Mutagenesis by Metal-induced Oxygen Radicals

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To assess the contribution of reactive oxygen species (ROS) to metal-induced mutagenesis, we have determined the spectrum of mutations in the lacZa gene after exposure of M13mp2 DNA to Fe3+, Cu+, and Ni2+. With iron and copper ions, mutations are clustered and are predominantly single-base substitutions. Fe, Cu, and phorbol ester-stimulated neutrophils also produced tandem double CC→TT mutations. This mutation may provide a marker for the role of oxidative damage in carcinogenesis. Mutagenesis by Ni2+ required the complexing of the metal to a tripeptide and the addition of H2O2. To assess the contribution of ROS in mammalian cells, we determined the spectrum of mutations produced when purified DNA polymerases-α and -β synthesized DNA using a template that had been damaged by ROS. The mutation spectra produced by the two polymerases indicates that these enzymes substitute different nucleotides opposite the same lesions. — Environ Health Perspect 102(Suppl 3):57-61 (1994).

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Introduction

Certain metals are ubiquitous, known to cause mutations, and have been demonstrated to cause cancer in a variety of animal species. Based on epidemiologic data some of these metals have been classified as human carcinogens (1,2). The mechanism by which they produce tumors is not completely understood, nor is it known whether they are initiating or promoting agents. The fact that most metal ions do not form covalent adducts with DNA suggests that metal mutagenesis may result via the generation of activated intermediates, in particular, reactive intermediates of reduced oxygen (3,4). Oxygen-free radicals have been implicated in a number of degenerative diseases including aging and cancer (5–7). Reactive oxygen species (ROS) arise through normal cellular processes, inflammatory events, ischemia, and xenobiotic metabolism (8,9). The primary species produced by these processes, the superoxide ion and hydrogen peroxide, can interact with transition metals such as iron and copper via the metal catalyzed Haber-Weiss reaction to form -OH radicals:

\[ \cdot \text{O}_2^- + \text{Fe}^{3+} \rightarrow \text{Fe}^{2+} + \cdot \text{O}_2 \]
\[ \text{Fe}^{2+} + \text{H}_2\text{O}_2 \rightarrow \text{Fe}^{3+} + \cdot \text{OH} + \text{OH}^- \]

The ability of ROS to damage cellular molecules including DNA is well documented (10,11). This damage has been hypothesized to be a factor in the initiation or promotion of malignancies, but as yet there is no direct proof of this. A major problem is that the multitude of lesions that oxygen radicals produce in DNA makes it difficult to assign a particular mutation to a given oxidative lesion. In contrast, many chemical carcinogens produce a limited number of DNA lesions and base changes, thus allowing a correlation between the mutational spectrum found in commonly mutated genes of tumors and the proposed etiologic agent (12,13).

To assess the contribution of ROS to metal-induced mutagenesis, we have systematically determined the spectrum of mutations produced by exposure of purified DNA to Fe, Cu, and Ni ions and compared these mutations to those produced by other agents known to generate reactive oxygen species.

Forward Mutation Assays

To catalogue a wide variety of mutations produced by metal-generated ROS we modified the M13 forward mutation assay described by Kunkel (14). Single-strand M13mp2 DNA contains a target for mutagenesis, the lacZa gene and its regulatory sequences. Upon transfection into E. coli the M13mp2 DNA provides the coding information needed to produce a functional gene product, β-galactosidase. E. coli expressing fully active β-galactosidase produces dark blue plaques on the indicator substrate, X-gal. Mutations within the lacZa segment of M13mp2 DNA reduce the level of production or the activity of the lacZa-encoded β-galactosidase yielding light blue or colorless plaques. Single-strand DNA is a particularly useful target since one can directly correlate damage at each template nucleotide with substitutions that occur opposite the damaged nucleotide.

The ability of Fe2+, Cu2+, and Ni2+ to induce mutations in this assay has been established (15,16; Tkishelashvili and Loeb, unpublished). Incubation of M13mp2 DNA with each of these metal ions results in an increase in mutagenesis compared to DNA incubated in the absence of metal ions. Mutagenesis by Ni2+ requires the complexing of the metal to a tripeptide (glycine-glycine-histidine) and
the addition of H₂O₂. Furthermore, the addition of scavengers of oxygen radicals to these assays reduces the levels of mutagenesis produced by these metals, providing indirect evidence for a role of metal-generated ROS as the causative species. A summary of the most prominent mutations produced by each metal is shown in Figure 1. It can be seen that C→T transitions make up a high percentage of the mutations produced by these metals, in agreement with other studies on the mutations produced by ROS. In addition, G→C transitions are a frequent mutation produced by Fe²⁺. The C→T transitions induced by metal-generated ROS are likely mediated by alterations at cytosine residues. Hydroxyl radicals react with cytosines primarily by addition to the 5,6 double bond and produce oxidative products similar to those seen from thymines including glycol, formamide, and hydroxanthine derivatives (10). Cytosine glycol is a major form of oxidized cytosine in DNA (17) but it is not as stable as thymine glycol and can undergo deamination and dehydration to yield uracil glycol and 5-hydroxy derivatives of cytosine and uracil (10). In solution, irradiated cytosine can be deaminated directly to uracil but this base has not been observed in intact DNA following irradiation (18,19).

The possibility that cytosine damage by ROS and the production of C→T transitions is mediated by cytosine deamination to uracil was assessed by introducing oxidatively damaged DNA into E. coli host cells that were without a functional uracil DNA glycosylase (Reid and Loeb, unpublished). If oxidative damage causes the production of uracil residues in DNA, then introduction of this DNA into a ung mutant should result in an increase in mutation frequency in this strain relative to the wild-type strain. Two systems were used. One, the M13 forward mutation assay, was described above. The second system, a reversion assay, allows detection of mutations specifically at two cytosine residues within a single codon of the lacZα gene. In both forward and reversion assays, the introduction of oxidatively damaged DNA into uracil glycosylase deficient host cells did not increase mutation frequency relative to an isogenic wild-type strain. In addition, there was no difference in the biological activity of the DNA in either strain. An analysis of the mutants produced by oxidative damage in the reversion assay was consistent with the lack of formation of uracil residues. The relative frequency of C→T transitions was not significantly greater in the ung strain than in the wild-type host, indicating that some other lesion is responsible for these mutations.

**Mutations Produced by Human Leukemia Cells**

Large amounts of ROS are produced by polymorphonuclear leukocytes (PMNs) as part of their role in defense against invading bacteria. These cells are normally activated to produce ROS when phagocytizing bacteria or other opsonized particles. However, they can also be activated to produce ROS by soluble stimuli such as tumor promoters. In addition, certain Ni and Cd complexes can cause PMNs to accumulate and stimulate them to generate ROS (3). This inflammatory response leads to the release of large amounts of damaging ROS into the surrounding tissue where they can interact with cellular membranes and macromolecules. The types of DNA damage produced by human neutrophils following stimulation by the tumor promoter PMA (12-O-tetradecanoylphorbol-13-acetate) has been extensively investigated. Altered DNA bases include hydroxymethyl uracil, thymine glycol, cytosine glycol, 8-hydroxyguanine (8-OH-dG), 8-hydroxyadenine, and formamido derivatives of altered purines (20–22). These types of damage are similar to those observed following exposure of DNA to ionizing radiation and oxygen radical generators (10, 19).

To determine the spectrum of mutations produced by ROS from stimulated PMNs, we made use of the human cell line HL-60. This cell line consists of promyelocytes that can be induced to differentiate to a neutrophil-like cell population. These cells have most of the functional characteristics of genuine PMNs, including the ability to release measurable amounts of ROS in response to the tumor promoter PMA. Mutagenesis induced by HL-60-generated free radicals was assessed using the M13mp2 forward mutation assay (23). Single-stranded M13mp2 DNA was co-incubated with phorbol ester-stimulated HL-60 cells, after which mutations were scored by transfecting the M13mp2 DNA into SOS-induced E. coli. The ability of PMA-stimulated HL-60 leukemia cells to induce mutations in M13mp2 DNA is shown in Table 1. The mutation frequency of HL-60-treated M13mp2 DNA in SOS-induced E. coli was 6-fold greater than untreated DNA. This increase in mutation frequency with the PMA-stimulated leukemia cells was linear with respect to time of incubation, consistent with an observed increase in production of ROS over time. Exposure of M13mp2 DNA to differentiated HL-60 cells without PMA stimulation led to a slight increase in mutation frequency (<2-fold over background). This may be due to a basal level of reactive oxygen species produced by these cells. In accord with our studies on iron and copper, the major types of mutations generated by exposure of M13mp2 DNA to PMA-stimulated HL-60 cells were single-base substitutions. These occurred primarily opposite guanine residues, leading to G→T and G→C substitutions. Approximately 40% of the remaining mutations occurred opposite cytosine and thymine residues. To determine whether HL-60-induced mutagenesis was mediated by ROS, M13mp2 DNA was co-incubated with PMA-stimulated cells in the presence of superoxide dismutase, catalase, or mannitol. The presence of mannitol, a -OH

**Table 1.** Mutation frequency induced by differentiated HL-60 cells

| Sample     | PMA | Mutants/total plaques | Mutation frequency |
|------------|-----|-----------------------|--------------------|
| M13 control | –   | 21/15784              | 1.3 x 10⁻⁸         |
| HL-60 (60') | –   | 27/11940              | 2.3 x 10⁻⁷         |
| HL-60 (30') | +   | 88/20520              | 4.3 x 10⁻⁸         |
| HL-60 (60') | +   | 241/30659             | 7.9 x 10⁻⁸         |

Single-stranded M13mp2 DNA was co-incubated with differentiated HL-60 cells for the indicated times. *Based on Reid and Loeb (23). †Time of incubation of single-stranded M13mp2 DNA with HL-60 cells.
radical scavenger, reduced mutation frequency by 75%.

A comparison of the frequency and location of the base substitution mutations observed in this study with those generated in studies using iron and copper (12,13) is displayed in Figure 2. In this region of the lacZα gene, the location of some of the mutational hotspots produced by the HL-60 cells and those produced by iron are similar. This suggests that an interaction between Fe and H₂O₂ may be responsible for generation of ·OH radicals by PMA-stimulated HL-60 cells. Neutrophils themselves probably do not generate OH radicals without a transition metal cofactor (24).

**Mammalian DNA Polymerases**

To determine the mechanisms by which mutations might occur in mammalian cells exposed to metal-generated ROS, we used the M13 forward mutation assay to measure the fidelity of mammalian DNA polymerases α and β following in vitro synthesis of ROS-damaged DNA templates (Feig and Loeb, unpublished). DNA polymerase α (pol α) is one of the principal enzymes involved in DNA replication while DNA polymerase β (pol β) is considered to be primarily involved in DNA repair. A combination of CuCl₂, H₂O₂, and ascorbic acid was used to damage a single-stranded region of the lacZα gene in gapped M13mp2 DNA. The region was then copied with purified calf thymus DNA pol α or recombinant rat DNA pol β. Following transfection of the copied DNA into E. coli, mutants were identified by loss of β-galactosidase activity as described above. Despite markedly different mutation frequencies on undamaged DNA, the increase in mutation frequency due to ROS damage for the two polymerases was quite similar, 3.3-fold for pol α and 3.8-fold for pol β. This suggests that the probability of misincorporation opposite oxidative lesions is more dependent on the inherent error frequency of the polymerase than on the nature of the lesions themselves.

The mutational spectra produced by the in vitro copying of ROS-damaged DNA consisted primarily of single-base substitutions or single-base deletions. Hot spots for mutagenesis due to ROS damage are at entirely different locations for the two polymerases. Furthermore, the damage-dependent substitutions by pol α were primarily C→A and A→C mutations whereas those produced by pol β were A→C, G→C and to a lesser extent, C→A and C→T. In addition to the single-base substitutions, approximately 13% of the mutations resulting from pol β copying of Cu-damaged DNA were non-tandem double mutations. This tendency toward non-tandem double mutations was also observed when pol β was used to copy DNA damaged by FeSO₄ and H₂O₂. Of 189 independently isolated mutants, 29 were non-tandem double mutations. A specific pair of base changes, C→A and T→G separated by eight bases, accounted for most (20 of 29) double mutations. Because the ROS-induced double mutants occur too frequently to be explained by independent events, they could represent an example of untargeted mutagenesis resulting from a conformational change in the DNA template. The mutation spectra obtained with mammalian DNA polymerases clearly demonstrate the frequency, location, and specificity of ROS-induced mutations is dependent upon the DNA polymerase that encounters the damaged DNA.

**Tandem Double Mutations**

A major goal in analyzing the spectrum of mutations produced by ROS is to identify specific type of mutation that was diagnostic of DNA damage by ROS. An intriguing similarity between the copper mutation spectrum (16) and that produced by the HL-60 cells is the presence of double CC→TT substitutions, which comprised 6% of the HL-60-induced mutations (23). To our knowledge, this mutation has not been observed in studies on the types of mutations produced by DNA polymerases (25,26), viral reverse transcriptases (27), or following exposure of DNA to chemical carcinogens. This mutation has only been reported in vitro following exposure to UV light (28–32) and has been thought to be a specific indicator of DNA damage by UV irradiation.

To determine the potential of reactive oxygen species to produce tandem double CC→TT mutations, we have adapted a reversion assay (33) that is specific for damage to cytosine residues and can detect both single and tandem double mutations at the same locus. We have used this assay to measure the frequency of tandem double CC→TT mutations produced by metal ions and to compare oxygen radical-induced mutagenesis with that produced by UV light (Reid and Loeb, unpublished). Essentially, the reversion assay is based on a derivative of M13mp2 (termed M13G*1) that has an altered codon (GCC→CCC) within the lacZα gene which, if not mutated, yields white plaques rather than the dark blue of the parental M13mp2 (Figure 3). Any single-base substitution at the first two positions of this altered codon causes a reversion to wild-type dark blue phenotype. Certain tandem double mutations can also be scored including CCC→TTT and CCC→CCTT mutations. Thus, this DNA construct allows detection of both single and double C→T mutations within the same codon of the lacZα gene.

The nucleotide sequence of approximately 200 of the revertants produced by exposure to metal ions or UV light was determined. All possible single-base substitutions were detected at the first two positions of the CCC codon (Figure 4). However, C→T transitions were by far the most prevalent mutation produced by either oxygen damage or UV irradiation. Approximately twice as many single C→T mutations occurred at the first position of the codon compared to the second for both oxygen and UV treatment. Whether this is due to a difference in the degree to which each base is modified or to a bias toward insertion of a serine (TCC) rather than a

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**Figure 3.** Principles of the M13G*1 reversion assay. The CCC codon at position 141-143 of the lacZα gene is the target for mutagenesis. Any single-base substitution at the first two positions of this codon and any tandem double CC→TT mutation causes a reversion to plaque phenotype from white to dark blue.

**Figure 4.** Single and tandem double mutations at the CCC locus of M13G*1. The sequence shown is the viral strand of M13G*1 from position 135 to 149 where position 1 is the first transcribed base. Single base substitutions are shown below the sequence. Tandem double mutations are shown above the sequence. Single and double mutations produced by exposure to Fe³⁺ are shown in italicized bold type; those produced by Cu + H₂O₂ are shown in regular type.
leucine (CTC) at this position is not known.

While single mutations occurred at a frequency of approximately 1 per 2000, tandem double CC→TT mutations were about 30-fold less frequent. No tandem double mutations were observed among the 349,000 plaques obtained with DNA that was not exposed to agents that generate reactive oxygen species. In contrast, DNA damaged by either Fe²⁺ or Cu + H₂O₂ resulted in the production of CC→TT substitutions. Fe²⁺ tended to produce mutations at the first two positions of the CCC codon, while Cu plus H₂O₂ did not show this specificity. When the hydroxyl radical scavenger mannositol was included in the incubation of DNA and Fe²⁺ the frequency of CC→TT mutations was decreased. However, we have observed that mannositol has no effect on the frequency of CC→TT mutations produced by UV light. The diminution of the Fe²⁺-induced mutations by the addition of mannositol provides evidence that these mutations are dependent on the production of ROS. The fact that mannositol did not inhibit the production of tandem double mutations by UV irradiation suggests that there is probably not a common ROS involved in UV and oxidatively induced double mutations. The ability of Fe, Cu, and phorbol ester-stimulated neutrophils to produce CC→TT mutations suggests that CC→TT mutations are a general manifestation of oxygen damage to DNA. The tandem double CC→TT mutation is a unique mutation that provides a marker of oxygen free radical-induced mutagenesis in cells that are not exposed to UV irradiation, and may serve as an indicator for assessing the involvement of oxidative damage to DNA in aging and tumor progression.

Conclusions

The fact that certain metals are carcinogenic may be due to their ability to generate ROS. ROS produced by metals that are not considered carcinogenic per se (e.g., Cu and Fe) may also play a role in multi-stage carcinogenesis during initiation or promotion phases (34). In fact, there is epidemiologic evidence for a correlation between an increased risk of cancer in men with elevated body iron stores (35). In an effort to better define the mechanisms by which mutations are produced by metals, we have cataloged mutations produced by metal ions in both forward and reversion assays and determined the specificity of mutations produced by two mammalian DNA polymerases when using ROS-damaged DNA as a template.

Three base substitutions appear to be a common result of oxidative damage by Fe²⁺, Cu²⁺, and Ni²⁺: G→C, G→T, and C→T substitutions were produced by all three metals. G→C was the predominant mutation produced by Fe²⁺ while C→T substitutions were most frequently due to Cu²⁺, and Ni²⁺. The G→T substitutions are likely caused by 8-OH-dG which is recognized as the most frequent lesion to arise from oxidative damage to DNA. Studies of the mutagenic specificity of 8-OH-dG, have shown that G→T transversions are the primary mutation produced by this lesion (36,37).

G→C substitutions are the primary mutation due to singlet oxygen damage of DNA (38). The mutational specificity of oxidative cytosine lesions has not yet been established. However, deaminated derivatives of cytosine glycol are a likely candidate for the C→T mutations produced by ROS (17). The observation that tandem double mutations result from ROS damage may finally allow a determination of the role of oxygen damage in carcinogenesis. To date the large number of different lesions produced in DNA by ROS has hampered the identification of unique mutations that might provide "molecular signatures" of ROS damage. The tandem double CC→TT mutation may be such a marker and as such its presence in cells that are not exposed to UV irradiation would be convincing evidence for the involvement of oxidative damage.

DNA polymerases obviously play a key role in all mutagenesis. It is interesting to note the contrast in mutation spectra produced when two DNA polymerases synthesize across the same damage. This suggests that an analysis of mutations found in cells exposed to ROS may provide clues to the identity of the polymerase involved in ROS-induced mutagenesis. Having established the types of mutations produced by ROS, much work remains to be done, in particular, to identify the chemical lesions responsible for C→T and CC→TT mutations. However, the ability to define the specific nature of mutations produced by ROS should give a greater insight into the role of metal-generated oxidants in carcinogenesis.

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