the degradation of DNA, may also contribute adducts; (iii) the oxidation of cytosolic and circulating nucleobase pools and DNA fragments are potential sources of excreted adducts; (iv) large amounts of oxo^{8}Gua are of dietary origin; (v) oxo^{8}dG and oxo^{8}Gua are sensitive to oxidation, and may be destroyed *in vivo* before excretion, or by enzymatically catabolized via salvage pathways which operate on intact nucleotides, resulting in loss of these analytes from the urinary pathway under study. All of these confounders would change the estimate of excreted adducts, and therefore lead to an inaccurate estimate of the number of damage sites ("hits") repaired each day.

In conclusion, methods for DNA isolation continue to improve, with minor modification of the chaotropic NaI technique producing the lowest oxo^{8}dG values reported to date using HPLC-EC. Although the use of urinary oxo^{8}Gua adducts to monitor DNA oxidation *in vivo* remains attractive, the results can be difficult to interpret. Comparison of carefully controlled groups can minimize some of the confounders (such as dietary contributions), but the effect of experimental treatments on, for example, the rate of cell turnover, will remain unknown and uncontrolled. Thus, given our ignorance about the dynamics of nucleotides and their oxidative adducts in whole-body metabolism, studies of urinary excretion must be interpreted with considerable caution.

Acknowledgment

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Methods Enzymol. 1998; 300:166-184

[19] Markers of Oxidative Damage to DNA: Antioxidants and Molecular Damage

By STEFFEN LOFT and HENRIK ENGHUSEN POULSEN

Cells are constantly exposed to oxidants from both physiological processes, such as mitochondrial respiration, and pathophysiological conditions, such as inflammation, ischemia–reperfusion, foreign compound metabolism, and radiation.[1] Failure of the system of enzymatic, endogenous, and nutritional antioxidants may lead to carcinogenesis and degenerative

[1] B. N. Ames, L. S. Gold, and W. C. Willett, *Proc. Natl. Acad. Sci. USA* **92,** 5258 (1995).

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diseases of aging. Thus, the concept of supplementing nutritionally based antioxidants in order to prevent cancer and postpone aging appears rational.[1,2] Descriptive epidemiological studies support the notion by a highly reproducible close relationship between a high dietary intake of vegetables and fruits rich in antioxidants and a low risk of epithelial cancers, particularly in the airways and upper gastrointestinal tract.[3,4] So far, however, the large-scale intervention studies of the effects of single and combinations of antioxidants, vitamin E and β-carotene have been negative and even indicated a cancer-promoting effect of the latter in smokers.[5-7] The enormous costs and the very limited number of compounds or combinations and doses that can be tested in only a few high risk population groups in such studies may warrant an alternative approach. Small-scale human intervention studies with mechanistically based biomarkers as intermediate end points and supported by animal and *in vitro* experiments may target the optimum intervention strategy for the large-scale intervention.[8] In addition, the use of such biomarkers may provide further proof of a causal relationship between oxidative damage to DNA and cancer and aging. Accordingly, oxidative DNA adducts may serve as valuable biomarkers in this context.

More than 100 different oxidative modifications have been observed in DNA.[9,10] However, so far only a few of the base modifications have been used as biomarkers, and of these, the oxidative C-8 adduct of guanine is by far the most studied as either the nucleoside or base. In principle, the level in DNA from target or surrogate tissues or cells or the excretion of repair products into the urine can be measured. Under the usual steady-state conditions, the latter will reflect the rate of damage, whereas the former will reflect the balance between damage and repair. The present

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[10] J. Cadet, J. L. Ravanat, G. W. Buchko, H. C. Yeo, and B. N. Ames, *Methods Enzymol.* **234,** 79 (1994).

review will focus on the involved assays, the interpretation of the methods, and some results from antioxidant intervention studies, particularly in humans.

Urinary Excretion of DNA Repair Products

The repair products from oxidative DNA damage, i.e., oxidized bases and nucleosides, are poor substrates for the enzymes involved in nucleotide synthesis; they are fairly water soluble and generally are excreted into the urine without further metabolism.[11,12] Among the possible repair products from oxidative DNA modifications, 8-oxo-7,8-dihydro-2′-deoxyguanosine (8-oxodG), 8-oxoguanine (8-oxoGua), thymine glycol (Tg), thymidine glycol (dTg), and 5-hydroxymethyluracil (5-OHmU) have so far been identified in urine.[11,13–18] Of these, 8-oxodG and the thymine derivatives are the most intensively studied. The levels of concentration and excretion of the oxidized bases and nucleosides obtained in different laboratories are in the same range (Table I).

The assays for the urinary DNA repair products include high-performance liquid chromatography (HPLC) with electrochemical detection (EC) for 8-oxodG and 8-oxoGua and with UV absorbance detection for dTg and Tg, whereas all the repair products can potentially be measured by gas chromatography-mass spectrometry (GC/MS).[9,14,16,17] The major problem with all these assays involves separation of the very small amounts of analyte from urine, which is a very complicated matrix. Thus, although several of the products are electrochemically active and high sensitivity is achievable, the HPLC methods require extensive cleanup procedures such as multiple solid-phase extractions, HPLC column switching techniques, or

[11] M. K. Shigenaga, C. J. Gimeno, and B. N. Ames, *Proc. Natl. Acad. Sci. USA* **86**, 9697 (1989).
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TABLE I
RANGE OF PUBLISHED VALUES REGARDING URINARY BIOMARKERS OF OXIDATIVE DNA DAMAGE IN HUMANS[a]

Lesion	Assay	Range of averages	Total number of subjects	Total number of publications
dTg	HPLC	390–435 pmol/kg 24 h	19	2
Tg	HPLC	100–174 pmol/kg 24 h	19	2
dTg	GC/MS	110–250 pmol/kg 24 h	3	2
5-OHmU	GC/MS	74 ± 9 nmol/24 h	14	1[b]
8-oxodG	HPLC	170–600 pmol/kg 24 h	360	6
		1.0–3.0 nmol/mmol creatinine	206	8
8-oxodG	GC/MS	110–345 pmol/kg 24 h	26	3
8-oxodG	ELISA	1600–4800 pmol/kg 24 h	4	1
		7.7 ± 3.4 nmol/mmol creatinine	52	1[c]
8-oxoGua	HPLC	1.5–5.0 nmol/mmol creatinine	13	1

[a] Data from Loft and Poulsen, *J. Mol. Med.* **74**, 297 (1996).
[b] From Faure *et al.*, *Free Rad. Biol. Med.* **20**, 979 (1996).
[c] From Erhola *et al.*, *FEBS Lett.* **409**, 287 (1997).

immunoaffinity columns.[11,13,19–25] The complicated extraction procedures cause recovery problems in both HPLC and GC/MS-SIM methods and may require labeled internal standards. Moreover, the complicated procedures limit the analytical capacity. An enzyme-linked immunosorbent assay (ELISA) based on monoclonal antibodies has been developed for estimation of 8-oxodG in urine samples.[26] However, the values obtained in rat and human urine samples[26,27] were 3–5 times higher than other published values.[18] Similarly, in 4 smokers studied before and after smoking cessation,

[19] S. Loft, K. Vistisen, M. Ewertz, A. Tjønneland, K. Overvad, and H. E. Poulsen, *Carcinogenesis* **13**, 2241 (1992).
[20] C. Tagesson, M. Källberg, and P. Leanderson, *Toxicol. Meth.* **1**, 242 (1992).
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the urinary 8-oxodG excretion values estimated by the ELISA method were 8 times higher than and showed only a weak correlation (r = 0.42) with the values obtained by HPLC.[28]

HPLC–EC Assay of 8-OxodG in Urine

Most data regarding urinary excretion of oxidative DNA repair products have been obtained on 8-oxodG using HPLC–EC assays with column switching techniques for both extraction and separation. In our laboratory an automated three-dimensional HPLC method with isocratic separation and electrochemical detection has been used for 6 years with minimum modifications for the analysis of 8-oxodG in several thousand urine samples.[19]

Urine can be stored frozen. For analysis, thawed or fresh urine samples e.g., 2 ml, are acidified with 40 μl 2 M HCl and frozen at −20° overnight for precipitation of uric acid and other solutes. After thawing and centrifugation for 5 min at 3000g, 34 μl NaOH 2 M is added to 1.7 ml of the supernatant. At this stage the samples can again be stored frozen for prolonged periods. To 95 μl aliquots of the treated urine, 5 μl of a solution containing 8-oxodG (Sigma, St. Louis, MO) 0, 800, or 8000 nM and 100 μl 1 M Tris buffer, pH 7.9, are added and 25 μl of the mixture is injected on the HPLC apparatus. This consists of the following Merck-Hitachi (San Jose, CA) components: L-6000 and L-6200 pumps, 655A-40 autosampler, column oven set at 40°, and D-6000 data handling and integration software, as well as an LP21 (Science Systems Inc., Mikrolab, Aarhus, DK) pulse dampener in the flow path, a Waters 440 UV absorbance detector at 254 nm, and an ESA Coulochem II electrochemical detector (Bedford, MA) equipped with a 5011 cell. The flow path diagram is shown in Fig. 1. In the first column (Spherisorb ODS2 15 cm 5 μm; Waters, Denmark), 8-oxodG is extracted from the urine sample with an alkaline mobile phase [2.5% acetonitrile and 1.5% methanol (v/v/v) in 10 mM borate buffer, pH 7.9]. The retention time of 8-oxodG in the extraction column is determined by UV absorbance after injection of a 4000 nM solution in the Tris buffer. Via a 6-port automatic Valco (Switzerland) valve, a 1 ml fraction of the effluent from urine samples containing the 8-oxodG is brought onto a 2 cm ion-exchange column PRP-X100 (Hamilton, Reno, NE). With a switch of the valve, this column is then flushed with an acidic eluent (100 mM phosphate buffer, pH 2.1) with 2.5–4% acetonitrile, bringing the contents onto a 25 cm Nucleosil C_{18} 3 μm column (Knaur, Germany). The effluent

[28] H. Prieme, S. Loft, R. G. Cutler, and H. E. Poulsen, *in* "Natural Antioxidants and Food Quality in Atherosclerosis and Cancer Prevention" (J. T. Kumpulainen and J. T. Salonen, Eds.), pp. 78–82. The Royal Society of Chemistry, London, 1996.

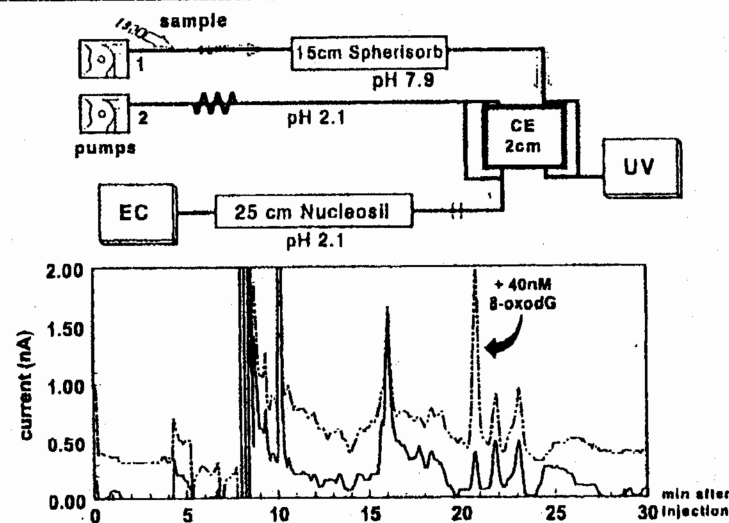

FIG. 1. Flow path (top) and chromatogram (bottom) of three-dimensional HPLC analysis of urinary 8-oxodG. The urine sample is introduced in the flow path of pump 1 and a fraction containing 8-oxodG is extracted by a Spherisorb column and brought on a cation exchange column (CE). The retained 8-oxodG is brought into the flow path of pump 2 by a six-port valve, separated on a Nucleosil column, and monitored by an EC-detector. In the chromatogram the broken and solid lines represent the tracing of a sample containing 11 nM 8-oxodG with and without addition of 40 nM.

of this column is monitored by the electrochemical detector in the oxidation mode set at 100–120 mV at electrode 1 and 200–300 mV at electrode 2 according to the voltammogram, which is the S-shaped relationship between the applied voltage and the response of the EC detector. The voltammogram is subject to change, particularly with wear of the cell, and should be checked frequently for optimum adjustment of the voltage to a response at the upper part of the S-shape (Fig. 2). The peak identity of 8-oxodG in urine samples has been ascertained by identical retention time and voltammogram as compared with the genuine compound.[19] Moreover, after acid hydrolysis in 0.5 M HCl at 140° for 1 hour, the 8-oxodG peak disappeared from the chromatogram of urine samples with and without the addition of 40 nM genuine compound (unpublished observations).

For quantification each urine sample is run with and without addition of at least two concentrations of genuine 8-oxodG and the concentrations calculated from the individual calibration curve based on the peak heights. The performance of the method includes linear calibration curves, an interassay coefficient of variation of 9–13%, and a limit of detection of 0.2

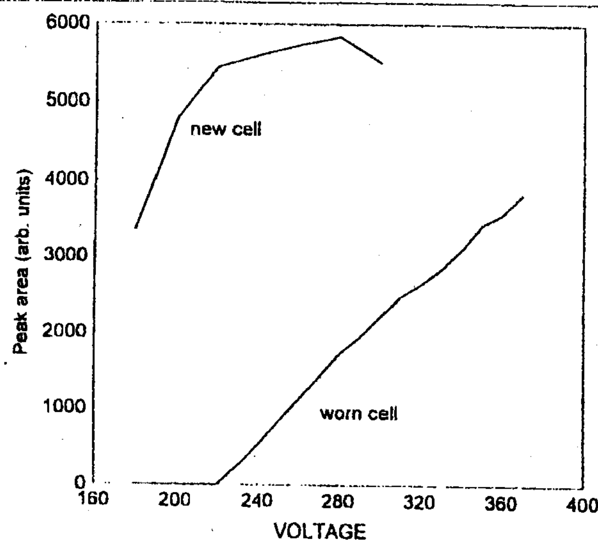

FIG. 2. Examples of voltammograms of 8-oxodG after HPLC analysis with an ESA Coulochem II detector equipped with a new or a worn 5011 cell.

nM as compared to concentrations ranging from 1 to 100 nM in human urine. We have reanalyzed 75 samples after 6 years of storage at −20° (Fig. 3). The two sets of values were highly correlated (r^2 = 0.75) and the residual standard deviation was 22% of the grand mean. The linear regression slope of 0.87 may be due to change of the source of calibration standard from solutions of 8-oxodG received as gifts from other researchers to the now commercially available crystallized compound; with the old calibration standards the slope would have been 1.09. The significant positive intercept may be related to a change in the mode of integration of the chromatographic peaks. Considering the long interval, the method appears reasonably robust and 8-oxodG is stable in storage for at least 6 years. Moreover, 20- to 25-year-old urine samples show concentrations in the range of fresh ones (unpublished observations). Although the method is relatively simple and automated, problems with separations frequently limit the number of samples that can be handled. In our hands, separation is primarily optimized by adjusting the acetonitrile concentration in the mobile phase of the Nucleosil column, and secondly by adjusting the corresponding pH. This column usually has a long lifetime, whereas the extraction columns wear out after only 4–8 weeks of use. In general, separation is more difficult with rat samples than with human samples.

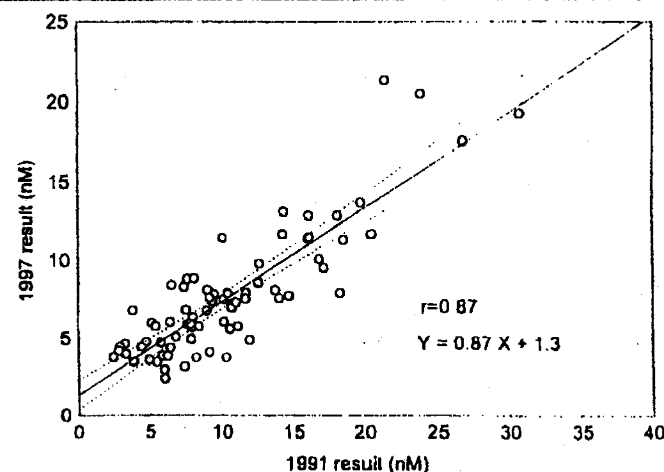

FIG. 3. Results of a three-dimensional HPLC analysis of 75 urine samples for 8-oxodG in 1991 and in 1997. Between the two assays the source of calibration standard and the method of peak integration were changed. The linear regression line and the equation are included.

A Swedish group has developed a similar column switching assay for 8-oxodG in urine.[24] However, in that assay the sample is concentrated on a Bondelut CH minicolumn before injection on a polymer reversed-phase column eluted with an acidic mobile phase (pH 4.0) with heptanesulfonic acid. A timed fraction of the effluent is brought on a C_{18} column eluted at pH 7.0 and monitored with a Waters 460 electrochemical detector. That assay seems to perform satisfactorily and has been used in some large-scale epidemiological studies.[29–31] The use of monoclonal antibody-based immunoaffinity columns for extraction of 8-oxodG, 8-oxoGua, and 8-oxoguanosine from biological fluids, including urine, has been extensively described in this series previously.[22]

For convenience, the use of spot urine samples corrected for creatinine would be simpler than 24 h collection of urine. However, creatinine production is variable, e.g., increased with exercise and decreased with age. Indeed, in 74 healthy subjects we collected urine for 24 h and a spot urine sample from the subsequent voiding. The correlation between the 8-oxodG to

[29] S. Lagorio, C. Tagesson, F. Forastiere, I. Iavarone, O. Axelson, and A. Carere, *Occup. Environ. Med.* **51**, 739 (1994).
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creatinine ratio in the spot samples and the 24 h excretion of 8-oxodG was rather poor ($r = 0.50$; unpublished).

For measurement of 8-oxoGua in human urine only one HPLC–EC method appears to have been used for published data.[15] It involves complicated strong cation exchange and cellulose partition extraction procedures before separation on a C_{18} column. No further data involving the method than those included in the original paper appear to have been published.

Interpretation of Urinary Oxidized Nucleobases and Nucleosides

For the use of oxidized bases and deoxynucleosides as urinary biomarkers, the repair pathways of the modifications, in particular 8-oxoGua, in DNA and other sources may be debated.[21] Thus, two different DNA repair enzymes, one with glycosylase activity and one excising single 8-oxodG as a nucleotide, have been isolated from nuclear extracts of a human cell line.[32] The human 8-oxoGua glycosylase was cloned by several groups,[33,34] whereas nucleotide excision repair was shown to contribute to the repair of 8-oxodG in DNA.[35] Moreover, the 8-oxodGTP phosphatase and 8-oxodGMP nucleotidase will selectively and rapidly convert the liberated oxidized nucleotide to a nucleoside ready for excretion.[36,37] These enzymes will also sanitize oxidized dGTP from the cellular pool and allow its excretion as 8-oxodG. In addition, digestion of damaged DNA from cell renewal and mitochondrial turnover will liberate 8-oxodG. Unpublished data from rat studies indicate that the induction of 8-oxodG in nuclear DNA in target organs corresponds to the increase in urinary excretion after administration of the carcinogen 2-nitropropane, supporting the idea that 8-oxodG is the primary repair product *in vivo*. Animal experiments have shown that injected 8-oxodG is readily excreted unchanged into the urine, whereas 8-oxodG in the diet or oxidation of dG during excretion does not contribute.[11,12,21] In rats, at least, dietary purines are an important determinant of the excretion of 8-oxoGua, which is far larger than the excretion of 8-oxodG.[21] However, in humans the excretion of 8-oxoGua and 8-oxodG are in the same range and both are increased by smoking.[15,19] Accordingly,

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although the exact relative importance of the repair pathways remain to be determined, the urinary excretion of 8-oxodG reflects the general average risk of a promutagenic oxidative adduct in DNA of all tissues and organs. Possibly, 8-oxoGua in the urine will be a valuable addition, allowing a complete account of the repair of 8-oxodG in DNA. The RNA oxidation product 8-oxoguanosine (8-oxoG) has also been used as a urinary biomarker.[38] The excretion is 3–4 times higher than of 8-oxodG.

For the use of the urinary excretion of repair products as biomarkers of oxidative DNA, extensive repair is assumed. Thus, after ionizing radiation the increase in urinary excretion of thymine glycol and 8-oxodG occurred within 24 hours in humans, whereas excess 8-oxodG was removed from mouse liver DNA after approximately 90 min.[39–41] In a study of 8-oxodG in human brain, the accumulation rate in the nuclear DNA corresponded to two lesions per cell per day.[42] In humans the reported values of the urinary excretion of the repair products, 8-oxodG, are in the range 15–50 nmol per 24 h[18] and the alternative repair product 8-oxoGua appears to be excreted in similar amounts.[15] The sum of these products thus corresponds to an average of 300–1000 lesions per day for each of the assumed 5×10^{13} cells in the body per day.[19,43] Accordingly, the calculated repair efficiency under these assumptions ranges from 99.4% to 99.8%.

Because of the extensive and rapid repair, the urinary excretion of the repair products will reflect the average rate of oxidative DNA damage in all the cells in the body. In contrast, the levels of oxidized bases in DNA lymphocytes or other accessible cells will reflect the steady-state levels, i.e., the balance between damage and repair, albeit only in a surrogate for target tissues. Accordingly, the two groups of biomarkers are supplementary.

Tissue/Cell Levels of Oxidized Bases in DNA

In tissue or cell samples, the level of oxidatively modified nucleobases can be measured by various techniques, including HPLC–EC (or MS or UV), GC/MS-SIM, TLC with ^{32}P-postlabeling, and various immunoassays

[38] H. Witt, A. Z. Reznick, C. A. Viguie, P. Starke-Reed, and L. Packer, *J. Nutr.* **122,** 766 (1992).
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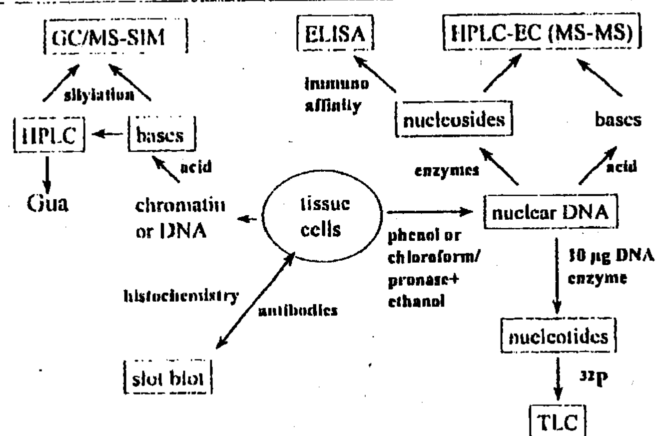

FIG. 4. Principles of assays for determination of tissue and cell levels of 8-oxodG and other modified nucleosides in DNA.

(Fig. 4). Except in the slot-blot technique and immunohistochemistry,[44,45] DNA or chromatin is isolated and hydrolyzed by enzymes or acid at high temperature. In all the assays the abundant unmodified nucleobases may be oxidized and thus cause artificially high values. Particularly, the derivatization with silyl groups for the GC/MS is prone to give rise to oxidation and should be carried out after removal of the unmodified base[46,47] or under controlled temperature and other conditions.[48] Similarly, the γ-radiation from the [^{32}P]phosphate used for postlabeling could oxidize guanine and thus explain the rather high values measured in human lymphocytes and rat organs by that method.[49,50] Even with the HPLC–EC method the reported values for 8-oxodG in leukocyte DNA varies from 0.3 to 13 per 10^5 dG.[18] There is no doubt that oxidation may occur during DNA extraction, particularly with the use of impure phenol and during drying the DNA after

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[49] V. L. Wilson, B. G. Taffe, P. G. Shields, A. C. Povey, and C. C. Harris, *Environ. Health Perspec.* **99**, 261 (1993).
[50] U. Devanaboyina and R. C. Gupta, *Carcinogenesis* **17**, 917 (1996).

ethanol precipitation.[51–53] The lowest values have been obtained with anaerobic DNA extraction.[54,55] Some of the immunoassays are calibrated by HPLC-EC values,[44,56] whereas others appear to yield much higher 8-oxodG values than the HPLC-EC assays,[57] but whether that is due to insufficient specificity of the antibodies is unknown.

HPLC–EC Assays of 8-Oxo(d)G and Other Products in DNA from Tissues and Cells

Chemicals. 8-OxodG, 8-oxoGua, 8-aminoguanosine, 2′-deoxyguanosine (dG), guanine, pronase E, and nuclease P1 are from Sigma (St. Louis, MO), and alkaline phosphatase is from Boehringer Mannheim (Germany).

DNA Extraction and Hydrolysis

Tissues. Samples of tissues, e.g., liver, kidney, spleen, brain, or heart (200–600 mg) are homogenized in 10–30 ml HEPES 5 m*M* buffer [*N*-(hydroxyethyl)piperazine-*N*′-(2-ethanesulfonic acid)], sucrose 70 m*M*, mannitol 250 m*M*, pH 7.4, on ice. After centrifugation at 1000*g*, the pellet containing the nuclei is resuspended in 0.15 *M* NaCl, 1 μl per mg tissue, on ice. Two hundred μl of suspension is transferred to 1.8 ml ice-cold TE buffer (150 m*M* NaCl, 10 m*M* Tris, 10 m*M* Na_2EDTA, pH 8.0), and 200 μl 10% sodium dodecyl sulfate (SDS) is added. After vortexing for 30 s, followed by rotation in an extraction bench for 15 min and incubation in a water bath at 37°C for 10 min, 200 μl 3 *M* sodium acetate (pH 5.2), 550 μl 5 *M* sodium perchlorate, and chloroform/isoamyl alcohol (24 : 1) 2 ml are added. After further rotation in an extraction bench for 10 min, the samples are centrifuged at 2100*g* and 4° for 10 min and the separated supernatant (nonorganic phase) is transferred to a 15 ml vial. The chloroform extraction step may be repeated before 6 ml of ice-cold 96% (v/v) ethanol is slowly added to the nonorganic phase.

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Lymphocytes, Sperm, Bone Marrow, or Other Cells. Lymphocytes are extracted from 5–7 ml blood diluted 1 : 1 with NaCl 0.9%. To 5 ml blood suspension in a 10 ml vial, 4 ml Lymphoprep (Nycomed Oslo, Norway) is carefully added to the bottom of the vial. After centrifugation for 30 min at 350*g* and 21° without brake, the lymphocyte layer is transferred to a 10 ml vial and 10 ml NaCl 0.9% is added. The pellet after centrifugation at 350*g* for 10 min, or approximately 20×10^6 sperm or other cells, is suspended in 7 ml of a buffer pH 7.6 containing Triton X-100 1% (v/v), sucrose 0.32 *M*, Trisma base 5 m*M*, and magnesium chloride 10 m*M*. After centrifugation at 1000*g* for 10 min, the supernatant is discarded. The pellet containing the nuclei is resuspended in 575 μl ice-cold buffer, pH 6.5, of sodium citrate 5 m*M* and sodium chloride 20 m*M*. Pronase E (1.2 mg/ml) with dithiothreitol (2.35 m*M*). *N*-lauroylsarcosine (0.75% w/v; Sigma), EDTA (10 m*M*), Trisma base (10 m*M*), and butylated hydroxyanisole (BHA, 0.3%, w/v) in methanol (6%, v/v) are added to a final volume of 1.4 ml, the stated concentrations, and pH 7.5. The samples are incubated overnight at 40°. Ammonium acetate to a final concentration of 0.5 *M* and 650 μl of an ice-cold 10 m*M* Tris–1 m*M* EDTA buffer are added and the DNA is precipitated by addition of 5 ml ice-cold ethanol (96%).

The DNA is allowed to precipitate at −20° overnight, if necessary followed by centrifugation at 2000*g* for 5 min. The DNA precipitate is washed with ice-cold 70% (v/v) ethanol in a petri dish, and dried in a vial with a stream of nitrogen gas or under vacuum in a centrifuge. It is crucial that the DNA be just barely dry, as further drying will cause artifactual oxidation of guanine, particularly in air and if phenol has been employed.[52] In fact, blotting the lump of DNA with paper tissue to remove ethanol/water is sufficient drying before hydrolysis. The DNA is dissolved in 200 μl 20 m*M* sodium acetate (pH 4.8), and digested to nucleotide level at 37° with 20 μl (5 U/sample) of nuclease P1 for 30 min followed by addition of 20 μl (1 U/sample) of alkaline phosphatase in 1 *M* Tris buffer (pH 8) for 60 min 37°. Two hundred μl is transferred to HPLC autosampler vials, for tissue samples after filtration through a Whatman (Clifton, NJ) filter (pore size 30 kDa) by centrifugation at 2600*g* for 35 min at 4°.

Alternatively, to the enzymatic hydrolysis the DNA sample can be hydrolyzed to nucleobases in acid, provided an RNAse digestion step has been included. The partly dried DNA is dissolved in 60% formic acid in screw cap tubes filled with nitrogen and subjected to 130° for 30 min (some laboratories use up to 150° for 45–60 min) in evacuated sealed Pierce (Rockford, IL) hydrolysis tubes.[58] After hydrolysis the formic acid is removed by freeze drying in a centrifuge and the sample reconstituted in mobile phase.

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HPLC Procedures

The HPLC apparatus consists of Merck-Hitachi components (San Jose, CA), L-6000 pump, AS-2000 autosampler, column oven set at 30°, D-6000 integration software, a Waters 440 UV absorbance detector at 254 nm, and an ESA Coulochem II electrochemical detector (Bedford, MA) equipped with a 5011 cell set 100–120 mV at electrode 1 and 200–300 mV at electrode 2 according to the voltammograms. A 25 cm Beckman Ultrasphere 5 μm column (Fullerton, CA) is eluted with acetonitrile 3.5–4.0% in phosphate buffer, pH 4. The UV detector is placed after the electrochemical cell in the flow path as the latter may cause back pressure, ruining the UV cuvette, and identical results are obtained in either sequence. dG is quantified by UV absorbance and 8-oxodG by electrochemical reactions (Fig. 5). The system is calibrated with injection of 8-oxodG 0, 4, 40, and 400 n*M* and dG 100 n*M*. Peak areas are used for calculations. Calibration curves and a quality control sample (rat liver pool) are run before and after each batch of samples. Each sample is extracted in duplicate and the enzymatic digest is injected three times, 20, 30, and 40 μl as the exact DNA content is not necessarily determined.

For nucleobases the mobile phase is a buffer of citric acid 12.5 m*M*, sodium acetate 25 m*M*, and EDTA 25 μ*M* at pH 2.5. The electrochemical 5011 cell is set at 0 mV at electrode 1 and 200–300 mV at electrode 2. Guanine can be quantified both by UV absorbance, along with the other unmodified bases, and by electrochemical detection, whereas 8-oxoGua is

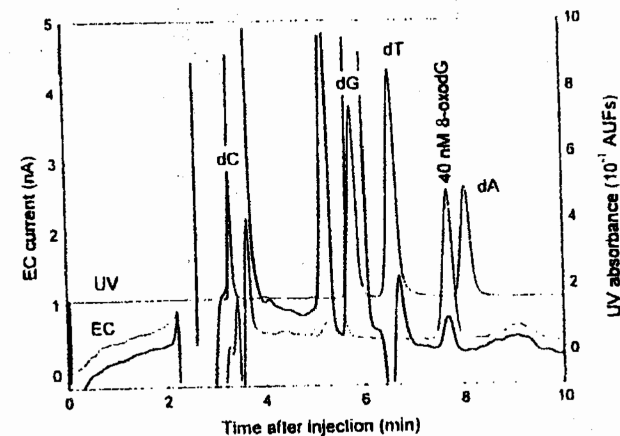

FIG. 5. Chromatogram of HPLC analysis of 8-oxodG in nuclear DNA. The 4 undamaged deoxynucleosides are shown in the UV tracing, whereas the electrochemical (EC) tracings show 8-oxodG in the hydrolyzed DNA sample (solid line) and as a standard (broken line).

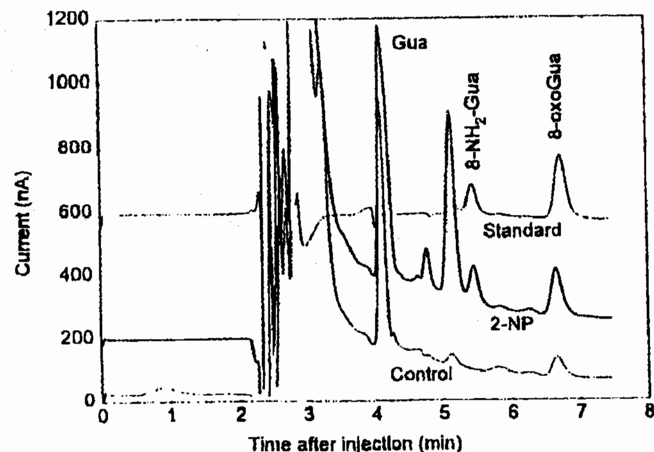

FIG. 6. Chromatogram (electrochemical tracing) of HPLC analysis of 8-oxoGua and 8-aminoGua in nuclear DNA from rats with or without pretreatment with 2-nitropropane (2-NP) and a standard solution.

quantified by electrochemical detection. If a higher pH is used, guanine elutes close to the 8-oxoGua and a guanase pretreatment is necessary to reduce the concentration.[59] Quantification is done as for 8-oxodG and dG. In addition to 8-oxoGua, the assay can measure other oxidatively modified nucleobases, 2,6-diamino-4-hydroxy-5-formamidopyrimidine (FapyGua), 5-OHmU, and 8-oxoadenine (8-oxoAde), which are electrochemically active.[58] Moreover, 8-aminoguanine, a reduction product of 8-nitroguanine that is a product of peroxynitrite attack on guanine and has received much attention, can be assayed by this method.[60,61] For the latter, calibration is achieved by subjecting 8-aminoguanosine (Sigma, St. Louis, MO) to the formic acid hydrolysis. For determination of 8-nitroguanine in DNA, the reconstituted sample, after freeze drying the formic acid hydrolyzate, is reduced with sodium hydrosulfite. Figure 6 shows electrochemical chromatograms of hydrolyzed liver DNA from rats with or without pretreatment with 2-nitropropane (100 mg/kg), which induces both 8-oxoGua and 8-aminoGua.[62]

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Artifacts and Other Problems. The main problems with the assay relate to artifactual formation of 8-oxodG from the large amounts of dG present. This can happen at any time during sample DNA extraction and hydrolysis, as well as in the autosampler. Even in the pipes or injection ports of the autosampler, residual dG from a previous injection may be oxidized and contaminate a later injection. Accordingly, all procedures should be performed at the lowest possible temperature and we use repeated and thorough washing procedures in the autosampler, which is equipped with a cooled sample tray. Some laboratories recommend the addition of desferrioxamine for binding iron during homogenization of, e.g., liver tissue and the addition of antioxidants.[22] Some laboratories use a digestion step with both RNase and pronase before extraction with both phenol and chloroform/isoamyl alcohol.[22] However, we have tried such steps without any reduction in variation or background levels. We have tried to use the pronase digestion procedure described for cells for the organ tissues, as well as the chloroform extraction method for sperm cells. In both cases the variation and background levels increased. In our hands the most important step is the drying of the DNA after precipitation as outlined above. With our present method we have systematically passed calf thymus DNA through the individual extraction steps and subjected solutions of dG to the enzymatic and acid hydrolysis steps, and we have seen no signs of oxidation of dG in terms of increasing 8-oxodG or 8-oxoGua levels. Another problem involves variability in the EC detector response and changes in the voltammogram, particularly low responses after an analytical pause and as wear-out. The former problem can be reduced by injecting a solution containing a high concentration, e.g., 400 n*M*, 5 times or more before a batch is run, and the voltammogram should be checked frequently. Since all these sources of variation may cause considerable shifts in the measured levels, it is strongly recommended that quality control samples, e.g., from a pool of rat liver, be run with every analytical batch.

With other methods such as GC-MS SIM, ^{32}P, and immunoaffinity cleanup with slot-blot quantitation, even larger variations in obtained values from tissue and cellular DNA have been obtained in different laboratories.[63] After recent improvements in the GC-MS/SIM techniques with removal of intact bases before and/or control of temperature and oxygen during sample derivatization, the results are similar to what is achieved with the HPLC-EC methods described here.[46-48] Currently, a European interlaboratory quality comparison study is underway in order to standardize the methods and results related to 8-oxodG/-8-oxoG in tissue DNA.

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Antioxidant Intervention Studies Involving Biomarkers of Oxidative DNA Damage

The 24-hour urinary excretion of 8-oxodG shows a seven-fold range within the studied populations and an intersubject coefficient of variation of 30–40% (Table I). The intraindividual coefficient of variation has been 22% in 8 subjects examined twice on a controlled diet for 10 weeks[43] and 27% in groups of smokers studied two or three times.[64] For Tg and dTg excretion the interindividual coefficients of variation range from 28% to 92% (Table I).

Antioxidant supplementation could be expected to reduce the rate of oxidative DNA modification. Indeed, in a controlled smoking cessation study, the decrease in 8-oxodG excretion was a mirror of the increase in plasma vitamin C concentration.[64,65] However, so far intervention studies have not provided support for the notion of a beneficial effect of antioxidants. In smokers daily administration of β-carotene, vitamin C, vitamin E, or coenzyme Q had no effect on the excretion rate of 8-oxodG[66,67] or the RNA damage product, 8-oxoG.[38] Depletion of dietary ascorbic acid in healthy men had no effect on the level of 8-oxodG in lymphocyte DNA or the urinary excretion of 8-oxoguanosine, whereas the level of 8-oxodG in sperm DNA increased substantially.[68,69] Upon replenishment of ascorbate, the 8-oxodG level in sperm returned to the initial values.[69] In guinea pigs, the levels of 8-oxodG in liver DNA were not found to be affected by either very low or very high levels of dietary ascorbic acid or vitamin E.[70] No significant effect of vitamin E supplementation on rat liver 8-oxodG[71] and on tumor incidence and 8-oxodG in the liver of rainbow trout[72] has been found.

Ascorbic acid inhibited 8-oxodG formation in calf thymus DNA and V79 Chinese hamster cells exposed to ultraviolet radiation.[73,74] Glutathione,

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cysteine, and vitamin C (but not vitamin E) also protected against increases in renal 8-oxodG caused by the kidney carcinogen potassium bromate in rats,[75] although tissue levels of ascorbate and glutathione, which are synthesized by rat tissues, were not measured in that study. On the other hand, vitamin E and ellagic acid, but not vitamin C, inhibited the increase in liver 8-oxodG induced by the hepatocarcinogen 2-nitropropane in rats.[76]

A potential DNA-protective effect of specific vegetable components was suggested by a 28% reduction in the rate of urinary 8-oxodG excretion after a diet with 300 g of Brussels sprouts in comparison with 300 g of noncruciferous vegetables.[77] This effect has been reproduced in rats in our laboratory (unpublished data). In a repeat experiment involving humans, however, no sign of reduced 8-oxodG excretion was seen in women and a reduction did not reach statistical significance in men after 300 g of Brussels sprouts per day for a week.[78] Nevertheless, a new method for 8-oxodG analysis was used in that study, giving extremely high values in some subjects. Indeed, cruciferous vegetables, such as Brussels sprouts and broccoli, contain certain phytochemicals which are anticarcinogenic possibly by inducing enzymes scavenging electrophiles and by mimicking the cellular protective response to oxidative stress.[79,80]

The differences between groups and effects of interventions seen in human studies of oxidized DNA bases are usually rather small, less than twofold, and for urinary excretion less than 50%. Moreover, for the nuclear steady-state levels, effects are obscured by the huge variation between and even within the various methods and laboratories. Thus, the above-mentioned negative results of antioxidant intervention trials could be due to the limited number of study subjects, i.e., a type 2 statistical error. For example, assuming that the variation is about 35%, a two-parallel-group controlled trial would need 190 persons in each group to detect a difference of 10%, 86 persons to detect a difference of 15%, and 47 persons to detect a difference of 20%, assuming $\alpha = 0.05$, $\beta = 0.20$. On the other hand, a crossover experiment with a homogeneous defined and controlled group

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would show considerable lesser variation and differences of 30–50% could easily be detected, e.g., in a study of Brussels sprouts in 5 subjects.[66]

Urinary excretion of oxidized nucleosides and nucleobases, so far mainly 8-oxodG, represent promising biomarkers of oxidative DNA damage with a potential for establishing a relation to carcinogenesis and aging and for detecting changes in intervention trials. It should be borne in mind that even small changes, e.g., 10–15%, in oxidation rate could change the balance with DNA repair to a biologically important degree, and that detection of such a change in DNA oxidation may require large-scale controlled clinical trials.

[20] Comet Assay for Nuclear DNA Damage

By STYLIANOS M. PIPERAKIS, EVANGELOS-E. VISVARDIS, and ASPASIA M. TASSIOU

Background

Single-cell gel electrophoresis (SCGE, comet assay) provides a very sensitive method for detecting strand breaks and measuring repair kinetics at the level of single cells. The technique was introduced in 1988 by N. P. Singh *et al.*, who modified other primary microgel electrophoresis techniques.[1,2] The unique design of the comet assay provides direct determination of the DNA damage in the responses of individual cells as well as examination of DNA damage and repair under a variety of experimental conditions. The SCGE assay is a particularly valuable technique because it allows the detection of intercellular differences in DNA damage and repair in any eukaryotic cell population. A variety of possible modifications of the assay facilitates the detection of single-stranded DNA breaks and alkali labile sites, double-stranded DNA breaks, incomplete excision repair sites, and interstrand cross-links, and increases the specificity and sensitivity of the assay. Moreover, it enables the study of different DNA repair pathways such as base excision and nucleotide excision repair.[3,4] In addition to the above, DNA fragmentation associated with cell death or related to apoptosis can be evaluated with the comet assay.[5] There is also a great variety of DNA damaging agents that can be used in order to study DNA damage and repair with the comet assay procedure. After their treatment, the cells are embedded in agarose layers, lysed, and electrophoresed. Under fluorescence microscopy, cells with increased DNA damage display increased DNA migration from the nucleus toward the anode, thus forming the shape of a "comet" after staining with a fluorescent DNA binding dye. The comet assay utilizes a relatively small number of cells. The results can be obtained in a relatively short period of time, thus enabling the successful biomonitoring of DNA damage and repair in human cells, which makes the comet assay a very valuable tool in molecular epidemiology.

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Cell Types

A great variety of cells isolated from many different sources[6] can be examined with the comet assay. Fresh or cryopreserved peripheral blood mononuclear cells are readily used from human, mouse, rat, and dog. (We have developed a method in which lymphocytes kept frozen for an indefinite period of time give the same response as fresh lymphocytes when treated with DNA damaging agents; they also show the same repair efficiency.)[8] Granulocytes from humans, epithelia (lens) from human or rat mucosal epithelia, human fibroblasts, human spermatocytes, human adenocarcinoma, lymphoma, and small cell carcinoma can be used. Other types of cells such as splenocytes, thymocytes, bone marrow cells from mouse, and brain cells from mouse and sheep have also been studied. Kidney, liver, pancreas, and testis cells from mouse and rat as well as embryos from rat have also been examined. Various cell lines can also be used in the comet assay that are derived from different types of cell culture. R_{aji} and TK6 from B-cell lines and HUT-78 from T-cell lines are currently used. From colon carcinoma cell lines, WiDr and HT-29 have been frequently used, from bladder carcinoma A1698, from cervix SiHa and HeLa, from prostate carcinoma DU-145, and from melanoma MeWo and HT-144 cell lines. Many cell lines from animal cell cultures have also been used, such as CHO and V79 from hamster, or L5178Y and SCCVII from mouse cell cultures.

Treatment

A significant number of DNA damaging factors have been studied with the comet assay and are summarized in Table I. The treatment conditions

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