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DNA Oxidation Products Determined with Repair Endonucleases in Mammalian Cells: Types, Basal Levels and Influence of Cell Proliferation

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Purified repair endonucleases such as Fpg protein, endonuclease III and IV allow a very sensitive quantification of various types of oxidative DNA modifications in mammalian cells. By means of these assays, the numbers of base modifications sensitive to Fpg protein, which include 8-hydroxyguanine (8-oxoG), were determined to be less than 0.3 per 10^6 bp in several types of untreated cultured mammalian cells and human lymphocytes and less than 10 per 10^6 bp in mitochondrial DNA from rat and porcine liver. Oxidative 5,6-dihydropyrimidine derivatives sensitive to endonuclease III and sites of base loss sensitive to endonuclease IV or exonuclease III were much less frequent than Fpg-sensitive modifications. Here, we summarize our indications that all Fpg-sensitive modifications are recognized under the assay conditions and that on the other hand there is no artifactual generation of oxidative damage during the analysis. In addition, we show that the steady-state levels of Fpg-sensitive modifications in human lymphocytes and in two mammalian cell lines were higher in proliferating than in resting (confluent) cells. Only some of the Fpg-sensitive base modifications induced by various oxidants are 8-oxoG residues, as demonstrated for the damage under cell-free conditions. The percentage was dependent on the species ultimately responsible for the DNA damage and was approx. 40% in the case of hydroxyl radicals and peroxynitrite, 75% for type II photosensitizers (reacting via singlet oxygen) and only 20–30% in the case of type I photosensitizers such as riboflavin and acridine orange, which are assumed to react directly with the DNA.

Keywords: Alkaline elution, oxidative DNA damage, Fpg protein, 8-hydroxyguanine, steady-state level

INTRODUCTION

There is good evidence that oxidative DNA damage is generated endogenously in all types of cells. The damage is attributed to reactive oxygen species (ROS) generated in the oxygen metabolism of the cells, although the relevant mechanisms (nature of the enzymes and species involved) remain to be established. There is no doubt that several types of oxidative DNA modifications, e.g. 8-hydroxyguanine (8-oxoG) are pre-mutagenic.[1–5] Therefore, the endogenous oxidative damage is expected to contribute to the spontaneous mutation rates in all cells

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and could play a role in carcinogenesis, the development of age-correlated degenerative diseases and the process of aging itself.[6–8]

Since oxidative DNA modifications are subject to specific repair, the balance between generation and removal of the lesions is expected to result in steady-state levels in the cellular DNA. The assessment of these levels as potential risk factors for the above-mentioned diseases is of major interest. However, the various techniques that are sensitive enough to allow a quantification of oxidative modifications in untreated cells, e.g. HPLC with electrochemical detection (HPLC/ECD),[9] gas chromatography/mass spectrometry (GC/MS)[10] and ^{32}P-postlabelling,[11] gave conflicting results (reviewed in Refs. [12,13]). An oxidation of guanine during isolation and derivatization was shown to be responsible at least in some cases.[14–21] Particularly low steady-state levels of oxidative DNA modifications were obtained by assays in which the recognition of oxidative modifications by repair endonucleases is exploited to determine the number of "endonuclease-sensitive modifications" in cellular DNA.[22–25] The assays make use of the fact that the repair endonucleases incise the DNA at their substrate modifications. The DNA single-strand breaks thus generated can be very sensitively quantified by a variety of techniques, e.g. in the chromosomal DNA of mammalian cells by alkaline elution (Figure 1) or in supercoiled DNA such as mitochondrial DNA (mt-DNA) by means of a relaxation assay.[26,27]

Here, we summarize our indications that the steady-state levels of endonuclease-sensitive oxidative modifications are neither underestimated nor overestimated by alkaline elution. For the damage induced by various oxidants under cell-free conditions, we report on the fraction of base modifications sensitive to the repair endonuclease Fpg protein that was identified to be 8-oxoG by HPLC/ECD. In addition, we provide evidence suggesting that the steady-state levels of oxidative DNA damage are higher in proliferating than in resting cells.

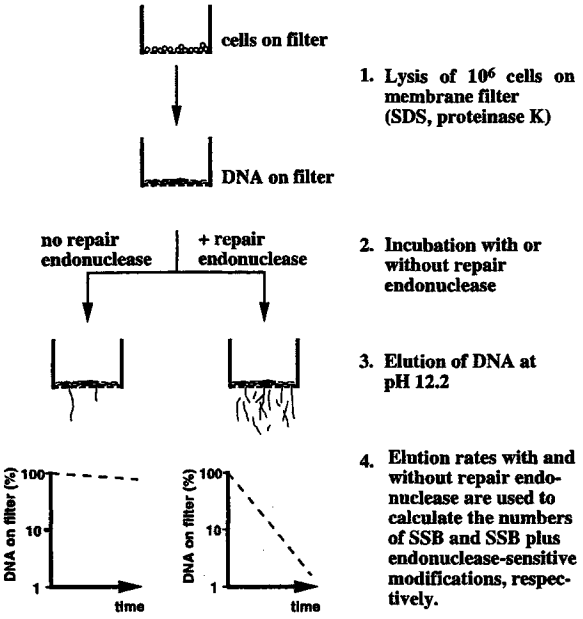

FIGURE 1 Quantification of endonuclease-sensitive modifications in mammalian cells by alkaline elution.

MATERIALS AND METHODS

Cells, DNA and Repair Endonucleases

Blood was collected from healthy donors in syringes containing heparin and then supplemented with catalase (315 U/ml). Lymphocytes were isolated by centrifugation with Histopaque-1077 separation medium (Sigma, Deisenhofen, Germany). AS52 Chinese hamster ovary cells, which carry the bacterial *gpt* gene for analysis of mutations,[28] were obtained from W.J. Caspary, Research Triangle Park, USA, and cultured in Ham's F12 medium with 5% fetal calf serum. HeLa cells were cultured in Dulbecco's modified Eagle's medium (DMEM) containing 4.5 g/l glucose and 10% fetal calf serum. HaCaT cells (spontaneously immortalized human keratinocytes[29]), obtained from N.E. Fusenig, Heidelberg, Germany, were cultured in DMEM medium with 10% fetal calf serum. All culture media were supplemented with penicillin (100 units/ml) and streptomycin (100 µg/ml).

DNA from bacteriophage PM2 (PM2 DNA) was prepared according to the method of Salditt *et al.*[30]

Formamidopyrimidine-DNA glycosylase (Fpg protein)[31] and endonuclease III from *E. coli* were kindly provided by S. Boiteux, Fontendy aux Roses, France. T4 endonuclease V was partially purified by the method described by Nakabeppu *et al.*[32] from an *E. coli* strain harbouring the *denV* gene on an inducible expression vector (kindly provided by L. Mullenders, Leiden, Netherlands). Exonuclease III was purchased from Boehringer, Mannheim, Germany. All repair endonucleases were tested under cell-free conditions by means of a relaxation assay for their incision at reference modifications (e.g. sites of base loss induced by low pH, 8-oxoG residues induced by methylene blue plus light, thymine glycols induced by osmium tetroxide) to determine at which concentration the correct substrate modifications are fully recognized and no incision at non-substrate modifications takes place.[26]

Treatments of Cells

The exposure of cells to the photosensitizer Ro19-8022 ([R]-1-[(10-chloro-4-oxo-3-phenyl-4H-benzo[a] quinolizin-1-yl)-carbonyl]-2-pyrrolidine-methanol), a gift from Hoffmann-LaRoche AG (Basel, Switzerland), in the presence of visible light from a 1000 W halogen lamp (Philips PF811) at a distance of 47 cm was carried out in PBSG (140 mM NaCl, 3 mM KCl, 8 mM Na_2HPO_4, 1 mM KH_2PO_4, 1 mM $CaCl_2$, 0.5 mM $MgCl_2$, 0.1% glucose, pH 7.4) on ice (10^6 cells/ml). Ten min illumination corresponded to 111 kJ/m^2 between 400 and 800 nm. The cells were pelleted by centrifugation and resuspended in PBSG three times.

To compare the levels of Fpg-sensitive modifications in proliferating and non-proliferating cells, AS52 and HaCaT cells were grown to yield confluent monolayers and either diluted and replated for 24 h in full medium (exponential growth conditions) or kept for the same time in the confluent state in fresh medium. Freshly prepared human lymphocytes were incubated for 24 h in RPMI 1640 medium containing 10% fetal calf serum in the presence or absence of 10 μg/ml phytohemagglutinin (PHA) (Sigma, Deisenhofen, Germany).

Quantification of 8-oxodG by HPLC/ECD

The isolation of DNA from mammalian cells after lysis on polycarbonate membrane filters according to the alkaline elution protocol was described previously.[22] For enzymatic hydrolysis, 50 μg DNA were incubated in 200 μl Tris-Mg-buffer (40 mM Tris, 10 mM $MgCl_2$, pH 8.5) with DNAse I (10 U/50 μg DNA), spleen exonuclease (0.0005 U/50 μg DNA), snake venom exonuclease (0.025 U/50 μg DNA), and alkaline phosphatase (0.5 U/50 μg DNA) for 2 h at 37°C.[33] Five μg PM2 DNA modified under cell-free conditions (see below) were redissolved in 100 μl Tris-Mg-buffer and hydrolysed under the same conditions.

HPLC analysis was performed with a Nucleosil 100-5 C18 250/4 column (Macherey-Nagel, Düren, Germany), a Kontron UV spectrophotometer Uvicon 730 LC (Kontron, Zürich, Switzerland) operated at 290 nm, and an ESA Coulochem II (Bischoff, Leonberg, Germany) coulometric electrochemical detector equipped with an analytical cell model 5011 at 0.3 V and 2 nA as described previously.[22] The amounts of deoxythymidine and deoxyguanosine (determined by means of the UV detector) were used as internal references for the amount of DNA hydrolysed.

Quantification of Endonuclease-sensitive Modifications by Alkaline Elution

The alkaline elution assay originally described by Kohn *et al.*[34] with modifications described previously[26] was used for the quantification of modifications sensitive to repair endonucleases in mammalian cells, as outlined in Figure 1.

Briefly, 10^6 cells were washed by centrifugation and resuspension in PBSCMF buffer (140 mM NaCl, 3 mM KCl, 8 mM Na_2HPO_4, 1 mM KH_2P0_4, pH 7.4), collected on a polycarbonate filter (2 μm pore size) and lysed by pumping a lysis solution (100 mM glycine, 20 mM Na_2EDTA, 2% SDS, 500 mg/l proteinase K, pH 10.0) through the filter for 60 min at 25°C. After extensive washing, the DNA remaining on the filter was incubated for 30 min at 37°C with a repair endonuclease. The concentration of the enzymes applied were the same as those required for full recognition of defined substrate modifications in the relaxation assay (see above). The DNA was washed again and then eluted at 25°C with an elution buffer [20 mM H_4-EDTA (acid form), adjusted to pH 12.2 with tetraethylammoniumhydroxide] at 2.3 ml/h for 10 h. The slopes of the elution curves were used to obtain the sum of endonuclease-sensitive modifications and single-strand breaks; elution curves obtained with γ-irradiated cells were used for calibration (6 Gy = 1 ssb/10^6 bp).[34] The numbers of endonuclease-sensitive modifications were obtained by subtraction of the number of single-strand breaks determined in a parallel experiment in which the incubation was carried out without repair endonuclease. With ionizing radiation, a linear dose response was observed between 0.3 and 9.0 Gy, equivalent to 0.05–1.6 single-strand breaks per 10^6 bp.

Damage Analysis in PM2 DNA

The exposure of PM2 DNA (10 μg/ml) to various photosensitizers and other oxidants was carried out in phosphate buffer (5 mM KH_2PO_4, 50 mM NaCl, pH 7.4) as described previously.[35–39] The DNA was precipitated by ethanol/sodium acetate and redissolved in Tris-Mg-buffer for damage analysis by HPLC/ECD as described above or in BE_1 buffer (20 mM Tris-HCl, pH 7.5, 100 mM NaCl, 1 mM EDTA) for the quantification of endonuclease-sensitive modifications by a relaxation assay as described previously.[26]

RESULTS

In untreated mammalian cells, base modifications recognized by Fpg protein are the most frequent type of endonuclease-sensitive oxidative DNA damage

By means of the alkaline elution technique (see Materals and Methods), the numbers of modifications sensitive to various repair endonucleases with different substrate specificities (Table I) were quantified in the nuclear DNA of untreated human lymphocytes, HeLa cells and AS52 Chinese hamster ovary cells. The results (Figure 2, upper panel) indicate that base modifications recognized by Fpg protein, which include

TABLE I Recognition of DNA modifications by repair endonucleases used in this study

Repair endonuclease	Recognition spectrum[a]	
	Sites of base loss[b]	Base modifications
Fpg protein	+	8-oxoG[c]; Fapy[d]
Endonuclease III	+	5,6-dihydropyrimidines; hyd[e]
T4 endonuclease V	+	CPD[f]
Exonuclease III	+	—
Endonuclease IV	+	—

[a]See Ref. [40–43].
[b]For the recognition of sites of base loss oxidized in the 1′ or 4′ position, see Ref. [44].
[c]7,8-Dihydro-8-oxoguanine (8-hydroxyguanine).
[d]Formamidopyrimidines (imidazole ring-opened purines), e.g. 4,6-diamino-5-formamidopyrimidine (Fapy-A) and 2,6-diamino-4-hydroxy-5-formamidopyrimidine (Fapy-G).
[e]5-Hydroxy-5-methylhydantoin and other ring-contracted and fragmented pyrimidines.
[f]Cyclobutane pyrimidine photodimers.

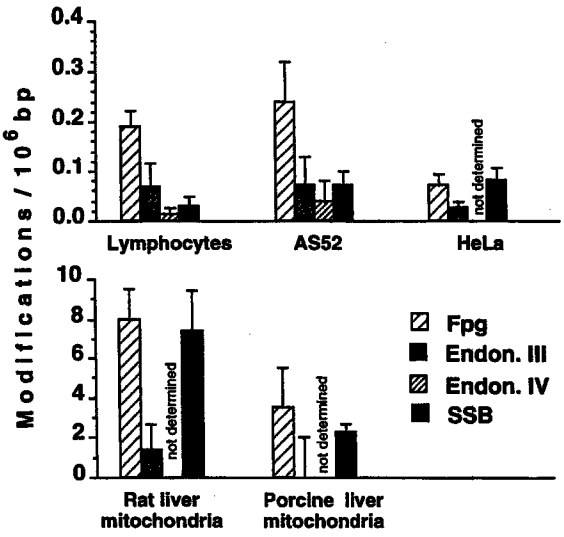

FIGURE 2 Steady-state (background) levels of DNA modifications recognized by various repair endonucleases (Table I) in chromosomal DNA from human lymphocytes (averaged from 3 donors), AS52 Chinese hamster cells and HeLa cells (upper panel) and in mt-DNA from rat and porcine liver (lower panel). Values for mt-DNA are taken from Ref. [27]. Data represent the means of 3–7 independent experiments (±SD).

8-oxoG, are most frequent in all types of cells. The numbers of sites of base loss (AP sites) recognized by endonuclease IV or exonuclease III and the numbers of oxidized pyrimidines recognized by endonuclease III are much lower and within or close to the detection limit of 0.05 modifications/10^6 bp. A similar dominance of Fpg-sensitive base modifications was also previously observed in mt-DNA from rat and porcine liver, in which the background levels of endonuclease-sensitive modifications was analysed by means of a relaxation assay (Figure 2, lower panel).

Also shown in Figure 2 are the numbers of single-strand breaks in untreated nuclear and mt-DNA, which are obtained from experiments without repair endonuclease. These values represent upper limits for the actual number of single-strand breaks in the cells, since a low level of DNA hydrolysis cannot be excluded under the (alkaline) conditions of cell lysis.

TABLE II Comparison of the steady-state levels of Fpg-sensitive modifications in proliferating and non-proliferating cells

Cells compared	Ratio of the lesions[a,b]
AS52 cells	1.33 ± 0.12 (3)
HaCaT cells	1.22 ± 0.33 (4)
Human lymphocytes[c]	1.36 ± 0.34 (8)

[a]Levels of Fpg-sensitive modifications in proliferating cells divided by the levels in non-proliferating (confluent) cells.
[b]Numbers of experiments indicated in parenthesis.
[c]Lymphocytes were from 3 different donors. Proliferation was stimulated by incubation with PHA.

The steady-state levels of oxidative damage are higher in proliferating cells than in arrested cells

The data presented in Figure 2 indicate that the cellular steady-state levels of Fpg-sensitive modifications depend on the cell type, as described previously.[22] To test whether also cell proliferation has an effect on the basal levels of oxidative damage, primary human lymphocytes were incubated with phytohemagglutinin (PHA) to induce mitosis. In the PHA-stimulated cells, the basal levels of Fpg-sensitive modifications were found to be significantly ($p = 0.036$) higher than in the unstimulated control lymphocytes (Table II). When the steady-state levels of Fpg-sensitive modifications in AS52 cells and immortalized keratinocytes (HaCaT cells) were compared under conditions of exponential growth and in the confluent (cell-cycle arrested) state, cell proliferation was again found to be associated with increased levels of oxidative damage (Table II).

The recognition of 8-oxoG residues is complete under the assay conditions

The cellular steady-state levels of Fpg-sensitive sites determined by alkaline elution and other techniques are much lower than the steady-state levels of 8-oxoG determined by techniques such as HPLC/ECD and GC/MS, when standard protocols of the latter techniques are employed.[13,22] Therefore, it is an important question whether all 8-oxoG residues are recognized by Fpg protein under the assay condition. The data in Table III indicate that this is indeed

TABLE III Levels of photosensitizer-induced 8-oxoG residues in mammalian cells (chromosomal DNA) and cell-free DNA from bacteriophage PM2 before and after incubation with Fpg protein

DNA	8-oxoG residues per 10^6 bp[a]	
	Induced[b]	Residual[c]
AS52	54 ± 4	8
L1210	22 ± 3	2.3 ± 1.0
HeLa	68	11
PM2 (cell-free)	74 ± 10	Not detectable[d]

[a]Determined by HPLC/ECD. Background (steady-state) levels in untreated controls were subtracted. Data for cellular DNA are taken from Ref. [22].
[b]By exposure to visible light (675 kJ/m^2 between 400 and 800 nm) in the presence of 2.5 μM Ro 19-8022 (AS52, HeLa), 1 μM Ro 19-8022 (L1210) or 50 μM Ro 19-8022 (PM2 DNA).
[c]After incubation with Fpg protein under the conditions of the alkaline elution assay (cellular DNA) or the relaxation assay (PM2 DNA).
[d]Detection limit for 8-oxoG in 5 μg PM2 DNA approx. 4 modifications per 10^6 bp.

TABLE IV Effects of the presence of the iron chelator DSF[a] and antioxidants on the level of Fpg-sensitive DNA modifications in AS52 cells determined by alkaline elution

Presence of DSF		Relative number of modifications (%)[b]
Control		100 ± 15
100 μM DSF[a]		106 ± 13
100 μM DSF	+5% DMSO	99 ± 15
	+10% DMSO	93 ± 17
	+5 mM GSH	94 ± 24
	+10 mM GSH	91 ± 19

[a]Desferrioxamine mesylate.
[b]Means of 2–3 experiments. Number of modifications observed in the absence of DSF and antioxidants defined as 100%.

the case for 8-oxoG residues induced by the photosensitizer Ro19-8022 plus light. The quantification of 8-oxoG by HPLC/ECD before and after the incubation with Fpg protein confirms that most of the lesions are removed by the enzyme, both under the conditions of the alkaline elution assay (cellular chromosomal DNA) and of the relaxation assay (supercoiled DNA).

The presence of desferrioxamine and DMSO during cell lysis has no influence on the determination of Fpg-sensitive modifications by alkaline elution

For the quantification of 8-oxoG by HPLC/ECD, the presence of iron chelator such as desferrioxamine and antioxidants such as glutathione during cell lysis and DNA hydrolysis has been demonstrated to reduce the amount of 8-oxoG detected.[19] This indicates that an artifactual generation of 8-oxoG can take place during damage analysis. To test, whether this is also a problem in the quantification of Fpg-sensitive modifications by alkaline elution, the iron chelator desferrioxamine alone or in combination with the antioxidants DMSO or glutathione was added before cell lysis and analysis by alkaline elution. The steady-state levels in AS52 cells determined under these conditions were not signficantly different from those obtained in the absence of the iron chelator and the antioxidant (Table IV).

Only some of the Fpg-sensitive modifications generated by oxidants are 8-oxoG residues

Many, but not all repair endonucleases have been shown to recognize more than one substrate (Table I). Moreover, the recognition of as yet unknown substrates is difficult to exclude. Therefore, the chemical nature of the Fpg-sensitive modifications has to be established by independent techniques.

In supercoiled DNA, endonuclease-sensitive modifications and 8-oxoG can be determined with the same sensitivity, e.g. at a level of approximately 1 modification per 10^4 bp. For various types of oxidative damage induced in bacteriophage PM2 DNA under cell-free conditions, we therefore determined the fraction of Fpg-sensitive modifications that 8-oxoG accounts for. The damage profiles induced by the various oxidants analysed are shown in Figure 3. The fractions of Fpg-sensitive modifications identified as 8-oxoG by HPLC/ECD are listed in Table V, together with the fractions supposed to be sites of base loss according to the number of lesions sensitive to exonuclease III and endonuclease IV, which recognize these lesions specifically. The species or mechanism that – according to previous studies[35–39] – are most probably

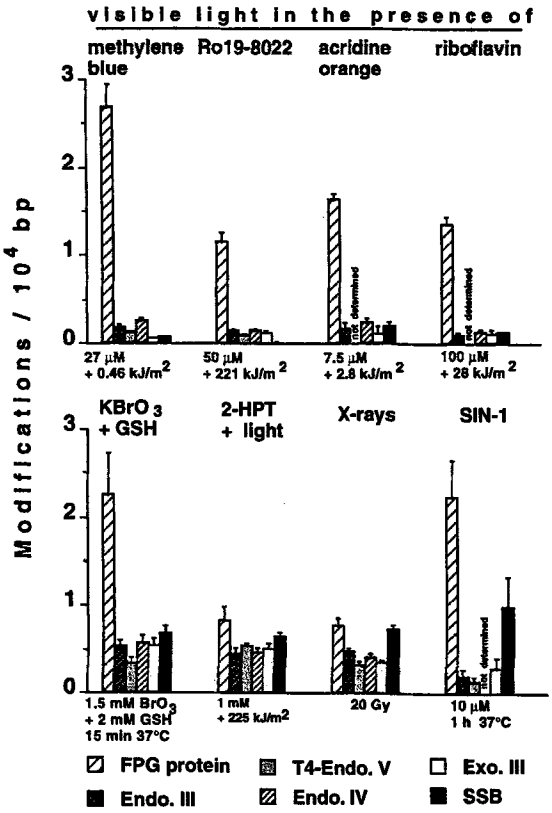

FIGURE 3 DNA damage profiles induced by various photosensitizers plus light (upper panel) and other oxidants (lower panel) in PM2 DNA under cell-free conditions (phosphate buffer). Data are from Ref. [35] (photosensitizers except Ro19-8022), Ref. [36] (bromate plus glutathione), Ref. [37] (ionizing radiation), Ref. [38] (2-HPT plus light) and Ref. [39] (SIN-1). Putative ultimate reactive species are listed in Table V.

directly responsible for the various types of damage are also indicated in Table V. With the exception of hydroxyl radicals, produced either by photodecomposition of 2-hydroxy-2-thiopyridone (2-HPT)[38] or by ionizing radiation, most reactive species generate Fpg-sensitive base modifications in much higher yields than all other types of modifications (Figure 3).

The results indicate that in the case of damage induced by hydroxyl radicals (generated either from 2-HPT or by ionizing radiation) 8-oxoG accounts for approximately 20% of the total number of modifications recognized by Fpg protein, which – due to the presence of sites of base loss – is equivalent to approximately 40% of the base modifications recognized by this enzyme. Interestingly, 44% of the Fpg-sensitive base modifications were also identified to be 8-oxoG in the case of the damage attributed to activated peroxynitrite, generated by thermal decomposition of 3-morpholinosydnonimine (SIN-1).[39] In contrast, in the damages induced by the photosensitizers methylene blue and Ro19-8022, which are to a large extent generated via singlet oxygen ($^1O_2$), 8-oxoG accounts for approximately 75% of the Fpg-sensitive base modifications. Surprisingly, the fraction represented by 8-oxoG is much lower in the case of the type I photosensitizers riboflavin and acridine orange, which are assumed to react directly with the DNA. A high

TABLE V Fractions of Fpg-sensitive modifications induced in PM2 DNA under cell-free conditions that was identified as 8-oxoG by HPLC/MS and as sites of base loss by endonuclease IV and exonuclease III

Damaging agent[a]	Species[b]	% of Fpg-sensitive modifications[c] recognized as		
		8-oxoG[d,e]	sites of base loss by	
			Endon. IV[c]	Exon. III[c]
Methylene blue + light	$^1O_2$	69 ± 11 (3)	10 ± 2	2 ± 1
Ro19-8022 + light	$^1O_2$	60 ± 5 (2)	14 ± 3	10 ± 3
Acridine orange + light	type I[f]	29 ± 5 (2)	15 ± 3	7 ± 6
Riboflavin + light	type I[f]	21 ± 13 (6)	10 ± 3	8 ± 4
Bromate + GSH	$Br^•$	70 ± 11 (3)	26 ± 4	24 ± 4
2-HPT + light	$OH^•$	19 ± 2 (2)	56 ± 6	58 ± 10
Ionizing radiation	$OH^•$	18 (1)	52 ± 8	44 ± 5
SIN-1	ONOOH	38 ± 10 (3)	not det.	13 ± 6

[a]For damaging conditions and references see Figure 3. [b]Ultimately DNA-modifying species according to experiments with scavengers etc. [c]Determined by means of a relaxation assay. [d]Determined by HPLC/ECD analysis. [e]Numbers of experiments given in parenthesis. [f]Triplet-excited photosensitizer.

fraction of 8-oxoG is observed in the damage induced by bromate plus glutathione, for which bromine radicals were suggested as ultimately reactive species.[36]

DISCUSSION

The determination of steady-state (background) levels of oxidative DNA modifications generally raises much more difficulties than the quantification of DNA modifications additionally induced by exposure to an oxidant, since the substraction of the background values allows a convenient correction for artifacts only in the latter case. Thus, the reported steady-state levels of 8-oxoG and Fpg-sensitive modifications in untreated lymphocytes differed by at least two orders of magnitude,[13,22] while on the other hand the photosensitizer-induced levels of 8-oxoG detected by HPLC/ECD were in reasonable agreement with the induced levels of Fpg-sensitive modifications determined in parallel, both in isolated DNA (Table V) and in cellular DNA.[22]

For the quantification of 8-oxoG by HPLC/ECD and GC/MS, an artifactual oxidation during cell lysis, DNA hydrolysis and – in the case of GC/MS – derivatization has been demonstrated to be a major source of error.[14–21] Its avoidance under oxygen-free conditions or by addition of antioxidants resulted in steady-state levels that were similarly low as the steady-state level of Fpg-sensitive modifications determined by alkaline elution and other techniques.[17,21]

The results shown in Table IV indicate that the presence of the iron chelator desferrioxamine and the antioxidants DMSO and glutathione have no effect on the determination of Fpg-sensitive modifications by alkaline elution. Thus, there is no indication for DNA damage via a Fenton-reaction during or after the disruption of the cell compartmentation that is associated with cell lysis. The absence of artifactual damage by hydroxyl radicals can also be concluded from the relatively low level of single-strand breaks observed in untreated lymphocytes and AS52 cells (Figure 2), since hydroxyl radicals induced a 1:1 ratio of Fpg-sensitive modifications both under cell-free conditions (Figure 2) and in cells.[38]

An overestimation of endonuclease-sensitive modifications could also result from an unspecific endonuclease activity of the enzyme preparation. The saturation curves obtained both with mt-DNA[27] and chromosomal DNA[22,45] when the Fpg protein concentration in the protocol was varied are indications that the incision by Fpg protein occured at a limited number of substrate modifications, i.e. were not unspecific. Furthermore, the differences observed between various cell types and between proliferating and non-proliferating cells argue against unspecific nuclease activity and other sources of artifacts.

It should be noted that mt-DNA is lost during cell lysis in the alkaline elution technique. Even high levels of damage in mt-DNA therefore would not influence the results.

Since the levels of Fpg-sensitive modifications in untreated cells determined by alkaline elution are lower than the levels of 8-oxoG measured by (standard protocols of) HPLC/ECD and GC/MS, it is important to exclude that only a fraction of the 8-oxoG residues are recognized. For the damage induced by photosensitization in cellular and cell-free DNA, the results shown in Table III demonstrate that the excision of 8-oxoG by Fpg protein is nearly complete under the assay conditions. There is no reason to assume that this should not be true for the levels of 8-oxoG in untreated cells as well. It should be noted, however, that the determination by alkaline elution is based on a random distribution of lesions and clustering of modifications could in theory cause errors.

The results shown in Figure 2 indicate that in untreated cells purine modifications sensitive to Fpg protein are much more frequent than pyrimidine modifications sensitive to endonuclease III, AP sites and – at least in most cases – single-strand breaks. The dominance of Fpg-sensitive

modifications is also observed in the damage profiles induced by many oxidants, both under cell-free conditions (Figure 3) and in cells.[35,36,46] Recently, the steady-state (background) level of thymine glycols, a well-known substrate of endonuclease III, in a human lung carcinoma cell line was determined to be approximately 0.05 modifications per 10^6 bp by means of an immunochemical assay,[47] in reasonable agreement with the low steady-state levels of modifications sensitive to endonuclease III observed in this study (Figure 2).

The steady-state levels of Fpg-sensitive modifications depend on the cell type (Figure 2) and were higher in proliferating than in non-dividing cells (Table II). The latter observation could indicate that the oxidative stress is reduced in non-proliferating cells. Further experiments with additional cell types are required to demonstrate that this is indeed a general phenomenon.

The data presented in Table V indicate that between 20% and 75% of the Fpg-sensitive base modifications induced by various oxidants under cell-free conditions are 8-oxoG residues. According to the present knowledge of the recognition spectrum of Fpg protein, the remaining modifications should be imidazole ring-opened purines (formamidopyrimidines) (Table I). However, a direct proof of this assumption is missing and the recognition of other oxidative purine modifications by Fpg protein, e.g. oxazolones,[48] remains to be established.

In summary, the results support the assumption that repair endonucleases allow a very sensitive and reliable quantification of several groups of oxidative DNA modifications in untreated mammalian cells. A problem is the limited substrate specificity of several enzymes; it can be partly overcome when results obtained with several enzymes are compared.

Acknowledgements

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