Topographic and Age-Dependent Expression of Heme Oxygenase-1 and Catalase in the Human Retinal Pigment Epithelium

Noriitake Miyamura, Tsukibiko Ogawa, Sharon Boylan, Lawrence S. Morse, James T. Handa, and Leonard M. Hjelmeland

PURPOSE. To investigate the hypothesis that there are topographic and age-related changes in the expression of heme oxygenase (HO-1) and catalase in the RPE.

METHODS. Cryosections of the macula and periphery of human eyes (n = 18; aged 27–87 years) were subjected to a high-sensitivity digoxigenin (DIG)-labeled cRNA in situ hybridization protocol to determine the expression of HO-1 and catalase. The immunoreactivity of HO-1 and catalase were also investigated in the same sample set. Specimens were examined by light microscopy, and images were captured with a digital camera. The total number of RPE cells and HO-1- and catalase-labeled RPE cells was counted in each section, and the ratio of labeled RPE cells to total RPE cells was calculated in both the macular and the peripheral regions of each donor eye.

RESULTS. There was a mosaic pattern of mRNA and protein expression of HO-1 and catalase in macular and peripheral RPE. Topographical differences in the expression of HO-1 at the mRNA level and catalase at both the mRNA and protein levels was also observed. The topographical differences between the expression of HO-1 in the macula and periphery protein were not statistically significant but showed similar trends. For HO-1, the only significant age-related decline in expression was observed in the macula and periphery. Expression of HO-1 at the protein level and that of catalase at both the mRNA and protein levels showed no significant decline with age.

CONCLUSIONS. There is a possible age-related decline in HO-1 expression, whereas catalase expression remains unchanged with aging. Both exhibit mosaic patterns in the RPE monolayer.

D escreased resistance to oxidative stress caused by a decline in the antioxidant defense system has been implicated in oxidation-related aging and possibly age-related macular degeneration (AMD).1–3 Two antioxidant enzymes have been proposed to have an age-related reduction in protein expression or enzyme activity. Frank et al.4 demonstrated, by quantitative electron immunocytochemistry, decreased immunoreactivity of catalase and heme oxygenase (HO-1) in RPE cell cytoplasm and lysosomes with age and in AMD. HO-1 is a heme-degradation enzyme that cleaves heme to biliverdin, which is then converted to the antioxidants bilirubin and carbon monoxide.5 Catalase was shown by Liles et al.6 to have decreased enzymatic activity with age and age-related disease in whole-cell preparations of the macular RPE. Frank et al.7 also showed an age-dependent decrease in immunoreactivity of catalase. Catalase, an enzyme with high levels of expression in the RPE, converts hydrogen peroxide to water and oxygen.7,8 The enzymatic activity of superoxide dismutase, however, did not change with respect to age or topography.6

These two studies differ in the methods used for quantitative analysis of age and topographic changes. In the study by Frank et al.,4 gold particles were counted by electron microscopy to quantify the extent of immunolabeling within individual cells. In the study by Liles et al.6 whole populations of cells from either the macula or periphery were assayed to determine enzymatic activity. One of the implicit assumptions made by both of these approaches is a uniform phenotype in all RPE cells with respect to the expression and function of these enzymes. Burke et al.9,10 however, showed that bovine RPE cells in situ have a nonuniform or mosaic pattern of cytoskeletal expression. Our laboratory has recently analyzed the expression of insulin-like growth factor binding protein-2 at the mRNA and protein levels for age and topographic variation.11 In the current study, we used a cell-counting method that takes into account the possibility of mosaic patterns of gene and protein expression. The purpose of this study was to address the hypothesis that the expression patterns of HO-1 and catalase are mosaic in the RPE, and these expression patterns exhibit age-related and topographic variation.

METHODS

Tissue Processing

Fresh globes from 11 male and 7 female donors, ranging in age from 27 to 87 years at the time of death, were obtained from the Sierra Tissue Eye Bank (Sacramento, CA) within 35 hours of death. The protocol for obtaining the donor eyes complied with the provisions of the Declaration of Helsinki for research involving human tissue. Table 1 outlines the age, sex, postmortem time, past systemic diseases, and past ophthalmic diseases of each donor. Inspection with a dissection microscope did not reveal any obvious posterior segment disease. The globes were fixed in phosphate-buffered saline (PBS; pH 7.4) containing 4% paraformaldehyde at 4°C. Using microscopic guidance, the anterior segment was removed, and the posterior segment was divided into five sections (6 × 6 mm): nasal (nasal side of disc), superior (superior to arcade vessels), inferior (inferior to arcade vessels), macular (centered around the foveola), and temporal (temporally outside of macula to equator). The tissue was cryoprotected using the technique of Barthel and Raymond12 and Mishima et al.13 All tissue blocks were stored at −80°C until used. Cryosections (10 μm) were cut with a cryotome (model CM3050; Leica Microsystems Inc., Bannockburn, IL), mounted on coated glass slides (Vectabond; Vector Laboratories, Burlingame, CA), and air dried at room temperature for 4 hours.

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**TABLE 1. Case Data**

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HT, hypertension; HD, heart disease; DM, diabetes mellitus; CHF, chronic heart failure; RF, renal failure.

**In Situ Hybridization**

The plasmid containing cDNA of human HO-1 was kindly provided by Lee-Young Chau and the plasmid containing the cDNA of human catalase was purchased from ATCC (Manassas, VA). The HO-1 cDNA was 335 bp and inserted into a vector (pCR II; Invitrogen, Carlsbad, CA). The catalase cDNA was inserted into a cloning vector (pBluescript II SK(+); Stratagene, La Jolla, CA) after digestion with XhoI (Promega, Madison, WI) and ApaI (Promega) to 450 bp. The antisense and sense digoxigenin (DIG)-labeled RNA riboprobes were synthesized according to the labeling protocol in a kit (DIG Labeling Kit; Roche, Indianapolis, IN). The length and integrity of the synthesized riboprobes were quantified by gel electrophoresis. The concentrations were estimated by the dot-spot test, using DIG-labeled control RNA.

In situ hybridization histochemistry was performed according to Braissant and Wahl with slight modification. After postfixation in 4% paraformaldehyde-PBS for 10 minutes, sections were immersed in 0.25% acetic anhydride for 10 minutes and 5× SSC for 15 minutes. Prehybridization was performed at 55°C (for HO-1) or 55°C (for catalase) for 2 hours in the hybridization mixture (50% formamide, 5× SSC, 40 μg/ml salmon sperm DNA). After denaturing the probes for 5 minutes at 80°C, hybridization was performed at 55°C (for HO-1) or 55°C (for catalase) for 40 hours with a cover (Parafilm; American Can Company, Greenwich, CT) in a chamber saturated with the hybridization mixture in a hybridization incubator (Fisher Scientific, Pittsburgh, PA). Sections were washed and equilibrated in 100 mM Tris, 150 mM NaCl, and 50 mM MgCl₂ (pH 7.5) for 5 minutes and incubated with a 0.5% DIG-blocking reagent (Roche), containing 100 mM Tris, 150 mM NaCl, and 50 mM MgCl₂ (pH 7.5), for 60 minutes. The sections were incubated with alkaline phosphatase-coupled anti-DIG antibody (Roche) diluted 1:5000 in the 0.5% DIG-blocking reagent (Roche) at room temperature for 2 hours. The sections were washed in 100 mM Tris, 100 mM NaCl, and 50 mM MgCl₂ (pH 9.5), for 5 minutes. Color was developed at room temperature with 0.0175% 5-bromo-4-chloro-3-indolyl-phosphate (BCIP; Roche), 0.045% nitroblue tetrazolium chloride (NBT; Roche), 100 mM NaCl, and 50 mM MgCl₂ (pH 9.5). Staining was stopped by TE buffer (pH 8.0) for 15 minutes. Nonspecific background staining was removed in 95% EtOH for 1 hour. Selected sections were bleached with potassium permanganate, as previously described. Sections were counterstained with nuclear fast red (Vector Laboratories), dehydrated, and mounted.

**Immunohistochemistry**

Immunohistochemistry was performed according to a previously published technique. After blocking with 3% goat serum and 3% blocking solution (Blotto; Santa Cruz Biotechnology, Santa Cruz, CA), sections were incubated at 4°C overnight with 1:1000 anti-HO-1 rabbit polyclonal antibody (StressGen Biotechnologies Corp., Victoria, British Columbia, Canada) or 1:500 anti-catalase rabbit polyclonal antibody (Calbiochem-Novabiochem Corp., San Diego, CA). Detection was performed using an antidouble medium (Vectastain ABC kit; AK-5001; Vector Laboratorties) and an alkaline phosphatase substrate (SK-5400, Vector Laboratories). Control sections were incubated with 1:1000 and 1:500 normal rabbit immunoglobulin fraction, respectively (Dako Co., Carpinteria, CA) instead of the primary antibody. Some sections were bleached with potassium permanganate, as described earlier. Sections were counterstained with nuclear fast red (Vector Laboratories), dehydrated, and mounted.

**Data Analysis**

Specimens were observed under a light microscope (model BH-2; Olympus Optical Co., Ltd., Tokyo, Japan) with a charged-coupled device camera (ProgRes 3012; Kontron Elektronik GmbH, Eching, Germany). Digitalized images were captured through the digital camera plug-in directly to graphic software (Photoshop 5.0; Adobe, Mountain View, CA). The number of total RPE cells and labeled RPE cells were counted on each section that contained at least 250 RPE cells, according to our previously published method. The ratio of labeled RPE cells to total RPE cells counted per section was calculated for both the macular and peripheral regions of each eye. Statistical significance was determined using the Wilcoxon signed rank test for the ratio of labeled macular cells versus the ratio of labeled peripheral cells. The Spearman rank correlation was used to determine the influence of age on each ratio. P < 0.05 was considered significant.

**Results**

**In Situ Hybridization and Immunohistochemistry for HO-1 in the RPE**

With the antisense HO-1 probe, the RPE showed nonuniform labeling that was characterized by clumps of labeled cells adjacent to unlabeled cells, in all 18 globes (Fig. 1). The HO-1 mRNA expression in the macular RPE showed a greater ratio of labeled cells to total RPE cells per section than in the periphery (P = 0.03, Wilcoxon signed rank test; Fig. 2A). The ratio of HO-1 mRNA-positive cells to total RPE cells decreased with age in both the macula (P = 0.0009, Spearman rank correlation) and periphery (P = 0.002, Spearman rank correlation), with a more pronounced decrease in the macula than in the periphery (Fig. 2A). Immunohistochemical staining of HO-1 in RPE cells showed an expression pattern similar to the pattern found by in situ hybridization (Fig. 3). No statistically significant difference in the HO-1-labeled cell ratio was detected between the macula and periphery of individual globes when all 18 globes were examined as a set. When these ratios were plotted as a function of age for the macula and periphery, the labeling of HO-1 mRNA by in situ hybridization in RPE cells by region and age. RPE sections from a 27-year-old (case 1) macula (A) and periphery (B) and a 69-year-old (case 11) macula (C) and periphery (D). The DIG-labeled in situ hybridization reaction with BCIP/NBT appears blue-purple. Arrowheads: labeled cells. No labeling was seen with the sense probe (E). Bar, 50 μm.
separate regression lines appeared, but each had a correlation coefficient of only $r = 0.5$. This data set lacks sufficient power to assess statistically significant variations of HO-1 protein expression as a function of age or topography.

In Situ Hybridization and Immunohistochemistry for Catalase in the RPE

The RPE in general showed nonuniform labeling, characterized by clumps of labeled cells adjacent to unlabeled cells in all 18 cases (Fig. 4). Catalase mRNA expression in macular RPE cells showed a greater ratio of labeled cells: total RPE cells counted per section compared with the same ratios counted for periphery ($P = 0.002$, Wilcoxon signed rank test; Fig. 5A). The ratio of catalase mRNA-positive RPE cells to total RPE cells showed no statistically significant correlation with age in either the macula or the periphery (Fig. 5A). Immunohistochemical staining of catalase in RPE cells showed a pattern similar to the pattern found by in situ hybridization (Fig. 6). No statistically significant age-related change in the catalase-labeled cell ratio was detected (Fig. 5B).
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Attempts to compare our studies with the work of Liles et al. are also difficult because of the dissimilarity of the methods. They used an enzymatic assay for catalase activity in whole samples of macular RPE. Total enzyme activity may be affected by a number of factors that would not be appreciated by immunohistochemistry, such as posttranslational modification of protein and loss of enzymatic activity with postmortem time. The approach of Liles et al. yielded an average value over all RPE cells in the macula rather than examining activity on an individual cell basis. Our methods investigated expression of both mRNA and protein by individual cells in both the macula and periphery. This approach allowed us to observe the mosaic nature of mRNA and protein expression of not only catalase, but also HO-1, in both macular and peripheral RPE. Our results, however, can be directly related to a measure such as total enzyme activity. Age-related changes have a nonuniform distribution within any macula. The mosaic pattern of expression for both HO-1 and catalase by the RPE could be a contributing factor to the heterogeneous appearance of age-related macular changes. Further investigation determining the role of chimeric expression of antioxidant enzymes in AMD is warranted.

References


DISCUSSION

In this study, we investigated age and topographic changes in the mosaic expression patterns of two antioxidant enzymes in human RPE cells by determining the number of labeled cells and calculating the ratio of labeled RPE cells to the total number of cells per section. We identified three important findings in the expression patterns of HO-1 and catalase in the RPE. First, the ratio of labeled to total cells was significantly different between the macula and periphery. Second, the ratio of HO-1 mRNA-labeled cells to total cells in the RPE decreased with age in both the macula and periphery. The ratio of labeled to total RPE cells observed by HO-1 immunostaining showed no significant decline with age or topography. Third, the ratio of labeled to total RPE cells for catalase mRNA did not show any age-related changes in either the macula or periphery. Our immunohistochemistry on HO-1 and catalase protein confirmed the mosaic expression pattern shown by in situ hybridization.

Our studies cannot be directly compared with the studies by Frank et al. The method of quantification used in the study by Frank et al. involved the counting of gold particles in 50-μm² areas within individual cells. This method has the advantage that the number of particles is a continuous variable capable of quantifying relative levels of protein within an individual cell. Frank et al. showed for example, that lysosomes were more heavily labeled than cytoplasm. A possible limitation of this method is the difficulty in quantifying results from a large number of cells. In contrast, our method scored each cell counted as either positive or negative, but sampled a larger number of cells in both the macula and periphery. The mosaic nature of HO-1 staining that we observed suggests that large deviations in HO-1 expression at both mRNA and protein levels occur among cells. This observation is consistent with the large standard deviations found by Frank et al. An obvious weakness of our method therefore is the simple quantification of cells as either stained or unstained. It is important to note that the mosaic pattern was not a general staining property for all the cells that we observed. Ganglion cells for example, displayed uniform staining regardless of topography or age (data not shown).

Our findings on catalase are also not directly comparable to those in the study by Frank et al. on protein expression or to the results in the study by Liles et al. on total enzyme activity. In normal eyes, Frank et al. found a slight decrease in the number of gold particles counted per 50-μm² area of cytoplasm or lysosome as a function of age. Once again, our own results most likely sampled a larger number of cells, but quantified cells only as positive or negative with respect to staining for mRNA or protein expression. Using this approach, we could not find an age-related change in the ratio of labeled to total RPE cells for catalase expression in the macula. We did find, however, a consistent topographic variation between the macula and the periphery in the ratios of stained cells to total cells counted for mRNA and possibly for protein.