Endogenous oxidative DNA base modifications analysed with repair enzymes and GC/MS technique

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ABSTRACT

GC/MS technique was used to identify endogenous levels of oxidatively modified DNA bases. To avoid possible artefact formation we used Fpg and Endo III endonucleases instead of acid hydrolysis to liberate the base products from unmodified DNA samples. Several different DNA preparations were used: (i) commercial calf thymus DNA, (ii) DNA isolated from rat liver, (iii) DNA isolated from human lymphocytes and (iv) nuclei isolated from rat liver. In all DNA samples used in our assays the most efficiently removed bases by Fpg protein are FapyG and FapyA although 8-oxoG was also detected in all preparations. The amount of 8-oxoG in human lymphocytes and in rat liver DNA was 3 and 2 per 10⁷ bases, respectively. It is reasonable to postulate that the presented method is one of the techniques which should be used to reveal the enigma of endogenous, oxidative DNA damage.

INTRODUCTION

Oxygen derived free radicals are produced continuously inside the living cell as well as being able to get into the cell from outside sources. These radicals such as •OH can damage biomolecules, among them DNA. When reacting with DNA, •OH radicals may produce oxidatively modified DNA bases and some of them have premutagenic potential (1,2).

Therefore, a reliable assay for measuring oxidative base damage would be a valuable tool to answer some very important questions about the involvement of such damage in carcinogenesis and other disorders.

There are several techniques that are currently used to analyse the base products, but GC/MS is the only one that can unequivocally identify a wide spectrum of oxidatively modified DNA bases. This technique was successfully applied in order to analyse the base products in DNA isolated from cancerous and non-cancerous human tissues (3,4). However, it has recently been reported that artificial oxidative damage to DNA bases could occur during sample preparation for GC/MS analysis mainly during acid hydrolysis that liberates purines and pyrimidines from DNA, and during the derivatisation step (5). Methods making use of repair enzymes are the most sensitive ones

(6). However, this type of measurement is both quantitatively and qualitatively indirect.

In the described experiments GC/MS technique was used to identify oxidatively modified purines and pyrimidines. To avoid possible artefact formation we used repair endonucleases (Fpg and Endo III) instead of acid hydrolysis to liberate the base products from unmodified DNA samples.

MATERIALS AND METHODS

DNA from lymphocytes and DNA from rat liver cells were isolated using the method described by Miller *et al.* (7), with some modifications according to Kvam and Tyrrell (8). Briefly, lymphocytes (and nuclei isolated from rat liver cells) were suspended in 3 ml of nuclei lysis buffer (10 mM Tris-HCl, 400 mM NaCl, 2 mM EDTA, 1 mM desferrioxamine, 4 mM histidine, 3 mM GSH, pH 8.2) and after addition of 0.2 ml of 10% SDS and 0.5 ml solution containing 1 mg proteinase K in 1% SDS, 2 mM EDTA were digested for 5 h in the dark. Then, proteins were precipitated by 1 ml of saturated NaCl followed by centrifugation for 15 min at 2000 g at 4°C. Supernatant containing nucleic acids was treated with 2 vol of cold absolute ethanol in order to precipitate high molecular weight DNA. The precipitate was removed with a plastic spatula, washed with 70% ethanol and after centrifugation dissolved in 220 µl of water. DNA concentration in the preparations was determined spectrophotometrically. DNA samples were stored at -70°C. Isolation of rat liver nuclei was carried out according to Lilja *et al.* (9).

Enzymatic assay

Escherichia coli Fpg protein and endonuclease III were purified to homogeneity from overproducing strains (a kind gift of Dr Jacques Laval, Institute G. Roussy, Villejuif, France) as described previously (10,11). Aliquots of DNA samples (150–250 µg of DNA) were dried in a SpeedVac under vacuum. Samples were then dissolved in 1 ml Eppendorf tubes in the incubation mixture containing 50 mM phosphate buffer (pH 7.4), 100 mM KCl, 1 mM EDTA, 0.1 mM dithiothreitol and bovine serum albumin (0.1 mg/ml). For incubation 4 µg of Fpg and Endo III proteins were added. Some samples contained no Fpg and Endo III proteins, but the equivalent volume of buffer (25 mM HEPES, 200 mM NaCl, 1 mM EDTA and 50% glycerol). Three independent samples of each DNA preparation were used and three replicates of each sample were incubated at 37°C in a water bath for 30 min.

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	5-OH-Ura	5-OH-Cyt	5,6-diHUrA	FapyAde	8-OH-Ade	FapyGua	8-OH-Gua
CT DNA	4,2±0,1	11,0±1,3	2,9±0,75	2,6±0,4	1,4±0,3	4,2±0,9	0,8±0,2
RL DNA	0,3±0,06	3,3±0,5	1,5±0,5	1,0±0,4	N/D	2,0±0,2	0,2±0,08
RN	N/D	2,9±0,8	1,6±0,5	N/D	N/D	1,8±0,5	0,3±0,1
HL DNA	N/D	0,2±0,06	0,9±0,4	0,9±0,3	N/D	1,0±0,3	0,3±0,03

Figure 1. Levels of oxidatively modified DNA bases (expressed as modified bases/ 10^6 bases) in different DNA samples. CT-DNA, calf thymus DNA; RT-DNA, rat liver DNA; RN, nuclei isolated from the rat liver cells; HL, DNA from human lymphocytes.

Following incubation, 100 μ l of cold (-20°C) chloroform was added to each sample. Samples were shaken vigorously and kept on ice for 10 min. Then samples were centrifuged at 4°C for 5 min at 8000 g and supernatant fractions containing DNA were separated. Then 540 μ l of cold ethanol (-20°C) was added to each supernatant. Samples were kept at -20°C for 2 h and then centrifuged at 4°C for 30 min at 8000 g . Supernatant fractions were separated and aliquots of stable isotope-labelled analogues of modified DNA bases were added as internal standards. Samples were not hydrolysed. The derivatisation of the lyophilised samples was performed in teflon sealed vials, in nitrogen bubbled 100 μ l mixture of BSTFA:acetonitrile for 30 min in 120°C . Subsequent analysis by GC/MS with selected ion monitoring was performed as described previously (12). The amount of modified bases (mb) in DNA was calculated as a number of mb molecules/ 10^6 bases.

RESULTS AND DISCUSSION

To avoid artificial formation of oxidatively modified bases we used Fpg protein and endonuclease III instead of acid

hydrolysis to liberate modified pyrimidines and purines from the DNA sample. Since both enzymes are specific for oxidative base damage there are no unmodified bases present at the derivatisation step.

Dr Dizdaroglu's group was the first to characterise the specificity of both enzymes. In their assays they used DNA samples irradiated or modified with $\text{Fe}/\text{H}_2\text{O}_2$ system (13). We analysed the background level of oxidatively modified DNA bases in different samples: (i) commercial calf thymus DNA, (ii) DNA isolated from rat liver, (iii) DNA isolated from human lymphocytes and (iv) nuclei isolated from rat liver. In all DNA samples used in our assays the most efficiently removed bases by the Fpg protein are Fapy G and Fapy A although 8-oxoG was also detected in all preparations (Fig. 1). Generally there was approximately the same amount of oxidatively modified purines and pyrimidines. In agreement with previous studies, calf thymus DNA contained more damages than freshly isolated DNA but our results are lower than the lowest value reported in the literature (14,15). However, enzymatic techniques give the values of the order of magnitude lower than HPLC/EC technique when DNA damage was measured in

the same sample (16,17). However, we cannot exclude the possibility that the enzymes do not recognise all the damages. It is also noteworthy that the ratio of 8-oxoG level in calf thymus DNA to that in DNA isolated from liver in our study (=4) is exactly the same as that detected by the J. Cadet group when they applied the newly developed HPLC/MS/MS assay (18).

The amount of 8-oxoG in human lymphocytes was 3 per 10⁷ bases and it was quite comparable with those of other enzymatic assays estimates (comet assay, alkaline elution or alkaline unwinding) (19,20). However, in all the above-mentioned methods, Fpg sensitive sites were calculated instead of real 8-oxoG. In our assay this corresponds to the total amount of both Fapys, 8-oxoG and 8-oxoA, which comprises 21 damages per 10⁷. Interestingly, the amount of 8-oxoG in rat liver DNA was in close agreement with the estimation of Ames *et al.* (21). The higher amount of modified bases recognised by Fpg protein in our assay than in other enzymatic methods may depend on several factors: (i) purification of DNA during the work-up of the sample may produce some of the oxidative DNA damage (however, we used antioxidants during the procedure); (ii) there may be some oxidative base damage which is inaccessible to the enzymes in the other enzymatic assays (comet or alkaline elution); (iii) there is clustering of oxidative DNA damage (22). Such clusters of oxidised purines and pyrimidines may produce one strand break which would be calculated as one Fpg or Endo III sensitive site for detection in alkaline elution or comet method. (iv) Since the conditions of the assays are different, the specificity of the enzymes may differ too.

It is also possible that the enzymes do not recognise and remove all modified bases (23). Therefore, all the methods based on enzymatic liberation of modified bases may underestimate their level. However, in our investigation the kinetics of enzymatic digestion demonstrated that under the applied conditions lesion removal is essentially complete, since no more bases were liberated with increased amount of the enzymes and longer incubation time (data not shown).

To avoid possible artefact formation during work-up of the sample the nuclei were digested with the enzymes instead of purified DNA. Surprisingly, the amounts of most of the base products were quite comparable in both preparations. (However, in the sample of nuclei we could not detect FapyAde although in the DNA sample the amount of this base was quite high.) These results suggest that the use of antioxidants efficiently protects DNA during purification of nuclei. It is also possible that the majority of artificial base modifications (if such artefacts are really produced) are introduced during cell disruption. Interestingly, these results pointed out that accessibility of the enzymes to the damaged bases is comparable in both nuclei and purified DNA (with the exception of FapyAde).

Oxidatively modified DNA bases have different mutagenic potential and some of them may be quite innocuous (24). Therefore, there is a need to know about the involvement of the particular type of base modification in the global DNA damage. The only method that offers such a possibility is GC/MS. Enzymatic methods such as comet assay, although very sensitive, can detect the global oxidative DNA damage recognised by the enzyme. Indeed, as we demonstrated, 8-oxoG, which is believed to be the main mutagenic modification of all bases, is only a small fraction of all the sites recognised by Fpg.

In recently described experiments no correlation has been found between individual values of 8-oxodG (measured with HPLC/EC technique) and Fpg sensitive sites (analysed by comet assay) in human lymphocytes (25). Our results pointed out that this lack of correlation may be explained, at least in part, by the fact that HPLC technique analyses real 8oxodG, while the majority of Fpg sensitive sites are the other modifications.

In conclusion we demonstrated that GC/MS technique based on the use of the repair enzymes may be applied to analysis of the background level of individual oxidatively modified DNA bases and the level of the damage is quite comparable with the lowest values obtained by the other methods. In view of the importance for accurate measurement of the background level of oxidatively modified bases, and taking into consideration the fact that measurement of single base product can give misleading results (26,27), it is reasonable to postulate that the above presented method is one of the techniques which should be used to reveal the enigma of oxidative DNA damage.

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