Mitochondrial Superoxide Dismutase in Mature and Developing Human Retinal Pigment Epithelium

Peter D. Oliver*† and David A. Newsome*‡

Human retinal pigment epithelium (RPE) contains two genetically distinct forms of superoxide dismutase (SOD) enzymes that scavenge harmful superoxide anions. Biochemical and immunochemical techniques were used to compare levels of copper-zinc- and manganese-containing forms of SOD (CuZn-SOD and Mn-SOD) in human adult and fetal RPE cells. It was found that Mn-SOD activity was higher in adult than fetal RPE cells, both in vivo and in vitro. Immunolocalization of Mn-SOD in cultured RPE cells showed a greater reactivity in the mitochondria of the adult cells. Primary cultures of adult RPE contained cells with various patterns of mitochondria as shown by immunolabeling for Mn-SOD. Adult RPE cells were more resistant to the effects of a superoxide generator, paraquat, which appeared to disrupt mitochondrial integrity as judged by staining with rhodamine 123. These results suggest that high levels of Mn-SOD protect mitochondria from oxidative damage that probably occurs with aging in the RPE. Invest Ophthalmol Vis Sci 33:1909-1918, 1992

One of the primary functions of the retinal pigment epithelium (RPE) is active phagocytosis and degradation of shed photoreceptor outer segments.1 Phagocytic activity in the RPE results in the generation of reactive oxygen species, including superoxide anions.2 Another potential oxidative insult to the RPE is the high levels of oxygen found in the adjacent choroid.3 Both superoxide anions and hydrogen peroxide can be elevated by increasing oxygen tension.4 Oxidative stress in the RPE is exacerbated by light exposure.5-9

As protection against oxygen-generated radicals, RPE cells contain superoxide dismutase (SOD) enzymes.1011 Two forms of SOD are found in human RPE cells, a copper-zinc-containing molecule (CuZn-SOD) found in the cytoplasm and a mitochondrial enzyme containing manganese (Mn-SOD).12 Recent evidence has shown that the RPE also expresses mRNA specific for extracellular SOD.13 Experimentally administered SOD has been shown to protect ocular tissues from hyperoxic damage, presumably by eliminating superoxide anions.14

The dismutation of superoxide anions by SOD results in the production of \( \text{H}_2\text{O}_2 \), which can form reactive hydroxyl radicals by the \( \text{Fe}^{2+} \)-mediated Haber-Weiss reaction.1516 The RPE contains peroxidases917-18 and catalase19-23 that have the potential to break down harmful \( \text{H}_2\text{O}_2 \) into water and oxygen.

Several antioxidant enzymes increase during development and cellular differentiation in numerous species and tissues.24-37 In cultured human fibroblasts, for example, a four- to fivefold greater Mn-SOD activity was found in adult cells compared with fetal cells, even when the cells were incubated with various oxygen tensions.36

By contrast with CuZn-SOD, mitochondrial Mn-SOD is inducible by several factors, including paraquat, a redox active compound that catalyzes the formation of superoxide anions.3839 In cultured cells, Mn-SOD has been shown to protect cells from superoxide-mediated paraquat toxicity.39 Certain immunoregulatory molecules, such as tumor necrosis factor4041 and interleukin-1,42 stimulate expression of Mn-SOD gene products. The specific factors that upregulate Mn-SOD expression during cellular differentiation remain to be elucidated. We compared SOD activities in adult and human fetal RPE cells in vivo and in vitro and investigated the role of antioxidant enzymes in resistance to paraquat-mediated oxidative stress.
Materials and Methods

Tissue

Adult human eyes were obtained within 24 hr of death through the National Disease Research Interchange (Philadelphia, PA) and used to isolate RPE cells as previously described. Adult RPE cells for in situ enzyme analysis were isolated by scraping with a small spatula (Moria, Paris, France); they were placed in phosphate-buffered saline (PBS, pH 7.3) and stored at −20°C. We used cell lines and freshly isolated adult RPE from eyes from 12 donors aged 52–83 yr. A total of ten samples of fetal eyes aged 16–20 weeks' gestation also were used, and they were obtained through the International Institute for the Advancement of Medicine (Washington, DC). After removing the cornea, lens, vitreous, and retina, the RPE was removed mechanically with a fine forceps.

Cell Culture

The cells were cultured in Coon’s modified Ham’s F12 (Sigma, St. Louis, MO) supplemented with L-glutamine (292 mg/l; GIBCO, Grand Island, NY), ascorbic acid (49 mg/l; GIBCO), streptomycin (100 mg/l; GIBCO), penicillin G (100,000 units/l; GIBCO), fetal bovine serum (FBS 5%; Hyclone, Logan, UT), and epidermal growth factor (10 ng/ml; Collaborative Research, Bedford, MA). They were subcultured by trypsinization when they reached confluence. For biochemical analysis, the cells were grown to confluence for at least 1 week and given fresh medium the day before collection. In general, they were collected between passages 3 and 5 to ensure that all cell lines were consistently in a growth phase for comparative purposes. In one series of experiments, paraquat (Sigma) was added daily (5–7 days) to two adult and two fetal cell lines at concentrations of 25, 50, 100, and 200 μM.

Preparation of Intracellular Proteins

Trypsin-ethylenediaminetetraacetic acid (GIBCO) was used to harvest cultured cells for preparation of proteins. Trypsin was blocked by adding culture medium containing FBS to the cell suspension. The cells were collected by centrifugation, rinsed twice in PBS, and the final pellet resuspended and sonicated in either PBS or a solution of Tris HCl (20 mmol/l, pH 7.8) with diethylenetriamine-pentaacetic acid (0.2 mmol/l). Unless otherwise specified, all chemicals were obtained from Sigma. The cell homogenate then was placed in an Eppendorf centrifuge (Brinkmann, Westbury, NY) at 13,000 rpm for 10 min, and the supernatant was collected. Protein was determined using the Biorad (Richmond, CA) microassay.

Gel Electrophoresis and SOD Activity

Biochemical studies were done on six fetal cell lines (ages, 16–20 weeks' gestation) and six adult cell lines (from donors ages, 64–83 yr). Enzymatic activities of CuZn-SOD and Mn-SOD were distinguished on gels under nondenaturing conditions. Identical amounts of protein (250 or 100 μg) were loaded onto each polyacrylamide gel (12%) in the absence of sodium dodecyl sulfate (SDS). After running at 55 V for 16 hr, the gels were incubated in a solution of nitro blue tetrazolium (NBT 2.45 mmol/l) for 40 min, rinsed briefly in water, and incubated 40 min in a solution of tetramethylthylene diamine (28 mmol/l) and riboflavin (0.028 mmol/l), buffered with potassium phosphate (36 mmol/l, pH 7.8). The gels then were placed in water on a fluorescent light box for approximately 30 min. The SOD activity was evident on the gels by its inhibition of the formation of the formazan blue reaction product.

Antisera

Antisera to CuZn-SOD and Mn-SOD and secondary donkey anti-sheep antisera conjugated to fluorescence isothiocyanate (FITC) or peroxidase were obtained commercially (The Binding Site, San Diego, CA). For immunoblotting, primary antibodies were used at a dilution of 1:600 and secondary antibodies, at 1:1,000. For indirect immunofluorescence, we used dilutions of 1:50 for both primary and secondary antisera.

Immunoblotting

Immunolabeling of cellular proteins was done by first subjecting cells to SDS-polyacrylamide gel electrophoresis (12% gels), followed by electrophoretic transfer onto nitrocellulose, as previously described. Extracted proteins were run at 50 and 200 μg/lane and labeled with antisera specific for CuZn-SOD and Mn-SOD. Before adding the first antiserum, the nitrocellulose was soaked in bovine serum albumin (BSA) 1% for 1–2 hr as a blocking step. The sheep anti-human CuZn-SOD or Mn-SOD antibodies then were applied overnight in PBS (pH 7.4, 22°C) with BSA 1% and Tween 20 0.05%. The membranes were rinsed briefly and incubated with the peroxidase-conjugated secondary antiserum for 1 hr in PBS (pH 7.4, 22°C), containing BSA 1% and Tween 20 0.05%. The reaction product was revealed in PBS with 0.5 mg/ml of 3,3’-diaminobenzidine and hydrogen peroxide 0.06% (5 min, pH 7.4).
Biochemistry of SOD

Both CuZn-SOD and Mn-SOD activities from cultured and noncultured RPE cells were revealed by subjecting the cellular proteins to electrophoresis under nondenaturing conditions. Compared with the activity in the freshly isolated tissue, there appeared to be a slight reduction in CuZn-SOD after the cells were cultured for three passages (Fig. 1). The Mn-SOD activity in situ was much higher in the adult cells than in the fetal RPE cells in all cell lines and persisted in vitro after the cells had been cultured for several passages.

We compared SOD levels in cultured cells by transferring cellular proteins onto nitrocellulose for immunolabeling (Fig. 2). The immunoreactivity associated with bands of CuZn-SOD was similar in protein preparations from adult and fetal cells. Lanes containing 50 μg of adult cell proteins showed a greater amount of immunoreactivity for Mn-SOD than the lanes of fetal cells loaded with 200 μg protein/lane. This indicates at least a fourfold greater amount of the enzyme per unit protein in the cultured adult cells.

Immunolabeling of Mn-SOD

Indirect immunofluorescence of cultured cells with antiserum directed against Mn-SOD revealed striking differences in labeling between adult and fetal cells (Fig. 3). Mitochondria in adult cells were numerous and generally had a filamentous or thread-like appearance with intermittent nodules with intense immunoreactivity (Fig. 3A). The fetal cells had less Mn-SOD immunoreactivity, and the labeling did not reveal as extensive a pattern of filamentous mitochondria (Fig. 3B).

To determine whether the variation in the immunolabeling pattern of Mn-SOD was caused by differences in the mitochondria themselves, we incubated cultured cells with a mitochondrial marker, rhodamine 123.
Fig. 3. Immunolabeling of Mn-SOD (A, B) and rhodamine 123 (C, D) in cultured RPE cells. Cultured adult cells (A) show greater Mn-SOD reactivity than do comparable (P3) fetal cells (B). Rhodamine 123 uptake into the mitochondria of comparable (P3) adult (C) and fetal (D) RPE cells shows no apparent differences in number or appearance of mitochondria. Bar = 33 µm.
Fig. 4. Effect of paraquat treatment (100 μmol/l, 7 days) on cultured adult and fetal RPE. (A) Phase contrast of paraquat-treated fetal cells. Note the cytoplasmic vacuoles. (B) Phase contrast of cultured adult cells showing normal morphology. (C) The rhodamine 123 in the fetal cells shows rounded-atypical mitochondria with less uptake of the dye. (D) Rhodamine 123 in treated adult cells shows normal mitochondrial distribution. Bar = 33 μm.
mine 123. Mitochondria in both fetal and adult RPE cells readily took up the dye, and the cellular distribution of the rhodamine 123 showed a similar filamentous pattern of mitochondria in both adult and fetal cells (Figs. 3C-D).

Paraquat Toxicity

We subjected the cultured adult and fetal cells to oxidative stress by exposing them to paraquat-induced generation of superoxide anions. After 5–7 days of exposure to 50–100 μM paraquat, the fetal cells contained numerous cytoplasmic vacuoles, and many of the cells had rounded up. The adult cells appeared to be unaffected (Figs. 4A–B). Paraquat-treated fetal cells had mitochondria that were rounded and generally exhibited a weaker intensity of rhodamine 123 labeling (Fig. 4C). At the same concentration of paraquat (100 μM), adult cells showed a normal array of filamentous mitochondria (Fig. 4D).

Biochemical analysis of SOD was done using NBT gels to detect CuZn-SOD and Mn-SOD levels in cultured adult and fetal cells that had been subjected to paraquat treatment (Fig. 5). The adult cells had increased levels of Mn-SOD after paraquat exposure. Although the fetal cells showed a slight increase in Mn-SOD, the levels of Mn-SOD remained much lower than those observed in the adult cells.

Primary Cultures of Adult Cells

During the course of establishing primary cultures of adult RPE cells, numerous large (apparently not dividing) cells were observed. Many of these retained their pigment granules. Primary cultures of adult RPE were established on glass cover slips to facilitate microscopic examination. After 1–2 weeks’ incubation, large nondividing cells were distinguished readily from a population of smaller cells, as shown by staining with rhodamine 123 (Figs. 6A–B).

After labeling primary cultures of RPE with antiserum to Mn-SOD, differences in the patterns of mitochondrial distribution were observed (Figs. 6C–F). Many of the large nondividing cells contained autofluorescent pigment granules localized in the perinuclear region. In most cells, the system of mitochondria was extensive, consisting of filamentous forms with intermittent larger, more intensely stained spheric structures (Figs. 6C, 6E). In other cells, the Mn-SOD localization showed a highly branched system of mitochondria with fewer nodules containing intense immunoreactivity (Fig. 6F).

Discussion

We found differences in SOD antioxidant enzyme activity between cells from adult and fetal sources. The level of Mn-SOD was notably higher in both freshly isolated (in vivo) and in vitro adult tissue in all tissue samples and cell lines examined, regardless of donor age or passage. In the case of adult cells, we previously showed that there was no age-related correlation with total SOD levels, although there was a significant decrease in catalase activity with age.23 In light of this, additional studies would be interesting to determine whether there are age-related changes between specific subgroups of SOD (ie, Mn- and CuZn-SOD).

The secondary band of Mn-SOD with increased electrophoretic mobility (Fig. 1) has been reported previously and appears to be the predominant form after chelation of the cellular proteins with diethyldithiocarbamic acid, a chelating agent that inactivates CuZn-SOD.12 Because corresponding differences in Mn-SOD gene expression were maintained in tissue culture, the higher level in the adult cells appears to be related to the differentiated state of the cells at the time of isolation. Our results were similar to a study of SOD activity in human fibroblasts, in which cultured cells from adult sources had a fivefold higher level of Mn-SOD than fetal cells.36 Mitochondrial activity tends to increase during development,46 and the higher levels of
Fig. 6. Adult RPE cells in primary culture. Cells stained with rhodamine 123 (A, B) showing a typical filamentous array of mitochondria. Note that the cell in (A) is much larger than those in (B). (C) Mn-SOD in a large cell. Note both the filamentous array and the large bright nodules (arrows). (D) Autofluorescence of cell in (C). (E, F) Mn-SOD localization of two large cells with abundant autofluorescent granules (AF) in the perinuclear region. In (F), the Mn-SOD reveals filamentous mitochondria with interspersed bright nodules. The immunoreactivity of Mn-SOD in the cell in (F) shows primarily a branched system of mitochondria. Bar = 33 µm.
Mn-SOD may reflect a cellular response to increased oxidative activity and concomitant superoxide production. An assay to test mitochondrial cytochrome c oxidase activity would have been useful to interpret our data.

The higher levels of mitochondrial SOD in the cultured adult RPE cells appeared to be correlated with a greater immunoreactivity of Mn-SOD in cells cultured on cover slips. The use of rhodamine 123 showed little difference between the mitochondria of fetal and adult cells (Figs. 3C-D), although no precise quantitation was made.

Nonetheless, our findings suggest the presence of intrinsic regulatory factors that influence levels of Mn-SOD gene expression during development. Certain factors (eg, interleukin-1 and tumor necrosis factor) stimulate expression of Mn-SOD gene products. Additional studies are required to determine whether these or other substances regulate Mn-SOD expression in an autocrine or paracrine fashion at different stages of development.

The higher levels of Mn-SOD in the adult cells correlated with greater resistance to superoxide-mediated paraquat toxicity, which caused alterations in mitochondrial morphology in the fetal cells. The observation that cultured fetal cells did not induce a major increase of Mn-SOD in response to the oxidative stress of paraquat indicates that the generation of superoxide anions alone may not be sufficient to induce the four- to fivefold increase in Mn-SOD that occurs during maturation of RPE cells.

Others have shown that Mn-SOD affords a greater resistance to superoxide-mediated paraquat toxicity in cultured cells. However, the greater resistance of adult RPE to the effects of paraquat we found may not be caused entirely by Mn-SOD. We also found higher levels of catalase and glutathione peroxidase in adult RPE cells, both in vivo and in vitro (manuscript in preparation).

Numerous reports have shown that oxidative stress and deficiencies of antioxidant systems can cause damage to ocular tissues. We do not know if the low levels of RPE antioxidant enzyme levels in fetal cells represent a potential deficiency that would compromise the well-being of RPE cells exposed to the normal range of oxygen tension experienced after birth. The exposure of premature infants to unusually high oxygen levels and possible lower levels of antioxidant enzymes may have a combined effect in the formation of clinical pathologic conditions associated with retinopathy of prematurity.

Our data on Mn-SOD localization in primary cultures of adult cells suggest that there may be some variability in the mitochondria from cell to cell. Additional studies, including ultrastructural examination, are required to determine whether there are senescence-related alterations in RPE mitochondria with respect to age. Observations of highly branched and condensed mitochondria in cultured cells passaged to the point of senescence have been described previously. Because mitochondria have no DNA repair mechanism, mitochondrial DNA may be susceptible to accumulative deleterious damage and mutations. Mitochondrial DNA damage has been linked to aging and certain diseases, including retinal pigmentary alterations.

In summary, antioxidant systems in RPE cells may be important in two ways. First, accumulative oxidative damage may be a crucial factor in age-related macular degeneration, a leading cause of visual loss in older persons. During the aging process, accumulative changes may occur in the mitochondria and contribute to the senescence of RPE cells. Second, lower levels of antioxidant defenses in premature infants subjected to high levels of oxygen could contribute to retinopathy of prematurity. Additional studies are warranted to explore the role of Mn-SOD and other antioxidants in protecting RPE cells from specific oxidative damage at the level of cellular organelles.

Key words: human retinal pigment epithelium, mitochondria, superoxide dismutase, aging, development, paraquat

Acknowledgments

The authors thank David J. Tate, Jr., for his technical support and Mary Sewell for her coordination of our endeavors.

References

8. Ruffolo JJ Jr, Ham WT, Mueller HA, and Millen JE: Photo-


