Oxidative Decay of DNA*

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The study of DNA oxidation has progressed from an exploratory phase, during which its basic biochemistry was established, into a field branching out into numerous areas. Early on, radiation biologists discovered that radiolysis of water generates oxygen free radicals, which are responsible for many of the consequences of irradiating living things. The characterization of radiation-induced oxidative DNA lesions, and the connection between radiation and cancer, caused a surge of interest in DNA oxidation per se and raised the possibility of DNA damage from biological oxidants. Nucleic acid biochemists, cancer biologists, and toxicologists then set out to ask key questions: “how much oxidative DNA damage is there, how does it get there, how and when is it removed, and what are the consequences?” A proliferation of techniques has resulted in the confirmation of the early hypotheses and also delivered some surprises. In this minireview, we have outlined some of the most interesting recent results. Extensive reviews on DNA oxidation published elsewhere have discussed the earlier work in detail (1–5). A companion minireview by Henle and Linn (6) covers in depth the biochemistry of DNA oxidation.

Methods for Measuring Oxidative DNA Damage

The steady-state amount of DNA oxidation appears to be massive, with oxidative adducts occurring at a frequency that is 1 or more orders of magnitude higher than non-oxidative adducts (1, 7). Despite their abundance, oxidative DNA adducts exist in a large background (10^−5–10^−10) of unaltered nucleotides, which may be prone to oxidation during sample preparation and analysis. Concerns about artificial oxidation, combined with the different values that have been generated by alternative methods, have fueled an ongoing debate over the most appropriate techniques for studying DNA oxidation.

Gas chromatography coupled with mass spectroscopy (GC-MS), initially used in characterizing oxidative adducts, is also a quantitative tool whose principal advantage is the simultaneous analysis of a number of different adducts (5). DNA is chemically hydrolyzed, derivatized, and injected onto GC-MS. In the absence of a mass spectrometer, an alternative approach is the enzymatic hydrolysis of DNA to nucleosides and chromatography of the hydrolysate by HPLC (8). The adducts 8-oxo-7,8-dihydro-2'-deoxyguanosine (oxo8dG) and its corresponding base 8-oxo-guanine (oxo 8Gua) are especially useful in this regard, since they are electrochemically active, lending themselves to sensitive electrochemical (EC) detection. The relative simplicity and high sensitivity of HPLC-EC detection of oxo8dG have made it the most popular method for monitoring DNA oxidation in vivo.

Generally speaking, GC-MS estimates of DNA oxidation have been higher than HPLC-EC estimates, by about a factor of 10 (9). The debate about the cause of the difference (an overestimate due to artifactual oxidation with GC-MS versus an underestimate due to inefficient enzymatic digestion with HPLC-EC) has now been settled; artifactual oxidation occurs during GC-MS derivatization, in the case of guanine/oxo8dG (10) and in the case of adducts formed from adenine, cytosine, thymine, and thymidine (9). The HPLC-EC method itself, however, has also been criticized on the grounds that the variability of the assay is unacceptable (11–15). Estimates of the ratio of oxo8dG to dG (for example, in rat tissues) have ranged from approximately 0.25 × 10^-5 to higher than 10^-4, and it has been suggested that artifactual oxidation is to blame.

Artifactual oxidation poses problems of accuracy and precision. For example, the total cellular burden of oxidative adducts has been estimated from HPLC-EC measurements of oxo8dG (1, 10), on the assumption that oxo8dG represents 5% of all adducts (it is one of about 20 major radiation adducts characterized by GC-MS (16)). This estimate, about a million oxidative adducts per rat cell (1), is a number which argues forcibly that oxidative mutagenesis must be important in vivo (2, 3, 7). To the extent that the initial measurement of oxo8dG may be artificially elevated, this estimate may also be inaccurate. Worse, perhaps, is the effect that artifactual oxidation has on the ability to detect an elevation of oxo8dG. If the DNA damage “signal” is obscured by background artifact “noise,” then real increases in DNA oxidation may be obscured or fail to achieve statistical significance. Fortunately, a number of incremental improvements have recently been introduced (12, 13), driving down the estimate of steady-state oxo8dG 2–5-fold from previous estimates. The current lowest estimates of the ratio of oxo8dG/dG (in rat hepatocytes and human lymphocytes) cluster around 0.25 × 10^-5, equivalent to approximately 7,500 oxo8dG or about 1.5 × 10^-4 oxidative adducts per human cell (if oxo8dG represents 5% of all such adducts) (12, 13).

150,000 oxidative adducts per cell represents a huge load of damage: is there solid evidence that this number is accurate? Although it is difficult to rule out some contribution by artifactual oxidation to these values (or indeed to values derived from any of the techniques that have been devised), emerging features of experiments with oxo8dG lend them credibility. Namely, recent studies show low sample-to-sample variance, as well as dramatic patterns of appearance and disappearance of oxo8dG following oxidant challenges, occurring in parallel with the induction of oxo8dG repair activity (13, 17–21). Together with the fact that radically different techniques (discussed below) have demonstrated similar degrees and patterns of induced DNA damage, these results suggest that the problem of artifactual noise has been tamed, if not eliminated. Elsewhere, we have discussed in detail how to avoid the artifacts that can occur with HPLC-EC.

The cloning and overexpression of repair enzymes continue to provide new ways to detect oxidative adducts. Enzymes such as Escherichia coli endonuclease III and formamidopyrimidine glyco- sylase (Fapy glycosylase), which recognize and excise oxidized pyrimidines and purines, respectively, possess associated lyase activities that result in strand cleavage (23). Treating DNA with these enzymes introduces nicks, which may then be measured by alkaline elution (24), nick translation (25), or ring opening of supercoiled molecules (26).

The polymerase chain reaction (PCR) has provided an approach called “quantitative PCR” (Q-PCR), which takes advantage of the fact that many DNA lesions block thermostable DNA polymerases, thereby decreasing the efficiency of amplification (27). As the length of the desired amplon increases, the probability that a strand-terminating adduct will occur also increases, as does the sensitivity of the method. With appropriate controls and calculations.

This paper is available on line at http://www.jbc.org

Minireview

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THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 272, No. 32, Issue of August 8, pp. 19631–19636, 1997
Printed in U.S.A.
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In reducing free ferric ions, although the types of DNA lesions resulting in decreased amplification are only known in general. There is potential that Q-PCR may be coupled with repair endonucleases, enabling the quantification of specific adducts.

All of the techniques discussed so far, from GC-MS to Q-PCR, require purified DNA. A technique for estimating the rate of oxo8dG formation, which does not require the isolation of DNA and its associated problems, is the measurement of its repair products excreted into urine or tissue culture medium (3, 28, 29). The daily flux of repaired adducts should reflect the intracellular rate of DNA damage, if not in a direct way. Although there is a different set of concerns concerning the accuracy of these methods (such as uncertainty about repair pathways and products, oxidation of free nucleotide pools, and the contribution of cell and mitochondrial turnover), an overwhelming advantage of these methods is that they are non-invasive and integrative. As a consequence, the measurement of repaired adducts in urine is one of the few techniques that has been routinely applied to humans. Recently, an elegant related approach has been reported: the measurement of oxo8dG in small amounts of muscle interstitial fluid, collected with a microdialysis probe. During a period of reperfusion following ischemia (a well established model of oxidative stress) a rapid increase in intercellular oxo8dG was observed (30).

Last, there exist two techniques that preserve cellular integrity: single-cell gel electrophoresis and immunohistochemistry with anti-DNA-adduct monoclonal antibodies (mAbs). Single-cell gel electrophoresis, also descriptively termed the “comet” assay, involves casting cells in a thin agarose gel on a microscope slide and running the DNA out of the nuclei by electrophoresis. The more fragmented the chromatin, the more it migrates, assuming (upon staining) the appearance of a comet’s tail streaking away from the nucleus in the direction of the anode. The analysis of the length and intensity of the tail (its “moment”) is achieved with the help of software (31). Modifications of the assay permit the analysis of specific lesions; alkaline conditions are used to study single-strand nicks, and by treating DNA “in vivo” or “in situ” with an enzyme, casting cells in agarose, and by treating DNA “in situ” with an enzyme, for instance, are used in enzyme-linked immunosorbent assays of purified DNA (34). The ultimate power of mAbs, however, may lie in their ability to detect DNA damage in fixed cells and tissues “in situ,” as was recently reported for a mAb to oxo8Gua (35).

There is no single ideal method for measuring oxidative lesions, as all have their strengths and weaknesses. The most important of these is the lack of a single method to rigorously quantitate but require relatively large quantities of pure nucleic acids. Molecular biological methods like Q-PCR require less DNA but are not as specific in their detection. Cellular assays are ideal for analyzing tiny samples (hundreds of cells) and avoiding cellular disruption but are semi-quantitative. What is encouraging about recent results is the growing congruence between studies using different approaches.

Mechanisms and Location of DNA Oxidation

Until the last 2 years, it had become almost accepted wisdom that the role of the superoxide anion radical (O2) in DNA oxidation was its ability to reduce ferric iron (Fe3+) to ferrous iron (Fe2+). Fe2+ catalyzes the formation of the hydroxyl radical ‘OH (from H2O2) which, according to the scheme (referred to as the Fenton chemistry), is the ultimate reactive species in DNA oxidation (6). Support for the roles of all three components of this model (O2, iron, and H2O2) continues to accumulate (36, 37). However, as is discussed in the companion minireview by Henle and Linn (6), the nuances of DNA oxidation have turned out to be more complex and interesting. For one, the nature of the ultimate oxidant responsible for DNA damage by H2O2 is unclear. Detailed experiments have illustrated that a model of freely diffusible ‘OH fails to account for the strikingly parallel dynamics of DNA strand scission in vitro and cytotoxicity of H2O2 to E. coli. Rather, multiple classes of oxidant appear to exist, associated with the DNA double helix to different extents (6). Moreover, the role of O2 in reducing free ferric ion has been challenged by experiments suggesting that its principal role is to release iron from protein-bound iron-sulfur clusters (38). Besides O2, there are other reductants (such as NADH) that effectively reduce Fe3+ to Fe2+ and that may be more relevant as reductants of free or DNA-bound iron than is O2 (39). Copper (40) and less well studied transition metals such as chromium (41) also take part in Fenton-like chemistry in DNA oxidation.

Reactive nitrogen intermediates such as peroxynitrite (ONOO-) also react with DNA, forming (among other lesions) the adduct oxo8dG (42–44). Interestingly, oxo8dG itself is far more susceptible to peroxynitrite than to peroxynitrous acid (9, 45), which emphasizes the fact that more stable oxidative end products than oxo8dG exist (45, 46). Also, oxidative DNA adducts may be formed indirectly; the peroxidation of membrane lipids results in various aldehyde breakdown products that are able to form covalent mutagenic adducts (47). Recently, we have reported that the concentration of protein-bound aldehyde accumulates with age in rats (48), suggesting that aldehyde-DNA adducts may also increase with age. A final complication is the evidence that DNA is not a homogeneous target of oxidative damage and repair. Internucleosomal DNA appears at least 3.5 times more susceptible than nucleosomal DNA to oxidation by physiological iron chelates (49), and repair of a number of adducts is more rapid in DNA in the nuclear matrix than in total chromatin (50).

A fascinating subtlety of DNA oxidation involves the possibility that damage may be mediated by long distance electron transport along the π stack of the DNA double helix. Experiments with synthetic double-stranded oligonucleotides have shown that long range oxidative damage may occur, resulting in the formation of oxo8dG in susceptible 5-GG-3’ at a distance from a covalently attached terminal oxidant (51). If such a phenomenon is important in vivo, it may mean that the topology of DNA serves to channel or trap oxidation in zones.

Oxidative Mutagenesis: GOing, GOing, GOOne

In E. coli, oxidative DNA damage is removed by pathways involving both nucleotide and base excision pathways. The latter include endonuclease III, which recognizes and removes oxidized pyrimidines, and Fapyglycine lyase (29), which removes the adduct FapyGua from RNA. The former includes the cognate “GO system” (mutM, mutT, mutY), a set of three repair enzymes that suppress mutagenesis by Guanine Oxidation, by removing oxo8Gua paired with cytosine (mutM), adenine paired with oxo8Gua (mutY), and by hydrolyzing the oxidized nucleotide oxo8dGTP to the nucleoside monophosphate (mutT), thereby preventing its incorporation into DNA. Although space constraints preclude a full discussion of oxidative repair enzymes here, it is important to note that mutT is highly conserved throughout eucaryotes. In S. cerevisiae, it has been shown that the loss of mutT results in over 100-fold increases in the rate of spontaneous mutagenesis and that homologous genes or activities have been identified in humans, including the cloning of human homologs of endonuclease III (52), mutY (53), and mutT (54) and the recent identification of a MutM-like activity (55). As would be expected of a fundamental type of DNA damage, repair of oxidative lesions appears widely conserved. The cloning of mouse genes involved in repair of oxidative damage and the subsequent generation of transgenic knockout mice by recombination will permit a powerful test of the “oxidative mutagenesis” hypotheses of cancer and aging (53).

Eukaryotes likely possess unique systems in addition to their homologs of prokaryotic enzymes. The Drosophila ribosomal S3 protein, which possesses an associated oxo8Gua glycosylase/AP lyase activity and is able to restore the wild-type phenotype to mutM mutants of E. coli, may be one such example (56). In addition to its involvement in protein synthesis, the S3 protein possesses a nuclear localization signal, hinting at communication between transcription and DNA repair. Moreover, aberrant levels of the human ribosomal S3 protein have been reported in xeroderma pigmentosum and Fanconi’s anemia, a disease associated with elevated levels of the adduct oxo8dG. Mammalian cells also face the additional burden of delivering DNA repair capacity to their mitochondria, an organelle which, despite early reports to the contrary, is able to process oxidative DNA damage (57). Therefore, in addition to phenotype resulting from loss of nuclear repair, there may be collateral or independent syndromes associated with inefficient repair of mtDNA. In xeroderma pigmentosum complementation group A, for instance, a deficiency in the repair of oxidative damage...
of both nuclear and mtDNA is observed (58).

The removal of oxidative adducts in human cells appears to be very rapid. In lymphoblasts, the half-lives of 
H$_2$O$_2$-induced adducts ranged from 8.5 to 62 min (59). In human respiratory tract epithelial cells, repair of some 
H$_2$O$_2$-induced adducts (for example, oxo$^8$dG) is so rapid that a narrow window of opportunity (approximately 
30 min) exists for their detection (60). The heterogeneity of DNA adducts should be noted; whereas oxo$^8$dG may rapidly appear 
and disappear with an hour, in the same cells thymine glycol and single-strand breaks (themselves a result of repair) may increase 
(60).

Hormesis or the “beneficial effect of a low level exposure to an 
agent that is harmful at high levels” (61) may be relevant for some 
oxidative stresses, as has been argued to be the case for low level 
radiation exposure (62). Hyperbaric oxygen therapy of humans (100% O$_2$ at 2.5 atm), for instance, induces significant oxidative 
DNA damage to peripheral blood cells on the first day of therapy 
but fails to cause damage on subsequent days (23); in fact, it results 
in a lower base-line level of total and oxidative DNA damage. 
Similarly, γ-irradiation of rats, which significantly elevates oxida-
tive adducts in hepatic chromatin, results in lower base-line levels 
of some oxidative DNA adducts 24 h after an acute exposure (50), 
and other observations of the lowering of base-line oxidative dam-
age by oxidants have appeared (27). These results are not surpris-
ing, since defense systems are often induced in response to oxida-
tive stress, a generalization that has recently been extended to 
oxo$^8$Guo glycosylase activity in E. coli (63) and rats (30). This implies that there is a degree of slack in oxidative defense and 
repair under “normal” circumstances and that cells may ordinarily 
tolerate a burden of oxidative adducts that contributes to the 
spon-
taneous rate of mutation.

**DNA Oxidation and Cancer**

Experimental and epidemiological evidence suggests that DNA 
oxidation is mutagenic and is a major contributor to human cancer through three main sources: smoking, inflammation, and 
endogenous oxidants such as leakage from mitochondria (1, 3, 7, 
64, 65). Cigarette smoke contains high levels of NO, and depletes 
the body’s antioxidants, and phagocytic cells recruited to sites of 
chronic infection abundantly generate reactive oxidants such as NO$\_2$ and HOCl. Oxidative stresses such as these may contribute to 
as much as half of all human cancers, and evidence of oxidative 
damage during experimental carcinogenesis is accumulating. 
Merely to cite the most recent results, elevated DNA oxidation has 
been measured during early Helicobacter pylori infection (stomach 
cancer (66)), ferric nitroproteinate administration (experimental 
rodent renal cancer (67)), smoking (lung cancer (17, 68)), and 
exposure to diesel exhaust particles (lung cancer (69)), asbestos (lung 
cancer (70)), benzene (leukemia (71, 72)), and aflatoxin (liver can-
cer (73)). Some studies have provided more than a simple associa-
tion between carcinogenic agents and oxidative DNA damage by 
measuring the specific induction of repair enzymes by oxidative 
carcinogens (74) and by demonstrating the suppression of carcino-
genesis by administration of antioxidants (21, 75). The latter re-
sults are consistent with the strong correlation between a high 
intake of fruits and vegetables, which are the principal source of 
dietary antioxidants, and reduction in cancer risk by as much as 
half (1, 7). We have reported elevated oxidative damage to sperm 
DNA in smokers and in men on low serum antioxidants (vitamin C) 
(76) and have hypothesized that oxidative damage to male germ 
cells contributes to cancer and birth defects in the children of male 
smokers (77). Indeed, new epidemiological evidence indicates that 
all types of childhood cancer studied are increased in offspring of 
male smokers; for example, the risks of acute lymphoblastic leukae-
mia, lymphoma, and brain tumors are increased three to four times 
(78).

A transgenic mouse model, in which somatic mutations occur-
ing in vivo can be measured ex vivo with the use of a shuttle vector 
incorporated into the mouse genome, has recently been used to 
quantify a 5-fold increase in mutant frequency in vivo in response 
to short-term ischemia-reperfusion, an oxidative stress (79). Tran-
genic in vivo mutagenesis models, the use of which is becoming 
routine (80), should soon permit the degree and spectrum of mu-
tagenesis to be measured in tandem with increases in oxidative 
damage. Already, the spectrum of alterations in the growing data 
bases of oncogenic human mutations provides some evidence of 
relevant oxidative mutagenesis, as shown by the high frequency of 
G-to-T transversions (a signature mutation resulting from 
oxo$^8$Guo) in human p53 and ras (81).

**Stress and Damage from Cradle to Grave**

The role of DNA oxidation in diseases of aging and in develop-
mental abnormalities is less well established but ripe for investi-
gation. A fundamental unanswered question is whether or not 
DNA oxidation is able to adversely affect quiescent and postmitotic 
cells in diseases in which uncontrolled proliferation is not an issue. 
There is evidence that the frequency of oxidative DNA adducts 
increases by as much as 2-fold with age in a number of species and 
tissues (19, 82, 83). We have recently found that mitochondria from 
senescent animals produce a greater flux of oxidants than young 
mitochondria, which is consistent with these results and suggests a 
mechanism for the observation (65). Even potent exogenous oxida-
dants result in a limited (several-fold at most) and rather short 
term increase in adduct frequency (60). Therefore, it may be that 
an age-related, persistent 50–100% increase in the steady-state 
level of adducts is physiologically relevant, representing an inability 
to prevent or repair oxidative damage. Unfortunately, the de-
tection of such a change in the steady-state frequency of adducts 
requires the virtual absence of artifactual background noise. Two 
recent and independent studies, in which the frequency of oxo$^8$dG 
in a variety of organs of Fisher 344 rats was studied, illustrate this 
point. In the first, a clear increase in the ratio of oxo$^8$dG/dG was 
noted (18), whereas in the second, the lack of a significant increase 
in the ratio of oxo$^8$dG/dG was associated with higher base-line 
values in young animals (19).

So far, we have not made a distinction between the oxidation of 
nDNA and that of mtDNA, but there is a body of work based on the 
HPLC-EC detection of oxo$^8$dG which suggests that the latter is 
higher by more than 10-fold (48) and that age-related mtDNA 
oxidation is particularly dramatic (84). These empirical observa-
tions have been attributed to a number of properties of mtDNA, 
including its proximity to metabolic oxidant generation and its 
established sensitivity to mutagens in general. It is worth stressing 
that measuring mitochondrial oxo$^8$dG by HPLC-EC represents a 
great methodological challenge, due to the difficulty in purifying it 
in quantity. The correspondingly greater potential for artifactual 
oxidation during the preparation and analysis of mtDNA may 
explain the large disparity between published values (85). The 
technique of Q-PCR, which requires neither DNA purification nor 
large amounts of DNA, has recently been used in studies of mtDNA 
oxidation and has confirmed the greater sensitivity of mtDNA than 
nDNA to exogenous oxidants (27, 68, 81). However, the small 
number of studies, pitfalls of analyzing mtDNA, and large range of 
credible values (85) indicate that more work on mtDNA oxidation is 
needed, particularly in light of the potential role of mitochondria in 
age-related human diseases (65).

Last (or perhaps first), there is evidence from the other end of 
the human lifespan that oxidative damage may interfere with develop-
ment. The elevation of oxo$^8$dG by teratogens has been observed 
(86), and the phenotype of Cockayne’s syndrome, which includes 
mental retardation, developmental defects, and (often) premature 
death in childhood, has been tightly associated with the specific 
lack of transcription-coupled repair of oxidative damage (34). Even 
the process of birth itself, which represents, among other things, 
the first direct oxidative stress encountered by newborn mammals, 
duces a measurable degree of genotoxic oxidative stress (87).

**Conclusion**

In this minireview, we have attempted to highlight the recent 
results in this fast moving field. A principal outstanding question is: “what proportion of carcinogenic and spontaneous mutations 
are caused by metabolic oxidants?” Although there is evidence that 
specific oxidative injury, such as that associated with reperfusion 
injury, chronic infection, or smoking, may result in mutagenesis, it 
is less clear that spontaneous mutations are oxidative. The coordi-
nated use of three different types of transgenic mouse models 
should soon make these tractable problems. Transgenic mice have 
been established that possess altered antioxidant activities (22)
