Potential Molecular Mechanisms of Oxidant-induced Carcinogenesis

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Chronic exposure to oxidants is associated with an increased incidence of malignancy; however, the mechanism(s) by which oxidants contribute to carcinogenesis is unknown. Since oncogene activation plays an important role in carcinogenesis, we hypothesized that hydroxyl radical-induced DNA damage might contribute to carcinogenesis by causing oncogene activation. The studies reported herein demonstrate that hydroxyl radical-induced DNA damage can activate the K-ras 4B and C-RafI oncogenes by causing point mutations and deletions, respectively. In addition, our results indicate that a) hydroxyl radical-induced DNA damage causes selective base substitutions; b) the four DNA bases have different susceptibilities to hydroxyl radical-induced mutations; and c) hydroxyl radical-induced mutations are not randomly distributed among oncogene codons. Our studies suggest that oncogene activation could be one potential mechanism by which oxidants contribute to carcinogenesis. — Environ Health Perspect 102(Suppl 1):159-158 (1994)

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Introduction

Chronic exposure to oxidants, such as those generated by inflammatory leukocytes or cigarette smoke, is associated with an increased incidence of malignancy (1). However, the mechanism(s) by which oxidants might contribute to carcinogenesis is unknown. In this report I will briefly summarize some of the recent studies from our laboratory aimed at determining potential molecular mechanisms by which oxidants might contribute to carcinogenesis.

Currently, there are believed to be two major mechanisms by which normal cells can be converted into malignant cells. These include the conversion of normal protooncogenes into activated oncogenes, and the inactivation of normal tumor suppressor genes. In general, protein products of protooncogenes transduce growth stimulating signals, while the protein products of tumor suppressor genes transduce growth inhibiting signals. In normal cells, the activity of protooncogene and tumor suppressor gene protein products can be reversibly switched on or off by precisely controlled cellular mechanisms. Accordingly, the growth and differentiation of normal cells can be carefully regulated by modulating the relative activity of these protein products. However, when the protooncogenes or tumor suppressor genes of normal cells undergo genetic alterations (such as point mutations, deletions, rearrangements, allelic loss, etc.), the normal regulation of cell growth and differentiation can become disrupted. For instance, if genetic damage converts normal protooncogenes into activated oncogenes, the protein products of these activated oncogenes can transduce a continuous growth-stimulating signal that cannot be turned off. Likewise, damage to tumor suppressor genes can render their protein products inactive and incapable of inhibiting cell growth. Therefore, the accumulation of activated oncogenes and inactivated tumor suppressor genes in a given cell could lead to disregulated cell growth, and this disregulated cell growth could ultimately contribute to carcinogenesis.

Given the important role of oncogene activation and tumor suppressor gene inactivation in carcinogenesis, we hypothesized that oxidants might contribute to carcinogenesis by causing oncogene activation or tumor suppressor gene inactivation. Previous studies from our laboratory, as well as others, have shown that leukocytes or cigarette smoke release superoxide and hydrogen peroxide that can, in the presence of catalytic amounts of iron, be converted into the very potent oxidant, hydroxyl radical (•OH); and this •OH appears to be the specific oxidant responsible for inducing DNA base damage and DNA strand breaks (2-20). We hypothesized, therefore, that •OH-induced DNA base damage and DNA strand breaks might cause point mutations and deletions, respectively, that could lead to oncogene activation and ultimately contribute to carcinogenesis.

•OH-induced DNA Base Damage and Oncogene Activation

Let us first consider the ability of •OH-induced DNA base damage to cause oncogene activation. Utilizing gas chromatography-mass spectroscopy with selected ion monitoring (GC-MS/SIM), we showed that approximately 7 out of every 1,000 DNA bases became modified when isolated DNA was exposed to physiologic concentrations of PMA-stimulated neutrophils and Fe3+. The specific base modifications detected included (in order of decreasing yield) 8-hydroxyguanine, 8-hydroxyadenine (more correctly referred to as 7,8-dihydro-8-oxoguanine and 7,8-dihydro-8-oxoadenine), cytosine glycol, 2,6-diamino-4-hydroxy-5-formamidopyrimidine, thymine glycol, and 4,6-diamino-5-formamidopyrimidine. In order to determine if these different types of DNA base modifications could cause DNA base mispairing, that could result in point mutations and lead to oncogene activation, we used the K-ras protooncogene as our model protooncogene, because this protooncogene is known to be activated into a transforming oncogene by point mutations (21). K-ras is also a very relevant model for studies of human carcinogenesis, because it is the most frequent oncogene activated in human malignancies (it is activated in 30 to 40% of lung adenocarcinomas; 40 to 50%
of colon carcinomas; and 90 to 95% of pancreatic carcinomas).

The K-ras protooncogene encodes a 21 kD GTP-binding protein. The function of the normal K-ras protooncogene is unknown, but it is believed to play a crucial role in cell growth and differentiation. The normal K-ras protooncogene can be converted into a transforming activated oncogene in vivo by point mutations at codons 12, 13, and 61, and in vitro by mutations at codons 12, 13, 59, 61, 63, 116 to 119, or 146 (21). The activity of K-ras proteins containing these mutations cannot be turned off, and therefore these mutant proteins continuously transduce growth stimulatory signals. To determine if ·OH could convert the normal K-ras protooncogene into an activated oncogene, we exposed the normal K-ras protooncogene cDNA (contained in a pZIP mammalian expression vector) to a variety of different ·OH-generating systems. Specifically these ·OH-generating systems included; PMA (250 ng/ml) + neutrophils (4 × 10⁶/ml); solubilized cigarette smoke (1/3000 of 1 cigarette in PBS); or urine (2 mM) + xanthine oxidase (0.002 µM); in the presence or absence of Fe²⁺/EDTA (10 µM/20 µM).

·OH-exposed K-ras protooncogene DNA was subsequently transfected into mouse fibroblast NIH 3T3 cells and cells were analyzed after 14 days for evidence of morphologically transformed foci. DNA from these transformed foci was extracted, amplified by the polymerase chain reaction, and sequenced. We found that approximately 25 to 30% of the dishes transfected with K-ras DNA that had been treated with PMA-stimulated leukocytes, cigarette smoke, or urine and xanthine oxidase, in the presence of Fe²⁺, developed transformed foci. In contrast, no transformed foci developed in the absence of Fe²⁺ or in the presence of Fe³⁺ alone. Sequence analysis of our K-ras transformants revealed mutations predominantly at codons 12 and 61 although a few mutations occurred at other sites including codons 13, 18, 117, and 146. In addition, sequence analysis of our K-ras transformants suggested that there may be several characteristics of ·OH-induced K-ras oncogene activation. First, ·OH-induced DNA damage appeared to cause selective base substitutions. Specifically, guanine bases were exclusively converted to adenines or thymines; ad- nine was exclusively converted to thymine; and cytosine was exclusively converted to adenine. Second, the four DNA bases appeared to have different susceptibilities to ·OH-induced mutations. Specifically, guanine and adenine bases appeared to be much more susceptible to ·OH-induced attack than cytosine and thymine bases. Third, the preponderance of mutations at codons 12 and 61 indicated that ·OH-induced mutations are not randomly distributed among the K-ras codons; and fourth, the pattern of mutations observed in our studies correlated well with the pattern of mutations detected in human and rodent carcinomas.

Although the aforementioned studies clearly indicate that ·OH can cause point mutations and activate the K-ras protooncogene, it was impossible to definitively conclude from these studies that point mutations were specifically due to DNA base modifications (rather than DNA strand breaks, etc.). Accordingly, to more firmly establish that DNA base modifications can cause point mutations and K-ras oncogene activation, we incorporated chemically synthesized 8-hydroxydeoxyguanosine [the most prevalent ·OH-modified DNA base in our studies (7)] into the first or second position of codon 12 of the normal K-ras protooncogene; transfected this 8-hydroxydeoxyguanosine-containing K-ras protooncogene DNA into NIH 3T3 cells; and analyzed these cells for the development of transformed foci. DNA from transformed foci was subsequently extracted, amplified by the polymerase chain reaction, and sequenced. We found that incorporation of 8-hydroxydeoxyguanosine into the first or second positions of codon 12 caused G to A transitions and G to T transversions at codon 12. Moreover, the pattern of these G → A and G → T transitions and transver- sions was identical to the pattern observed in our aforementioned studies of ·OH-treated K-ras DNA. Similar results, utilizing the H-ras protooncogene, have been obtained by Kamiya et al. (22). These studies indicate, therefore, that ·OH-modified DNA bases can cause point mutations that can lead to K-ras oncogene activation.

·OH-induced DNA Strand Breaks and Oncogene Activation

In light of the ability of ·OH-modified DNA bases to activate oncogenes, we were also interested in determining if ·OH-induced DNA strand breaks might also cause oncogene activation. Specifically, we hypothesized that DNA strand breaks might cause deletions in K-ras codons; and, if so, we could test our hypothesis. We used the C-Raf-1 protooncogene, because this protooncogene is known to be activated into a transforming oncogene by deletion of its N-terminus (23). C-Raf-1 is believed to be an important downstream component of the Ras signal transduction pathway, and activated C-Raf-1 oncogenes are frequently detected in human malignancies.

The C-Raf-1 protooncogene encodes a 74 kD protein. The N-terminus of C-Raf-1 contains a regulatory domain; while its C-terminus contains a Ser/Thr kinase domain. The Ser/Thr kinase activity of C-Raf-1 proteins that have lost their N-terminal regulatory domain, cannot be turned off; therefore these proteins continuously transduce growth stimulatory signals. In order to determine if ·OH could cause N-terminal deletions and convert the normal C-Raf-1 protooncogene into an activated oncogene, we exposed the normal C-Raf-1 protooncogene cDNA (contained in a mammalian expression vector) to the same ·OH-generating systems that we used in the aforementioned isolated K-ras protooncogene cDNA studies. ·OH-treated C-Raf-1 protooncogene cDNA was transfected into NIH 3T3 cells and DNA from transformed foci was extracted and PCR amplified, as outlined above. We found that approximately 20 to 25% of dishes transfected with ·OH-treated C-Raf-1 DNA developed transformed foci. No transformed foci occurred in the absence of Fe²⁺ or in the presence of Fe³⁺ alone. We have not completed our analysis of the C-Raf-1 transformants, however preliminary analysis by Southern blots suggest that C-Raf-1 oncogene activation occurred as a result of N-terminal deletions. These studies suggest, therefore, that ·OH-induced DNA strand breaks can cause deletions and result in oncogene activation.

The aforementioned studies indicate that ·OH-induced DNA base damage and ·OH-induced DNA strand breaks can cause oncogene activation and it is likely that this oncogene activation could ultimately contribute to carcinogenesis. It will be important to determine if ·OH-induced DNA base damage and DNA strand breaks can similarly cause tumor suppressor gene inactivation.

Other Mechanisms of Oxidant-induced Carcinogenesis

There exists another potential mechanism by which oxidants might contribute to carcinogenesis. Previous studies (24,25) have shown that cells containing the normal tumor suppressor gene p53 undergo DNA damage and
G1 cell cycle arrest when they are treated with gamma irradiation. This G1 cell cycle arrest allows the cells to repair their damaged DNA, and only after the damaged DNA is repaired do the cells resume their cell cycle. Therefore, this G1 cell cycle arrest prevents cells containing damaged DNA from replicating their damaged DNA. Interestingly, cells that contain a mutant or absent p53 tumor suppressor gene fail to undergo G1 cell cycle arrest when exposed to gamma irradiation (24, 25). These cells continue to undergo gamma irradiation-induced DNA damage, the lack of G1 cell cycle arrest enables these cells to replicate damaged DNA. In preliminary studies from our laboratory, we have found that leukocyte-derived •OH also causes G1 cell cycle arrest in cells with normal p53, but fails to cause G1 cell cycle arrest in cells with mutant or absent p53. Since the lack of G1 cell cycle arrest enables cells that contain mutant p53 to replicate damaged DNA, the fidelity of replication is likely to be significantly reduced. Errors in replication can result in oncogene activation as well as inactivation of additional tumor suppressor genes. Therefore, in addition to the possibility that •OH-induced DNA damage might directly inactivate tumor suppressor genes, it is possible that cells containing inactivated tumor suppressor genes (whether inactivated by •OH or some other factor) are essentially “set up” to undergo oncogene activation and additional tumor suppressor gene inactivation when exposed to •OH, because of their inability to undergo G1 cell cycle arrest following DNA damage.

**Conclusion**

In summary, our studies support the following model of oxidant-induced carcinogenesis: leukocytes or cigarette smoke release superoxide and hydrogen peroxide; hydrogen peroxide diffuses into the nucleus of cells and reacts with iron that is either bound to or close to DNA and generates •OH; •OH causes DNA base damage and DNA strand breaks that lead to oncogene activation or tumor suppressor gene inactivation; tumor suppressor gene inactivation (caused by •OH or some other factor) further predisposes cells to develop oncogene activation and tumor suppressor gene inactivation, following •OH exposure; the accumulation of activated oncogenes and inactivated tumor suppressor genes ultimately results in carcinogenesis.

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