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Measuring Oxidative Damage to DNA; HPLC and the Comet Assay Compared

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Depending on the analytical method employed estimates of background levels of base oxidation in human DNA vary over orders of magnitude. It is now realised that oxidation of guanine *in vitro* can result in serious overestimation of the nucleoside by HPLC (with electrochemical detection). We have modified procedures of isolation, hydrolysis and storage of DNA with the aim of eliminating this artefact. Vacuum- or freeze-drying, and dialysis, tend to encourage oxidation. We compare results obtained with HPLC and with the comet assay, which employs lesion-specific enzymes to introduce breaks in DNA at sites of oxidative damage. Although estimates of background levels of DNA oxidation using the comet assay are several-fold lower than the estimates by HPLC, both approaches have been used successfully to detect differences between human subjects or population groups that seem to relate to human disease and nutritional factors.

Keywords: DNA oxidation, HPLC, comet assay

INTRODUCTION

The idea that endogenous oxidation of cellular DNA by free radicals released during normal respiration might contribute to carcinogenesis is popular, as it provides a ready explanation of the epidemiological finding that fruit and vegetables protect against cancer. Many phytochemicals can act as antioxidants, and so if they are absorbed from the gut they may significantly limit the amount of oxidative damage incurred by biomolecules including DNA.

Testing this hypothesis depends on being able accurately to measure oxidative damage in DNA. Gas chromatography-mass spectrometry (GC-MS) has been particularly valuable in identifying oxidation products of the radiolysis of DNA, and so it is not surprising that it was applied also to the measurement of background levels of damage in mammalian cells. The values revealed by this method were startlingly high – up to 0.1% or more of guanine was supposedly oxidised to 8-oxoguanine.[1,2] However, preparation of samples for GC-MS is particularly prone to oxidation, especially during the derivatisation step, which involves a silylation reaction at elevated temperature.[3] Another widely used method is HPLC, with electrochemical detection of 8-oxo-deoxyguanosine (and simultaneous UV detection of

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deoxyguanosine). Values for background 8-oxo-dG concentrations determined by HPLC typically range between 0.5 and 8 per 10^5 dG. Much attention has been given to the possibility that HPLC, too, might be subject to oxidation artefacts, and with the technique modified in various ways to control this problem, estimates have become progressively lower; a recently described anaerobic method gives a mean background level of only 0.2–0.3 8-oxo-dG per 10^5 dG.[4]

There is a quite different approach to the measurement of oxidative DNA damage, based on the use of lesion-specific bacterial DNA repair endonucleases which create strand breaks in the DNA at sites of oxidative damage – endonuclease III at oxidised pyrimidines, and formamidopyrimidine glycosylase (FPG) at 8-oxoguanine. (FPG can also recognise other altered purines – ring-opened products known as formamidopyrimidines.) The strand breaks are measured by a variety of techniques including alkaline elution[5] and single cell gel electrophoresis (SCGE), also known as the "comet assay".[6] Alkaline elution depends on the unwinding of DNA from strand breaks which enables single DNA strands to elute through the pores of a filter membrane. SCGE allows the visualisation of loops of DNA in which supercoiling has been relaxed by a strand break; the relaxed DNA forms a comet-like tail on electrophoresis. These enzymic methods consistently give estimates of background DNA oxidation that, at about 0.05 FPG-sensitive sites per 10^5 dGs, are several times lower than suggested by HPLC measurements.

Here we describe our attempts to identify artefactual sources of DNA oxidation as detected by HPLC, and to reconcile the HPLC and enzymic approaches to estimation of background levels of DNA oxidation.

MATERIALS AND METHODS

Isolation of Nuclear DNA from Rat Liver

Briefly, rat liver (stored at −80°) was homogenised on ice in 10 mM Tris-HCl, 0.4 M NaCl, 2 mM Na_2EDTA, pH 8.0, 0.5% Triton X-100. The homogenate was centrifuged, the pellet suspended in buffer without Triton, and centrifuged again. The pellet, suspended in buffer with 0.6% sodium lauryl sulphate, was heated at 55° for 10 min before incubating with ribonuclease IIIA and ribonuclease T_1 for 30 min at 37°. Proteinase K was then added, and incubation continued for 1 h at 55°. An equal volume of chloroform : isoamyl alcohol (24 : 1) was added; the mixture was shaken for 1 min, centrifuged, and the aqueous fraction re-extracted with chloroform : isoamyl alcohol. To precipitate residual protein, the aqueous fraction was made up to 1.7 M NaCl, vortexed, and centrifuged. DNA was precipitated from the supernatant by addition of two volumes of ethanol (−20°); the DNA precipitate was washed 3× with ice-cold 70% ethanol. Excess ethanol was removed and the DNA was dissolved in 40 mM Tris-HCl, pH 8.5 before storage at −80° under N_2. (Full details of the method will be published elsewhere; S.G. Wood, C.M. Cedik and A.R. Collins, in preparation.)

Isolation of Total DNA from Rat Liver

Liver was homogenised as above, but with no Triton X-100 added to the homogenisation buffer; sodium lauryl sulphate was added without prior centrifugation.

Isolation of DNA from HeLa Cells and Leukocytes/Lymphocytes

HeLa cells were suspended at 10^7/ml in 10 mM Tris-HCl, 0.4 M NaCl, 2 mM Na_2EDTA, pH 8.0, and frozen under N_2 at −80° (for total DNA); or, for nuclear DNA, suspended in buffer plus 0.5% Triton X-100, kept on ice for 5 min, centrifuged (1200× *g*, 10 min, 4°), washed with Triton-free buffer, centrifuged (1200× *g*, 5 min, 4°), and the pellet of nuclei suspended in Triton-free buffer and frozen under N_2 at −80°. Isolation of DNA from cells or nuclei was then essentially as described for liver.

Human leukocytes isolated from 12–15 ml of blood were suspended in 1 ml of 10 mM Tris-HCl, 0.4 M NaCl, 2 mM Na_2EDTA, 1 mM 1,10-phenanthroline, pH 8.0, and stored under N_2 at −80° until DNA was isolated by a high salt precipitation method.[6]

DNA Hydrolysis

Hydrolysis for 2 h at 37° with deoxyribonuclease I, phosphodiesterases I and II, and alkaline phosphatase was based on the method of Richter *et al.*[7]

HPLC with Electrochemical Detection

Samples of DNA hydrolysate were applied to an Apex ODS 3 μm C18 150 × 4.6 mm column with a 20 mm pellicular LC18 guard column. The mobile phase was 50 mM K phosphate buffer, 8% methanol, pH 5.5; the flow rate was 0.5 ml/min. A Coulochem II electrochemical detector (ESA) was used with an ESA 5021 conditioning cell and 5011 analytical cell. All samples were analysed in triplicate.

Comet Assay

This has been described in detail.[6] Briefly, lymphocytes were embedded in a thin layer of a agarose on a microscope slide, lysed for 1 h in 2.5 M NaCl, 0.1 M Na_2EDTA, 10 mM Tris-HCl, pH 10, 1% Triton X-100, and then washed with 0.1 M KCl, 0.5 mM Na_2EDTA, 40 m HEPES-KOH, 0.2 mg/ml bovine serum albumin, pH 8 and incubated with endonuclease III or FPG in this buffer at 37° to convert oxidised bases to strand breaks. Control gels were incubated with buffer but no enzyme. Slides were placed in 0.3 M NaOH, 1 mM Na_2EDTA, pH 13 for 40 min before electrophoresis at 25 V for 30 min. After electrophoresis, the gels were treated with a neutralising buffer, the DNA stained with DAPI, and comets scored by visual examination or computer image analysis.[8]

RESULTS AND DISCUSSION

Using DNA isolated from rat liver nuclei (or in one experiment calf thymus DNA), we looked for evidence of oxidation occurring at different stages in the processing of DNA for HPLC analysis. The effects of modifying the standard procedure described in Materials and Methods are described.

Homogenisation

It is possible that redox-active iron released during homogenisation might catalyse production of hydroxyl radicals by the Fenton reaction. In an attempt to improve the chelation of free iron, we replaced EDTA in the homogenisation medium with deferoxamine mesylate (DF) – with variable results. (DF is light-sensitive, and so DNA extraction was carried out in the dark.) Whereas 1 mM DF had no effect, at 5 mM there was sometimes a substantial decrease in 8-oxo-dG (Figure 1).

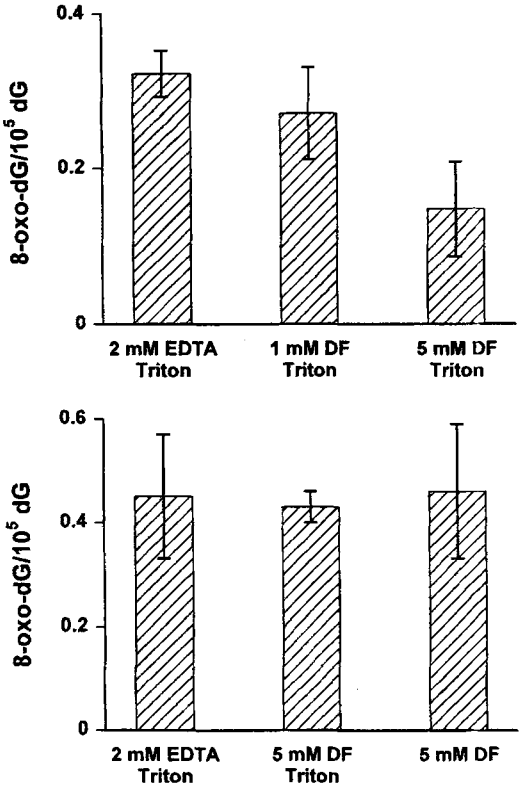

FIGURE 1 Substitution of DF for EDTA during homogenisation of tissue. 8-oxo-dG levels were determined in nuclear DNA isolated from rat liver, homogenised in the presence of EDTA or DF (with or without Triton X-100). Two separate experiments. Bars indicate SD of triplicate samples.

Omission of Triton X-100 did not reduce the level of 8-oxo-dG.

Histidine, which can act as an antioxidant, was added to the homogenisation buffer in place of EDTA, and – at 4 mM – sometimes decreased the amount of 8-oxo-dG detected (Figure 2). At 10 mM it appears to act as a pro-oxidant; 4 mM histidine together with 5 mM DF did not provide any more protection against oxidation than either alone.

TEMPO is a nitroxide that maintains transition metals in an unreactive oxidised form. Substituting 5 mM DF with 0.1 mM TEMPO[9] in the homogenisation buffer did not alter the level of 8-oxo-dG detected (0.47 per 10^5 dG in each case). Replacing DF with the spin-trap reagent POBN (α-[4-pyridyl-1-oxide]N-t-butylnitrone) at 50 μM and 5 mM, or adding it at these concentrations with DF (5 mM), also had no effect.

Isolation of DNA

In our standard procedure, after addition of sodium lauryl sulphate, the nuclear pellet is incubated at 55° for 10 min and for a subsequent period of 1 h at this temperature with proteinase K. Decreasing the incubation temperature to 37°, and the incubation time with proteinase K to 30 min, had no effect on 8-oxo-dG levels.

Preparation of DNA for Storage

Nuclear DNA from a single liver sample was treated in various ways after the ethanol precipitation step (Table I). As much ethanol as possible was removed from all samples to reduce subsequent drying time to a few minutes. Whether the DNA was dried in air or under nitrogen, or simply frozen, the same level of 8-oxo-dG was observed. Heating in Tris buffer for 20 min at 55° (in order to evaporate ethanol) had no effect. However, drying under vacuum or lyophilisation did lead to an increase in 8-oxo-dG. The greatest effect was from dialysis, which increased the concentration of 8-oxo-dG, in some cases, by more than 10-fold. It seems that metal ions present on dialysis tubing can catalyse the production of reactive oxygen; pre-treating the tubing with EDTA abolishes the increase.

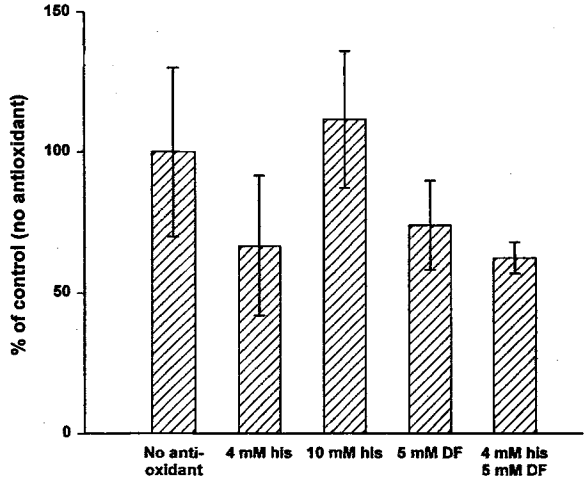

FIGURE 2 Effect of histidine present during homogenisation of tissue. 8-oxo-dG levels were determined in nuclear DNA isolated from rat liver, homogenised in the presence of histidine, compared with DF. For each condition, there were between 3 and 6 samples. Bars indicate SD of mean values for each sample.

TABLE I Preparation of DNA for storage. After ethanol precipitation, DNA was treated in various ways before storage at −80°. All samples were stored under nitrogen (except for the air-dried sample, stored under air). 8-oxo-dG was then measured by HPLC. Duplicate samples (with range indicated) except for the DNA dialysed in untreated dialysis tubing, of which there were 5 samples

	8-oxo-dG/10^5 dG
Vacuum-dried	0.43 ± 0.06
Nitrogen-dried	0.29 ± 0.13
Air-dried	0.28 ± 0.04
Frozen	0.26 ± 0.08
Blotted dry, then + Tris buffer	0.24 ± 0.02
Tris buffer, heated 20 min at 55°	0.25 ± 0.01
H_2O, freeze-dried	0.38 ± 0.10
Tris buffer, dialysed in untreated dialysis tubing against H_2O, freeze-dried	0.7–4.38
Tris buffer, in dialysed EDTA-treated dialysis tubing against H_2O, freeze-dried	0.44 ± 0.01

Hydrolysis

Inclusion of 4 mM histidine or 0.1 mM DF (at 1 mM, DF interferes with the electrochemical trace) during enzymic hydrolysis of DNA to nucleosides did not decrease the yield of 8-oxo-dG, while 10 mM histidine, as before, apparently acted as a pro-oxidant (Figure 3). When a sample of dG was incubated in the presence of rat liver nuclear DNA during hydrolysis, there was no increase in 8-oxo-dG content. Thus the hydrolysis step is not susceptible to spurious oxidation.

Post-hydrolysis Stability

When an auto-sampler is employed, hydrolysed samples can remain for many hours at 4°. Measuring 8-oxo-dG in identical samples of hydrolysed calf thymus DNA, we found no significant change in 8-oxo-dG content over a period of 10 h (results not shown). This was true also for rat liver DNA. In some experiments, at times later than 12 h, an increase was seen; routinely, therefore, we do not run the auto-sampler for longer periods.

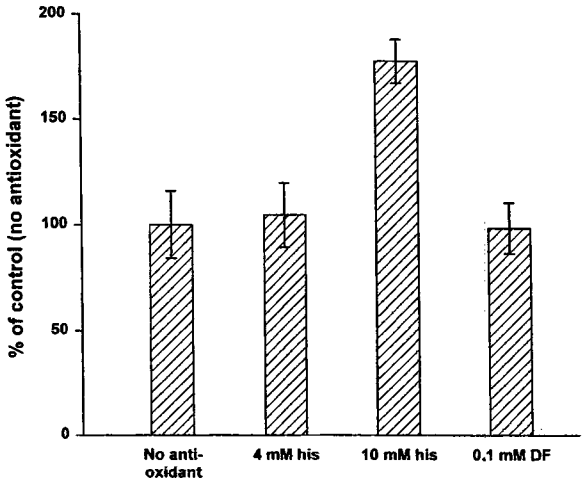

FIGURE 3 Histidine or DF present during hydrolysis. 8-oxo-dG levels were determined in nuclear DNA isolated from rat liver. Enzymic hydrolysis was carried out in the presence of histidine or DF. Bars indicate SD.

Total Liver DNA Compared with Nuclear DNA

In HeLa cells, whether DNA is prepared from whole cell lysate or from isolated nuclei does not greatly affect the amount of 8-oxo-dG detected (Figure 4). However, DNA isolated from whole hepatocytes has ten times as much 8-oxo-dG as DNA from hepatocyte nuclei. It is likely that the high content of transition metal ions in hepatocyte cytoplasm promotes the production of 8-oxoguanine via the Fenton reaction during preparation.

Estimating Background Levels of Oxidative DNA Damage

The level of damage seen in nuclear DNA with our HPLC method is much lower than suggested in most previous reports (discussed in Ref. [10]) and is close to that reported by Nakajima *et al.*[4] with the anaerobic technique. Even lower values are obtained with the comet assay – around 0.04 endonuclease III-sensitive sites and the same number of FPG-sensitive sites.[11] Individual values of 8-oxo-dG (in leukocytes) and of FPG sites (in lymphocytes) do not show any correlation (Figure 5) – not surprising in view of the order-of-magnitude discrepancy between the mean values.

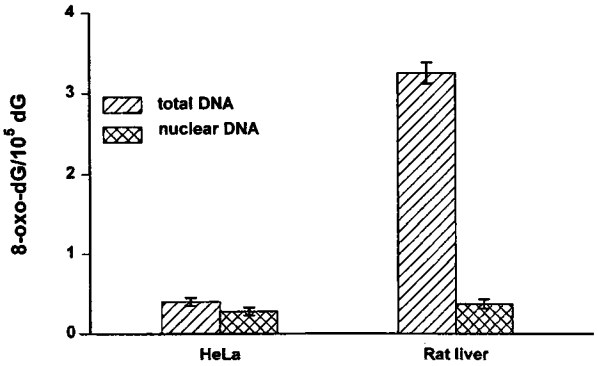

FIGURE 4 Whole cell and nuclear DNA. DNA was prepared from total cell homogenate (i.e. including mitochondria) or from isolated nuclei. Bars indicate SD for 3 samples (HeLa) or 2 samples (liver) analysed in triplicate.

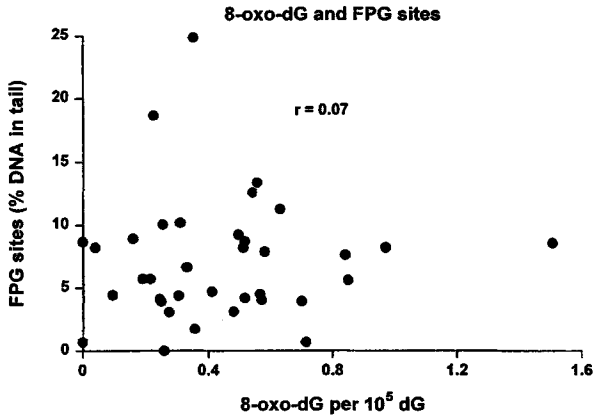

FIGURE 5 Estimation of 8-oxo-dG by HPLC and by the comet assay. Leukocytes or lymphocytes isolated from 37 samples taken from normal healthy men were analysed by HPLC or by the comet assay respectively.

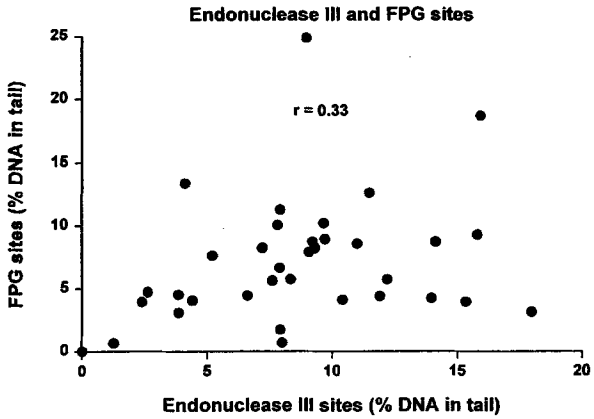

FIGURE 6 Oxidised bases estimated with the comet assay. FPG-sensitive sites and endonuclease III-sensitive sites were measured in lymphocytes from 37 men by the comet assay.

Which approach should we trust? It is possible that still in the preparation of samples for HPLC some oxidation of dG is occurring and so our values are over-estimates. It is also possible that the comet assay under-estimates DNA damage. For example, FPG or endonuclease III might recognise only a fraction of the oxidised bases present in the DNA, because of steric interference. However, T4 endonuclease V, an enzyme of similar size to FPG, detects the expected number of cyclobutane pyrimidine dimers induced by UV;[12] and supercoiled, protein-free DNA is known to be a good substrate for FPG.[13] Another possibility is that base damage occurs in clusters in DNA after oxidative attack. Although individual 8-oxo-dGs would be measured as such by HPLC, a group of 8-oxo-dGs in close proximity would be equivalent to a single DNA break as detected with the comet assay. Clustering has been reported for a fraction of the damage induced by ionising radiation;[14] whether it occurs to a significant extent after attack by reactive oxygen is not known. On the other hand, the estimates obtained with the comet assay might themselves be over-estimates, since FPG recognises other altered purines which may be present in the DNA as well as 8-oxo-guanine. Endonuclease III recognises oxidised *pyrimidines*, and the number of breaks it induces in the comet assay is similar to the number of altered purines detected with FPG. There is a correlation between FPG-sites and endonuclease III-sites (Figure 6).

Whether HPLC over-estimates damage, or the comet assay under-estimates it, the two approaches have been used successfully to detect biologically significant differences in baseline levels of oxidative damage. We recently showed several-fold higher levels of 8-oxo-dG in lymphocytes from men, compared with women, in northern European countries, whereas levels in men and women of southern Europe were similarly low.[15] With the comet assay, we have detected effects of dietary antioxidant supplements,[16] and substantial differences between oxidative DNA damage levels in normal subjects and in patients with diabetes and with ankylosing spondylitis.[17] It seems reasonable to conclude that the true level of oxidative base damage in human cells will be somewhere between that determined by the current HPLC technique and the estimated value from the comet assay.

CONCLUSIONS

Modification of the procedures for preparing DNA samples for HPLC analysis by adding

chelators/antioxidants does not seem greatly to decrease the concentration of 8-oxo-dG measured in nuclear DNA – suggesting that our estimates may be relatively free of artefacts. We have identified dialysis with freeze-drying, which is often employed in preparation of DNA especially for GC-MS analysis, as a step that is particularly prone to oxidative artefacts.

The downward revision of estimated levels of oxidative DNA damage in normal human cells prompts a re-assessment of the importance of DNA oxidation in terms of human carcinogenesis. It seems likely that antioxidant defences and DNA repair systems have evolved that maintain a steady state level of damage that is tolerable in terms of the mutagenic load. Following on from this it is worth asking whether antioxidants present in fruit and vegetables, even if they further decrease the level of DNA oxidation, can contribute significantly to cancer prevention. Attention should also be directed to other components of these foods that may play a protective role against cancer – quite apart from effects on oxidation of DNA.

Acknowledgments

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