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Development of an enzymatic DNA repair assay for molecular epidemiology studies: Distribution of OGG activity in healthy individuals

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Abbreviations:

95% CI, 95% confidence interval;

ACTB, β -actin; CV, coefficient of

variation; HPRT1, hypoxanthine

phosphoribosyltransferase 1; OGG,

8-oxoguanine DNA glycosylase;

8-oxoG, 8-oxoguanine; PBMC,

peripheral blood mononuclear cells;

RT, reverse transcriptase; STDEV,

standard deviation; TBP,

TATA-binding protein

ABSTRACT

While the role of reduced DNA repair in susceptibility to hereditary cancers is well established, its role in sporadic cancer is less understood. One of the reasons is the lack of specific DNA repair assays that are suitable for epidemiology studies. Here we describe the development of the OGG test, an epidemiology-grade enzymatic assay for the activity of the base excision repair enzyme 8-oxoguanine DNA glycosylase, in protein extracts prepared from human blood cells. The assay is robust and reproducible, with a coefficient of variation of 10%. Using the OGG test we determined OGG activity in 120 healthy individuals. Our results show an inter-individual variation of 2.8-fold in OGG activity, from 3.6 up to 10.1 units/ μ g protein, with a mean value of 7.2 units/ μ g protein. There was no significant difference in OGG activity between males and females, or between smokers and non-smokers. Interestingly, there was a gender-specific effect of age: OGG activity was slightly but significantly lower in males older than the age of 55 years compared to younger males, but not in females at the same age groups. Analysis of OGG1 mRNA by quantitative real-time RT-PCR showed a group trend of an increase in OGG enzymatic activity with increasing mRNA expression, but the correlation between activity and mRNA in individuals was poor, indicating the importance of factors other than mRNA expression. The OGG test described is expected to be useful in studying the role of 8-oxoguanine repair in cancer, as recently demonstrated for non-small cell lung cancer [T. Paz-Elizur, M. Krupsky, S. Blumenstein, D. Elinger, E. Schechtman, Z. Livneh, *J. Natl. Cancer Inst.* 95 (2003) 1312–1319]. In addition, it may serve as a paradigm for the development of additional functional DNA repair tests, which are needed in order to gain further insight into the role of DNA repair in cancer risk and pathology.

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1. Introduction

Cancer is caused by multiple somatic mutations in oncogenes and tumor suppressor genes that lead to aberrant cell growth [1]. A major source of mutations is replication errors caused by misinsertion at normal and damaged nucleotides [2]. Thus, DNA repair mechanisms, which function to eliminate DNA damage and reduce mutations, are expected to be an important component in the defense of organisms against cancer. This was clearly illustrated in several human cancer predisposition syndromes where mutations in DNA repair genes were shown to cause high susceptibility to hereditary cancers. The MYH adenine DNA glycosylase, which is involved in base excision repair, is mutated in patients with familial adenomatous polyposis (FAP) colon cancer [3]; nucleotide excision repair genes are mutated in patients with xeroderma pigmentosum, a syndrome characterized by increase sensitivity to sunlight and high susceptibility to skin cancer [4], and mismatch repair genes are mutated in patients with hereditary nonpolyposis colorectal cancer (HNPCC) [5]. Similarly, DNA repair is likely to play an important role also in sporadic cancers, which account for the vast majority of human cancers. Indeed, several studies support this hypothesis [6–13], but in general more information is needed.

Several methods were employed to study the role of DNA repair in sporadic cancer. The comet assay is a generalized assay for DNA repair, based on measuring DNA breaks. In this assay cells are treated for a short time with a DNA damaging agent (e.g., γ -rays, benzo[a]pyrene diol epoxide), followed by a period of time that enables DNA repair to occur, after which chromosomal DNA breaks are determined by gel electrophoresis [14]. The host cell reactivation assay measure DNA repair capacity indirectly, as the reactivation of gene expression from a damaged plasmid introduced into stimulated lymphocytes [15]. Another approach involves measuring the amount of specific DNA lesions, which is an indicator of the combined effects of the exposure level and the DNA repair activity [16]. Finally, there are gene-specific approaches, mainly the analysis of single nucleotide polymorphism in DNA repair genes (e.g., Refs. [17,18]).

In general the study of the role of DNA repair in sporadic cancer is more complex than in hereditary cancer. Unlike hereditary cancer, in which the activity of the DNA repair gene is abolished by a single key mutation, in sporadic cancer the protein activities involved are expected to be moderately different, and the final cancer susceptibility is greatly affected by complex gene–environment interactions. This complexity suggests that single nucleotide polymorphisms in particular DNA repair genes may not be informative enough, and functional DNA repair assays are needed to provide useful tools for exploring the role of DNA repair in the etiology of sporadic cancer. In particular, activity assays of specific DNA repair enzymes or pathways are potentially powerful since they measure the relevant enzymatic activity involving the sum of possible effects of dozen of factors, including the enzyme expression, stability, polymorphisms, effect of inhibitors and stimulators, as well as environmental and lifestyle factors that affect its activity.

A key requirement for DNA repair assays to be useful in cancer research in humans is suitability to epidemiologic studies, implying simplicity, robustness, and high reproducibility. When considering enzymatic activity of a DNA repair enzyme this translates to establishing reproducible procedures for blood collection and processing, efficient extraction of proteins from peripheral blood mononuclear cells (PBMC; usually the tissue of choice), developing protocols that will enable long-term storage of frozen PBMC and protein extracts, optimizing the enzymatic reaction, and minimizing possible biases that may interfere with the epidemiologic study. Here we present the development of an epidemiology-grade assay for the enzymatic activity of the DNA repair enzyme 8-oxoguanine DNA glycosylase (OGG) in protein extracts from PBMC. OGG removes 8-oxoG from DNA [19], a common lesion that is formed by multiple pathways including intracellular metabolism, oxidative stress, ionizing radiation, and tobacco smoke [20–22]. 8-OxoG is highly mutagenic, frequently mispairing with A to yield GC \rightarrow TA transversions [23–26]. Using this assay we determined the OGG activity in PBMC from 120 healthy individuals, and analyzed it by gender, smoking status, and age group. We have also compared OGG activity and OGG1 mRNA expression in a group of individuals. The power of the OGG assay was recently demonstrated in a case-control study, which indicated that reduced OGG activity is associated with the risk of non-small cell lung cancer [10].

2. Materials and methods

2.1. Isolation of PBMC

For the development stages 500 ml bags of whole blood with CPDA-1 (citrate-phosphate-dextrose-adenine; Teva medical, Ashdod, Israel) as anticoagulant, obtained from healthy donors, were purchased from the National Blood Bank at the Sheba Medical Center (Tel Hashomer, Israel). The blood was divided to 10 ml portions (10–20 samples) and processed in parallel. For analysis of inter-individual variation of OGG activity in healthy individuals, samples of 10 ml peripheral blood were collected each in a 50 ml tubes containing 1.4 ml of CPDA-1. All samples were processed within 18–24 h of blood collection. PBMC were isolated essentially as described [10]. It involved fractionation of a diluted blood sample by centrifugation on ficoll, followed by lysing red blood cells, washing, and resuspending the PBMC at a concentration of 20,000 cells/ μ l in a hypotonic buffer solution (LTGO) containing 50 mM Tris-HCl (pH 7.1), 1 mM EDTA, 0.5 mM spermidine, 0.1 mM spermine, 0.5 mM dithiothreitol, and a protease inhibitor cocktail (Sigma, St. Louis, MO). The PBMC were incubated on ice for 30 min, after which they were frozen in liquid nitrogen and stored at -80°C . Since thawing the frozen cells causes them to lyse, this procedure is suitable for preparing protein extracts, but not for retrieving live cells. To optimize the procedure for freezing PBMC various buffers and cell concentration were examined, as described in Section 3.

2.2. Preparation of protein extract

Frozen PBMC were lysed by quickly thawing them in a water bath at 30°C , followed by one of several treatments that were

examined in order to obtain protein extracts with the highest OGG activity. These included additional one to three freeze-thaw cycles, shearing the cells by passage through a syringe needle (25gx5/8"), sonication in a water bath, and the addition of detergents (NP40 or triton X-100 each at 0.12%, 0.06%, and 0.03%, and CHAPS at 0.17%, 0.09%, and 0.04%). To determine the most efficient extraction conditions of OGG activity from the nucleus, the lysed cells were incubated on ice with KCl concentrations of 25–300 mM for 0.5, 1, and 1.5 h. The optimal extraction was with 222 mM KCl for 30 min, which was set as the standard. Extracts were cleared by centrifugation at $11,000 \times g$ for 15 min at 4 °C, after which glycerol (final concentration of 10%) was added, and the samples were frozen in liquid nitrogen. Protein concentrations in the extracts were determined with the bicinchoninic acid assay kit (Pierce, Rockford, IL), using bovine γ -globulin as a standard.

2.3. Substrates preparation

The standard substrate used for the OGG activity assay was substrate T5 (32 bp long, Fig. 2C). It was prepared as described [10]. In short, the oligonucleotide with the sequence 5'-CCGGTGCATGACACTGTXACCTATCCTCAGCG-3' (X, 8-oxoG) was 32 P-labeled using T4 polynucleotide kinase, and annealed to a complementary oligonucleotide 5'-CGCTGAGGATAGGTCACAGTGTCATGCACCGG-3' in the presence of 150 mM NaCl for 10 min at 70 °C, followed by cooling in a water bath kept at room temperature. The radiolabeled duplex DNA was separated from non-annealed oligonucleotides by PAGE on a native 10% gel, followed by extraction from the gel, and concentration determination by measuring its OD₂₆₀ using a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, Delaware, USA).

Eight additional substrates were similarly prepared. The substrates differ by their sequences and lengths (Fig. 2C): Substrate T1 (45 bp) is a longer version of a substrate previously used to assay repair of 8-oxoG [27]; Substrates T2 (50 bp) and T2A (32 bp), were driven from the p53 gene region containing the hotspot mutation at codon 249 (p53 nucleotides 722–771 and 729–760, respectively); Substrates T3 (48 bp) and T3A (32 bp) were driven from a different region of the p53 gene (p53 nucleotides 406–453 and 415–446, respectively); Substrates T4, T5, T5B, and T5C (45, 32, 28, and 24 bp, respectively) are randomly selected substrates, which share a common, randomly selected, DNA sequence context in the vicinity of 8-oxoG.

2.4. OGG activity assay

The OGG assay monitors the ability of OGG present in the PBMC protein extract to remove an 8-oxoG residue from a radiolabeled synthetic duplex oligonucleotide containing a single site-specific 8-oxoG. Following OGG removal the resultant abasic site is cleaved, generating a shorter radiolabeled DNA product that can be distinguished on the basis of its size. The standard reaction mixture (20 μ l) contained 50 mM Tris-HCl (pH 7.1), 1 mM EDTA, 115 mM KCl, 20 μ g of bovine γ -globulin, 2 pmol of polydA.polydT, 0.5 pmol of radiolabeled duplex DNA, and 8–12 μ g of protein extract. Assay conditions were rigorously optimized including buffer type and

pH (Tris-HCl pH 7.09–8.1; Hepes-NaOH pH 7.8; MOPS-KOH pH 6.86–7.64; Na-Phosphate pH 7.13–8.08), salts (KCl and NaCl) at concentration of 75–250 mM, metal ions at 0.1 or 1 mM (Ni^{2+} , Co^{2+} , Ca^{2+} , Mn^{2+} , Fe^{3+} , Fe^{2+} , Zn^{2+}) and 7 mM Mg^{2+} , stabilizing agents like bovine serum albumin (0–80 μ g), bovine γ -globulin (0–40 μ g), and polyethylene glycol 6000 (0–7.5%), and in addition polydA-polydT (0–24 pmol), EDTA (0–5 mM), the amount of DNA substrate (0.2–3 pmol), and the amount of protein extract (2–20 μ g total proteins). Reaction kinetics was performed at 10–120 min. Standard reactions were incubated at 37 °C for 30 min. Reactions were stopped by the addition of 15 mM EDTA and 0.2% sodium dodecyl sulfate. The proteins in the reaction mixture were then digested with proteinase K (20 μ g) for 1 h at 37 °C. Before fractionation samples were incubated with 80 mM NaOH for 30 min at 37 °C to ensure complete cleavage at the abasic site resulting from OGG activity, and the denatured DNA products were subjected to 15% PAGE-urea in 89 mM Tris-borate and 2.5 mM EDTA (pH 8.0) at 1500 V for 2 h at 45–50 °C. The distribution of the radiolabeled DNA products was visualized and quantified using a Fuji BAS 2500 PhosphorImager. One unit of OGG activity is defined as the amount of protein that cleaves 1 fmol of DNA substrate in 1 h at 37 °C, under standard reaction conditions. Specific OGG activity was calculated by dividing the activity (in units) by the amount of protein (in micrograms) in the reaction mixture. The activity in each sample was measured in duplicates, unless otherwise stated.

2.5. Competition experiments

In order to establish whether AAG (MPG) activity in PBMC extracts is involved in the removal of 8-oxoG from DNA, competition experiment were performed with an unlabeled duplex oligonucleotide containing a site-specific hypoxanthine (5'-CCGGTGCATGACACTGTHxACCTATCCTCAGCG-3'; Hx, hypoxanthine). The assay was performed under standard reaction conditions, except that the reaction mixture contained either 0.5 pmol or 2 pmol of radiolabeled substrate, and up to 25-fold excess of unlabeled competitor. Similarly, control experiments were performed with an excess of unlabeled duplex oligonucleotide containing a G instead of 8-oxoG (5'-CCGGTGCATGGACTGTGACCTATCCTCAGCG-3'), and with the unlabeled substrate T5.

2.6. Analysis of OGG1 mRNA by quantitative RT-PCR

PBMCs ($\sim 5 \times 10^6$) were stored in 100 μ l RNAlater (Ambion Inc., Austin, TX, USA) overnight at 4 °C followed by storage at –80 °C for 1–2 weeks. Before RNA extraction, an equal amount of PBS was added, cells were pellet by centrifugation, resuspended in 100 μ l PBS, and then homogenized in Tri-Reagent (Molecular Research Center Inc., Cincinnati, OH, USA). Following phase-separation an equal volume of 70% ice-cold ethanol was added to the aqueous layer, and total RNA was further extracted using the RNeasy Mini Kit (Qiagen GmbH, Hilden, Germany). Genomic DNA was eliminated by RNase-free DNase I (Qiagen GmbH) digestion during the isolation procedure. RNA concentration was measured with the NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA), and its integrity assessed (as RNA integrity number, RIN) by the Agilent 2100 Bioanalyzer (Agilent Technologies, Palo

Alto, CA). The threshold inclusion values for RNA samples were $A_{260}/A_{280} > 1.90$ and $RIN > 7$.

First strand cDNA was synthesized from 500 ng total RNA in 20 μ l reaction volume using random primers (Promega Corporation, Madison, WI, USA) and SuperScriptII reverse transcriptase (Invitrogen Corporation, Carlsbad, CA, USA) according to the manufacturer's protocol. The expression level of four genes was measured by quantitative PCR performed on ABI7300 Real-Time PCR System (Applied Biosystems, Foster City, CA, USA). The genes tested included the target gene OGG1, and three reference genes from different biological pathways: hypoxanthine phosphoribosyltransferase 1 (HPRT1), TATA-binding protein (TBP), and β -actin (ACTB). The PCR amplification mixture contained 1/10 volume of the RT reaction, Universal PCR Master kit (Applied Biosystems), and primers and MGB-Taq-Man probes labeled with FAM reporter dye at the 5' end and non-fluorescent quencher at the 3' end (Applied Biosystems). The PCR cycling conditions were set according to manufacturer's instructions. The linearity of the PCR reaction was tested using a series of cDNA dilutions (up to 1:256), giving 85–95% PCR efficiency with a correlation coefficient of 0.99. Linearity of RT reaction was determined by using cDNA made from increasing amounts of total RNA (63 ng–2 μ g RNA), yielding an efficiency of 107–119% with a correlation coefficient of 0.985.

To assess the presence of contamination in the PCR reaction, the reaction for each gene assay was performed without adding cDNA (No Template Control—NTC). Genomic DNA contamination was checked by PCR amplification of RNA samples without cDNA synthesis (-RT). In addition, an RT reaction without RNA, and PCR without the Universal PCR Master kit was checked for reaction products contaminations. All controls gave negative results, indicating no contaminations. The relative expression of OGG1 was normalized to each of the three endogenous reference genes, or to their combination, further normalized to an OGG1 calibrator sample analyzed in all experiments, and finally given as $2^{-\Delta\Delta C_T}$. Samples were assayed in duplicates, and experiments were repeated two to four times with independent cDNA preparations.

2.7. Study subjects

The healthy subjects were volunteers from the Sheba Medical Center, and the Weizmann Institute, including employees, retired employees and their relatives, and healthy individuals who were undergoing routine check-up at the Sheba Medical Center. The study involved only people who never smoked (non-smokers) or who currently smoked (current smokers). Exclusion criteria were prior cancer and previous smoking. Of the 129 healthy subjects who were enrolled, 9 were excluded (3 had cancer; 6 were ex-smokers). Of the 120 subjects, 68 were included in the case-control study published previously, and the additional 52 were individuals whose OGG activity was not reported previously. Among the additional 11 individuals whose blood was assayed for both OGG activity and OGG1 mRNA expression, 8 were healthy volunteers, and 3 were patients with non small-cell lung cancer. Each subject was interviewed by one of the team physicians (MK or AB). A standard questionnaire was used to collect information on demographic data, including smoking status and first-degree

relatives with cancer. All subjects provided written informed consent. The study was approved by the institutional ethics committees of the Sheba Medical Center, and the Weizmann Institute of Science.

2.8. Statistical analysis

A three-way ANOVA was used to compare mean OGG activity values, with gender, age (<55, >55 years), and smoking status as fixed effects. Since a significant interaction was detected between age and gender, a two-way ANOVA was employed for the groups of males and females separately. In addition, to adjust for possible effects originating from the difference in the mean ages between males and females, OGG activity means were compared between males and females, and between smokers and non-smokers, using ANCOVA, with age (treated as a continuous variable) as a covariate. The tests were performed using Statistical Analysis System (SAS) software (Version 8e for Windows; SAS Institute, Cary, NC).

3. Results

3.1. Outline of the OGG activity assay

The OGG test is based on an enzymatic assay that measures the specific activity of the enzyme(s) that remove 8-oxoG from DNA. The source of the enzyme is a protein extract prepared from PBMC (which are mostly lymphocytes), and the substrate is a 5'-labeled synthetic duplex oligonucleotide carrying a single site-specific 8-oxoG. OGG activity removes the 8-oxoG from the substrate, leaving an abasic site, which is then cleaved by an AP endonuclease activity present in the extract. To ensure that all the abasic sites created by OGG are cleaved, the substrate was treated with alkali prior to analysis. Analysis was done by urea-PAGE of the reaction mixture, followed by visualization and quantification of the substrate oligonucleotide, and the shorter product oligonucleotide. Similar assay types are routinely used in the study of DNA N-glycosylases (e.g., Refs. [27,28]).

3.2. Development of a protocol for the preparation of protein extracts from PBMC

In the ideal case, a protein extract prepared to assay a specific enzyme, accurately represents the activity of the enzyme in the cell. This may be difficult to achieve since extraction may be incomplete, and/or it may extract cellular components that are inhibitory to the enzymatic assay in the test tube (but not *in vivo*). Nevertheless, by carefully optimizing the extraction procedure reliable enzyme assays in crude extracts can be developed. Here we describe the development of the extraction procedure for the OGG1 enzymatic activity.

3.2.1. Cell lysis

Several methods of lysing PBMC were examined, and the extracts obtained were tested for specific activity of OGG. Those included freeze-thaw cycles in a hypotonic buffer, shearing by passage through a syringe needle, sonication, and detergent treatment (NP40, triton X-100, and CHAPS) (Fig. 1A).

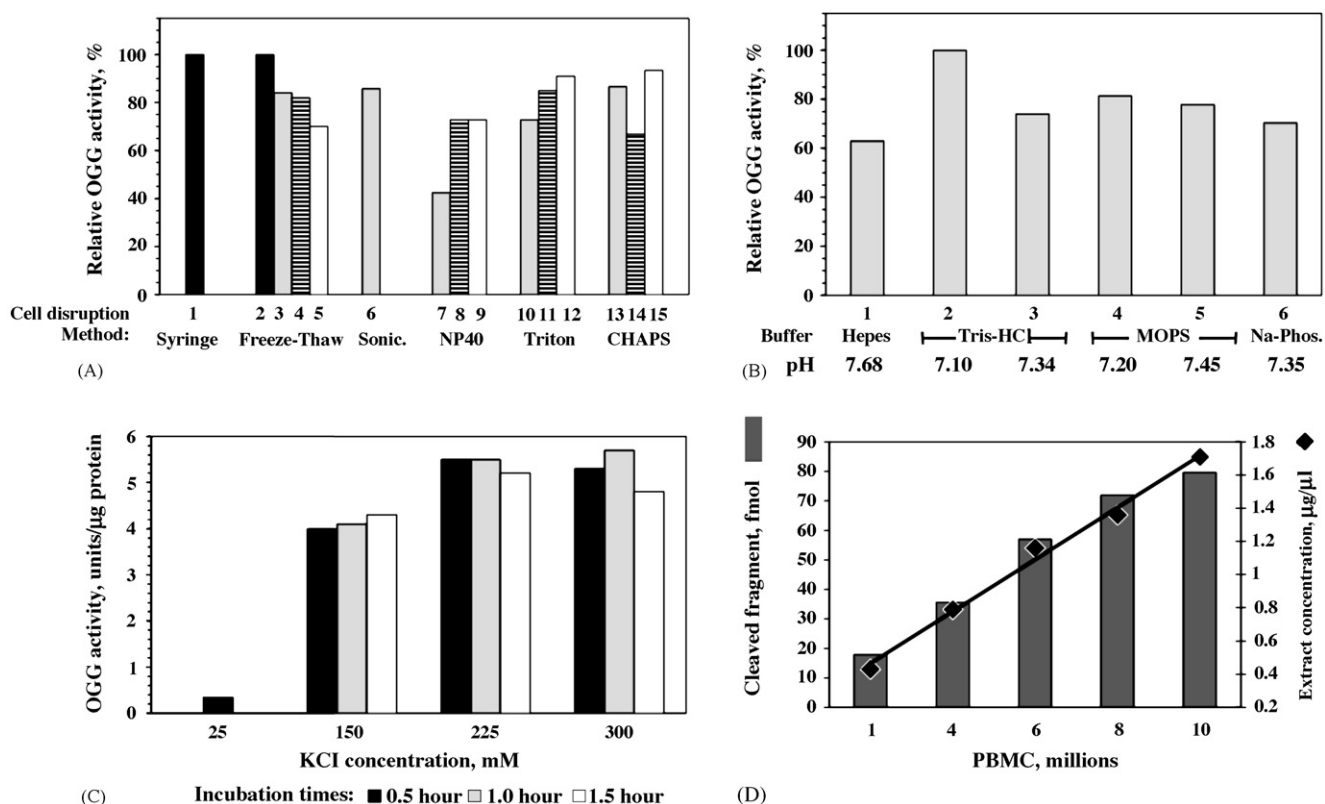


Fig. 1 – Optimizations of the preparation of protein extracts. Protein extracts were prepared under various conditions and assayed for OGG activity. (A) PBMC frozen in hypotonic buffer were quickly thawed in a water bath at 30 °C followed by extraction using either shearing by passage (column 1), additional 0–3 freeze-thaw cycles (columns 2–5, respectively), sonication (column 6), or detergent treatment (columns 7–15). Columns 7–9, NP40 at final concentrations of 0.12, 0.06, and 0.03%, respectively; columns 10–12, triton X-100 at final concentrations of 0.12, 0.06, and 0.03%, respectively; columns 13–15, CHAPS at final concentrations of 0.17, 0.09, and 0.04%, respectively. (B) Extracts were prepared from PBMC samples each frozen in a hypotonic solution with one of the indicated buffers at a concentration of 50 mM, and the indicated pH. (C) PBMC thawed at 30 °C were incubated with KCl at the indicated concentrations and time periods. (D) PBMC at concentrations of 10,000–50,000 cells/μl were suspended in hypotonic buffer to a final number of 2–10 million cells, after which extracts were prepared and assayed for protein concentration (black diamonds) and OGG activity (grey columns). The details are presented under Section 2.

The highest specific activity was obtained with extracts prepared by the syringe and freeze-thaw procedures, which are the mildest of all the extractions examined (Fig. 1A, columns 1 and 2). OGG specific activity slightly decreased with increasing the number of freeze-thaw cycles from 1 to 3 (Fig. 1A, columns 2–5). Extracts prepared by sonication, a more harsh extraction method, yielded 10–20% lower specific activities (Fig. 1A, column 6). The three detergents were inhibitory, yielding extracts with OGG activity that decreased with increasing concentration of the detergent used for cell lysis (Fig. 1A, columns 7–15). NP40 extraction yielded the lowest OGG activity (Fig. 1A, columns 7–9). These results mean that once the isolated PBMC were frozen in the hypotonic solution, no other treatment is required for their lysis, except thawing them when starting the protein extraction stage. This method is simple and convenient, and safer than the syringe method, a significant advantage when working with human blood samples.

3.2.2. Choice of buffer and salt concentration

To select the buffer to be used in the extraction procedure we first examined the effects of various buffers on OGG nicking reaction, using an extract prepared in 25 mM HEPES-NaOH pH 7.8. The buffers used included Tris-HCl, MOPS-KOH, Na-Phosphate, and HEPES-NaOH, at a pH range of 6.9–8.1. The buffers that yielded the highest OGG activity were then each used to extract a separate sample of PBMC (from the same donor), and the extracts obtained were assayed for OGG activity (Fig. 1B). The highest OGG activity was obtained by extracting the PBMC in a buffer containing 50 mM Tris-HCl pH 7.1 (Fig. 1B, column 2). Salt is used to facilitate the extraction of nuclear proteins. Addition of KCl during extract preparation increased OGG activity, with little dependence on extraction time in the range of 0.5–1.5 h (Fig. 1C). A 30 min extraction period with 222 mM KCl was selected for the final extraction protocol.

3.2.3. Effect of PBMC concentration on protein extraction

Since the number of PBMC in human blood samples may vary, we examined whether the protein extraction procedure is affected by cell concentration and amount. To that end we used 2–10 million PBMC that were isolated from the same donor, and suspended them at increasing cell concentrations from 10,000 to 50,000 cells/ μ l. As can be seen in Fig. 1D, both the protein concentration and OGG activity of these extracts were linear with the initial number of cells. The specific activity of OGG in each of these extracts was calculated yielding a mean value of 6.1 ± 0.7 units/ μ g protein, representing a CV of 12%.

3.2.4. Reproducibility of protein extraction

The reproducibility of the protein extraction protocol was examined by comparing multiple blood samples from the same individual on 3 different days. This was performed with blood obtained from two blood bank donors. A volume of 120 ml blood was obtained from each donor, the PBMCs isolated, divided into 12 equal portions (each containing 4 million cells at 20,000 cells/ μ l), and stored at -80°C . For each donor protein extracts were prepared from three to four samples in parallel, on 3 different days. The extracts were stored at -80°C , and OGG activity was assayed on the same day for the multiple samples obtained from the same donor. As can be seen in Table 1 the mean OGG activity values obtained were 4.7 ± 0.13 and 3.8 ± 0.23 for donors 1 (11 extracts) and 2 (12 extracts), respectively. The low CV values of 3% and 6% obtained in these extractions indicate a highly reproducible extraction procedure.

3.3. Optimization of assay conditions

Assay conditions were rigorously examined for the development of a quantitative, reproducible, and robust assay, suitable for epidemiological studies. This included analysis of the effects of substrate concentration, salts concentration, metal ions, stabilizing agents like bovine serum albumin, bovine γ -globulin and polyethylene glycol, and nuclease inhibitors like EDTA. Here we present the key optimization experiments. Our criteria for the optimization procedure were: (1) the highest OGG activity level, aiming at 5–10% substrate conversion within a 30 min reaction time. (2) Linear kinetics within a broad reaction time range. (3) A linear

range of activity within a reasonably broad range of protein extract.

3.3.1. Nuclease inhibition

A common problem in assays such as the OGG assay when performed with crude protein extracts is the presence of interfering non-specific nucleases, which may degrade the substrate, and phosphatases, which may remove the $5'$ - ^{32}P radioactive label of the substrate. Under the OGG assay conditions, we found no loss of radioactivity from the substrate, suggesting lack of interfering phosphatase activity in the extract. The extracts do contain a non-specific nuclease activity, evident by degradation of the substrate when assayed in the presence of Mg^{2+} (data not shown). However, as OGG activity does not require Mg^{2+} , assays can be performed in the presence of 1 mM EDTA, conditions under which the interfering nuclease activity was essentially eliminated. Addition of synthetic polynucleotides to the reaction mixture can be used as an additional mean to reduce non-specific nuclease activity. In the presence of EDTA, the addition of polydA-polydT had no effect on non-specific nuclease background activity, or on OGG activity. However, we decided to add 2 pmol polydApolydT to the standard reaction mixture as a preventive mean to potentially cover the possibility of increased nuclease activity in some human blood samples, due to inter-individual variability.

3.3.2. Salt and DNA substrate concentrations

Titration of KCl concentrations showed an optimal OGG activity at 115 mM (Fig. 2A). Lower KCl concentrations slightly reduced OGG activity, whereas higher concentrations caused a strong inhibition (Fig. 2A). Excision of 8-oxoG by purified OGG1 does not follow Michaelis-Menten kinetics, because the enzyme does not turn over. However, in the presence of human AP endonuclease (APE1) the specific activity of OGG1 increases ~ 5 -fold, and the enzyme follows Michaelis-Menten kinetics [29]. The K_M for the excision of 8-oxoG by human OGG1 is 9.9 nM [29,30]. We therefore examined the effect on OGG activity of DNA concentration range of 25–150 nM, representing concentrations of up to 15-fold higher than the K_M . As can be seen in Fig. 2B, the rate of cleavage approached saturation at the higher concentration range, consistent with a significant excess of substrate concentration. Under these conditions less than 2% of the substrate is converted to product, causing a

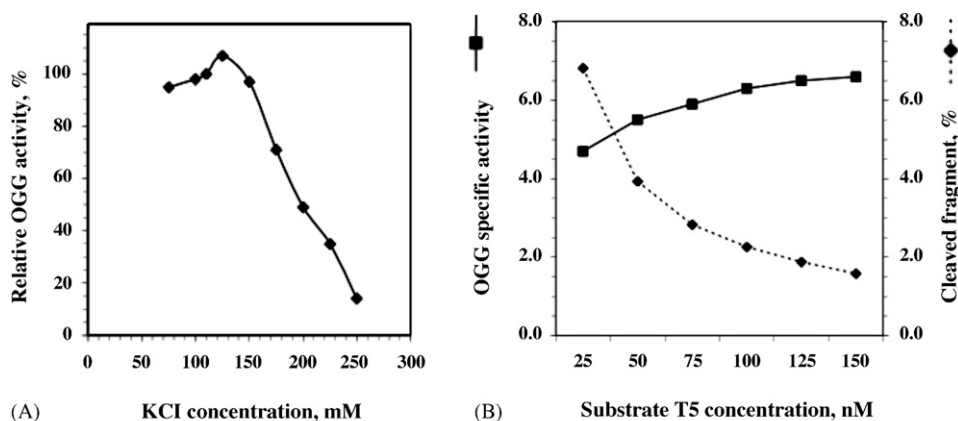
Table 1 – Analysis of the reproducibility of protein extraction

Extraction day	Samples from Donor 1				Samples from Donor 2			
	No.	Average OGG activity	STDEV ^a	CV ^b (%)	No.	Average OGG activity	STDEV ^a	CV ^b (%)
1	4	4.6	0.15	3	4	3.6	0.35	10
2	4	4.7	0.13	3	4	3.7	0.10	3
3	3	4.7	0.06	1	4	3.9	0.08	2
Average	11	4.7	0.13	3	12	3.8	0.23	6

PBMC isolated from 120 ml blood obtained from each of two blood bank donor were divided to 12 identical portions each containing 4 million cells. For each donor extracts were prepared from three to four samples in parallel, on 3 different days.

^a Standard deviation.

^b Coefficient of variation.



Substrate	Length (bp)	Sequence	Relative activity (%)
T1	45	5' -GCCTAGTGACTGCAGCCAATCA <u>X</u> TGCACCATCCATTCGCTCGTG	84
T2	50	5' -CCTGCATGGGCGGCATGAACCGGA <u>X</u> GCCCATCCTCACCATCATCACACTG	72
T2A	32	5' -GGGCGGCATGAACCGGA <u>X</u> GCCCATCCTCACCA	24
T3	48	5' -CAACTGGCCAAGACCTGCCCTGTGCA <u>X</u> CTGTGGGTTGATTCCACACCC	38
T3A	32	5' -AAGACCTGCCCTGTGCA <u>X</u> CTGTGGGTTGATTTC	3
T4	45	5' -GTCATCCGGTGCATGACACTGT <u>X</u> ACCTATCCTCAGCGAGATCGTG	31
T5	32	5' -CCGGTGCATGACACTGT <u>X</u> ACCTATCCTCAGCG	100
T5B	28	5' -GGTGCATGACACTGT <u>X</u> ACCTATCCTCAG	24
T5C	24	5' -TGCATGACACTGT <u>X</u> ACCTATCCTC	61

(C)

Fig. 2 – Effects of salt and oligonucleotide substrate on OGG activity. (A) Effect of KCl concentration on OGG activity, using substrate T5. (B) Effect of concentration of substrate T5. (C) Effect of substrate length and sequence. Nine different oligonucleotides (35 nM each) were used as indicated. OGG specific activities with the various substrates are presented relative to substrate T5, which was set to 100%. Reactions were performed at 37 °C for 1 h. See Section 2 for details.

rather low signal to noise ratio. Therefore, the standard substrate concentration was set to 25 nM, under which 5–10% of the substrate is converted to product.

3.3.3. Effect of substrate sequence and length on OGG activity

The DNA sequence context frequently affects the activity of DNA repair enzymes, although the basis for this effect is usually not fully understood (e.g., Refs. [31–34]). We therefore studied OGG activity on 8-oxoG in nine oligonucleotides covering four different sequence contexts (Fig. 2C). T1 was derived from an oligonucleotide previously used as a substrate for OGG activity, T2 and T2A were derived from the p53 gene, T3 and T3A were derived from a different region of the p53 gene, and T4, T5, T5B, and T5C were oligos of different lengths, all sharing a common, randomly selected, DNA sequence context in the vicinity of the 8-oxoG (Fig. 2C). OGG assays were performed

with these substrates, using several different extracts. As can be seen in Fig. 2C, the different substrates yielded big differences of up to 33-fold in OGG activity. These changes were caused by DNA sequence context effects (compare substrate T3A to T5, both 32 bp long), as well as substrate length effects (compare substrates T5 to T5B, and T3 to T3A, each pair having a common sequence). However, no simple rules could be deduced from this collection of nine substrates. The best substrate for OGG activity was substrate T5, a 32 bp long oligonucleotide with a randomly selected DNA sequence. This substrate was selected as standard substrate for the OGG assay.

3.3.4. OGG assay kinetics, titration, and specificity

Representative OGG activity kinetics and titration experiments are presented in Fig. 3. DNA cleavage occurred only in the substrate containing 8-oxoG and not in the control substrate containing an unmodified guanine residue instead of

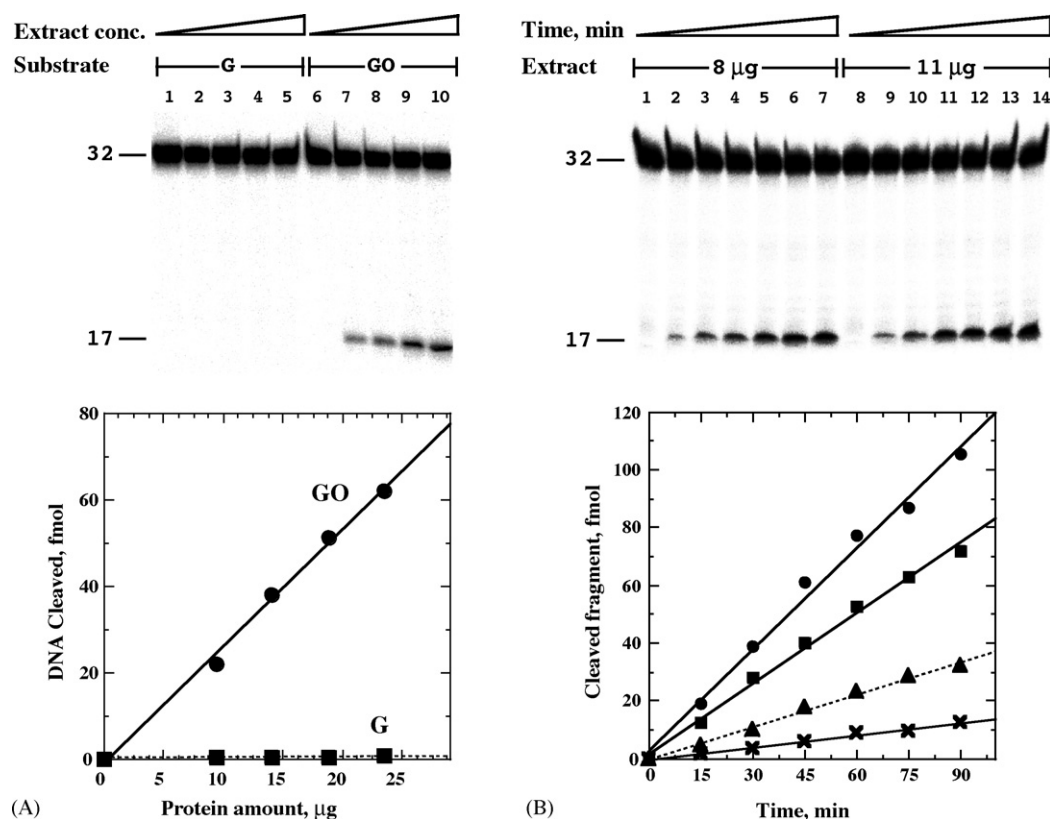


Fig. 3 – Protein extract titration and time course of the OGG activity reaction. Reactions were performed with a protein extract prepared from PBMC and substrate T5. The upper panels show gel images of ^{32}P -labeled reaction products. The 32-mer oligonucleotide band corresponds to the non-cleaved substrate, and the 17-mer oligonucleotide band corresponds to the OGG reaction product. The lower panels present quantification of gel images similar to those presented in the upper panels. (A) The effect on OGG activity of increasing protein extract amount. Reactions were performed with a control substrate having a G instead of 8-oxoG (lanes 1–5, marked G), and with substrate T5 (lanes 6–10, marked GO). Protein extract amounts of 0, 9.4, 14.1, 18.8, and 23.5 μg were used in each group of lanes 1–5, and 6–10, respectively. Reactions were performed with 25 nM substrate at 37 °C for 1 h. (B) Time course of OGG activity. Lanes 1–7 represent reaction times of 0, 15, 30, 45, 60, 75, and 90 min, respectively, for a reaction with 8 μg extract, and lanes 8–14 represent the respective times of a reaction performed with 11 μg extract. The lower panel shows the kinetics of reactions performed with various protein concentrations, derived from the gel image in the upper panel for 8 μg (squares) and 11 μg (circles) extract, and similar gels obtained with 2 μg (x) and 4 μg (triangles) extract/reaction.

8-oxoG (Fig. 3A, compare lanes 1–5 to lanes 6–10). Cleavage was linear with protein extract concentration up to 25 μg /reaction (Fig. 3A). In addition, a linear increase in DNA cleavage was observed with increasing reaction time up to 90 min (Fig. 3B).

The specificity of the OGG assay is determined by the damaged nucleotide in the substrate, namely 8-oxoG. Therefore, the assay measures total 8-oxoguanine DNA glycosylase activity in the extract, and not specifically OGG1. However, OGG1 was shown to be responsible for most of OGG activity in extracts prepared from human cells [35]. In addition to OGG, APNG (alkylpurine DNA N-glycosylase), also termed AAG (alkyladenine DNA glycosylase), or MPG (N-methylpurine glycosylase), was reported to act on 8-oxoG [36]. *In vivo* this protein has no significant role in removing 8-oxoG from DNA, at least in mice [37,38]. In order to establish whether MPG is involved in the removal of 8-oxoG from DNA by lymphocyte extracts, we have performed a competition experiment with an unlabeled duplex oligonucleotide containing

a site-specific hypoxanthine (a substrate of MPG but not for OGG1 [37,38]). As can be seen in Fig. 4, this duplex oligonucleotide did not inhibit the incision of the 8-oxoG-containing DNA by the extract, suggesting that MPG is not involved in the incision reaction. A control experiment with an excess of unlabeled duplex oligonucleotide containing a G instead of 8-oxoG showed no inhibition either, whereas a duplex oligonucleotide containing 8-oxoG-DNA did cause inhibition, as expected (Fig. 4). These competition experiments suggest that MPG is not involved in the observed OGG activity in our assay.

3.4. Reproducibility of the OGG test

High reproducibility is critical to the usefulness of the OGG assay. We examined reproducibility by processing in parallel 12 blood samples from the same donor, and assaying OGG specific activity for each sample independently. As can be seen in

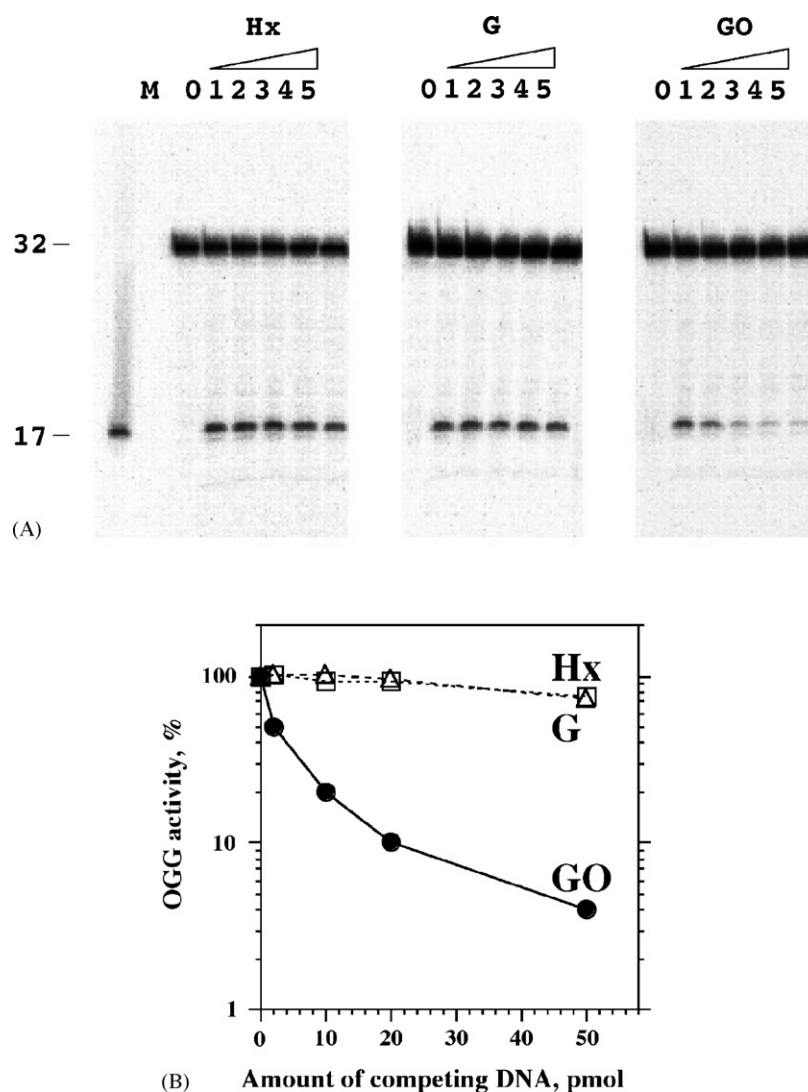


Fig. 4 – Analysis of the specificity of the OGG reaction by competition experiments. OGG reactions were performed under standard reaction conditions, except that the reaction mixture contained 2 pmol radiolabeled T5 substrate and increasing amounts of unlabeled competitor, as indicated. The reactions were incubated at 37 °C for 1 h. Hx, a duplex oligonucleotide similar to T5, except that it had a hypoxanthine instead of 8-oxoG; G, the control oligonucleotide with a G instead of 8-oxoG; GO, unlabeled substrate T5. (A) Gel image of the competition experiments. Lanes marked 0 contained no extract, and lanes marked 1 contained no competitor. Each group of lanes marked 2–5 contained reactions with 2, 10, 20, and 50 pmol of a competitor, respectively. (B) Quantification of the gel image shown in A performed using phosphorimaging.

Table 2A, similar OGG specific activity was obtained in all 12 samples. The mean value was 6.7 ± 0.4 units OGG/ μ g protein, representing a coefficient of variation of 6%. To further determine specifically the reproducibility of the nicking reaction we processed in parallel six different blood samples from a single donor, prepared protein extract from each sample, and assayed OGG activity of these six extracts repeatedly on 3 different days. Highly reproducible results were obtained for the different samples assayed in the same experiment (average CV = 5%; Table 2B), and for the same sample assayed on 3 different days (average CV = 7%; Table 2B). The overall coefficient of variation for all the samples in all the experiments was 7%. These results indicate that the OGG test is highly reproducible in all of its stages, with a coefficient of variation of 10% or

lower. In addition, we found that OGG activity is stable over at least 3 years in the same individual [10].

3.5. Stability of stored frozen PBMC and protein extracts

The stability of frozen PBMC as a source for protein extracts for the OGG assay was examined by comparing extracts prepared from PBMC, and assayed for OGG activity within 1–14 days, and after an extended storage period at -80°C . As can be seen in Table 3A, storing PBMC at -80°C did not significantly affect their OGG activity. Similarly, examination of the activity of protein extracts stored at -80°C for up to 3 years showed essentially no loss of activity in the first 2 years, but there

Table 2 – Reproducibility of the OGG activity assay(A)^a

Blood sample #	OGG activity (units/ μ g protein)	Blood sample #	OGG activity (units/ μ g protein)
1	6.8	7	6.9
2	6.9	8	6.5
3	6.4	9	6.6
4	6.5	10	6.7
5	6.7	11	5.9
6	7.4	12	6.9

Average OGG activity: 6.7 ± 0.4 (CV = 6%)(B)^b

Blood sample #	OGG activity (units/ μ g protein)				
	Day 1	Day 2	Day 3	Average \pm STDEV by sample	CV (%)
1	6.7	7.9	6.9	7.2 ± 0.6	8
2	7.2	6.9	7.0	7.0 ± 0.2	3
3	6.7	7.8	7.1	7.2 ± 0.6	8
4	6.8	8.2	7.3	7.4 ± 0.7	9
5	7.1	8.1	7.9	7.7 ± 0.5	6
6	6.7	7.8	7.2	7.2 ± 0.6	8
Average \pm STDEV by day	6.9 ± 0.2	7.8 ± 0.5	7.2 ± 0.4		
CV (%)	3	6	6		

Overall average OGG activity: 7.3 ± 0.5 (CV = 7%)^a Twelve blood samples from the same donor were processed in parallel, and analyzed for OGG activity.^b Extracts were prepared in parallel from six blood samples from a single donor. The OGG activity assay was repeated with these extracts on 3 different days.**Table 3 – Stability of stored frozen PBMC and protein extracts**(A) Stability of frozen PBMC^a

Blood sample #	PBMC storage time (months)	OGG activity (units/ μ g protein)		
		Before storage	After storage	Ratio
1	16	5.7	5.1	0.89
2	16	4.9	4.9	1.00
3	16	5.3	5.0	0.93
4	17	6.3	6.0	0.96
5	17	6.4	6.0	0.93
6	18	6.9	7.1	1.03
7	23	7.5	6.8	0.90
8	25	5.9	6.2	1.05
9	26	5.8	6.0	1.04
10	26	6.0	5.3	0.89
				0.96 ± 0.06^b

(B) Stability of protein extracts^c

Extract storage time (years)	Number of samples	Mean OGG activity ratio after/before storage
0	8	1.00 ± 0.04
≤ 1	33	1.00 ± 0.10
1-2	42	0.97 ± 0.08
2-3	11	0.93 ± 0.09
		0.98 ± 0.09^b

^a PBMC isolated from 10 blood samples were frozen in two portions. Extracts were prepared from the PBMC either within 1-14 days (before storage) or after the indicated storage time at -80°C , and assayed for OGG activity.^b Overall average.^c Protein extracts were prepared from PBMC isolated from 120 ml blood obtained from two blood bank donors, divided into $30\mu\text{l}$ aliquotes, frozen in liquid nitrogen and stored at -80°C . OGG activity was determined within a month (before storage), or periodically during storage of up to 3 years at -80°C . The ratio of OGG activity after/before storage was calculated for each sample, and the average for each year of storage is presented.

might be a slight loss of activity during the third year (Table 3B). Thus, both PBMC and proteins extracts can be stored for prolonged periods at -80°C , without a significant loss in OGG activity.

3.6. Distribution of OGG activity in a group of healthy individuals

OGG activity was determined in PBMC obtained from a group of 120 healthy individuals. As can be seen in Fig. 5A and Table 4, there was a distribution of OGG activity, with a mean value of 7.2 (95% CI 7.02–7.39) units/ μg protein. The lowest OGG value obtained was 3.6 units/ μg protein and the highest was 10.1 units/ μg protein, defining a 2.8-fold range of inter-individual variability in OGG activity. In order to analyze the effects of smoking status (smoker/non-smoker) and age (≤ 55 , >55 years) on OGG activity, a two-way ANOVA was performed separately for males and females (due to an interaction between gender and age). The model for males was significant ($P=0.0021$), while the model for females was not ($P=0.77$). Interestingly, there was a gender-specific age effect on OGG activity (Table 4 and Fig. 6). Men at the age of 55 years or younger had a mean OGG activity value of 7.7 (95% CI 7.39–8.05), compared to a value of 6.8 (95% CI 6.44–7.23) in younger men. This represents a small (11.7%), but statistically significant ($P=0.0064$), decrease with age in the mean OGG activity values of males (Table 4 and Fig. 6A). In contrast, we found no difference between the mean OGG activity values of women at the age of 55 years or younger (7.2; 95% CI 6.91–7.56) compared to older women (7.0; 95% CI

Table 4 – Distribution of selected characteristics and OGG activity values among healthy subjects					
Variable	No.	OGG values (95% CI) ^a	P	Min ^b	Max ^c
All	120	7.2 (7.02–7.39)		3.6	10.1
Age (years)					
Males					
≤ 55	26	7.7 (7.39–8.05)		5.7	9.1
>55	26	6.8 (6.44–7.23)	0.0064 ^d	5.6	10.1
Females					
≤ 55	37	7.2 (6.91–7.56)		3.6	9.2
>55	31	7.0 (6.65–7.40)	0.88 ^d	4.4	9.3
Sex					
Male	52	7.3 (7.00–7.57)		5.6	10.1
Female	68	7.1 (6.89–7.39)	0.12 ^e	3.6	9.3
Smoking status					
Non-smoker	85	7.1 (6.92–7.37)		4.4	10.1
Smoker	35	7.3 (7.00–7.69)	0.84 ^e	3.6	9.2

OGG activity was determined using 10 ml blood samples obtained from 120 healthy subjects.

^a Mean OGG activity and 95% confidence interval.

^b The minimal OGG activity in the group.

^c The maximal OGG activity in the group.

^d P by two-way ANOVA.

^e P by covariate analysis. The P value of the model was significant (0.0475).

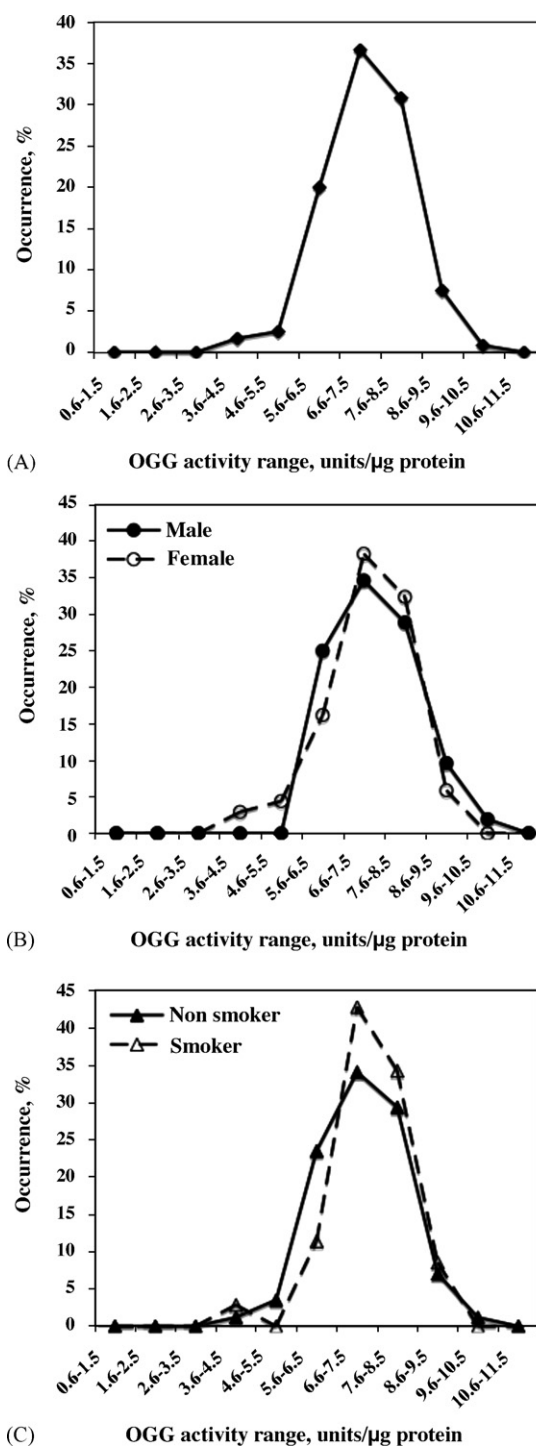


Fig. 5 – Distribution of OGG activity in a group of 120 healthy individuals. OGG activity was measured in protein extracts prepared from 10 ml blood samples obtained from 120 healthy subjects. (A) Distribution of OGG specific activity values of all 120 healthy subjects. (B) Sub-group comparison of OGG activity in 68 female subjects (white circles) and 52 male subjects (black circles). (C) Sub-group comparison of OGG activity in 85 non-smokers (black triangles) and 35 smokers (white triangle).

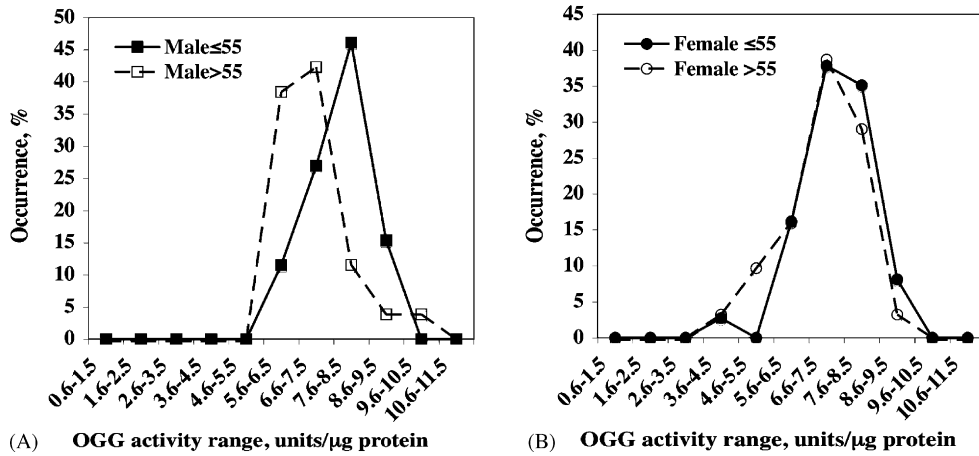


Fig. 6 – Age sub-group analysis of OGG activity in females and males. OGG activity values of the 120 individuals were plotted for females 55 years old or younger ($N=37$), compared to older females ($N=31$) (panel A), and for males 55 years old or younger ($N=26$), compared to older males ($N=26$) (panel B).

6.65–7.40), where $P=0.88$ (Table 4 and Fig. 6B). Smoking had no significant effect on OGG activity, neither in females nor in males.

Since the mean age of the males group (58.9 ± 13 years) was slightly different from the mean age in the females group

(54.6 ± 14 years; $P=0.09$), analysis of covariance was used to compare age-adjusted mean OGG activity values. There was no significant difference between the mean OGG activity values of men (7.3; 95% CI 7.00–7.57) and women (7.1; 95% CI 6.89–7.39), where $P=0.12$ (Fig. 5B and Table 4), or between

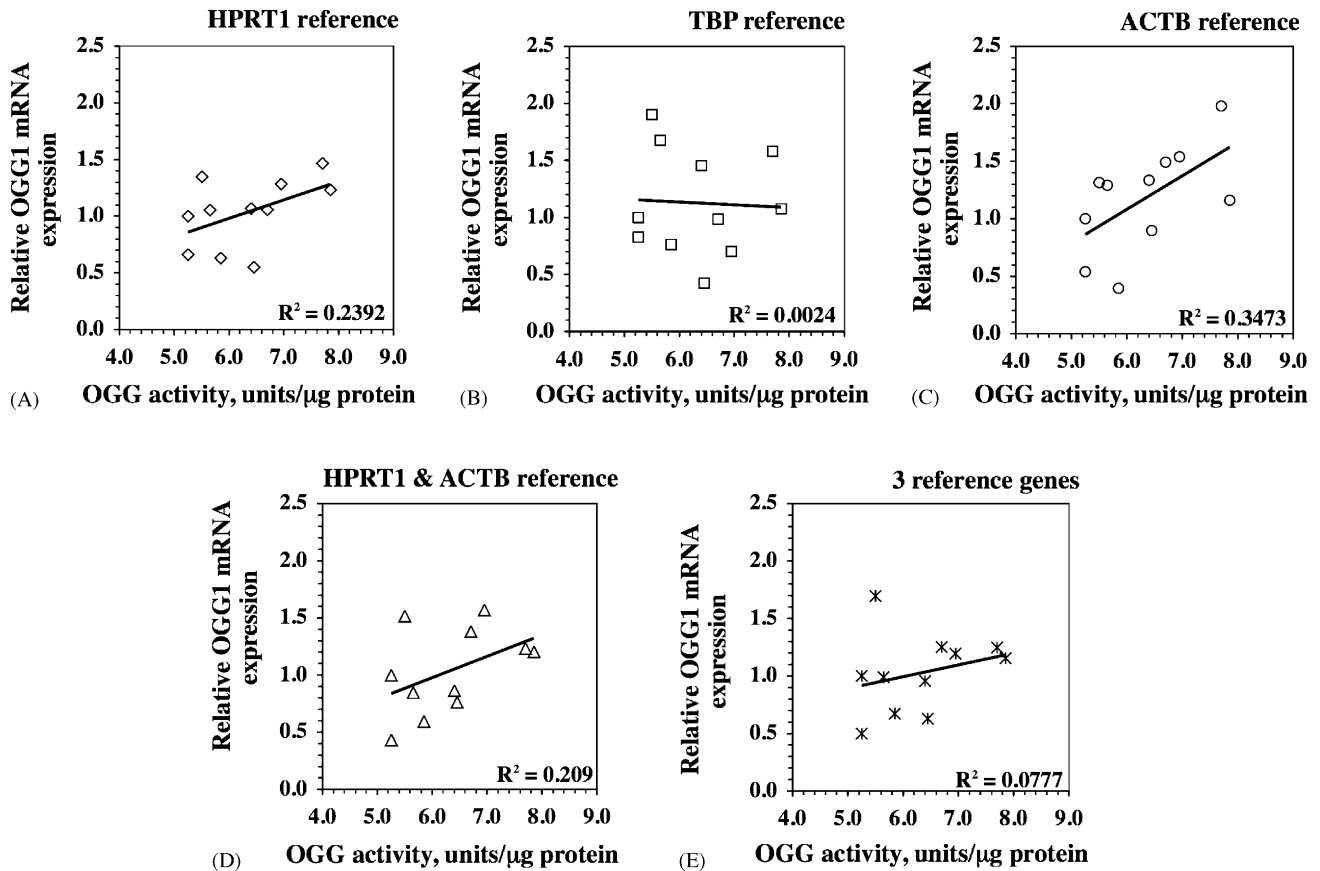


Fig. 7 – The relationship between OGG1 mRNA expression and enzymatic activity in human PBMC. Relative expression of OGG1 mRNA in PBMC from 11 individuals (8 healthy, 3 lung cancer patients) was determined by quantitative real-time RT-PCR, and plotted against their OGG activity. The detailed procedure is described under Section 2, and the numerical data are presented in Table 5.

Table 5 – Relationship between OGG enzymatic activity and expression of OGG1 mRNA

No.	Health status ^a	OGG activity ^b	Relative OGG1 mRNA expression					
			Ref. gene ^c	HPRT1	TBP	ACTB	HPRT1 & ACTB	HPRT1 & TBP & ACTB
1	H	5.3 ± 0.3		1.00	1.00	1.00	1.00	1.00
2	L	5.3 ± 0.3		0.66	0.83	0.54	0.43	0.50
3	H	5.5 ± 0.1		1.35	1.90	1.32	1.52	1.70
4	H	5.7 ± 0.1		1.05	1.68	1.29	0.85	0.99
5	L	5.9 ± 0.3		0.63	0.76	0.40	0.60	0.67
6	H	6.4 ± 0.2		1.07	1.45	1.33	0.87	0.96
7	L	6.5 ± 0.3		0.55	0.42	0.90	0.76	0.63
8	H	6.7 ± 0.3		1.06	0.99	1.49	1.38	1.25
9	H	7.0 ± 0.2		1.29	0.70	1.54	1.57	1.19
10	H	7.7 ± 0.3		1.47	1.58	1.98	1.24	1.25
11	H	7.9 ± 0.1		1.24	1.08	1.16	1.20	1.16

OGG enzymatic activity and OGG1 mRNA were determined in PBMC obtained from 11 individuals. Relative mRNA expression levels are given based on the indicated reference genes, or their combinations. See Section 2 for details.

^a H, healthy individual; L, lung cancer patient.

^b OGG activity in units/μg protein extract.

^c The following reference genes were used: HPRT1, hypoxanthine phosphoribosyltransferase 1; TBP, TATA-binding protein, and ACTB, β-actin.

smokers (7.3; 95% CI 7.00–7.69) and non-smokers (7.1; 95% CI 6.92–7.37), where $P = 0.84$ (Fig. 5C and Table 4).

3.7. OGG1 mRNA expression poorly correlates with OGG enzymatic activity

To examine whether the inter-individual variation in OGG enzymatic activity results from differences in OGG1 mRNA expression we compared the two in PBMC from 11 individuals. Expression of OGG1 mRNA was determined using real-time quantitative RT-PCR, with three different reference genes: hypoxanthine ribosyl transferase (HPRT), TATA-binding protein (TBP), and β-actin (ACTB). The measurements of the C_T values for each of the genes were highly reproducible, with a coefficient of variation of 1–2%. Fig. 7 and Table 5 show the relationship between OGG activity and the relative expression of OGG1 mRNA in the 11 individuals, calculated separately relative to each reference gene, as well as two or three reference genes combined. With two of the reference genes, namely HPRT1 (Fig. 7A) and ACTB (Fig. 7C), the group trend was an increase in OGG enzymatic activity with increasing OGG1 mRNA expression. A similar result was obtained based on the mean of these two reference genes (Fig. 7D), or the mean of all three reference genes (Fig. 7E). In contrast, with TBP as a reference, there was no such correlation (Fig. 7B). Regardless of the reference gene, when compared in each individual separately, OGG enzymatic activity correlates very poorly with the expression of OGG1 mRNA (Table 5), indicated by the very low R^2 values of the linear fits (Fig. 7).

4. Discussion

Based on our current understanding of the molecular mechanisms underlying cancer, and in particular the essential role of mutations in oncogenes and tumor suppressor genes, DNA repair is expected to play a major role in cancer. This is indeed well established in hereditary cancer, as discussed in Sec-

tion 1, and an increasing body of research indicates that the same is true also for sporadic cancer [6–13], which accounts for approximately 85% of all cancer cases. Clearly, additional experimental tools, namely reliable and epidemiology-grade DNA repair assays will provide the means for further mechanistic insight into the role of DNA repair in human cancer. The term DNA repair represents a multitude of pathways involving over 100 proteins [39], directed to both specific DNA lesions, as well as groups of DNA lesions [2]. The complexity of DNA repair makes it unlikely that a single ‘general DNA repair’ assay be developed, and in any case general assays might obscure involvement of specific pathways or enzymes. Instead, specific DNA repair assays, targeting either particular DNA lesions, or specific DNA enzymes or pathways are expected to be more powerful.

Analysis of genetic polymorphism is widely used as a tool for elucidating the role of particular DNA repair genes in human cancer [17,18,40]. From the standpoint of cancer risk assessment this approach has two main advantages: (a) polymorphism is a germ line trait, and therefore expected to be present in all tissues. This means that a blood test faithfully represents the situation in other target organ, (b) analysis of genetic polymorphism is epidemiology compatible: it is simple, rapid, reliable, and high-throughput. However, genetic polymorphism is often a poor predictor of complex traits, such as the efficiency of DNA repair, because of two main reasons [41]: (a) efficiency of a particular DNA repair enzyme or pathway is determined by dozens of proteins, most of which unknown yet, that affect protein expression, stability, and activity, (b) environmental and lifestyle factors are not taken into account. This seems to be an important variable in human biology, although it is usually poorly defined, and difficult to analyze.

Functional specific DNA repair assays can potentially overcome the limitations of the genetic polymorphism approach, because they measure the actual performance of particular DNA repair enzymes or pathways, thereby integrating the effects of a large number of genetic polymorphisms, as well

as potential environmental and lifestyle effects on DNA repair activity. The two main limitations of the functional assay approach are its higher complexity, and the need to use a surrogate tissue, often blood. Since many proteins exhibit tissue specific variation, the concern is that measuring a particular repair activity in the blood might not necessarily be a good surrogate for an internal organ, which is the target of research. The critical parameter is not the absolute level of the DNA repair activity, which does vary from tissue to tissue. It is sufficient that the repair activity in PBMC and in an internal organ in a group of individuals show the same *relative* distribution. Namely, if the activity of a repair enzyme in PBMC from an individual A, is higher than in PBMC from an individual B, than the activity in the lung of individual A is higher than in the lung of individual B. Such a condition may indeed be fulfilled for some, but not necessarily all tissues and repair activities. This concern can be addressed, for some cancers, by measuring whether the activity under study in the blood is linearly correlated to the activity in the target organ in a group of individuals. Such an approach indicated that OGG enzymatic activity in PBMC is linearly correlated to OGG activity in the non-tumor lung tissue in non-small cell lung cancer [10].

We took the single DNA repair enzyme approach, since it is expected to be simpler, and at the same time specific. We focused on the repair of 8-oxoG, a common lesion formed in DNA by oxidative stress, radiation, and tobacco smoke. The main pathway for removing 8-oxoG from DNA is base excision repair, initiated by OGG1 [2]. It was shown that OGG1 activity is the rate-limiting step in the BER of 8-oxoG in cell extracts ([42] and our unpublished results). Therefore, we chose to assay OGG, based on its activity to remove a site-specific 8-oxoG from a synthetic duplex oligonucleotide, an assay routinely used in basic DNA repair research. The assay specificity is dictated by the lesion in the substrate, rather a specific enzyme, implying that any DNA glycosylase that acts on 8-oxoG will contribute to the observed nicking activity. It was reported that OGG1 activity is responsible for 90% of OGG activity in human cell extracts [35]. However, there might be a contribution from NEIL1, which was reported to act on 8-oxoG [43]. AAG (ANPG, MPG) does not contribute to the removal of 8-oxoG under our assay conditions. It was reported that APE1 (HAP1) [29] and NEIL1 [44] stimulate the activity of OGG1, by increasing its turnover. Thus, OGG activity may be modulated not only by the level of OGG1, but also by inter-individual variations in APE1 and NEIL1, underscoring the advantage of using an activity assay, which integrates also stimulators and activators present in the cell.

Developing an epidemiology-grade assay required special considerations, which are usually not addressed in regular basic research laboratory practice. The critical features of such an assay are simplicity, reproducibility, stable storage of cells and extracts, and a potential for high throughput application. This required considerable optimization work, and validation of reproducibility by several laboratory workers performing the assay with the same blood samples, on different days. The goal was to obtain similar OGG assay results with a coefficient of variation of no more than 10%. This is critical, since differences among individuals are often small. Indeed, as presented in this work, OGG activity, as assayed in 120 healthy individ-

uals, spans a 2.8-fold range, from 3.6 to 10.1 units/ μ g protein. This narrow range is much smaller than previously reported ranges of inter-individual variations in DNA repair activities in humans (e.g. 7–300-fold [16]), and is likely to reflect lower variation in the assay. We found no difference in OGG activity between males and females. Interestingly, we did find a gender-specific effect of age on OGG activity, with a small, but statistically-significant decrease in men older than 55 years, compared to younger men. Such a difference was not observed for women. In addition, we found also no difference between smokers and non-smokers, suggesting no effect of smoking on OGG activity in PBMC. Of course, this does not imply that smoking has no effect on OGG in the lung, however this issue was not addressed in the current study.

The sources for inter-individual variations in OGG activity are not fully understood. They are likely to involve inter-individual differences in expression levels of OGG1 mRNA and protein, but also other parameters such as protein stability and modification, presence of inhibitors or stimulators, and perhaps environmental and lifestyle factors as mentioned above. A special challenge would be to develop highly accurate assays for these variables, such that the sources of the variations in OGG activity could be identified. As a first step towards this goal we have examined the relationship between OGG enzymatic activity and the expression of OGG1 mRNA. While the quantitative real-time RT-PCR technique is highly reproducible, the relative values obtained show significant dependence on the choice of the reference gene. We therefore chose three housekeeping genes from three independent pathways, which serve as common references in real-time PCR studies. When TBP mRNA was used as a reference, there was no correlation between OGG1 mRNA and OGG activity. Nevertheless, based on the other two reference genes (HPRT1 and ACTB), or on the mean obtained with these two or all three genes, the overall trend in the group of 11 individuals was that OGG activity increased with increasing relative OGG1 mRNA expression. This suggests that the expression of OGG1 mRNA important in determining the level of OGG activity, as previously reported for several cell lines [45]. However, the correlation was poor, as shown by the very low R^2 of the linear fits (Fig. 7), indicating that the expression of OGG1 mRNA is a very poor predictor of the OGG enzymatic activity in a particular individual. This suggests that other factors that were mentioned above, such as protein expression, stability, etc., play a major role in determining the final level of OGG enzymatic activity. A similar lack of correlation between OGG1 mRNA expression and enzymatic activity was reported for rat liver tissue [46].

The current assay is based on a radioactive DNA substrate, whereas generally, non-radioactive assays are preferable for large-scale epidemiology assays. A possible further development of the OGG assay would be to use a substrate labeled with a fluorescent tag, rather than radioactivity. Such an approach was reported for basic research on DNA repair enzymes [47], but is not yet widely used. A possible complication in applying such an approach to DNA repair assays in general, and the OGG assay in particular, is the recognition of the fluorescent tag as a 'DNA lesion' by cellular proteins. However, the potential advantages of using a fluorescent substrate in terms of simplification and potential for high-throughput analysis warrant the effort to apply it to the OGG test.

The usefulness of the OGG test was illustrated in a case-control study that we have conducted on the role of OGG activity in the risk of lung cancer [10,48]. In that study we found that reduced OGG activity is a risk factor for non-small cell lung cancer in both smokers and non-smokers, and that the estimated relative risk for developing the disease increases as the activity of OGG decreases. Moreover, we found that the combination of low OGG and smoking caused an extra-high estimated relative risk for lung cancer [10,49], suggesting that screening of smokers for low OGG activity might be used as a tool for lung cancer prevention. Our assay should be a useful tool to study the involvement of OGG in other types of human cancer. It may serve also as a paradigm for the development of additional epidemiology-grade DNA repair tests, which are needed to further our understanding on the role of DNA repair in human cancer, and other human disorders.

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