HHS Public Access

Author manuscript

Methods Mol Biol. Author manuscript; available in PMC 2018 April 24.

Published in final edited form as:

Methods Mol Biol. 2010 ; 610: 419–434. doi:10.1007/978-1-60327-029-8_25.

Detection and Localization of Markers of Oxidative Stress by In Situ Methods: Application in the Study of Alzheimer Disease

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Abstract

Oxidative stress is a key factor involved in the development and progression of Alzheimer disease (AD), and it is well documented that free radical oxidative damage, particularly of neuronal lipids, proteins, nucleic acids, and sugars, is extensive in brains of AD patients. The complex chemistry of peroxynitrite has been the subject of intense study and is now evident that there are two principal pathways for protein modification: the first one involves homolytic hydroxyl radical-like chemistry that results in protein-based carbonyls and the second involves electrophilic nitration of vulnerable side chains, in particular the electron-rich aromatic rings of Tyr and Trp. In the presence of buffering bicarbonate, peroxynitrite forms a CO_2 adduct, which augments its reactivity. Formation of 3-nitrotyrosine by this route has become the classical protein marker specifically for the presence of peroxynitrite. Protein-based carbonyls can be detected by two methods: (i) derivatization with 2,4-dinitrophenylhydrazine (DNPH) and detection of the protein-bound hydrazones using an enzyme-linked anti-2,4-dinitrophenyl antibody and (ii) derivatization with biotin-hydrazide and detection of the protein-bound acyl hydrazone with enzyme-linked avidin or streptavidin. Glycation of proteins by reducing sugars (Maillard reaction) results in a profile of time-dependent adduct evolution rendering susceptibility to oxidative elaboration. In addition, oxidative stress can result in oxidized sugar derivatives which can subsequently modify protein through a process known as glycoxidation. Of more general importance, oxidative stress results in lipid peroxidation and the production of a range of electrophilic and mostly bifunctional aldehydes that modify numerous proteins. The more important protein modifications are referred to as advanced glycation end products (AGEs) and advanced lipoxidation end products (ALEs). Protein modification can result in both non-cross-link and cross-link AGEs and ALEs, the latter arising from the potential bifunctional reactivity, such as that of the lipid-derived modifiers 4-hydroxy-2-nonenal (HNE) and malondialdehyde (MDA). Oxidative damage to nucleic acids results in base modification, substitutions, and deletions. Among the most common modifications, 8-hydroxyguanosine (8OHG) is considered a signature of oxidative damage to nucleic acid.

Cells are not passive to increased oxygen radical production but rather upregulate protective responses. In neurodegenerative diseases, heme oxygenase-1 (HO-1) induction is coincident with the formation of neurofibrillary tangles. This enzyme thatconverts heme, a prooxidant, to biliverdin/bilirubin (antioxidants) and free iron has been considered an antioxidant enzyme. But

[4]In these experiments, it is important to recognize that DAB is supplied as the tetrahydrochloride and that DAB free base is only sparingly soluble in water. Thus, it is crucial that the tissue incubation solution remains at a low enough pH that the DAB stays in solution. In this manner, the insoluble brown DAB oxidation product formed at tissue sites of redox activity is readily visualized without interference from precipitated unoxidized DAB.

seen in the context of arresting apoptosis, HO-1 and tau may play a role in maintaining the neurons free from the apoptotic signal (cytochrome *c*), since tau has strong iron-binding sites. Given the importance of iron as a catalyst for the generation of reactive oxygen species, changes in proteins associated with iron homeostasis can be used as an index of cellular responses. One such class of proteins is the iron regulatory proteins (IRPs) that respond to cellular iron concentrations by regulating the translation of proteins involved in iron uptake, storage, and utilization. Therefore, IRPs are considered to be the central control components of cellular iron concentration.

Keywords

Advanced glycation end products; advanced lipoxidation end products; glycation; glycoxidation; heme oxygenase-1; 8-hydroxyguanosine; 4-hydroxy-2-nonenal; iron regulatory proteins; malondialdehyde; 3-nitrotyrosine; protein carbonyls

1. Introduction

Oxidative damage is relevant in the brain (1) for several reasons: (1) it is a post-mitotic tissue with a high energy demand; (2) it is exposed to high oxygen concentration and utilizes about one-fifth of the oxygen consumed by the body; (3) it contains relatively poor concentrations of antioxidants and related enzymes; (4) it is rich in polyunsaturated fatty acids that are more prone to oxidation; and (5) it is rich in iron content, which accumulates as a function of age and can be a source of potent catalyst for oxidative species formation.

Oxidative stress is characterized by a compromise in the physiological removal of reactive oxygen species (ROS) and/or an increase in their production that results from irreversible damage to biomacromolecules. Oxidative damage of biomacromolecules has been described in the brains of Alzheimer disease (AD) patients: (1) DNA and RNA oxidation is marked by increased levels of 8-hydroxy-2-deoxyguanosine (8OHdG) and 8-hydroxyguanosine (8OHG) (2–4); (2) protein oxidation is marked by elevated levels of protein carbonyl and nitration of tyrosine residues (5, 6). Moreover, cross-linking of proteins by oxidative processes may contribute to the resistance of the lesions, senile plaques, and neurofibrillary tangles (NFT) to intracellular and extracellular removal despite being extensively ubiquitinated (7). This resistance of NFT to proteolysis might play an important role in the progression of AD (8); (3) lipid peroxidation is marked by high levels of thiobarbituric acid-reactive substances (TBARS), malondialdehyde (MDA), 4-hydroxy-2-nonenal (HNE), and isoprostanes and altered phospholipid composition (9, 10); and (4) modification to sugars is marked by increased glycation and glycoxidation (11, 12). While intracellular oxidative balance is tightly regulated, the activation of multiple signaling pathways and the upregulation of compensatory mechanisms would be an anticipated outcome in neurons from AD patients.

The importance of in situ methods over bulk analysis cannot be overplayed when considering the structural and cellular complexity of the nervous system. First, the affected neurons comprise a small percentage of the tissue, and second, it is unknown how other cells such as glial cells are affected by the disease. For instance, while neurons may show one type of damage or response, the glial cells may show a compensatory response unrelated to

or opposing responses to that of neurons. Further, bulk analysis of oxidative damage will always be hampered by long-lived proteins modified during physiological aging. Extracellular matrix proteins of major vessels provide a record of long-term oxidative insult and therefore are sensitive markers of pathological changes. These properties that render vessels as ideal monitors for aging limit their sensitivity during the detection of disease-specific changes. While isolation of neurons or other cell fractions can address the issue of specificity, limitation exists for poorly represented cell types with the concerns of vascular contamination. Vascular contamination of insoluble proteins can interfere because even glass bead columns or collagenase treatments seldom quantitatively remove all vascular elements if the initial isolation procedure does not exclude them.

This chapter provides details of in situ detection methods that can be used for the qualitative and semi-quantitative measurement of various oxidative stress markers in tissue sections obtained at autopsy. While oxidative stress-related damage detected implicates general or specific cytotoxicity, it is not the purpose of this chapter to suggest that the appearance of certain markers correlates with a particular mechanism of toxicity. Indeed, as discussed in the last section, any such correlation requires a consideration of whether the damage apparent represents a primary or a secondary event in the cascade of biochemical processes leading to neuronal death.

2. Materials

2.1. Pathological Tissue

Liquid nitrogen/isopentane, methacarn (60% methanol, 30% chloroform, 10% acetic acid), and paraffin.

2.2. Direct Histochemical Methods

2.2.1. 2,4-Dinitrophenylhydrazine Reactivity of Protein-Bound Versus Protein-Based Carbonyls—2,4-Dinitrophenylhydrazine (DNPH), pyruvate 2,4-dinitrophenylhydrazone, enzyme-linked anti-2,4-dinitrophenyl antibody, biotin-hydrazide, enzyme-linked avidin or enzyme-linked streptavidin, xylene, ethanol, methanol, sodium chloride (NaCl), Tris-buffered saline (TBS; 50 mM Tris–HCl, 150 mM NaCl, pH 7.6), normal goat serum (NGS), rat monoclonal antibody (LO-DNP-2; Zymed Laboratories, San Francisco, CA), rat IgG (Boehringer-Mannheim, Indianapolis, IN), anti-rat peroxidase–antiperoxidase complex (ICN Pharmaceuticals, Costa Mesa, CA), hydrogen peroxide (H_2O_2), 3,3′-diaminobenzidine (DAB; Sigma, St. Louis, MO), sodium borohydride ($NaBH_4$), and sodium cyanoborohydride ($NaBH_3CN$).

2.2.2. Redox-Active Transition Metals; Cytochemical Detection of Iron(II)/(III)—Xylene, ethanol, potassium ferrocyanide [$K_4Fe(CN)_6 \cdot 3H_2O$], potassium ferricyanide $K_3Fe(CN)_6$], HCl, DAB, Tris–HCl (50 mM, pH 7.6), H_2O_2, and ultrapure water.

2.2.3. Metal Chelation/Binding—Deferoxamine (DFX) or diethylenetriaminepentaacetic acid (DTPA), ethylenediaminetetraacetic acid (EDTA), ferrocyanide, ferricyanide, H_2O_2, DAB, iron citrate ($FeC_6H_8O_7$), iron chloride ($FeCl_3$), and copper(II) chloride ($CuCl_2$).

2.3. Immunocytochemical Methods

2.3.1. Immunocytochemistry Using a Peroxidase-Coupled Secondary Antibody —Xylene, ethanol, H_2O_2, methanol, NGS, TBS, DAB, goat anti-mouse antibody, goat anti-rabbit antibody, mouse- and rabbit-specific peroxidase–antiperoxidase complexes, sodium hydroxide (NaOH), trypsin, Tris, NaCl, calcium chloride ($CaCl_2$), citrate buffer, guanidine, formic acid (HCOOH), antigen retrieval solution (Biogenix Laboratories), proteinase K (Boehringer Mannheim), and phosphate buffer (PBS; pH 7.4).

2.3.2. Nitrotyrosine Antibody for Determination of Protein Tyrosine Nitration by Peroxynitrite—Affinity-purified rabbit antiserum or mouse monoclonal antibodies (7A2 or 1A6) raised to nitrated keyhole limpet hemocyanin, NGS, NaCl, Tris, HCl, nitrated bovine serum albumin (BSA-NO_2), nitrated Gly-Tyr-Ala peptide, sodium hydrosulfite ($Na_2S_2O_4$), and carbonate buffer.

2.3.3. Antibodies to Advanced Glycation End Products—Keyhole limpet hemocyanin (KLH), pentosidine–KLH conjugate, Freund's complete adjuvant, Freund's incomplete adjuvant, mouse monoclonal pyrraline antibodies raised to keyhole limpet hemocyanin–caproyl pyrraline conjugate, free pentosidine, and pyrraline haptens.

2.3.4. Antibodies to Lipid Peroxidation End Products—Antiserum raised to keyhole limpet hemocyanin modified by 4-oxononanal (ON-KLH), antiserum raised to keyhole limpet hemocyanin conjugated with preformed 6-(2-pentylpyrrol-1-yl)caproic acid (PPC-KLH), DAB, H_2O_2, Tris–HCl, Congo red, BSA-derived antigens (ON-BSA, PPC-BSA).

2.3.5. Antibodies to DNA Damage—Anti-8OHG (QED Bioscience, San Diego, CA) and 1F7 (Trevigen, Gaithersburg, MD), NGS, proteinase K, DAB, H_2O_2, Tris–HCl, PBS, DNase (Boehringer Mannheim), S1 DNase (Boehringer Mannheim), and RNase (Boehringer Mannheim).

2.3.6. Antibodies to Cellular Response Factor, Heme Oxygenase-1—Rabbit antisera to heme oxygenase-1 (HO-1; Stressgen Biotechnologies, Victoria, Canada) and purified HO-1 protein (Stressgen Biotechnologies, Victoria, Canada).

2.3.7. Antibodies to the Cellular Response Factor; Iron-Response Element—Rabbit polyclonal antibodies to iron-response protein-2 (IRP-2) and purified IRP-2 protein.

3. Methods

3.1. Pathological Tissue

Tissue can either be flash frozen in liquid nitrogen/isopentane and cryostat sections prepared at 20 nm or be fixed in methacarn, paraffin embedded, and sections cut at 6 μm (*see* Note 1).

[1]It is essential that the modifications analyzed be both stable and not produced as a result of postmortem or other time-dependent post-experimental changes. The latter is a particular concern if initial lipid oxidative adducts are analyzed. One way we have addressed this issue is to analyze and compare results of localizing early lipoxidation adducts versus ALE. Material fixed in glutaraldehyde or even paraformaldehyde is problematic since it precludes the analysis of lipoxidative and other modifications that share the property of carbonyl condensation-dependent cross-links. The latter concern is the most significant limitation in practice since the majority of

3.2. Direct Histochemical Methods

3.2.1. 2,4-Dinitrophenylhydrazine Reactivity of Protein-Bound Versus Protein-Based Carbonyls—Two related methods for the detection of protein-based carbonyls are (i) derivatization with DNPH and detection of the protein-bound hydrazones using an enzyme-linked anti-2,4-dinitrophenyl antibody and (ii) derivatization with biotin-hydrazide and detection of the protein-bound acyl hydrazone with enzyme-linked avidin or enzyme-linked streptavidin (5, 13) (*see* Note 2).

After deparaffinization in xylene and rehydration through graded ethanol for methacarn-fixed material or brief fixation in ice-cold acetone for cryostat material, sections are covered with 0.1–0.001% DNPH in 2 N HCl. All incubations are conducted at room temperature, unless otherwise indicated, in a plastic box in which water-saturated paper towels are placed in the bottom over which the slides are placed on a platform. After 1 h incubation, sections are exhaustively rinsed in TBS followed by incubation for 30 min in 10% NGS to block non-specific binding sites. After rinsing with 1% NGS/TBS, a rat monoclonal antibody (LO-DNP-2) to DNP is diluted at 1:100 in 1% NGS/TBS and incubated with the sections at 4°C for 16 h. Sections are then rinsed with 1% NGS/TBS, followed by incubation with goat antiserum to rat IgG diluted at 1:50 with 1% NGS/TBS for 30 min. After rinsing in 1% NGS/TBS, rat peroxidase–antiperoxidase complex (1:250) in the same buffer is incubated with the section at room temperature for 1 h, after which it is rinsed with 1% NGS/TBS. Peroxidase activity is localized by development with 0.015% H_2O_2 in 50 mM Tris–HCl, pH 7.6, and 0.75 mg/mL DAB for 5–10 min (*see* Note 3). The development of the sections is directly monitored for maximum contrast under the 10× objective of a Zeiss Axioskop 20 microscope.

Chemical and immunochemical controls are used to define carbonyl-specific binding. Chemical reduction of free carbonyls and Schiff bases is performed by incubating sections with 25 mM $NaBH_4$ in 80% methanol for 30 min before incubation with DNPH. Selective reduction of Schiff bases as opposed to free carbonyls can be achieved by incubation with 50 mM sodium cyanoborohydride in 0.1 M phosphate buffer, pH 6.0, for 1 h. Immunochemical specificity is demonstrated by omission of the DNPH treatment or the antibody to DNP. Immunoabsorption of the antibody to DNP is performed by incubating the antibody (1:100)

pathological tissue available for retrospective study is fixed in formalin. If formalin-fixed tissue must be used, it is best to focus on highly defined products, unlikely to share homology with formalin or nucleic acid modifications.

[2]Although we have utilized only the DNPH method in situ, the biotin-hydrazide method should perform similarly and may be preferable in cases where there appears to be excessive non-specific binding of DNPH. In either case, it is important to recognize that protein-bound carbonyls can represent either oxidized side chains or the univalent adduction of a bifunctional glycoxidation or lipoxidation product such that the adduct still contains a free carbonyl group. Modification of unoxidized lysine amino groups by monofunctional lipid- or sugar-derived carbonyl compounds will not interfere because DNPH or biotin-hydrazide will displace the carbonyl compound into bulk solution. In these cases, one can learn about the nature of the lysine modification through direct LC-MS analysis of the DNP derivatives in solution. If it is important to localize and/or quantify total protein-associated carbonyls (both protein-based carbonyls and carbonyl-modified lysines), one approach is to carry out an in situ determination of protein-bound tritium using [^{3}H]-$NaBH_4$ with detection by fluorography.

[3]When the peroxidase–antiperoxidase method is used for visualization of bound DNP antibody, we have typically utilized a 20-min pre-incubation of tissue sections with 3% H_2O_2 in methanol to prevent artifacts of staining arising from endogenous peroxidase activity in the tissue (18). Although H_2O_2 itself, or synergistically with tissue-bound transition metals, might create oxidative modifications through hydroxyl radical formation (e.g., carbonyl residues), deletion of this step had no effect upon subsequent DNPH labeling conducted in our laboratory, though it did convert Fe(II) to Fe(III) (16). Therefore, a control that omits the H_2O_2 treatment step should be performed with each experimental paradigm. Alternatively, utilization of a secondary antibody coupled to alkaline phosphatase, with *p*-nitrophenyl phosphate as chromogen, obviates the necessity of the prior H_2O_2 treatment.

with 5 mM pyruvate 2,4-dinitrophenylhydrazone (stock 1 mg/mL in ethanol) at 4°C for 16 h and comparing the resulting immunoreactivity with unabsorbed antibody that had been similarly treated with ethanol.

3.2.2. Cytochemical Detection of Iron(II)/(III) and In Situ Oxidation—This technique incorporates three key modifications of the established histological method (14, 15) that greatly increase the detection sensitivity of this technique: (i) increasing the concentrations of potassium ferrocyanide (7%) and hydrochloric acid (3%); (ii) lengthening the incubation time and/or increasing the temperature (15 h at room temperature or 1 h at 37°C); and (iii) using methacarn versus formalin-fixed tissue. The last modification is perhaps the most critical to iron detection in human tissue where formaldehyde-based fixation for extended periods is routine. Indeed, we found that even a brief post-fixation with formaldehyde (3.7%, 5–60 min at room temperature) reduced labeling in a time-dependent manner (16). The tesults with methacarn fixation are similar to those found in sections made from frozen blocks, which were neither fixed nor embedded, suggesting that the iron localization is not a result of tissue fixation or embedding. This method takes advantage of forming the intensely blue mixed valence Fe(II)/Fe(III)–cyanide complex, which can be observed directly as a pale blue stain on tissue sections or can be enhanced by taking advantage of the complex to catalyze the H_2O_2-dependent oxidation of DAB, giving an insoluble brown precipitate localized to bound iron. For the latter case, the tissue sections are deparaffinized in xylene, rehydrated through graded ethanol, and then incubated for 15 h in 7% potassium ferrocyanide (for iron(III) detection) or 7% potassium ferricyanide (for iron(II) detection) in aqueous HCl (3%) and subsequently incubated with a solution containing 0.75 mg/mL DAB tetrahydrochloride in 50 mM Tris–HCl, pH 7.6, and 0.015% H_2O_2 for 5–10 min.

3.2.3. Metal Chelation/Binding—Redox-active metals bound adventitiously to proteins in tissue sections can be removed by exposure to up to 0.1 M DFX or DTPA at room temperature for a period of time depending on the affinity of the tissue sites. For example, whereas for 0.1 M DFX, 20 min was sufficient to remove ferrocyanide-detectable iron from certain sites, a 15 h incubation was required to completely remove iron bound to others and the use of 0.1 M EDTA for 15 h was only partly effective in removing iron (16). Observed differential efficiency of the removal of H_2O_2-dependent DAB oxidizing activity by, e.g., DFX vs. DTPA provides information on the nature of endogenous metals present, since DFX and DTPA are somewhat selective for iron and copper, respectively. The fact that following chelation of iron, incubation of AD tissue sections with ferrocyanide or ferricyanide did not result in H_2O_2-dependent oxidation of DAB on the tissue indicated that formation of the mixed valence complex represents real tissue-bound iron and is not an artifact of the use of an iron-containing reagent.

Potential sites for binding of redox-active transition metals in tissue sections or attempted rebinding of metals following chelation-dependent removal of endogenous metals can be carried out, for example, by incubating with 0.01 mM iron(III) citrate plus 0.01 mM iron(II) chloride or with 0.01 mM copper(II) chloride at room temperature for 3 h, followed by detection (*see* Note 5).

3.2.4. In Situ Oxidation of Redox-Active Transition Metals—Protein-bound redox-active transition metals can be directly demonstrated by incubation of sections with 3% H_2O_2 and 0.75 mg/mL DAB in 50 mM Tris–HCl, pH 7.6. This protocol does not involve preformation of a mixed valence iron complex, and so it does not directly identify the nature of the tissue-bound metal (17). However, iron and copper are the metals most likely to be bound adventitiously in redox-active form (*see* Note 5).

3.3. Immunocytochemical Methods

3.3.1. Immunocytochemistry Using a Peroxidase-Coupled Secondary Antibody —Following deparaffinization with xylene, sections are rehydrated through graded ethanol (100, 95, 70, 50, and 30%). Endogenous peroxidase activity in the tissue is inactivated by incubation with 3% H_2O_2 in methanol for 20 min at room temperature and non-specific binding sites are blocked in a 30-min incubation at room temperature with 10% NGS in TBS. Immunostaining is accomplished by the peroxidase–antiperoxidase technique using DAB as co-substrate chromogen (18). Adjacent sections can be immunostained with antisera to known proteins and to the location of specific structures.

There are a great variety of protocols available for the pretreatment of tissue sections to facilitate antigen retrieval (i.e., exposure of hidden epitopes at the protein–protein interface). For example, in previous studies we, and others, have used pretreatments with (i) NaOH (0.2 M, 15 min, room temperature); (ii) trypsin (400 μg/mL in 0.05 M Tris, 0.3 M NaCl, 0.02 M $CaCl_2$, pH 7.6 for 10 min, room temperature); (iii) microwave oven or pressure cooker in citrate buffer (100 mM); (iv) guanidine (8 M for 15 min, room temperature); (v) formic acid (70% for 15 min, room temperature); (vi) antigen retrieval solution; or (vii) proteinase K [10 μg/mL in PBS (pH 7.4), 40 min at 37°C].

3.3.2. Nitrotyrosine Antibody for Determination of Protein Tyrosine Nitration by Peroxynitrite—Affinity-purified rabbit antiserum or mouse monoclonal antibodies (7A2 or 1A6), raised to nitrated keyhole limpet hemocyanin (19), are used at a 1/100 dilution in 1% NGS, 150 mM NaCl, 50 mM Tris–HCl, pH 7.6.

Controls consist of the following: (i) omission of the primary antibody; (ii) absorption of the antibody with 50 μM nitrated BSA or 15 μM nitrated Gly-Tyr-Ala peptide at 4°C overnight prior to application to the section; and (iii) chemical reduction of nitrotyrosine by treating sections with 15 mM sodium hydrosulfite (dithionite) in 50 mM carbonate buffer, pH 8.0, for 15 min at room temperature (20) prior to immunostaining (*see* Note 6).

3.3.3. Antibodies to Advanced Glycation End Products—Polyclonal antibodies against pentosidine were raised in rabbits. Briefly, 250 μg of pentosidine–KLH conjugate prepared by the carbodiimide coupling technique (21) was emulsified with an equal volume of Freund's complete adjuvant and injected intradermally and intramuscularly into New

[5]By altering the parameters of metal chelation and/or rebinding, one can obtain valuable information regarding the avidity and affinity of various metal sequestration sites.

[6]These procedures are performed in parallel with the anti-sera to known markers as controls against artifacts of inactivation of either primary or secondary antibodies from the use of sodium hydrosulfite-reduced sections and against non-specific adsorption with nitrated BSA or nitrated Gly-Tyr-Ala.

Zealand Wistar rabbits. At 2–3 week intervals, booster injections of pentosidine–KLH emulsified in Freund's incomplete adjuvant were made. Serum was obtained 7–10 days post-injection and antibody titer assessed by ELISA. When antibody levels plateau, the rabbits were exsanguinated by cardiac puncture, and the collected serum was stored at –80°C until required. The production of monoclonal antibodies against pyrraline has been described previously (22).

Labeling was by the peroxidase–antiperoxidase technique described earlier. Adsorption experiments can be performed in parallel by incubation of primary anti-pentosidine or anti-pyrraline antibody with either free pentosidine or pyrraline hapten (10 nM) for 3 h at 37°C. Cross adsorption of pentosidine antisera with free pyrraline and pyrraline antibodies with free pentosidine was also performed as a control against artifactual adsorption. The preparation of pyrraline and pentosidine has been described previously (22, 23).

3.3.4. Antibodies to Lipid Peroxidation End Products—Two different antisera to HNE–pyrrole have been used, one raised to ON-KLH (24, 25), which generates as the major product the same 2-pentylpyrrole on lysine ε-amino groups formed (in low yield) upon direct exposure to HNE, but which also gives some side products. The other antiserum was raised to PPC-KLH, which generates the pure pyrrole epitope, but with a longer tether than when the pyrrole is formed from HNE (24). Thus, the coincidence of labeling with both antisera was deemed to be most convincing of specific immunocytochemical recognition of the HNE-derived pyrrole.

HNE pyrrole antisera were used at a dilution of 1:10–1,000. We found a 1:100 dilution to be optimal and this dilution was used throughout for immunostaining with the exception of the immunoadsorption experiment, see below. Immunostaining utilized the peroxidase–antiperoxidase procedure and was developed under a microscope by using 0.75 mg/mL DAB in 0.015% H_2O_2, 50 mM Tris–HCl, pH 7.6. The sections were dehydrated through ethanol and xylene solutions and then mounted in Permount (Fisher). The location of aberrant protein aggregates such as NFT and senile plaques can be determined following immunostaining by counterstaining the sections with Congo red and viewing them under crossed polarized light. Antisera specific to the protein constituents of aberrant aggregates can be used on adjacent sections in order to determine the microscopic localization of the lipoxidation-dependent adduct.

The specificity of both HNE–pyrrole antibodies was confirmed by omitting the primary antibody or by performing an adsorption. The two antisera to HNE–pyrrole were diluted at 1:150 and then incubated with 0.1 mg/mL of ON-BSA or PPC-BSA (9) for 16 h at 4°C prior to immunocytochemistry. The results of adsorbed immunostaining were compared with adjacent sections labeled with 1:150 dilution of unabsorbed antibody.

3.3.5. DNA Damage—Two mouse monoclonal antibodies to 8OHG have been used (4): anti-8OHG (1:250) and 1F7 (1:30; Trevigen) (26), which recognize RNA- as well as DNA-derived 8OHG. For 1F7, sections were treated with proteinase K prior to incubation with 10% NGS.

Immunostaining was developed by the peroxidase– antiperoxidase procedure (18) using 0.75 mg/mL DAB as co-substrate in 0.015% H_2O_2, 50 mM Tris–HCl, pH 7.6.

The specificity of both antibodies to 8OHG was confirmed by comparison with sections in which (i) the primary antibody was omitted or (ii) absorption with purified deoxyribo-8OHG (0.24 mg/mL) was compared to deoxyribo-G (4). Following the proteinase K treatment, additional sections were pretreated with DNase I (10 U/μL, PBS, 1 h at 37°C), S1 DNase (10 U/μL, PBS, 1 h at 37°C), or RNase (5 μg/μL, PBS, 1 h at 37°C) prior to incubation with 8OHG antibody.

3.3.6. Antibodies to the Cellular Response Factor, Heme Oxygenase-1—Rabbit antisera to HO-1 are available from Stressgen Biotechnologies Corporation. In addition, the MAP system described by Posnett et al. (27) was employed for the production of a rabbit antibody against a peptide corresponding to residues 1–30 from the sequence reported for rat HO-1 (28). Details of the preparation and characterization of the rabbit antibody to HO-1 peptide as well as its ability to react with human HO-1 have been described (29). Use of this antibody required a trypsin pretreatment of the section (see antigen retrieval on section 2.3) prior to the immunocytochemical protocol described above (*see* Note 7). Adsorption experiments were performed on anti-HO-1 to confirm the specificity of antibody binding. The immunostaining protocol was repeated, except here using adsorbed antisera in parallel. Adsorbed antisera were generated by incubation of primary antisera with purified HO-1 protein diluted to a final con-centration of 10 μg/mL for 3 h at 37°C.

3.3.7. Antibodies to the Cellular Response Factor, Iron-Response Element—Rabbit polyclonal antibodies to IRP-2 raised to a 75 amino acid peptide representing the N-terminal sequence unique to IRP-2 (30) or IRP-1 (31) can be used as previously described.

Adsorption experiments were performed with the antibody to IRP-2 to confirm the specificity of antibody binding (32). The immunostaining protocol was repeated, except here

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[7]For immunostaining with HO-1 antisera (Stressgen), we found that methacarn-fixed tissue was superior to formalin-fixed tissue, requiring shorter incubation times and lower titers of antisera.

General Note: In assessing markers of oxidative damage, there are a number of potential complications that investigators should be aware of and, if possible, control against processing artifacts since oxidative processes continue after death and therefore it is imperative to minimize the time before fixation. In this respect, it is important to realize that iron, a potent catalyst of oxidative chemistry, is frequently liberated following death. Additionally, one needs to pay particular attention to the fixation protocol employed since oxidation-related modifications could either be destroyed or created by the fixative. In this regard, we routinely use fixation in methacarn, which is relatively inert and appears to optimize labeling by immunocytochemical and histochemical techniques. The variability of oxidative changes also serves as an important control: by destroying or altering the oxidative modification with specific reagents, one can readily assert whether a particular technique is selective. Examples are reduction of free carbonyls with sodium borohydride, reduction of nitrotyrosine with sodium dithionite, and enzymatic removal of oxidized nucleic acids with DNase or RNase.

Finally, perhaps the most important aspect that one has to tackle is that oxidative modifications are fundamental aspects of both aging and disease. Indeed, cell death, both by necrosis and apoptosis, involves alterations in redox chemistry. Therefore, if one is looking at a pathological process, it is highly unlikely that one would not detect oxidative changes. In order to put such changes into context, it is important to understand the relevance of these changes with respect to other detrimental events. Thus, it is extremely important to empirically determine the conditions required for the detection of selective changes relative to the appropriate control. The definition of “selective” depends on the goal of the study. For example, if one is interested in observing immunocytochemical evidence for oxidative damage in a particular age-related neurodegenerative disease, then the antibody response should be titrated to a level such that age-matched control tissue exhibits changes at the background threshold level. On the other hand, for conclusions regarding the effect of aging itself, the immunocytochemistry should be titrated such that young controls exhibit only background levels. Overall, it is important that the researcher takes into consideration the various factors thought, at the time, to underlie the changes of interest, in order to permit an informed evaluation of the experimental observations. This will pave way for a proper perspective in evaluating whether oxidative damage represents a primary or a secondary phenomenon.

using adsorbed antisera in parallel. Adsorbed antiserum was generated by incubation of primary antisera with purified IRP-2 protein (30) diluted to a final concentration of 100 μg/mL overnight at 4°C. Adsorption of antibodies to tau with IRP-2 protein was also performed as a control against artifactual adsorption.

3.4. Application of In Situ Methods to AD Tissue Sections to Visualize Senile Plaques and NFT

The methods outlined in this chapter have been applied in an effort to localize markers of oxidative damage in AD. We and others (11, 33, 34) determined that advanced glycation end product (AGE) modifications are present in both NFT and senile plaques in AD. These studies employed not only antibodies to the specific structures pentosidine and pyrraline but also less defined AGE antibodies raised to carrier proteins treated with reducing sugars for long periods of time. AGE modification is likely an early in vivo event since both diffuse senile plaques (11) and paired helical filaments (33, 34), considered two of the earliest pathological changes in AD (35, 36), were stained by the anti-AGE antibodies.

Immunostaining using antibodies to lipoxidation-derived modifications has exhibited more distinctive patterns. Whereas some antibodies (e.g., to MDA adducts (33) or to ill-defined HNE adducts) display variable staining of both NFT and senile plaques, antibodies to characterized HNE adducts are exclusively localized to neuronal cell bodies and neurofibrillary pathology (9, 25). In fact, antibodies to the HNE–lysine-derived pyrrole, an advanced lipoxidation end product (ALE), were found to stain not only intraneuronal and extraneuronal NFT but also apparently normal hippocampal neurons in AD but not in controls (9). Counterstaining with Congo red on the same sections or antisera to tau (37) on the adjacent serial sections was used to assess relationships to NFT. The same profile of staining seen for HNE–pyrrole was seen by using antibodies to markers of direct protein oxidation, including nitrotyrosine and protein-based carbonyls (5, 6, 13). Again, the heightened sensitivity of immunochemical follow-up subsequent to derivatization of protein-based carbonyls with 2,4-dinitrophenylhydrazine permitted the in situ detection of carbonyl reactivity not only within NFT but also within vulnerable neurons in AD (5, 13). Two key objectives in the development of multiple in situ immunochemical markers for oxidative stress damage are the following: (i) to obtain clues to the temporal aspects of pathogenesis and (ii) to help track down the sources of oxidative damage. In the former case, it is of interest to determine whether, and at what time point, there is a biochemical response to oxidative damage revealed by induction of stress proteins such as HO-1. We found that HO-1 is associated with neurofibrillary pathology (38) at the same (early) stage of degeneration as is revealed by the antibody (Alz50) to abnormal conformation of tau. As far as sources of oxidative stress are concerned, the modified Perl's stain for protein-bound iron showed iron accumulation in both NFT and senile plaques. In the absence of the formation of the mixed valence iron complex through employment of hexacyanoiron (II/III), direct utilization of the H_2O_2-dependent DAB oxidation protocol localized endogenously bound redox-active transition metals at the same sites on NFT and senile plaques. Overall, the panel of in situ methods for localizing oxidative stress damage in AD is permitting an assessment of the spatio-temporal aspects to neuronal degeneration that define the disease process. One question that remains to be answered has to do with better characterization of

the cellular response to AD lesions since certain oxidative stress markers show up only in neurofibrillary pathology, whereas others are associated with both NFT and senile plaques.

3.5. Quantification and Statistical Analyses

The intensity of immunoreaction on tissue sections can be evaluated by measuring the optical density with a suitable imaging system (4, 39) (e.g., Zeiss Axiocam and Axiovision software). All measurements are done under the same optical and light conditions as well as using an electronic shading correction to compensate for any un-evenness that might be present in the illumination. Statistical analysis for the differences in the corrected OD value among subgroups is by the analysis of variance (ANOVA) using StatView program (Abacus Concepts, Inc., Berkeley, CA). Fisher's protected least significant difference can be applied in the post hoc analysis.

Acknowledgments

Work in the authors' laboratories is supported by the National Institutes of Health, the Alzheimer's Association, and Philip Morris USA and Philip Morris International.

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