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Oxidative damage in age-related macular degeneration

J.K. Shen[1,2], A. Dong[1,2], S.F. Hackett[1,2], W.R. Bell[1], W.R. Green[1] and P.A. Campochiaro[1,2]

The Departments of [1]Ophthalmology and [2]Neuroscience,

The Johns Hopkins University School of Medicine Maumenee, Baltimore, Maryland, USA

Summary. Epidemiologic studies have suggested that elderly patients who consumed diets rich in antioxidants throughout their lives are less likely to be afflicted with age-related macular degeneration (AMD). This led to the Age-Related Eye Disease Study, which showed that supplements containing antioxidant vitamins and zinc reduce the risk of progression to severe stages of AMD. Despite these data that indirectly implicate oxidative damage in the pathogenesis of AMD, there has not been any direct demonstration of increased oxidative damage in the retinas of patients with AMD. In this study, we used biomarkers of oxidative damage in postmortem eyes from patients with AMD and comparably aged patients without AMD to directly assess for oxidative damage. Sections from 4 eyes with no pathologic features of AMD showed no immunofluorescent staining for markers of oxidative damage, while sections from 8 of 12 eyes with advanced geographic atrophy showed evidence of widespread oxidative damage in both posterior and anterior retina. Only 2 of 8 eyes with choroidal neovascularization and 2 of 16 eyes with diffuse drusen and no other signs of AMD showed evidence of oxidative damage. These data suggest that widespread oxidative damage occurs in the retina of some patients with AMD and is more likely to be seen in patients with advanced geographic atrophy. This does not rule out oxidative damage as a pathogenic mechanism in patients with CNV, but suggests that a subpopulation of patients with geographic atrophy may have a major deficiency in the oxidative defense system that puts the majority of cells in the retina at risk for oxidative damage.

Key words: Acrolein, Choroidal neovascularization, Geographic atrophy, Reactive oxygen species

Offprint requests to: Peter A. Campochiaro, M.D., Maumenee 719, The Johns Hopkins University School of Medicine, 600 N. Wolfe Street, Baltimore, MD 21287-9277, USA. e-mail: pcampo@jhmi.edu

Introduction

Oxidative damage has been implicated in aging and in several neurodegenerative diseases (Beal, 2002). Some cases of familial amyotrophic lateral sclerosis (FALS) occur due to mutations in superoxide dismutase 1 (SOD1) that alter SOD1 in a way that causes it to generate reactive oxygen species (ROS) resulting in enhanced protein nitrosylation (Rosen et al., 1993; Shefner et al., 1999). Degeneration of cortical neurons in patients with Alzheimer's disease has been linked to cleavage products of the ß-amyloid precursor protein, including amyloid ß-protein. One potential toxic effect of amyloid ß-protein is generation of ROS (Iadecola et al., 1999). Patients with Parkinson's disease have selective degeneration of neurons in the substantia nigra with reduced levels of glutathione peroxidase in the substantia nigra, but not in other regions of the brain, suggesting localized oxidative damage (Alam et al., 1997; Bowling and Beal, 1995; Yoritaka et al., 1996; Selley, 1998; Zhang et al., 1999). Mitochondrial complex 1 inhibitors, such as 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) and rotenone, uncouple oxidative phosphorylation, generate ROS, and induce a Parkinson disease-like syndrome in humans, monkeys, mice, and rats (Lai et al., 1993; Betarbet et al., 2000). Metabolites of these agents are mitochondrial uncouplers that generate large amounts of ROS. Therefore, oxidative damage may be an important pathogenic feature of several neurodegenerative diseases.

Oxidative damage results in adducts on macromolecules that act like fingerprints at a crime scene indicating that oxidative damage has occurred. These biologic markers of oxidative damage include 3-nitrotyrosine residues on proteins (Beal, 2002), acrolein and 4-hydroxynonenal (HNE) adducts on lipids (Benedetti et al., 1980; Esterbauer et al., 1991), and 8-hydroxy-2'-deoxyguanosine (8-OHdG) modifications in DNA (Toyokuni et al., 1997, 1999). When a tissue shows immunostaining with antibodies that specifically recognize these adducts, it means that the tissue has undergone oxidative damage. Post mortem spinal cord, substantia nigra, and cortex from patients with FALS,

Parkinson's Disease, and Alzeheimer's Disease, respectively show specific staining for these biomarkers (Yoritaka et al., 1996; Ando et al., 1998; Calingasan et al., 1999).

Age-related macular degeneration is a neurodegenerative disease of the retina. It has been suspected for quite some time that oxidative damage may play a role in the pathogenesis of AMD (for reviews see (Winkler et al., 1999; Beatty et al., 2000)). This hypothesis is strongly supported by results of the Age-Related Eye Disease Study (AREDS) which demonstrated that administration of antioxidant vitamins and/or Zn, significantly slows the rate of progression from high risk non-neovascular AMD to neovascular AMD (Group, 2001). Another compelling piece of evidence stems from mass spectroscopy of drusen from postmortem eyes of patients with AMD, which shows many oxidized proteins (Crabb et al., 2002).

In this study, we explored the hypothesis that oxidative damage plays a role in age-related macular degeneration by evaluating postmortem eyes from patients with AMD for the presence of biomarkers for oxidative damage.

Materials and methods

Postmortem eyes

Most of the specimens used in this study were derived from eyes submitted to the Ocular Pathology Laboratory of the Wilmer Eye Institute. A pathologic diagnosis was assigned after detailed examination by one of the authors (WRG). Paraffin sections from eyes with the following pathologic diagnoses were studied: early non-neovascular AMD with diffuse drusen (n=16), advanced AMD with geographic atrophy (n=11), advanced AMD with choroidal neovascularization (CNV, n=8), or eyes with no pathologic changes from elderly individuals (n=11). One eye was obtained through the National Disease Research Interchange (NDRI, Philadelphia, PA) and was processed for frozen sections. The eye had drusen and atrophy of the retina, RPE and choroicapillaris centrally and was assigned a diagnosis of advanced geographic atrophy.

Immunohistochemical staining

Indirect immunofluorescence staining was done using antibodies specific for biomarkers of oxidative damage, including rabbit anti-HNE (1:100, Alpha Diagnostics International, Inc., San Antonio, TX), rabbit anti-nitrotyrosine IgG (1:300, Upstate Biotechnologies, Lake Placid, NY), goat anti-8-OHdG (1:150, Chemicon, Temecula, CA), and rabbit anti-acrolein (1:150, Cell Sciences, Canton, MA). A protein-acrolein conjugate (Cell Sciences) was also obtained for blocking studies. These antibodies are well-characterized and have proven useful for specific recognition of the respective markers for oxidative damage in previous studies (Kooy et al., 1995; Nyhlin et al., 2002; Cauwelss et al., 2003; Kono et al., 2003; Tuder et al., 2003; Isayama et al., 2004; Serviddio et al., 2004; Uchida et al., 2004; Usuki et al., 2004; Van Everbroeck et al., 2004) De-paraffinized sections or frozen sections were dried at room temperature and post-fixed in cold acetone for 15 minutes. Sections were washed with phosphate-buffered saline (PBS), and antigen retrieval was done by covering sections with 0.02 M citrate buffer, pH 6.0 and heating them in a microwave oven for 3 minutes. After cooling for 3 minutes, additional buffer was added and the slides were reheated; this process was repeated until 4 cycles of heating/cooling had been performed. Slides were cooled to room temperature for 20 minutes, rinsed with PBS, and blocked with 7% normal goat serum in PBS for 30 minutes at room temperature. The sections were incubated overnight at 4°C with one of the above primary antibodies diluted with PBS and containing 3% goat serum. After washing 3 times with PBS/0.05% Tween-20, sections were incubated for 1 hour at room temperature with a fluorescently labeled secondary antibody: (1) slides first incubated in goat anti-8-OHdG were incubated in 1:120 donkey anti-goat IgG conjugated with FITC (Jackson ImmunoResearch Lab, West Grove, PA), (2) slides first incubated in rabbit anti-nitrotyrosine were incubated in 1:1200 goat anti-rabbit IgG conjugated with Cy3 (Jackson ImmunoResearch Lab), and (3) slides first incubated in rabbit anti-acrolein or anti-HNE were incubated in 1:150 goat anti-rabbit IgG conjugated with FITC (Jackson ImmunoResearch Lab). Sections were thoroughly washed with PBS/Tween-20 and counterstained for 3 minutes at room temperature with the nuclear dye Hoechst 33258 (1:1,200; Sigma, St. Louis, MO). Slides were mounted with Aquamount solution and viewed with a Nikon Fluorescence Microscope (Nikon Instruments Inc., NY, USA). Using the same exposure time for each section, images were captured using a Nikon digital camera and SPOT RT 3.4 software. Pictures were obtained by merging an image of a Hoechst-stained slide and the fluorescent image from the same field. The following controls were performed: (1) autofluorescence controls in which sections were not stained with primary or secondary antibodies and were examined using either the green or red channel of the fluorescence microscope, (2) controls in which primary antibody was eliminated and sections were incubated with one of the secondary antibodies, and (3) blocking controls in which the anti-acrolein antibody was incubated with a 3M excess of acrolein-protein conjugate for 1 hour prior to incubation with sections.

Results

Autofluorescence in ocular sections

Unstained sections from all eyes were examined by fluorescence microscopy to identify autofluorescence. Most eyes showed autofluorescence in the RPE, which is

commonly seen in eyes from normal adults due to accumulation of lipofuscin. Choroidal neovascularization sometimes showed autofluorescence easily visualized with settings optimal for viewing fluorescein (green, Fig. 1A) and less conspicuous when viewed with settings optimal for viewing rhodamine (red, Fig. 1B). In contrast, many drusen showed faint autofluorescence when viewed with the green channel (Fig. 1C), but

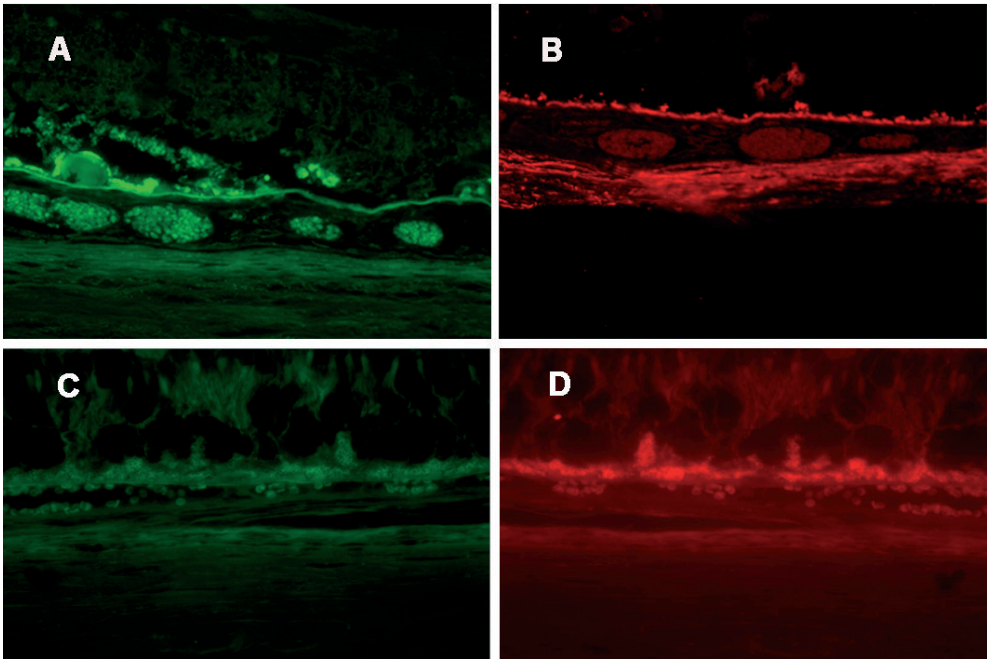

Fig. 1. Autofluorescence in choroidal neovascularization and drusen. Some areas of choroidal neovascularization showed autofluorescence that was most prominently seen with the green channel **(A)** and was less when viewed with the red channel **(B)**. Drusen often showed faint autofluorescence when viewed with the green channel **(C)** that was substantially stronger when viewed with the red channel **(D)**. x 400

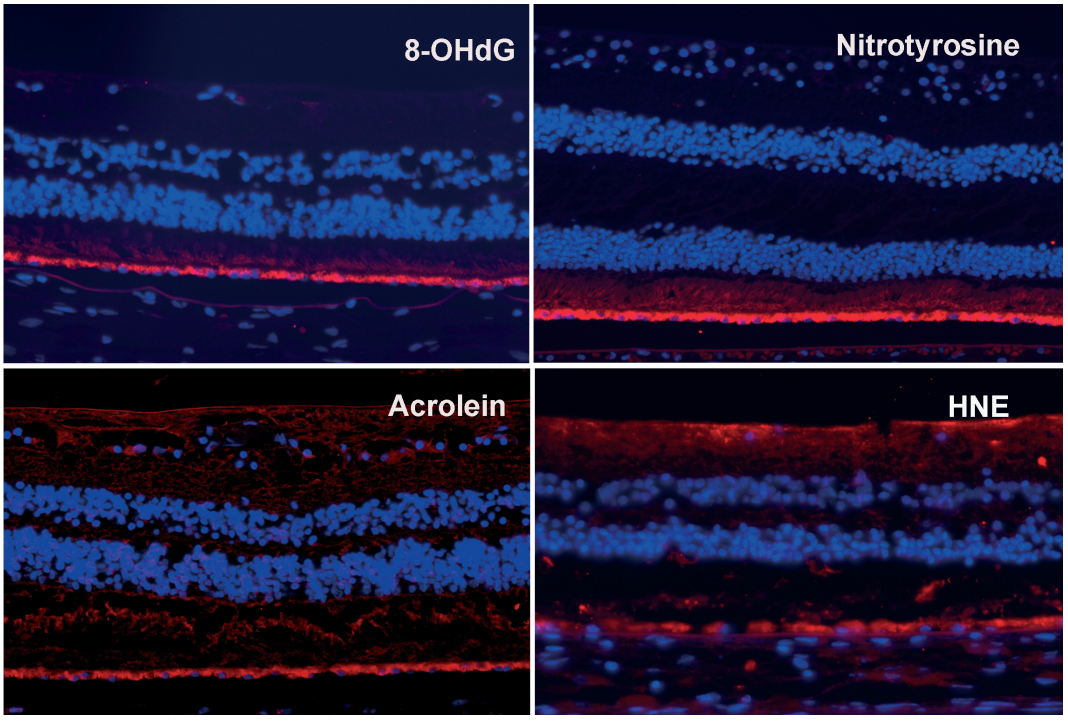

Fig. 2. Retinas from elderly patients without pathologic evidence of age-related macular degeneration show only mild evidence of oxidative damage in the retina. Ocular sections were immunohistochemically stained with antibodies specific for 8-hydroxy-deoxyguanosine (8-OHdG), nitrotyrosine, acrolein, or 4-hydroxynonenal (HNE) and secondary antibodies coupled to rhodamine (**A-D**, red). Nuclei were stained with Hoechst (blue). Autofluorescence in the retinal pigmented epithelium (RPE) is detected with the red channel and accounts for the signal in the RPE in A-D. **A.** Mid-peripheral retina from a 70 year old African-American male shows only faint background staining for 8-OHdG. **B.** Posterior retina from an 80 year old Caucasian female shows only faint staining in outer and inner segments of photoreceptors for nitrotyrosine. **C.** Posterior retina from the same patient shown in B stains faintly for acrolein in photoreceptor inner segments and along the inner surface of the retina. **D.** Midperipheral retina from an 89 year old Caucasian male shows some mild staining along the inner surface of the retina and in occasional inner or outer segments of photoreceptors. x 300

fluoresced strongly when viewed with the red channel (Fig. 1D), which was also true for the RPE (B and D).

Eyes from elderly patients with no sign of AMD do not show oxidative damage in the retina

Advanced age is a strong risk factor for AMD and oxidative damage is part of the aging process. Eyes from patients who died at an advanced age, but showed no drusen or other pathologic evidence of AMD were stained with antibodies specific for acrolein or HNE, markers of lipid peroxidation, nitrotyrosine, a marker of oxidation of proteins, and 8-OHdG, an indicator of oxidative damage to DNA. Ten of eleven of these

Table 1. Staining for markers of oxidative damage.

	n	Age	SD	ONL	INL	GCL
No signs of AMD	11	87.1	4.8	1/11	0/11	0/11
Soft or diffuse drusen	16	77.9	13.5	2/16	2/16	0/16
Geographic atrophy	12	81.3	10.3	7/12	8/12	1/12
CNV	8	78.4	11.2	0/8	0/8	2/8

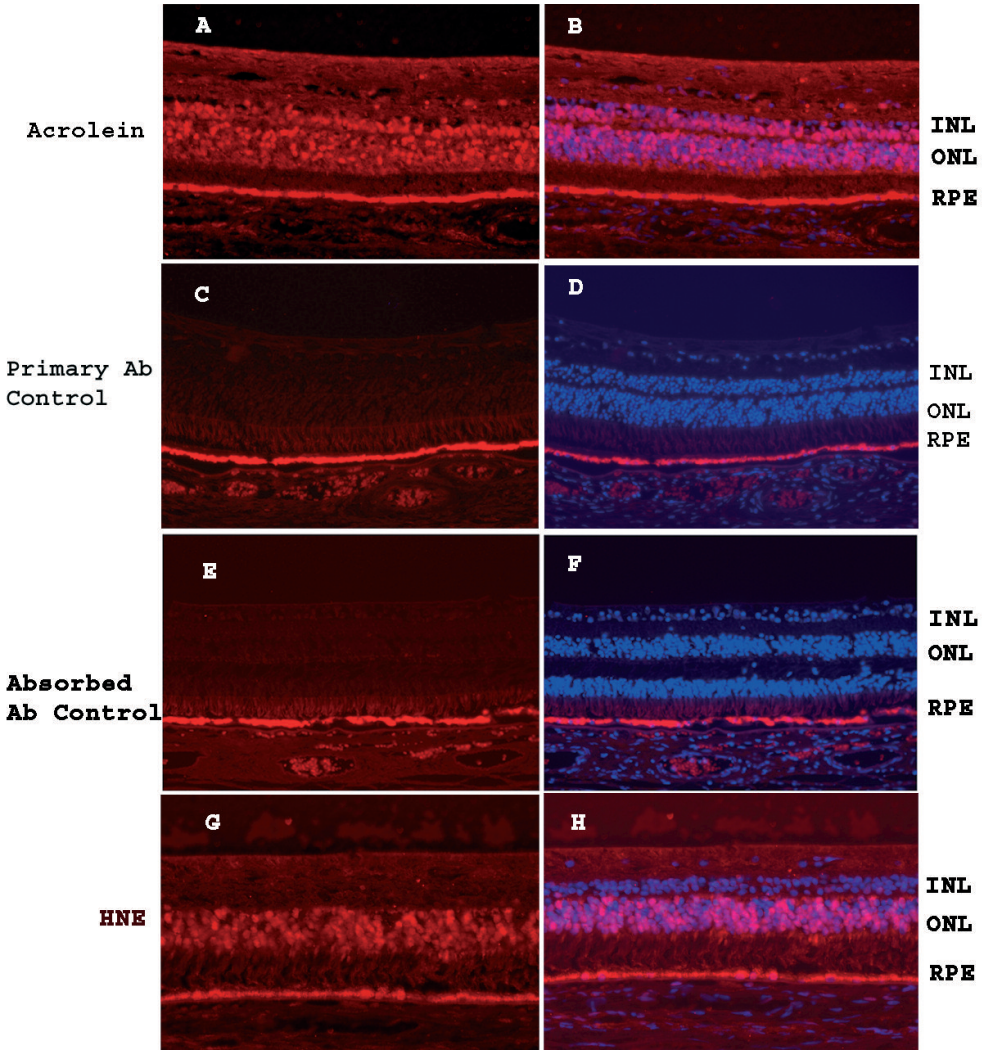

Fig. 3. Prominent lipid peroxidation in the retina from a patient with geographic atrophy. Ocular sections from a 97 year old Caucasian male with advanced geographic atrophy were stained with a primary antibody directed against acrolein or 4-hydroxynonenal (HNE) and a secondary antibody coupled to rhodamine (red) or nuclei were stained with Hoechst. Merged Hoechst-stained and immunohistochemically-stained images are shown in the right column. **A and B.** A section from the peripheral retina shows intense staining for acrolein in the majority of cells in the inner nuclear layer (INL) and outer nuclear layer (ONL). There is also staining in the inner plexiform layer and along the surface of the retina. The red stain in the retinal pigmented epithelium (RPE) is due to autofluorescence. **C and D.** A section adjacent to the one shown in A and B was stained using the same procedure except that the primary antibody was eliminated. The red stain in the RPE is still present, because it is due to autofluorescence and the faint stain in the photoreceptor inner and outer segments is nonspecific, but the staining in the remainder of the retina has disappeared indicating that it is specific. **E and F.** Another adjacent section was stained with anti-acrolein antibody after it had been preabsorbed with acrolein. The staining in the ONL, INL and the remainder of the inner retina seen in A and B was eliminated confirming its specificity. **G and H.** Another section from the peripheral retina was stained with an antibody directed against HNE, a different adduct that occurs from peroxidation of lipids. The pattern was different from that seen with anti-acrolein. There was intense staining of most of the cells in the ONL, but little staining in the INL or elsewhere in the retina. x 200

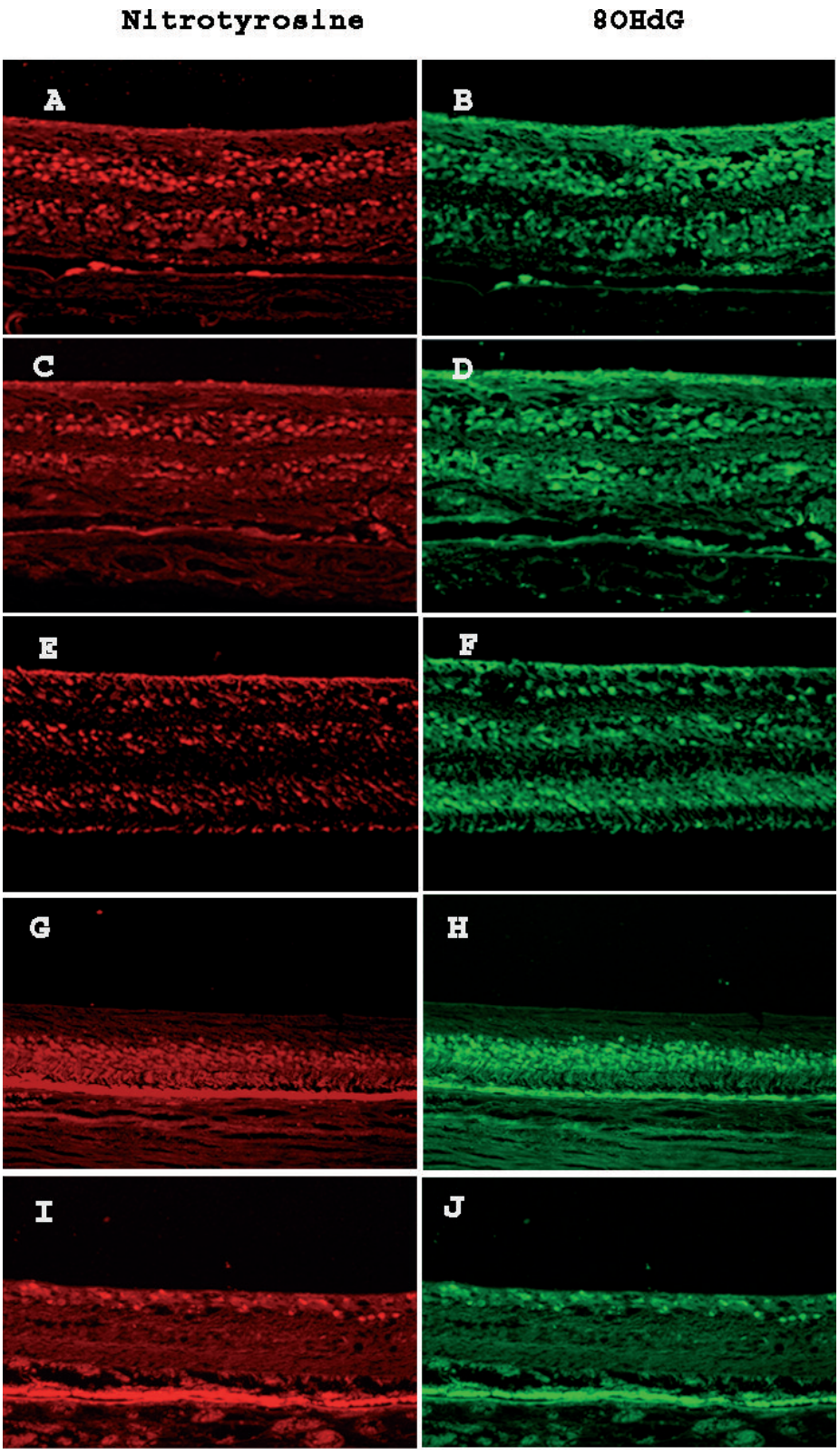

Fig. 4. Oxidation of proteins and DNA in retinas from patients with widespread diffuse drusen (**A-F**), geographic atrophy (**G, H**), or choroidal neovascularization (**I, J**). Ocular sections from patients with diffuse drusen were stained with antibodies directed against nitrotyrosine (rhodamine-labeled secondary antibody, red) or 8-hydroxy-deoxyguanosine (8-OHdG, fluorescein-labeled secondary antibody, green). **A and B.** A section through the parafoveal retina of a 96 year old Caucasian female shows widespread staining for nitrotyrosine (**A**) and 8OHdG (**B**) throughout the inner and outer retina. **C and D.** A section through retina in the posterior pole, but well outside the fovea of the same patient shown in A and B also shows widespread staining for nitrotyrosine (**C**) and 8OHdG (**D**) throughout the inner and outer retina. **E and F.** A section through the midperipheral retina of the patient shown in A and B shows staining for nitrotyrosine **(E)** and 8-OHdG **(F)** in both the inner and outer retina, but the percentage of stained cells appears slightly less than that seen in the sections from the parafoveal retina (**A and B**). **G and H.** A section from the peripheral retina of a 76 year old Caucasian female with geographic atrophy shows intense staining for nitrotyrosine in photoreceptor cell bodies, inner, and outer segments (G). There was intense staining for 8OHdG in the photoreceptor cell bodies, but not the inner and outer segments. **I and J.** A section through the peripheral retina of a 63 year old Caucasian female with choroidal neovascularization showed strong staining for nitrotyrosine (**I**) and 8-OHdG (**J**) in ganglion cells and along the inner surface of the retina, but not in the outer retina. x 200

control patients showed no staining for any of the markers and one patient showed staining for nitrotyrosine in cones. Figure 2 shows representative ocular sections from 3 elderly patients who lacked signs of AMD and they show minimal staining for the 4 markers. There is autofluorescence in the RPE and mild staining for HNE along the inner surface of the retina (D), but otherwise the retinas are remarkable for lack of fluorescence.

Lipid peroxidation in retinas of patients with AMD

Figure 3A,B show strong staining for acrolein in most of the cells in the inner and outer nuclear layer of the midperipheral region of the retina of a patient with advanced geographic atrophy. There was also staining in the inner plexiform layer and along the surface of the retina. Staining using the secondary antibody without the primary antibody (Fig. 3C,D) or using primary antibody that had been pre-absorbed with acrolein (Fig. 3E,F) showed that all of the retinal staining was specific except the faint staining in photoreceptor inner and outer segments. The signal in the RPE was similar in these controls and in stained sections confirming that it represents autofluorescence. Other sections from the peripheral or posterior retina of the same patient with geographic atrophy showed strong staining for HNE the majority of cells in the outer nuclear layer, but not the inner nuclear layer and staining along the surface of the retina was very light (Fig. 3G,H). Thus, this patient with geographic atrophy showed widespread lipid peroxidation even in the peripheral retina with acrolein adducts throughout the inner and outer retina and HNE adducts primarily in the outer nuclear layer.

Oxidation of proteins and DNA in the retinas of patients with AMD

Figure 4 A-F shows staining for nitrotyrosine and 8-OHdG in different regions of the retina of a patient with diffuse drusen. There is widespread staining for both of these markers of oxidative damage in all layers of the retina in the parafoveal retina (A and B), other regions of the posterior retina (C and D), and peripheral retina (E and F). There was no staining when primary antibodies were eliminated. In patients with advanced geographic atrophy, their was increased staining in the posterior retina, but the severe disruption of the tissue makes interpretation somewhat difficult. However, the peripheral retina is intact and shows prominent staining for both nitrotyrosine and 8-OHdG; the peripheral retina of the patient with geographic atrophy shown in Figure 3G and H has staining for both markers confined to the outer nuclear layer. In contrast, a section through the peripheral retina of a patient with choroidal neovascularization showed strong staining for nitrotyrosine (Fig. 3I) and 8-OHdG (J) in ganglion cells and along the inner surface of the retina, but not in the outer retina.

Frequency and localization of oxidative damage in the retinas of patients with AMD

With the observer masked with respect to pathologic grading, ocular sections were graded as to the presence or absence of unequivocal staining for any of the markers of oxidative damage in each of the 3 cellular layers of the retina. Sections from 10 individuals who had no pathologic signs of AMD showed no staining in the ONL, INL, or GCL (Table 1). One age-matched control had staining for nitrotyrosine only in cone photoreceptors. Only 2 of 16 eyes with soft drusen, but no other signs of AMD, showed staining for markers of oxidative damage in the ONL and INL. Oxidative damage was more frequent in eyes with geographic atrophy, occurring in 8 of 12 eyes with that diagnosis, and in all but one in which staining was limited to the ONL (Fig. 4), the staining occurred in both the INL and

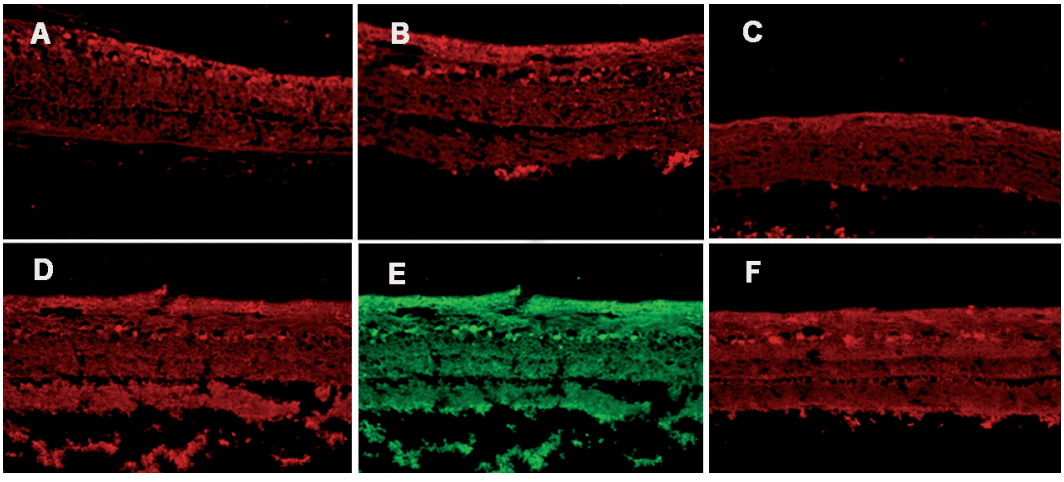

Fig. 5. Oxidative damage in the retinas of patients with choroidal neovascularization. The retina from a 89 year old Caucasian male with choroidal neovascularization shows staining for 4-hydroxynonenal in the ganglion cell layer (GCL) and along the inner surface of the retina in the parafoveal area (**A**) and other parts of the posterior pole (**B**), but minimal staining above background in the peripheral retina (**C**). There was also staining for nitrotyrosine (**D**), 8-hydroxy-deoxyguanosine (**E**), and acrolein (**F**) in the GCL of the posterior retina. x 200

ONL. In one eye, all 3 cellular layers of the retina showed staining. Oxidative damage was less prominent in eyes with choroidal neovascularization, occurring in 2 of 8 eyes with that diagnosis. In both cases the staining occurred in the GCL, but not the INL nor ONL (Fig. 5).

Discussion

Oxidative damage has been suggested to play a role in the pathogenesis of AMD. In this study, we tested that hypothesis by performing immunofluorescent staining for markers of oxidative damage in the retinas of patients with pathologically characterized AMD and comparably aged individuals with no signs of AMD. Ten individuals that lacked evidence of AMD also lacked evidence of oxidative damage and one showed some evidence of protein oxidation only in cones. In contrast, 8 of 12 patients with geographic atrophy showed widespread oxidative damage in the retina, primarily in the INL and ONL. A much lower percentage of patients with CNV or only drusen showed oxidative damage in the retina.

These data are consistent with the hypothesis that oxidative damage plays a role in the pathogenesis of AMD, but suggest that it may play a very prominent role in some patients and a less prominent role in others. AMD is a multigenic disease and gene defects that make patients more susceptible to oxidative damage in the retina may constitute one group of conditions that we lump together as AMD. Patients with this biochemical phenotype appear prone to develop the pathologic phenotype of advanced geographic atrophy. None of the patients with CNV showed widespread oxidative damage, but 2 of 8 showed oxidative damage in ganglion cells. Only 2 of 16 patients with diffuse drusen showed extensive oxidative damage in the retina; it is reasonable to hypothesize that these patients would have developed advanced geographic atrophy had they lived longer, but there is no way to test the hypothesis.

Although we did not see widespread oxidative damage in the retinas of patients with CNV, we cannot rule out a role for oxidative damage in such patients. Autofluorescence in RPE cells and drusen prevent assessing oxidative damage in those locations by immunofluorescent staining for markers of oxidative damage. Other techniques, such as proteomics, suggest that oxidized proteins occur in drusen and RPE (Crabb et al., 2002), so it is likely that genetic defects that result in susceptibility to oxidative damage in the RPE, but not generalized damage throughout the retina, may also result in a phenotype that we recognize as AMD. Furthermore, the AREDS study showed that patients with high risk characteristics treated with antioxidants and zinc had a lower incidence of conversion to neovascular AMD, directly implicating oxidative damage in the development of CNV. Therefore, oxidative damage may contribute to the different phenotypes that we lump together as AMD in several different ways. As we gain more understanding regarding how particular gene defects make individuals more susceptible to developing AMD, we may identify patients most likely to benefit from antioxidant therapy. When such patients are identified, more effective, albeit more invasive approaches, such as bolstering the endogenous antioxidant defense system with gene therapy may be considered. In the mean time, noninvasive approaches, such as diets rich in natural antioxidants should continue to be recommended in all patients with AMD.

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