MORPHOLOGIC ASSESSMENT OF OXIDATIVE DAMAGE: A REVIEW

Terry D. Oberley^{1,2} and Theodor A. Zainal³

¹ Pathology and Laboratory Medicine Service,
Veterans Administration Hospital

² Department of Pathology and Laboratory Medicine,
University of Wisconsin Medical School

³ Department of Nutritional Sciences
University of Wisconsin, Madison, WI

ABSTRACT

Biochemical studies have indicated changes in antioxidant enzyme activities and increased oxidative damage products in many disease states, particularly aging and diseases associated with aging, such as neurodegenerative diseases and cancer. To try to determine cellular and subcellular localization of oxidative damage, our laboratory has developed quantitative light and electron microscopy immunogold techniques using specific antibodies to oxidative damage products. Results from studies of different pathologic processes are presented, illustrating that both localization and quantitation of oxidative damage products is possible. These analyses give important insights into the nature of various pathologic processes.

INTRODUCTION

One of the major theories of aging is that it is due to the accumulation of oxidative damage (1). Oxidative stress occurs in normal cells as a result of normal aerobic metabolism (2). It is well documented that reactive oxygen species (ROS) are a byproduct of oxidative phosphorylation in mitochondria, but ROS are also produced throughout the cell by other cellular reactions which utilize molecular oxygen. Although increased ROS production has been demonstrated in mitochondria from old compared to young animals (3), it has not been conclusively demonstrated that increased ROS production occurs during aging *in vivo*. Studies have also compared oxidative damage products in homogenates of tissues from young versus old organs and have reported increased oxidative damage with aging (4-5); problems with these latter studies are that one cannot determine from the results either the cell types(s) in which injury has occurred or the subcellular localization of oxidative damage. Insights into the cellular and subcellular origin of ROS will provide useful new information about the role of ROS in various physiologic and pathologic processes, including aging. Our labora-

tory has begun this analysis by studying localization of antioxidant enzymes and oxidative damage products using morphologic tools. In the present review, we present results from both normal tissues and tissues injured by several pathologic processes to illustrate the specificity of the results obtained.

Standard morphologic techniques

Routine light and electron microscopy analysis may be of great use in analysis of aging experiments. As one example, we have conducted a study of the effect of caloric restriction on lifespan in mice, and found a significant increase in lifespan in calorie-restricted animals (6). However, pathologic analysis of tissue sections using routine light microscopy, following staining with hematoxylin and eosin, showed that the majority of mice died due to a particular cancer, multiple myeloma; classification of this tumor was confirmed by electron microscopy. This study illustrates that changes associated with aging must be distinguished from age-associated diseases such as cancer. Morphologic techniques are invaluable in making this distinction.

Immunomorphologic techniques

The techniques used for immunomorphologic analyses are standard immunoperoxidase and immunogold techniques at the light microscopic level and standard immunogold techniques at the ultrastructural level. For light microscopy, tissues are fixed in neutral-buffered formalin, embedded in paraffin, slides with tissue sections stained with primary antibodies, then developed with commercial secondary antibody color development kits (horseradish peroxidase, alkaline phosphatase, colloidal gold etc.) to produce visible precipitates. For electron microscopy, tissues are fixed in Carson-Millonig's fixative, embedded in LR White, grids stained with primary antibodies, and developed with commercial immunogold reagents.

The innovation in these techniques came with the development of informative primary antibodies to antioxidant enzymes (AEs) and oxidative damage products. Antibodies to manganese and copper, zinc superoxide dismutases, catalase, and glutathione peroxidase have been made in individual laboratories and some are now commercially available (listed in reference 7). Antibodies to lipid peroxidation products, such as 4-hydroxy-2-nonenal (4HNE) protein adducts, have been made in

To whom all correspondence should be addressed.

Terry D. Oberley, M.D., Ph.D.

Room A-35, VA Hospital

2500 Overlook Terrace

Madison, WI

Phone: 608-256-1901, ext. 11722

Fax: 608-280-7087

individual laboratories (8-10). Antibody to 8-hydroxy-2'-deoxyguanosine (8OHdG) has been generated by one laboratory (11) and is also available commercially (QED Bioscience, San Diego, CA). Antibody to nitrotyrosine is available commercially (Upstate Biotechnology, Lake Placid NY) and its utility and specificity has been demonstrated (12). Antibody to 2,4-dinitrophenylhydrazine (DNP) is available commercially (Zymed, San Francisco, CA) and can be used to detect protein carbonyls (13). These antibodies can be used to identify AEs and oxidative damage products within individual cell types and at the subcellular level.

Quantitation of light microscopy immunogold and immunoperoxidase techniques

Our laboratory and others have developed techniques to perform relative quantitation of light microscopic images. In our laboratory, for quantitation of 4HNE protein adducts, carbonyls, and nitrotyrosine using image analysis, black and white prints for each slide are prepared utilizing a 2x objective lens. Black and white images are then obtained as PICT files with a Microtek ScanMaker V300 flatbed scanner. Image analysis is performed using NIH Image freeware (version 1.61, available for download on the Internet from <http://rsh.info.nih.gov/nih.image>). The PICT files are opened in gray scale mode by NIH Image. To make a manual area measurement, a region of interest (0.3mm²) is outlined using the rectangular selection tool. The measure command, which will compute the area, mean gray value, and the minimum and maximum gray value, is then selected. Ten regions are randomly selected for each PICT file. Mean gray value for each PICT file is obtained by taking the mean value of the ten measurements.

Using this technique, we have demonstrated a linear increase in 4HNE protein adducts in rhesus monkey skeletal muscle with age (see below; manuscript submitted). Toyokuni (14) has used similar techniques with immunoperoxidase protocols to measure relative 8OHdG accumulation in rodent kidney after treatment with iron nitrilotriacetate compared to normal kidney, and Kondo et al. (15) has used these techniques to compare 8OHdG levels in normal and malignant human colonic epithelium.

Immunogold electron microscopy quantitation

Tissues are prepared for immunogold electron microscopy using standard protocols (16). For quantitation using counting of gold beads, a magnification of 25,000x is used. To make a measurement, a region of interest (0.1mm²) is outlined using the picture field. Gold beads within the region are then counted. The regions are randomly selected for each sample. Mean gold bead count for each sample is obtained by taking the mean value of the measurements.

Using this technique, we have demonstrated a linear increase in 4HNE protein adducts in aging rhesus monkey skeletal muscle (see below; manuscript submitted).

Localization of antioxidant enzymes

AEs are the primary defense against oxidative stress, and their presence in large amounts suggests the presence of oxidative stress. *Thus, localization of AEs may be used as an indirect measure of the location of oxidative stress.* AEs include superoxide dismutases, catalases, and glutathione peroxidases. Superoxide dismutases convert superoxide anion into hydrogen peroxide and molecular oxygen. There are three forms of superoxide dismutase (SOD): manganese superoxide dismutase (MnSOD) found in mitochondria (17), copper, zinc superoxide dismutase (CuZnSOD) found primarily in cell cytoplasm (18), and extracellular superoxide dismutase (ECSOD) found in the extracellular spaces (19). Catalase (CAT) is found primarily in peroxisomes (20); its function is to convert hydrogen peroxide into the nontoxic products molecular oxygen and water. Glutathione peroxidase (GPX) is found in all subcellular compartments (21) and converts hydrogen peroxide and lipid hydroperoxides to nontoxic products. Several laboratories have documented altered AE activities in specific organs with age (22-24); however, these studies were performed in homogenates and so reflect average activities of all cell types present.

Our laboratory has analyzed AE immunoreactive protein levels in organs of several species at both the light and electron microscopic levels. Immunoperoxidase or immunogold studies at the light microscopic level allows determination of AE levels in different cell types, while immunogold analysis at the ultrastructural level allows examination of subcellular location of specific AEs. Well characterized antibodies to MnSOD, CuZnSOD, CAT, and GPX were used for these analyses (25-26). Each cell type studied was found to have a specific AE profile. In general, MnSOD was found to be high in epithelial cells, while GPX was high in mesenchymal cells. The subcellular distribution of each AE studied was the same regardless of cell type. For example, MnSOD was always found in mitochondria; all cells that had mitochondria showed immunogold labeling of mitochondria at the ultrastructural level, even if light microscopy showed only trace cytoplasmic labeling. CuZnSOD was found in nuclei and cytoplasm, but not in mitochondria. Cells with peroxisomes (liver, renal proximal tubule) exhibited high levels of CAT in this organelle. Other epithelia also showed high levels of CAT protein. In some cases, such as transitional epithelium of the urinary tract, this immunoreactive protein appeared to be in microperoxisomes (21). GPX was found in all subcellular organelles; GPX was found in high levels in mesenchymal cells including vascular smooth muscle cells throughout the body. CuZnSOD, CAT, and GPX also localized to cell surface microvilli in lung and kidney (16,21), though one cannot rule out that this labeling was actually due to the presence of enzymes present in extracellular fluids adherent to microvilli.

We have mapped the anatomic distribution of AE immunoreactive protein levels in the kidney of several

species, including hamster, rat, mouse, and human, and have found similar distribution of enzymes in all four species. The proximal and distal tubules as well as the transitional epithelium had relatively high levels of all four AEs, while levels in glomeruli were virtually undetectable by light microscopy immunoperoxidase or immunogold techniques (21). However, immunogold ultrastructural studies revealed labeling for MnSOD, CuZnSOD, and GPX, but not CAT, in all four glomerular cell types (visceral and parietal epithelium, mesangial, and endothelial cells), because of the greater sensitivity and higher resolution of immunogold ultrastructural studies (21). We have mapped several isoenzymes of glutathione S-transferase in normal rat kidney (27-28) and have found these are also expressed in high levels in proximal and distal tubules and transitional epithelium.

While high levels of AEs infer the presence of oxidative stress, the physiologic and/or pathologic significance of these findings remain to be elucidated. First, there are many antioxidant proteins and compounds which we did not evaluate. These other antioxidants may influence the overall antioxidant/prooxidant balance and thus determine whether oxidative stress results in oxidative damage. Examples of these proteins/compounds not measured include other antioxidant enzymes (phospholipid-dependent glutathione peroxidase (29), cytoplasmic catalase (30), and ECSOD), glutathione, thioredoxin, antioxidant vitamins, DNA repair enzymes, ubiquitin ligase (31), metal-complexing proteins (such as metallothionein), and nitric oxide synthase. A second major problem with trying to interpret whether AE levels correlate with oxidative damage is that cells have mechanisms to repair oxidized proteins and DNA and to replace oxidized lipids. These repair mechanisms must be evaluated in order to assess whether oxidative stress truly leads to oxidative damage. Finally, even if oxidative damage were present, cells with the capability of turnover can replace injured cells. Therefore, tissue and organ damage might not occur, even in the presence of intrinsic or extrinsic injury.

Antioxidant enzyme levels during pathologic processes

One disease associated with aging, cancer, has had extensive study of AE levels. In general, it has been shown that AE levels, especially MnSOD, are lower in cancer than their cell of origin (reviewed in reference 32).

Localization of oxidative damage products

Due to the caveats of evaluating oxidative damage by evaluation of selected AEs, we have begun analysis of normal and pathologic tissues with antibodies to oxidative damage products, specifically antibodies to 8-hydroxy-2'-deoxyguanosine (anti-8OHdG), 4-hydroxy-2-nonenal protein adducts (anti-4HNE), a fluorophore derived from the reaction of HNE with lysine (anti-Lys-HNE) (33), and nitrotyrosine (anti-nitrotyrosine). Anti-8OHdG and anti-nitrotyrosine antibodies are commercially available, while anti-4HNE and anti-Lys-HNE

antibodies were produced and characterized by Dr. Luke Szweda, Case Western Reserve University. The anti-4HNE and anti-Lys-HNE antibodies recognize products of lipid peroxidation, and as described below, co-localize with lipofuscin. Carbonyl content, a measure of protein oxidation, was also assessed with antibodies to DNP, which are commercially available.

Correlation of immunohistologic results with biochemical analyses

The antibodies used have been shown to be highly specific in terms of antigen recognized. However, our initial studies were focused on proving that antibody results obtained at the light and ultrastructural levels correlated with biochemical analyses of oxidative modifications and were truly indicative of oxidative damage. To accomplish this, iron nitrilotriacetate (Fe-NTA)-induced kidney injury was studied (34). Rats were treated with Fe-NTA and lipid peroxidation and protein carbonyl content assessed biochemically. Lipid peroxidation-derived aldehydes (malondialdehyde [MDA] plus 4-hydroxy-2-alkenals [4-HDA]) increased 20-fold one hour after Fe-NTA treatment, while protein carbonyls increased 5-fold at this same time point. Staining with anti-HNE protein adduct antibody showed increased staining in proximal tubules at this time; this cell type is the segment of the nephron injured by this compound, as revealed by routine light microscopy and ultrastructural studies. Other studies have shown a strong correlation between biochemical measurements of 8OHdG and morphologic quantitation in this same model (14-15). Thus, in this latter study, rats treated with Fe-NTA showed increased 8OHdG by both morphologic quantitation using a specific antibody and by biochemical measurements with high pressure liquid chromatography techniques.

Positive controls

Any immunohistologic technique should have positive controls. To date, appropriate controls for anti-8OHdG and anti-nitrotyrosine antibodies have not been rigorously demonstrated, though Toyokuni et al. (15) suggest normal lymphocytes are strongly positive for 8OHdG. My laboratory has studied a potential positive control for anti-4HNE and anti-Lys-HNE antibodies, which is utilization of the knowledge that the human prostate and seminal vesicle glands are known to contain large amounts of lipofuscin (35-36), which is a substance thought to derive from lipid peroxidation products. Indeed, immunoperoxidase analysis using anti-4HNE protein adduct and anti-Lys-HNE antibodies and histochemical stains for lipofuscin demonstrated co-localization of antibodies and histochemical staining for lipofuscin (manuscript submitted). Further, immunogold ultrastructural analysis of normal mouse brain using anti-HNE protein adduct or anti-Lys-HNE antibodies demonstrated co-localization of immunogold beads and lipofuscin granules (unpublished results). Thus, prostate, seminal vesicle, and brain may serve as positive controls for tissue studies.

Negative controls

Preincubation of antigen with antibody has been shown to abolish immunolabeling in the case of the antigens nitrotyrosine, 4HNE protein adducts, and Lys-HNE fluorophore (unpublished results). Pretreatment of tissue sections with DNase abolished 8OHdG staining in the tumors described below (unpublished observations).

Analysis of oxidative damage products in selected cell types

Studies of normal kidney and prostate have documented oxidative damage products in normal cells. For instance, normal kidney was demonstrated to contain significant levels of 4HNE protein adducts in proximal and distal tubular and transitional epithelial cells (37), which are the same cells that have significant levels of AE immunoreactive proteins. Interestingly, immunogold ultrastructural analysis in both human (37) and rat kidney (34) showed 4HNE protein adducts in both nucleus and mitochondria. Studies of oxidative damage products in normal human prostate and seminal vesicle have recently been completed (manuscript submitted). These studies show localization of moderate levels of 8OHdG, 4HNE protein adducts, Lys-HNE fluorophore, and nitrotyrosine in basal cells of prostate epithelium, with almost no oxidative damage products identified in prostate stroma (manuscript submitted). Prostatic stroma has been identified to have very high levels of GPX (manuscript submitted), and this may account for the low levels of oxidative damage products. It should be emphasized that oxidative damage products were identified in many normal organs without significant pathology being present. Thus, oxidative damage products do not necessarily cause significant pathology, probably because repair systems at both the organelle and cell level can replace injured molecules and cells. *Identification of oxidative damage products morphologically can be assumed to be important in a disease process only if subsequent physiologic or pathologic alterations can be identified.*

Oxidative damage products during pathologic processes

My laboratory has begun analyses of cellular and sub-cellular localization of oxidative damage products during different pathologic processes. *If oxidative damage products are causally associated with distinct pathologic processes, one would predict that each pathologic entity studied would have distinct oxidative damage profiles.*

Ischemia-reperfusion injury in heart

Immunogold ultrastructural analysis using anti-4HNE protein adduct antibody showed greatly increased labeling in mitochondria of hearts following ischemia-reperfusion compared to normal cardiac tissues (unpublished observations). This is consistent with the hypothesis that ischemia-reperfusion damage results from overproduction of ROS in mitochondria.

In addition, we have studied cardiac ischemia-reperfusion injury in transgenic mice overexpressing CuZnSOD. The results demonstrated that overexpression of CuZnSOD protected the heart against ischemia-reperfusion injury by pathophysiologic criteria (manuscript submitted). Interestingly, immunogold ultrastructural analysis demonstrated that CuZnSOD was significantly overexpressed in vessels, not in myocytes. We suggest that the site of ischemia-reperfusion injury must be the vasculature rather than the cardiac muscle tissue.

Cancer

Our laboratory has studied two types of human cancer in some detail: kidney and prostate cancer. There are several morphologic variants of human kidney cancer, and all types of primary kidney cancers showed lower levels of 4HNE protein adducts compared to normal kidney tissue (37). Ultrastructural analysis showed the same distribution of 4HNE protein adducts in benign and malignant renal epithelium, with both nuclei and mitochondria being positive; however, in the type of renal cancer examined (renal adenocarcinoma), the mitochondria were much smaller in malignant cells.

Prostate cancer has one predominant morphologic type (adenocarcinoma), and primary and metastatic prostate cancer showed lower levels of 4HNE protein adducts and Lys-HNE fluorophore than normal prostatic epithelium (manuscript submitted). 8OHdG and nitrotyrosine were elevated in metastatic prostate cancer compared to normal prostate epithelium (manuscript submitted); elevated 8OHdG and nitrotyrosine were observed primarily in nuclei in metastatic lesions.

Kondo et al. (15) reported higher nuclear 8OHdG and nitrotyrosine levels in human colon cancer when compared to adjacent normal epithelium. The authors suggest that 8OHdG and nitrotyrosine levels correlate with tumor cell proliferation, as assessed immunohistochemically with antibody to proliferating cell nuclear antigen. It is of note that oxidative damage occurred primarily in nuclei, since it is genetic changes in nuclei that are hypothesized to cause cancer.

A recent study of experimental chemically-induced liver cancer demonstrated increased inducible nitric oxide synthase and nitrotyrosine levels in nuclei of preneoplastic and neoplastic lesions using immunoperoxidase techniques (38). The authors conclude that chronic tissue damage by chemical carcinogenesis may act to induce inducible nitric oxide synthase and peroxynitrite formation.

Neurodegenerative diseases

Oxidative damage is postulated by many investigators to be involved in several neurodegenerative diseases. Elevated levels of 4HNE protein adducts have been detected in Parkinson's disease (39), and elevated 4HNE protein adducts (40) and protein carbonyls (13) have been detected in Alzheimer's disease.

A model of familial amyotrophic lateral sclerosis is available in transgenic mice in which mutated CuZnSOD (the mutation is not in the enzymatic active site) is overexpressed and causes pathology in motor neurons similar to that observed in human disease (41). Whether oxidative stress/damage is present in this model is much debated since enzymatic activity is not greatly altered. Future studies in our laboratory will examine levels of oxidative damage products in motor neurons of this mouse model. CuZnSOD knockout mice showed pathology and altered physiology at the neuromuscular junctions (42), illustrating that the pathologic consequences of absence of CuZnSOD enzyme activity has a different target tissue specificity than mutated CuZnSOD.

Aging

We have studied oxidative damage products in aging rhesus monkey skeletal muscle (manuscript submitted). Lipid peroxidation products (MDA plus 4-HDA) increased 80% in skeletal muscle from 34 year old compared to 2 year old rhesus monkeys, while carbonyl content increased 2-fold. Using quantitative immunogold electron microscopic analysis, HNE-protein adducts were increased 4-fold in these same animals, while protein carbonyl content was increased two-fold. Thus, the carbonyl data shows agreement between biochemical and morphologic measurements. The 4HNE data is not directly comparable, since biochemical measurements determine free HNE, while morphologic measurements detect 4HNE bound to protein (protein adducts). However, both biochemical and morphologic measurements showed an increase in lipid peroxidation products during aging in skeletal muscle. Interestingly, morphologic assessment of nitrotyrosine levels in skeletal muscle showed a decrease during these same time periods; the significance of this change remains to be determined.

At the ultrastructural level, oxidative damage using anti-4HNE antibody and anti-DNP antibody was primarily localized to myofibrils. Oxidative damage accumulation is the sum of several processes, including oxidant production, antioxidant defense, molecular repair, and organelle and cell turnover. Thus, our results suggest that in a postmitotic tissue such as skeletal muscle, the nucleus and mitochondria do not seem to be the primary site of pathologic accumulation of oxidative damage, even though these sites have been demonstrated to be important in other pathologic processes (mitochondria:ischemia-reperfusion injury; nucleus: cancer). A recent study showed no increase with aging in liver of either nuclear or mitochondrial oxidative DNA base changes and suggest that various antioxidant defenses may be sufficient to protect these organelles (43). Mitochondria are extremely well protected against oxidant injury, and have enzymes such as MnSOD and phospholipid-dependent GPX, which are specifically targeted to this organelle. Thus, subcellular localization of oxidative damage products depends on both the cell type and the kind of pathologic injury studied.

Other disease processes

Other disease processes in which oxidative damage product accumulation has been demonstrated include systemic amyloidosis (44), alcoholic liver disease (45), and chronic hepatitis C infection (46).

Future Directions

The technology described should prove to be very valuable when complementing biochemical and molecular biology studies. In a recent study of mouse skeletal muscle, oligonucleotide microarrays were used to analyze gene changes with aging (47). Several of the genes altered were nerve genes, illustrating the point that even the most homogeneous tissues such as skeletal muscle is composed of more than one cell type. While one hypothesis is that skeletal muscle atrophy with aging is due to oxidation of key molecules in skeletal muscle, a second possibility is that oxidative damage occurs in other cell types such as peripheral nerve cells or cells of blood vessels (endothelial and/or smooth muscle cells), which then secondarily results in skeletal muscle damage. By quantitating oxidative damage as a function of age, one should be able to determine which comes first, skeletal muscle, nerve, or vascular injury.

Similarly, subcellular localization of oxidative damage products can give important clues concerning etiology of pathologic events. Primary location of 4HNE protein adducts was in mitochondria in ischemia-reperfusion injury, nuclei and mitochondria in cancer, and myofibrils in sarcopenia. The latter observation suggests decreased protein turnover may be a pivotal event in aging, since it is known that myofibrillar proteins undergo slow turnover (48). Many investigators have suggested mitochondria as the source of oxidant production in aging, yet we saw almost no oxidative damage products in mitochondria in old rhesus monkey skeletal muscle. One possibility is that sarcopenia is not a uniform process, and we simply did not analyze the fibers that were affected, namely atrophic muscle fibers. However, it may be that powerful antioxidant defense mechanisms and increased protein turnover in mitochondria may account for a lack of staining in mitochondria. A histochemical method for detecting reactive oxygen species at the ultrastructural level is available (49), and we intend to use this method in the future to correlate sites of oxidant production with sites of oxidant damage. Oxidant production can also be assessed by confocal microscopy in living cells, and sites of oxidant generation in living cells compared to sites of oxidative damage in fixed cells by ultrastructural immunogold analysis.

We have detected oxidative damage products in normal tissues. One challenge will be to map oxidative damage products in normal tissues to provide baselines for pathologic studies. A second important area will be to try to determine whether oxidative damage products can have physiologic functions. For instance, it has been demonstrated that 4HNE inhibits cell proliferation through effects on signal transduction pathways (50),

and it has been demonstrated that nitric oxide regulates microtubule assembly (51).

The present review focuses on selected oxidative damage products. Many investigators have studied advanced glycation endproducts and believe they are involved in several pathologic processes, including aging. Future studies in our laboratory will focus on other oxidative damage products to determine their role in the aging process.

A final area of important study will be the use of genetically-engineered mice. Mice overexpressing MnSOD and CuZnSOD and "knockouts" of these genes have been produced by other laboratories. These mice should be invaluable in determining the effect of reactive oxygen species in various pathologic processes. Studies using these mice are currently underway in our laboratory. We have demonstrated that transgenic mice overexpressing MnSOD are resistant to adriamycin cardiotoxicity (52-53). Studies are in progress to determine the role of MnSOD in chemically-induced skin carcinogenesis and CuZnSOD in liver carcinogenesis.

CONCLUSIONS

Immunohistology techniques at the light microscopic level and immunogold techniques at the ultrastructural level allow relative quantitation of antioxidant proteins and oxidative damage products. Such techniques will provide important ancillary information to complement biochemistry and molecular biology studies.

ACKNOWLEDGMENTS

The authors would like to thank Dr. Larry W. Oberley for helpful suggestions concerning this manuscript. This work was supported by the Veterans Administration Research Service.

ABBREVIATIONS

AE(s)- antioxidant enzyme(s)
CAT- catalase
CuZnSOD- copper, zinc superoxide dismutase
DNP- 2,4-dinitrophenylhydrazine
ECSOD- extracellular superoxide dismutase
Fe-NTA- iron nitrilotriacetate
GPX- glutathione peroxidase
4-HDA- 4-hydroxy-2-alkenel
4HNE- 4-hydroxy-2-nonenal
Lys-HNE- fluorophore derived from reaction of HNE with lysine
MDA- malondialdehyde
MnSOD- manganese superoxide dismutase
8OHdG- 8-hydroxy-2'-deoxyguanosine
ROS- reactive oxygen species
SOD- superoxide dismutase

REFERENCES

1. Sohal, R.S., and Weindruch, R.: Oxidative stress, caloric restriction, and aging. *Science*, 273: 59-63, 1996.
2. Chance, B., Sies, H., and Boveris, A.: Hydroperoxide metabolism in mammalian organs. *Physiol. Rev.*, 59: 527-605, 1979.
3. Sohal, R.S., Ku, H.H., Agarwal, S., Forster, M.J., and Lal, H.: Oxidative damage, mitochondrial oxidant generation and antioxidant defenses during aging and in response to food restriction in the mouse. *Mech. Ageing Dev.*, 74: 121-133, 1994.
4. Pansarasa, O., Bertorelli, L., Vecchiet, J., Felzani, G., and Marzatico, F.: Age-dependent changes of antioxidant activities and markers of free radical damage in human skeletal muscle. *Free Radic. Biol. Med.*, 27: 617-622, 1999.
5. Mecocci, P., Fano, G., Fulle, S., MacGarvey, U., Shinobu, L., Polidori, M.C., Cherubini, A., Vecchiet, J., Senin, U., and Beal, M.F.: Age-dependent increases in oxidative damage to DNA, lipids, and proteins in human skeletal muscle. *Free Radic. Biol. Med.*, 26: 303-308, 1999.
6. Pugh, T.D., Oberley, T.D., and Weindruch, R.: Dietary intervention at middle age: caloric restriction but not dehydroepiandrosterone sulfate increases life span and lifetime cancer incidence in mice. *Cancer Res.*, 59: 1642-1648, 1999.
7. Yan, T., Jiang, X., Zhang, H.J., Li, S., and Oberley, L.W.: Use of commercial antibodies for detection of primary antioxidant enzymes. *Free Radic. Biol. Med.*, 25: 688-693, 1998.
8. Uchida, K., Itakura, K., Kawakishi, S., Hiai, H., Toyokuni, S., and Stadtman, E.R.: Characterization of epitopes recognized by 4-hydroxy-2-nonenal specific antibodies. *Arch. Biochem. Biophys.*, 324: 241-248, 1995.
9. Uchida, K., Szweda, L.I., Chae, H-Z., and Stadtman, E.R.: Immunochemical detection of 4-hydroxy-2-nonenal protein adducts in oxidized hepatocytes. *Proc. Natl. Acad. Sci. USA*, 90: 8742-8746, 1993.
10. Toyokuni, S., Miyake, N., Hiai, H., Hagiwara, M., Kawakishi, S., Osawa, T., and Uchida, K.: The monoclonal antibody specific for the 4-hydroxy-2-nonenal histidine adduct. *FEBS Lett.*, 359: 189-191, 1995.
11. Toyokuni, S., Tanaka, T., Hattori, Y., Nishiyama, Y., Yoshida, A., Uchida, K., Ochi, H., and Osawa, T.: Quantitative immunohistochemical determination of 8-hydroxy-2'-deoxyguanosine by a monoclonal antibody N45.1: its application to ferric nitrilotriacetate-induced renal carcinogenesis model. *Lab. Invest.*, 76: 365-374, 1997.

12. MacMillen-Crow, L.A., Crow, J.P., Kerby, J.D., Beckman, J.S., and Thompson, J.A.: Nitration and inactivation of manganese superoxide dismutase in chronic rejection of renal allograft. *Proc. Natl. Acad. Sci. USA*, 93: 11853-11858, 1996.
13. Smith, M.A., Sayre, L.M., Anderson, V.E., Harris, P.L.R., Beal, M.F., Kowall, N., and Perry, G.: Cytochemical determination of oxidative damage in Alzheimer disease by immunochemical enhancement of the carbonyl reaction with 2,4-dinitrophenylhydrazine. *J. Histochem. Cytochem.*, 46: 731-735, 1998.
14. Toyokuni, S.: Reactive oxygen species-induced molecular damage and its application in pathology. *Path. Int.*, 49: 401-410, 1999.
15. Kondo, S., Toyokuni, S., Iwasa, Y., Tanake, T., Onodera, H., Hiai, H., and Imamura M.: Persistent oxidative stress in human colorectal carcinoma, but not in adenoma. *Free Radic. Biol. Med.*, 27: 401-410, 1999.
16. Coursin, D.B., Cihla, H.P., Oberley, T.D., and Oberley, L.W.: Immunolocalization of antioxidant enzymes and isozymes of glutathione S-transferase in normal rat lung. *Am. J. Physiol.*, 263: L679-L691, 1992.
17. Weisiger, R.A., and Fridovich, I.: Mitochondrial superoxide dismutase. Site of synthesis and intramitochondrial location. *J. Biol. Chem.*, 248: 4793-4796, 1973.
18. McCord, J.M., and Fridovich, I.: Superoxide dismutase. An enzymic function for erythrocuprein (hemocuprein). *J. Biol. Chem.*, 244: 6049-6055, 1969.
19. Marklund, S.L., Holme, E., and Hellner, L.: Superoxide dismutase in extracellular fluids. *Clin. Chim. Acta*, 126: 41-51, 1982.
20. Peeters-Joris, C., Vandervoorde, A.M., and Bandhuin, P.: Subcellular localization of superoxide dismutase in rat liver. *Biochem. J.*, 150: 31-39, 1975.
21. Muse, K.E., Oberley, T.D., Sempf, J.M., and Oberley, L.W.: Immunolocalization of antioxidant enzymes in adult hamster kidney. *Histochem. J.*, 26: 734-753, 1994.
22. Vertechy, M., Cooper, M.B., Ghirardi, O., and Ramacci, M.T.: Antioxidant enzyme activities in heart and skeletal muscle of rats of different ages. *Exp. Gerontol.*, 24: 211-218, 1989.
23. Ji L.L., Dillon, D., and Wu, E.: Alteration of antioxidant enzymes with aging in rat skeletal muscle and liver. *Am. J. Physiol.*, 258: R918-R923, 1990.
24. Oh-Ishi, S., Kisaki, T., Yamashita, H., Nagata, N., Suzuki, K., Taniguchi, N., and Ohno, H.: Alterations of superoxide dismutase iso-enzyme activity, content, and mRNA expression with aging in skeletal muscle. *Mech. Ageing Dev.*, 84: 65-76, 1995.
25. Oberley, T.D., Oberley, L.W., Slattery, A.F., Lauchner, L.J., and Elwell, J.H.: Immunohistochemical localization of antioxidant enzymes in adult Syrian hamster tissues and during kidney development. *Am. J. Pathol.*, 137: 199-214, 1990.
26. Oberley, T.D., Oberley, L.W., Slattery, A.F., and Elwell, J.H.: Immunohistochemical localization of glutathione S-transferase and glutathione peroxidase in adult hamster tissues and during kidney development. *Am. J. Pathol.*, 139: 355-369, 1991.
27. Oberley, T.D., Friedman, A.L., Moser, R., and Siegel, F.L.: Effects of lead administration on developing rat kidney. II. Functional, morphologic, and immunohistochemical studies. *Toxicol. and Applied Pharmacol.*, 131: 94-107, 1995.
28. Daggett, D.A., Oberley, T.D., Nelson, S.A., Wright, L.S., Kornguth, S.E., and Siegel, F.L.: Effects of lead on rat kidney and liver: GST expression and oxidative stress. *Toxicology*, 128: 191-206, 1998.
29. Arai, M., Imai, H., Koumura, T., Yoshida, M., Emoto, K., Umeda, M., Chiba, N., and Nakagawa, Y.: Mitochondrial phospholipid hydroperoxide glutathione peroxidase plays a major role in preventing oxidative injury to cells. *J. Biol. Chem.*, 274: 4924-4933, 1999.
30. Bulitta, C., Ganea, C., Fahimi, H.D., and Volkl, A.: Cytoplasmic and peroxisomal catalases of the guinea pig liver: evidence for two distinct proteins. *Biochim. et Biophys. Acta*, 1293: 55-62, 1996.
31. Swaroop, M., Bian, J., Aviram, M., Duan, H., Bisgaier, C.L., Loo, J.A., and Sun, Y.: Expression, purification, and biochemical characterization of SAG, a ring finger redox-sensitive protein. *Free Radic. Biol. Med.*, 27: 193-202, 1999.
32. Oberley, T.D., and Oberley, L.W.: Antioxidant enzyme levels in cancer. *Histol. Histopathol.*, 12: 525-535, 1997.
33. Tsai, L., Szveda, P.A., Vinogradova, O., and Szveda, L.I.: Structural characterization and immunochemical detection of a fluorophore derived from 4-hydroxy-2-nonenal and lysine. *Proc. Natl. Acad. Sci. USA*, 95: 7975-7980, 1998.
34. Zainal, T.A., Weindruch, R., Szveda, L.I., and Oberley, T.D.: Localization of 4-hydroxy-2-nonenal-modified proteins in kidney following iron overload. *Free Radic. Biol. Med.*, 26: 1181-1193, 1999.
35. Brennick, J.B., O'Connell, J.V., Dickerson, G.R., and Young, R.H.: Lipofuscin accumulation (so-called "melanosis") of the prostate. *Am. J. Surg. Pathol.*, 18: 446-454, 1994.

36. Amin, M.B., and Bostwick, D.B.: Pigment in prostatic epithelium and adenocarcinoma: a potential source of diagnostic confusion with seminal vesicle epithelium. *Modern Pathol.*, 9: 791-795, 1996.
37. Oberley, T.D., Toyokuni, S., and Szweda, L.I.: Localization of hydroxynonenal protein adducts in normal human kidney and selected human kidney cancers. *Free Radic. Biol. Med.*, 27: 693-703, 1999.
38. Ahn, B., Han, B.S., Kim, D.J., and Oshima, H.: Immunohistochemical localization of inducible nitric oxide synthase and 3-nitrotyrosine in rat liver tumors induced by N-nitrosodiethylamine. *Carcinogenesis*, 20: 1337-1344, 1999.
39. Yoritaka, A., Hattori, N., Uchida, K., Tanaka, M., Stadtman, E.R., and Mizuno, Y.: Immunohistochemical detection of 4-hydroxynonenal protein adducts in Parkinson's disease. *Proc. Natl. Acad. Sci. USA*, 93: 2696-2701, 1996.
40. Montine, K.S., Olson, S.J., Amarnath, V., Whetsell, W. Jr., Graham, D.J., and Montine, T.J.: Immunohistochemical detection of 4-hydroxy-2-nonenal adducts in Alzheimer's disease is associated with inheritance of APOE4. *Am J. Pathol.*, 150: 437-443, 1997.
41. Gurney, M.E., Pu, H., Chiu, A.Y., Dal Canto, M.C., Polchow, C.Y., Alexander, D.D., Caliendo, J., Hentati, A., Kwon, Y.W., Deng, H-X., Chen, W., Zhai, P., Sufit, R.L., and Siddique, T.: Motor neuron degeneration in mice that express a human Cu,Zn superoxide dismutase mutation. *Science*, 264: 1772-1775, 1995.
42. Flood, D.G., Reaume, A.G., Gruner, J.A., Hoffman, E.K., Hirsch, J.D., Lin, Y-G., Dorfman, K.S., and Scott, R.W.: Hindlimb motor neurons require Cu/Zn superoxide dismutase for maintenance of neuromuscular junctions. *Am. J. Pathol.*, 155: 663-672, 1999.
43. Anson, R.M., Senturker, S., Dizdaroglu, M., and Bohr, V.: Measurement of oxidatively induced base lesions in liver from Wistar rats of different ages. *Free Radic. Biol. Med.*, 27: 456-452, 1999.
44. Ando, Y., Nyhlin, N., Suhr, O., Holmgren, G., Uchida, K., Sahly, M.E., Yamashita, T., Terasaki, H., Makamura, M., Uchino, M., and Ando, M.: Oxidative stress is found in amyloid deposits in systemic amyloidosis. *Biochem. Biophys. Res. Comm.*, 232: 497-502, 1997.
45. Ohhira, M., Ohtake, T., Matsumoto, A., Saito, H., Ikuta, K., Fujimoto, Y., Ono, M., Toyokuni, S., Kohgo, Y.: Immunohistochemical detection of 4-hydroxy-2-nonenal-modified-protein adducts in human alcoholic liver disease. *Alcohol Clin. Exp. Res.*, 22: S145-S149, 1997.
46. Kageyama, F., Kobayashi, Y., Koide, S.: Enhanced lipid peroxidation in chronic hepatitis C. *Jpn. Pharmacol. Ther.*, 26: S405-S408, 1998.
47. Lee, C-K., Klopp, R.G., Weindruch, R., and Prolla, T.A.: Gene expression profile of aging and its retardation by caloric restriction. *Science*, 285: 1390-1393, 1999.
48. Bates, P.C., and Millward, D.J. Myofibrillar protein turnover. *Biochem. J.*, 214: 587-592, 1983.
49. Kerver, E.D., Vogels, I.M., Bosch, K.S., Vreeling-Sindelavara, H., van den Munckhof, R.J.M., and Frederiks, W.M.: In situ detection of spontaneous superoxide anion and singlet oxygen production by mitochondria in rat liver and small intestine. *Histochem. J.*, 29: 229-237, 1997.
50. Uchida, K., Shiraishi, M., Naito, Y., Torii, Y., Nakamura, Y., and Osawa, T.: Activation of stress signaling pathways by the end product of lipid peroxidation: 4-hydroxy-2-nonenal is a potent inducer of intracellular peroxide production. *J. Biol. Chem.*, 274: 2234-2242, 1999.
51. Eiserich, J.P., Estevez, A.G., Bamberg, T.V., Ye, Y.Z., Chumley, P.H., Beckman, J.S., and Freeman, B.A.: Microtubule dysfunction by posttranslational nitrotyrosination of α -tubulin: A nitric oxide-dependent mechanism of cellular injury. *Proc. Natl. Acad. Sci. USA*, 96: 6365-6370, 1999.
52. Yen, H-C., Oberley, T.D., Vichitbanda, S., Ho, Y-S., and St. Clair, D.K.: The protective role of manganese superoxide dismutase against adriamycin-induced acute cardiac toxicity in transgenic mice. *J. Clin. Invest.*, 98: 1253-1260, 1996.
53. Yen, H-C., Oberley, T.D., Gairola, C.G., Szweda, L.I., and St. Clair, D.K.: Manganese superoxide dismutase protects mitochondrial complex I against adriamycin-induced cardiomyopathy in transgenic mice. *Arch. Biochem. Biophys.*, 362: 59-66, 1999.