; n.d., not detected.

†To whom correspondence should be addressed.

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INTRODUCTION

Free radicals generated *in vivo* by endogenous or exogenous sources have been implicated to play a role in biological processes such as mutagenesis, carcinogenesis and cell death (reviewed).[1] Reactions of free radicals *in vivo* may cause damage to biological molecules including DNA. Thus, free radicals may be mutagenic (reviewed),[2] and may act as promoters of carcinogenesis.[3,4] Ionizing radiation exerts the bulk of its effects in cells indirectly through free radicals [i.e., hydroxyl radical (· OH), hydrated electron (e^-_{aq}), H atom] produced by reactions with cellular water, in particular by · OH. In addition, ionizing radiation directly causes ionization within the DNA itself (reviewed).[5] There is evidence that the critical target for ionizing radiation-induced damage in cells is the nuclear DNA (reviewed).[6] Studies done with radical scavengers have shown that the contribution of scavengeable free radicals to the lethal action and DNA damage by ionizing radiation amounts to $\approx 70\%$ in oxic cells.[7-9] Radiation-generated free radicals, especially · OH, produce a large number of base-derived and sugar-derived products in DNA, and DNA-protein cross-links in nucleoprotein (reviewed).[5,10-12] The understanding of the repair and biological consequences of radiation-induced DNA modifications in cells depends on the knowledge of their chemical nature at the molecular level and the quantitation of each species.

In the past, a number of modified bases in the DNA of cells exposed to ionizing radiation have been identified and quantitated by the use of various measurement techniques.[13-23] Generally, one modified base or a small number of modified bases have been measured at a time with no specific structural evidence. Overall, there exist no comprehensive studies on the simultaneous measurement with structural evidence of both pyrimidine-derived and purine-derived lesions in chromatin DNA of irradiated cells.

In the present work, we report on the measurement of pyrimidine- and purine-derived lesions in chromatin of γ-irradiated cultured human cells. The technique of gas chromatography/mass spectrometry with selected-ion monitoring (GC/MS-SIM) was used for this purpose. Unlike other techniques that are available for measurement of a limited number of modified DNA bases, this technique permits the structural identification and the quantitation of various products of all four bases in DNA, and also directly in chromatin (reviewed).[24]

MATERIALS AND METHODS

Materials†

Authentic compounds were purchased or synthesized as described previously.[25] 5-Hydroxy-5-methylhydantoin (5-OH-5-Me-Hyd) was a gift from Dr. W.F. Blakely of the Armed Forces Radiobiology Research Institute, Bethesda, Maryland. RPMI medium (#R5507), L-glutamine, fetal bovine serum, isoguanine [2-hydroxyadenine (2-OH-Ade)], thymine, cytosine, adenine, guanine and calf thymus DNA were purchased from Sigma Chemical Co. Penicillin and streptomycin were from Gibco

†Certain commercial equipment or materials are identified in this paper in order to specify adequately the experimental procedure. Such identification does not imply recommendation or endorsement by the National Institute of Standards and Technology, nor does it imply that the materials or equipment identified are necessarily the best available for this purpose.

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Lab. Acetonitrile and bis(trimethylsilyl)trifluoroacetamide (BSTFA) containing 1% trimethylchlorosilane were obtained from Pierce Chemical Co. Formic acid was from Mallinckrodt. Dialysis membranes with a molecular weight cutoff of 3500 were purchased from Fisher Scientific Co.

Cell Culture

The cells used were K562 human cells (courtesy of Dr. S.A. Akman of the City of Hope National Medical Center, Duarte, California). Suspension cultures of this cell line were incubated at 37°C under an atmosphere of 3% CO_2 mixed with room air. The growth medium consisted of RPMI medium supplemented with 10% fetal bovine serum, L-glutamine (4 mM), penicillin (50 units/mL) and streptomycin (50 μg/mL).

Irradiations

Cells in air-saturated culture medium (4×10^6 cells/mL) were irradiated in a ^{60}Co γ-source while their container was submerged in an ice bath. The dose range used was 42–420 Gy ($J \cdot kg^{-1}$). The dose rate of the ^{60}Co γ-source (98.4 $Gy \cdot min^{-1}$) was determined by Fricke dosimeter.[26] Immediately after irradiation, cells were frozen in liquid nitrogen. Subsequently, chromatin was isolated and characterized as described previously.[25] Except for irradiation, unirradiated cells were treated in the same manner. Chromatin was obtained in 1 mM Tris buffer (pH 7.4). No difference was observed between the yields of chromatin isolated from unirradiated cells and irradiated cells. The DNA content of isolated chromatin was determined both by measurement of the absorbance at 258 nm using a molar extinction coefficient of $6.6 \times 10^3 M^{-1} \cdot cm^{-1}$ and by Burton's assay.[27] The RNA content of chromatin was $\leqslant 5\%$ of the amount of DNA as measured according to Schneider.[28] Calf thymus DNA was dissolved in 10 mM phosphate buffer (pH 7.4) at a concentration of 0.35 mg/mL and then dialyzed extensively against 10 mM phosphate buffer (pH 7.4). Dialyzed solutions were bubbled with N_2O for 30 min and then γ-irradiated at a dose of 240 Gy. Irradiated and unirradiated solutions of DNA were dialyzed against 10 mM phosphate buffer (pH 7.4) before use.

Hydrolysis and Trimethylsilylation

To aliquots of chromatin samples (in 1 mM Tris buffer) containing 0.1 mg of DNA or to aliquots of calf thymus DNA samples (0.1 mg), 0.5 nmol of 6-azathymine and 2 nmol of 8-azaadenine were added as internal standards. Samples were then lyophilized. Samples of calf thymus DNA were hydrolyzed with 0.5 mL of formic acid at various concentrations (50%, 60%, 70% and 88%) in evacuated and sealed tubes at 140°C for 30 min. Samples of chromatin were hydrolyzed with 0.5 mL of 60% formic acid in the same manner. Samples were lyophilized and then trimethylsilylated with 0.1 mL of a BSTFA/acetonitrile (4/1; v/v) mixture in poly(tetrafluoro-ethylene)-capped hypovials under nitrogen at 130°C for 30 min. The determination of the relative molar response factors of modified bases were performed as described previously.[29] For this purpose, an aliquot of the mixtures of the modified bases and the internal standards was lyophilized and then derivatized. Another aliquot of these mixtures was treated with formic acid, and subsequently lyophilized and derivatized as described above for DNA samples.

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TABLE I
Dependence on formic acid concentration of relative molar response factors of modified bases

Base (ion used)		Formic acid concentration			
		No acid (1)	60% (2)	70% (3)	88% (4)
5,6-diHThy	(*m/z* 257)	0.530 ± 0.012[a]	0.514 ± 0.017	0.526 ± 0.018	0.581 ± 0.006
5-OH-5-Me-Hyd	(*m/z* 331)	0.463 ± 0.050	0.627 ± 0.060	0.857 ± 0.032	1.34 ± 0.01*†
5-OHMe-Ura	(*m/z* 358)	0.431 ± 0.016	0.604 ± 0.034*	1.05 ± 0.03*	1.95 ± 0.07*†
5-OH-Ura	(*m/z* 329)	0.361 ± 0.020	0.293 ± 0.013	0.317 ± 0.007	0.357 ± 0.028
Thy glycol	(*m/z* 259)	0.204 ± 0.013	0.312 ± 0.024*	0.345 ± 0.006*	0.413 ± 0.005*†
5,6-diOH-Ura	(*m/z* 417)	3.12 ± 0.35	3.84 ± 0.26	4.98 ± 0.96	5.28 ± 0.48
FapyAde	(*m/z* 354)	1.10 ± 0.09	0.960 ± 0.090	1.08 ± 0.04	1.10 ± 0.12
8-OH-Ade	(*m/z* 352)	0.296 ± 0.024	0.276 ± 0.013	0.315 ± 0.028	0.347 ± 0.015
2-OH-Ade	(*m/z* 352)	0.188 ± 0.013	0.164 ± 0.012	0.197 ± 0.008	0.203 ± 0.015
FapyGua	(*m/z* 442)	0.637 ± 0.059	1.03 ± 0.17	1.60 ± 0.12*	1.41 ± 0.15*
8-OH-Gua	(*m/z* 440)	0.415 ± 0.030	0.398 ± 0.046	0.395 ± 0.049	0.452 ± 0.008

[a]Each value represents the mean ± standard error from three independent experiments.
*Significantly different from the value in column 1 ($p \leq 0.05$).
†Significantly different from the value in column 2 ($p \leq 0.05$).

Gas Chromatography/Mass Spectrometry

Analysis of derivatized samples was performed by GC/MS-SIM as described previously.[25] An aliquot (4 μL) of each derivatized sample was injected without any further treatment into the injection port of the gas chromatograph by means of an autosampler. A split ratio of 1 : 20 was used, resulting in ≈0.2 μg of hydrolyzed and derivatized DNA going through the GC column for each analysis.

RESULTS

The objective of this work was to identify and quantitate modified DNA bases formed in chromatin of γ-irradiated cultured cells by means of the GC/MS technique. The preparation of DNA samples for an analysis by GC/MS involves hydrolysis with formic acid followed by derivatization. A recent study from our laboratory has reported on the stability in formic acid of a number of modified bases.[29] In the present work prior to analysis of chromatin from γ-irradiated cells, we examined the stability of modified bases and their release from DNA at different concentrations of formic acid in a quest for further optimizing hydrolysis conditions. In the first step, the relative molar response factor (RMRF)† of each modified base was determined,[29] without the use of formic acid and with the use of formic acid at various concentrations. Table I shows the measured values. 5-OHMe-Ura and Thy glycol were destroyed to an extent of approximately 20–25% by treatment with 60% formic acid as indicated by their significantly greater RMRFs in column 2 than those in column 1 of Table I. Other modified bases showed no significant difference within the indicated error limits between their respective RMRFs measured without the use of formic acid and with the use of 60% formic acid. This indicates that these compounds

†RMRF = (amount of the analyte)/(amount of the standard) × (peak area of the ion of the standard)/(peak area of the ion of the analyte).

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underwent no significant destruction by treatment with 60% formic acid. At higher formic acid concentrations. 5-OH-5-Me-Hyd, 5,6-diOH-Ura and FapyGua were also destroyed significantly. The use of a lower formic acid concentration (50%) resulted in RMRFs similar to those obtained with 60% formic acid (not shown). It should be pointed out that the stability of the internal standards under all conditions of formic acid treatment given in Table I is a prerequisite for the validity of these results. The stability of 6-azathymine and 8-azaadenine under the conditions of hydrolysis at the highest formic acid concentration (88%) used here has been shown previously.[29] This was also confirmed in the present work.

Having determined the extent of stability of modified bases, we examined the release of modified bases from DNA at various formic acid concentrations. Calf thymus DNA exposed to ionizing radiation in N_2O-saturated aqueous solution (dose 240 Gy) and unirradiated calf thymus DNA were used for this purpose. Results are shown in Table II. Fifteen modified bases were identified. 5-OH-Ura and 5-OH-Cyt result from acid-induced modification of Cyt glycol, the former by deamination and dehydration, and the latter by dehydration; 5,6-diOH-Ura is formed by deamination of 5,6-diOH-Cyt.[30,31] Similarly, 5-OH-6-HUra is thought to result from acid-induced deamination of 5-OH-6-HCyt. The RMRFs given in Table I were used for calculation of the amounts shown in Table II. There were no authentic compounds available for 5-OH-Hyd, 5-OH-6-HThy, 5-OH-6-HUra and 5-OH-Cyt. Their gas chromatographic retention times and their mass spectra were obtained using trimethylsilylated samples of γ-irradiated thymine (for 5-OH-6-HThy) and cytosine (for 5-OH-Hyd, 5-OH-6-HUra and 5-OH-Cyt after treatment with formic acid). The RMRF of the trimethylsilyl (Me_3Si) derivative of 5-OH-Hyd was assumed to be the same as that of the Me_3Si derivative of 5-OH-5-Me-Hyd because of the similarity of their mass spectra.[32] A similar assumption was made for the RMRF of the Me_3Si derivative of 5-OH-Cyt, which has a mass spectrum similar to that of the Me_3Si derivative of 5-OH-Ura.[33,34] The RMRFs of Me_3Si derivatives of 5-OH-6-HThy and 5-OH-6-HUra were estimated from their mass spectra,[29,30,33] and therefore may have an error associated with them.

Results in Table II indicate that, under all four hydrolysis conditions, similar amounts were obtained for each modified base in irradiated DNA except for a few cases. This means that a compensation for losses of labile modified bases such as 5-OHMe-Ura, Thy glycol and FapyGua even at formic acid concentrations higher than 60% was achieved by the use of corresponding RMRFs given in Table I. Similar background amounts for each modified base were obtained in unirradiated DNA at different formic acid concentrations except for a few cases at 50% formic acid (Table II).

Table II indicates that some of the modified bases already were present in unirradiated DNA. Occurrence of these modified bases in isolated DNA and in isolated chromatin, which were not exposed to free radical-generating systems, has been shown previously using the GC/MS technique after hydrolysis with formic acid of DNA or of chromatin followed by derivatization of hydrolyzates (reviewed).[24] There is a possibility that these modified bases may be formed in DNA by acidic treatment. We investigated this possibility by analyzing individual DNA bases, which were treated with formic acid under the conditions used for hydrolysis of DNA. For this purpose, equimolar amounts of commercial thymine, cytosine, adenine and guanine were mixed. Aliquots of this mixture were lyophilized and then treated with formic acid at various concentrations. As a control, another aliquot was treated with water by the conditions of acidic hydrolysis without formic acid. All samples including

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TABLE II
Dependence on formic acid concentration of the release of modified bases (molecules/10^5 DNA bases) from DNA

	Formic acid concentration							
	50%		60%		70%		88%	
Base	Control (1)	Irrad. (2)	Control (3)	Irrad. (4)	Control (5)	Irrad. (6)	Control (7)	Irrad. (8)
5,6-diHThy	n.d.[a]	36.8 ± 1.7[b]	n.d.	31.8 ± 3.5	n.d.	34.2 ± 1.0	n.d.	35.8 ± 3.4
5-OH-5-Me-Hyd	2.14 ± 0.16	19.9 ± 0.9	2.24 ± 0.13	19.5 ± 0.9	2.82 ± 0.22	23.4 ± 1.1	3.84 ± 0.54	20.9 ± 0.5
5-OH-Hyd	1.60 ± 0.19	9.06 ± 0.74	1.57 ± 0.19	8.93 ± 0.32	1.38 ± 0.35	10.7 ± 1.1	1.76 ± 0.16	10.3 ± 0.80
5-OH-6-HThy	n.d.	88.0 ± 1.7	n.d.	87.0 ± 1.8	n.d.	85.1 ± 2.3	n.d.	84.5 ± 3.1
5-OH-6-HUra	n.d.	19.4 ± 0.5	n.d.	19.8 ± 0.7	n.d.	19.0 ± 0.9	n.d.	18.4 ± 0.7
5-OHMe-Ura	0.93 ± 0.06	18.6 ± 1.0	2.43 ± 0.45	17.5 ± 1.5	2.69 ± 0.26	23.7 ± 3.3	2.69 ± 0.51	24.5 ± 0.5*†
5-OH-Ura	n.d.	29.1 ± 0.7	n.d.	30.1 ± 1.8	n.d.	30.1 ± 3.6	n.d.	30.9 ± 2.7
5-OH-Cyt	6.11 ± 1.15	40.3 ± 0.9	7.20 ± 0.86	45.0 ± 1.6	8.26 ± 0.16	46.4 ± 5.3	8.90 ± 1.54	48.5 ± 4.8
Thy glycol	0.19 ± 0.02	22.4 ± 1.5	0.74 ± 0.10	27.5 ± 2.1	0.68 ± 0.06	33.0 ± 4.5	1.85 ± 0.16	45.1 ± 1.0*†
5,6-diOH-Ura	0.14 ± 0.02	10.6 ± 1.3	0.14 ± 0.02	9.98 ± 0.42	0.18 ± 0.03	12.0 ± 0.7	0.18 ± 0.02	12.6 ± 0.4
FapyAde	0.67 ± 0.10	55.7 ± 6.1	0.90 ± 0.03	60.2 ± 7.3	0.67 ± 0.10	66.2 ± 7.5	1.06 ± 0.19	81.6 ± 6.1
8-OH-Ade	11.7 ± 1.1	86.7 ± 4.6	14.2 ± 2.9	93.4 ± 3.9	18.0 ± 0.6	90.6 ± 5.7	19.4 ± 2.1	81.3 ± 2.2
2-OH-Ade	0.65 ± 0.07	4.00 ± 0.19	0.70 ± 0.06	4.83 ± 0.16	0.64 ± 0.06	5.98 ± 0.13*†	0.74 ± 0.10	8.38 ± 1.31*†
FapyGua	0.67 ± 0.06	38.4 ± 1.6	1.12 ± 0.10	42.6 ± 2.6	0.86 ± 0.13	49.6 ± 7.7	0.61 ± 0.08	57.6 ± 1.5*
8-OH-Gua	48.9 ± 7.7	152.6 ± 12.6	50.9 ± 9.9	214.4 ± 18.8	52.5 ± 3.2	241.9 ± 22.8*	55.0 ± 11.8	251.2 ± 4.2*

[a]Not detected.
[b]Each value represents the mean ± standard error from three independent experiments.
*Significantly different from the value in column 2 ($p \leq 0.05$).
†Significantly different from the value in column 4 ($p \leq 0.05$).

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an aliquot of the mixture without any treatment were lyophilized, trimethylsilylated and then analyzed by GC/MS-SIM for measurement of modified bases listed in Table II. The data in Table III show that commercial DNA bases contained some of the modified bases dealt with in the present work, and that the amounts of these modified bases were not increased by the acidic treatment under the conditions used for DNA hydrolysis.

As a conclusion of the results presented in Tables I, II and III, hydrolysis with 60% formic acid was chosen to be optimal for DNA hydrolysis.

DNA Base Damage in Chromatin of Irradiated Cells

For measurement of modified DNA bases in cells, chromatin was isolated from irradiated cells instead of DNA alone, because DNA may not be extracted efficiently from chromatin, due to formation of DNA-protein cross-links in chromatin,[35] and to possible fragmentation of DNA. In addition, unextracted DNA may contain a significant portion of modified bases. Chromatin isolated from unirradiated or γ-irradiated cells was hydrolyzed with 60% formic acid. The hydrolysates were derivatized and analyzed by GC/MS-SIM. Twelve modified bases were identified in chromatin samples from cells irradiated at five different radiation doses as well as in those from unirradiated cells. Of the modified bases identified in calf thymus DNA irradiated in aqueous solution (Table II), 5,6-diHThy, 5-OH-6-HThy and 5-OH-6HUra were not detected in chromatin. An example of identification by GC/MS-SIM of modified bases in chromatin isolated from irradiated and unirradiated cells is illustrated in Figure 1. Shown are selected-ion current profiles of three characteristic ions of the Me_3Si derivative of FapyGua from GC/MS-SIM analyses of hydrolyzed and derivatized chromatin samples. Several more ions were monitored in the same time interval; however, profiles of three ions are illustrated in Figure 1. Signals of the three monitored ions are seen at the expected retention (indicated with an arrow) of the Me_3Si derivative of FapyGua in both Figure 1A and Figure 1B. A partial mass spectrum was obtained on the basis of the signals of the monitored ions and their relative abundances. This mass spectrum was then compared with that of the authentic material for unequivocal identification (for a detailed description of this technique see Ref. 36).

The measured amounts of modified bases in control chromatin and in chromatin from irradiated cells are given in Table IV. The yields of 5-OH-Hyd, Thy glycol, 5,6-diOH-Cyt, FapyGua and 8-OH-Gua were increased with increasing doses of radiation up to 420 Gy. As examples, Figure 2 illustrates the dose-yield plots of two modified bases. The yields of 5-OHMe-Ura, 5-OH-Ura and FapyAde were increased with radiation dose up to 214 Gy. At 420 Gy, no further significant increase in the yields of these modified bases was observed. A dose of 822 Gy was also applied to check whether higher yields would be obtained at high radiation doses. At this dose, the yields of modified bases were not increased significantly over the levels obtained at 420 Gy. The amounts of 5-OH-Cyt and 8-OH-Ade were not increased over the control levels below 420 Gy. The amount of 5-OH-5-Me-Hyd observed in control chromatin was not increased significantly in chromatin of irradiated cells. The yields of products were increased over the background levels in different ratios. For example at 214 Gy, the highest ratio of increase was $\approx$8–9-fold for 5,6-diOH-Cyt and FapyGua followed by $\approx$4-fold for 5-OH-Ura and Thy glycol.

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TABLE III
Dependence on various treatments of the amounts[a] (molecules/10^5 DNA bases) of modified bases in individual DNA bases

	Treatment					
	Water		Formic acid			
Base	No incub. (1)	With incub. (2)	50% (3)	60% (4)	70% (5)	88% (6)
5,6-diHThy	n.d.[b]	n.d.	n.d.	n.d.	n.d.	n.d.
5-OH-5-Me-Hyd	3.93 ± 0.33	3.87 ± 0.63	3.83 ± 0.53	3.75 ± 0.79	3.08 ± 0.39	3.35 ± 0.47
5-OH-Hyd	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
5-OH-6-HThy	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
5-OH-6-HUra	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
5-OHMe-Ura	5.80 ± 0.58	6.46 ± 1.17	5.43 ± 0.85	4.92 ± 1.15	4.26 ± 0.94	4.09 ± 0.58
5-OH-Ura	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
5-OH-Cyt	10.27 ± 0.41	11.89 ± 3.17	13.54 ± 2.35	10.73 ± 2.72	14.98 ± 2.44	14.89 ± 2.59
Thy glycol	1.61 ± 0.46	1.40 ± 0.36	1.80 ± 0.23	1.28 ± 0.28	1.67 ± 0.28	1.84 ± 0.24
5,6-diOH-Ura	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
FapyAde	24.63 ± 4.82	34.97 ± 3.43	34.20 ± 7.20	33.75 ± 7.16	36.41 ± 2.31	27.21 ± 3.34
8-OH-Ade	43.98 ± 6.38	44.65 ± 8.69	40.56 ± 7.30	46.03 ± 7.50	43.67 ± 9.52	47.20 ± 7.30
2-OH-Ade	2.05 ± 0.24	2.30 ± 0.50	3.06 ± 0.82	3.19 ± 0.79	3.30 ± 0.71	2.60 ± 0.27
FapyGua	6.27 ± 1.10	7.44 ± 1.69	7.28 ± 1.11	8.22 ± 0.68	7.12 ± 1.52	6.93 ± 0.96
8-OH-Gua	76.40 ± 11.28	80.64 ± 14.11	82.55 ± 5.15	74.85 ± 11.27	68.67 ± 5.81	78.00 ± 25.90

*Each value represents the mean ± standard error from five independent experiments.
[b] Not detected.

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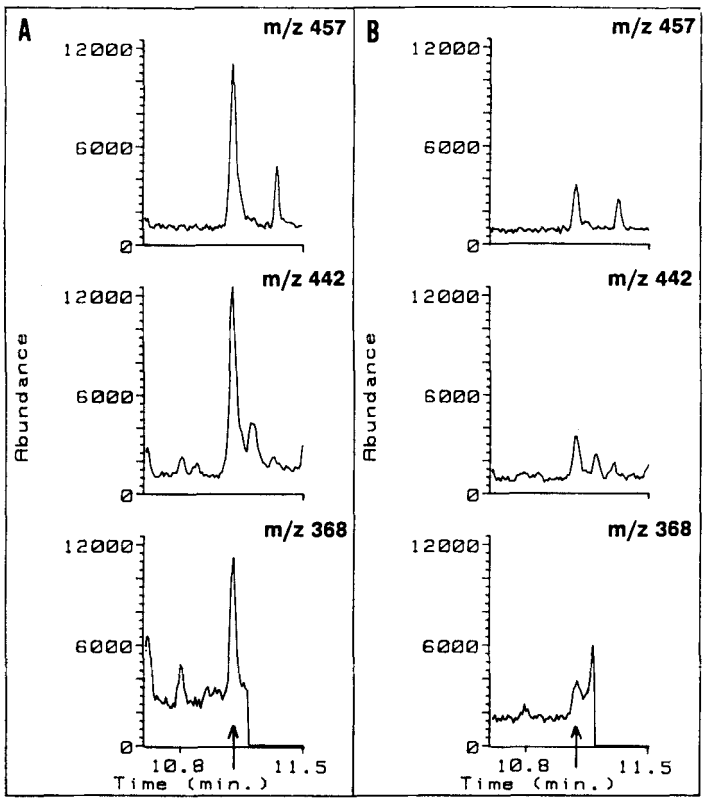

FIGURE 1 Selected-ion current profiles of the ions at *m/z* 368, 442, and 457 obtained during GC/MS-SIM analysis of trimethylsilylated hydrolysates of chromatin. (A) Chromatin isolated from γ-irradiated cells (dose, 116 Gy); (B) chromatin isolated from unirradiated cells. The temperature of the GC column was programmed from 150 to 260°C at 8°C/min after 2 min at 150°C. For other details see Materials and Methods.

DISCUSSION

The results of the present work show the formation of a number of modified bases in chromatin DNA of γ-irradiated cells. Of the modified bases, 5-OH-5-Me-Hyd, 5-OH-Me-Ura, Thy glycol and 8-OH-Gua have been identified previously in DNA of irradiated cells (see Introduction). As for the remaining modified bases, the present work represents the first demonstration of their formation in chromatin of irradiated cells. Modified bases identified here (except for 2-OH-Ade) have also been shown to be formed in isolated chromatin upon γ-irradiation in aqueous suspension.[25] 2-OH-Ade has recently been identified in chromatin treated *in vitro* with H_2O_2 in the presence of metal ions.[37] The yields of modified bases measured here were lower than their yields measured in isolated chromatin, which was γ-irradiated in air-saturated aqueous suspension.[25] In the previous study, chromatin fully expanded in a low ionic strength buffer has been used. DNA in fully expanded chromatin is expected to be more susceptible to free radical attack than DNA in chromatin of the intact cell,

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TABLE IV
Yields[a] of modified bases (molecules/10^5 DNA bases) formed in chromatin of γ-irradiated cultured human cells

Base	Radiation dose				
	Control (1)	42 Gy (2)	116 Gy (3)	214 Gy (4)	420 Gy (5)
5-OH-5-Me-Hyd	2.91 ± 0.38	4.19 ± 0.64	3.04 ± 0.45	2.78 ± 0.32	3.07 ± 0.39
5-OH-Hyd	10.40 ± 1.38	–	15.74 ± 1.73*	17.50 ± 1.44*	23.23 ± 2.88*
5-OHMe-Ura	0.77 ± 0.09	1.28 ± 0.22	1.89 ± 0.51*	2.21 ± 0.19*	2.85 ± 0.35*
5-OH-Ura	0.38 ± 0.05	0.76 ± 0.14*	1.08 ± 0.19*	1.59 ± 0.27*	1.77 ± 0.06*
5-OH-Cyt	2.44 ± 0.38	3.03 ± 0.59	3.01 ± 0.22	3.26 ± 0.51	4.67 ± 0.54*
Thy glycol	1.63 ± 0.22	3.65 ± 0.51*	6.56 ± 0.61*	6.72 ± 0.96*	10.24 ± 0.83*
5,6-diOH-Cyt	0.32 ± 0.09	1.15 ± 0.08*	1.89 ± 0.22*	2.78 ± 0.11*	4.13 ± 0.86*
FapyAde	3.26 ± 0.51	4.48 ± 1.18	6.98 ± 0.99*	8.33 ± 0.67*	10.02 ± 0.16*
8-OH-Ade	2.98 ± 0.58	4.51 ± 0.99	6.08 ± 0.64*	4.22 ± 0.74	5.54 ± 0.77*
2-OH-Ade	1.95 ± 0.27	3.03 ± 0.10*	4.42 ± 0.38*	3.84 ± 0.64*	4.86 ± 0.58*
FapyGua	2.56 ± 0.48	5.34 ± 0.54*	14.91 ± 3.33*	20.90 ± 2.08*	34.24 ± 1.60*
8-OH-Gua	7.71 ± 1.18	12.54 ± 2.43	15.74 ± 2.24*	15.58 ± 1.22*	23.30 ± 3.07*

[a]Each value represents the mean ± standard error from five independent experiments.
*Significantly different from the value in column 1 ($p \leqslant 0.05$).

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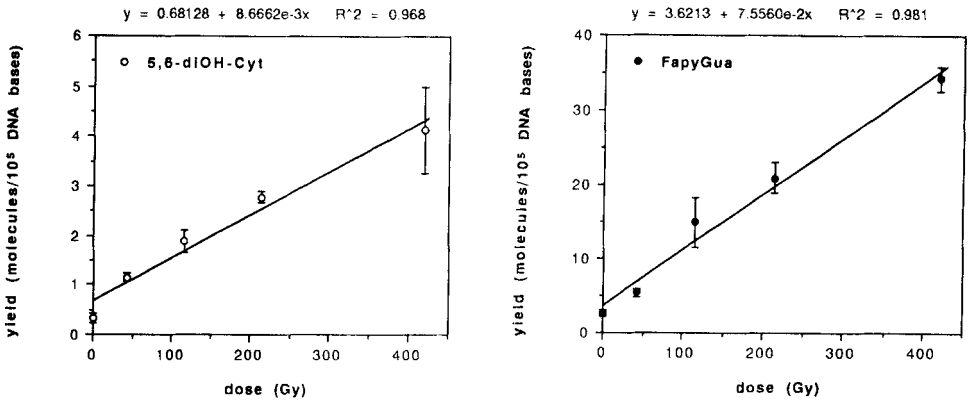

FIGURE 2 Dose-yield plots of 5,6-diOH-Cyt and FapyGua in chromatin of γ-irradiated cells. Error bars represent standard errors of the means from five independent experiments. Lines were drawn by linear regression analysis.

which has a higher order structure. In fact, the susceptibility of isolated chromatin to ionizing radiation-induced damage has been reported to be greater than that of chromatin in cells with respect to DNA lesions such as base damage, DNA-protein cross-links, and strand breaks.[35,38–42] Our results are consistent with the results reported in those studies.

The product yields were increased by increasing doses of radiation and then approached a plateau at radiation doses higher than either 214 Gy or 420 Gy, depending on the product. This deviation from linearity may be a result of generation of hypoxic conditions due to radiation consumption of oxygen. A recent *in vitro* study done with isolated chromatin has shown that most of the products measured in the present work are formed more abundantly in the presence of oxygen than in its absence.[25] A difference in the yields of DNA isolated from unirradiated and irradiated cells may also contribute to a deviation of dose-yield relationships from linearity. It is well known that DNA may not be extracted efficiently from damaged chromatin of irradiated cells due to the formation of covalent DNA-protein cross-links in chromatin.[12,35] In the present work, however, chromatin rather than DNA alone was isolated, and no difference was observed between the chromatin yields from unirradiated and irradiated cells as indicated in Materials and Methods.

Modified bases detected in chromatin of irradiated cells are known to be the typical · OH-induced products of DNA bases (reviewed).[5,24] Recently, they have also been identified in chromatin of H_2O_2-treated mammalian cells.[43] Their formation in chromatin of irradiated cells is most likely due to reactions with DNA bases of · OH produced from cellular water by ionizing radiation. Earlier measurements have shown that $\approx 70\%$ of radiation-induced lethality and DNA damage in oxic cells is caused by hydroxyl radical.[7–9] Because of its high reactivity toward organic molecules, however, · OH has a short diffusion distance in cells, and must be generated in close proximity to DNA in order to cause any DNA damage.[8,44] The track model of energy deposition of ionizing radiation in an aqueous medium provides the concept of free radical formation in track entities such as spurs and blobs, which may be formed in the vicinity of DNA bases (reviewed).[45] The direct effect of radiation on DNA may also account in part for the formation of modified bases through ionization of DNA

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bases.[46–49] Three modified bases namely 5,6-diHThy, 5-OH-6-HThy and 5-OH-6-HCyt identified in DNA γ-irradiated in N_2O-saturated solution were not detected in chromatin of irradiated cells. The absence of 5,6-diHThy, which is induced by e^-_{aq} and/or H atom, is likely due to scavenging of e^-_{aq} and H atom by oxygen. Hydroxyl radical-induced 5-OH-6-HThy and 5-OH-6-HCyt are known to be formed only in deoxygenated systems.[5,10] The absence of these three modified bases has also been shown in chromatin γ-irradiated in air-saturated aqueous suspension.[25]

Modified bases identified in chromatin of irradiated cells were found to be present in chromatin of unirradiated cells. Intrinsic DNA damage may occur as a result of free radical production by cellular metabolic processes (reviewed).[1] Various steps in the procedure for isolation of chromatin from cells may also contribute in part to the formation of modified bases in chromatin. However, this procedure involves mild treatments of cells, isolated nuclei and various forms of chromatin, and does not use phenol extraction. Thus it should be milder than the procedures generally used for DNA isolation. Formic acid treatment may also contribute to background levels of modified bases in chromatin. However, the results obtained in this work indicate that modified bases are not significantly formed from DNA bases by treatment with formic acid. It should be emphasized that the background levels of modified bases in chromatin, and not the sensitivity of the technique used are the limiting factor in measurement of modified bases in irradiated cells at lower doses than those used here. The methodology incorporating the GC/MS-SIM technique is capable of measuring any of the modified bases at levels of ≈ 1–3 molecules per 10^6 DNA bases. For example, the lowest amount of a modified base detected in unirradiated DNA [i.e., 5,6-diOH-Ura (Table II)] correspond to ≈ 1.4 molecules per 10^6 DNA bases. Despite the limiting factor mentioned above, significant increases in the yields of a number of modified bases in chromatin over background levels were observed at a dose of as low as 42 Gy.

Of the modified bases measured in chromatin of irradiated cells, the yields of guanine-derived bases were the highest (e.g., $\approx 45\%$ of the net total yield of modified bases, when calculated from the yields at 116 Gy after subtraction of background values). The yields of adenine-, cytosine- and thymine-derived bases constituted the rest of the net total yield in almost equal percentages. These results are similar to those previously obtained with isolated chromatin γ-irradiated in aqueous suspension.[25] This may indicate the high reactivity with $\cdot$OH of guanine residues in chromatin in cells and *in vitro*. On the other hand, guanine-derived bases FapyGua and 8-OH-Gua may also result in part from reactions of the guanine radical cation formed by direct ionization of guanine residues in chromatin of cells. Thus, the high yield of guanine-derived bases is also in accord with the well-known property of guanine being the most easily oxidized DNA base by the direct effect of ionizing radiation and by other oxidants (reviewed).[5,48] The yields of formamidopyrimidines (FapyAde and FapyGua) were higher than those of 8-hydroxypurines (8-OH-Ade and 8-OH-Gua) in chromatin of irradiated cells. Formamidopyrimidines and 8-hydroxypurines result from one-electron reduction and oxidation of C-8 OH-adduct radicals of purines, respectively (reviewed).[48] The overall pattern of the yields of these compounds is in contrast to that obtained with isolated chromatin γ-irradiated in air-saturated aqueous suspension, and rather resemble that obtained with isolated chromatin γ-irradiated in deoxygenated aqueous suspensions.[25] This may indicate the inability of oxygen to completely interfere with reactions of C-8 OH-adduct radicals of purines in cellular chromatin and/or the hypoxic nature of the environment of the cell nucleus (reviewed).[50]

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A number of modified bases have been investigated *in vitro* and *in vivo* for their biological consequences.[2,51–56] The variety of modified bases in DNA of chromatin of irradiated cells makes it difficult to assess their role in biological end-points such as mutagenesis, carcinogenesis and cell death. The contribution of the modified bases, which have herein been identified in the chromatin of cells, to the biological effects of ionizing radiation is as yet unknown. The measurement of modified bases in chromatin of γ-irradiated cells provides the basis for studies for the understanding of their biological significance.

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