

# Commentary

## Oxidative Damage and Cancer

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Considerable evidence has linked oxidative damage and cancer. This Commentary will briefly review this area in light of new data linking specific gene modification with oxidative damage.

### *Reactive Oxygen Species (ROS)*

ROS are generated in mitochondria of normal mammalian cells as a byproduct of normal respiration and in other subcellular locations as a function of biochemical reactions using oxygen.<sup>1</sup> ROS at high levels are toxic to the cell, but at low levels, ROS have physiological functions, including activation and modulation of signal transduction pathways,<sup>2</sup> modulation of activities of redox-sensitive transcription factors,<sup>3,4</sup> and regulation of mitochondrial enzyme activities.<sup>5</sup> Levels of ROS are reduced by antioxidant defenses, but increased by transition metals such as iron or copper and by exogenous agents such as ionizing radiation or ozone.

### *Antioxidant Defenses*

To protect against toxic effects of ROS and to modulate physiological effects of ROS, the cell has developed an intricately regulated antioxidant defense system. The antioxidant enzyme system is very complex, being composed of small molecular weight antioxidant compounds (vitamins E, C, A, and so forth); primary (manganese, copper, zinc superoxide dismutases, catalase, glutathione peroxidase) and secondary antioxidant enzymes (enzymes such as glutathione reductase and glucose-6-phosphate dehydrogenase); and the glutathione,<sup>6</sup> glutaredoxin,<sup>7</sup> and thioredoxin<sup>8</sup> systems. Protein and DNA repair enzymes may be considered part of the antioxidant system. Proteins that sequester metals are important in modulating cell redox state. Nitric oxide modulates levels of ROS in part by its reaction with superoxide

anion. Finally, proteins involved in response to cell stressors, such as the heat shock system, are important in modulating oxidant damage. Each component of the antioxidant system is specifically compartmentalized to specific subcellular locations.<sup>9,10</sup>

### *Identification of Oxidative Damage*

Methods for identification of oxidative damage have recently been reviewed by Toyokuni.<sup>11</sup> Just as the identification of antioxidant enzymes proved the existence of ROS, the presence of DNA repair enzymes specific for oxidative DNA damage documented the inherent toxicity of ROS for cellular DNA.<sup>12</sup> In general, ROS have a short half-life because of their high reactivity and are therefore difficult to detect with standard biochemical methods. Advanced physical-chemical techniques such as electron spin resonance with spin traps allow detection and identification of free radicals. However, these techniques require expensive instrumentation, and quantification of the amount of oxygen-free radicals in tissues is difficult. ROS can be detected by methods allowing detection of fluorescence signals, eg, luciferase assays with a fluorometer or use of redox-sensitive dyes with flow cytometry or confocal microscopy. These fluorescent techniques suffer from a lack of specificity, but have high sensitivity. Histochemical techniques for identification of oxidant stress have been described, and these techniques have recently been reviewed;<sup>13</sup> an example is the direct Schiff's reaction for the localization of lipid peroxidation in tissues.

Because of the caveats of the aforementioned techniques, investigators have turned to analyzing the footprints of oxidative damage, eg, analysis of oxidative damage products, including products of lipid, protein, and DNA oxidation. Oxidative damage products can be measured with biochemical assays. Antibodies to oxidative damage products have been developed and may be used in either biochemical or morphological assays. In addition, nitrosative damage can be detected with antibody to nitrotyrosine.

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### *Analysis of Antioxidant Enzymes and Oxidative Damage Products in Human Cancer*

It has been demonstrated both *in vivo* and *in vitro* that antioxidant enzyme levels are altered in cancer. The most consistent finding in biochemical studies has been that manganese superoxide dismutase (MnSOD), a mitochondrial antioxidant enzyme, is lowered in most types of primary cancers and cancer cell lines examined.<sup>14</sup> Our laboratory has focused on antioxidant enzymes in human lung,<sup>15</sup> renal,<sup>16,17</sup> and prostate cancers;<sup>18,19</sup> immunoperoxidase studies demonstrated low levels of antioxidant enzymes in primary tumors, although small groups of cancer cells, often on the invading edge of the tumor, did occasionally show strong positivity. These results agree with the well-developed paradigm from numerous biochemical studies that MnSOD levels are low in cancer. However, primary cancers from some organs have been shown to have high levels of MnSOD. In all cases, after comparison to normal tissue controls, MnSOD has been shown to be altered (activities elevated or depressed) in primary cancers. In our laboratory, metastatic prostate cancer was shown to have significantly elevated levels of MnSOD.<sup>19</sup> One possible explanation of this finding is that primary prostate cancer cells undergo a selection process, with those cells destined for metastasis having high levels of MnSOD. Future studies will be necessary to test this hypothesis.

Some progress has been made in understanding the reason for abnormal regulation of MnSOD in cancer. Polymorphisms in the leader sequence of the MnSOD gene have been correlated with susceptibility to breast cancer,<sup>20</sup> whereas additional mutations have been identified in the 5'-untranslated region, although the significance of these latter mutations is not known.<sup>21</sup> The coding sequences for both manganese and copper, zinc superoxide dismutases were not mutated in colon cancer<sup>21</sup> and prostate<sup>18</sup> cancer, respectively, although a polymorphism in the enzyme has been described.<sup>22</sup> Therefore, abnormalities in mRNA regulation or post-translational modifications need to be studied more intensely to explain the altered levels of MnSOD in these forms of cancer. Additional studies of other cancers will be necessary to determine whether mutations in the coding region can be found. It has yet to be determined whether abnormal antioxidant enzyme regulation is because of abnormalities in gene expression or some more fundamental property of cancer cells, ie, degree of cell differentiation.

One possible consequence of antioxidant enzyme imbalance is alterations in cellular redox state. Several laboratories have analyzed changes in cell redox state in cancer. Okamoto and colleagues<sup>23</sup> demonstrated with biochemical techniques that renal cell carcinomas showed higher levels of DNA oxidation compared with corresponding normal tissue controls, as determined by measurements of 8-hydroxy-2'-deoxyguanosine (8-OHdG). Using immunoperoxidase techniques with specific antibodies to 4-hydroxy-2-nonenal (4HNE)-modified

proteins, our laboratory demonstrated significant lipid peroxidation products in clear cell variant of renal carcinoma,<sup>24</sup> but little lipid peroxidation in other types of renal cancer (papillary carcinoma, Wilms' tumor, and transitional cell carcinoma of the renal pelvis). Kondo and colleagues<sup>25</sup> used antibodies against 8-OHdG, 4HNE-modified proteins, and 3-nitro-L-tyrosine (3-NT) to demonstrate oxidative stress in human colorectal carcinoma, but not in adenoma. High levels of DNA oxidation were confirmed with high-performance liquid chromatography measurements.

Our laboratory has studied human prostate cancer with immunohistology techniques using specific antibodies to oxidative and nitrosative damage products and have demonstrated low levels of oxidative stress (as measured with immunoperoxidase techniques using antibodies to 4HNE-modified proteins), but high levels of nitrosative (3-NT) stress in primary prostate cancer compared to normal prostate epithelium.<sup>19</sup> Focal areas of primary prostate cancer showed very high levels of 8-OHdG. In contrast, metastatic prostate cancer had higher levels of oxidative (4HNE-modified proteins and 8-OHdG) and nitrosative (3-NT) damage products than either primary cancer or normal prostate epithelium. These results confirm abnormalities in oxidative and nitrosative metabolism in one human cancer. Interestingly, Kondo and colleagues<sup>25</sup> also demonstrated high levels of 8-OHdG and 3-NT in primary colon cancer, suggesting that this phenotype is present in more than one type of cancer. It is highly attractive to speculate that high levels of 8-OHdG and 3-NT are because of low levels of MnSOD, a possibility that is being currently tested in our laboratory in cell culture systems. In contrast to our study of human prostate cancer,<sup>19</sup> the study of Kondo and colleagues<sup>25</sup> in human colon cancer showed increased levels of 4HNE-modified proteins in malignant compared to adjacent normal epithelium. Our laboratory has demonstrated that 4HNE-modified proteins are present in both nucleus and mitochondria in human renal cancer.<sup>24</sup> The amount of 4HNE-modified proteins is thus specific to cell type, because each cell type will have varying number and size of mitochondria; thus, differences in mitochondrial number and metabolism may explain the differences in levels of 4HNE-modified proteins in prostate *versus* colon cancer. In support of mitochondria being a major source of 4HNE-modified proteins is the finding that human renal oncocytoma, which is a tumor with large numbers of mitochondria per cell, demonstrated large amounts of 4HNE-modified proteins in cell cytoplasm.<sup>24</sup>

A major criticism of many studies to date is that levels of antioxidant enzymes and oxidative damage products have not been determined in preneoplastic lesions. However, Kondo and colleagues<sup>25</sup> examined human colon adenomas, which demonstrated low levels of oxidative damage products compared to colon carcinomas, suggesting that oxidative stress may be a feature of neoplastic lesions only. Future studies of other preneoplastic lesions will be necessary to determine the generality of this finding.

## In Vitro Model of Oxidative Damage and Cancer

*In vitro* models have studied the effects of oxidative damage on cancer cells, and so provide information about the biochemistry of cancer cells. Unfortunately, because the cancer phenotype is already present, little can be learned about the carcinogenesis process. However, *in vitro* models do allow studies of differences between normal, malignant, and derived metastatic cancer cells, and therefore may provide important information for the development of possible therapeutic regimens.

One of the most studied model systems is transfection of MnSOD cDNA into cancer cells. MnSOD is a mitochondrial antioxidant enzyme with both antioxidant (removal of superoxide anion) and pro-oxidant (enzymatic generation of hydrogen peroxide) functions. The conditions under which antioxidant or pro-oxidant functions predominate are not certain, but in either case the mitochondrial redox state is modulated by MnSOD overexpression. It has been demonstrated *in vitro* that many cancer cells produce hydrogen peroxide,<sup>26</sup> and one possible explanation for this is that cancer cells could have low levels of hydrogen peroxide-detoxifying enzymes.

It has been shown that overexpression of MnSOD inhibits cell growth *in vitro* in at least 13 malignant cell types.<sup>27</sup> In two cell types, hydrogen peroxide was strongly implicated in growth inhibition because double transfection with MnSOD plus mitochondrial catalase or glutathione peroxidase reversed growth inhibition. Further, strong evidence for the enzymatic activity of MnSOD causing growth inhibition and not functions of the 3'- or 5'-untranslated regions of the MnSOD gene was provided by studying a polymorphism of the subunit MnSOD interface that has low MnSOD activity in comparison with wild-type protein; transfection of the polymorphic MnSOD cDNA, resulting in low MnSOD activity compared to activity resulting from transfection with normal MnSOD cDNA, showed little growth inhibition.<sup>22</sup>

The cellular mechanism by which MnSOD inhibits tumor growth is not certain, because MnSOD overexpression has pleiotropic effects on cells, including modulation of transcription factors,<sup>3,4</sup> signal transduction pathways,<sup>2</sup> mitochondrial function,<sup>28</sup> and microtubule organization.<sup>29</sup> Numerous studies have demonstrated that the mechanism of growth inhibition does not involve cell death, ie, necrosis or apoptosis. In addition, studies in our laboratory have demonstrated that MnSOD inhibits cell-cycle progression.<sup>29,30</sup> It has been well documented that ROS at low levels stimulate cell proliferation, whereas higher levels of ROS cause cell injury. Thus, effects of MnSOD on cell cycle probably reflect net antioxidant and pro-oxidant functions on each individual cell type. Because many cancer cells have low levels of hydrogen-peroxide detoxifying enzymes, inhibition of cell growth may reflect the effects of hydrogen peroxide on cell growth. In fact, our laboratory has demonstrated that prostate carcinoma cells (DU145) overexpressing MnSOD have cell growth rates that correlate with ROS levels.<sup>29</sup>

## In Vivo Model of Oxidative Damage and Cancer: Multistage Mouse Skin Carcinogenesis

A multistage carcinogenesis model has been used to study skin cancer in the mouse for many years.<sup>31</sup> Stages of this model have been defined as initiation, promotion, and progression. 7-12-dimethylbenz(a)-anthralene (DMBA) has been used as a tumor initiator, whereas 12-O-tetradecanoylphorbol-13-acetate (TPA) has often been used as a tumor promoter. Although DMBA is known to result in DNA damage with resultant mutations, the role of TPA in tumor promotion is less well understood, although it has been demonstrated that TPA binds to the enzyme protein kinase C and hence regulates phosphorylation within cells.<sup>32</sup> However, TPA may have other roles in tumor promotion. Keratinocytes treated with TPA *in vitro* show increased levels of ROS,<sup>33</sup> and SOD mimetics, which reduce levels of superoxide anion, have been shown to reduce skin papilloma formation after DMBA/TPA treatment.<sup>34</sup>

To study the role of a mitochondrial antioxidant enzyme in papilloma formation, transgenic mice overexpressing MnSOD have been developed.<sup>29</sup> Transgenic mice that overexpressed MnSOD were shown to have reduced papilloma formation compared to their nontransgenic littermates.<sup>35</sup> To study the relationship between DMBA/TPA treatment and oxidative damage, our laboratory analyzed oxidative damage products in keratinocytes from transgenic and nontransgenic mouse skin (data submitted). Nontransgenic and transgenic mice overexpressing MnSOD were topically treated with one dose (20 nmol/L) of DMBA and a subsequent dose (4  $\mu$ g) of TPA. At selected times after TPA treatment, mouse skin keratinocytes were analyzed for levels of MnSOD and levels of the oxidative damage product 4HNE-modified proteins using specific antibodies and immunogold electron microscopy with computerized image analysis. At all time points analyzed after TPA treatment, there was more MnSOD immunoreactive protein in mitochondria of transgenic mouse keratinocytes treated with DMBA/TPA than in mitochondria of nontransgenic mouse keratinocytes similarly treated. To study the functional consequences of MnSOD overexpression, intact skin was analyzed for the presence of 4HNE-modified proteins. Compared to control groups, there was a large increase in 4HNE-modified proteins at 6 to 24 hours after TPA in DMBA/TPA-treated mouse keratinocytes, and this increase was larger in nontransgenic than transgenic mice. Proteins modified by lipid peroxidation were detected in significant levels in keratinocyte mitochondria and nuclei, but at only low levels in cytoplasm. Mitochondrial injury was detected by electron microscopy as loss of cristae caused by inclusion formation; these inclusions were shown by specific antibody and immunogold techniques to contain 4HNE-modified proteins. The appearance of mitochondrial inclusions was delayed in keratinocytes of transgenic compared to nontransgenic mice. Thus, reduction in papilloma formation observed in transgenic mice overexpressing MnSOD is correlated with oxidative damage and mitochondrial events.

### *Oxidative Damage and Cancer: Iron Nitrotriacetate (FeNTA)-Induced Rat Kidney Cancer*

FeNTA has been shown to cause kidney cancer in the rat.<sup>36</sup> Presumably, iron is delivered to the proximal tubule by the nitrotriacetate moiety of FeNTA. Once iron is present in nonprotein-bound forms in tissues, it can react in Fenton chemistry reactions to produce highly toxic ROS. Oxidative damage has been shown to be present in this model by the detection of lipid peroxidation products<sup>37</sup> and 8-hydroxy-2'-deoxyguanosine<sup>38</sup> using both biochemical and immunohistology techniques with specific antibodies.

Studies in our laboratory have demonstrated accumulation of large amounts of 4HNE-modified proteins in nuclei and mitochondria of proximal tubules of rat kidney after FeNTA treatment.<sup>39</sup> Thus, although the oxidative damage seems to be cell-type-specific, oxidative damage appears in more than one subcellular compartment. It thus becomes difficult to understand specificity as a result of oxidative damage because injury is localized to more than one compartment of the cell. Because cancer is thought to involve sequential genetic changes in the nucleus, the question arises as to whether oxidative damage affects the genome equally or whether specific genes are targeted.

Analysis of renal cancers arising from FeNTA treatment has shown specificity in genes modified. No mutations were observed in *H-ras*, *K-ras*, and *N-ras* oncogenes or *p53* tumor suppressor gene in kidney cancer<sup>40</sup> resulting from FeNTA treatment, whereas *p15<sup>INK4B</sup>* and *p16<sup>INK4A</sup>* tumor suppressor genes were shown to be inactivated.<sup>41</sup> Because a prominent feature of cancer is chromosomal instability, it is difficult to determine whether genetic changes observed in cancer are causal in nature or simply a result of subsequent genetic changes. To begin to answer this question, two groups have analyzed genetic changes shortly after FeNTA treatment. Nomoto and colleagues<sup>42</sup> assessed the frequency of oxidative base damage hours after FeNTA treatment using the ligation-mediated polymerase chain reaction technique. They assessed the frequency of 8-hydroxy-guanine (8-OH-Gua) formation in three genes, the tumor suppressor gene *p53*, the heat shock protein 70 (*HSP 70*) gene, and the *Na, K-ATPase  $\alpha$ 1* subunit gene. Changes in 8-OH-Gua were not detected in *p53* or *HSP 70* genes after FeNTA treatment. In contrast, time-dependent alterations, corresponding to the time course of overall 8-OH-Gua formation and repair, were detected in the promoter region of the *Na, K-ATPase  $\alpha$ 1* subunit gene. Thus, oxidative damage was not distributed uniformly along the whole genome, but seemed to be restricted to particular genes.

These results did not indicate which gene changes are actually important in the development of the rat renal cancer. In an article in the present issue of *The American Journal of Pathology*, Hiroyasu and colleagues<sup>43</sup> determined whether oxidative damage can cause allelic loss 1 to 3 weeks after FeNTA administration. Using fluorescent *in situ* hybridization with imprint cytology at single cell

resolution, these investigators found that the number of renal tubular cells with aneuploidy (one or three fluorescent signals as opposed to the expected two signals) at the *p16<sup>INK4A</sup>* locus was specifically increased 1 to 3 weeks after administration of FeNTA. No increase in aneuploidy was observed at the loci of either the *p53* or the *vhl* tumor suppressor genes. Therefore, the *p16<sup>INK4A</sup>* loss is specifically vulnerable to oxidative damage, leading to its allelic loss within weeks.

### *Specificity of Oxidative Damage Reactions*

Oxidative damage results from relatively nonspecific chemical reactions, yet, Hiroyasu and colleagues<sup>43</sup> demonstrate specificity of gene damage. How can this occur? Most importantly, the specificity probably results from the specialized and localized redox environment of each cell type. That is, reactions generating pro-oxidants occur in specific locations within cells, antioxidant defenses are localized in specific subcellular compartments, and each cell type has unique redox state biochemistry. As a few examples, plasma membranes generate superoxide anion via NADPH oxidase, whereas smooth endoplasmic reticulum generates ROS via reactions involving cytochromes P450. Rough endoplasmic reticulum actually has a reducing environment. Antioxidant enzymes are localized in varying locations in cells.<sup>9</sup> MnSOD is in mitochondria, whereas CuZnSOD is located in cytoplasm, including lysosomes, and nucleus. Catalase is located primarily in peroxisomes, whereas glutathione peroxidase is found in all subcellular compartments. In addition, each mammalian cell type has unique amounts of antioxidant enzymes. As just one example, kidney proximal tubules have high levels of mitochondrial MnSOD, whereas kidney glomerular cells immediately adjacent have low levels of mitochondrial MnSOD.<sup>9</sup> Components of the glutathione,<sup>6</sup> glutaredoxin,<sup>7</sup> and thioredoxin<sup>10</sup> systems are also specifically compartmentalized within the cell. The unique amount and distribution of individual antioxidant proteins and compounds are a major determinant of the large variation in susceptibility of individual cell types to oxidative damage.

ROS generated also have different chemical properties. Thus, hydroxyl radical is so reactive that it immediately reacts with cellular components and thus cannot reach distant subcellular targets. Hydrogen peroxide and nitric oxide are much less reactive and more able to diffuse throughout the cell. Chemical reactions that ROS can participate in generate different products that form derivatives with varying degrees of toxicity. It must be re-emphasized that ROS and nitric oxide at low concentrations perform physiological functions, whereas at high concentrations these compounds are toxic.

Understanding the specificity of reactions will thus require a knowledge of the localized redox environment of each cell type and a more complete understanding under which specific oxidative and nitrosative chemical reactions occur. Such studies will form the basis for a better understanding of specific oxidative damage reactions.

## Oxidative Damage and the Genome

Understanding how specific genes are inactivated after oxidative damage is of great interest and importance. Possibilities for explaining specific gene loss include physical gene structure, chromosomal location, and/or physiological function of the gene. The latter possibility is especially intriguing because evidence is accumulating that genes modulating cell redox are, in turn, modulated by redox-regulated proteins that modulate specific mRNA stability.<sup>44</sup> It is also known that redox state regulates specific transcription factors.<sup>3,4</sup> It is possible that redox modulation may control accessibility of ROS to DNA. For example, ROS may cause removal of proteins from DNA and thus allow ROS attack of DNA.

## Conclusions

Oxidative damage has long been implicated in both the malignant phenotype and carcinogenesis. Enthusiasm for these correlative studies has been tempered by the perceived lack of specificity of chemical reactions involving oxidative damage. In fact, specificity is probably achieved by the localized redox state biochemistry within each cell type. Understanding this localized redox micro-environment will be an exciting challenge for the future.

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