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8-Oxo-7,8-dihydro-2′-deoxyguanosine is not salvaged for DNA synthesis in human leukemic U937 cells

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Abstract
8-Oxo-7,8-dihydro-2′-deoxyguanosine (8-oxo-dG), the most common oxidatively modified nucleoside, is released from oxidized DNA and oxidized nucleotide pool. However, little information is available regarding the metabolic pathway of free 8-oxo-dG. In this study, we generated radiolabeled 8-oxo-dG to track its metabolic fate. We report that 8-oxo-dG is neither phosphorylated to 8-oxo-dGMP nor degraded to the free base, 8-oxo-7,8-dihydroguanine (8-oxo-Gua), indicating that 8-oxo-dG is not a substrate for nucleotide synthesis. This result was confirmed by the finding that no radioactivity was detected in the DNA of U937 cells after incubating the cells with radiolabeled 8-oxo-dG. These observations indicate that 8-oxo-dG produced by oxidative stress is not reutilized for DNA synthesis.

Keywords: *Oxidative stress, 8-oxo-7, 8-dihydro-2′-deoxyguanosine, salvage pathway*

Introduction

8-Oxo-7,8-dihydroguanine (8-oxo-Gua) is one of the most abundant oxidative DNA adducts [1]. The formation of 8-oxo-Gua in DNA results not only from the direct oxidation of guanine base in DNA but also from the incorporation of 8-oxo-dGTP, an oxidized form of dGTP in the cytosolic nucleotide pool. 8-Oxo-Gua in DNA is a detrimental lesion because of its mutagenic and oxidative damage-inducing effects [2,3].

However, mammalian cells have multiple repair systems that counteract the detrimental effects of 8-oxo-Gua. 8-Oxo-Gua in DNA, which is paired with cytosine, is predominantly removed by base excision repair (BER) enzyme, OGG1 [4]. Besides, OGG2 removes 8-oxo-Gua from 8-oxo-Gua: A pair [5], and NTH1 repairs 8-oxo-Gua: G pair [6]. 8-Oxo-Gua in DNA also seems to be eliminated by nucleotide excision repair (NER), which is mediated by excision repair proteins which is found to be mutated in xeroderma pigmentosum patients [7]. The 8-oxo-Gua-containing oligomer generated by NER may be finally cleaved to mononucleoside 8-oxo-dG [8]. Free 8-oxo-dG may also be originated by an endonuclease lacking a glycosylase activity and subsequent hydrolysis [9].

Moreover, 8-oxo-dGTP, a mutagenic substrate for DNA polymerase, is sanitized from DNA precursor pool by MTH (8-oxo-dGTPase), which hydrolyzes this 8-oxo-Gua-containing triphosphate to its monophosphate form [10–13], or in the absence of MTH, by human Nudix type 5 (NUDT5), which hydrolyzes 8-oxo-Gua-containing diphosphate to its monophosphate form [13]. The repair product 8-oxo-dGMP is then dephosphorylated to 8-oxo-dG presumably by nucleotidase or specific phosphatase [14]. This uncharged form can be easily excreted from cells. Overall, these defence mechanisms cooperate to minimize the harmful effects of 8-oxo-Gua in DNA.

Hence, 8-oxo-Gua released from oxidized DNA by BER, or 8-oxo-dG either degraded from oxidized oligonucleotides by NER and endonuclease or dephoshorylated from oxidized nucleotides in the cytosolic pool appear in extracellular fluids such

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as urine and blood [15]. Moreover, these repair products, 8-oxo-Gua and 8-oxo-dG are used as a biological marker of oxidatively generated damage [16,17]. The urinary excretion levels of 8-oxo-Gua and 8-oxo-dG have been detected about 1.5 and 0.3 nmol/kg/day, respectively, in human [18,19].

However, it has not been demonstrated yet as to how 8-oxo-dG is metabolized. In this study, we investigated the metabolic fate of 8-oxo-dG, and found that 8-oxo-dG is not phosphorylated into 8-oxo-dGMP or degraded into 8-oxo-Gua, indicating that it is not reutilized as a substrate for DNA synthesis.

Materials and methods

Reagents and cell lines

2′-Deoxyguanosine (dG), 8-oxo-7,8-dihydro-2′-deoxyguanosine (8-oxo-dG), 2′-deoxyguanosine 5′-triphosphate (dGTP), and purine nucleoside phosphorylase were obtained from Sigma. 2′-Deoxy[1′,2′-^{3}H]guanosine 5′-triphosphate (dGTP) (40 Ci/mmol) was purchased from Amersham Pharmacia Biotech. Alkaline phosphatase was purchased from Roche Molecular Biochemicals. U937 cells were obtained from the American type culture collection and maintained in RPMI 1640 supplemented with 10% heat-inactivated fetal bovine serum at 37°C in 5% CO_2.

Preparation of ^{3}H-labeled 8-oxo-dG

^{3}H-labeled 8-oxo-dG was prepared by oxidizing dG according to a previously described procedure [17,20]. Briefly, [1′,2′-^{3}H]-dGTP was evaporated to dryness under vacuum to remove ethanol and then hydrolyzed with alkaline phosphatase (8 mU/1 nmole triphosphate) in 50 mM Tris–HCl, pH 8.0 at 37°C for 1 h. The hydrolyzed [1′,2′-^{3}H]-dG was then purified on a J'sphere ODS-H80 column (YMC Co. Ltd 4 μm, 4.6 × 250 mm) using 5% methanol/water at a flow rate of 0.5 ml/min. The purified [1′,2′-^{3}H]-dG was evaporated under vacuum and hydroxylated at the C-8 position as follows. A solution (100 μl) containing 6 mM dG (including 600 μCi [1′,2′-^{3}H]-dG), 30 mM ascorbate and 100 mM hydrogen peroxide was incubated in the dark for 2 h at 37°C. Immediately, the mixture was applied to a J'sphere ODS-H80 column and eluted with 5% methanol/water at 0.5 ml/min. Fractions containing 8-oxo-dG eluted after unreacted dG were pooled and concentrated under vacuum. The obtained [1′,2′-^{3}H]-8-oxo-dG was dissolved in H_2O and the concentration of [1′,2′-^{3}H]-8-oxo-dG was determined by measuring specific radioactivity.

Preparation of cytosolic and mitochondrial fractions

Cytosolic and mitochondrial fractions of U937 cells were prepared as follows [21]. Briefly, 2×10^8 cells were suspended in 300 μl of hypotonic buffer containing 1.5 mM $CaCl_2$, 10 mM NaCl and 10 mM Tris–HCl, pH 7.5, incubated on ice for 15 min, and homogenized. The homogenate was mixed with 100 μl of 4 × a mannitol–sucrose buffer to give a final concentration of 210 mM mannitol, 70 mM sucrose, 5 mM Na_2EDTA, 5 mM Tris–HCl, pH 7.5. The mixture was centrifuged at 1200*g* for 15 min at 4°C. The supernatant obtained was recentrifuged at 20,000*g* for 1 h at 4°C and used as a cytosolic fraction. The pellet was resuspended in 2 volume of 1 × the mannitol–sucrose buffer, washed twice by centrifuging at 20,000*g* for 10 min at 4°C and lysed in a solution of 150 mM NaCl, 1% Nonidet P-40 and 50 mM Tris–HCl, pH 8.0. The lysate was centrifuged at 20,000*g* for 1 h at 4°C and the supernatant obtained was used as a mitochondrial fraction. The separation of cytosolic and mitochondrial fractions was verified by Western blotting for cytochrome c oxidase, which is present only in mitochondria.

Deoxynucleoside kinase assay

The activity of deoxynucleoside kinase was measured radiochemically as previously described [22,23]. Briefly, cytosolic or mitochondrial fraction (10 μl protein) was incubated with 10 μM of [1′,2′-^{3}H]-dG or [1′,2′-^{3}H]-8-oxo-dG in a reaction mixture (50 μl) of 5 mM $MgCl_2$, 5 mM ATP, 2 mM DTT, 15 mM NaF, 100 mM KCl and 50 mM Tris–HCl, pH 7.6 at 37°C. Aliquots (10 μl) were taken at the times indicated and spotted on anion exchange Whatman DE81-filter discs (Whatman), which were allowed to air-dry and then were washed three times with 50 mM ammonium formate with shaking at room temperature. The filter-bound monophosphate product was eluted from the filters with 0.5 M KCl/0.1 M HCl for 30 min, and radioactivity was determined by scintillation counting.

Purine nucleoside phosphorylase assay

Cell pellet was suspended in 2 volume of a lysis buffer containing 20 mM Tris–HCl, pH 7.4, 0.5 mM phenylmethanesulfonyl fluoride (PMSF), and 0.5 μg/ml leupeptin and pepstatin A and lysed by three cycles of freezing and thawing. Cell lysate was cleared by ultracentrifugation at 100,000*g* for 1 h at 4°C and the supernatant was used as a crude cell extract. Purine nucleoside phosphorylase activity was measured using a modified HPLC method [24]. Briefly, 60 μg of the crude cell extract or 2 U purine nucleoside phosphorylase was incubated with dG or 8-oxo-dG (0.4 mM each) at 37°C for up to 60 min in 150 μl of a reaction mixture containing 1 mM EDTA, 80 mM potassium phosphate and 30 mM Tris–HCl, pH 7.4. At the times indicated, reaction mixtures were loaded onto a reverse phase J'sphere ODS-H80 column and eluted with 50 mM NaH_2PO_4/6% methanol at a flow rate of 0.5 ml/min. The amount

of guanine or 8-oxo-Gua was determined by comparing the peak height of each compound to that of a known amount of its respective authentic compound.

Quantitation of intracellular uptake and DNA incorporation of dG and 8-oxo-dG

Cells (5×10^6) were incubated with 400 μM of dG or 8-oxo-dG plus 2 μCi of [1′,2′-^{3}H]-dG or 2 μCi of [1′,2′-^{3}H]-8-oxo-dG, respectively, for 48 h and then washed with cold PBS. Intracellular uptake was detected by measuring the radioactivity of harvested cells. To detect the incorporation into DNA, nuclear DNA was isolated by NaI extraction technique using a cDNA extractor WB kit according to the manufacturer's instructions (Wako Pure Chemical Industries, Ltd) and its radioactivity was measured by liquid scintillation counting [25,26].

Results

Purification of [1′,2′-^{3}H]-8-oxo-dG

The hydrolysis of dGTP produced dG (Figure 1A). 8-Oxo-dG, a hydroxylation product of dG was monitored using a UV detector (254 nm) and was found to elute 10 min later than dG on a reverse phase column (Figure 1B and C). The purity of 8-oxo-dG was ascertained by a single peak in HPLC (Figure 1D) and its identity was confirmed by UV spectral analysis (Figure 1F), which showed the spectral characteristics with the maxima at 245 and 293 nm. This purified [1′,2′-^{3}H]-8-oxo-dG was used in this study.

8-Oxo-dG was not salvaged for nucleotide synthesis

It has been reported that 8-oxo-dGMP cannot be anabolized to its triphosphate nucleotide, 8-oxo-dGTP, but that it is easily degraded to its nucleoside form, 8-oxo-dG [14,27]. Therefore, the final repair product of 8-oxo-dGTP *in vivo* may be 8-oxo-dG. In order to determine the metabolic fate of 8-oxo-dG, deoxynucleoside kinase, a rate-limiting enzyme in phosphorylation steps for a synthesis of DNA precursors [28], was assayed using 8-oxo-dG as a substrate. Since major deoxynucleoside kinases are expressed in cytosol and mitochondria [28], cytosolic or mitochondrial fraction was incubated with

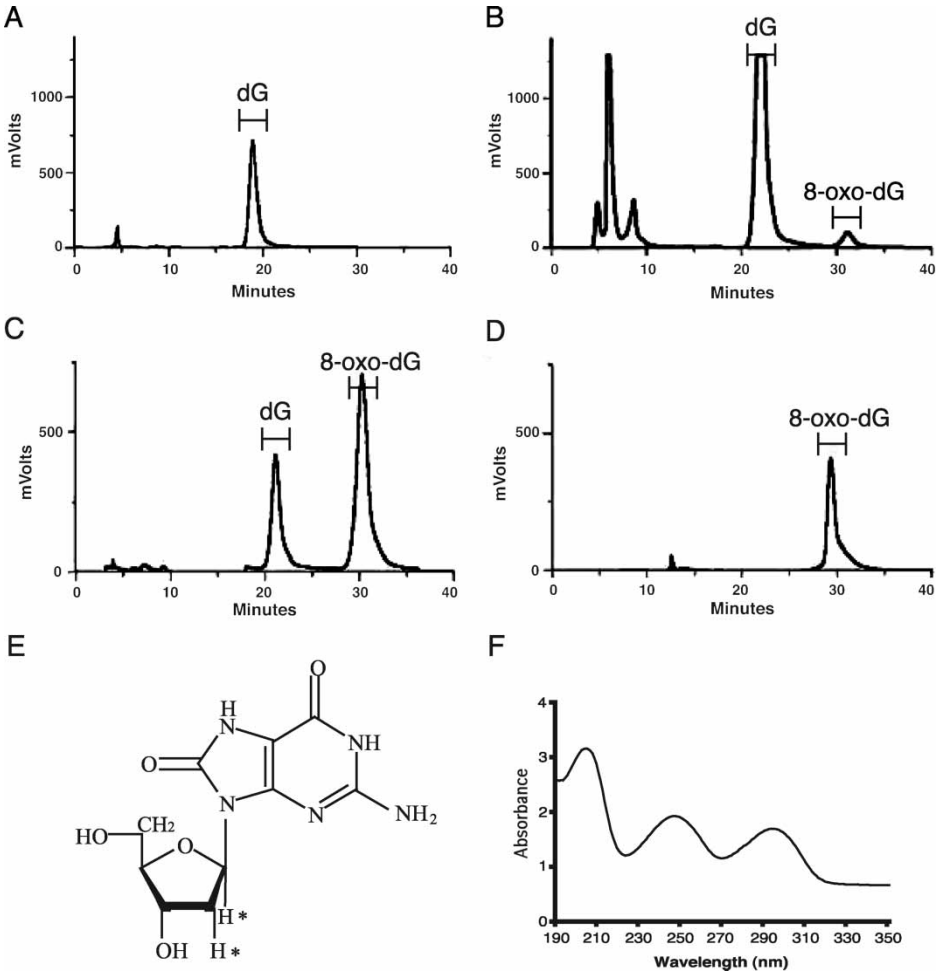

Figure 1. [1′,2′-^{3}H]-8-oxo-dG was prepared by dG oxidation and purified by column chromatography. (A) [1′,2′-^{3}H]-dGTP was hydrolyzed to dG by alkaline phosphatase; (B) dG was hydroxylated at the C-8 position; (C) and (D) 8-oxo-dG was purified sequentially by HPLC until a single peak of 8-oxo-dG was obtained; (E) Structure of [1′,2′-^{3}H]-8-oxo-dG, *, radiolabeling; (F) UV spectrum of 8-oxo-dG.

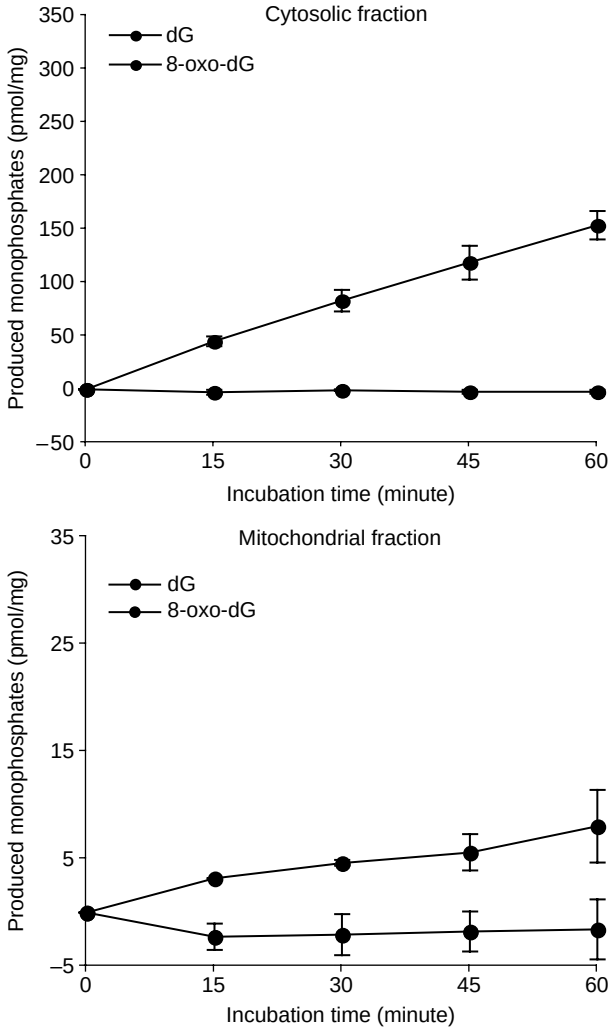

Figure 2. 8-Oxo-dG was not phosphorylateled to 8-oxo-dGMP by U937 cells. [1′,2′-^{3}H]-dG or [1′,2′-^{3}H]-8-oxo-dG (10 μM each) was incubated with 10 μg of cytosolic (upper panel) or mitochondrial (lower panel) fraction of U937 cells at 37°C. At the times indicated, aliquots of reaction mixtures were spotted onto anion-exchange filters and the filter-bound monophosphate product was quantified by measuring the radioactivity on filter. Details are described in "Materials and methods" section. The values are presented as the mean of three independent experiments + standard error.

[^{3}H]-labeled dG or 8-oxo-dG. As shown in Figure 2, both cytosolic and mitochondrial fractions phosphorylated dG to its monophosphate. In contrast to dG, neither fraction produced a detectable monophosphate form from 8-oxo-dG, indicating that 8-oxo-dG is not salvaged for nucleotide synthesis.

8-Oxo-Gua base was not produced from 8-oxo-dG

Deoxynucleoside containing 8-oxo-7,8-dihydroguanine moiety is not used as a substrate for nucleotide synthesis. Next, we sought to determine whether 8-oxo-dG is metabolized to its base form, a possible substrate for the salvage pathway. The salvage pathway utilizes bases which are produced from degradation of nucleic acids, for nucleotide synthesis

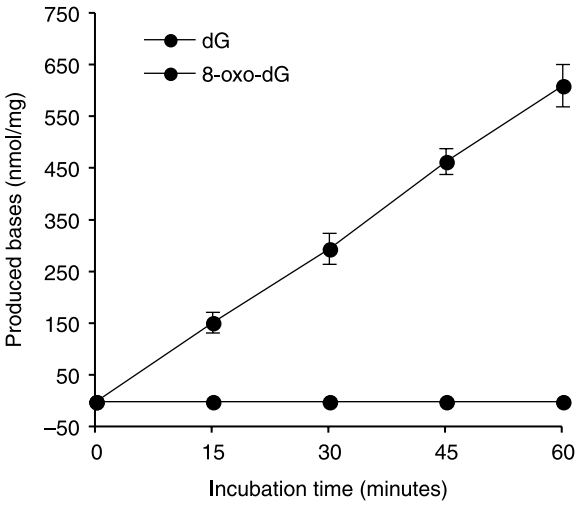

Figure 3. 8-Oxo-dG was not phosphorolysed to 8-oxo-Gua by U937 cells. dG or 8-oxo-dG (0.4 mM each) was incubated with 60 μg of crude cell extract of U937 cells at 37°C. The reaction product, guanine or 8-oxo-Gua was detected and measured by a reverse phase HPLC. Details are described in "Materials and methods" section. The values are presented as the mean of three independent experiments + standard error.

[29,30]. In order to test this possibility, the activity of purine nucleoside phosphorylase, which catalyzes the phosphorolysis of purine 2′-deoxy ribonucleosides to the free bases and 2-deoxyribose 1-phosphate [31], was assayed using 8-oxo-dG as a substrate. Whereas, guanine base was produced from dG by U937 lysates, 8-oxo-Gua was not produced from 8-oxo-dG (Figure 3). A purified purine nucleoside phosphorylase also showed no activity in terms of decomposing 8-oxo-dG to 8-oxo-Gua (data not shown). This indicates that 8-oxo-dG does not undergo phosphorolysis and suggests that 8-oxo-dG, once it is formed *in vivo*, is excreted without being metabolized.

8-Oxo-dG was not incorporated into DNA

In order to further verify that 8-oxo-dG is not used as a substrate for DNA synthesis, [^{3}H]-radiolabeled 8-oxo-dG was exogenously added to U937 cell culture media for 48 h, and radioactivity was measured in the DNA isolated from the U937 cells. As shown in

Table I. Incorporation of dG or 8-oxo-dG into DNA of U937 cells.

	dG	8-Oxo-dG
Intracellular uptake (μCi)	0.71(±0.06)	0.35(±0.03)
Nuclear DNA (μCi)	0.13(±0.03)	$5.2 \times 10^{-5}(\pm 1 \times 10^{-5})$

Cells (5×10^6) were incubated with 400 μM of dG or 8-oxo-dG including 2 μCi [1′,2′-^{3}H]-dG or 8-oxo-dG, respectively, for 48 h and then washed with cold PBS. Intracellular uptake of dG or 8-oxo-dG was measured by counting the radioactivities (μCi) of harvested cells. Incorporation into DNA was measured by the radioactivities of DNAs isolated from U937 cells treated with dG or 8-oxo-dG. Details are described in "Materials and methods" section.

Table I, radioactivity was detected in the nuclear DNA of U937 cells incubated with radiolabeled dG, but radioactivity was found to be very little in the DNA of 8-oxo-dG-treated U937 cells, indicating that 8-oxo-dG is not directly incorporated into DNA. The intracellular uptake of 8-oxo-dG was lower than that of dG. Probably, that might be due to some cellular mechanism to excrete 8-oxo-dG to the extracellular compartment.

Discussion

The present study shows that 8-oxo-dG is neither phosphorylated to 8-oxo-dGMP nor degraded to 8-oxo-Gua base. In addition, we show that radioactivity is not detected in DNA from cells incubated with radiolabeled 8-oxo-dG. These findings indicate that 8-oxo-dG is not salvaged and thus, is not a physiological substrate for DNA synthesis.

Natural nucleosides or bases are reutilized by the Salvage pathway. However, 8-oxo-dG originated from oxidized DNA by repair process or from cytoplasmic 8-oxo-dGTP, is a poor substrate for deoxyguanosine kinase (Figure 2). Moreover, 8-oxo-dG is not decomposed into 8-oxo-Gua base which would be a possible substrate for DNA synthesis (Figure 3). The inability of cell-free extracts to decompose 8-oxo-dG has also been shown by Bialkowski [32]. In addition, it was reported that a ribonucleotide reductase that reduces ribonucleotide diphosphate to deoxyribonucleotide diphosphate, cannot reduce 8-oxo-GDP to 8-oxo-dGDP [27]. The existence of these multiple mechanisms designed to prevent the incorporation of 8-oxo-dG into DNA reflects the potentially harmful nature of the 8-oxo-Gua residue *in vivo*.

Therefore, 8-oxo-dG is not salvaged for DNA synthesis. However, the hydrophilic nature of 8-oxo-dG allows it to be readily excreted into the extracellular compartment [17,33], a point which has led several studies to claim that elevated levels of 8-oxo-dG in extracelullular fluids including blood and urine are a good marker of oxidative damage not only in DNA but also in cellular nucleotides.

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