MARKERS OF OXIDATIVE DAMAGE TO DNA

The degradation of DNA may also contribute adducts; (iii) the oxidation of cysteine and circulating nucleotide pools and DNA fragments are potential sources of reactive intermediates that can be detected by the histochemical technique producing the lowest oxiGal values reported to date using HPLC-EC. Although the use of rat liver RNA and DNA adducts to monitor oxidative DNA damage in vivo remains attractive, the results can be difficult to interpret. Comparison of carefully controlled groups can minimize the confounders (such as dietary contributions), but the effect of experimental treatments, such as the cell cycle, remains unknown and uncontrolled. Thus, the results must be interpreted with caution.

Acknowledgment
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MARKERS OF OXIDATIVE DAMAGE TO DNA AND MOLECULAR DAMAGE TO DNA

By STEVAN LEBL AND HENNICK EMBRICH JOINSON

Cells are constantly exposed to oxidative stress from both physiological processes, such as mitochondrial respiration, and pathophysiological conditions, such as inflammation, ischemia-reperfusion, foreign compound metabolism, and radiation. Failure of the system of enzymes, endogenous, and nutritional antioxidants may lead to carcinogenesis and degenerative disease. Hence, the concept of supplementing nutritionally based antioxidants in order to prevent cancer and postpone aging appears rational. Descriptive epidemiological studies support the notion of a high risk of colorectal cancer. A high intake of fresh vegetables and fruits rich in antioxidants and a low risk of epithelial cancers, particularly in the airways and upper gastrointestinal tract. Small-scale human intervention studies with chemically based biomarkers as intermediate end points and supported by animal and in vitro experiments may target the optimal intervention strategy for the large-scale intervention. 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. However, so far only a few of the basic modifications have been used as biomarkers, and of these, the oxidative C8 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 most steady-state conditions, the latter will reflect the rate of damage, whereas the former will reflect the balance between damage and repair. The present...
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 nucleoside synthetases; 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-hydroxyuracil (5-OHUr) have so far been identified in urine.11,12,15 Of these, 8-oxoGua 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 urine DNA repair products include high-performance liquid chromatography (HPLC) with electrochemical detection (EC) for 8-oxoGua 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).11,12,13,15 The major problem with all these assays involves separation of the very small amounts of analyte from urine, which is a very complex 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

<table>
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<th>Assay</th>
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<td>100-274 pmol/g</td>
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<td>200</td>
<td>6</td>
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<td>6.5-34 pmol/g</td>
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immunoassay column.18,19,20 The complicated extraction procedures cause recovery problems in both HPLC and GC/MS-SIM methods and may require labeled internal standards. Moreover, the complicated procedure limits the analytical capacity. An enzyme-linked immunosorbent assay (ELISA) based on monoclonal antibodies has been developed for estimation of 8-oxoGua in urine samples.13 However, the values obtained in rat and human urine samples20 were 3-5 times higher than other published values.13 Similarly, in 4 smokers studied before and after smoking cessation,
the urinary 8-oxoG 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.  

**HPLC-EC Assay of 8-OxoG in Urine**

Most data regarding urinary excretion of oxidative DNA repair products have been obtained on 8-oxoG 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-oxoG in several thousand urine samples.  

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°C overnight for precipitation of uric acid and other solutes. After thawing and centrifugation for 5 min at 3000 g, 34 μl NaOH 2 M is added to 1.7 ml of the supernatant. At this stage the samples can again be stored for prolonged periods. At 95 μl aliquots of the treated urine, 5 μl of a solution containing 8-oxoG (Sigma, St. Louis, MO) 0.80, or 8000 μM and 100 μl 1 M Tris buffer, pH 7.9, are added and 25 μl of the mixture is injected on the HPLC apparatus. The rest of the sample is brought to the flow path of pump 2 by isocratic elution, separated on a Nucleosil column, and monitored by an EC detector. In the chromatogram the broken and solid line represent the running of a sample containing 8-oxoG at 10 μM 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 in 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-oxoG in urine samples has been ascertained by identical retention time and voltammogram as compared with the genuine compound. Moreover, after acid hydrolysis in 0.5 M HCl at 140°C for 1 h, the RosaiG 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-oxoG and the concentrations calculated from the individual calibration curve based on the peak heights. The performance of the method includes linear calibration curve, an intercept coefficient of variation of 9–13%, and a limit of detection of 0.2
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°C (Fig. 3). The two sets of values were highly correlated (r² = 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-oxoG received as gifts from other researchers to the now commercially available crystallized compound; with the old calibration standards the slope would have been 1.0. 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-oxoG 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 electrokinetic 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.

A Swedish group has developed a similar column switching assay for 8-oxoG in urine.27 However, in this assay the sample is concentrated on a Bondesil C18 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 C18 column eluted at pH 7.0 and monitored with a Waters 400 electrochemical detector. This assay seems to perform satisfactorily and has been used in some large-scale epidemiological studies.28-29 The use of monoclonal antibody-based immunofluorescent columns for extraction of 8-oxoG, 8-oxoG-caffeic acid, and 8-oxo-guanosine from biological fluids, including urine, has been extensively described in this series previously.30

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-oxoG to

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

For the use of the urinary excretion of repair products as biomarkers of oxidative damage to DNA, extensive repair is assumed. Thus, after limiting radiation the increase in urinary excretion of thyminic glycol and 8-oxoG occurred within 24 hours in humans, whereas excess 8-oxoG was removed from mouse liver DNA after approximately 90 min. In a study of 8-oxoG in human brain, the accumulation rate in the nuclear DNA corresponded to two lesions per cell per day. In humans the reported values of the urinary excretion of the repair products, 8-oxoG, are in the range 15-50 nmol per 24 h and the alternative repair product 8-oxoG appears to be excreted in similar amounts. The sum of these products thus corresponds to an average of 300-1000 lesions per day for each of the assumed 5 x 10^12 cells in the body per day. Accordingly, the calculated repair efficiency under these assumptions range from 99.4% to 99.89%.

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 level, i.e., the balance between damage and repair, albeit only in a surrogate for target tissues. Accordingly, the two groups of biomarkers are complementary.

Tissue/Cell Levels of Oxidized Bases in DNA

In tissue or cell samples the level of oxidatively modified nucleotides can be measured by various techniques, including HPLC (in MS or UV), GC/MS-SIM, TLC with "P, pulselabeling, and various immunoblot

References:
ethanol precipitation.** The lowest values have been obtained with non-ionic DNA extraction.

Some of the immunostains are calibrated by HPLC-EV values,** whereas others appear to yield much higher 8-oxoG values than the HPLC-EC assays,*** but whether that is due to insufficient specificity of the antibodies is unknown.

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

Chemicals. 8-OxodG, 8-oxoGuA, 8-aminoquinoline-2'-deoxyquinacine (dG), guanine, guanine E, and nucleases P1, P2 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 HIPEPS 5 mM buffer [N-hydroxyethylpiperazin-N'-2-chetalosulfonic acid], sucrose 70% w/v, mannitol 250 mM, pH 7.4, on ice. After centrifugation at 1000g, the pellet containing the nuclei is resuspended in 0.15 M NaCl, 1% w/v I% agarose, on ice. Two hundred μl of suspension is transferred to 1.8 ml ice-cold TE buffer (150 mM NaCl, 10 mM Tris, 10 mM Na2EDTA, pH 8.0), and 200 μl 10% sodium dodecyl sulfate (SDS) is added. After vortexing for 30 s, followed by rotation in an extraction buffer 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 buffer for 10 min, the samples are centrifuged at 21000 g for 15 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.


Lymphocytes, Spore, 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 (Nycodenz Oslo, Norway) is carefully added to the bottom of the vial. After centrifugation for 20 min at 350g and 21°C 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 350g for 10 min or approximately 20 x 10⁷ sperm or other cells, is suspended in 7 ml of a buffer pH 7.6 containing Triton X-100 1% (v/v), sucrose 37.3 M, Tris HCl 5 M, and magnesium chloride 10 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 mM and sodium chloride 20 mM. Promisc U (1 mg/ml) with dithiothreitol (2.5 mM), N,N-hydroxyethylethane (0.75% w/v, Sigma), EDTA (10 mM), Tris base (10 M), and butylylated hydroxyanisole (BHA, 0.3%, w/v) in methanol (95%, 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 4°C. An ammonium acetate to a final concentration of 0.5 M and 50 µl of an ice-cold 10 mM Tris-1 mM EDTA buffer are added and the DNA is precipitated by addition of 5 ml ice-cold ethanol (90%).

The DNA is allowed to precipitate at -20°C 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 artificial oxidation of guanine, particularly in pur and 7-hydroxymethyl has been employed. 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 mM sodium acetate (pH 4.8), and digested to mononucleotide level at 37°C with 20 µl (5 U/sample) of Alcalase P (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°C). Two hundred µl is transferred to HPLC autosampler vials, for tissue samples after filtration through a Whirlpak (Clifton, NJ) filter (prepurse 30 kDa) by centrifugation at 2600g for 25 min at 4°C.

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


HPLC Procedures

The HPLC apparatus consists of Merck-Hitachi components (Sun Liscal, CA), L-4000 pump, AS-2000 autosampler, column oven set at 30°C. D-6000 integration software, a Waters 440 UV absorbance detector at 254 nm, and an ESA Coulomb II electrochemical detector (Bedford, MA) equipped with a 911 cell set 108-120 mV at electrode 1 and 200-300 mV at electrode 2 according to the voltammogram. A 25 cm Beckman Ultrasphere 5 μm column (Fullerton, CA) is eluted with n-octane:trifluoroacetic acid:water (5:3.3:0.9) 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. dA is quantified by UV absorbance and 8-oxoG by electrochemical reactions (Fig. 5). The system is calibrated with injection of 8-oxoG-0.4, 4, 40, and 400 nM and dG 100 nM. Peak areas are used for calculations. Cathode peaks used as quality control sample (rat liver pool) are run before and after each batch of samples. Each sample is extracted in duplicate and the enzymatic digester 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 mM, sodium acetate 25 mM, and EDTA 25 mM at pH 2.5. The electrochemical 5011 cell is set at 600 mV at electrode 1 and 280-300 mV at electrode 2. Guanine can be quantified both by UV absorbance, along with the other unmodified bases, and by electrochemical detection, where 6-oxoguanine is

![Fig. 5. Chromatogram of HPLC analysis of 8-oxoG in mammalian DNA. The undamaged deoxynucleotides are shown in the UV tracing, whereas the electrochemical (EC) tracings show 8-oxoguanine in the hydrolyzed DNA sample (solid line) and in a standard (dashed line).](image-url)
quantified by electrochemical detection. If a higher pH is used, guanine elutes closer to the 8-oxoGua and a guanine pretreatment is necessary to reduce the concentration.\textsuperscript{59} Quantification is done as for 8-oxoGua and 8Q.

In addition to 8-oxoGua, the assay can measure other oxidatively modified nucleobases, 2,6-diamino-4-hydroxy-5-formamidopyrimidine (FapyGua), 5-OHU, and 8-oxoquinone (8-Q), which are electrochemically active.\textsuperscript{60} 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 quantitated by this method.\textsuperscript{60} For the latter, calibration is achieved by subjecting 8-aminoguanine (Sigma, St. Louis, MO) to the formic acid hydratase. For determination of 8-nitroguanine in DNA, the reconstituted sample, after freeze drying the formic acid hydratase, is reduced with sodium hydrosulfite. Figure 6 shows electrochemical chromatograms of hydroxylated liver DNA from rats with or without pretreatment with 2-nitropropane (100 mg/kg), which induces both 8-oxoGua and 8-aminoguanine.\textsuperscript{61}


\textsuperscript{61} O. Yarom, J. Tamei, M. Ben-Dor, M. D. Formos, B. F. Pignataro, and H. Ozasa, Carcinogene

Artifacts and Other Problems. The main problems with the assay relate to artefactual formation of 8-oxoGua from the large amounts of 8Q 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 8Q 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 deferox-amine for binding iron during homogenization of, e.g., liver tissue and the addition of antioxidants.\textsuperscript{20} Some laboratories use a digestion step with both RNase and protein prior to extraction with both phenol and chloroform/isoctyl alcohol.\textsuperscript{22} However, we have tried such steps with no additional problems in the form of increased 8-oxoGua or 8-oxoGua levels. Another problem involves variability in the EC detector response and changes in the voltammogram, particularly low response after an analytical pause and at wash-out. The former problem can be reduced by injecting a solution containing a high concentration, e.g., 400 nM, 5 times or more before a batch is run, and the voltammogram should be checked frequently. Since all of these sources of variation may cause considerable shifts to 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,\textsuperscript{58} and immunoassay cleanup with shot-lot quantitation, even larger variations in obtained values from tissue and cellular DNA have been obtained in different laboratories.\textsuperscript{53} After recent improvements in the GC-MS SIM techniques with removal of intact bases before and control of temperature and oxygen during sample derivatization, the results are similar to what is achieved with the HPLC-EC methods described here.\textsuperscript{60} Currently, a European interlaboratory quality comparison study is underway in order to standardize the methods and results related to 8-oxoGua/8-oxoG in tissue DNA.\textsuperscript{60}
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 interindividual coefficient of variation of 33%-106% (Table I). The intraclass coefficient of variation has been 22% in 8 subjects examined twice on a controlled diet for 10 weeks in 27% in groups of smokers studied two or three times. For 79 and 125 excretion the interindividual coefficients of variation range from 28% to 97% (Table 1).

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. However, so far intervention studies have not provided support for the notion of a beneficial effect of antioxidants. In smokers daily administration of vitamin C, vitamin E, or coenzyme Q had no effect on the excretion rate of 8-oxodG or on the DNA damage product, 8-oxodG. Depletion of dietary ascorbic acid in healthy men had no effect on the level of 8-oxodG in lymphocyte DNA of the urinary excretion of 8-oxoguanine, whereas the level of 8-oxodG in sperm DNA increased substantially. Upon replenishment of ascorbic acid, the 8-oxodG level in sperm returned to the initial value. In guinea pigs, the level 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. No significant effect of vitamin E supplementation on rat liver 8-oxodG and on tumour incidence and 8-oxodG in the liver of rainbow trout has been found.

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


References

Cysteine, and vitamin C (but not vitamin E) also protected against increases in renal 8-oxodG caused by the kidney carcinoma oxygenation, and in rats, although tissue levels of ascorbic and glutathione, which are synthesized by rat tissues, were not measured in that study. On the other hand, vitamin E andolic acid, but not vitamin C inhibited the increase in liver 8-oxodG induced by the hepatocarcinogen 2-acetoxyprene in rats.

A potential DNA-protective effect of specific vegetable components was suggested by a 20% reduction in the rate of urinary 8-oxodG excretion after a diet with 300 g of Brussels sprouts in comparison with 300 g of nonconcrete vegetables. 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 population did not reach statistical significance in men after 300 g of Brussels sprouts per day for a week. 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 the yellow pigments called by inducing enzymes scavenging electrophiles and by mimicking the cellular protective response to oxidative stress. 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 level, 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 II 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 α = 0.05, β = 0.20. On the other hand, a crossover experiment with a homogeneous and controlled group
COMET ASSAY FOR NUCLEAR DNA DAMAGE

[20] Comet Assay for Nuclear DNA Damage
By Stavroula M. Pipiras, Evangelos E. Vouvakis, and Aristide M. Tassouri

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. The unique design of the comet assay provides direct determination of the DNA damage in the response of individual cells as well as examination of DNA damage and repair under a variety of experimental conditions. The SCGE assay is particularly valuable 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 facilitate the detection of single-stranded DNA breaks and alkali labile sites, double-stranded DNA breaks, in vivo alkaline labile 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. In addition to the above, DNA fragmentation associated with cell death or relaxed to

apoptosis can be evaluated with the comet assay. 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 bio-monitoring of DNA damage and repair in human cells, which makes the comet assay a very valuable tool in molecular epidemiology.

Cell Types
A great variety of cells isolated from different sources 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.) Granulocytes from humans, epithelia (lens) from human or rat mucosal epithelia, human fibroblasts, human spermatocytes, human adenocarcinomas, 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 embryonic 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, B1 and T6 from B-cell lines and HT-1177 from T-cell lines are currently used. From colon carcinomas cell lines, WiDr and HT-29 have been frequently used, from breast adenocarcinoma A-150, from cervix SiHa and HeLa, from prostrate 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 SCCVIII from mouse cell cultures.

Treatment
A significant number of DNA damaging factors have been studied with the comet assay and are summarized in Table 1. The treatment conditions