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Extracellular 8-oxo-dG as a sensitive parameter for oxidative stress *in vivo* and *in vitro*

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Abstract

8-oxo-7,8-dihydro-2′-deoxyguanosine (8-oxo-dG) is one of the mutagenic base modifications produced in DNA by the reaction of reactive oxygen species. The biological significance of 8-oxo-dG is shown by the existence of repair pathways that are able to recognize and remove this lesion from both DNA and the nucleotide pool. The final outcome of these evolutionarily conserved repair mechanisms in man is excretion of 8-oxo-dG/8-oxo-Gua from the intracellular to extracellular milieu including the blood plasma and urine. The aim of this investigation was to establish dose response relations for radiation-induced appearance of extracellular 8-oxo-dG in cellular model systems. Here we report on excretion of 8-oxo-dG after *in vitro* irradiation of whole blood and isolated lymphocytes with clinically relevant doses. We find that this excretion is dependent on dose and individual repair capacity, and that it saturates above doses of 0.5–1 Gy of gamma radiation. Our data also suggest that the nucleotide pool is a significant target that contributes to the levels of extracellular 8-oxo-dG; hence the mutagenic target for oxidative stress is not limited to the DNA molecule only. We conclude that extracellular 8-oxo-dG levels after *in vitro* irradiation have a potential to be used as a sensitive marker for oxidative stress.

Keywords: *8-oxo-dG, oxidative stress, ionizing radiation, nucleotide pool, blood serum, cell medium*

Abbreviations: *8-oxo-dG, 8-oxo-7,8-dihydro-2′-deoxyguanosine; 8-oxo-Gua, 8-oxo-7,8-dihydro-guanine; 8-oxo-Guo, 8-oxo-Guanosine; Gy, Gray; ROS, reactive oxygen species; LET, linear energy transfer; HPLC-ECD, high-performance liquid chromatography with electrochemical detection; ELISA, enzyme-linked immunosorbent assay; FBS, foetal bovine serum; BER, base excision repair; NER, nucleotide excision repair; NIR, nucleotide incision repair*

Introduction

Reactive oxygen species (ROS) are formed continuously in living cells as a result of cell metabolism as well as external factors such as ionizing radiation. Oxidative stress occurs when the production of ROS overwhelms the scavenging capacity of the antioxidant defence systems. The steady-state level of endogenous oxidative DNA damage is estimated to be in the range of 10^2–10^5 modifications per cell [1–3]. Oxidative damage to cellular components has been proposed to play an important role in the development of a number of pathological processes including cancer [4,5]. In addition, an accumulation of damages in nuclear and mitochondrial DNA often appear in aging and inflammatory processes [6].

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Low LET (linear energy transfer) ionizing radiation produces a wide spectrum of DNA damages, such as double strand breaks (~40 breaks/Gy/cell), single strand breaks (~1000 breaks/Gy/cell) and various types of chemical changes of the 2′-deoxynucleotides (~1000 modifications/Gy/cell) [7–10]. Ionizing radiation may give rise to clustered lesions (several lesions within one helix turn), a unique molecular signature of ionizing radiation exposure, through both direct (ionization and excitation of DNA) and indirect effects (production of ROS during radiolysis of cellular water). As the majority of the primary radiation effects are mediated by free radicals, the spectrum of radiation-induced DNA damages overlaps with that caused by oxidative stress. The dominant forms of radiation-induced base modifications include 8-oxo-7,8-dihydroguanine (8-oxo-Gua) [11] as well as thymine glycols [12]. 8-oxo-Gua is produced by hydroxyl radical reaction of deoxyguanosine at the C8 position [13]. It has also been indicated that direct effects of radiation lead to higher yields of guanine lesions in DNA through charge transfer ability in the DNA molecule [14]. Elimination of ROS and their deleterious reaction products with DNA is ensured by multiple defence mechanisms, ranging from low molecular weight antioxidants, antioxidant enzymes to DNA repair networks and is essential for the maintenance of genome [15]. The main enzymatic route of 8-oxo-Gua removal from DNA is that of base excision repair (BER) [16–18]. Although recent studies implicate the involvement of several other DNA repair mechanisms i.e. nucleotide excision repair (NER) and nucleotide incision repair (NIR) pathways in coping with free radical induced DNA damage [15,17], their biological significance (when compared to BER) is not yet fully elucidated [19].

During the recent years it became clear that the nucleotide pool of cells is an important target for oxidative stress, as the free nucleotide forms of guanine are prone to oxidation resulting in formation of 8-oxo-dGTP [20] that is a mutagenic substrate for DNA synthesis. DNA replication errors due to incorporation of 8-oxo-dGTP are prevented by sanitization of the nucleotide pool by the MutT enzyme in bacteria and 8-oxo-dG-triphosphatase (hMTH) in mammals [21]. A schematic picture of repair pathways with possible contribution to the appearance of 8-oxo-dG in extracellular fluids is provided in Table I.

The first report on the presence of 8-oxo-dG in urine of human, rat and mouse came from the work of Ames and his colleagues in 1989 [22]. Since then, both 8-oxo-Gua and 8-oxo-dG have been extensively used as markers of oxidative stress elicited by agents of both endogenous and exogenous origin [11,23–26]. Albeit published data indicate about 3–5 fold higher urinary levels of 8-oxo-Gua than those of 8-oxo-dG [22,27,28], the analysis of the former is, however, associated with technical difficulties and interference from RNA degradation processes [28–33].

We have previously investigated the feasibility of use of urinary 8-oxo-dG as a marker for individual radiosensitivity in breast cancer patients undergoing radiotherapy [25]. The results of this study showed significant differences of urinary 8-oxo-dG levels in patients with pronounced acute radiosensitivity as compared to those with minor reactions. We suggested that the observed individual variations in the urinary levels of 8-oxo-dG in breast cancer patients undergoing radiotherapy may reflect individual variations in DNA repair capacity [25]. It should be noted, however, that urinary levels of 8-oxo-dG most likely reflect the systemic effects of oxidative stress with undefined contributions from various tissues. We have been interested whether similar variations of individual ability to cope with the effects of radiation-induced oxidative damage may be demonstrated at cellular level. In the study presented herein, we have therefore investigated the excretion of 8-oxo-dG from cells irradiated *in vitro*, including leukocytes (whole blood irradiated in test tubes) and isolated G_0 lymphocytes (irradiated in cell culture media) from healthy individuals.

Materials and methods

Irradiation of whole blood samples

Early morning venous blood from four healthy individuals was collected in tubes without anticoagulant. The tubes (10 ml of whole blood in each) were irradiated at 4°C with 0, 1 and 2 Gy, respectively, with a ^{137} Cs source (Scanditronix, Uppsala, Sweden) at a dose rate of 0.56 Gy/min. After exposure the samples were quickly brought to 37°C using water bath and incubated for 1 h at this temperature in a CO_2 incubator. One of the controls, 10 ml of whole

Table I. Schematic picture of repair pathways with possible contribution to the appearances of 8-oxo-dG in extracellular fluids.

Target	Nucleotide pool	DNA		
Pathway	8-oxo-dGTPase hMTH	BER	NER	NIR
Intermediate repair products	8-oxo-dGMP	8-oxo-Gua	oligonucleotides with 8-oxo-dG	oligonucleotides with 8-oxo-dG 8-oxo-dGMP
Marker in extracellular fluids	8-oxo-dG	8-oxo-Gua	8-oxo-dG	8-oxo-dG

BER: Base Excision Repair, NER: Nucleotide excision Repair, NIR: Nucleotide Incision Repair. Extracellular 8-oxo-dG and oxidative stress.

blood, was irradiated by 2 Gy and kept on ice during the experiment. After incubation, the samples were centrifuged at 180*g* for 40 min at 4°C. The supernatants (blood serum) were collected and stored at −18°C, until processed by HPLC-ECD. This study was in accordance with the ethical standards of the Swedish ethical committee (Dnr 03-621).

Isolation of lymphocytes

Lymphocytes were isolated from fresh buffy coats by density gradient centrifugation in Ficoll-Paque (Pharmacia LKB, Sweden) according to the manufacturer's protocol. The purified lymphocytes were pre-incubated for one hour in RPMI 1640 medium (Gibco, UK) containing 1% foetal bovine serum (FBS, Gibco, UK) and 1% gentamicin (Gibco, UK). The non-adherent cells were carefully decanted into centrifuge tubes and centrifuged at 180*g* for 15 min at room temperature. The pelleted cells were resuspended in RPMI containing 10% FBS and 1% gentamicin and incubated over night at 37°C in 5% CO_2.

Following the incubation, the lymphocytes were divided to 5 cell culture plates with a cell density of 6×10^6/ml in RPMI medium without phenol red (GIBCO, UK) containing 5% FBS. The cells were irradiated by 0, 0.16, 0.5, 1 and 2 Gy at 4°C and then incubated at 37°C. Two ml of medium was removed from each dish after 30 and 60 min of incubation. The samples were centrifuged at 180*g* for 15 min at 4°C and the supernatants were analyzed for 8-oxo-dG content.

Scoring of apoptotic/necrotic cells in whole blood/isolated lymphocytes

Changes in levels of leukocytes with apoptotic and necrotic phenotypes upon irradiation of whole blood samples and lymphocyte cultures were visualised and scored essentially as described earlier [34]. Briefly, 40 μl of either whole blood or lymphocyte suspension was mixed with a mixture of fluorescent dyes Hoechst H-33342 and propidium iodide (PI) at final concentrations 5 and 1 μg/ml, respectively. At least 400 cells were scored per sample and classified into normal, apoptotic and necrotic cells as described earlier [34].

Analysis of extracellular 8-oxo-dG by HPLC-ECD

The analysis of 8-oxo-dG was carried out by a two-step HPLC method as previously described [24,25], with introduction of some modifications to adapt the method to the tested protein-rich biological fluids. Two ml of serum was freeze-dried overnight, the dried pellet was dissolved in 1 ml PBS, pH 7.4 and pre-purified by solid-phase-extraction according to our previously published method [25]. 8-oxo-dG was eluted by 2 ml of PBS, pH 7.4, containing 20% methanol. The eluate was freeze–dried, dissolved in water and separated by HPLC (Gilson) on a C18-reverse phase column (250 × 4.6 mm, 5 μ, Hichrom Ltd, UK) at a flow rate of 1 ml/min [24,25]. The mobile phase consisted of citric acid (12.5 mM), sodium acetate (25 mM), NaOH (30 mM), acetic acid (10 mM), and heptanesulfonic acid sodium monohydrate (5 mM), pH 5.1. A 5 ml fraction was collected at the same retention time at which the 8-oxo-dG standard eluted in a parallel run (Figure 1, upper panel). The collected fraction was freeze–dried, then dissolved in 170 μl of distilled water and separated by HPLC-ECD again (Figure 1, lower panel). The mobile phase in this run consisted of citric acid (12.5 mM), sodium acetate (25 mM), NaOH (30 mM), acetic acid (10 mM), and 5% methanol, pH 5.1. 8-oxo-dG was quantified by an electrochemical detector (BAS LC-400) with a glassy carbon electrode. An Ag/AgCl electrode was used as a reference (applied potential = +0.7 v).

The chromatograms were analyzed and quantified by ELDS software package (Chromatography Data Systems AB, Stockholm, Sweden) comparing the peak areas with those obtained from the external standards. The quantity of 8-oxo-dG in the serum was normalized to the number of white blood cells per ml and was expressed as ng/10^7 leukocytes under assumption that the extracellular 8-oxo-dG in irradiated samples is produced by the exposed leukocytes. Background values, on the other hand, were not adjusted for leukocyte counts under assumption that they reflect the systemic levels of 8-oxo-dG.

Analysis of extracellular 8-oxo-dG by ELISA

We have applied a competitive ELISA for detection of 8-oxo-dG using a commercially available mouse anti-8-oxo-dG monoclonal antibody (Japan Institute for the Control of Aging, Japan). We settled for ELISA-based detection of 8-oxo-dG in cell culture medium because it is both less time consuming and more sensitive compared to the HPLC-ECD method. The monoclonal antibody used here recognizes 8-oxo-Guanosine (8-oxo-Guo) as well [35] but with a much lower (about two orders) specificity. To abolish the possible interference of 8-oxo-Guo, the samples were cleaned up prior to ELISA by a solid-phase-extraction step according to our previously published method [25] with some modification. Briefly, one ml of cell culture medium was loaded on a solid-phase-extraction column, followed by a washing step (PBS, pH 7.4), and elution (PBS, pH 7.4, containing 20% methanol). The efficiency of the clean-up procedure was checked by adding 10 pmol of 8-oxo-Guo standard (OXIS International Inc, USA) and 3 pmol of 8-oxo-dG standard (SIGMA) to the same sample. After a solid-phase-extraction as described above, no

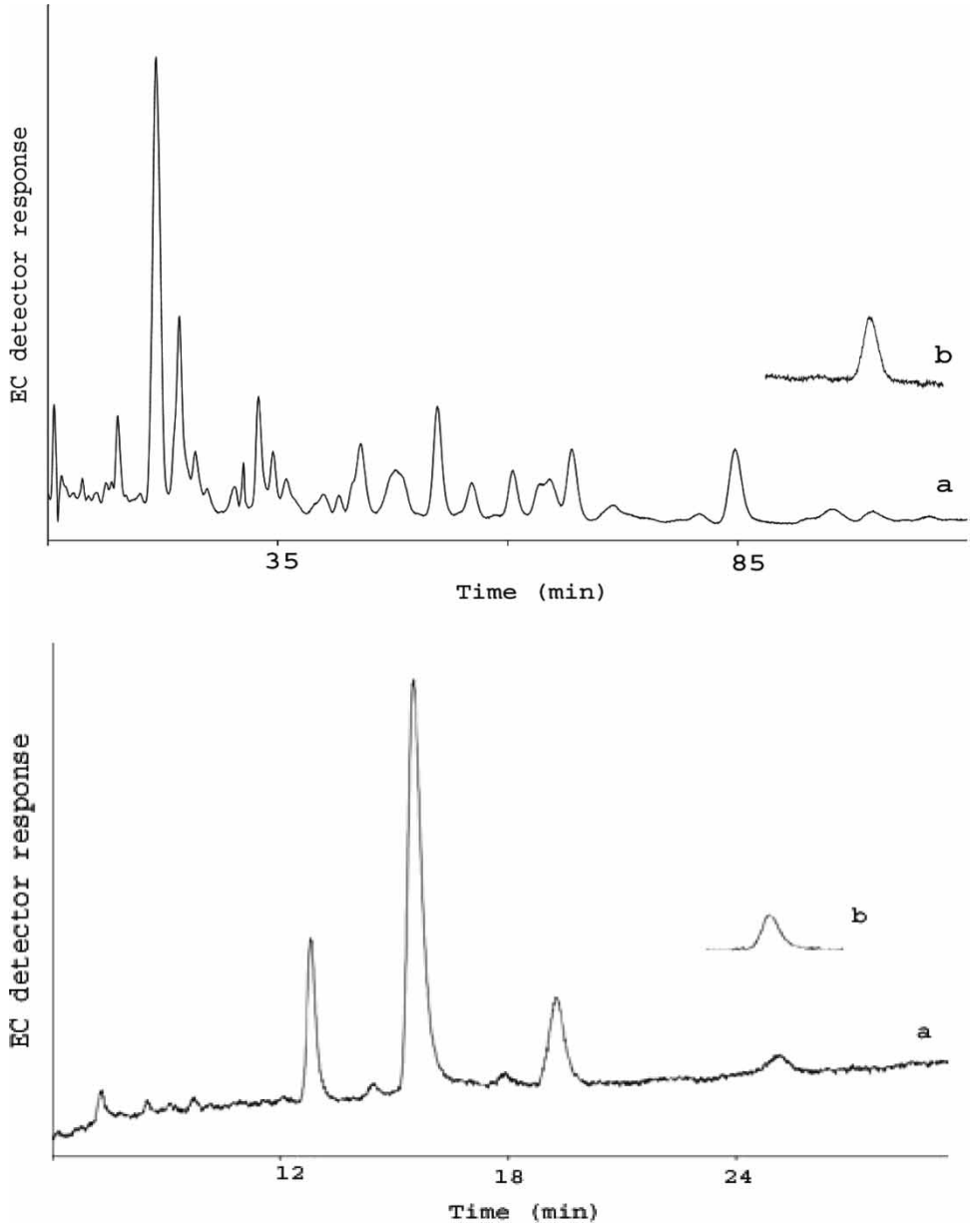

Figure 1. Analysis of 8-oxo-dG in blood serum by two-step HPLC-ECD. The upper panel shows a representative profile of 8-oxo-dG analysis in solid phase extracted serum by HPLC-ECD (a) with a superimposed chromatogram of standard 8-oxo-dG (3.8 pmol) (b). The lower panel depicts analysis of 8-oxo-dG by HPLC-ECD in a fraction collected in the first run at the retention time of standard 8-oxo-dG (a) with a superimposed chromatogram of standard 8-oxo-dG (2.8 pmol) (b).

detectable 8-oxo-Guo was seen in the eluate while the recovery of 8-oxo-dG was practically 100% (checked by HPLC-ECD).

The cleaned-up samples were concentrated by freeze-drying and dissolved in PBS, pH 7.4, to a volume of 500 μl. To half of this sample an internal standard of 8-oxo-dG (3.8 pmol) (Sigma, USA) was added. Ninety microliter aliquots of samples both with and without internal standard, in duplicates, were mixed with 50 μl of the primary antibody and transferred to 96-well ELISA plates that were pre-coated with 8-oxo-dG. After overnight incubation at 4°C the plates were washed 3 times by 250 μl of washing solution (PBS, pH 7.4, 0.02% Tween 20 and 0.1% bovine serum albumin). An amount of 140 μl of HRP-conjugated secondary antibody (goat anti-mouse IgG-HRP, Scandinavian Diagnostic Services, Sweden) was added to each well and incubated for 2 h at room temperature. The wells were washed by 3 × 250 μl of washing solution and finally with PBS, pH 7.4. Then 140 μl of tetramethylbenzidine liquid substrate (ICN Biomedicals Inc, USA) was added to each well and the wells were incubated for 15 min at room temperature; the reaction was terminated by adding 70 μl of 2 M H_3PO_4 (MERCK, Germany). The absorbance was read at 450 nm using

an automatic ELISA plate reader. Both samples with and without internal standard were analyzed in duplicate. Standard curves for 8-oxo-dG (from 0.05 to 60 ng/ml) were established for each plate and the quantity of 8-oxo-dG was normalized to the corresponding sample with internal standard.

Statistical methods

The obtained values for excreted 8-oxo-dG in blood samples were compared by a 2-way ANOVA, considering the experiment as the elementary unit of repetition and two random factors, donor and dose. A 1-way ANOVA test with pair-wise comparison for contrasts and Tukey's method of error protection was then performed to find significant differences among the donors at a particular dose. Student's paired *t*-test was used for testing statistical significance for excreted 8-oxo-dG from lymphocytes at 2 different post irradiation incubations time.

Results

Dual staining of whole blood samples and isolated G_0 lymphocytes with Hoechst H-33342 and PI indicated consistently low levels of apoptotic and membrane damaged cells, which accounted for 2–3% in the whole blood samples and were slightly higher in lymphocyte cultures (4–5%). No detectable increase above these values was observed upon irradiation in the tested dose range during 5 h of post-irradiation incubation (data not shown).

Irradiation of whole blood resulted in an increase of 8-oxo-dG levels in blood serum that levelled of between 1 and 2 Gy, as determined by HPLC-ECD. The measurements are summarized in Table II as mean values of background and radiation-induced 8-oxo-dG (4 individuals, 3 independent experiments per each donor). In each experiment an aliquot of the blood sample was irradiated by 2 Gy and kept on ice during the experiment (negative control). The mean of the individual serum background values was 0.40 ng/ml ($n = 4$) ranging from 0.24 to 0.67, an almost three fold significant difference.

Irradiation of the samples with 1 or 2 Gy resulted in slight relative increases of 8-oxo-dG levels (Table II). This increase is most likely repair-related, as incubation of irradiated samples on ice did not result in changes in 8-oxo-dG levels. As marked variations in the leukocyte counts among the donors were noted, the observed levels of radiation induced 8-oxo-dG in serum were normalised to the total number of leukocytes present in the irradiated blood (Table II). The rationale for this approach is that the radiation induced extracellular 8-oxo-dG in whole blood originates from the leukocytes (DNA and nucleotide pool) with the assumption that the yield is proportional to the total target.

Table II. Blood serum 8-oxo-dG levels after low LET irradiation.

	8-oxo-dG							
		1 Gy (ng/ml)		2 Gy (ng/ml)				
Donor	0 Gy (ng/ml of whole blood)	Whole blood	10^7 Leukocytes	Whole blood	10^7 Leukocytes	2 Gy on ice (ng/ml of Whole blood)	Leukocyte counts $\times 10^{-6}$ (per ml of whole blood)
1	0.31 ± 0.05 **10**	0.35 ± 0.09 (13)	0.51 ± 0.13 (65)	0.37 ± 0.02 (19)	0.52 ± 0.03 (67)	0.28 ± 0.06	7.1–6.9
2	0.24 ± 0.03 **0**	0.26 ± 0.04 (8)	0.54 ± 0.05 (125)	0.27 ± 0.05 (13)	0.55 ± 0.1 (129)	0.24 ± 0.05	4.5–4.9
3	0.67 ± 0.01 **5**	0.75 ± .02 (11)	1.24 ± 0.03 (85)	0.69 ± 0.01 (3)	1.15 ± 0.02 (71)	0.64 ± 0.016	5.8–6.2
4	0.39 ± 0.10 **-3**	0.48 ± 0.12 (23)	0.69 ± 0.17 (77)	0.49 ± 0.01 (25)	0.77 ± 0.08 (97)	0.41 ± 0.1	7.1–6.9
Mean	0.4 ± 0.18 **3**	0.46 ± 0.2 (15)	0.74 ± 0.34 (85)	0.45 ± 0.18 (13)	0.75 ± 0.29 (88)	0.39 ± 0.18	

The values are means ± SD from three independent experiments per donor.
Relative increases (%) of extracellular 8-oxo-dG in the unirradiated samples (37°C) as compared to the corresponding negative controls (2 Gy, on ice) are indicated in bold numbers.
The values in the parentheses are relative increases (%) of extracellular 8-oxo-dG in irradiated samples as compared to the un-irradiated samples kept at 37°C.

Normalisation of values to the total leukocyte counts revealed large individual variations in the relative increase of 8-oxo-dG in blood serum after irradiation with 1 Gy, ranging from 65 to 125% with a mean of 85% (Table II). The significance of individual variations in both background and radiation-induced levels of excreted 8-oxo-dG at the 95% CI level was further confirmed by ANOVA (Table III).

With the assumption that the 8-oxo-dG values observed in serum from whole blood reflect the collective response of the leukocyte subpopulations we repeated these experiments with isolated lymphocytes in order to establish how much of the extracellular 8-oxo-dG is excreted by lymphocytes. Here we used competitive ELISA for determination of the 8-oxo-dG excreted to the culture medium that we found more sensitive and less time-consuming than the traditionally used HPLC-ECD method. 8-oxo-dG levels in cleaned-up samples were between 1.8 and 35 ng 8-oxo-dG per ml medium, well within the sensitivity range of the applied ELISA method. Significant radiation effects on 8-oxo-dG excretion were observed, both after 30 ($p < 0.001$) and 60 ($p < 0.001$) min for all the studied doses as compared to the non-irradiated controls. On the other hand, no significant differences among the applied doses and times (Figure 2) were demonstrated; neither were the levels of 8-oxo-dG measured at 60 min significantly different from those detected at 30 min of postirradiation time.

Discussion

In our previous study we have shown that increase of 8-oxo-dG in urine of breast cancer patients receiving radiotherapy significantly differs between patients showing severe skin reactions and those with minor skin reactions [25]. The studies presented herein were initiated with the aim to investigate the dose response and kinetics for radiation induced extracellular 8-oxo-dG in cellular experimental model systems with relevance to the observed *in vivo* exposures.

The experiments were done on both whole blood samples and isolated lymphocytes from healthy donors. Our results show a radiation-induced increase of 8-oxo-dG both in blood serum and cell culture media. Albeit no simple dose response relation was observed for the investigated dose range and post irradiation times, the results suggest individual variations in response.

The excretion of 8-oxo-dG was temperature-dependent, pointing at involvement of DNA/nucleotide pool repair. The majority of oxidative DNA base damages are repaired by base excision repair pathway (BER) resulting in 8-oxo-Gua (damaged base without 2′-deoxyribose) that can be detected in urine [28,32]. It seems therefore unlikely that BER would contribute to extracellular 8-oxo-dG in our test system. Even though removal of 8-oxo-dG from DNA by means of NER could hypothetically lead to excretion of 8-oxo-dG, the relevance of NER to occasionally reported presence of 8-oxo-dG-containing oligonucleotides in urine [29] is still discussed [16,36,37]. Indeed, it has been demonstrated that human urine contains only limited amounts of oligonucleotides and/or mononucleotides; moreover they do not contain oxidative modification in form of 8-oxo-dG [37]. An alternative DNA repair pathway, based on endonuclease activity, has been recently described and termed nucleotide incision repair (NIR) [17,38]. Albeit the action of NIR on damaged DNA may lead to a single nucleoside or oligonucleotides containing 8-oxo-dG and serve as a backup repair activity for BER [38], this repair mechanism is still poorly characterized and its biological significance needs to be further evaluated [19].

As indicated above, DNA repair mechanisms involved in removal of 8-oxo-dG from oxidatively damaged DNA will mainly produce 8-oxo-Gua; hence their contribution to the extracellular levels of 8-oxo-dG observed in our experiments will be rather low. Consequently, this leaves the nucleotide pool as one of the major sources of 8-oxo-dG in our experimental system. This conclusion seems to be plausible for several reasons. The chromatin structure of DNA in the nucleus with the association to nuclear proteins reduces its accessibility to free radicals and offers additional protection. The nucleotide pool, on the other hand, is mainly located in the cytoplasm and lacks direct structural protection resulting in its higher vulnerability to the attack of free radicals. Free radicals may produce a wide spectrum of changes in the free nucleotide pool including the formation of highly mutagenic 8-oxo-dGTP. Both prokaryotes [39] and eukaryotes [40,41] have developed repair systems to avoid the incorporation of 8-oxo-dGTP by an 8-oxo-dG/TPase enzyme that dephosphorylates 8-oxo-dGTP to 8-oxo-dGMP. It should be stressed, that the yield of extracellular 8-oxo-dG per cell after irradiation of whole blood with 1 Gy, 60 min post irradiation incubation, exceeds about 35 times the directly radiation induced yield of 8-oxo-dG in nuclear DNA [8]. Thus a large part of the extracellular 8-oxo-dG originates clearly from other source than DNA and could result from dephosphorylation of 8-oxo-dGMP followed by transport through the plasma membrane.

Oxidative cellular damage (oxidation of thiols, increase in lipid peroxidation, oxidative DNA damage) is frequently associated with both the initiation and progression of apoptotic/necrotic mode of cell death in a number of cellular models [42]. Measurements of extracellular (urinary, plasma) 8-oxo-dG have long been considered to be no more than a marker of generalised oxidative stress, with major contribution from dying cells [43] from various tissues. However there is no experimental evidence

Table III. ANOVA analysis of dose and donor-dependent variations in amount of excreted 8-oxo-dG.

	Results of the 2-way ANOVA analysis (12 experiments, 4 donors, 4 dose treatments)				
Source of variation	Sum of squares	Degrees of freedom	Mean of squares	F	p
Dose	1.451	3	0.484	72.24	<0.0001
Donor	2.224	3	0.741	110.72	<0.0001
Dose × Donor	0.195	9	0.022	3.23	0.0067
Within cells	0.214	32	0.007		
Total	4.084	47			

	Results of *post-hoc* tests (matrix of probabilities for pair-wise comparisons)							
	D1 (0 Gy)	D1 (1 Gy)	D2 (0 Gy)	D2 (1 Gy)	D3 (0 Gy)	D3 (1 Gy)	D4 (0 Gy)	D4 (1 Gy)
D1 (0 Gy)	1							
D1 (1 Gy)	**<0.05**	1						
D2 (0 Gy)	>0.05	**<0.05**	1					
D2 (1 Gy)	**<0.05**	>0.05	**<0.05**	1				
D3 (0 Gy)	**<0.05**	**<0.05**	**<0.05**	**<0.05**	1			
D3 (1 Gy)	**<0.05**	**<0.05**	**<0.05**	**<0.05**	**<0.05**	1		
D4 (0 Gy)	>0.05	>0.05	**<0.05**	**<0.05**	**<0.05**	**<0.05**	1	
D4 (1 Gy)	**<0.05**	>0.05	**<0.05**	>0.05	>0.05	**<0.05**	**<0.05**	1

1-way ANOVA test with pairwise comparisons for contrasts (Tukey's correction);
statistically significant differences ($P < 0.05$) are in bold.

ANOVA analysis of dose and donor-dependent variations in amount of excreted 8-oxo-dG.
The 1-way ANOVA analysis with pair-wise comparison for contrasts and Tukey's method of error protection was applied on background and 1 Gy values only. D1 to D4 designate individual donors.

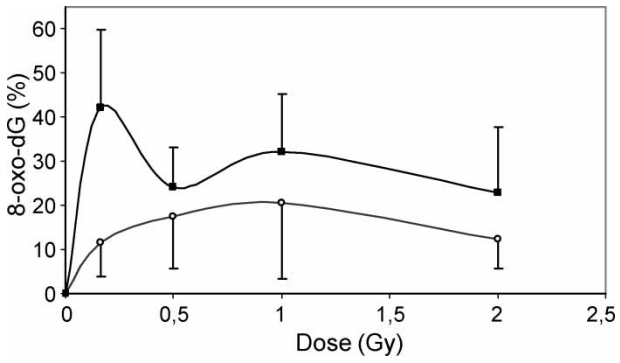

Figure 2. Excretion of 8-oxo-dG from lymphocytes irradiated *in vitro*. Isolated lymphocytes (6 healthy donors) were irradiated by 0, 0.16, 0.5, 1 and 2 Gy and then incubated 30 (○) and 60 (■) min at 37°C. 8-oxo-dG in the medium was analyzed by ELISA. The increase of extracellular 8-oxo-dG is normalized to the individual background levels and data shown are mean values ± SD ($n = 6$).

supporting this hypothesis (for review see ref [29]). We have therefore carefully investigated the background as well as radiation induced levels of apoptotic/necrotic cells in our samples with dual fluorescent staining, as described earlier [34]. Our measurements revealed consistently low background levels of both types of cells in either of the tested cell models, ranging from two to four percent of the total cell population and representing mainly apoptotic mode of cell death. Neither negative controls nor the irradiated samples displayed an increase of cell death in either form during the time of sampling (up to 2 h post-irradiation) for 8-oxo-dG in extracellular fluids. The first detectable increase of cell death rate was observed after 5–6 h of post-irradiation incubation, in accordance with our previously published results [34,44]. Even though apoptotic cells, under cell culture conditions, may proceed to secondary necrosis and eventually release DNA that has been cleaved, such events take place a considerably long time (48–72 h) after irradiation [34,44]. Hence, the eventual contribution of necrotic and/or apoptotic cells (as defined by morphological changes) to the radiation-induced levels of extracellular 8-oxo-dG (by release of highly fragmented oxidised DNA) in our experimental model should be minimal.

Both the initiation (no apparent morphological changes) and progression of apoptosis is associated by generation of ROS. It has also been demonstrated that ionizing radiation in the therapeutic dose range stimulates a transient (3–5 min after irradiation) cellular generation of ROS that coincides with hallmarks of the mitochondrial permeability transition [45]. Owing to their place of generation a plausible target for these secondary ROS products is the nucleotide pool where they could amplify the initial ionization events resulting in formation of 8-oxo-dG. The plateau in the dose response for extracellular 8-oxo-dG in the range of 1–2 Gy of gamma radiation both for whole blood samples (Table II) and cultures of isolated lymphocytes (Figure 2) may reflect the saturation of mitochondrial response induced by radiation [45]. Alternative explanations for the observed plateau could be the energy dependency of 8-oxo-dG excretion pathway and/or limitations by the amount and turnover rate of accessible repair enzyme(s) [46] which seem to be supported by nonsignificant increase of excreted 8-oxo-dG levels between 30 and 60 min post irradiation sampling times (Figure 2).

Ionizing radiation is a potent inducer of immediate inflammatory reactions already at low doses [47]. These responses are intimately associated with production of various oxidants of mainly neutrophile origin which may inflict oxidative damage upon DNA, RNA and the nucleotide pool [48]. Of note, while 1 Gy of radiation resulted in about 80% increase of extracellular 8-oxo-dG in whole blood, at similar conditions (during one hour of postirradiation incubation) the average increase of 8-oxo-dG in the culture media of irradiated isolated lymphocytes was about 30%. These data indicate a pronounced contribution from other leucocytes population than lymphocytes to the observed levels of extracellular 8-oxo-dG in blood serum after irradiation.

In this study, the blood samples were obtained from healthy donors with no documentation of clinical radiosensitivity. Interestingly, marked variations of both background and radiation-induced levels of extracellular 8-oxo-dG were observed between the donors (Table III). This level of individual response supports in principle our observations on marked individual urinary levels of 8-oxo-dG in response to tumour therapy [25]. For obvious reasons we cannot draw conclusions on the significance of these observations at population level. Still, these variations hint at a wide span of heterogeneity in radiation-induced stress response among individuals that could be influenced by both genetic and environmental (life style, smoking etc.) factors.

In summary, we suggest that the main target for production of extracellular 8-oxo-dG in irradiated leukocytes is the nucleotide pool. The formation of extracellular 8-oxo-dG seems to be enhanced by secondary oxidative events associated with the stress responses elicited by irradiation. We suggest that extracellular 8-oxo-dG is a sensitive biomarker of individual stress response. Furthermore, the availability of damage-specific antibodies, or HPLC techniques of appropriate sensitivity, opens up novel possibilities for studies of oxidative stress under *in vivo* and *in vitro* conditions.

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