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Salvage of oxidized guanine derivatives in the (2'-deoxy)ribonucleotide pool as source of mutations in DNA

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Abstract

Recent evidence suggests that salvage of 8-oxodG and 8-oxoGua can contribute substantially to levels of 8-oxoGua in DNA and RNA. However, it remains to be determined if this mechanism contributes to mutagenesis and disease. This review covers the predominant methods for detecting 8-oxoGua and its derivatives, summarizes some of the relevant recent DNA repair studies and discusses the mechanisms for metabolism of oxidized guanine derivatives in the (2'-deoxy)ribonucleoside and (2'-deoxy)ribonucleotide pools.

Keywords

8-oxodG; nucleotide pool oxidation; salvage; accelerator mass spectrometry; DNA repair

1. Introduction

Cellular metabolism results in the production of reactive oxygen and reactive nitrogen species (ROS and RNS), which at low concentrations can serve as signaling molecules. In contrast, high levels of ROS/RNS lead to modification of cellular biomolecules, such as proteins, lipids and DNA. Inflammation, exposure to toxins and ionizing radiation can result in sufficiently high concentrations of ROS and RNS products as to overwhelm cellular antioxidant defenses, leading to mutagenic DNA nucleobase damage.

Of the 70 DNA lesions characterized from cells, over 30 are nucleobase modifications, which includes 8-oxo-7,8-dihydro-2'-deoxyguanosine (the 2'-deoxyribonucleoside 8-oxodG is shown in Figure 1)[1]. Of these DNA adducts, 8-oxodG has received the greatest amount of attention, owing to its high endogenous concentrations and ease of detection compared to most other known DNA adducts [2]. There is a rapidly growing body evidence which suggests that 8-oxodG and other nucleic acid-derived lesions arising from oxidative stress have been implicated in the onset and development of several pathological processes

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including cancer and age-related neurodegenerative diseases such as Parkinson's Disease [3–5]. Recently, evidence has emerged from our group that 8-oxodG, and its analogous DNA repair product, 8-oxo-7,8-dihydro-guanine (8-oxoGua) are recognized by salvage pathways and can be incorporated into DNA and RNA (Figure 1). The detection of 8-oxodG and biochemistry of cellular responses to this nucleoside and its nucleotide analogs has been reviewed exhaustively (recent reviews include [6–11]. This review will summarize the prevailing methods measuring 8-oxodG and the biologically relevant (2'-deoxy)ribonucleotide derivatives, and discusses recent progress in understanding the related DNA repair, (2'-deoxy)ribonucleotide pool "cleansing" and cellular metabolism.

A wide variety of approaches have been employed to study oxidatively damaged DNA. However, findings from the European Standards Committee on Oxidative DNA Damage (ESCODD [12]), have suggested that a number of these techniques suffer from artefactual oxidation during sample workup and analysis (reviewed by Guetens et al. [13] and Cooke et al. [10]). Recently, effective methods have been developed to minimize this risk (recent advances include [14;15].

The prevailing thought is that direct oxidation of DNA results in the formation of 8-oxodG, which leads to G-to-T transversion mutations during replication unless the damage is repaired by base excision repair (BER)(Figure 2). Increases in 8-oxoGua levels in DNA can also occur after •OH radical attack to the cellular 2'-deoxyguanosine 5'-triphosphate (dGTP) pool producing oxidized 8-oxodGTP, which can then be incorporated into DNA during cellular replication or during DNA repair leading to A-to-C transversions (Figure 2)[16]. The dGTP nucleotide pool is mainly located in the cytoplasm; therefore, it is more available for attack by ROS, compared to DNA, which is protected by histones and tightly packaged in the nucleus. Recent evidence demonstrated that 8-oxodG in the nucleotide pool can be metabolized to form 8-oxodGTP, which implies that salvage can contribute to 8-oxodG-induced mutations [17–19]. These observations have been made possible, in part, by the use of ultrasensitive methods of detecting oxidatively modified (2'-deoxy)ribonucleosides and (2'-deoxy)ribonucleotides and by tracking the metabolism of radiolabeled analogues of these compounds.

2. Methods of analysis

Determination of cellular and urinary nucleoside and nucleotide concentrations has been investigated by several methodologies including gas chromatography (GC), bioluminescence, nuclear magnetic resonance spectroscopy, and high-performance liquid chromatography (HPLC) [20]. It has been generally established that the quantification of NTPs and dNTPs is most reliably assessed via ion-pair HPLC with UV detection [21].

Methods for the measurement of oxidatively damaged DNA are based on either chromatography combined with electrochemical detection or mass spectrometry or, alternatively, immunoassay, although the presence of these adducts in DNA is inferred from the single cell gel electrophoresis (comet) assay. The majority of assays focus upon 8-oxodG as the analyte of choice.

Chromatographic techniques

A commonly used assay of oxidatively damaged DNA is the measurement of 8-oxodG by HPLC with electrochemical detection (HPLC-ECD) after enzymatic hydrolysis of DNA [22;23]. This procedure exploits the fact that 8-oxodG has a substantially lower oxidation potential compared to the four canonical nucleobases, and can be selectively detected with proper adjustment of the detector. However, problems can arise because of incomplete enzymatic hydrolysis (e.g. oxidatively modified nucleobases can interfere with the action of

nucleases [24]) and deglycosylation of 8-oxodG during sample handling (discussed in [25]). Although these problems have been adequately addressed for 8-oxodG analysis, a fundamental limitation of HPLC-ECD is that many DNA damage products do not have relatively low oxidation potentials, and are not suitable for quantitation by this method.

Mass spectrometry instrumentation and methods have recently advanced to the point where sensitivity and precision rival or exceed HPLC-ECD, but with the advantage of detecting a larger number of nucleic acid damage products. An advantage of HPLC-MS methods is the use of isotopically-labeled internal standards. These standards simplify quantification by accounting for loss during sample workup, differences in ionization efficiencies due to matrix effects and phosphorylation, and allow confirmation of analyte identity by detection of molecular ions and fragmentation patterns from co-eluting analytes. Notably, the HPLC-MS/MS assay described by Weimann et al. [26] was the first report for the simultaneous analysis of 8-oxoGua, 8-oxoguanosine and 8-oxodG as well as the parent Gua, guanosine and dG moieties. Similarly, HPLC-GC/MS requires HPLC pre-purification of the compounds of interest, which again includes native compounds; 8-oxoGua, 8-oxodG, Gua, dG and others [27;28], which are then derivatized prior to GC-MS analysis in order to increase analyte volatility. Solid phase extraction (SPE) has been used for the analysis of urinary 8-oxodG by GC-MS [29] and HPLC-MS/MS [30–32]. Accelerator mass spectrometry (AMS) is an emerging technology for measuring radiocarbon- and tritium-labeled compounds that was originally developed for biomedical research at Lawrence Livermore National Laboratory [33]. Briefly, AMS works by breaking down the molecules in a sample and isolating an element of interest, such as carbon in the form of graphite, which is then quantified in a small particle accelerator. If the drug is labeled with a rare isotope, such as ^{14}C , the ratio of radiocarbon to total carbon in the particle beam is used to calculate the concentration of drug in blood, tissue, cells and sub cellular components such as protein and DNA. Although carbon is the most commonly assayed element owing to the low natural background of ^{14}C , other elements exist as mixtures with rare isotopes so as to be useful for biomedical AMS applications including ^3H and ^{41}Ca [34–37]. For radiocarbon studies, AMS has attomole sensitivity for DNA samples as small as a microgram. This technology is currently used with HPLC to measure pharmacokinetics and metabolism of drugs, vitamins, xenobiotics and, more recently, 8-oxodG metabolism studies [18;33;38–62]. AMS does not quantify 8-oxodG directly, but is useful for metabolism studies in which a tracer of [^{14}C]8-oxodG or other related metabolites is tracked at physiologically relevant concentrations [17–19]. A similar tracer study was reported for M₁G metabolism [63] at the MIT BEAMS Lab [64]. Direct detection of DNA damage by AMS requires the utilization of specific "postlabeling" chemistries that are still in development for DNA adduct studies [55;65;66].

Immunoassay

The competitive enzyme-linked immunosorbent assay (ELISA) has received widespread use for the analysis of nucleic acid lesions. Predominantly, 8-oxodG has been the lesion of choice for commercially available (e.g. from the Japanese Institute for the Control of Aging, JaICA), and custom made ELISAs. The benefits of ELISA include ease of use, high throughput and applicability to numerous extracellular matrices (serum, plasma, saliva, urine, cell culture medium, sputum and others [67–79]). The kit from JaICA has received the most published use.

However, some antibodies are notorious for their cross-reactivity with 8-oxoGua, Gua, and 2'-deoxyguanosine [67;69]. Immunohistochemical studies using antibodies for 8-oxodG have also displayed high background reactivity in both the nucleus and cytosol in various cell types. This demonstrates unreliable discrimination between oxidatively modified nucleobases in DNA and in the free guanosine ribonucleoside or guanosine nucleotide

triphosphate forms, at least for a group of the antibodies reported [68;70]. Therefore, the use of ELISA may be more qualitative than quantitative, depending on the antibody used and the experimental conditions employed.

3. Sources of guanine oxidation products in vivo

DNA repair

The prevailing thought is that BER is the most important mechanism for the removal of the majority of oxidatively-derived DNA lesions [2;80]. BER utilizes DNA glycosylases that remove the modified base, leaving an apurinic-apyrimidinic (AP) site, which may be cleaved to form a single strand gap (AP lyase activity), depending upon the specific glycosylase (Figure 2). The AP lyase activity may result in the removal of one nucleotide (short patch repair), or 2–6 nucleotides (long patch repair, followed by gap filling and ligation).

The DNA glycosylase considered to have the primary responsibility for the removal of 8-oxoG from DNA in human cells, is the human 8-oxoGua DNA glycosylase 1 (hOGG1 [81;82]). This enzyme acts via short patch repair, and has a specificity for 8-oxoG:C pairs present in double-stranded DNA (Figure 2). The activity of hOGG1 may be complemented by another enzyme, denoted OGG2, which removes the 8-oxoGua from the nascent strand in 8-oxoGua:A or 8-oxoGua:G pairs, arising from misincorporation of 8-oxodGTP [83], although the reported results have yet to be reproduced independently. Other glycosylases have been reported to have some activity towards 8-oxodG or for 2'-deoxyribonucleotides mispaired with 8-oxodG including NEIL1, NEIL2, NTH1 and MYH1 [84–87].

The levels of urinary 8-oxoGua have been measured in *OGG1* $-/-$ mice, which showed a 26 % reduction in the deficient strain, compared to wild type, indicating that, in mice at least, OGG1 is a significant, but not exclusive, source of urinary 8-oxoGua [88]. The results support the existence of back up DNA glycosylase(s), such as the Nei-like glycosylases [89], but which cannot entirely compensate for OGG1 deficiency. Alternatively, the urine data support the existence of enzymes such as purine nucleoside phosphorylase (PNP) that cleave 8-oxodG at the glycosidic bond, resulting in 8-oxoGua formation that is not related to BER of DNA damage [90].

The initial enzymatic event in nucleotide excision repair (NER) is the removal of an oligonucleotide from duplex DNA that contains one or more damaged nucleotides. NER primarily removes bulky lesions, such as cyclobutane thymine dimers (T<>T), although there is some evidence for minor activity towards 8-oxodG [91]. Therefore, NER is, in principle, capable of producing oligonucleotides containing 8-oxodG, which could potentially be degraded to free 8-oxodG. However, there is also evidence that some proteins involved in NER regulate OGG1 activity [92;93]. The latter pathway may be the more biologically relevant contributor to the repair of 8-oxodG in duplex DNA.

Evidence has emerged that mismatch repair is synergistic with both (2'-deoxy)ribonucleotide pool 'cleansing' and BER in lowering mutation frequencies due to 8-oxodG-derived species [94–96].

Oxidation of the (2'-deoxy)ribonucleotide pool by ROS

GTP is required for RNA synthesis and can be modified by ROS. GTP concentrations in the cytoplasm are hundreds of times larger than dGTP [97]. This suggests that under conditions of high ROS levels, significantly more 8-oxoGTP than 8-oxodGTP could be produced in the cell. However, due to the lack of a reliable way to quantify these oxidation products, little is known of the degree of oxidation to dGTP or GTP pools after ROS attack and the possible

impact of these oxidation products to cellular physiology. Recently, it has been shown that the dGTP pool incurs greater levels of oxidation after irradiation compared to DNA [78]. Levels of 8-oxodG in cells, tissue, and whole animal have been reported as an important biomarker for oxidative stress when evaluating disease pathologies ranging from cancer to diabetes [4;98]. However, most of this evidence has been accrued by assessment of 8-oxodG via potentially questionable antibody technology. Therefore, it is necessary to rigorously investigate the relative contributions of oxidatively modified dGTP and GTP as potential biomarkers of oxidative stress.

Nucleotide pool cleansing

The origins, presence and metabolism of free 2'-deoxyribonucleosides and 2'-deoxyribonucleotide lesions in cells and urine is not well-defined. For example, there are no reports of a DNA repair enzyme whose activity yields 8-oxodG, although oxidation of dG is a likely source of 8-oxodG [99]. For the oxidized (2'-deoxy)ribonucleotides, several Nudix hydrolases mediate cellular concentrations of 8-oxodGTP, 8-oxodGDP and 8-oxodGMP (Figure 3). The best characterised enzyme that performs such a role is NUDT1 (also known as the human MutT homologue, MTH1), which hydrolyzes 8-oxodGTP to 8-oxodGMP (Figure 3). Further processing of 8-oxodGMP, perhaps by nucleotidases, may give rise to 8-oxodG [16]. The roles of other Nudix hydrolases such as NUDT15 (MTH2) and NUDT5, which include 8-oxodGTP and 8-oxodGDP among their substrates respectively, remain to be defined [100;101]. A recent paper by Hori et al. suggests that all three of these enzymes are required to suppress 8-oxodGTP-induced mutations in nuclear DNA [102].

Other sources

Other potential sources of free oxidatively modified (2'-deoxy)ribonucleosides and (2'-deoxy)ribonucleotides may include cell death/turnover and diet (reviewed in [10]). However, the role(s) of these sources in causing mutations via 8-oxodG formation is likely to be minor or negligible..

4. Salvage of 8-oxodG as a potential source of mutations

In contrast to 8-oxodG in DNA, little information is available on the mutagenic potential and cellular responses to the presence of free 8-oxodG. The cellular metabolism of 8-oxodG is the subject of an emerging and sometimes conflicting literature. In human cells, dG predominantly undergoes phosphorolysis by PNP to form the free nucleobase Gua and 2'-deoxyribose-1'-phosphate. The resulting free nucleobase is a substrate for hypoxanthine-guanine phosphoribosyltransferase (HGPRT), resulting in formation of GMP. Two additional phosphorylation steps afford GTP, which serves as a substrate for RNA polymerase-dependent incorporation into RNA. This pathway can also feed into DNA synthesis, since GDP can be reduced to dGDP by ribonucleotide reductase (RR) by replacement of the 2'-OH with a hydrogen. Alternatively, cellular deoxycytidine kinase (dCK) or mitochondrial deoxyguanosine kinase (dGK) phosphorylate dG to form dGMP, which can then be serially phosphorylated to dGTP prior to incorporation into DNA during replication or repair synthesis.

Based upon the known salvage pathways for dG [103–105], 8-oxodG may also be metabolized by two distinct pathways (Figure 3):

(1) If 8-oxodG is a substrate for PNP, the nucleobase portion of 8-oxodG can be hydrolyzed from the 2'-deoxyribose ring. The resulting 8-oxoGua is a substrate for salvage, resulting in the ribonucleotide and 2'-deoxyribonucleotide triphosphates 8-oxoGTP and 8-oxodGTP, respectively, which contribute to RNA and DNA synthesis.

(2) Direct mono-, di- and triphosphorylation of 8-oxodG would result in formation of 8-oxodGTP, a substrate for DNA synthesis. Both possible pathways reflect the known metabolism of the canonical 2'-deoxynucleoside dG described above. However, salvage of 8-oxodG is complicated by contributions from BER via hOGG1, the putative hOGG2, nucleotide pool "cleansing" by nudix hydrolases and possibly 5'-nucleotidase activity.

In an effort to determine whether 8-oxodG is a substrate for the human salvage pathways that utilize dG, we measured [^{14}C]8-oxodG metabolism in MCF-7 cells [17–19]. The [^{14}C]8-oxodG was labeled on the nucleobase in order to probe both possible salvage mechanisms. AMS was required for the study since the levels of ^{14}C -labeled metabolites were too low to be detected by other mass spectrometry methods and well below the limit of detection for liquid scintillation counting. Specifically, the ability of MCF-7 cells to import 8-oxodG and employ either PNP-dependent salvage or direct phosphorylation for nucleic acid synthesis was investigated. Incorporation of exogenous 8-oxodG was observed at a concentration of approximately one 8-oxoG nucleobase per 10^7 nucleotides in DNA and one 8-oxoG per 10^6 nucleotides in RNA, in cells exposed to concentrations of 8-oxodG approximating those present in human plasma (~ 100 fmol per mL) [106]. Resulting levels of 8-oxoGua incorporated into DNA and RNA, were at concentrations within an order of magnitude of those typically reported for mammalian cells as measured by HPLC-EC [19].

These initial observations were accompanied by inhibitor-based experiments aimed at determining the mechanism of incorporation. The [^{14}C]8-oxodG experiments were repeated in the presence of inhibitors of PNP, RR and dCK [19]. The inhibition of PNP almost completely abrogated incorporation of radiocarbon into both DNA and RNA, supporting PNP as a gatekeeper of 8-oxodG catabolism. Inhibition of RR resulted in reduced radiocarbon incorporation into DNA, indicating that 8-oxoGDP is a substrate for RR. This was a particularly surprising result, since 8-oxodG was previously described as being a poor substrate for RR by Hayakawa and coworkers [107], who used tested purified 8-oxoGDP as a potential substrate for recombinant mouse RR. The *in vitro* experiment showed no reduction of 8-oxoGDP. Our data were obtained from intact cells, in which the entire salvage pathway was functioning. This means that another ribonucleotide reductase subunit may have been active, such as p53R2 [108;109]. Clearly, additional research is needed to resolve the difference between these two results. Inhibition of dCK had no effect on radiocarbon incorporation into DNA and RNA compared to controls with no inhibitor, supporting, at best, a minimal contribution by direct phosphorylation of 8-oxodG to the overall metabolism, which is in contrast to catabolism of dG [19].

An intriguing possible consequence of salvage for DNA and nucleotide pool repair pathways is the production of both 8-oxodG and 8-oxoGua that may ultimately be excreted in urine. The recently published data on exogenous 8-oxodG metabolism is in partial agreement with the literature. For example, Zeng et al. [110] demonstrated that HGPRT can catalyze the formation of 8-oxoGuo monophosphate from 8-oxoGua, which is consistent with PNP-dependent salvage of 8-oxodG. However, this process has not been specifically demonstrated to occur *in vivo*, even in a cell culture system. The extracellular addition of $400\text{ }\mu\text{M}$ 8-oxodG to a number of leukemic cell lines (KG-1, H9, CEM-CM3, Molt-4), in which hOGG1 activity is absent or low, resulted in cytotoxicity and the accumulation of 8-oxoG in their nuclear DNA [111;112], which is consistent with the PNP-mediated pathway. However, the increased 8-oxoG in DNA was not due to the direct incorporation of the extracellular 8-oxodG [113]. This study reported the use of tritium-labeled 8-oxodG in which the radioactive label resided on the 2'-deoxyribose ring, which would be precluded from incorporation into DNA unless there was direct phosphorylation by dCK. It has previously been reported that 8-oxodG is not a substrate for dCK or dGK [16;112], but these enzymes are not required by the PNP-mediated pathway for incorporation of 8-oxoGua into

DNA or RNA. The report by Kim et al. claimed that the 8-oxodG was not substantially degraded to 8-oxoGua. However, we would have to question the biological relevance of using an extracellular concentration of 400 μ M 8-oxodG, since we demonstrated that increasing concentrations of 8-oxodG in the cell culture medium above approximately 2 μ M resulted in saturation of the salvage pathway [18].

Our data indicate that PNP and RR enable salvage of 8-oxodG in MCF-7 cells, a previously unrecognized mechanism that may contribute to mutagenesis and carcinogenesis. These data indicate the need to answer several questions including: To what extent does this phenomenon occur *in vivo*, especially in the body composed of largely non replicating cells? What is the relative contribution of salvage to 8-oxodGTP levels in the 2'-deoxyribonucleotide pool, compared with direct oxidation of dGTP? What fraction of 8-oxodG in cellular DNA is derived from the 2'-deoxyribonucleotide pools after salvage? Answering these questions will lead to further fundamental investigations of the impact of ROS on genomic integrity, and may ultimately lead to better diagnostics and treatments for multiple diseases.

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Figure 1.

The proposed salvage of oxidized guanine derivatives that constitute a recently characterized source of mutations, RNA alterations and a contributor to 8-oxodG and 8-oxoGua in urine.

Base Excision Repair:

Nucleotide Pool Cleansing:

Figure 2.
Base excision repair and (2'-deoxy)ribonucleotide pool cleansing mechanisms.

Figure 3.

Proposed salvage pathways for 8-oxodG and 8-oxoGua. Importantly, our data indicate that 8-oxodG is metabolized to 8-oxoGua exclusively via the PNP-mediated pathway.