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Oxyradicals and DNA damage

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A major development of carcinogenesis research in the past 20 years has been the discovery of significant levels of DNA damage arising from endogenous cellular sources. Dramatic improvements in analytical chemistry have provided sensitive and specific methodology for identification and quantitation of DNA adducts. Application of these techniques to the analysis of nuclear DNA from human tissues has debunked the notion that the human genome is pristine in the absence of exposure to environmental carcinogens. Much endogenous DNA damage arises from intermediates of oxygen reduction that either attack the bases or the deoxyribosyl backbone of DNA. Alternatively, oxygen radicals can attack other cellular components such as lipids to generate reactive intermediates that couple to DNA bases. Endogenous DNA lesions are genotoxic and induce mutations that are commonly observed in mutated oncogenes and tumor suppressor genes. Their mutagenicity is mitigated by repair via base excision and nucleotide excision pathways. The levels of oxidative DNA damage reported in many human tissues or in animal models of carcinogenesis exceed the levels of lesions induced by exposure to exogenous carcinogenic compounds. Thus, it seems likely that oxidative DNA damage is important in the etiology of many human cancers. This review highlights some of the major accomplishments in the study of oxidative DNA damage and its role in carcinogenesis. It also identifies controversies that need to be resolved. Unraveling the contributions to tumorigenesis of DNA damage from endogenous and exogenous sources represents a major challenge for the future.

A major development over the past 20 years has been the realization that DNA damage and mutation arise from endogenous products of cellular metabolism (1). Oxygen radicals generated during reduction of O_2 can attack DNA bases or deoxyribose residues to produce damaged bases or strand breaks (2). Alternatively, oxygen radicals can oxidize lipid or protein molecules to generate intermediates that react with DNA to form adducts (3). Attempted replication of this damage leads to mutation or apoptosis (4).

Discovery of background damage to DNA from oxyradicals

The development of analytical methods sensitive enough to detect background damage to DNA was critical to the appreciation of the importance of endogenous oxidative damage in carcinogenesis (5–8). Scientists tend to study what they can see and until the mid-1980s the major focus of attention was DNA damage arising from xenobiotics such as polycyclic hydrocarbons, aromatic amines, nitrosamines, etc. Sensitive methods such as ^{32}P-post-labeling were developed to detect and quantify adducts arising from these exogenous carcinogens and numerous studies were conducted relating adduct levels to tumorigenesis in animal models (9). In the absence of treatment, no adducts were detected in the DNA from target tissues so it was assumed that the genomic DNA of normal animals and humans was undamaged. This opinion was held not only by scientists studying carcinogenesis but also by regulatory agencies that interpreted the linearity of carcinogen-induced DNA damage as justification for a non-threshold policy regarding chemical exposure. Radiation chemists had provided an extensive characterization of the panoply of products generated when DNA is subjected to X-rays or γ-rays but this was assumed to be a minor source of background DNA damage in most people arising from exposure to cosmic rays (10). The exception to the widely held opinion about the pristine condition of genomic DNA was an appreciation that spontaneous deamination and depurination occurred at some frequency (11). The rate of spontaneous deamination of cytosine is slow in duplex DNA and although the rate of depurination is high, the latter is difficult to measure in cells or tissues because of the rapid rate of repair of abasic sites (however, see ref. 12).

The original version of ^{32}P-post-labeling was optimized for detection of hydrophobic adducts such as those derived from polycyclic hydrocarbons (5). Unmodified deoxynucleoside-3′,5′-*bis*-phosphates were washed from the thin layer plates in the first two elutions of the four-dimensional chromatography. However, this also removed polar adducts from the plates, which accounted for the ‘cleanliness’ of the control DNA samples from carcinogen-treated animals. When Randerath *et al.* altered the polarity of the initial washes to retain all the *bis*-phosphates, they found a significant number of spots in DNA from untreated animals that did not coelute with unmodified deoxynucleoside-3′,5′-*bis*-phosphates (13). These spots also did not coelute with the *bis*-phosphate of 5-methyldeoxycytidine nor were they due to RNA contamination (14). Randerath suggested these adducts arose from indigenous compounds so he coined the term ‘I-spots’ to describe the *bis*-phosphates detected on thin layer chromatography plates (13). The patterns of I-spots in rodents and humans varied with tissue, species, gender, diurnal cycle and diet (15,16). The levels in rodents increased with age and their formation could be altered by administration of hormonal agents, cytochrome P450 inducers or peroxidation stimuli (17–19). The small quantities of adducts that are detectable by ^{32}P-post-labeling has made it difficult to identify individual I-spots.

At approximately the same time, Ames and co-workers demonstrated that oxidized DNA bases are present at high

Abbreviations: GC/MS, gas chromatography/mass spectrometry; HNE, 4-hydroxynonenal; HO-PdGs, hydroxy-propanodeoxyguanosines; MDA, malondialdehyde; 8-oxo-dG, 8-oxo-deoxyguanosine.

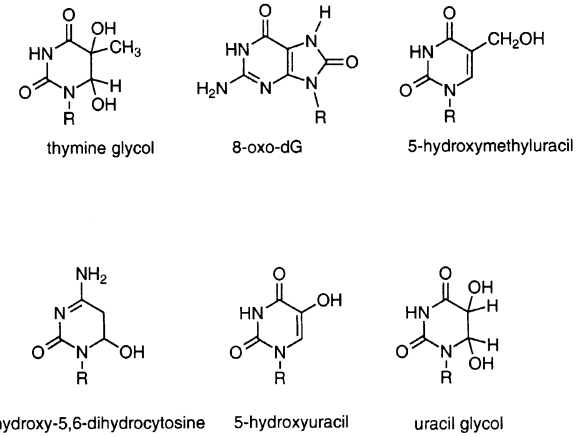

Fig. 1. Oxidized DNA bases discussed in this review.

levels in a wide range of human and rodent tissues and that the species variation in adduct levels correlates roughly with metabolic rate (20). Floyd *et al.* reported that one of these lesions, 8-oxo-deoxyguanosine (8-oxo-dG), could be easily measured by HPLC with electrochemical detection (Figure 1) (21). 8-Oxo-dG had been detected previously in DNA treated with a variety of oxidants so the availability of a facile method for its detection made it the adduct of choice for measuring DNA oxidation (22). Eventually, Dizdaroglu and Gajewski applied gas chromatography/mass spectrometry (GC/MS) for the simultaneous detection of multiple oxidized DNA bases which expanded the pantheon of damaged bases detectable in the genome (6). Ironically, the analytical data that provided much of the driving force for the study of endogenous DNA damage are now the focus of intense and acrimonious debate (see below).

These reports of background DNA damage had a dramatic effect on carcinogenesis research. Attention shifted from exogenous compounds as the sole source of DNA damaging agents to endogenous compounds. The ease with which 8-oxo-dG could be measured in tissues, body fluids, etc. rapidly led to hundreds of reports of its detection in animal and human DNA. Had *Science* magazine initiated its 'molecule of the year' feature in the late 1980s, a strong case could have been made for 8-oxo-dG.

Nature of the oxidants

The identity of the oxidants responsible for the production of oxidized DNA bases is still the focus of study. The hydroxyl radical (HO·) is an obvious candidate because it is extremely reactive and adds to DNA bases or abstracts hydrogen atoms to produce many of the products that occur in the genome (10,23). It is likely that HO· plays a role in the endogenous oxidation of DNA but it is certainly not free HO· generated in one component of the cell and diffusing to the nucleus. The reactivity of HO· is so great that it does not diffuse more than one or two molecular diameters before reacting with a cellular component (24). To the extent that HO· oxidizes DNA, it must be generated immediately adjacent to the nucleic acid molecule. It is likely that H_2O_2 serves as a diffusible, latent form of HO· that reacts with a metal ion in the vicinity of a DNA molecule to generate the oxidant (25,26).

Another oxidant that can generate many of the products observed with HO· is peroxynitrite (ONO_2^-) (27,28). Peroxynitrite is the coupling product of nitric oxide and superoxide and is an extremely strong oxidant. Although ONO_2^- does generate small quantities of HO·, the protonated form of ONO_2^- (peroxynitrous acid, ONO_2H; pK_a = 6.8) is an extremely reactive oxidant capable of oxidizing DNA independent of its ability to generate HO· (29,30). The pattern of products generated by DNA oxidation by ONO_2^- is complex and mirrors the diversity of oxidized DNA detected in tissues (31). Interestingly, ONO_2^- is more reactive toward 8-oxo-dG than to unmodified DNA bases (31). So as 8-oxo-dG levels build up they can compete with unmodified bases present in much higher concentrations for reaction with ONO_2^-. Nitric oxide and superoxide are produced simultaneously in macrophages so one anticipates that elevated levels of ONO_2^- would be produced in activated inflammatory cells. In contrast to HO·, ONO_2^- has the ability to diffuse within cells and may be taken up by some cells via anion transporters (32). This may provide a link between inflammation and the induction of mutation by virtue of the ability of ONO_2^- to oxidize DNA. In fact, Ambs *et al.* recently demonstrated an association between the occurrence of G→T transversions in the *p53* gene of human colorectal cancers and the level of expression of the inducible form of nitric oxide synthase (33).

Genotoxic effects of DNA damage by oxyradicals

8-Oxo-dG is not necessarily the most abundant oxidized DNA product, but it has been the most extensively studied because it is the most easily measured. Its biological properties have been extensively investigated. Shibutani and Grollman demonstrated that 8-oxo-dG causes miscoding by DNA polymerase *in vitro*, and site-specific mutagenesis experiments by Loeb, Moriya and Sarasin verified that 8-oxo-dG is mutagenic in bacterial and mammalian cells (34–37). 8-Oxo-dG is a relatively weak premutagenic lesion in these assays but induces G→T transversions, which are widely seen in mutated oncogenes and tumor suppressor genes (38). Site-specific approaches also have been used to demonstrate that 8-oxo-adenine, thymine glycol, 5-hydroxyuracil and uracil glycol are mutagenic (39). In fact, the latter two lesions are highly mutagenic (40).

Analysis of the mutagenic potential of adducts by site-specific approaches represents a unique challenge for each lesion because of the varying strategies for chemical synthesis, vector construction, vector characterization, etc. The plethora of lesions formed by oxidation of DNA indicates that it is difficult to comprehensively evaluate all of them by site-specific mutagenesis. Therefore, Loeb and co-workers developed a technique called reverse chemical mutagenesis as an approach to identifying the most mutagenic lesions in a complex series (41). By oxidizing deoxycytidine-triphosphate, then separating the oxidation products and incorporating them into vectors for mutagenic screening, they identified 5-hydroxy-deoxycytidine as a highly mutagenic lesion (39). The summation of biological information on oxidized DNA bases indicates that they are mutagenic and induce mutations commonly observed in mutated human genes.

Repair of oxyradical damage to DNA

A critical discovery was the finding that an elaborate repair system exists in *Escherichia coli* to reduce mutagenesis by 8-oxo-dG. This system is termed the GO system and utilizes glycosylases to remove 8-oxo-dG (FAPY glycosylase/*mutM* gene product) or to remove dA residues misincorporated

opposite it (*mutY* gene product) as well as a hydrolase to cleanse the nucleoside pool of 8-oxo-dGTP (*mutT* gene product) (42–44). Mutations in any of these genes has a significant effect on the spontaneous mutation rate in *E.coli* implying that oxidized DNA, and specifically 8-oxo-dG, plays a major role in the induction of spontaneous mutations (45,46). Glycosylases play an important role in the removal of other oxidized lesions as well (47,48). Endonucleases III and VIII of *E.coli* remove a spectrum of oxidized pyrimidines such as thymine glycol, 6-hydroxy-5,6-dihydrocytosine, uracil glycol and others (49–51). Homologs of these repair glycosylases exist in eukaryotic organisms (47,52,53).

An intriguing discovery was made recently by Leadon and co-workers who reported that thymine glycol is a substrate for transcription-coupled repair in mouse embryonic stem cells but not in embryonic stem cells bearing homozygous deletions of the breast cancer susceptibility gene, *BRCA1* (54). This finding not only indicates that glycosylases play an important role in strand-specific repair of oxidized DNA bases but also implicates endogenous DNA oxidation as a contributing factor in the development of one form of hereditary breast cancer. Another intriguing observation about oxygen radicals and DNA repair is that nitric oxide inhibits several DNA repair enzymes including the FAPY glycosylase that removes 8-oxo-G (55). This suggests there may be synergy between the ability of nitric oxide to stimulate DNA damage through formation of peroxynitrite (*inter alia*) and to inhibit repair of that damage.

Biomarkers of oxidative DNA damage

The existence of repair systems that appear to have evolved to remove oxidized DNA bases is strong supporting evidence for their involvement in spontaneous DNA damage across the range of life forms. This is important to recall because the extent to which oxidized DNA actually exists in healthy cells has become the subject of controversy. The principal methods employed for analysis are HPLC/EC, GC/MS and ^{32}P-post-labeling. Reports of the levels of 8-oxo-G or 8-oxo-dG in human tissues range from one adduct in 10^7 nucleotides to one adduct in 10^3 nucleotides (56). The baseline levels reported by different laboratories to exist in the same tissues vary by orders of magnitude (57). Concerns have been expressed about potential artifacts generated during the isolation of DNA, derivatization for mass spectrometry, etc (26,56). For example, 8-oxo-G can be generated from dG during the silylation reactions used to produce volatile derivatives for GC/MS (58). Similar reactions have been reported for oxidized bases (59). Thus, it is essential to incorporate a pre-purification step to remove unmodified deoxynucleosides or bases prior to analysis. Artifactual generation of oxidized bases appears to be more serious when low amounts of DNA are analyzed, regardless of the method of analysis employed (57). Even factors as apparently insignificant as the speed of the centrifuge used to pellet nuclei can lead to variation in the background levels of 8-oxo-dG. This has been a focus of pointed debate within the field. Each investigator seems to feel that his or her assays are reliable, but when these reliable methods are applied to the same samples, widely disparate values are determined. This has cast a pall on whether 8-oxo-dG is actually produced in human DNA. Clearly, an intricate series of repair enzymes would not have evolved to remove a lesion that does not exist, but exactly how much 8-oxo-dG is present in the steady-state is considered uncertain at best.

One way to resolve this dilemma might be to develop a different biomarker for oxidative stress. Le *et al.* recently reported what appears to be an extraordinarily sensitive and reliable method for quantitating thymine glycol residues in DNA (60). This method utilizes binding of a monoclonal antibody specific for thymine glycol to DNA followed by binding of a fluorescently labeled secondary antibody to the initial antibody–DNA complex. The ternary complex of primary and secondary antibodies with DNA is separated from other proteins or protein–DNA complexes by capillary gel electrophoresis with fluorescence detection. This method is extremely sensitive, exhibiting a detection limit of 3×10^{-21} mol thymine glycol. Analysis of DNA from human lung carcinoma (A549) cells reveals extremely low background levels of thymine glycol that can be increased by exposure to clinical doses of radiation (0.01–1 Gy). Theoretically, this method should be applicable to all types of DNA lesions if a suitable monoclonal antibody is available. It will be very exciting to see how this approach progresses but at the least, the existence of this assay may make thymine glycol the lesion of choice as a biomarker of oxidative damage in DNA.

DNA damage by lipid peroxidation

Oxygen radicals attack all cellular macromolecules, not just DNA. In fact, estimates suggest DNA may be the least significant target from a quantitative standpoint (61). Attention has focused on it because of the genetic consequences of DNA damage. But oxygen radical damage to other cellular components may be an equally important source of damage. The polyunsaturated fatty acid residues of phospholipids are extremely sensitive to oxidation. Every phospholipid in every membrane of the cell contains an unsaturated fatty acid residue esterified to the 2-hydroxyl group of the glycerol moiety of the phospholipid. Many of these are polyunsaturated and the presence of a methylene group between two double bonds renders the fatty acid sensitive to oxidation (62). The high concentration of polyunsaturated fatty acids in phospholipids not only makes them prime targets for reaction with oxidizing agents but also enables them to participate in long free radical chain reactions. It is estimated that 60 molecules of linoleic acid (the most common polyunsaturated fatty acid in cells) are consumed per oxidant that reacts with the phospholipid bilayer (63). Likewise, ~200 molecules of arachidonic acid react per oxidation event. The proclivity of polyunsaturated fatty acid molecules to be oxidized has resulted in the evolution of an extensive system of small molecule antioxidants (vitamin E/vitamin C, etc.) and enzymes (superoxide dismutase, catalase, glutathione peroxidase, etc.) to prevent membrane oxidation or to minimize the damage by removing it. Despite this fact, lipid peroxidation is an ongoing process, biomarkers of which can be detected in all healthy human beings. The best of these biomarkers is a set of arachidonic acid oxidation products termed isoprostanes (64). These compounds are reliably detectable by GC/MS and their use has been validated in numerous animal models of oxidative stress and in clinical trials (65,66).

Lipid hydroperoxides are the initial products of unsaturated fatty acid oxidation but they are relatively short-lived. They are either reduced by glutathione peroxidases to unreactive fatty acid alcohols or they react with metals to produce a variety of products which are themselves reactive (e.g. epoxides, aldehydes, etc.). The major aldehyde products of lipid peroxidation are malondialdehyde (MDA) and 4-hydroxynonenal

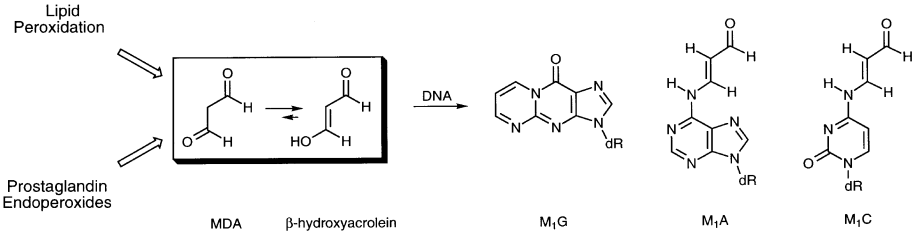

Fig. 2. MDA synthesis and reaction with DNA bases.

(HNE) (67). MDA is mutagenic in bacterial and mammalian cells and carcinogenic in rats (68–70). HNE is weakly mutagenic but appears to be the major toxic product of lipid peroxidation (71). In addition, HNE has powerful effects on signal transduction pathways which have a major effect on the phenotypic characteristics of cells. Some of the effects of HNE on signaling appear independent of DNA damage (see below).

MDA-induced damage

MDA reacts with DNA to form adducts to dG, dA and dC (Figure 2) (72). M_1G and presumably M_1A and M_1C also can be made by the reaction of the corresponding bases with base propenal, providing an alternate route for their generation by direct oxidation of DNA (73). Our laboratory developed a GC/MS method for the determination of M_1G and demonstrated the existence of the adduct in human liver, white blood cells and pancreas (8). M_1G residues were detected in all of these tissues at levels ranging from below the limit of detection to as high as 1.2 adducts per 10^6 nucleosides (approximately 6500 adducts per cell). M_1G also has been detected in human breast tissue by ^{32}P-post-labeling and in rodent tissues (74,75). The identity of M_1G in human liver and white blood cell DNA samples was independently verified by liquid chromatograpy/tandem mass spectrometry (76,77). The recent development of a highly sensitive and specific immunoslotblot assay that utilizes relatively small amounts of DNA offers a method for high throughput analysis amenable to population-based studies (78).

Modification of a single-stranded bacteriophage genome with MDA followed by transformation of SOS-induced *E.coli* strains causes frameshift and base pair substitution mutations in the *lacZ*α gene carried on the vector (79). Base pair substitutions are observed at G, A and C residues which are assumed to arise from the corresponding MDA–DNA adduct at that position (G→T, A→G and C→T). Site-specific experiments confirm that M_1G is mutagenic in *E.coli*, inducing transversions to T and transitions to A (80). The mutation frequencies are comparable with those reported for 8-oxo-dG in similar systems. Transformation of adducted genomes into repair-deficient strains suggest that M_1G is repaired by both bacterial and mammalian nucleotide excision repair pathways and is also repaired in *E.coli* by mismatch repair (81,82). Attempts to detect glycosylases that remove M_1G as the base have been unsuccessful.

High field NMR analysis indicates that M_1G undergoes rapid and quantitative ring-opening to N^2-oxopropenyl-G when it is present in duplex DNA but not when it is present in single-stranded DNA (Figure 3) (83). Thermal denaturation of the duplex leads to rapid cyclization of N^2-oxopropenyl-G to M_1G. Reannealing of the separated strands leads to quantitative ring-opening. Thus, the ring-opening and ring-closing is recapitulated at each cycle of renaturation/denaturation. The presence

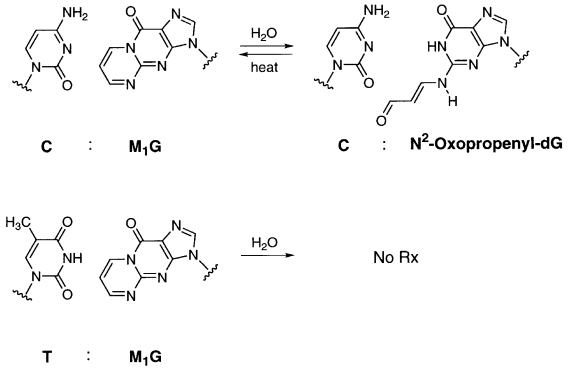

Fig. 3. Ring-opening of M_1G in duplex DNA when positioned opposite C but not when opposite T.

of the 4-amino group of dC opposite M_1G appears to be important for ring-opening because when M_1G is present in duplexes opposite T residues, ring-opening does not occur. Thus, it appears that DNA plays an active role in catalyzing the ring-opening of M_1G.

This and other studies suggest that M_1G is a reactive electrophile in the genome. It not only undergoes DNA-catalyzed hydrolysis to N^2-oxo-propenyl-dG but adds nucleophiles such as amines or hydroxylamines (84). Both M_1G and N^2-oxo-propenyl-dG are electrophilic but their reactive centers are present in different regions of the DNA. The reactive functionality of M_1G is present in the major groove whereas the reactive functionality of N^2-oxo-propenyl-dG is present in the minor groove. The interconversion of M_1G and N^2-oxo-propenal-dG may present reactive functional groups within the DNA that could lead to the formation of DNA–DNA interstrand crosslinks or DNA–protein crosslinks. DNA–protein crosslinks have been detected in cells exposed to oxidative stress but the molecular events responsible for the crosslinking are not well understood (85).

Etheno adducts

Other exocyclic DNA adducts that arise from lipid peroxidation have been detected in DNA from healthy human beings. Etheno-dA, etheno-dC and etheno-dG have been detected by both ^{32}P-post-labeling and GC/MS (86,87). The precise pathway of their formation in DNA is uncertain but the adducts can be generated *in vitro* by exposure of DNA to peroxidizing lipid (88). The epoxide of HNE reacts with dA to form etheno-dA so it is a potential intermediate leading to etheno adducts (Figure 4) (89).

The biological activity of etheno adducts has been extensively investigated by site-specific mutagenesis experiments. Loeb and co-workers (90) reported that N^2,3-etheno-dG induces transitions to A, and Langouet *et al.* (91) reported that 1,N^2-etheno-dG induces transitions to A and transversions to T in

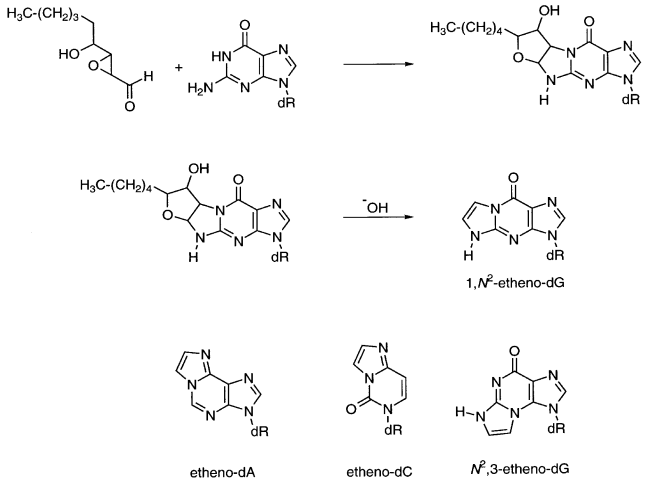

Fig. 4. Reaction of 2,3-epoxy-4-hydroxynonenal with DNA bases to make etheno adducts.

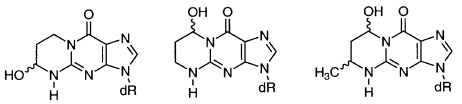

Fig. 5. Hydroxy-PdGs.

E.coli. Basu *et al.* (92) demonstrated that etheno-dA and etheno-dC are strongly genotoxic but weakly mutagenic when introduced on single-stranded vectors in *E.coli*. Likewise, Moriya *et al.* (93) demonstrated that etheno-dA and etheno-dC are weakly mutagenic in *E.coli*. However, when the same adducted shuttle vector is transfected into monkey kidney (COS-7) cells, both adducts are highly mutagenic. Etheno-dA induces mainly transitions to G whereas etheno-dC induces transversions to A and transitions to T (94). The differentials in mutagenic potency between bacterial and mammalian cells are striking and highlight the importance of the cell system used to evaluate the mutagenic activity of a given lesion.

Investigation of the repair of etheno adducts has focused on the base excision repair pathway. Several laboratories have demonstrated that etheno-dA is removed by the action of 3-methyladenine glycosylase and its mammalian homolog AAG (also called ANPG) (95–97). Recently, Saparbaev and Laval reported that etheno-dC is a substrate for the G:T mismatch glycosylase from both *E.coli* and human cells (98). Despite the fact that etheno adducts are good substrates for removal by glycosylases, other repair pathways should be considered. For example, the mutagenicity of 1,N^2-etheno-dG increases when vectors containing it are transfected into *E.coli* strains deficient in nucleotide excision repair (91). As mentioned above, the structurally-related adduct, M_1G, also is removed by the nucleotide excision repair pathway (80).

Propano adducts

Chung and colleagues have demonstrated that hydroxypropanodeoxyguanosines (HO-PdGs) are present in human and rodent liver DNA (Figure 5) (99,100). These adducts appear to be derived by reaction of DNA with acrolein and crotonaldehyde generated by lipid peroxidation. Acrolein and crotonaldehyde are mutagenic in bacteria and mammalian cells but the mutagenic potency of HO-PdGs has not been evaluated by site-specific approaches (101,102). This relates to the instability of these adducts, which renders their incorporation into oligonucleotides or shuttle vectors challenging. A novel, post-oligomerization strategy for the synthesis of oligonucleotides containing the acrolein-derived HO-PdG was reported recently, which should make it possible to construct the requisite adducted vectors (103). The unsubstituted adduct, PdG, has been evaluated by site-specific approaches. PdG is not a naturally occurring adduct but has been used as a model for several unstable exocyclic adducts, including the HO-PdGs. PdG induces base pair substitution mutations in *E.coli* with high efficiency (93,104,105). Interestingly, transformation of PdG-adducted shuttle vectors into COS-7 cells reveals that PdG is much less mutagenic than in *E.coli* (93). This is opposite to the effects described above with etheno-dA and etheno-dC.

Little is known about the repair of HO-PdGs. PdG is a substrate for the nucleotide excision repair complex of *E.coli* as well as mammalian cells and it is also recognized and repaired by the mismatch repair system (81,82). The possibility that PdG or HO-PdGs are substrates for base excision repair enzymes need to be evaluated in a comprehensive fashion.

DNA damage associated with oxidative stress/inflammation

Numerous studies have been conducted on the levels of oxidized bases or exocyclic adducts in tumor tissue or in chronic inflammatory states. A variety of assays have been employed so it is instructive to compare them. Malins and Haimanot first reported an association of oxidized DNA bases with breast cancer, demonstrating a 9-fold elevation in the levels of 8-oxo-G, 8-oxo-A and a formamidopyrimidine in tumor tissue compared with surrounding normal tissue (106). Subsequently, Shimoda *et al.* reported that the levels of 8-oxo-dG in liver tissue from individuals with chronic inflammatory diseases (hepatitis, cirrhosis) are elevated compared with control liver (107). Similar findings were reported in a transgenic mouse model that expressed the hepatitis B virus large envelope protein in the liver (108). Elevated 8-oxo-dG was detected early in life and increased progressively with the progression of disease. The levels of 8-oxo-dG in the DNA from gastric tissue of patients infected with *Helicobacter pylori* are elevated relative to the levels in uninfected individuals (109). The levels are highest in patients with chronic atrophic gastritis and reduced in patients with chronic non-atrophic gastritis and patients with gastric cancer. Individuals with hyperplastic diseases of the stomach exhibit higher levels of 8-oxo-dG than healthy controls (109).

Several analyses have been conducted of the levels of 8-oxo-G or 8-oxo-dG excreted in urine. Oxidized base and deoxynucleoside adducts are presumed to represent the products of repair of oxidized DNA but there is some concern that they may reflect damage to the nucleoside pool or dietary oxidized bases. Surprisingly, no obvious association appears between 8-oxo-dG levels in urine and the incidence of liver inflammation or gastric inflammation triggered by *Helicobacter* infection (110). Likewise, in clinical studies, no effect on the level of 8-oxo-dG is observed with age or the presence of plasma antioxidants (vitamin E, vitamin C, β-carotene, lycopene, coenzyme Q or glutathione transferase) (111). Only a relatively modest effect of cigarette smoking has been reported in one study and no effect in another (112,113). In contrast, urinary 5-(hydroxymethyl)uracil levels are increased in smokers compared with non-smokers and in patients treated

with adriamycin (112,114). This may be a better urinary biomarker than 8-oxo-dG.

The inability to detect increases in urinary 8-oxo-dG with inflammation and oxidative stress may be due to continual whole body oxidation of the guanine nucleotide pool that produces a significant background level of urinary 8-oxo-dG. This background may make it difficult to detect increases associated with repair of 8-oxo-G or 8-oxo-dG from genomic DNA in a particular tissue. Continual oxidation of the polyunsaturated fatty acid pool occurs as reflected in the levels of plasma and urinary isoprostanes detected in all human beings so it is likely that the nucleotide pool also undergoes continual oxidation (66). However, it should be noted that despite the continual spontaneous production of isoprostanes, an ~3-fold increase in their urinary levels is detectable in cigarette smokers and is reversible on smoking cessation (115). This increase is considerably larger than the modest increases in urinary 8-oxo-dG reported in smokers.

The levels of exocyclic adducts have been investigated in clinical disorders believed to be associated with enhanced oxidative stress. Wilson's disease is a copper storage disease that leads to abnormal copper accumulation and oxidative stress in the liver. The levels of etheno-A and etheno-C in liver DNA are elevated ~3-fold in patients with Wilson's disease (116). An increase of similar magnitude is detected in individuals with primary hemochromatosis, an iron-storage disease that also causes oxidative stress in the liver (116). HO-PdGs are detectable in oral tissue DNA of humans and their levels are significantly higher in smokers than in non-smokers (117).

An interesting dietary study has been reported by Nair *et al.* (118). Men and women administered a diet high in ω-6 fatty acids exhibit an ~10-fold increase in M_1G levels in their white blood cells, relative to individuals fed a diet high in ω-3 fatty acids. Interestingly, an even larger increase is detected in the levels of etheno-A and etheno-C (~40-fold) in white cell DNA but only in women; the levels in men are not elevated. This finding underscores the potential for dietary modulation of DNA damage from endogenous sources but illustrates that variations will be observed in the levels of individual adducts. This and other studies indicate that one cannot assume that trends in the production of all oxyradical-derived DNA adducts will correlate (119).

Animal models of chronic inflammatory diseases also have been probed for the levels of exocyclic adducts. The levels of both etheno adducts and HO-PdGs are significantly increased in the livers of Long–Evans Cinnamon rats relative to Long–Evans Agouti rats (117,120). Long–Evans Cinnamon rats have an abnormality in copper metabolism that renders them highly sensitive to copper-induced oxidative stress (121). Thus, they are a model for Wilson's disease and there appears to be an excellent correlation between the increases in the levels of etheno adducts in the livers of patients with Wilson's disease and in the livers of Long–Evans Cinnamon rats. Etheno adducts also have been demonstrated to increase in the spleens of SJL mice stimulated to produce high levels of nitric oxide (122). Finally, Nath *et al.* have demonstrated that depletion of glutathione by administration of buthionine sulfoximine to F344 rats leads to a significant increase in the levels of the HO-PdGs in the livers of treated animals (123).

Perspective

There is compelling evidence that oxidative metabolism generates a diverse range of adducts in DNA that are efficient premutagenic lesions and that repair systems have evolved to remove some of them. Thus, it seems reasonable to presume that this background DNA damage contributes to the origin of human genetic diseases such as cancer. However, providing a smoking gun to conclusively establish this is not easy. All of the evidence relating adduct levels to malignancy or treatments associated with malignancy are correlative in nature. Although some of these correlations are impressive, it is difficult to make the leap from adduct levels to mutations in critical genes causative for cancer. In fact, this is a problem that plagues carcinogenesis research in general, regardless of whether the DNA damage originates from exogenous or endogenous sources. Attempts have been made to use mutation spectra to perform retroanalyses of the adducts that lead to mutations. There is a complication of too many mutagens and too few mutations for this approach to be generally applied. For example, many mutagens, derived from either exogenous or endogenous sources, induce G→T transversions because they react with G to form replication blocking lesions. Thus, there are many roads to a G→T transversion and most other mutations for that matter. The notable exception is the CC→TT double mutation, which is a virtual signature for UV-induced DNA damage in sunlight-related cancers (124). Reid and Loeb (125) have shown that CC→TT double mutations also are induced by oxygen radicals, and have proposed this may be a useful marker for oxygen radical involvement in internal organs. However, this double mutation has only been seen in a few tumors of internal organs, at least as judged from the mutation spectra of *p53* genes from human cancers (38).

It may be possible to use second-order effects (e.g. sequence-context effects) to implicate individual DNA adducts. For example, Wallace and co-workers (126) have shown recently that the sensitivity to repair and the miscoding potential of 8-oxo-dG varies 100–1000-fold in different DNA sequences and that these sequence contexts are strongly correlated with G→T transversion hotspots in the *E.coli lacI* gene and the human *p53* and factor IX genes (126). This approach may provide a useful strategy for probing the involvement of other adducts.

Our understanding of the proteins involved in DNA replication and repair is expanding rapidly. Concomitant with this expansion of knowledge is an increasing availability of mice bearing targeted deletions in the genes that code for these proteins. These animals will be extremely useful for defining which repair enzymes are primarily responsible for the removal of individual DNA adducts. Most DNA adducts derived from oxygen radicals are small and relatively polar and there appears to be overlap in their removal by various repair systems. For example, M_1G is removed by both nucleotide excision repair and mismatch repair. It will be interesting to measure the endogenous level of M_1G in knockout mice that are deficient in genes that code for proteins in this pathway. It also will be interesting to assess whether animals with deletions in DNA repair enzymes, for example coding for specific glycosylases, are more sensitive to mutations and cancer.

Of course, mice deficient in DNA repair genes may not exhibit increased cancer incidence if mutation is not rate-limiting for the development of cancers at particular sites. Abundant evidence indicates that cell proliferation is a critical component of carcinogenesis and can be rate-limiting even in the presence of a significant adduct load in a given tissue (127,128). Thus, it is possible that steady-state adduct levels could be increased by an order of magnitude or more in a particular tissue without any detectable increase in tumor

incidence if proliferation or some other event is critical for development of the tumor.

This leads into the final disclaimer of this article. The entire focus of this presentation has been on DNA damage and mutagenesis but oxygen radicals, like all electrophiles, react with many molecules in a cell and can induce a range of responses that are not necessarily dependent on DNA damage (129). Oxygen radicals are known to affect various signal transduction pathways and transcription factors (e.g. NFκB) and to alter cell cycle kinetics (130). Some of these effects may be the result of DNA damage-induced signaling but others may not. For example, HNE, an important product of lipid peroxidation, inhibits signalling by NFκB (131). The target for the effects of HNE are not in the nucleus but rather the upstream events that lead to activation of IκB kinases. Inhibition of IκB kinase activation prevents dissociation of IκB from NFκB in the cytosol so it cannot diffuse to the nucleus to initiate gene expression. The development of microarrays that allow large-scale and simultaneous profiling of gene expression should provide a facile mechanism for evaluating the epigenetic effects of oxygen radicals and their endogenous products. This approach should be especially powerful when it employs cell lines from genetically defined animals (e.g. from knockout mice with DNA repair defects). Thus, one may be able to separate events initiated by reactions in the nucleus from those initiated by reactions elsewhere.

Conclusions

In roughly 20 years, the study of oxygen radical-dependent damage to DNA has become a major thrust of carcinogenesis research. To some extent, this reflects a natural shift in emphasis for researchers as new opportunities open up. But it also reflects the more basic consideration that oxygen radical generation is an inevitable consequence of aerobic life. It is the price we pay for extracting an extra 12 ATP molecules from every molecule of acetyl CoA that we combust. Oxygen radicals conduct an unremitting assault on our genomes that can be augmented or reduced by environmental, nutritional or hormonal influences. Our bodies construct Star Wars-like defenses to scavenge incoming oxidants and Civil Defense teams to repair the damage. But these defenses are not perfect and some damage persists until the genome needs to be copied. Then mutations arise that can contribute to the development of cancer. We do not yet know the precise role that oxygen radical damage plays in carcinogenesis and how it synergizes with other forms of genetic and epigenetic events to accelerate cell transformation and malignant progression. Research in the last 20 years has identified the molecular players, the defenses against them and the consequences of their attack on the genome. The challenge for the next 20 years is to integrate that knowledge with expanding road maps of intracellular and intercellular signal transduction to define the key steps in the carcinogenic process triggered by oxygen radicals.

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